Isolation and characterization of glycosaminoglycans and a study of its bioactive potential in two commercially important species of cephalopods, *Loligo duvauceli* and *Sepia pharaonis*

Thesis submitted to

Cochin University of Science and Technology

In partial fulfilment of the requirements for the degree of

Doctor of Philosophy

by

Manjusha, K.P.

Reg. No. 2823

School of Industrial Fisheries
Cochin University of Science and Technology
Kochi - 682 016

April 2011

Dedicated to

…my Parents

…my husband and daughter

Dr. Saleena Mathew

Professor
School of Industrial Fisheries
Cochin University of Science and Technology
Kochi – 16

Certificate

This is to certify that the Doctoral Thesis entitled **"Isolation and characterization of glycosaminoglycans and a study of its bioactive potential in two commercially important species of cephalopods, *Loligo duvauceli* and *Sepia pharaonis*"** is an authentic record of the original research work carried out by Ms. **Manjusha, K.P.**, under my supervision and guidance at the School of Industrial Fisheries, Cochin University of Science and Technology, in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** of Cochin University of Science and Technology and that no part thereof has been submitted before for any degree.

Kochi
26-04-2011

Prof. (Dr.) Saleena Mathew
Supervising guide

Declaration

I, Ms. Manjusha, K.P. do hereby declare that this Doctoral Thesis entitled **"Isolation and characterization of glycosaminoglycans and a study of its bioactive potential in two commercially important species of cephalopods, *Loligo duvauceli* and *Sepia pharaonis*"** is an authentic record of the original research work carried out by me under the guidance and supervision of **Dr. Saleena Mathew, Professor,** School of Industrial Fisheries, Cochin University of Science and Technology, Kochi – 16 in partial fulfillment of the requirements for the Degree of **Doctor of Philosophy**, and that no part has been submitted earlier for any other degree, diploma or similar titles of this or any other university.

Kochi
April, 2011

Manjusha, K.P.

Acknowledgements

It is with profound joy that I express my heartfelt gratitude to my supervising guide, and mentor Prof. (Dr.) Saleena Mathew, School of Industrial Fisheries, CUSAT, for her inspiring and excellent guidance, valuable suggestions, ceaseless encouragement and intellectual support. She has been my constant inspiration throughout the investigation and I am deeply obliged to her for providing all the necessary facilities and above all the absolute freedom provided in doing the experiments. I record my deep sense of gratitude to her for all the efforts she has put in and the moral support extended for the successful completion of this thesis.

I wish to thank Prof. (Dr.) A. Ramachandran, Director, School of Industrial Fisheries, CUSAT, for providing the prospect, encouragement and support.

I wish to express my gratitude to Prof. (Dr.) H.S. Ram Mohan, Dean, Faculty of Marine Sciences for the prospect and encouragement.

I feel extremely privileged to place on record my sincere gratitude and respect to Prof. (Dr.) Babu Philip, Head, Department of Marine Biology, Microbiology and Biochemistry, CUSAT. I am deeply indebted to him throughout my career, for being instrumental in providing the opportunity to commence my Doctoral program at the Cochin University of Science and Technology. Prof. (Dr.) Babu Philip was a source of inspiration and I sincerely thank him for the valuable suggestions, encouragement and constant support rendered.

I gratefully acknowledge the University Grants Commission, New Delhi for awarding the financial support as Junior and Senior Fellowship for the successful completion of this Ph D work.

I take this opportunity to thank Dr. Lissy.K.Krishnan, Scientist and Head, Thrombosis Research Unit, SCTIMST, Thiruvananthapuram, for initiating and motivating me into the fascinating world of scientific research. I express my heartfelt gratitude to Dr. Resmi. K.R., Scientist, TRU, SCTIMST, Thiruvananthapuram, for the inspiration and encouragement extended to me in pursuing a research career while I did my M.Sc dissertation work at the Thrombosis Research Unit, SCTIMST, Thiruvananthapuram.

I wish to thank all teachers at the School of Industrial Fisheries, CUSAT, for their encouragement and support.

I keep in record my sincere gratitude to Dr. John Mohan, Assistant Professor, School of Industrial Fisheries, CUSAT for the support, encouragement, valuable comments and inspiration.

I am highly grateful to Prof. (Dr.) A.V. Saramma, and Dr. Rosamma Philip, Department of Marine Biology, Microbiology and Biochemistry, CUSAT for providing the necessary facilities, support and encouragement leading to the successful completion of this work.

I am immensely thankful to Prof. (Dr.) Prathapan S., School of Applied Chemistry, CUSAT., for the valuable discussions and guidance rendered in the FT-IR and NMR spectroscopic analysis. I am deeply indebted to him for providing valuable suggestions in preparing the manuscript and evaluating the same.

My sincere thanks are due to Dr. T.K. Srinivasa Gopal, Director, Central Institute of Fisheries Technology (CIFT), and Senior Scientists, Dr. T.V. Sankar, and Dr. Ravishankar. C.N. of CIFT, Kochi for providing the necessary facilities at various stages of my work. I am grateful to Dr. P. K. Surendran, former Head, Microbiology and Fermentation Division, CIFT for the valuable advice, suggestions and inspiration rendered during my Ph.D work. I extend my sincere gratitude to Dr. T.K. Thankappan, Dr. P.T. Mathew, Dr. Jose Joseph and Dr. George Ninan, CIFT, Kochi for their encouragement and support.

I wish to express my hearty thanks to Sri. H. Krishna Iyer, former Scientist, CIFT and Dr. V. Geethalakshmi, CIFT, Kochi for providing valuable suggestions and guidance in the statistical analysis of the data. My thanks are due to Dr. A. Gopalakrishnan, Head, NBFGR Unit, CMFRI Campus, Kochi, for the generous encouragement and support.

I thank Mr. P.J. Manuel, Librarian, and the supporting staff of the Lake Side Campus Library, CUSAT for their valuable co-operation and encouragement. Thanks are due to Mr. Prabhakaran, R., Librarian, and Mr. K. N. Vimal Kumar, Deputy Director, MPEDA, Kochi, for providing valuable data on the export details. I extend my sincere thanks to the Librarian and staff of the libraries of SCTIMST, Thiruvananthapuram; CIFT and CMFRI Kochi; CFTRI, Mysore, and the University of Mysore, for their kind support and consideration during my literature survey.

Thanks are due to Dr. Jacob Philip, Director, Sophisticated Test and Instrumentation Centre (STIC), Main Campus, CUSAT, for providing the instrumental facilities available at the Center, enabling the successful completion of my work. I am thankful to Dr. Shibu M Eappen for XRD analysis, Mr. Melbin Baby for SEM analysis, Mr. Saji. T. K. for NMR analysis, Mr. Shyam Kumar for FT-IR analysis and Mr. Adarsh. K.J. for DSC analysis.

I sincerely thank Dr. Latha Unnikrishan, Regional Laboratory, Kochi for the services rendered in collecting the human blood samples required for the study.

Mere words of thanks will be inadequate for the timely help, love, strong support, and concern rendered by my friends Dhanya. P.R., Pearly Tony, Abhilash, S. and Prasanth. K.M. I wish to remember the beautiful moments of humor, fruitful discussions, the wit and wisdom we all shared together at the School of Industrial Fisheries, CUSAT, which has served considerably in providing a smooth working environment and a homely atmosphere in the lab.

I am grateful to Dr. Maya Raman, my senior for the wholehearted support and I sincerely thank her for providing innumerable references during her tenure as post doctoral fellow at the Lund University, Sweden. I immensely thank my senior Mr. John Chembian, Scientist, Fisheries Survey of India, Kochi, for providing me with the raw materials for the study. I extend my thanks to my seniors Dr. Beena Sulochanan, Dr. Femeena Hassan, and Dr. Mohanan P for their encouragement and goodwishes.

My collective and individual acknowledgements are also owed to the technical staff, Ms. Bindu. M.S. and Sri. Achuthan Pilla V at the School of Industrial Fisheries, CUSAT. Special thanks to 'Pilla Chettan' for making a number of 'self designed tools' that has aided in the smooth and successful completion of some experiments.

I am extremely privileged to have the care, moral support and words of inspiration of Dr. Mini Sekharan, Lecturer, School of Industrial Fisheries, CUSAT. I would also like to place on record Dr. Sreedevi, C. Guest Lecturer, School of Industrial Fisheries, CUSAT and Dr. Valsamma Joseph, Lecturer, School of Environmental Studies, CUSAT.

I sincerely thank my friends Shubasree Sankar, Sarita Thomas, Rosewine Joy, Baiju, K, K., George MA, Sindhu MC, Liya Jayalal, Joinee Jacob M, Soumya SubraDe and Antony V.T. at the School of Industrial Fisheries, CUSAT, for their encouragement and support. Special thanks to Baiju K, K for the help rendered in statistical analysis. I would like to place on record the help, friendship and support of Jones Varkey, Biji K B, Dhanya K, Nabajyothi Biswas, Arya Raveendran, Veena MG, Ginson Joseph, Sibin PB and Antony Libin former M. Sc and M. Phil students at the School.

I always cherish the sincere support and warm friendship of my colleague Manjusha, K., for the help and assistance rendered in some of the experiments and unparallel friendship ever since our graduate days at St.Teresa's college, Ernakulam. My special thanks to Dr. Selvan, S. and Ms. Giji Poulose for their valuable suggestions and wholehearted support. I extend my sincere gratitude to Anit M. Thomas and Dr. Sanil Kumar M. G. for their help rendered in taking the photographs of the histochemical sections. I also express my thanks to H.S. Harisankar and P. R. Anil Kumar for the help and support extended at various stages of

my Ph.D work. I extend my sincere thanks to Dr. Srideevi N Kutty, Jisha Maria Jose, Simi Joseph P, Swapna P Antony, Deepthi Augustine, Priyaja P, and Vrinda S, at the sister Departments of the Lake Side Campus, CUSAT.

I sincerely thank Mr. Jayashankar, M, Section Officer and Ms. Susan Joseph, and all other staff of the School of Industrial Fisheries, CUSAT, for their timely service.

I record my gratitude to Electrical staff, CUSAT Lake Side Campus, for their timely help.

I would like to put on record and thank one and all who directly and indirectly extended their kind co-operation and support throughout my research work.

I take this opportunity to compliment the affectionate friendship and bond of love shared for many years with my friends Dr. Sumi S. and Adarsh. S. for their boundless support and encouragement.

My joy knew no bounds in recording my deep sense of gratitude and regard all along my life, to my parents, my grandmother and brothers for their prayers, affection and love. My beloved parents have provided me with the best of all in life, and the encouragement, inspiration, patience and support smoothly paved my path towards the successful completion of this research work. My younger brother Vivek Lal has always been my regular companion to the sample collection sites. I extend my deep sense of gratitude to all other family members for their prayers and good wishes.

I express my sincere gratitude to my parents in law for the encouragement and moral support extended.

Last but not least, thanks are due to my beloved husband Dr. Pramod M.S. for his unreserved and unconditional love, constructive criticism, prayers, and motivation, at all stages of my work up to the final formatting of the Thesis, for without his generous support this work would not have been successfully completed.

My heartfelt apologies to my babe Ammu, for unknowingly sacrificing the beautiful moments we would otherwise have shared together.

Finally, I humbly bow before the almighty God for showering his blessings upon me and giving me the strength, wisdom, health and luck to accomplish this important milestone in my academic life.

Manjusha, K. P.

Contents

List of figures

List of tables

List of plates

Abbreviations

µg	microgran
µl	microliter
µm	micrometer
ANOVA	Analysis of variance
AOAC	Association of the Official Analytical Chemists
ATR	attenuated total reflectance
AU	absorbance unit
BSA	bovine serum albumin.
BSE	bovine spongiform encephalopathy
CBB	coomassie brilliant blue
cDNA	complimentary deoxyribonucleic acid
cFCS	control fish collagen sheet
CF- FCS	cuttlefish GAG impregnated fish collagen sheet
cm	centimeter
CNS	central nervous system
Conc	concentration
CPC	cetlypyridinium chloride
CS	chondroitin sulfate
CsCl	caesium chloride
CTAB	cetyltrimethyammonium bromide
D	dimensional
D_2O	deuterium oxide (heavy water)
Da	Dalton
DEAE	diethyl amino ethyl
DI	deionized
Dia	Diameter
DMMB	dimethyl methylene blue
DPX	dibutyl pthalate in xylene
DS	dermatan sulfate
DSC	Differential scanning calorimetry
DSPG	dermatan sulfate proteoglycans
EC	enzyme commission
ECM	extracellular matrix
EDTA	ethylene diamine tetra acetic acid
EEO	electro endo osmosis

EEZ	Exclusive Economic Zone
EGF	epidermal growth factor
EULAR	European League against Rheumatism
FCS	fish collagen sheet
FDA	Food and Drug Administration
FID	Free induction decay
Fig	figure
FMD	Foot and mouth disease
FTIR	Fourier Transform Infra Red
g	gram
GAG	glycosaminoglycan
Gal	galactose
GalN	galactosamine
GalNAc	N-acetylgalactosamine
GdnHCl	guanidine hydrochloride
GlcA	glucuronic acid
GlcN	glucosamine
GlcNAc	N-acetylglucosamine
GlcNAcT	N-acetylglucosamine glycosyltransferase
h	hour
HA	hyaluronic acid
HABPs	hyaluronic acid binding proteins
HACCP	Hazard Analysis Critical Control Point
HAS	hyaluronan synthase
HMWM	high molecular weight markers
HPLC	High performance liquid chromatography
HR-NMR	high resolution-nuclear magnetic resonance
HS	heparan sulfate
HSPG	heparan sulfate proteoglycans
i.d	internal diameter
ICAM-1	Intracellular adhesion molecule-1
IdoA	iduronic acid
IQF	individually quick frozen
IR	infrared
KDa	Kilo Dalton
km	kilometer
KS	keratan sulphate

kV	kilo volt
L	litres
LF-NMR	low field - nuclear magnetic resonance
m	meter
M	molar
ME	mercaptoethanol
mg	milligram
min	minutes
ml	milliliter
mm	millimetre
mM	millimolar
MT	metric ton
n	number of times repeated
N	normality
nm	nanometer
NMR	Nuclear magnetic resonance
No	number
NOESY	nuclear overhauser spectroscopy
NSAID	non–steroid anti inflammatory drug
OD	optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PG	proteoglycans
pH	hydrogen ion concentration
PITC	phenyl isothiocyanate
PMSF	Phenyl methyl sulphonyl fluoride
ppm	parts per million
R_f	retention factor
ROESY	rotating frame nuclear overhauser effect spectroscopy
RP–HPLC	Reverse phase–High performance liquid chromatography
rpm	revolutions per minute
RT	room temperature
s	seconds
SAX	Strong anion exchange
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM- EDAX	Scanning electron microscopy - energy dispersive X ray

SEM	scanning electron microscope
SPSS	Scientific Package of Social Science
SQ-FCS	squid GAG impregnated fish collagen sheet
TBS	tris buffered saline
TE	tissue engineering
TEA	triethylamine
TEMED	tetra methyl ethylene diamine
TMS	tetramethylsilane
TNFα	tumor necrosis factor α
TRIS	tris hydroxyl methyl aminomethane
TX100	triton x 100
UA	uronic acid
UV	ultraviolet
V	volt
v/v	volume by volume
Vs	versus
w/v	weight by volume
WHO	World Health Organisation
XANES	X-ray absorption near edge structure
XRD	X ray diffraction

Chapter1: Introduction and Review of Literature

Contents

1.1 General Introduction

The Indian Exclusive Economic Zone (EEZ) encompassing approximately 2.02 million km^2 abounds in rich marine flora and fauna and includes a wide variety of commercially important finfish, crustaceans and molluscs. Seafood forms an important part in the fisheries sector with regard to employment, livelihood, food security and human nutrition. There is also an increased worldwide concern about the use and over exploitation of natural seafood resources demanding greater utilization of fish landings including the seafood processing discards which are hitherto considered as waste. The utilization of processing discards for the production of commercially viable products can have enormous economic implications in a developing country like India.

Over the past several decades scientists have given increasing attention to marine organisms and the secondary metabolites they produce. Investigations on marine natural products have focused on areas like marine nutraceuticals, biopolymers, biofilms, CNS – membrane active toxins and ion channel effectors, antifouling, anticancer and antiviral agents, tumor promoters, anti – inflammatory agents, as well as metabolites that control micro – filament mediated processes. The marine natural products field is encouraging a multidisciplinary approach between biologists, chemists and pharmacologists. It is also important to note that the number of compounds reported annually is increasing steadily, indicating that marine organisms will continue to be sufficient sources of natural bioactive substances.

1.1.1 Cephalopods

The Cephalopoda is an ancient and very successful class of the phylum Mollusca and have been among the dominant predators in the ocean at various times in geological history. Cephalopods are soft-bodied, bilaterally symmetrical animals with a well-developed head and a body that consists of the muscular mantle enclosing, a mantle cavity that houses the internal organs. The head bears an anterior circumoral (surrounding the mouth) crown of mobile appendages (arms, tentacles). This characteristic feature reflects the origin of the name Cephalopoda, which derives from the union of the two Greek words: 'kefale', head, and 'pous', feet.

There are about 700 living species that are diverse in form, size and nature, and occupy a great variety of marine habitats in all of the world's oceans, distributed mainly in the regions of Arabian Sea, Indo-Pacific, along the coast line of the African coast to the Red Sea, and China Sea. Although there are relatively few species of living cephalopods, individual species are often very abundant and provide major targets for marine fisheries in the Indian waters (Silas, 1985).

Squids, cuttlefishes and octopus in India are represented by several genera. The economically important cephalopods in India are, the squids represented mainly by *Loligo duvauceli*, *Sepioteuthis lessoniana*, *Doryteuthis sp*., and cuttlefish species, mainly *Sepia pharaonis*, *S. aculeata*, *S. brevimana*, *S. elliptica*, *S. prashadi* and *S. inermis*. The major species of octopus include *Octopus dollfusi, Octopus membranaceus, Octopus lobensis, Octopus vulgaris and Cistopus indicus.* More than 75% of the cephalopod landings along the south-west coast are generally represented by the squid, *Loligo duvauceli* and cuttlefish, *Sepia pharaonis*.

1.1.2 Description and Biology of the species selected for the study

The cephalopod species selected for the study are squid, *Loligo duvauceli* and cuttlefish, *Sepia pharaonis*. A detailed account of these species was given by Roper *et al*. (1984).

1.1.2.1. Squid: *Loligo duvauceli* Orbigny, 1848 [Plate1.1 (a)]

1.1.2.1(a) Diagnostic Features: *Loligo duvauceli,* commonly known as the 'Indian squid' is characterized by a relatively short, stout mantle. Fins are rhombic, broad, short, just over 50 % of mantle length. Tentacular clubs expanded; large median manal suckers 1 ½ times larger than marginals, with 14 to 17 short, sharp teeth around ring. Arm suckers of female of about equal size on arms II and III, rings smooth proximally, toothed with about 7 broad, blunt teeth distally (the central one pointed). In males, suckers of arm II and III are greatly enlarged, with 9 to 11 broad, squared to rounded, truncate teeth in the distal 2/3 of ring, proximal 1/3 smooth; left arm IV of male hectocotylized for more than 1/2 its length, with rows of large papillae, some with minute suckers on tip; ventral rows larger, turned outward, comblike; an oval photophore on each side of rectum and ink sac and a chitionus internal shell known as the 'pen or gladius' embedded under the mantle, mid-dorsally.

1.1.2.1(b) Geographical Distribution: Indo Pacific-Indian Ocean periphery, including the Red Sea and the Arabian Sea, extending eastwards from Mozambique to the South China Sea and the Philippines Sea, northward to Taiwan (Province of China).

1.1.2.1(c) Habitat and Biology: *Loligo duvauceli,* is a neritic shallow water species occurring in depths between 30 and 170 m, forming large aggregates during spawning season. Spawning occurs throughout the year, but usually peaks when water temperature increases, i.e. in February and from June to September off Chennai; from February to March, from May through July and from September to October off Kochi. The smallest mature individuals are one year old and longevity is estimated at 2 - 3 years. This species feeds on crustaceans (such as mysids, euphasids and ostracods), fishes and squids. Cannibalism is common. The size in Indian catches ranges from 6 to 28 cm mantle length.

1.1.2.1(d) Morphological features: In fresh condition especially in squid, immediately upon capture, the body is colourless and mantle is transparent showing the internal visceral organs. The skin contains pigment cells or

chromatophores scattered all over the mantle, fins, head and arms and are capable of rapid chameleon like colour changes that harmonies with the colour of the background. On the ventral side chromatophores are less dense and appear whitish.

1.1.2.2. Cuttlefish: *Sepia pharaonis,* Ehrenberg, 1831 [Plate 1.1(b)]

1.1.2.2(a) Diagnostic Features: Mantle broad; fins wide nearly as long as mantle and separated by a cleft at the posterior end. Tentacular clubs moderately long, protective membranes not meeting at base; 8 suckers in transverse rows with 5 or 6 median ones (3rd and 4th in the series) quite enlarged. Left arm IV hectocotylized: basal 12 quadriserial rows normal, next 10 rows with ventral suckers (2 rows) normal but those in dorsal 2 rows are minute and separated from ventral rows by a fleshy, transversally grooved ridge. Colour: a vivid transverse tiger stripe pattern (especially in males) on dorsal mantle and head; a narrow, light, interrupted line along bases of fins. The internal shell, the cuttlebone is a highly calcified structure which is secreted by the epithelial lining of the mantle sac.

1.1.2.2(b) Geographical Distribution

Indo Pacific: Red Sea, Arabian Sea to South China Sea, East China Sea and northern and northwestern Australia.

1.1.2.2(c) Habitat and Biology: This cuttlefish commonly named as the 'Pharaoh Cuttlefish', lives in warm water (30°C), and is fast growing. It is a neretic; demersal species occurring from the coastline to about 110 m depth, but more abundant in the upper 40 m, particularly during the reproductive season. Longevity is estimated at about 2 - 3 years and feeds on crustaceans and a variety of small demersal fishes. Spawning takes place in very shallow waters, at temperatures ranging from 18°C to 24°C where the eggs are attached in clusters to plants, shells, etc. They lay about 1500 eggs which take 14 days to develop at 28°C. Males are larger than females and often, weigh more than 1 kg. Common sizes in landings range from 15 to 30 cm mantle length.

Kingdom: Animalia

Phylum: Mollusca

Class: Cephalopoda

Order: Teuthida

Family: Loliginidae

Genus: *Loligo*

Species : *duvauceli*

Binomial Name: *Loligo duvauceli*, D'Orbigny, 1835

Kingdom: Animalia

Phylum: Mollusca

Class: Cephalopoda

Order: Sepiida

Family: Sepiidae

Genus: *Sepia*

Species: *pharaonis*

Binomial Name: *Sepia pharaonis* , Ehrenberg, 1831

Plate 1.1: (a) *Loligo duvauceli* (squid) and (b) *Sepia pharaonis* (cuttlefish)

1.1.2.2(d) Morphological features: The cuttlefish has a bilaterally symmetrical and dorso – ventrally flattened body, which is shorter and flatter than that of squid (*Loligo),* and tapers towards the posterior end. The body in general is divisible into an anterior 'head' protion and a posterior tapering 'trunk or visceral hump' joined together by a narrow neck. The head bears a pair of large highly developed eyes at the sides and a mouth at the free extremity, surrounded by five pairs of tapering, muscular circumoral appendages, eight of which are short, stumpy and non - retractile known as arms and two long, slender and retractile, tentacles. The inner surfaces of each arms/tentacles bears pedicellate and cup like suckers that are stalked and equipped with armature.

1.1.3 The Cephalopod Export Scenario

Seafood export forms a significant entity for overseas trade. The export of marine products has a long history in India, since the existence of the age-old dry-fish trade with neighbouring countries of South Asia. Sustainable development and empowerment of the marine fisheries sector in India is taken up with a focus on augmenting seafood production for domestic and export markets. The fish processing industry is well developed in the country with HACCP certification for all export-oriented processing plants. Development of adequate infrastructure for harvest and post-harvest operations is embarked upon with ample attention by the central and state governments with due consideration on minimizing post-harvest losses and ensuring enhanced food safety.

The marine products export from India has been rising over the years, and the export during the year 2010 was about US$ 2132.84 million. India's estimated marine resource potential is 3.93 million tons as per the previous update of 2000. Even though molluscan landings constitute only about 4% of the total marine landings in India, the cephalopods *viz,* squid and cuttlefish are prized seafood export items of our country (Fig 1.1). The export of octopus from our country is in a nominal range.

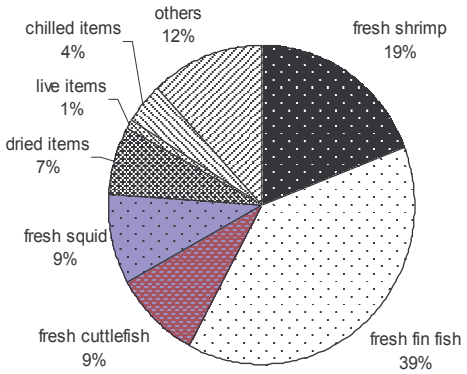


Fig 1.1: Major seafood items of export during the year 2009 – 2010.

[*Source*: MPEDA records, Head Quarters, Kochi].

During the last decade there has been a spectacular growth in the export of non – conventional items such as cuttlefish and squid. The trend of export development of these products in terms of volume and value during 2000 -2010 is shown in Fig 1.2

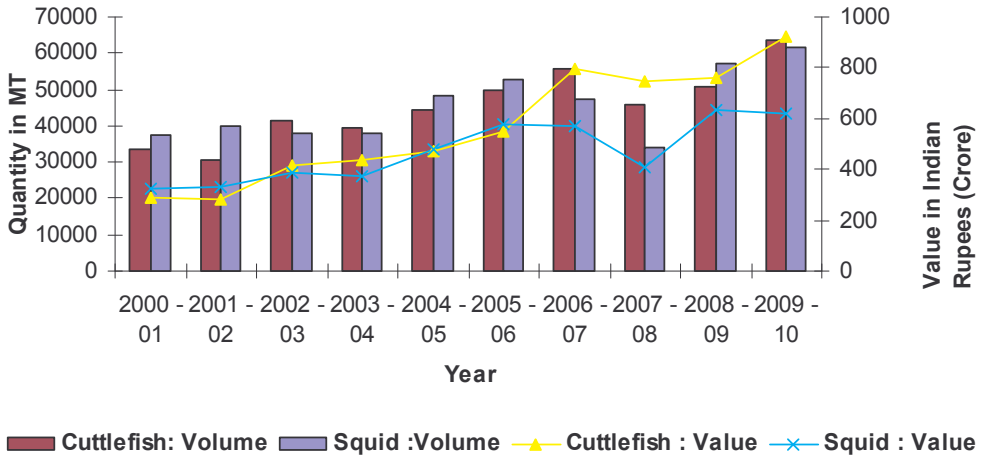


Fig 1.2: Export details of cuttlefish and squid during 2000 – 2010.

[*Source*: MPEDA records, Head Quarters, Kochi].

The export picture of frozen cephalopods over the last decade shows that the export market has remained quite diversified upto date. During year 2009 -10 the export of frozen cuttlefish recorded a growth of 25.13%, 21.39% and 16.30%

in quantity, rupee value and dollar terms respectively compared to 2008-09, even though there was a decline in the unit value realization (7.06%). Export of frozen squid showed an increase in quantity but there was a fall in rupee and US dollar realization. Unit value realization also declined by 13.95% compared to the previous year.

The major importers of frozen cephalopods from India are European Union, China, Japan, U.S.A, countries of South East Asia and the Middle East (Fig 1.3). As per the latest update of 2009 – 2010 the export of frozen cephalopods is worth about US$ 327.94 million. European Union (EU), continued to be the largest market with a share of 66.2% in US$ realization. During the year 2009 – 2010, South East Asia maintained the second place followed by China, USA, Japan, Middle East, and Other Countries.

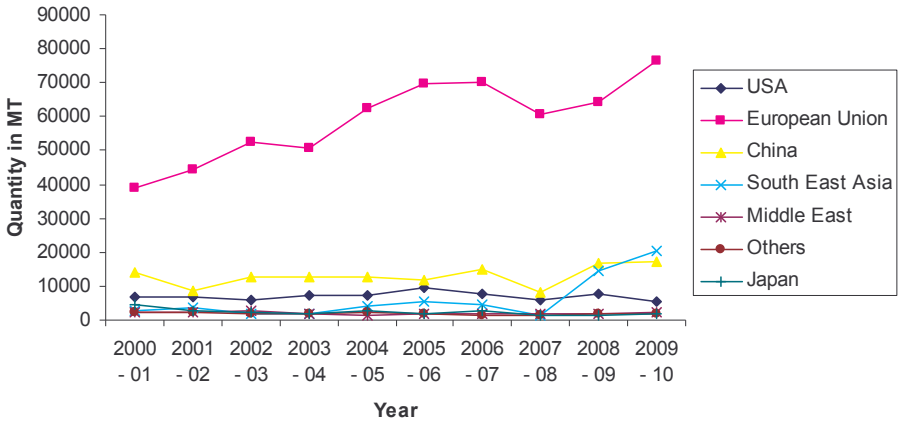


Fig 1.3: The major export markets of frozen squid and cuttlefish

[*Source*: MPEDA records, Head Quarters, Kochi].

1.1.4 Squid and Cuttlefish Processing

Cephalopods comprising mainly of the squid, *Loligo duvauceli* and the cuttlefish, *Sepia pharaonis* are the most abundant cephalopods along the west coast of India. Squid and cuttlefish were considered to be a poor man's food for long time. Studies proved that both squid and cuttlefish meat has great nutritive value with high protein and low fat content which makes them most suitable for human consumption (Roper *et al.*, 1984). Processing of squid and cuttlefish has shown a

rising trend in the recent years due to greater world demand. Though cephalopods can be processed in canned, dried and smoked forms, in India freezing is the predominant method adopted for export. Squid and cuttlefish are processed in several styles for export. The major frozen items of cuttlefish are whole, whole cleaned, rings, fillet, tentacles, IQF (individually quick frozen) and new product forms like wings and tray-packed cuttlefish. The major frozen items in the case of squid are squid whole, whole cleaned, tubes, rings, fillet, tentacles, peeled whole, stuffed, IQF tubes/rings/tentacles, and wings and tray-packed.

1.1.5 Waste Utilization Strategy in the Processing Sector

Processing of seafood as well as fish leaves large percentage of tissue as discards. An important waste reduction strategy for the processing industry is the recovery of marketable by-products from processing discards. In addition to the manufacturing of various marketable by-products, utilization of processing discards has the dual benefit in diminishing the deleterious aspects of environmental pollution as well. Some of the important products obtained from fish waste and trash fish in general are minced fish, surimi, fish protein concentrate, fish meal, fish oil, fish protein hydrolysate, caviar, fish sauce, fish silage, fish collagen, fish gelatin, fish glue, isinglass, leather from fish skin, fish maws, enzymes (proteases), biodiesel/biogas, antioxidants, pigments, pearl essence as well as shark fin, fin rays and squalene from shark. The most important by-products obtained from shell fish waste is chitin and chitosan. The second most abundant biopolymer in the biosphere after cellulose is chitin. It is a versatile homopolysaccharide composed of a long polymer of N – acetyl glucosamine units linked by β, 1-4 bonds; and chitosan is the deacetylated derivative of chitin. Both these by–products have a great variety of applications like clarification and purification, chromatography, in paper and textile industry, photography, agriculture, food and nutrition, as well as in the medical and pharmaceutical fields. The extortion of biologically functionalized polymers that can elicit specific biological responses and the development of methods to fabricate these functionalized polymers are of great interest for the scientific community. In addition to these homopolysaccharides its relative, the heteropolysaccharides of

which the simplest and best known are the 'glycosaminoglycans' are a promising entity in this aspect.

1.1.6 Glycosaminoglycans

The extracellular matrix (ECM) is a gel-like material filling the extracellular space in animal tissues that holds cells together and provides a porous pathway for the diffusion of nutrients and oxygen to individual cells. Extracellular matrix includes the interstitial matrix and the basement membrane. The structure is composed by fibrous proteins (collagen, elastin, fibronectin and laminin) and glycosaminoglycans (GAGs).

Glycosaminoglycans (GAGs), formerly named as mucopolysaccharides are important components of the extra cellular matrix of connective tissues and also on the cell surface of many cell types and in intracellular granules. They occur as long chains of linear acidic polysaccharides which are generally associated with matrix proteins (core proteins) *via* covalent bond to form proteoglycans (PGs) and thereby involved in a wide range of biological processes. These heteropolysaccharides are unbranched polysaccharide chains, generally composed of repeating disaccharide units that are highly negatively charged, due to the presence of sulphate or carboxyl groups on most of their sugar residues. They are called glycosaminoglycans because one of the two sugar residues in the repeating disaccharide is always an amino sugar (N-acetylglucosamine or N - acetylgalactosamine), which in most cases is sulphated. Depending upon the hexosamine unit present, GAGs can be classified mainly into galactosaminoglycans (GalGs) and glucosaminoglycans (GlcGs). Chondroitin sulphates A and C and dermatan sulphate, also termed chondrotin sulphate B (CS-B), are the galactosaminoglycans (GalGs). The glucosaminoglycans include hyaluronic acid, heparin, heparan sulphate and keratan sulphate. The structure and organization of glycosaminoglycans and proteoglycans from tissues of several vertebrates have been extensively studied. However, detailed studies on glycosaminoglycans from tissues of invertebrates, including marine cephalopods are rather limited.

1.1.7. Scope and Relevance of Glycosaminoglycans

Glycosaminoglycans (GAGs) are a fascinating group of molecules that are attracting increasing interest amongst scientists working in Cell Biology, Biochemistry and Medicine, owing to their several applications in the biomedical, veterinary, pharmaceutical and cosmetic field. GAGs such as hyaluronic acid, heparin, and chondroitin sulphate (CS) have wide therapeutic potential because of their outstanding properties. Heparin has been widely used as an injectable anticoagulant for over half a century, and is also in use as an inner anticoagulant surface on various experimental and medical devices. The main field of application of CS and hyaluronic acid is in the treatment of osteoarthritis, and are therefore regarded as nutraceuticals. Other potential uses are as viscosupplements, antiviral, anti-infective and anti-inflammatory agents. Novel documented areas of interest in particular are the suitability of hyaluronan and chondroitin sulphate for tissue engineering and regenerative medicine. Both these polymers are gaining popularity as a biomaterial scaffold which can significantly be improved with crosslinking of collagen, gelatin, chitosan etc producing a hydrogel, for wound healing applications.

1.1.8. Rationale of Glycosaminoglycan Isolation

The larger demand of glycosaminoglycans has switched to the development of biotechnological production processes either based on enzymatic biotransformation or on fermentation processes for glycosaminoglycans, which in fact is in the puerile stage. The non template driven nature of the biosynthesis has also made it challenging to develop techniques to amplify biological GAGs. Eventhough, hyaluronic acid is currently produced *via Streptococci* fermentations; large scale biotechnological production processes for other glycosaminoglycans including heparin and chondrotin sulphate have not yet been successful (Schiraldi *et al.,* 2010).

Most of the commercially available glycosaminoglycans have so far been extracted from higher vertebrates, which often points to the recent health concern issues on interspecies viral infections as well as some lower vertebrates *viz* sharks,

rays etc that are potentially endangered. Since marine natural products continue to be viewed as one of the few *de novo* sources of bioactive substances, the utilization of seafood processing discards for the extraction of glycosaminoglycans will be a promising account so as to fulfill the needs without the overexploitation of other endangered resources.

The present study is aimed at the isolation and characterization of glycosaminoglycans from selected tissues of two commercially important species of cephalopods; squid, *Loligo duvauceli* and cuttlefish, *Sepia pharaonis,* keeping in view of the aforementioned benefits on the utilization of waste generated during processing. The cephalopod GAGs may also be expected to have an effect on various physiological functions based on the results obtained from GAGs from other sources. In addition, knowledge of the chemical structure of macromolecules that constitute major components of extracellular matrix (ECM) will be helpful in understanding their interactions with other matrix components.

1.2 Objectives of the study

- Isolation of glycosaminoglycans from selected tissues of the two commercially important species of cephalopods, *Loligo duvauceli* and *Sepia pharaonis.*
- Qualitative and quantitative analysis of the glycosaminoglycan isolates by electrophoretic techniques and chromatographic methods including SAX-HPLC.
- To evaluate the physico - chemical properties of the GAGs by using spectroscopic techniques like FT- IR and NMR.
- To study the bioactive potential of glycosaminoglycans as exemplified by their role in biomineralization.
- To illustrate the potential role of the isolated glycosaminoglycans as biomaterials by the preparation of glycosaminoglycan impregnated fish collagen sheets.

1.3 Review of Literature

PROTEOGLYCANS

Proteoglycans (PGs) are a family of complex macromolecules distinguished by the covalent attachment of one or more glycosaminoglycan (GAG) chain(s) (N- and /or O- linked oligosaccharides) with matrix proteins (core proteins) (shown in Appendix 1.1). They are strategically located at the cell surface and in the extracellular matrix or ground substance of all multicellular organisms and thereby are involved in a wide range of biological processes (Iozzo, 1998; Esko and Lindahl, 2001). They are associated with each other and also with other major structural components of the matrix, *viz.,* collagen and elastin and play important roles in determining the structural organization of the matrix and in fundamental biological processes such as cell growth and development (Esko and Zhang, 1996; Esko and Selleck, 2002; Kramer and Yost, 2003; Lin, 2004; Hacker *et al.*, 2005; Bulow and Hobert, 2006). Although originally named and categorized on the basis of the GAG substituent, they are increasingly being viewed as products of gene families that encode the different core proteins.

Proteoglycan core proteins are indispensable for the correct development of almost all metazoan organisms that have been studied. The core proteins of the known proteoglycans vary in size from 11,000 to about 220,000 daltons (Doege *et al*., 1987). The number of glycosaminoglycan chains attached to the core varies from one (Glossl *et al.,* 1984) to about 100 (Roden *et al.,* 1985). The GAG chains consist of unbranched, repeated disaccharides that are modified by sulphation and epimerization during synthesis in the golgi apparatus. The classification into core proteins that carry KS (keratan sulphate), CS (chondroitin sulphate), DS (dermatan sulphate), or HS (heparin sulphate) is not always strict because there are a considerable number of proteins that bear more than one kind of GAG side chain.

In addition to their structural functions, proteoglycans and GAGs play different roles in diverse processes such as enzyme regulation and cellular adhesion, growth, migration or differentiation (Selleck, 2000; Turnbull *et al.,* 2001). These processes are mediated by their binding capacities and focal

sequestering of a number of biologically active proteins (David and Bernfield, 1998). GAGs and their core proteins have important physiological and homeostatic roles, for e.g. during inflammation and immune response (Wang *et al*., 2005), as well as a number of other roles like anticoagulant activity, anti–atherosclerotic activity, as fat clearing agents and also as binding substrates for infectious agents. Proteoglycans function also as tissue organizers, influence cell growth and the maturation of specialized tissues, play a role as biological filters and modulate growth-factor activities, regulate collagen fibrillogenesis and skin tensile strength, affect tumor cell growth and invasion, and influence corneal transparency and neurite outgrowth (Iozzo, 2001; Yamada *et al*., 2009). The involvement of GAGs/PGs in oncogenesis is particularly intriguing since changes in matrix composition of these molecules are a hallmark of several tumors and tumor-derived cell lines (Iozzo, 1997). Additional roles, derived from studies of mutant animals, indicate that certain proteoglycans are essential to life whereas others might be redundant (Toyoda *et al.,* 2000b; Ackley *et al.,* 2001; Minniti *et al.,* 2004).

GLYCOSAMINOGLYCANS: General Aspects

Structurally the simplest and best known of the heteropolysaccharides are the glycosaminoglycans (GAGs). They are multifunctional polysaccharides composed of repeating disaccharide units. Variations are imposed on the disaccharide repeats by post-synthetic modifications in the form of sulphation and epimerization. These modifications often dictate the protein recognition properties and biological activities of GAGs. The disaccharide repeating units of the mucopolysaccharides are similar in structure but varied in composition.

Based on the disaccharide composition, linkage type and on the presence of sulphate groups, GAGs have been divided into six main groups: (1) hyaluronic acid (HA), (2) chondroitin sulphate (CS), (3) dermatan sulphate (DS), (4) heparin, (5) heparan sulphate (HS), and (6) keratan sulphate (KS). Most sulphated GAG chains are covalently linked to core proteins to form proteoglycans. Hyaluronan shows no modifications and is not attached to a protein backbone (Spicer *et al.,*

2002; Toole, 2004). The amount of carbohydrate in a proteoglycan is usually much greater than found in a glycoprotein and may comprise upto 95 % of its weight. The GAGs present in the proteoglycans are highly negatively charged because of the presence of carboxyl or sulphate groups on many of the sugar residues and hence binds polycations and cations such as sodium or potassium. Modifications of sugar residues create enormous molecular diversity, and GAGs have been suggested to be the most information-dense biological molecules (Turnbull *et al.,* 2001). For example, a mere octasaccharide allows for more than one million theoretical combinations of modifcation patterns. Because the GAG side chains found *in vivo* are usually 50–150 disaccharide repeat units long, the combinatorial possibilities are rendered nearly boundless (Esko and Lindahl, 2001; Esko and Selleck, 2002).

The biosynthesis of GAGs generates significant diversity in the primary sequence of GAGs as defined by the chemical structure of the polysaccharide backbone and the sulphation patterns. The GAG attachment sites on the core protein of the PG have a consensus Ser-Gly/Ala-X-Gly motif, where chain initiation begins from serine. Heparan sulphate GAGs and chondroitin sulphate GAGs are synthesized in golgi apparatus, where the individual GAG chains are *O*-linked to a core protein, forming a proteoglycan (PG) (Sugahara and Kitagawa, 2002; Silbert and Sugumaran, 2002). Keratan sulphate, on the other hand, can be either *N*-linked or *O*-linked to the core protein of the PG (Funderburgh, 2002). Hyaluronic acid is not synthesized in the golgi but rather by an integral plasma membrane synthase, which secretes the nascent chain immediately (Itano and Kimata, 2002). The biosynthesis of GAGs is non template driven and involves several enzymes and their tissue-specific isoforms (Kusche-Gullberg and Kjellen, 2003). The complex biosynthesis and lack of proof reading machinery lead to inherent heterogeneity and large diversity of GAG structures. Understanding the functions of such diversity in glycosaminoglycans is one of the major goals of glycobiology.

GAGs such as hyaluronic acid, heparin, and chondroitin sulphate have several applications in the medical, veterinary, pharmaceutical and cosmetic field

because of their outstanding properties. The structure, biosynthesis, functional significance and applications of diverse GAGs with emphasize to studies on higher vertebrates are mentioned in the following sections.

1.3.1 Hyaluronic acid

Hyaluronic acid (also called Hyaluronan or hyaluronate) is an anionic, non-sulphated glycosaminoglycan distributed widely throughout connective, epithelial, and neural tissues. It is a biodegradable, biocompatible, non-immunogenic and linear polysaccharide and is abundant in synovial fluid and extracellular matrix (ECM) (Knudson and Knudson, 1993). It is unique among glycosaminoglycans in that it is unsulphated, not attached to core proteins, forms in the plasma membrane instead of the golgi, and has a wide molecular weight (MW) range from 1000 Da to 10,000,000 Da (Fraser *et al.,* 1997). Being one of the chief components of the extracellular matrix, hyaluronan contributes significantly to cell proliferation and migration, and may also be involved in the progression of some malignant tumors. The average 70 kg (154 lbs) person has roughly 15 grams of hyaluronan in their body, one-third of which is turned over (degraded and synthesized) every day (Stern, 2004).

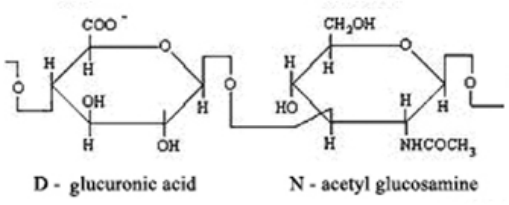
Hyaluronic acid is derived from the term *hyalos* (Greek for vitreous) and uronic acid, because it was first isolated from the vitreous humour and possesses a high uronic acid content. The term *hyaluronate* refers to the conjugate base of hyaluronic acid. Because the molecule typically exists *in vivo* in its polyanionic form, it is most commonly referred to as *hyaluronan*.

Hyaluronic acid was isolated from the vitreous humor of bovine eyes (Meyer and Palmer, 1934) and later from umbilical cord and many other sources such as rooster comb. In mammals, HA is abundant in heart valves, skin, vitreous of the eye, umbilical cord, synovial fluid and skeletal tissues, (Fraser *et al*., 1997). A significant property of HA is its capacity to bind huge amounts of water (1000-fold of its own weight) by virtue of its high number of negative charges along the molecule. Therefore, HA so-called as "biological glue" holds together gel-like connective tissue leading to a considerable water binding capacity and can serve as

a space filler, and functions as a biological lubricant in joints, reducing friction during movement and providing resiliency under static conditions (Laurent and Fraser, 1992).

1.3.1.1 Structure

The chemical structure of hyaluronan (Scheme 1.1) was determined in the 1950s in the laboratory of Karl Meyer. Hyaluronan is a polymer of disaccharides, composed of D-glucuronic acid and D-N-acetylglucosamine, linked together *via* alternating β-1,4 and β-1,3 glycosidic bonds (Weissman and Meyer, 1954). The number of this type of disaccharide units varies from hundreds to tens of thousands resulting in a molecular mass of millions of daltons for one hyaluronan molecule *in vivo*. The average molecular weight in human synovial fluid is 3−4 million Da, and hyaluronan purified from human umbilical cord is 3,140,000 Da (Saari *et al.,* 1993).



Scheme 1.1: Structure of hyaluronic acid disaccharide unit

1.3.1.2 Biosynthesis

HA is synthesized by membrane-bound synthases (HASs) at the inner surface of the plasma membrane, and the chains are extruded through pore-like structures into the extracellular space (Itano and Kimata, 2002). Vertebrates have three types of hyaluronan synthases: HAS1, HAS2, and HAS3. These enzymes lengthen hyaluronan by repeatedly adding glucuronic acid and N-acetylglucosamine to the nascent polysaccharide as it is extruded through the cell membrane into the extracellular space (Kakizaki *et al*., 2004). The structure of the polymerization product is [(1 → 4)-, β - D-glucuronosyl-(1→3)-, β -D-N-acetylglucosaminyl]$_n$. No further modification takes place.

1.3.1.3 Functions

HA has been known to play different roles in the body depending on its molecular weight. Hyaluronan binds to extracellular proteins, proteoglycans and

cell surface receptors. Hyaluronan is an important component of articular cartilage, where it is present as a coat around each cell (chondrocyte). When aggrecan monomers bind to hyaluronan in the presence of link protein, highly negatively-charged large aggregates form. These aggregates imbibe water and are responsible for the resilience of cartilage i.e., its resistance to compression (Papakonstantinou, 1998; Sugahara *et al.,* 2003; Seyfried *et al.,* 2005; Stern *et al.,* 2006; Stern, 2007). HA is likely to play a multifaceted role in mediation of these cellular and matrix events. Tammi *et al.* (1989) found that HA content increases in the presence of retinoic acid. The high molecular weight HA, which typically occurs in loose connective tissues, has a role in maintaining cell integrity and water content in the extracellular matrix by degrading itself to small molecules through several metabolic pathways thereby maintaining the homeostasis. Increased HA concentration in blood circulation occurs during disturbed cell behaviors such as inflammatory response, immune response, and tumor-genesis (Toole, 2004). Degraded fragments like oligosaccharide HA, are known to induce angiogenic signals in endothelial cells (Prevo *et al.,* 2001). Termeer and colleagues (2000) demonstrated that HA oligomers of tetra- and hexasaccharide size induced immunophenotypic maturation of human monocyte-derived dendritic cells.

Hyaluronan is actively synthesized during embryonic development and in tissue repair and regeneration processes, and contributes to cell growth, migration, and differentiation (Laurent & Fraser, 1992; Noble, 2002). Hyaluronan is also a major component of skin, where it is involved in tissue repair (Averbeck *et al.,* 2007; Stern, 2007). HA is abundant in the cumulus layer and zona pellucid of most mammalian oocytes, and gets hydrolyzed by the hyaluronidase enzyme located on the sperm surface and inner acrosomal membrane during most mammalian fertilization events, thus enabling conception (Cherr, *et al.,* 1996). HA covalently binds to a variety of proteins HABPs (hyaluronic acid binding proteins), also referred to as hyaladherins (Toole, 1990) and functions in growth, development, inflammation, and immune responses.

1.3.1.4 Medical applications

The first hyaluronan biomedical product, *Healon,* was developed in the 1970s by Pharmacia, Co. Ltd and is approved for use in eye surgery like corneal transplantation, cataract surgery, glaucoma surgery and surgery to repair retinal detachment. The FDA approved *Healon* as a surgical material in 1980. Hyaluronan may also be used postoperatively to induce tissue healing, notably after cataract surgery (Santos *et al.,* 1994). By the year 2009, an estimated quarter of a billion patients had benefited from the useful properties of *Healon* in eye surgery. Hyaluronan is also used to treat osteoarthritis of the knee (Puhl and Scharf, 1997). Such treatments, called *viscosupplementation*, are administered as a course of injections into the knee joint and are believed to supplement the viscosity of the joint fluid, thereby lubricating the joint, cushioning the joint, and producing an analgesic effect. It has also been suggested that hyaluronan has positive biochemical effects on cartilage cells. However, some placebo controlled studies have cast doubt on the efficacy of hyaluronan injections, and hyaluronan is recommended primarily as a last alternative to surgery. Oral use of hyaluronan has been lately suggested, although its effectiveness needs to be demonstrated (Price *et al.,* 2007). Hyaluronan is often used as a tumor marker for prostate and breast cancer. It may also be used to monitor the progression of the disease. Hyaluronan synthesis has been shown to be inhibited by 4-Methylumbelliferone, a 7-Hydroxy-4-methylcoumarin derivative (Yoshihara *et al.,* 2005). This selective inhibition (without inhibiting other glycosaminoglycans) may prove useful in preventing metastasis of malignant tumor cells.

1. 3.1. 5 Other applications

Hyaluronan is a common ingredient in skin care products. Dry, scaly skin (xerosis) caused by atopic dermatitis (eczema) may be treated using skin lotion containing sodium hyaluronate as its active ingredient. In 2003 the FDA approved hyaluronan injections for filling soft tissue defects such as facial wrinkles. *Restylane* is a common trade name for the product. Hyaluronan injections temporarily smooth wrinkles by adding volume under the skin, with effects typically lasting for six months.

Due to its high biocompatibility and its common presence in the extracellular matrix of tissues, hyaluronan is gaining popularity as a biomaterial scaffold in tissue engineering research by crosslinking, producing a hydrogel. Hyaluronan can be crosslinked by attaching thiols (trade names: Extracel, HyStem) (Shu *et al.,* 2004b); methacrylates, (Gerecht *et al.,* 2007) and tyramines (trade name: Corgel) (Dar and Calabro, 2009). Hyaluronan can also be crosslinked directly with formaldehyde (trade name: Hylan-A) or with divinyl sulphone (trade name: Hylan-B). Hyaluronan has also been used in the synthesis of biological scaffolds for wound healing applications. These scaffolds typically have proteins such as fibronectin attached to the hyaluronan to facilitate cell migration into the wound. This is particularly important for individuals with diabetes who suffer from chronic wounds (Shu *et al.,* 2004 a).

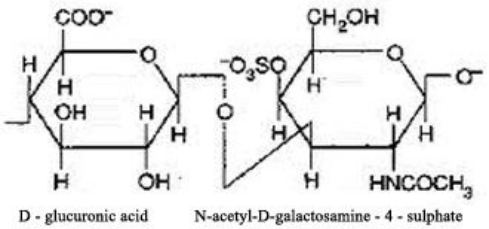
1.3.2 Chondroitin sulphate

Chondroitin sulphate (CS) is a major component of extracellular matrix, and is important in maintaining the structural integrity of the tissue. As an important structural component of cartilage it provides much of its resistance to compression (Baeurle *et al.,* 2009). Glucosamine and chondroitin sulphate products act as valuable symptomatic therapies for the treatment of knee and hip osteoarthritis with some potential structure-modifying effects (Zhang and Moskowitz, 2007; Bruyere and Reginster, 2007).

1.3.2.1 Structure

Chondroitin sulphate chains are unbranched polysaccharides of variable length containing two alternating monosaccharides: D-glucuronic acid (GlcA) and sulphated N-acetyl-D-galactosamine (GalNAc) (Scheme 1.2). Some GlcA residues are epimerized into L-iduronic acid (IdoA) and the resulting disaccharideis then referred to as *Dermatan sulphate*. "Chondroitin sulphate B" is an old name for dermatan sulphate, and is no longer classified as a form of chondroitin sulphate. Chondroitin sulphates are classified as CS-O, A, C, D, E, F, H, K (CS-O, chondroitin,[GlcAβ1-3GalNAc]; CS-A, chondroitin sulphate A,[GlcAβ1-3GalNAc(4 S)]; CSC, chondroitin sulphate C, [GlcAβ1-3GalNAc(6 S)]; CS-D,

chondroitin sulphate D,[GlcA(2S)β1-3GalNAc(6 S)]; CS-E, chondroitin sulphate E, ,[GlcAβ1-3GalNAc(4 S,6 S)]; CS-F, a fucosylated chondroitin sulphate, [GlcA (α1-3Fuc) β1-3Gal-NAc (4 S)] (Schiraldi *et al.,* 2010), CS-H [a dematan polysulphate from hagfish notochord (Anno *et al.,* 1971)] and CS–K [an unusual penta saccharide containing chondroitin sulphate from King crab cartilage (Kitagawa *et al.,* 1997)] according to their sulphation pattern mediated by specific sulphotransferases. Sulphation in these different positions confers specific biological activities to chondroitin GAG chains. Nakagawa *et al.* (2007) reported on the occurrence of a nonsulphated chondroitin proteoglycan in the dried saliva obtained from the 'edible nest' of *Collocalia* swiftlets birds. CSs extracted from animal sources (human, pig, shark, squid) are often a combination of the different types. CSs are abundant and widely distributed in humans, other mammals and invertebrates reflecting a central role in biological processes. They may function as regulators of growth factors, cytokines, chemokines, lipoproteins and adhesion molecules through interactions with the ligands of these proteins *via* specific saccharidic domains (Malavaki *et al.,* 2008).



Scheme 1.2: Structure of chondroitin sulphate (CS-A) disaccharide unit

Although the name "chondroitin sulphate" suggests a salt with a sulphate counter-anion, this is not the case, as sulphate is covalently attached to the sugar. Rather, since the molecule has multiple negative charges at physiological pH, a cation is present in salts of chondroitin sulphate. Commercial preparations of chondroitin sulphate typically are the sodium salt. Barnhill *et al.* (2006) have suggested that all such preparations of chondroitin sulphate be referred to as "sodium chondroitin" regardless of their sulphation status.

1.3.2.2 Biosynthesis

Chondroitin sulphate chains are linked to hydroxyl groups on serine residues of certain proteins. Glycosylated serines are often followed by a glycine

and have neighboring acidic residues. The structure of the polymerization product, chondroitin, is [(1→ 4)-, β -D-glucuronosyl- (1→3)-, β -D-N-acetylgalactosaminyl]n. This polymer is sulphated by sulphotransferases that utilize 3'-phosphoadenylylsulphate (PAPS) as sulphate donor and catalyze the formation of ester sulphate (O-sulphate) groups at C4 and C6 of the N-acetylgalactosamine residues. The sulphated product usually contains about one sulphate group per disaccharide residue although considerable deviations from a 1:1 ratio have been noted. Individual chondroitin sulphate chains may contain sulphate groups in both 4- and 6-positions but may also be exclusively 4-sulphated or 6-sulphated (Kusche-Gullberg and Kjellen, 2003).

Attachment of the GAG chain begins with a tetrasaccharide linker in a fixed pattern: Xyl-Gal-Gal-GlcA. Specific enzymes, namely xylosyl transferase, β4-galactosyl transferase (GalTI), β3-galactosyl transferase (GalT-II), and β3-GlcA transferase (GlcAT-I) transfer the four monosaccharides, respectively. The first modification involves the transfer of a GalNAc (CS-GAG) monosaccharide to the linker region by a GalNAcT. In the case of CS-GAGs, chondroitin synthase, which is a multidomain enzyme, transfers GalNAc and GlcA successively for chain elongation. The three main sulphation events of the CS-GAG chains are 4-*O* and/or 6-*O*-sulphation of GalNAc and 2-*O*-sulfation of uronic acid. Xylose begins to be attached to proteins in the endoplasmic reticulum, while the rest of the sugars are attached in the golgi apparatus (Silbert and Sugumaran, 2002).

1.3.2.3 Functions

CS has been isolated from various tissues obtained from a large number of animal species including both vertebrates and invertebrates (Theocharis *et al.,* 1999; Pinto *et al.,* 2004; Bao *et al.,* 2005; Sugahara and Mikami, 2007; Yamada *et al.,* 2007). Chondroitin's functions depend largely on the properties of the overall proteoglycan of which it is a part and can be broadly divided into structural and regulatory functions. Structural function is typical of the large aggregating proteoglycans: aggrecan, versican, brevican, and neurocan, collectively termed as the lecticans. The tightly packed and highly charged sulphate groups of chondroitin

sulphate generate electrostatic repulsion that provides much of the resistance of cartilage to compression (Yamaguchi, 1996). Loss of chondroitin sulphate from the cartilage is a major cause of osteoarthritis (Michel *et al.,* 2005). The lecticans are a major part of the brain extracellular matix, where the chondroitin sugar chains function to stabilize normal brain synapses as part of perineuronal nets. The levels of chondroitin sulphate proteoglycans are vastly increased after injury to the central nervous system where they act to prevent regeneration of damaged nerve endings (Fraser *et al.,* 2001).

Oversulphated CS-D and CS-E are involved in neuroregulatory functions (Ueoka *et al.,* 2000; Rolls *et al.,* 2004) and the binding of growth factors in mammalian systems (Deepa *et al.,* 2002). Chondroitinase ABC (cABC), which depolymerizes CS-GAGs, has been shown to promote functional recovery following central nervous system injury in adult rats (Bradbury *et al.,* 2002). A similar set of GAG-mediated neuronal path finding is evident in the visual system during development (Ichijo and Kawabata, 2001; Inatani and Tanihara, 2002). Studies in zebrafish suggest that regrowing retinal axons are excluded from some midbrain nuclei *via* a CS barrier (Carulli *et al.,* 2005).

Cartilage degradation manifests in osteoarthritis, a prevalent degenerative joint disease. Differences in CS-GAG sulphation profiles have been observed in arthritic tissues. Particularly striking is a decrease in the abundance of 6-*O*-sulphated disaccharides accompanying an increase in 4-*O*-sulphated CS disaccharides (Plaas *et al.,* 1998; West *et al.,* 1999; Sauerland *et al.,* 2003). In addition, chondroitin sulphate has recently shown a positive effect on osteoarthritic structural changes occurred in the subchondral bone (Kwan *et al.,* 2007). PG core proteins interact with collagen fibrils, and CS side chains provide electrostatic forces, enabling joints to function amid dynamic compressive, shear, and tensile forces (Ragan *et al.,* 2000; Bathe *et al.,* 2005).

1.3.2.4 Medical applications

The main field of application of CS is the treatment of osteoarthritis and also for its anti-inflammatory action. Chondroitin along with glucosamine, is used

as an alternative medicine for treatment of osteoarthritis and in fact it is approved and currently recommended by the European League Against Rheumatism (EULAR) in the treatment of knee and hip osteoarthritis, as a symptomatic slow-acting drug for this disease in Europe and some other countries (Jordan and Arden, 2003) and also by the U.S. Food and Drug Administration as a dietary supplement (Baeurle *et al.,* 2009). Chondroitin and glucosamine are also used in veterinary medicine (Forsyth *et al.,* 2006). CS is therefore regarded as a nutraceutical, a nutritional supplement with proven efficacy and pharmaceutical properties.

Both *in vitro* and *in vivo* studies have shown that chondro-protective property of CS results from an increase in the biosynthesis of connective tissue components (collagen, proteoglycans, and hyaluronan) and an increase of the viscosity of the synovial fluid at disease sites (Belcher *et al.,* 1997; McCarty *et al.,* 2000). Low-molecular weight CS is, in fact, applied for the treatment and prevention of osteoarthritis because it competitively inhibits some cartilage degradative enzymes. Chondroitin sulphate had been thought to decrease pain, improve functional disability, reduce NSAID (non steroid anti inflammatory drug) or acetaminophen consumption, and provide good tolerability with an additional carry-over effect (Uebelhart *et al.,* 1998; McAlindon *et al.,* 2000).

Pharmacokinetic studies performed on humans and experimental animals after oral administration of chondroitin sulphate revealed that it can be absorbed orally. Orally administred Condrosulf® was studied for its bioavailability and pharmaco kinetics by Conte and coauthors (Conte *et al.,* 1991). Several studies have been published on the biochemical activities of orally administered CS and was found bioavailable in dogs (Adebowale *et al.,* 2002), in horses (Du *et al.,* 2004) and in human (Volpi, 2002).

1.3.2.5 Other applications

Novel documented areas of interest for other potential use of CS are as antiviral, anti-infective and in tissue regeneration and engineering. It was recently shown that CS chains containing high amounts of oversulphated E units, [CS-E, GlcAβ1-3GalNAc (4, 6-*O*-disulphate)] are potent inhibitors of Herpes simplex

virus infections (Yamada and Sugahara, 2008). A potential target for the development of novel vaccines against maternal malaria was discovered by Alkhalil and co-workers (2000). CS based hydrogel preparations also proved to shorten the wound healing process. Gilbert *et al.* (2004) proved on animal models that CS-hydrogels accelerate wound-healing in the sinonasal mucosa. CS-C was found to improve palatal wound healing by regulating cell adhesion, cell proliferation, and cell migration (Zou *et al.,* 2004). Another research report describes the application of bi-layer gelatin –chondrointin-6-sulphate – hyaluronic acid (gelatin–C6S–HA) biomatrices in tissue engineered skin substitutes. Results demonstrate the feasibility of such scaffolds to overcome the shortage of skin autograft by culturing keratinocytes and dermal fibroblasts *in vitro* (Wang *et al.,* 2006). This bi-layer gelatin–C6S–HA skin substitute not only promotes wound healing, but also has a high rate of graft uptake. A proposed application regards the treatment of the extensively and profoundly burned patients who suffer from severe skin defects.

Highly sulphated CS/DS were found to bind the heparin binding growth factor, which has a key role in hepatocytes progression during liver regeneration (Mitchell *et al.,* 2005). Exogenous administration of either CS/DS or of their functional domains might be useful for the development of therapeutics for liver regeneration by regulating the physiology of hepatic tissues (Toyoki *et al.,* 1997; Yamada and Sugahara, 2008). Sulphated monosaccharides and disaccharides were shown to be effective inhibitors of GAG-induced amyloid fibre formation (Fraser *et al.,* 2001).

A biological function in cancerous cells and tissues was also established and therefore applications of CS as biomarker are foreseen. Pothacharoen *et al.* (2006) determined the value of serum chondroitin sulphate epitope WF6 for early detection of ovarian epithelial cancer. In addition, it was recently demonstrated by Sakko *et al.* (2008) that advanced stage prostrate cancer tissues exhibited higher levels of CS-O in comparison to benign prostatic hyperplasia tissues, whereas sulphated disaccharide levels were unaffected when analyzed for their

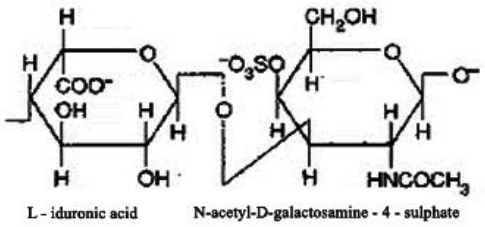
electrophoretic mobility.

I.3.3 Dermatan Sulphate

Dermatan sulphate (formerly named as chondroitin sulphate B or β-heparin) was first isolated from pig skin by Meyer and Chaffee (1941). Dermatan sulphate is the predominant GAG expressed in the skin and makes up as much as 0.3% of its dry weight. DS is also present in blood vessels, heart valves, tendons and lungs. Emerging evidence outlined later suggested that DS, like heparin and HS, serves a variety of roles. DS adds flexibility to many normal and pathological responses, such as development, growth, wound repair, infection and tumorigenesis. Dermatan sulphate derives its name from the dermis, its primary source.

1.3.3.1 Structure

Dermatan sulphate (DS) is composed of linear polysaccharides assembled as disaccharide units containing N-acetyl galactosamine (GalNAc) and iduronic acid (IdoA) joined by β-1, 4 and β-1, 3 linkages respectively (Scheme 1.3).



Scheme 1.3: Structure of dermatan sulphate disaccharide unit

DS was formerly defined as a chondroitin sulphate due to the presence of GaINAC. The presence of iduronic acid (IdoA) in DS distinguishes it from chondroitin sulphates-A (4-0-sulphated) and -C (6-0-sulphated) and likens it to heparin and HS, which also contain this residue. It is no longer classified as a form of chondroitin sulphate by most sources (Trowbridge and Gallo, 2002). IdoA appears to play a key role in binding site specificity for GAG-binding proteins. A clue to the importance of IdoA residues for biological effects has been suggested by data showing that GAG chains containing high amounts of IdoA inhibit the proliferation of normal fibroblasts more than GAGs with high GIcA content (Westergren-Thorsson *et al.,* 1991). DS is attached covalently *via* an O-xylose linkage to serine residues of core proteins to form DS-PGs.

1.3.3.2. Biosynthesis

The polymerization product is presumably identical to that formed during biosynthesis of chondroitin sulphate. However, the modification of this polymer is not restricted to *O*-sulphation reactions but includes also formation of L-iduronic acid residues by C5-epimerization of *O*-glucuronic acid units. Considerable structural variability has been demonstrated. The *O*-glucuronic acid content may thus range from negligible amounts to more than 90% of the total uronic acid. Some preparations have a comparatively simple structure. For example, pig skin dermatan sulphate consists, essentially, of iduronosyl – N – acetylgalactosaminyl – 4 - sulphate disaccharide units. However, others are extremely complex with extensively hybridised polysaccharide backbone and, in addition, both 4- and 6-0-sulphated galactosamine residues. Oversulphated regions may be due to the occurrence of sulphated L-iduronic acid units, whereas sulphated *O*-glucuronic acid has not been detected (Silbert and Sugumaran, 2002; Kusche-Gullberg and Kjellen, 2003).

1.3.3.3 Functions

Dermatan sulphate may have roles in coagulation, cardiovascular disease, carcinogenesis, infection, wound repair, fibrosis, and stimulation of cell growth (Petaja *et al.,* 1997; Fernandez *et al.,* 1999; Denholm *et al.,* 2000). One particularly well-studied DS binding interaction occurs with heparin cofactor II (HCII) (Maimone and Tollefsen, 1990), a serpin homolog of antithrombin which acts by inhibiting the procoagulative effect of thrombin, which is enhanced 1000-fold in the presence of DS or Heparin. It is postulated that the GAGs forms a stable ternary complex between the serpin and the protease (Liaw *et al.,* 2001). DS may also influence coagulation by enhancing the effects of activated protein C (APC), as in the case of heparin (Petaja *et al.,* 1997) as well as HS, CS-A, and CS-C. DS oligosaccharide size and sulphation determine binding affinity and lead to different physiological consequences (Fernandez *et al.,* 1999).

Dermatan sulphate accumulates abnormally in several of the

mucopolysaccharidosis disorders. An excess of dermatan sulphate in the mitral valve is characteristic of myxomatous degeneration of the leaflets leading to redundancy of valve tissue and ultimately, mitral valve prolapse (into the left atrium) and insufficiency. DS is released at high concentrations during wound repair and makes it a particularly interesting topic for evaluation. DS derived from wounds activates endothelial leukocyte adhesion through stimulation of ICAM-1 (Inter cellular adhesion molecule 1) and wound DSPGs are a potent promoter of the activity of FGF-2, (Fibroblast growth factor -2) a growth factor important for several aspects of the repair response (Penc *et al.,* 1999). Finally, a close relation between innate immune defense of wounds and PG synthesis has been shown. Antimicrobial peptides induced by injury protect against bacterial invasion (Nizet *et al.,* 2001) but can also contribute to the induction of syndecans (Gallo *et al.,* 1994).

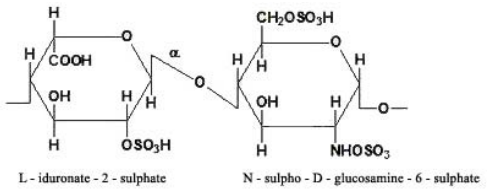
1.3.4 Heparin

Heparin is a naturally-occurring anticoagulant produced by basophils and mast cells. Heparin is known as a highly-sulphated biologically active glycosaminoglycan and has the highest negative charge density of any known biological molecule (Linhardt, 1991) and is well known for its ability to prevent the coagulation of blood (Bjork and Lindahl, 1982). Heparin is one of the oldest drugs still in widespread clinical use. Its discovery can be attributed to the research activities of two scientists, Jay McLean and William Henry Howell in 1916 (Copeland and Six, 2009), although it did not enter clinical trials until 1935 (Linhardt, 1991). It is widely used as an injectable anticoagulant for over half a century, and can also be used to form an inner anticoagulant surface on various experimental and medical devices such as test tubes and renal dialysis machines. Pharmaceutical grade heparin is derived from mucosal tissues of slaughtered meat animals such as porcine intestine or bovine lung (Linhardt and Gunay, 1999).

1.3.4.1 Structure

The term ‘Heparin’ is derived from *hepar* (Greek for "liver"), because it was originally isolated from canine liver cells. Heparin has also been extracted and

characterised in humans (Linhardt *et al.,* 1992) and other veretebrates *viz*., whale(Ototani *et al.,* 1981), mouse (Bland *et al.,* 1982), turkey (Warda *et al.,* 2003a) and dromedary camel (Warda *et al.,* 2003 b). Native heparin is a polymer with a molecular weight ranging from 3 kDa to 50 kDa, although the average molecular weight of most commercial heparin preparations is in the range of 12 kDa to 15 kDa. The most common disaccharide unit is composed of a 2-O-sulphated iduronic acid and 6-O-sulphated, N-sulphated glucosamine, IdoA(2S)-GlcNS(6S) (Scheme 1.4). Rare disaccharides containing a 3-O-sulphated glucosamine [(GlcNS(3S,6S)] or a free amine group ($GlcNH_3^+$) are also present. Under physiological conditions, the ester and amide sulphate groups are deprotonated and attract positively-charged counter ions to form a heparin salt. It is in this form that heparin is usually administered as an anticoagulant (Bjork and Lindahl, 1982).



Scheme 1.4: Structure of heparin disaccharide unit

One unit of heparin (the "Howell Unit") is an amount approximately equivalent to 0.002 mg of pure heparin, which is the quantity required to keep 1.0 ml of cat's blood fluid for 24 hours at 0°C.

1.3.4.2 Biosynthesis

The structure of the polymerization product that serves as intermediate in the biosynthesis of heparin and, presumably, heparan sulphate, is $[(1\rightarrow4)$-, β -D-glucuronosyl-$(1\rightarrow 4)$-α -D-N-acetylglucosaminyl$]_n$. Polymer-modification reactions resembes the biosynthesis of dermatan sulphate and in addition, two reactions, deacetylation of N-acetyl-*O*-glucosamine residues, followed by sulphation of the resulting free amino groups, appear to be unique to the formation of heparin-like polysaccharides (Sugahara and Kitagawa, 2002). The L-iduronic acid: D-glucuronic acid ratio generally increases with increasing sulphate content, therefore in broad terms low-sulphated, *O*-glucuronic acid rich polysaccharides are classified as heparan sulphate whereas high-sulphated, L-iduronic acid rich species

are designated heparin. Hence from a structural view point both polymers are considered as members of the same family of heparin-like polysaccharides.

1.3.4.3 Functions

Although used principally in medicine for anticoagulation, the true physiological role in the body remains unclear, because blood anti-coagulation is achieved mostly by endothelial cell-derived heparan sulphate proteoglycans (Kojima *et al.,* 1992). Originally heparin was thought to be produced exclusively by connective tissue mast cells, as the polysaccharide component of the intracellular proteoglycan serglycin, but it has also been discovered as a component of cell surface proteoglycans (Stringer *et al.,* 1999). Heparin is usually stored within the secretory granules of mast cells and released only into the vasculature at sites of tissue injury. It has been proposed that, rather than anticoagulation, the main purpose of heparin is in a defensive mechanism at sites of tissue injury against invading bacteria and other foreign materials (Nader *et al.,* 1999). This natural polymer also has antiviral activity, binds to a variety of growth factors, inhibits complement activation, and regulates angiogenic activity (Folkman *et al.,* 1983; Weiler *et al.,* 1992). In addition, it is conserved across a number of widely different species, including some invertebrates that do not have a similar blood coagulation system.

1.3.4.4 Medical applications

Heparin acts as an anticoagulant, preventing the formation of clots and extension of existing clots within the blood. While heparin does not break down clots that have already formed (unlike tissue plasminogen activator), it allows the body's natural clot lysis mechanisms to work normally to break down clots that have formed. Heparin has the advantage over EDTA of not affecting levels of most ions. Heparin and its derivatives are used for anticoagulation for conditions like acute coronary syndrome, pulmonary embolism, atrial fibrillation and deep-vein thrombosis (Agnelli *et al.,* 1998; Bergqvist *et al.,* 2002).

Heparin gel (topical) may sometimes be used to treat sports injuries. It is known that the diprotonated form of histamine binds site specifically to heparin

(Chuang *et al.,* 2000). The release of histamine from mast cells at a site of tissue injury contributes to an inflammatory response. The rationale behind the use of such topical gels may be to block the activity of released histamine, and so help to reduce inflammation.

1.3.4.5 Other applications

Heparin-coated blood oxygenators are available for use in heart-lung machines (Edmunds, 2004). These specialized oxygenators are thought to improve overall biocompatibility and host homeostasis by providing characteristics similar to native endothelium. Lithium salt of heparin (lithium heparin) or sodium salt of heparin (sodium heparin) is used as an anticoagulant on various experimental and medical devices such as test tubes, stents, vacutainers, and capillary tubes; and renal dialysis machines (Fukutomi *et al.,* 1996; Sylvie *et al.,* 2003). Heparin-coated intraocular lens is currently in use to prevent complications following cataract surgery with intraocular lens implantation. Modification of the surface of the intraocular lens with a layer of heparin may provide a more biocompatible surface.

Immobilized heparin can be used as an affinity ligand in protein purification (Banyard *et al*., 2003). The format of immobilized heparin can vary widely from coated plastic surfaces for diagnostic purpose to chromatography resin. The third use for immobilized heparin is group-specific purification of RNA and DNA binding proteins such as transcription factors and/or virus coat proteins (Astrid and Jean-Pierre, 2005). Heparin has been successful in fabrication of organic microspheres/ nanostructures constructed from organic NP (nanoparticle) clusters. Heparin/poly(l-lysine) nanoparticles coated onto PLGA (Poly-lactide-co-glycolide) microsphere surfaces were prepared by Na *et al.* (2007) as cell delivery vehicles of human mesenchymal stem cells (hMSCs) adding in osteogenic differentiation.

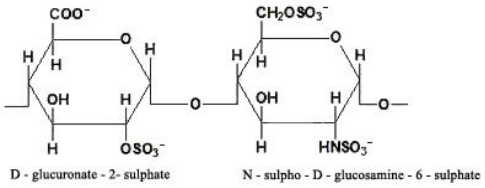
1.3.5 Heparan sulphate

Heparan sulphate (HS) is a member of the glycosaminoglycan family and is very closely related in structure to heparin. It is a linear polysaccharide found in all animal tissues and comprises 50% to 90% of the total endothelial proteoglycans

attached in close proximity to cell surface or extracellular matrix proteins (Iozzo, 1998). In addition to sequence diversity, its size ranges from 5–70 kDa (Lindblom *et al.,* 1991). Although it is initially produced in a cell-surface-bound form, it can also be shed as a soluble GAG, a process which completely transforms its function. HS binds to a variety of protein ligands and regulates a wide variety of biological activities (Andres *et al.,* 1992).

1.3.5.1 Structure

The most common disaccharide unit within heparan sulphate is composed of a glucuronic acid (GlcA) linked to *N*-acetylglucosamine (GlcNAc) (Scheme 1.5). Rare disaccharides containing a 3-O - sulphated glucosamine (GlcNS (3S, 6S) or free amine groups ($GlcNH_3^+$) are also present. Under physiological conditions the ester and amide sulphate groups are deprotonated and attract positively charged counter ions to form a salt. It is in this form that HS is thought to exist at the cell surface.



Scheme 1.5: Structure of heparan sulphate disaccharide unit

1.3.5.2 Biosynthesis

Many different cell types produce HS chains with many different primary structures and hence there is a great deal of variability in the way HS chains are synthesised. Regardless of primary sequence a range of multiple glycosyltransferases, sulphotransferases and an epimerase are essential to the formation of HS. Many of these enzymes have now been purified, molecularly cloned and their expression patterns studied. From the studies on the fundamental stages of HS/heparin biosynthesis, Lindahl *et al.* (1998) has explained about the order of enzyme reactions and specificity, using a mouse mastocytoma cell free system.

The initial steps in the chain-building process involve the attachment of a tetrasaccharide linker -GlcAβ1–3Galβ1–3Galβ1–4Xylβ1-*O*-(Ser)-, where four different enzymes, including xylosyl transferase, β4-galactosyl transferase (GalTI),

β3-galactosyl transferase (GalT-II), and β3-GlcA transferase (GlcAT-I) transfer the four monosaccharides, respectively. After the attachment of the linker, the first modification to the chain determines whether the chain matures into a CS-GAG or HS-GAG chain. The enzyme GlcNAcT-I transfers GlcNAc to the tetrasaccharide linker, initiating HS-GAG biosynthesis. This enzyme is distinct from the glycosyltransferase GlcNAcT-II involved in the elongation of the HS-GAG chain.

1.3.5.3 Functions

Heparan sulphate proteoglycans ((HS-PGs) consists of two or three HS chains attached in close proximity to cell surface or extracellular matrix proteins and hence binds to a variety of protein ligands and regulates a wide variety of biological activities, including developmental processes, angiogenesis, blood coagulation and tumour metastasis. HS are thought to affect proliferation, differentiation and maintenance of the stem-cell niche (Cool and Nurcombe, 2006).

HS-PGs also have direct roles in regulating the transport of solutes across the GBM (glomerular basement membrane, 100–200 nm) in the urinary system. The acidic GAG chains prevent negatively charged substances, such as proteins in the blood, from passing through the GBM and entering the filtrate. Consistent with this, loss of HS from the GBM results in proteinuria in cases of diabetic nephropathy (Raats *et al.,* 2000).

HS-PGs also contribute to pathophysiology (Fuster and Esko, 2005; Iozzo, 2005). Many other organisms (parasites, bacteria and viruses) are thought to use HS-PGs as adhesion receptors for infection (Dinglasan and Jacobs-Lorena, 2005). Heparan sulphate proteoglycans also serves as cellular receptor for a number of viruses (Hallak *et al.,* 2000). The function of HS in wound repair is an active area of investigation (Sher *et al.,* 2006). Altering the HSPGs in the wound environment could affect growth-factor sequestration or activation, cell migration and proliferation, and angiogenesis (Alexopoulou *et al.,* 2007).

1.3.6 Keratan sulphate

Keratan sulphate (KS), also called keratosulphate, is one of several sulphated glycosaminoglycans that have been found especially in the cornea,

cartilage, bone and the horns of animals. It is also synthesized in the central nervous system where it participates both in development (Miller *et al.,* 1997) and in the glial scar formation following an injury (Zhang *et al.,* 2006). Keratan sulphates are large, highly hydrated molecules which in joints can act as a cushion to absorb mechanical shock.

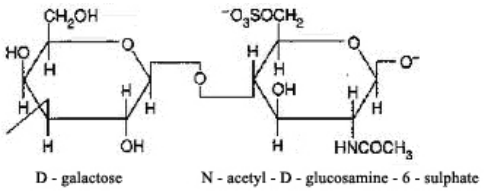
Keratan sulphate was identified in 1939 by Suzuki in extracts of cornea (Suzuki, 1939). By the 1950s the efforts of Karl Meyer (who coined the name keratosulphate) and others had characterized this material as a linear polymer of galactose and N-acetylglucosamine, (-3Galβ1-4GlcNAcβ1-) sulphated at the C6 of both hexose moieties (Meyer *et al.,* 1953). Keratan sulphates have variable sulphate content and unlike many other GAGs, contain a galactose unit instead of uronic acid/iduronic acid. KS core proteins include lumican, keratocan, mimecan, fibromodulin, osteoadherin and aggrecan. The amount of KS found in the cornea is 10 fold higher than it is in cartilage and 2-4 times higher than it is in other tissues (Funderburgh *et al.,* 1987). It is produced by corneal keratocytes (Funderburgh *et al.,* 2003), and is thought to play the role of a dynamic buffer in corneal hydration. Osteoadherin and fibromodulin are core proteins found in bone and cartilage, which are modified by N-linked KS chains.

Keratan sulphates have been grouped into three main classes namely, KSI, KSII and KSIII. The designations KSI and KSII were originally assigned on the basis of the tissue type from which the keratan sulphate was isolated. KSI was isolated from corneal tissue (Meyer *et al.*, 1953) and KSII from skeletal tissue (Meyer *et al.*, 1958). Major differences occur in the way each KS type is joined to its core protein and the designations KSI and KSII are now based upon these protein linkage differences. KSI is N-linked to specific asparagine amino acids *via* *N*-acetylglucosamine and KSII is O-linked to specific serine or threonine amino acids *via* N-acetyl galactosamine (Nieduszynski *et al.,* 1990). The tissue based classification of KS no longer exists as KS types have been shown to be non tissue specific (Tai *et al.,* 1996). A third type of KS (KSIII) has also been isolated from brain tissue that is O-linked to specific serine or threonine amino acids *via* mannose (Krusius *et al.,* 1986). The monosaccharide mannose is also found within

the linkage region of keratan sulphate type I (KSI). Disaccharides within the repeating region of KSII may be fucosylated and N-acetylneuraminic acid caps the end of all keratan sulphate type II (KSII) chains and up to 70% of KSI type chains (Funderburgh, 2000).

1.3.6.1 Structure

The basic repeating disaccharide unit within Keratan sulphate is galactose and N –acetyl glucosamine (-3Galβ1-4GlcNAcβ1-). This can be sulphated at carbon position 6 (C6) of either or both the Gal (galactose) or GlcNAc (N-Acetyl glucosamine) monosaccharides. However, the detailed primary structure of specific KS types is considered to be composed of three regions (Tai *et al.,* 1996). A linkage region at one end of which the KS chain is linked to the core protein; a repeat region composed of the -3Galβ1-4GlcNAcβ1- repeating disaccharide unit and a chain capping region, occurring at the opposite end of the KS chain to the protein linkage region.



Scheme 1.6: Structure of keratan sulphate disaccharide unit

1.3.6.2 Biosynthesis

KS is elongated *via* the action of (GnT) glycosyltransferases that alternately add Gal and GIcNAc to the growing polymer. In recent years a family of at least seven β 4Gal-T genes has been identified, and roles for each are defined (Amado *et al.,* 1999). A number of GnT enzymes are known, of which two seem potential candidates. A widely distributed enzyme (iGnT) has been shown to participate in synthesis of linear polylactosamine (known as "i" antigens) (Sasaki *et al.,* 1997). RNA transcripts for this enzyme are enriched in brain, a tissue in which KS is actively synthesized. A second GnT enzyme (β3GnT) active in synthesis of linear polylactosamine has been identified and cloned (Zhou *et al.,* 1999).

Sulphation of KS in cornea is carried out by at least two sulphotransferase enzymes. Two enzymes have been identified and cloned that add sulphate to KS (Habuchi *et al.,* 1996; Fukuta *et al.,* 1997). KS is also sulphated on the GlcNAc

moieties and presumably a specific enzyme is responsible for this activity. Nakazawa *et al.* (1998) have demonstrated that G1cNAc-6-sulphotransferase (Gn6ST) activity in keratocyte extracts specifically sulphates non reducing terminal GIcNAc (β 1-3) Gal-R. Partially desulphated KS received sulphation only on Gal moieties by these extracts. The cDNAs for two Gn6ST enzymes with specificity for nonreducing terminal GlcNAc have been identified and cloned (Uchimura *et al.,* 1998; Lee *et al.,* 1999). Patients with macular corneal dystrophy, a disease in which KS lacks GlcNAc sulphation throughout the body, had unaltered levels of this enzyme activity in their serum (Hasegawa *et al.,* 1999). The implication of these findings is that an enzyme of similar specificity with restricted tissue localization may be responsible for KS synthesis.

1.3.6.3 Functions

One of the main functions of keratan sulphate is the maintenance of corneal transparency. The high abundance of KS in cornea appears to be related to maintenance of a level of tissue hydration critical for corneal transparency. KS and dermatan sulphate-linked proteoglycans of the cornea have distinct water-binding properties (Hassell *et al.,* 1980). KS also is an active participant in the cellular biology of the tissues in which it is located and is also associated with a number of epithelial tissues. Keratinocytes, uterine endometrial cells, corneal endothelium, sebaceous gland, salivary gland, and sweat gland epithelia exhibit KS immunoreactivity in adult tissues (Fullwood *et al.,* 1996). KS is also found in a variety of epithelia-derived carcinoma cells (Ito *et al.,* 1996; Leygue *et al.,* 1998). After cornea and skeletal tissues, brain appears to exhibit the most abundant KS and is one of the tissues rich in enzymes of KS biosynthesis.

The important property of KS is its anti-adhesive character. Antiadhesive molecules function in complex and sometimes paradoxical roles during cell attachment and motility (Greenwood and Murphy-Ullrich, 1998). The anti-adhesive properties of KS may well play significant roles in implantation and endothelial cell migration as well as in other biological processes. Abundance of the cell-associated KS in the endometrial uterine lining varies markedly during

the menstrual cycle, reaching a peak at the time at which embryo implantation occurs (DeLoia *et al.,* 1998). These findings suggest a potential role for KS in the implantation process.

KS has also been implicated in motility of corneal endothelial cells, a single layer epithelium that lines the posterior surface of the cornea. These cells normally display a mosaic distribution of KS at their apical surface, but after wounding the KS is reduced or absent on migrating cells. KS returns in abundance to the cell surface when the cells cease migration (Davies *et al.,* 1999). KS-containing molecules constitute a barrier to neurite growth *in vitro* and appear to direct axon growth patterns during development or regeneration *in vivo* (Olsson *et al.,* 1996). Guerassimov *et al.* (1999) demonstrated that the "barrier" character of KS is also implicated in the findings that KS chains on aggrecan block the development of an immune response *in vivo* and *in vitro* to the G1 domain of this protein (aggrecan), suppressing development of antigen-induced osteoarthritis.

1.4 Glycosaminoglycans: Distribution in the Animal Kingdom

Even though most interest among scientific community centers on the structures and functions of mammalian glycosaminoglycans, investigations on the occurrence of GAGs in tissues of other vertebrates and invertebrates have also shown a wide distribution in the animal kingdom suggesting that GAGs are phylogenetically conserved through evolution, and play fundamental biological roles in animal development.

1.4.1 Glycosaminoglycans in aquatic vertebrates

Glycosaminoglycans in terrestrial vertebrates including mammalian sources have been extensively studied and characterized. Studies on glycosaminoglycans in aquatic/marine vertebrates are relatively scanty. Ototani *et al.* (1982) isolated heparin octasaccharide from the finback-whale (*Balanoptera physalua* L.) and reported on its structure and biological activity. Sugahara *et al*. (1991) performed the structural studies on sulphated oligosaccharides in chondroitin sulphate proteoglycans of whale cartilage. The use of powdered shark cartilage for wound

healing and as anti - angiogenic, anti –arthritic and anti–tumor agent from ancient times has been attributed to the presence of glycosaminoglycans as evidenced by the studies of Lee and Langer (1983); Uchiyama (1987); Oikawa *et al.* (1990); Sugahara *et al.* (1992b); Ronca and Conte (1993); Simone *et al.* (1998); Dupont *et al*. (1998); Liang and Wong (2000). Sugahara *et al.* (1996a) performed the structural analysis of chondroitin ABC lyase susceptible unsaturated hexasaccharides isolated from shark cartilage chondroitin sulphate D (CS-D). Karamanos *et al*. (1991a) isolated and characterized glycosaminoglycans from the skin of ray (*Raja clavata*) using HPLC technique. Chatziioannidis *et al.* (1999) isolated and characterized a small dermatan sulphate proteoglycan from *Raja clavata* skin. Summers *et al.* (2003) studied on the glycosaminoglycan and proteoglycan components in the fibrocartilaginous tendon of an elasmobranch fish (*Rhinoptera bonasus*) and reported the similarity in its biochemical traits to the fibrocartilaginous meniscus of mammals. Nandini *et al.* (2005) isolated chondroitin/dermatan sulphate (CS/DS) hybrid chains from shark skin with an aim to develop therapeutic agents. Li *et al.* (2007) isolated novel CS/DS preparations from the liver of *Prionace glauca* (blue shark) by anion exchange chromatography and compared with the neuritogenic activity of CS/DS hybrid chains of embryonic pig brain.

Acid mucopolysaccharides in the tissues of different fishes have been studied by many authors. Prame (1967) isolated glycosaminoglycans from the retrobulbar tissue of the crucian carp (*Carassius carassius* L.). Anno *et al.* (1971) isolated a novel dermatan sulphate polysulphate (CS–H) from hagfish notochord. Wasserman *et al.* (1972) reported on the composition of acidic glycosaminoglycan in the gills of *Cyprinus carpio*. Glycosaminoglycans have been isolated and characterized in the skins of *Labeo rohita* (Sikder and Das, 1979), *Mastacembalus armatus* (Sikder and Das, 1980) and Pacific mackerel (Ito *et al.*, 1982). Uchisawa *et al.* (2001) studied on the binding of calcium ions with the chondroitin sulphate glycosaminoglycan chains of salmon nasal cartilage. Pfeiler *et al.* (2002) analysed the structure and function of hyaluronan in developing fish larvae (leptocephali). Sakai *et al*. (2003) purified and characterized dermatan sulphate from the skin of

the eel, *Anguilla japonica*. Bernhardt and Schachner (2000) demonstrated the role of chondroitin sulphates in the formation of the segmental motor nerves in zebrafish embryos. Bink *et al.* (2003) reported on the importance of heparin sulphate sulphotransferases during muscle development in zebrafish. Souza *et al.* (2007) showed that chondroitin sulphate and keratan sulphate are the major glycosaminoglycans present in the adult zebrafish *Danio rerio* (Chordata-Cyprinidae). Laparra *et al.* (2008) studied on the role of glycosaminoglycans isolated from cooked haddock in nonheme iron uptake.

1.4.2 Glycosaminoglycans in invertebrates

Studies show that invertebrate species are a rich source of sulphated polysaccharides with novel structures (Vieira *et al.,* 1991; Santos *et al.,* 1992; Mulloy *et al.,* 1994; Nader *et al.,* 1999; Medeiros *et al.,* 2000).

Glycosaminoglycans have been extensively studied and characterized in ascidians, a specialized group of primitive Invertebrate-Chordates. The external supportive tissue called the tunic, in ascidians contain large amounts of sulphated polysaccharides which is different from all previously described mammalian glycosaminoglycans and also from the sulphated polysaccharides from marine algae (Santos *et al.,* 1992; Mourao *et al.,* 1997). The preponderant polysaccharide in the ascidian tunic is a high molecular weight sulphated α-L-galactan (Mourao and Perlin, 1987). The occurrence of glycosaminoglycans in the body of the ascidian, *Styela plicata* was also reported by Pavao *et al.* (1995). Small amounts of heparan sulphate and a large quantity of a dermatan sulphate-like glycosaminoglycan were found in the body of this ascidian. Preliminary analysis of this dermatan sulphate using degradation with chondroitin ABC lyase suggested structural differences when compared with mammalian dermatan sulphate. The distribution of glycosaminoglycans in different tissues like intestine, heart, pharynx, and cloak of *Styela plicata* was reported by Gandra *et al.* (2000). Dermatan sulphate like glycosaminoglycans have also been isolated and studied in other tunicates like *Ascidia nigra* (Pavao *et al.,* 1995) and *Halocynthia pyriformis* (Pavao *et al.,* 1998). Xu *et al.* (2008) investigated on the effects of chondroitin

sulphate (CS) extracted from *Styela clava* tunic on TNFα (tumor necrosis factor–alpha) induced inflammation and elucidated the mechanism of CS on the regulation of inflammatory factors.

Glycosaminoglycans in invertebrates were studied extensively by Cassaro and Dietrich (1977). The following list represents the species of invertbrates selected for their study. Insecta: *Cornitermis cumulans* (termite), *Camponotus rufipes* (ant), and *Periplaneta americana* (cockroach); Crustacea: *Penaeus brasiliense* (shrimp), *Scyllarides brasiliense* (lobster), *and Callinectes sapidus* (crab); Arachnida: *Nephila clavipes (spider); Mollusca: order* Filobranchia*, Aulocombia ater and Perna perna,* order Eulamellibranchia, *Mesodesma donacium* and *Anomalocardia brasiliense,* order Dibranchia, *Lolligo brasiliense* and Octopus sp.; Annelida: *Pheretima hawayana;* Tunicata: *Ascidia nigra and Styella plicata;* Echinodermata: *Holothuria grisea* and *Lytechinus uariegatus;* Coelenterata: two species from the order Actiniaria, sub-order Actiniaria; Porifera: two species from the order Spongiaria. Their results showed that the total amount and type of sulphated mucopolysaccharides varied from one species to another within the same class. For example in termites, chondroitin and heparitin sulphate were the main mucopolysaccharides, contrasting with cockroach in which heparitin sulphate and chondroitin sulphate A or B, or both, were the main mucopolysaccharides present.

Heparin is present in most invertebrates like lobster, *Homarus americanus* (Hovingh and Linker, 1982), prawn, *Macrobrachium birmanicum* (Dey and Varman, 1983), fresh water mussel, *Anodonta californiensis* (Hovingh and Linker, 1993), clams *Anomalocardia brasiliana* and *Tivela mactroides* (Pejler *et al.,* 1987), mangrove crab and sand dollar (Medeiros *et al.,* 2000). Sugahara *et al.* (1996b) isolated novel sulphated oligosaccharides containing 3-*O*-sulphated glucuronic acid from king crab chondroitin sulphate K. Kitagawa *et al.* (1997) isolated a novel chondroitin sulphate (CS –K) with a unique pentasaccharide sequence GlcA(3 – sulphate) (β1 -3)GalNAc(4- sulphate)(β1 -4)(Fuc-α -3) – GlcA(β 1-3)GalNAc(4 – sulphate) from king-crab cartilage and studied its differential susceptibility to chondroitinases and hyaluronidase. The occurrence of glycosaminoglycans in the

body of the shrimp *Penaeus brasiliensis* was shown by Nader *et al*. (1999) and Dietrich *et al*. (1999). Studies conducted by Vilela – Silva *et al.* (2001) on the sea urchin, *Strongylocentrotus purpuratus* suggested that the embryos synthesize a dermatan sulphate enriched in 4–*O*– and 6–*O*-disulphated galactosamine units.

The body wall of various sea cucumber species have been shown to contain heterogeneous fucose-branched chondroitin sulphate, heparin, and heparan sulphate (Vieria *et al.,* 1991; Mulloy *et al.,* 1994; Kariya *et al.,* 1997; Mourao *et al.,* 2001). Structure of fucose branches in the glycosaminoglycan from the body wall of the sea cucumber *Stichopus japonicus* was studied by Kariya *et al.* (1997). The antithrombotic activity of a fucosylated chondroitin sulphate extracted from the body wall of a sea cucumber was assessed by Pacheco *et al.* (2000), using a stasis thrombosis model in rats and found that intravenous administration of the polysaccharide reduced thrombosis in a dose-dependent manner compared to the antithrombotic action of standard mammalian glycosaminoglycans, mainly heparin and dermatan sulphate. GAGs were extracted from a commonly consumed sea cucumber, *Metriatyla scabra*, and administered to rats fed a cholesterol-supplemented diet for six consecutive weeks to study the effects on cholesterol and lipid metabolism (Liu *et al.,* 2002). Borsig *et al.* (2007) studied on the anti-selectin activity of a fucosylated chondroitin sulphate extracted from the body wall of the sea cucumber, *L. grisea.*

Yamada *et al.* (1999) demonstrated the presence of heterogenous non – sulphated glycosaminoglycans in a nematode *Caenorhabditis elegans*. Hwang *et al.* (2003) and Mizuguchi *et al.* (2003) have also shown the importance of chondroitin GAGs involved in the cell division of nematodes. The progress made in the *C. elegans* genome project has revealed the existence of multiple genes that are related to GAG-synthesizing enzymes and appear to be ancestors of vertebrate counterparts (Toyoda *et al.,* 2000a; Ackley *et al.,* 2001; Minniti *et al.,* 2004; Rhiner *et al.,* 2005).

Several studies have shown the presence of glycosaminoglycans or proteoglycan in insects. Hoglund (1976) found in fly, *Calliphora erythrocephala*

the presence of both chondroitin sulphates A and B. Proteoglycans or proteoglycan-like proteins, in the fruit fly, *Drosophila melanogaster* were studied by Spring *et al.* (1994) and Nakato *et al.* (1995). Mutations in genes encoding proteoglycan core proteins and GAG biosynthetic enzymes produce drastic morphological effects in the fly embryo (Haerry *et al.,* 1997; Sen *et al.,* 1998) indicating their significance in invertebrate development. Cambiazo and Inestrosa (1990) described the incorporation of ^{35}S-sulphate into extracellular HS. Pinto *et al.* (2004) studied the biosynthesis and metabolism of GAGs during the development of *D. melanogaster*. They developed a methodology to radiolabel sulphated GAGs in adult flies with ^{35}S-sulphate and followed the ^{35}S- sulphate GAGs in the embryo and in the three larval stages. Despite the extensive genetic analysis of proteoglycans and GAGs in *D. melanogaster*, biochemical analysis of these polymers in the fly has been less explored. More recently, biochemical studies described the disaccharide composition of HS and CS in *D. melanogaster* by Toyoda *et al.* (2000b).

A heparin-like substance able to bind antithrombin III (ATIII) was isolated from the marine clams *Anomalocardia brasiliana* (Dietrich *et al.*, 1985; Pejler *et al.,* 1987) and *Tivela mactroides* (Pejler *et al*., 1987), and heparin having an AT-binding region similar to that of mammalian heparin was also purified from another clam species, *Mercenaria mercenaria* (Jordan and Marcum, 1987; Ulrich and Boon, 2001). Santos *et al.* (2002) characterized heparin present in epithelial layers of different tissues of the mollusc *Anomalocardia brasiliana* and gave a correlation of heparin and histamine concentration. Heparin with high anticoagulant activity possessing high content of antithrombin III binding site was also isolated and characterized from the clam *Tapes phylippinarum* by Cesaretti *et al.* (2004). Heparin with anticoagulant activities varying from 150 to 320 IU/mg by the USP assay have also been isolated and characterized in the bivalves, *Spissula solidissima* (Burson *et al.,* 1956), *Marcia opima (*Gmelin) (Pandian and Thirugnanasambandan, 2008), and *Amussium pleuronectus* (Linne) (Saravanan and Shanmugam, 2010). Glycosaminoglycans in *Anodonta californiensis*, a freshwater mussel (Hovingh and Linker, 1993) and the giant African snail, *Achatina fulica*

(Kim *et al.,* 1996) have also been studied.

Mucopolysaccharides and glycoproteins are produced in considerable amounts by gastropods and serve a wide range of functions including mechanical or protective support and lubrication, and as components of egg gels and capsules (Park *et al.,* 2008). Nader *et al*. (1984) purified CSs with different structures from a gastropod (*Pomacea sp.*) and two bivalves (*Tagelus gibbus* and *Anomalocardia brasiliana*) with the former comprising of a large amount of non-sulphated disaccharide. Volpi and Mucci (1998) characterized a low-sulphated chondroitin sulphate from the body of *Viviparus ater* (mollusca - gastropoda) and demonstrated the modifications caused in its structure due to lead pollution. Gomez *et al.* (2010) identified a unique extracellular matrix heparan sulphate from the bivalve *Nodipecten nodosus* with potent anti-thrombotic activity.

Cephalopods including cuttlefish, squid and octopus are important marine resources in greater world demand due to their rich taste. Anno *et al.* (1964); Srinivasan *et al.* (1969); Radhakrishnamurthy *et al.* (1970), as well as Isobe and Seno (1971) isolated and characterized chondroitin-protein complexes from squid skin. Hjerpe *et al.* (1983) examined acidic polysaccharides in head cartilage of the squid *Illex illecebrosus coidentii*. Karamanos *et al.* (1986, 1990, 1991b, 1992) isolated and characterized the glycosaminoglycans in skin and cornea of squid with peculiar sulphation pattern. Vynios *et al.* (1990) assessed the proteoglycans in the cranial cartilage of *Illex illecebrosus coidentii* using immunochemical methods. Kinoshita *et al.* (1997) and Deepa *et al.* (2007) isolated novel chondroitin sulphate E glycosaminoglycans from the head cartilage of different squids. Falshaw *et al.* (2000) compared the skin and head cartilage glycosaminoglycans of Gould's arrow squid, (*Nototodarus gouldi*). Shetty *et al.* (2009) isolated and characterized a novel chondroitin sulphate from squid liver integument. Even though various studies on the biochemical composition, fractionation and characterization of sarcoplasmic, myofibrillar and connective tissue proteins, have been performed in various cephalopod species, studies on the occurrence of glycosaminoglycans in cephalopods belonging to the Indian waters are meager.

Chapter 2: Isolation of Glycosaminoglycans in Squid and Cuttlefish

Contents

2.1 Introduction

Carbohydrate-containing structures are amongst the most complex, heterogeneous, and abundant biomolecules on earth (Koeller and Wong, 2000). Glycans are central to many fundamental biological processes including cell–cell recognition, detection, and evasion of immune responses, cell attachment, and detachment, cell fate, development, and morphogenesis. In contrast to proteins, carbohydrates are not directly encoded but are constructed by the concerted activity of synthesizing and modifying enzymes. This indirect linkage between genome and glycome presents a barrier to unraveling the complex biology of glycans. The importance of glycans for health is exemplified by their central roles in cancer metastasis and in mediating immune responses (Feizi, 2000; Koeller and Wong, 2000; Hirabayashi, *et al.,* 2001).

Polymeric carbohydrates such as the glycosaminoglycans constitute major components of the extracellular matrix. There have been rapid advances in technology, ranging from whole-organism genetics to evaluate functional effects of knocking-out enzymes involved in GAG biosynthesis, to the development of enzymatic and analytical tools for structural characterization of GAGs. These advances have resulted in a paradigm shift of the GAG field from an integrated glycomics approach (similar to genomics and proteomics) to GAG structure-function relationships.

Advances in the technology to analyze GAGs and in whole-organism genetics have led to a dramatic increase in the known important biological role of these complex polysaccharides. Owing to their ubiquitous distribution at the cell-

ECM interface, GAGs interact with numerous proteins and modulate their activity, thus impinging on fundamental biological processes such as cell growth and development. GAGs show considerable heterogeneity with regard to their molecular size (ranging from 3 to 100 kDa), the identity of their monosaccharide residues and the degree of sulphation of their chains (Casu and Torri, 1999). The inherent heterogeneity in the chain length and the sulphation pattern of GAGs has complicated their isolation and structural characterization. The non template driven nature of the biosynthesis has also made it challenging to develop techniques to amplify biological GAGs. As a result, there is a practical requirement for the analytical tools to be able to handle minute sample amounts of GAGs isolated from various biological sources. These issues pose important challenges to the characterization of biologically active GAG species, which are typically found in low abundance within a heterogeneous mixture. Over the last decades, considerable knowledge has been gained regarding the structure and organization of these extremely large polyanionic molecules, especially in vertebrates, compared to that of invertebrates.

Marine organisms are a rich source of structurally novel and biologically active metabolites. So far, many chemically unique compounds of marine origin with different biological activities have been isolated and a number of them are investigated and/or being developed as new pharmaceuticals (Ely *et al*., 2004). The marine molluscs show extensive species diversity and their byproducts have received much attention from the beginning of the 20th century. Among the molluscs, some have pronounced pharmacological activities or other properties useful in the biomedical area. Some of these pharmacological activities are attributed to the presence of sulphated polysaccharides, particularly glycosaminoglycans (Hovingh and Linker, 1993; Kim *et al.,* 1996; Ulrich and Boon, 2001; Santos *et al.,* 2002; Cesaretti *et al.,* 2004; Pandian and Thirugnanasambandan, 2008; Saravanan and Shanmugam, 2010; Gomes *et al.*, 2010). Hence an attempt has been made in this study to isolate and characterize the GAGs from selected tissues of the two commercially important species of cephalopods namely, squid (*Loligo duvauceli*) and cuttlefish (*Sepia pharaonis*),

where such information is lacking.

The present chapter describes the protocol developed for the isolation of glycosaminoglycans, the percentage yield of GAGs obtained from the different tissues of squid and cuttlefish as well as some baseline data regarding the proximate composition and amino acid profile of these tissues.

2.2 Materials and methods

2.2.1 (a) Raw materials

The cephalopods, squid (*Loligo duvauceli*) and cuttlefish (*Sepia pharaonis*) were collected from Vypeen harbour, Cochin, India. The samples from the last haul of the trawl catch were selected for the study. The collected samples were kept in separate polythene covers and stored in crushed ice without direct contact and were transported to the laboratory without delay for further processing and analyses.

The samples were washed thoroughly with potable water to remove dirt, slime and ink. The different tissues of squid and cuttlefish namely mantle, tentacles/arms, and fin represented the edible portions; and the skin, gladius/pen, and head/cranial cartilage represented the non-edible portions. The mantle and fins were deskinned and filleted before use. The skin thus removed from the mantle and fins served as samples for the representative non-edible skin tissues. All the other representative tissues namely the tentacles and cranial cartilages in both the cephalopod species and the gladius in case of squid (*Loligo duvauceli*) were carefully separated from the remaining tissues and used as such. Since the pen/cuttlebone of *Sepia pharaonis* is a kind of calcium carbonate mineralized structure, corresponding pieces of the mineral free cuttlebone portions were used for all isolation and analyses.

The carefully dissected sample tissues were homogenized using a tissue homogenizer (Yorco Micro tissue homogenizer), at 2000 rpm for 10 minutes under chilled conditions for all aspects of isolation and analyses. In the case of gladius/pen air dried samples were powdered using a domestic blender and utilized for the preparation of acetone dry powder and subsequent glycosaminoglycan

isolation and analyses.

2.2.1 (b) Reagents, chemicals, and glasswares

All chemicals, acids, stains and reagents used were of analytical grade. The ethanol (food grade) used was redistilled before use. Deionized (DI) ultrapure water (Rios and Elix Millipore water purification system), which gave a water conductivity of 18.2 mΩ was used for the preparation of all reagents, buffers, calibration standards and as dilution water in all aspects of analysis. All glass wares and plastic wares were washed first with water and further cleaned by soaking in laboratory grade detergent, followed by repeated rinsing in water and finally rinsed thoroughly with copious amount of deionised water and dried in a hot air oven before use. Milligram quantities of chemicals and standards were weighed on a Mettler Toledo weighing balance (model PL303). All pH adjustments of reagents and buffers were made using a Cyber Scan 500 model pH meter.

2.2.2 Proximate composition analysis

The selected tissues of both the cephalopod species were analyzed for the proximate composition. The content of moisture, protein, fat and ash were analyzed by the method of AOAC (1995).

2.2.2.1 Determination of moisture

The moisture content was determined by drying 10.0 g sample at 103°C in a thermostatically controlled hot air oven. The samples were taken in a pre - weighed petri dish and kept in oven and the reduction in weight was checked by repeatedly heating and then cooling the sample in desiccators till the weight became constant. Moisture content was expressed as percentage.

2.2.2.2 Determination of crude protein

2.2.2.2. (a) Micro-Kjeldahl distillation method for crude protein

One gram of the homogenized samples was accurately weighed into a digestion tube. About 2.0 g of digestion mixture ($CuSO_4$ and K_2SO_4 as in the ratio of 1: 8) and 10.0 ml of concentrated H_2SO_4 (AR) were added to the samples taken in the digestion tube. The samples were digested to a clear solution in a KEL 12

PLUS KES digestion unit. The sample solution thus obtained was made up with 100 ml of distilled water by intermittent cooling. Five millilitres of this sample solution was pipetted out into the Kjeldahl Micro distillation apparatus, followed by the addition of 10.0 ml of 40% NaOH. The ammonia produced on steam distillation was absorbed into 2% boric acid solution with Tachiros indicator. The distillate collected was back titrated against N/70 H_2SO_4. Crude protein content in the sample was calculated by multiplying the nitrogen content by the factor 6.25.

2.2.2.2. (b) Determination of crude protein content of chitinous tissues

In the case of chitinous tissues, total nitrogen content is contributed by the chitin nitrogen as well as protein nitrogen (Total nitrogen content = Chitin nitrogen + Protein nitrogen). The gladius/pen samples (1.0 g) were analyzed for the total nitrogen content by Microkjeldahl method as mentioned in section 2.2.2.2 (a). Simultaneously, chitn nitrogen was determined after deproteinisation of similar quantities of the gladius/pen using 5% NaOH. Briefly, 1.0 to 2.0 g of the chitinous tissue was heated using 5% NaOH in a boiling water bath for 15 minutes. Allowed to cool for 15 minutes and washed with 3 or 4 changes of distilled water and filtered. The deproteinsed residues thus obtained were further utilized for the determination of chitin nitrogen by Microkjeldahl method (Section 2.2.2.2 (a)). Finally protein nitrogen was calculated using the formula, Protein nitrogen = Total nitrogen content - Chitin nitrogen.

2.2.2.3 Determination of crude fat

Fat content of the moisture free sample was determined by extracting the fat using petroleum ether (boiling point: 40 - 60°C), by soxhlet extraction method (AOAC, 1995). About 2.0 g of the moisture free sample was accurately weighed into an extraction thimble and was placed in the extractor. The extractor was connected to a pre - weighed dry receiving flask and water condenser. The unit was placed on a water bath and temperature was maintained at 40°C - 60°C so that solvent boiled continuously and siphoned at a rate of 5 or 6 times/h and continued until the solvent in the extractor became colorless and fat free. The solvent in the

receiving flask was evaporated completely and weighed for fat content. The result was expressed as percentage of crude fat.

2.2.2.4 Determination of ash

The ash content was measured by the incineration of the sample according to AOAC (1995). About 2.0 g of moisture free sample taken in a pre - weighed clean dry silica crucible was charred on low heat, followed by incineration in a muffle furnace at 550°C to get a white ash. Silica crucibles were finally cooled in desiccators and weighed. Ash content was expressed as percentage.

2.2.3 Amino acid composition analysis by HPLC: (PICO -TAG method)

Quantitative determination of the amino acids was conducted using a Reverse Phase –High Performance Liquid chromatography (RP – HPLC) after Precolumn derivatization using PITC (phenyl isothiocyanate) according to the method of Shang and Wang (1996).

2.2.3.1 Reagents

All reagents used were of HPLC grade.

(a) PICO – TAG Eluent A

Pico-Tag Eluent A was prepared by dissolving 19.0 g sodium acetate trihydrate in one liter of deionized Milli Q water. Added 0.5 ml triethylamine (TEA) to the above solution and titrated to pH 6.4 using glacial acetic acid. To the resulting solution added 63.8 ml of acetonitrile and filtered using sterile membrane filters (cellulose nitrate, 47mm Dia, 0.45 μm pore size, WCN type, Whatman Ltd, Maidstone, England).

(b) PICO – TAG Eluent B

Pico-Tag Eluent B was prepared by mixing HPLC grade acetonitrile and deionized milli Q water in the ratio 6: 4 by volume.

(c) Sample diluent solution

The diluent solution was prepared as follows: 710 mg of Na_2HPO_4 was added to one liter of water and titrated to pH 7.4 with 10 % phosphoric acid (H_3PO_4). The resulting solution was mixed with HPLC grade acetonitrile, so that

acetonitrile was 5 % by volume.

(d) Sample redrying Solution

The redrying solution consisted of HPLC grade methanol, millipore water, and triethylamine (TEA) in the ratio 1:1:0.5 by volume.

(e) Sample derivatization Solution

The derivatization reagent consisted of HPLC grade methanol, triethylamine, millipore water, and phenyl-isothiocyanate (PITC) in the ratio 7:1:1:1 by volume.

2.2.3.2 Sample preparation for Pico-Tag amino acid analysis

Samples (100 – 200 mg tissue) were hydrolysed using 10.0 ml of 6 M HCl containing 2 % phenol at 110°C for 24 hours in heat sealed tubes after flushing with nitrogen and were finally filtered using Whatman No 1. The hydrolyzed sample solutions thus obtained were subjected to derivatization using PITC (Phenyl-isothiocyanate). Briefly, 5µl each of the above filtered sample solutions were transferred to a 1.0 ml sample tube and dried under vacuum to remove all traces of HCl. The samples in the tubes were treated with 20µl of redrying solution (Reagent 2.2.3.1 (d)), by gentle vortexing and dried under vacuum. Subsequently, the amino acids were derivatized by addition of 10µl of derivatization solution (Reagent 2.2.3.1(e)), to the above dried samples by gentle vortexing for 20 minutes at room temperature. The sample derivatisation solution was further removed by drying under vacuum for 30 minutes. After derivatisation, one ml of sample diluent solution (2.2.3.1(c)) was added and mixed by vortexing for a few seconds and filtered using 0.45µm Nylon filters (Whatman, Maidstone, England). For chromatographic analysis 10µL each of the above samples were injected into the HPLC.

2.2.3.3 Apparatus

HPLC system (Waters Model 2487) equipped with a Binary pump model M 515, a 600 Gradient mixer solvent delivery system and a 5 µm Pico-Tag Reversed Phase column (3.9 mm i.d. x 150 mm length) was used for the analysis of amino acids. The equipment is provided with column oven (TCM Waters), a

dual λ absorbance detector (UV/VIS Model 484) and a manual injector. Data analysis was performed using EMPOWER 2 chromatography software.

2.2.3.4 Chromatographic conditions

Chromatographic separation made use of continuous gradient elution with Pico Tag eluant A and Pico Tag eluant B for hydrolysate amino acid analysis. The gradient started at 100% eluant A and was decreased to 54% and finally increased to 100% in 14.5 min. The total separation time was less than 14 min and the gradient was run for 22.5 min to ensure full separation. Detection was monitored at 254 nm. The concentrations of all the standard amino acids were 2.5 μmoles/ml, except for cysteine with 1.25 μmoles/ml. Quantification of the peak areas of individual amino acids were compared with standard amino acids and concentration was calculated after injecting 20 μl amino acid solutions. HPLC gradient profile developed for the separation of amino acids is given in Table 2.1.

Table 2.1: HPLC gradient profile developed for the analysis of amino acids.

Sl. No.	Flow (mL min^{-1})	Time (min)	% Eluant A	% Eluant B	%C	Curve
1	1.00	0.01	100.0	0.0	0.0	6
2	1.00	2.00	100.0	0.0	0.0	5
3	1.00	12.00	54.0	46.0	0.0	5
4	1.00	12.50	0.0	100.0	0.0	6
5	1.00	13.50	0.0	100.0	0.0	6
6	1.50	14.00	0.0	100.0	0.0	6
7	1.50	14.50	100.0	0.0	0.0	6
8	1.50	22.00	100.0	0.0	0.0	6
9	1.00	22.50	100.0	0.0	0.0	6

2.2.4 Isolation of glycosaminoglycans

2.2.4.1 Preparation of acetone dry powder

Acetone dry powder of the sample tissues *viz*; mantle, fin, tentacle/arms, skin, gladius/pen, and the head/cranial cartilage of squid and cuttlefish were prepared by the method of Mathew *et al*. (1982). The homogenized/powdered

tissue samples (prepared as mentioned in (Section 2.2.1 (a)) were immediately placed in chilled acetone 1:5 w/v (20.0 ml acetone/g tissue) at 0°C for 72 hours with change of acetone every 12 hours. The samples were then filtered off and dried in a vacuum oven equipped with a single stage oil free vacuum pump and further extracted using 3:1 (v/v) of diethyl ether: acetone at 37°C for one hour. The samples were again filtered off and dried under vacuum and further extracted using diethyl ether for another one hour at 37°C, filtered and dried under vacuum. The acetone dry powder of the samples thus prepared was stored at room temperature.

2.2.4.2 Enzyme digestion

The dry defatted tissues were digested with papain (20U/g dry weight) in 0.2 M sodium acetate buffer, pH 7 containing 2mg/ml cysteine – HCl at 65°C for 72 hours (Roden *et al.,* 1972). Fresh papain was added every 24 hours. Appropriate controls (without any tissue) containing same amount of papain in sodium acetate buffer were also set.

2.2.4.3 Extraction of glycosaminoglycans

The samples (digests) after papain digestion were subjected to centrifugation (5000 x g) for 5 min at room temperature. The clear supernatants thus obtained were precipitated with 4 to 5 volumes of 95 % (v/v) ethanol and maintained at -10°C for 24 hours (Albano and Maurao, 1986). The crude precipitates thus formed were collected by centrifugation (5000 x g) for 15 min at room temperature. The above crude precipitates containing a mixture of GAGs and papain were then dissolved in deionized MilliQ water and the supernatants containing GAGs alone were collected by centrifugation (10000 x g) for 10 min at room temperature. The GAGs thus obtained were lyophilized in a Freeze Dryer (Model Lyolab DHPG -222). The papain controls were also subjected to ethanol precipitation and subsequent centrifugation and lyophilization as mentioned above. Details of the protocol for GAG extraction is shown in Fig. 2.1.

2.2.4.4 Determination of crude GAG yield

To determine the yield of GAGs from the selected tissues, the crude ethanol

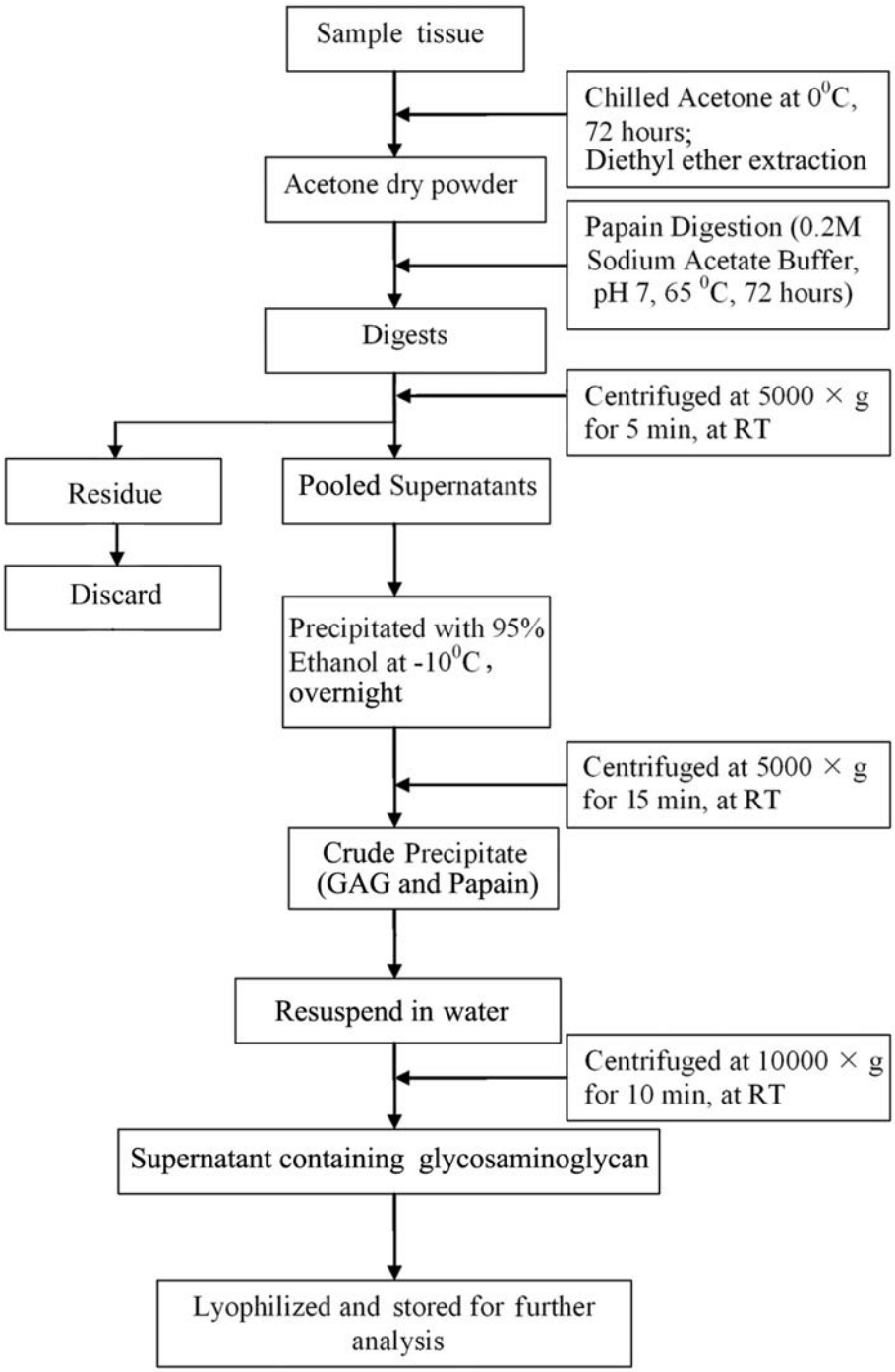


Fig. 2.1 Flow chart for the isolation of glycosaminoglycan

precipitates of the sample tissues and the control papain were freeze dried to a dry powder as mentioned in section 2.2.4.3., and finally the yields were calculated by subtracting the weight of control papain from that of the dry product (crude GAG precipitates) and expressed as percentage. Samples were analyzed in quadruplicate for each tissue.

2.2.5 Histochemical localization of the intact GAGs

2.2.5.1. Hematoxylin and eosin (H&E) staining

Histological observation was carried out essentially as described by Zhao *et al.* (2007). The samples, (a) mantle, (b) tentacle, (c) fin, (d) skin, (e) gladius and (f) cranial cartilage, were cut into pieces (0.5 cm × 0.5 cm) and fixed in 5% buffered formalin over 24 h. The tissues (e) and (f) were decalcified by stirring them for 72 h at room temperature in 5% formic acid. The samples were then dehydrated in a series of graded ethanol solutions (70%, 80%, 90%, and 100% ethanol for 10 min each), clarified in xylene and finally embedded in paraffin. Paraffin infiltration was performed with molten paraffin (60 – 70°C) for 1 h and 15 min. Approximately 7 µm sections were cut longitudinally using a rotary microtome (Lipshaw type Yorco model). The slides (with the sections) were cleared off paraffin with xylene and rehydrated with water through graded alcohols (100%, 90%, 80%, and 70% ethanol for 10 min each).

The sections were mounted on slides coated with Mayer's albumin and stained with hematoxylin and counter stained with eosin. The stained sections were permanently fixed using dibutylphthalate in xylene (DPX mountant) and observed using light microscopy. Digital images were taken using a Leica DM 2000 microscope fitted with a digital camera, at an original magnification of 10X.

2.2.5.2. Dimethyl methylene blue (1, 9 – DMMB) staining

Another set of samples were fixed and sectioned as described in Section 2.2.5.1. The rehydrated tissue sections were stained using 0.05 M 1, 9-dimethyl methylene blue in 0.1 M HCl, by the method of Gandra *et al.* (2000) for the visualization of intact GAGs.

2.2.6. Statistical analysis

All statistical calculations were performed in the SPSS 11.0 software package (SPSS, Chicago, IL). Data analysis was performed using one-way analysis of variance (ANOVA) with post-hoc multiple comparison analysis performed using Tukey's HSD test. p values less than or equal to 0.05 were considered statistically significant. Data are presented as mean $\pm$ standard deviation (SD).

2.3 Results

2.3.1 Proximate composition analysis

The proximate composition analysis was performed in order to obtain data regarding the major components present in the different edible tissues (mantle, tentacle, fin) and non-edible tissues (skin, gladius/pen, cranial cartilage) of squid and cuttlefish. The results obtained for proximate composition of the various tissues of squid and cuttlefish are depicted in table 2.2 and 2.3 respectively. Data obtained in this study was analyzed statistically using SPSS (Scientific Package of Social Science) version 11.0. One way ANOVA test was used to compare differences in the means of the moisture content, crude protein content, fat content and ash content, of different tissues of squid and cuttlefish, selected for the isolation of GAGs. This was followed by Tukey post-hoc analysis to determine in more detail how the different tissues of the cephalopod samples differed.

One way ANOVA showed that there were significant differences among the different edible and non-edible portions analyzed for squid (Appendix 2: Table 2.1), in the components (moisture, proein, fat and ash) analyzed. Similar results were obtained for the tissues of cuttlefish (Appendix 2: Table 2.2) on analysis of variance. The results showed that the tissues consisted of high protein and low fat content. Among the edible tissues (mantle, tentacle, and fin) analyzed the mantle of both the cephalopod species were high in protein content and are considered as good sources of seafood protein. In the case of non-edible tissues, the gladius/pen of both cephalopod species contained significantly higher amount of protein nitrogen, than all other tissues analyzed, owing to the presence of chitin-protein complexes in these tissues. The fat content of all the tissues were below 4%.

Table 2.2 Proximate composition analysis of the edible and non-edible tissues of squid (*Loligo duvauceli)*

		Moisture	Protein	Fat	Ash
Edible Tissues (%)	Mantle	83.83 ±1.05^{a}	14.20 ± 0.79 ^{bc}	0.46± 0.07^{bc}	0.46 ± 0.03^{b}
	Tentacle	81.84 ± 2.29^{a}	13.04 ± 0.04^{c}	2.00 ± 0.77^{a}	0.85 ± 0.05^{b}
	Fin	83.31 ± 0.91^{a}	14.23 ± 1.06 ^{bc}	1.07 ± 0.37^{abc}	0.37 ± 0.17^{b}
Non edible Tissues (%)	Skin	83.02 ± 0.99^{a}	13.92 ± 0.23 ^{bc}	1.30 ± 0.52^{ab}	0.75 ± 0.03^{b}
	Gladius	18.34 ± 1.19^{c}	*41.82 ± 1.33^{a}	0.03 ± 0.01^{c}	3.91 ± 0.83^{a}
	Cranial	77.10 ± 2.45^{b}	15.76 ± 1.10^{b}	0.85 ± 0.66^{bc}	1.29 ± 0.08^{b}

* Content of protein and chitin nitrogen

All values expressed as mean ± standard deviation, n = 4. Different superscripts in the same column indicate significant differences ($p<0.05$).

Table 2.3 Proximate composition analysis of the edible and non-edible tissues of cuttlefish (*Sepia pharaonis*)

		Moisture	Protein	Fat	Ash
Edible Tissues (%)	Mantle	80.39±0.09^{ab}	17.71±0.40 ^{a}	0.14±0.01^{c}	0.54±0.06 ^{d}
	Tentacle	79.19±2.74 ^{ab}	13.97±1.35^{b}	3.18±0.76^{a}	1.82±0.44^{c}
	Fin	82.96±1.81^{a}	14.50±2.12^{b}	0.40±0.12^{c}	0.89±0.10 ^{cd}
Non edible Tissues (%)	Skin	78.03±3.43 ^{ab}	19.59±1.58^{a}	1.45±0.70 ^{b}	0.95±0.10^{cd}
	Pen	23.45±2.65^{c}	*18.30±0.96^{a}	0.02±0.01^{c}	17.43±0.36^{a}
	Cranial	75.63±0.64^{b}	18.92±0.72^{a}	0.76±0.02^{bc}	3.2± 1.15 ^{b}

* Content of protein and chitin nitrogen

All values expressed as mean ± standard deviation, n = 4. Different superscripts in the same column indicate significant differences ($p<0.05$).

2.3.2 Amino acid composition analysis

HPLC chromatograms of the amino acid analyses of the randomly sampled portions of the representative edible and non edible tissues of squid and cuttlefish are given in Panels a-f of Fig. 2.2 and 2.3 respectively. The amino acid analysis shows that the protein of squid and cuttlefish are of high quality, containing an

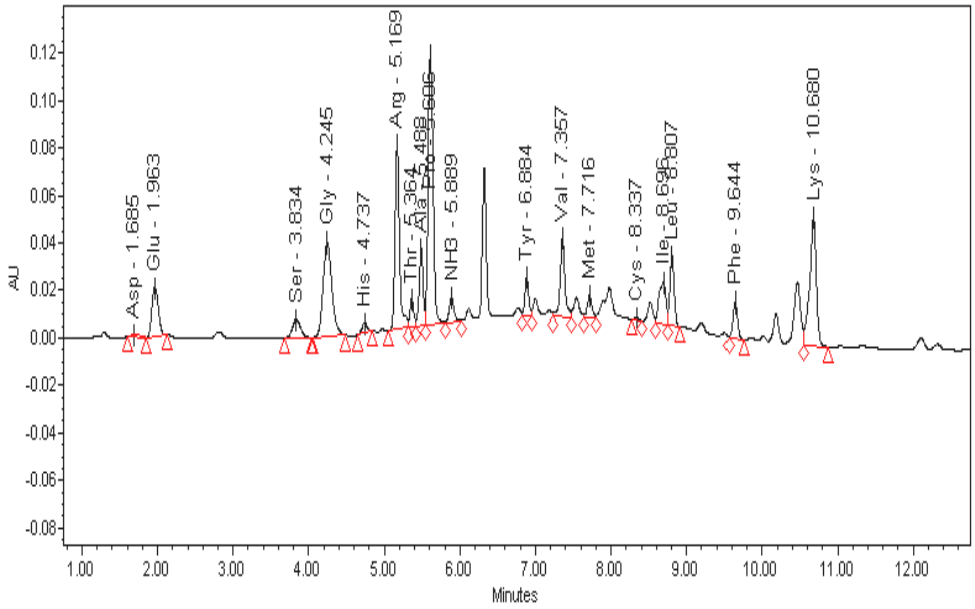


Figure 2.2(a): Amino acid profile of the mantle of squid

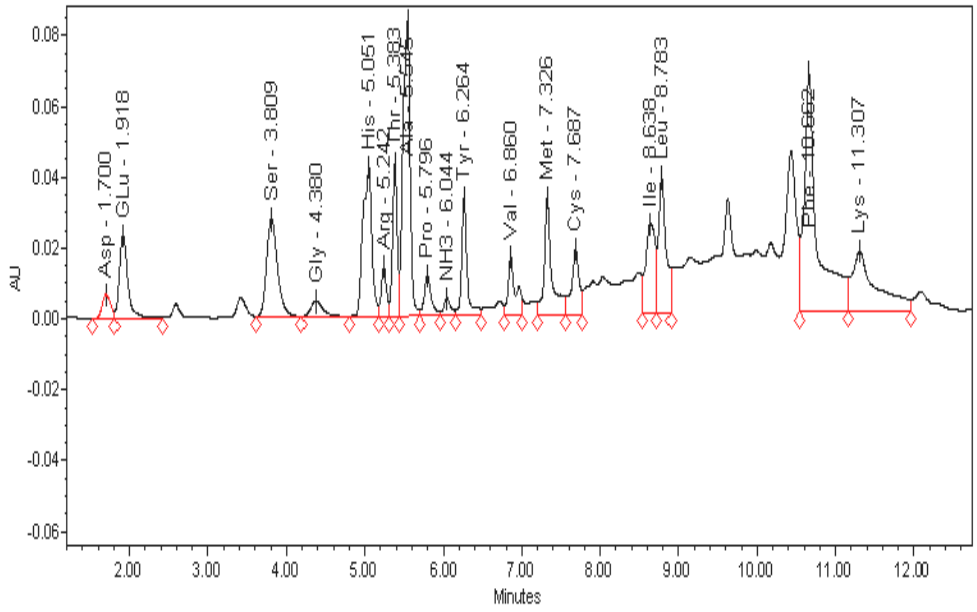


Figure 2.3(a): Amino acid profile of the mantle of cuttlefish

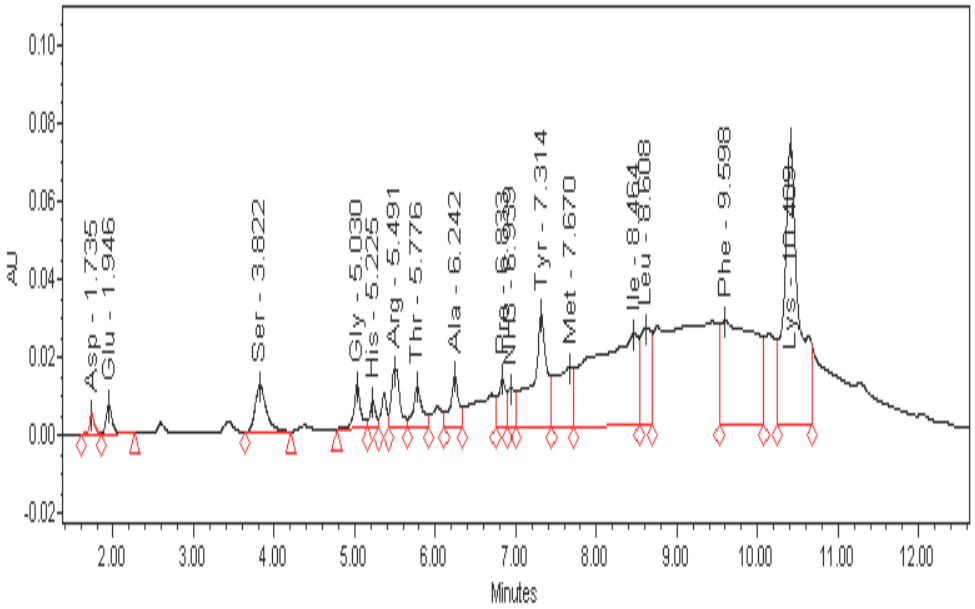


Figure 2.2(b): Amino acid profile of the tentacle of squid

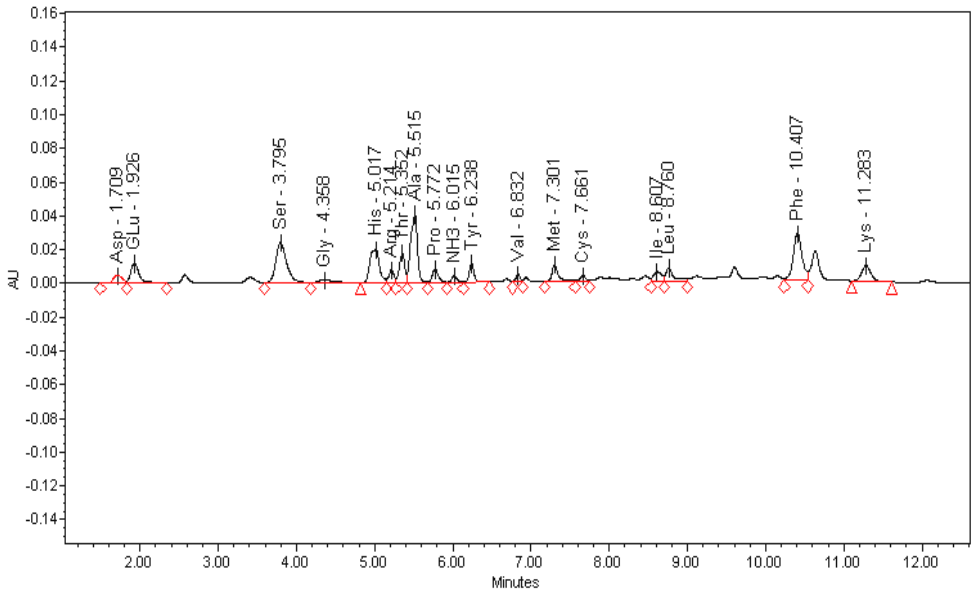


Figure 2.3(b): Amino acid profile of the tentacle of cuttlefish

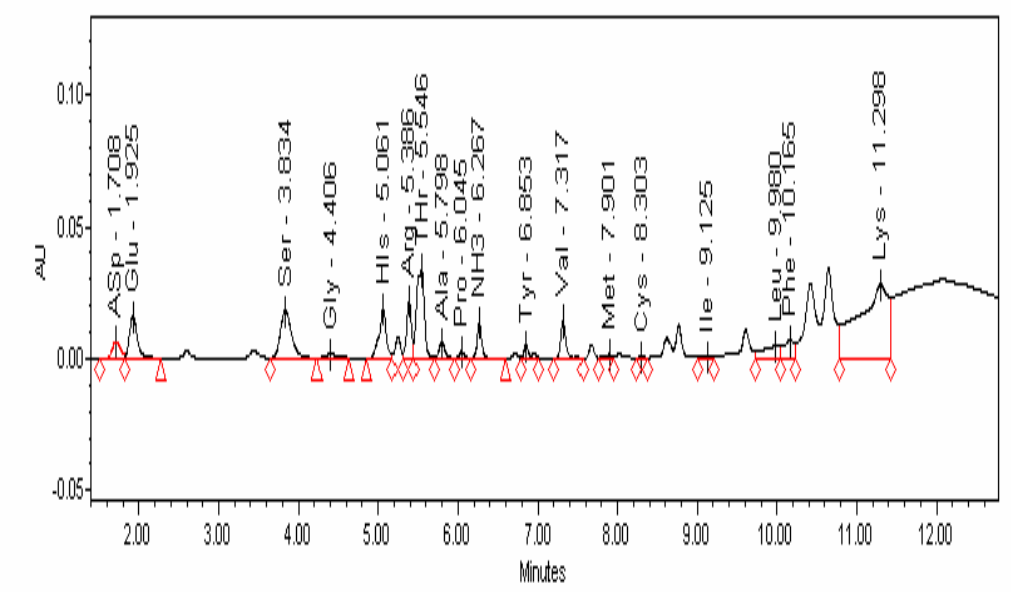


Figure 2.2(c): Amino acid profile of the fin of squid

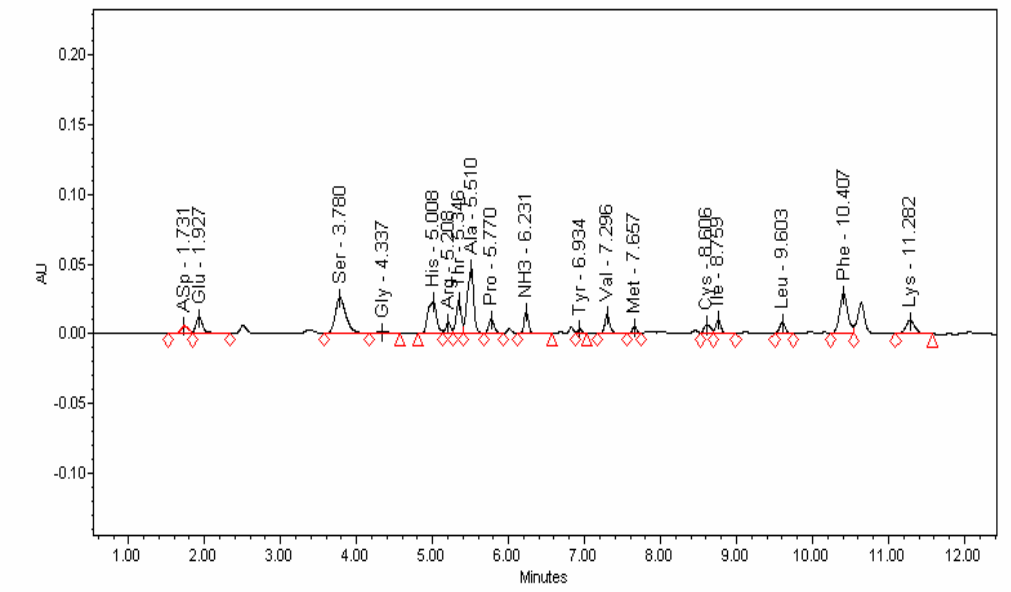


Figure 2.3(c): Amino acid profile of the fin of cuttlefish

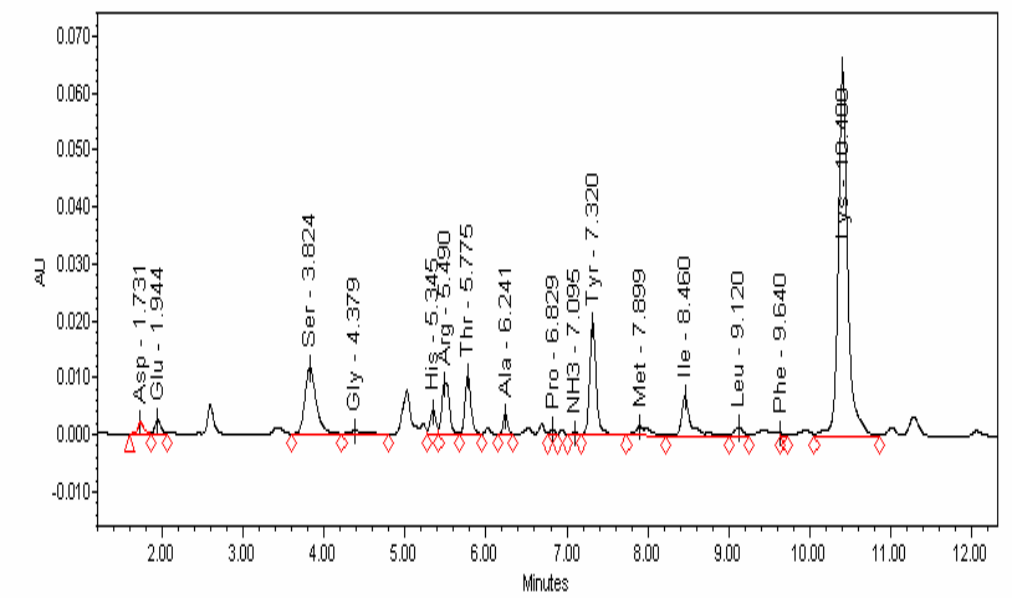


Figure 2.2(d): Amino acid profile of the skin of squid

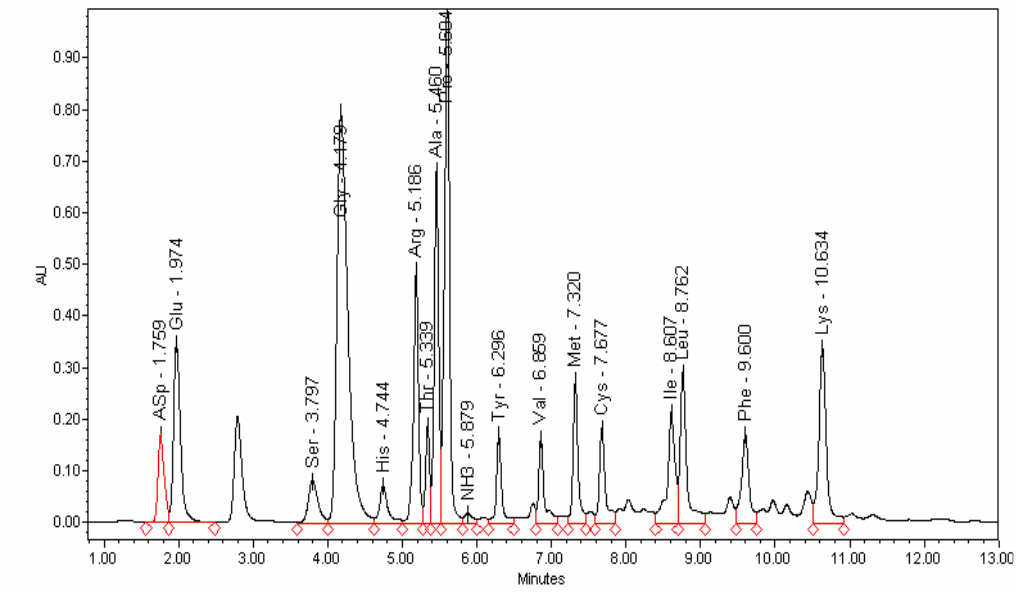


Figure 2.3(d): Amino acid profile of the skin of cuttlefish

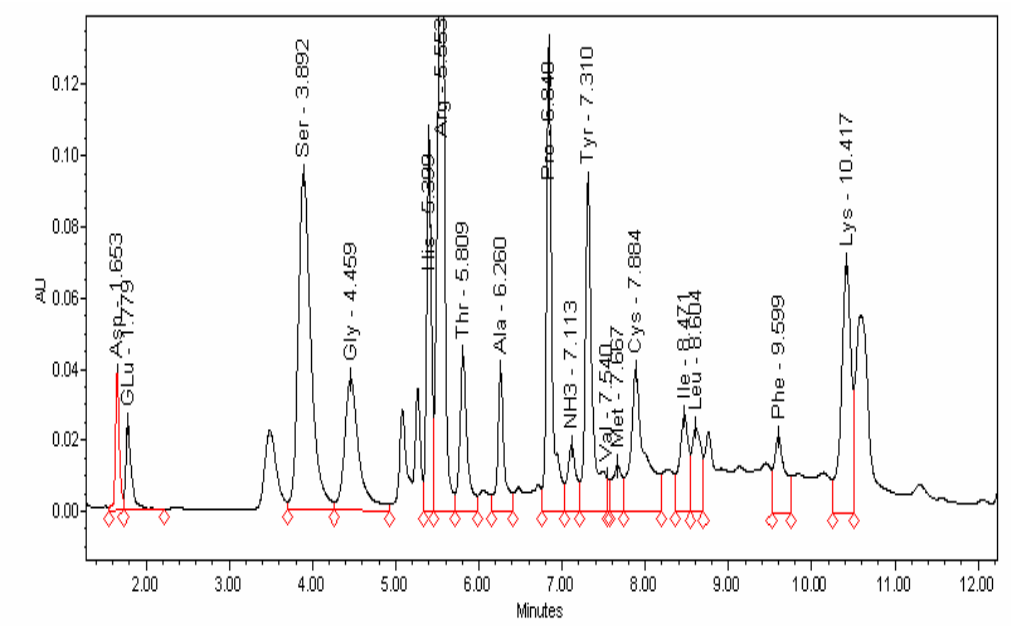


Figure 2.2(e): Amino acid profile of the gladius of squid

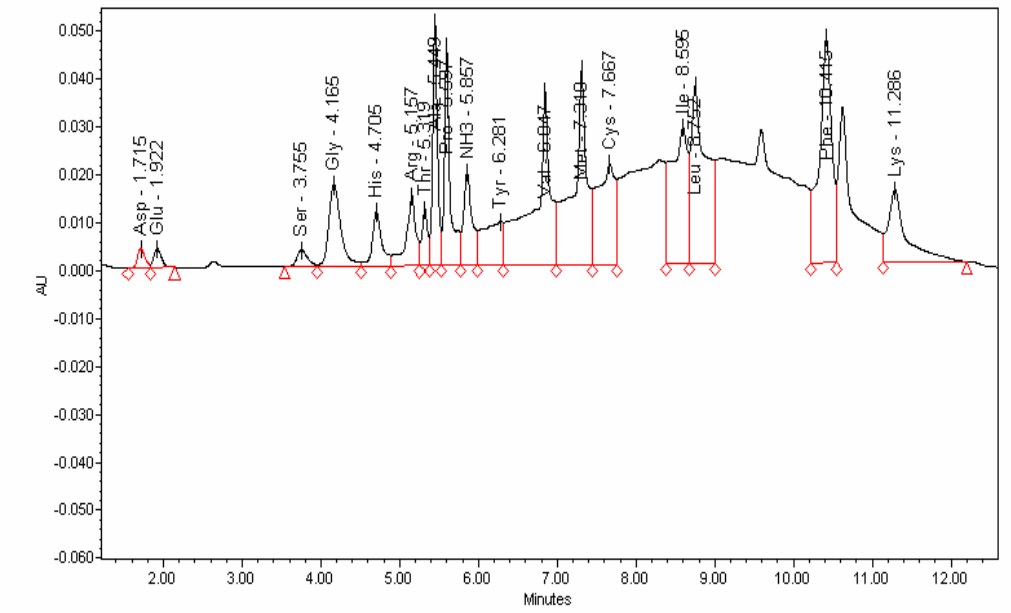


Figure 2.3(e): Amino acid profile of pen of cuttlefish.

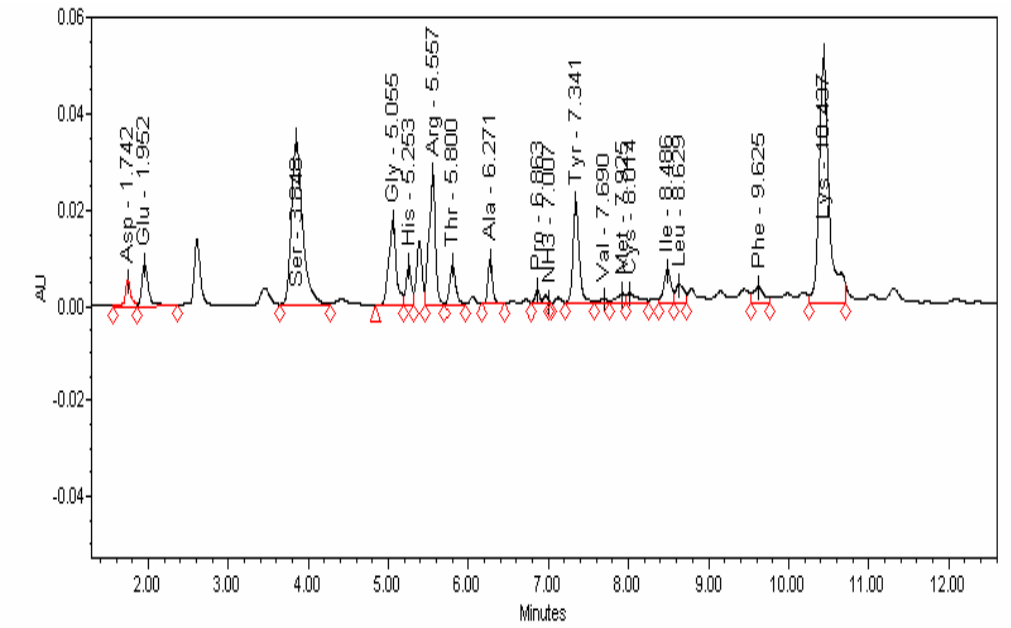


Figure 2.2(f): Amino acid profile of the cranial cartilage of squid

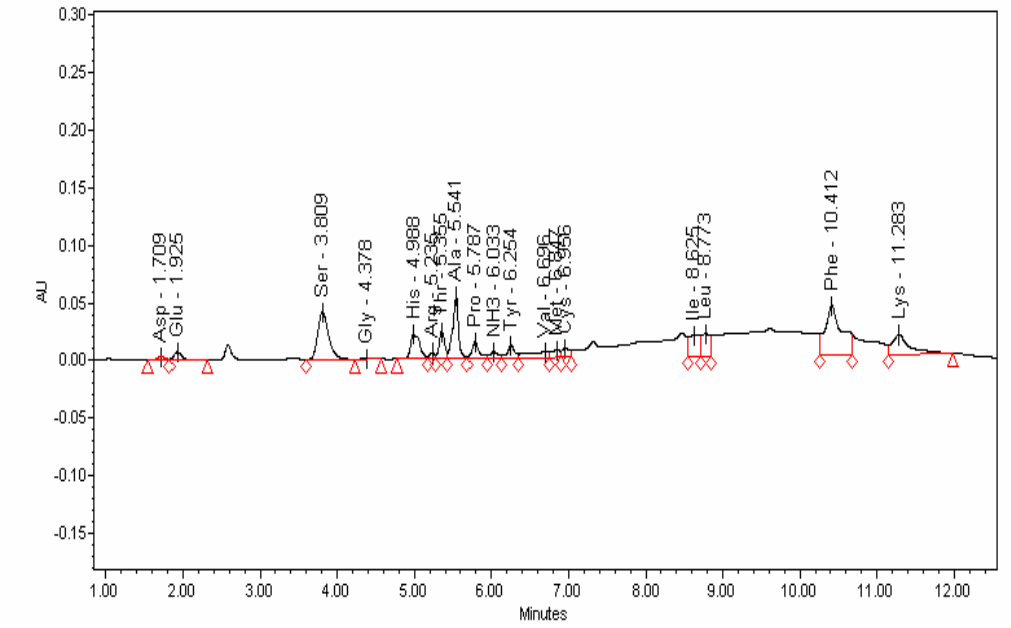


Figure 2.3(f): Amino acid profile of the cranial cartilage of cuttlefish.

abundance of the essential amino acids *viz*, histidine, isoleucine, lysine, methionine, phenylalanine, arginine, tyrosine and valine. Tyrosine also occurred in high amounts. Amino acid analyses showed that the sample tissues in the two specimens differed in their amino acid composition, particularly in the content of glutamic acid, glycine, alanine, methionine, phenylalanine, proline and lysine. Aspartic acid contributed to only a small portion of the total amino acids in all of the edible tissues analyzed. The amino acid profile of samples were assigned by comparing with the profile obtained for Pico-Tag standard amino acid standard (Appendix 2: Fig 1). Quantitative data of the individual amino acids of the different tissues of squid and cuttlefish are given in Appendix 2: Tables 2.3 and 2.4 respectively. The amount of tryptophan and taurine cannot be estimated by the Pico-Tag method and hence not reported in this study.

The results obtained from the proximate composition analysis and the amino acid analysis show that these diverse tissues of the respective edible and non-edible portions of squid and cuttlefish specimens are proteinaceous structures with significantly low proportion of lipids. The protein present in these tissues may, in part be contributed by connective tissue proteoglycans also, which are seen in association with glycosaminoglycans (GAGs) in the extracellular matrices. Thus the selected tissues namely mantle, tentacle and fin representing the edible portions and the non edible portions represented by the skin, gladius/pen and cranial cartilage were thus finalized for the subsequent isolation and characterization of glycosaminoglycans (GAGs), using the protocol designed in this study.

2.3.3 Isolation of glycosaminoglycans

GAGs can be easily extracted from animal tissues after proteolytic digestion and ethanol precipitation. The sample tissues selected for the isolation of GAGs were first subjected to dehydration and delipidation using chilled acetone and finally subjected to papain digestion and ethanol precipitation as described in the section 2.2.4. The yield of acetone dry powder and the amount of crude glycosaminoglycans obtained from the respective tissues using the developed protocol are shown in Table 2.4 and Fig 2.4, respectively. The percentage yield of

ADP of both gladius and pen showed high values due to lower moisture of these tissues. The ANOVA for ADP yield of both squid and cuttlefish tissues are shown in Appendix 2: Tables 2.5 and 2.6 respectively.

Table 2.4: Yield of acetone dry powder (ADP) from tissues of squid and cuttlefish

	Edible Tissue			Non Edible Tissue		
	Mantle	**Tentacle**	**Fin**	**Skin**	**Gladius/ Pen**	**Cranial cartilage**
Squid	17.54 ± 0.92^{b}	17.63± 0.79^{b}	16.47± 0.96^{b}	16.08± 1.39^{b}	82.45± 2.53^{a}	14.82± 0.55^{b}
Cuttlefish	20.78± 1.57^{b}	20.21± 0.71^{bc}	17.56± 1.22^{cd}	19.28± 0.62^{bc}	76.95± 2.43^{a}	15.64± 0.42^{d}

All values expressed as mean ± standard deviation, n = 4. Different superscripts in the same row indicate significant differences ($p<0.05$).

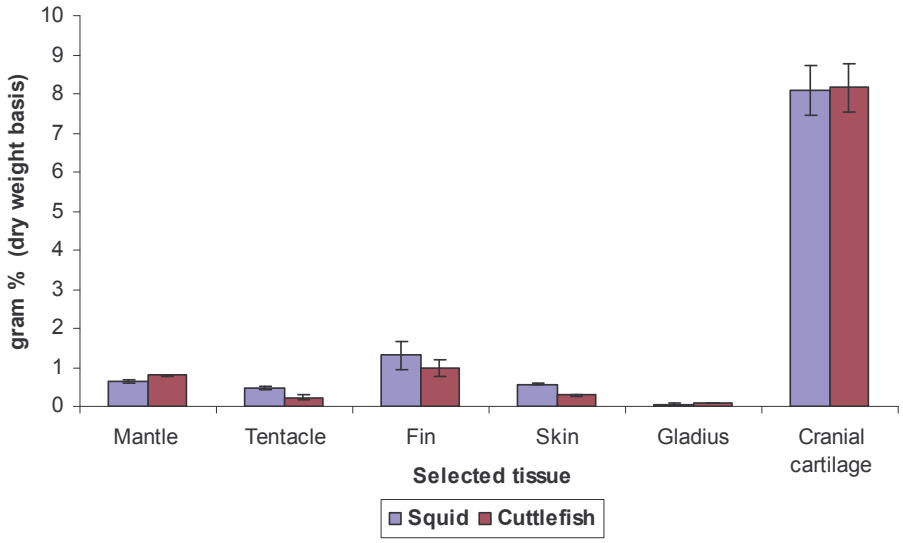


Fig 2.4 Histogram showing the percentage yield of glycosaminoglycans obtained from the different tissues of squid and cuttlefish.

Each estimation was performed in quadruplicate. Closed rectangles indicate mean values, and bars indicate standard deviation. The error bars indicate one standard deviation above and below the averaged curve.

Enzymatic extraction (papain digestion) of tissue glycosaminoglycans yielded a pale yellow solution after 72 hours at 65°C. The tissue debris and connective tissue components were removed by simple centrifugation. The GAGs in the clear supernatants were precipitated using 95% (v/v) ethanol at -10°C for 24 hours and collected by centrifugation. Considerable precipitation was obtained in the control papain also, when treated with 95% (v/v) ethanol. Thus the tissue glycosaminoglycans thus precipitated were a mixture of crude GAGs and papain, as evidenced from the precipitates obtained in the case of control. The crude GAGs were further re-dissolved in millipore water and centrifuged to remove the papain precipitates, which remain undissolved in solution. The GAGs were further recovered from the solution by freeze drying and were used for further assay. Since the GAGs are stable at elevated temperatures the GAGs could also be obtained with much ease by vacuum drying of the water resuspended ethanol fractions. The GAGs obtained from the tissue samples after lyophilization had a snowy white appearance and exhibited a hygroscopic nature at ambient temperatures. The yield of crude GAGs in the respective tissues of squid and cuttlefish were in the order, cranial cartilage > fin > mantle > skin > tentacle and gladius/pen.

One way ANOVA (Appendix 2: Tables 2.7 and 2.8) for the yield of GAGs from different sample tissues indicated that the cranial cartilage in both the species of cephalopods gave significantly higher yield compared to all the other tissues analyzed. The yield of crude GAGs from the cranial cartilage of squid was 8.09g% and cuttlefish gave a yield of 8.12g% on dry weight basis. The gladius/pen of both the cephalopods gave the least values for yield. Among all the edible tissues analyzed the fin followed by mantle tissue gave significant yields compared to the tentacle in both the species. The two way ANOVA (Appendix 2: Table 2.9) result shows that there are no significant differences in the yield (%) of GAGs from the cranial cartilages of both the cephalopod species.

2.3.4 Histochemical localization of the intact GAGs

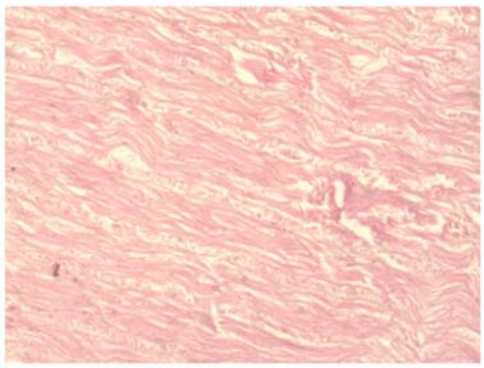
Inorder to determine the tissue distribution of glycosaminoglycans in the cephalopod samples, different tissues selected viz., mantle, tentacle, fin, skin,

gladius/pen and cranial cartilage were subjected to histochemical study by using two different staining methods. The results of the histochemical analysis are shown in Plates 2.1, 2.2 and 2.3. The Plates, 2.1 and 2.2 shows the histochemical images of the tissue sections of squid and cuttlefish stained using hematoxylin and eosin respectively. Plate 2.3 shows the tissue sections stained with a basic metachromatic dye, 1, 9 – dimethyl methylene blue (DMMB).

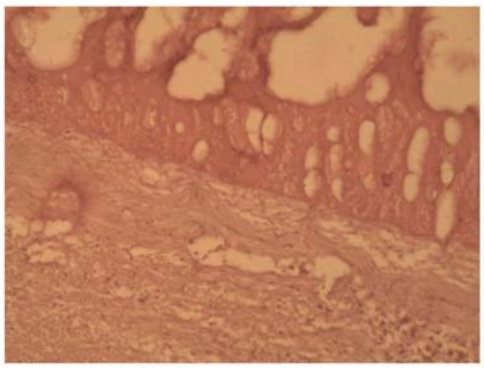
The hematoxylin and eosin staining shows a clear staining pattern representing the collagen fibres and myofibrillar components of the various tissues studied. The results obtained from the histochemical studies supports the data obtained on the percentage yield of GAGs. Surprising results were obtained for the sections of fin and cranial cartilage tissues (Plates 2.1 and 2.2, (c) and (f) respectively), with a staining pattern (purplish blue in colour) probably representing the acidic polysaccharides or more particularly the glycosaminoglycans. The study proves that glycosaminoglycans occur at different concentrations in the different tissues analyzed and that the GAGs are abundant in the cranial cartilages of both the cephalopod species studied (Plates 2.1(f) and 2.2 (f)), followed by the fins (Plates 2.1(c) and 2.2 (c)). The metachromatic dye, DMMB is used particularly for the identification and quantification of sulphated GAGs and the results obtained point out that the GAGs occurring in these tissues may be sulphated. The amount of GAGs in all the other samples analyzed were not sensitive enough to get stained using the 1, 9-DMMB staining protocol employed.

2.4 Discussion

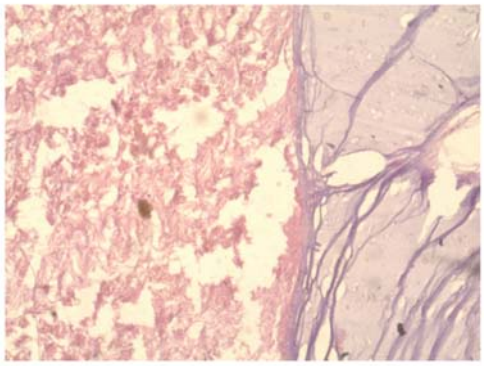
Biochemical studies are very important from the nutritional point of view. Seafood can make a significant contribution to the nutrient needs due not only to the high consumption *per capita* in several countries, but also to the increasing scientific evidence of its importance in a healthy diet. Seafood is a source of top-quality protein food that is low in calories, total fat, and saturated fat when compared to other protein-rich animal foods (Nunes and Batista, 2003). In addition seafood is a rich source of a variety of structurally novel and biologically active metabolites that are under investigation with the objective of developing new



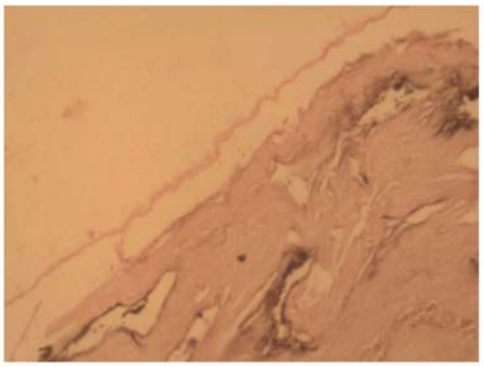
(a) mantle



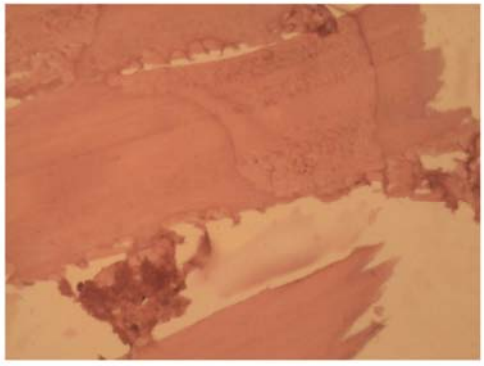
(b)tentacle



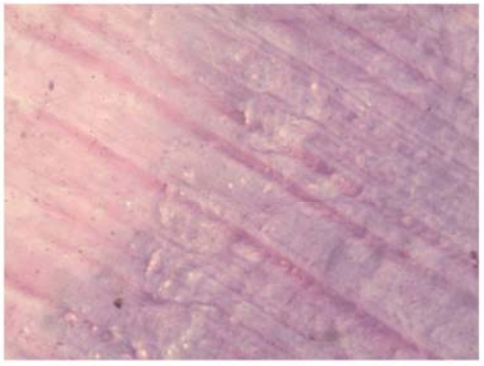
(c)fin



(d)skin

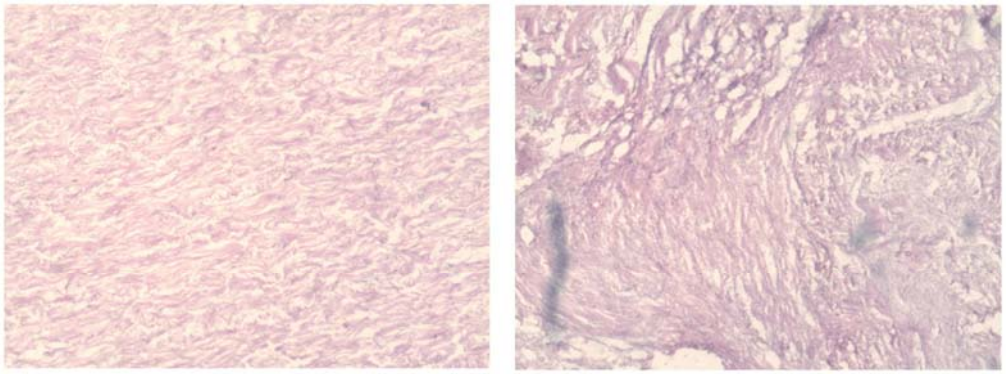


(e)gladius

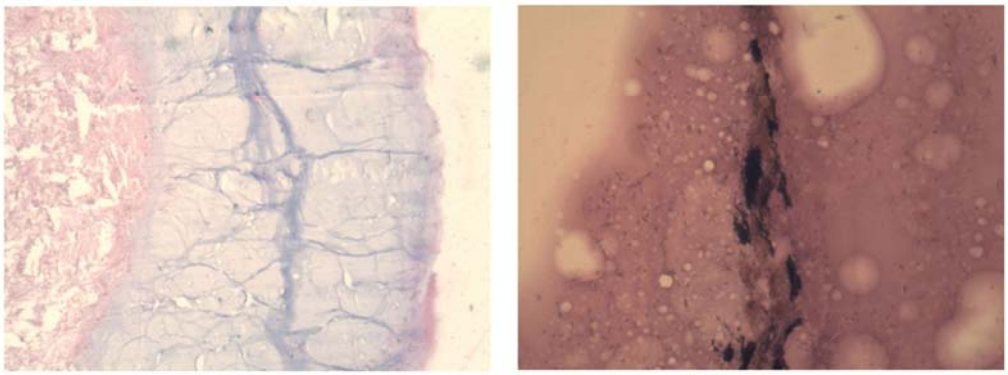


(f)cranial cartilage

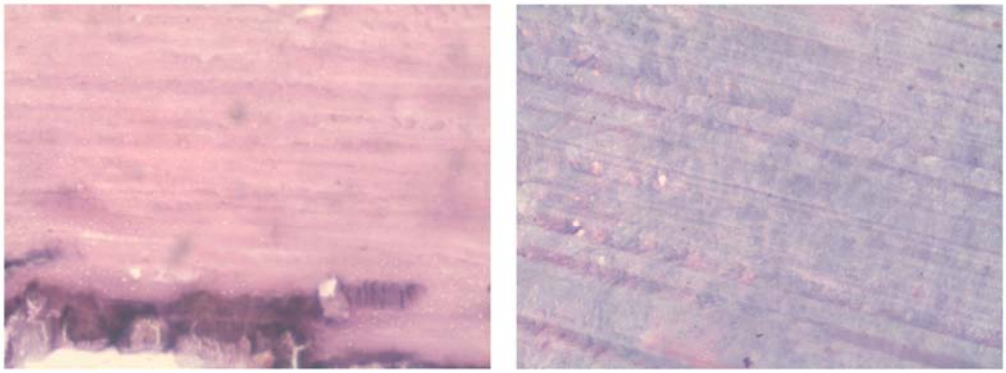
Plates 2.1: Histochemical sections of the selected tissues of squid (Hematoxyline and eosin staining)



(a) mantle **(b) tentacle**

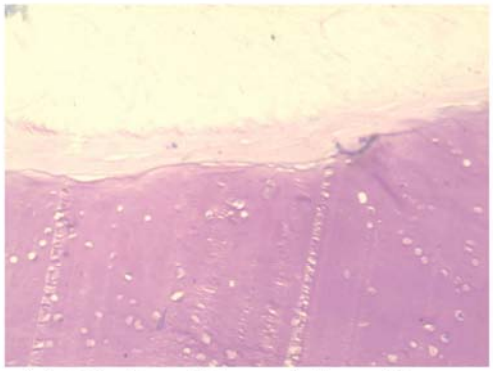


(c) fin **(d) skin**

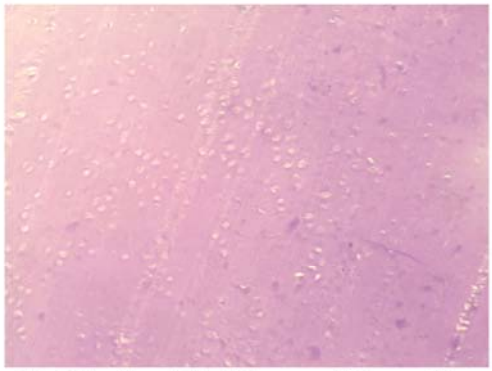


(e) gladius **(f) cranial cartilage**

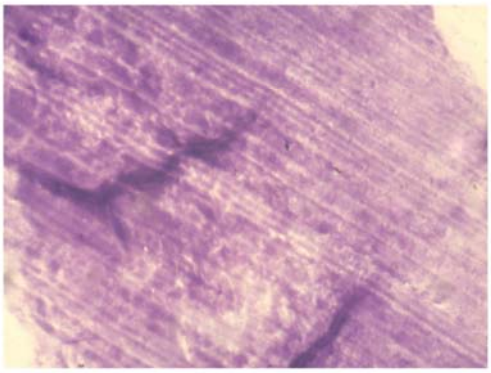
Plates 2.2: Histochemical sections of the selected tissues of cuttlefish (Hematoxyline and eosin staining)



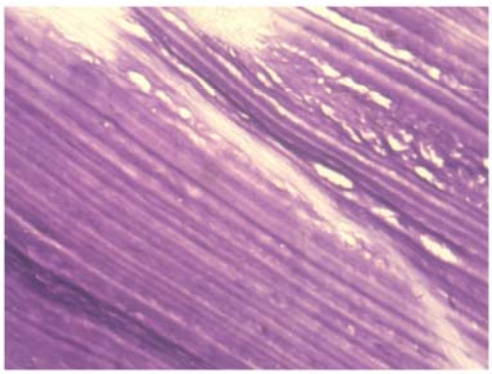
(a) LS of squid cranial cartilage



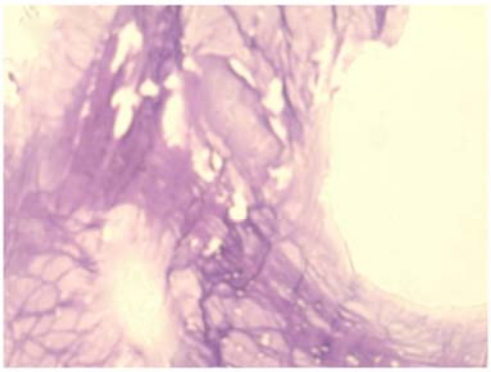
(b) LS of cuttlefish cranial cartilage



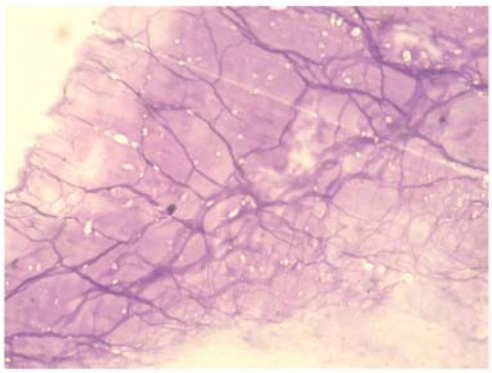
(c) CS of squid cranial cartilage



(d) CS of cuttlefish cranial cartilage



(e) Fin tissue of squid



(f) Fin tissue of cuttlefish

Plates 2.3: Histochemical sections of the tissues stained with 1, 9 - DMMB

pharmaceuticals and bioactive substances (Ely *et al*., 2004). Several studies have shown that marine molluscs are a rich source of sulphated polysaccharides, particularly glycosaminoglycans, with pronounced pharmacological activities or other properties useful in the biomedical area (Hovingh and Linker, 1993; Kim *et al.,* 1996; Ulrich and Boon, 2001; Santos *et al.,* 2002; Cesaretti *et al.,* 2004; Pandian and Thirugnanasambandan, 2008; Saravanan and Shanmugam, 2010; Gomes *et al.*, 2010). The present study reveals that *Loligo duvauceli* and *Sepia pharaonis,* the two cephaolods species possessing great economic importance in the Indian seafood industry are also a comparatively rich source of these biologically active metabolites, the glycosaminoglycans.

2.4.1. Proximate composition analysis

A general comparison of the major components present in the raw materials selected for the study (Table 2.2 and 2.3) indicates that the tissues contained varying amounts of moisture, protein, ash, and fat; which can be attributed to the structure associated functional properties of these tissues. According to Silas *et al*. (1985) the edible portions of cephalopods consisting of the mantle, arms/tentacle and fins forms 60-80% of the body weight and this is much higher than that in finfish or other shellfish. The high protein and low fat content of the meat of cephalopods makes them suitable for human consumption (Roper *et al*., 1984). The proximate composition analysis of the edible tissues of *Loligo duvauceli* and *Sepia pharaonis* shows that the results were in accordance to the earlier reports with significantly high amount of protein and low fat content (Thanonkaew *et al*., 2006; Nurnadia *et al*., 2011). Although the contents can vary from one fishing place to another, it remains that the main differences comes from the considered animal species. The biochemical constituents in animals are known to vary with season, size of the animal, stage of maturity, temperature and availability of food etc.

The composition analyses of the non-edible portions such as the skin, gladius/pen and cranial cartilages were done in order to assess their suitability in using as samples for GAG isolation and characterization. The results revealed that these tissues also contain significant amount of protein, which may, in part be

contributed by connective tissue proteoglycans, seen in association with glycosaminoglycans (GAGs). The major component analysis of the gladius/cuttlebone revealed that the shell of the both the species of cephalophods were a chitinous structure co-existing with proteins and certain minerals as reported by Kurita *et al*. (1993). The component analysis of the non-mineralized squid gladius infact showed that the proportion of protein was higher than chitin content and the results were in accordance to the reports of Chaussard and Domard (2004). According to recent reports the chitin present in these type of molluscan shell is of the β type which exhibits a higher accessibility to water and thus to water soluble reagents. It is also characterized by low mineralization, especially by the absence of calcium carbonate which is in contrast to that of *Sepia pharaonis*, as well as the α- chitin of arthropods. Reports by several authors reveal that the insoluble organic matrix of mollusc shells contain protein and carbohydrates (Xiao *et al*., 2005; Nudelman *et al*.,2006) as well as many important molecules involved in the interactions controlling crystal growth (Saleuddin, *et al*., 1969; Manoli and Dalas, 2000; Goldberg, 2001; Miyashita *et al*., 2001). Thus in the present study gladius/cuttlebone non-mineralized portions were also included as representative non – edible samples for screening the presence of GAGs.

2.4.2 Amino acid composition analysis

Amino acids are the basic components of the muscle protein structure of animals. Protein is essential for the sustenance of life and accordingly exists a major component of all nutrients. However, not all proteins have the same nutritional value, because protein quality strongly depends on its amino acid composition and digestibility. Fish, and in general, sea food proteins are considered as high quality proteins because of their balanced content in amino acids, especially of all the essential amino acids necessary for physical and mental well-being. Seafood is a source of high-quality protein that contains all of the nine essential amino acids, which are the building blocks needed by the body to make new proteins (Nunes and Bastista, 2003). Amino acids may also be found in free form, which contribute to fish taste and indirectly to aroma by generation of volatile compounds. Branched chain essential amino acids (valine, isoleucine, and

leucine), sulphur containing amino acids (methionine and cystine/cysteine), and aromatic amino acids (phenyl alanine and tyrosine) are the most important from this point of view. Free amino acids initiate important changes at early postmortem and during storage and can be very useful as quality indices of processing and storage. Thus, the analysis of amino acids in seafood, especially those considered essential, is important for the evaluation of both the nutritive value and the sensory quality of seafood.

HPLC is one of the preferred techniques to analyze amino acids. Amino acids, in their native form, absorb at 210nm and thus cannot be used for spectroscopic detection as it is a very unspecific detection wave length. Thus, the spectroscopic detection of amino acids requires their previous derivatisation to obtain an UV absorbing or fluorescent molecule. The derivatisation reaction can be performed after separation of the amino acids (post-column derivatisation) or before separating them (pre-column derivatisation). Pre column derivatisation methodology using phenyl-isothiocyanate (PITC) involves the conversion of primary and secondary amino acids to their phenyl-thiocarbamyl (PTC) derivatives, which are detectable at UV (254 nm) with detection limits around 5 – 50 picomoles (Shang and Wang, 1996). The PTC amino acids are moderately stable at room temperature for 24 hours and much longer when kept under frozen storage. All PTC amino acids have similar response factors, which constitutes an added advantage. The only limitation is the determination of PTC cystine that gives a poor linearity, which makes the quantitation of free cystine nonfeasible with this method.

The amino acid composition of the skin of cuttlefish was with surprisingly striking differences compared to other tissues, which can be attributed to the presence of specialized proteins termed 'Reflectins'. *Reflectins* are a recently identified protein family that is enriched in aromatic and sulphur containing amino acids that are used by certain cephalopods to manage and manipulate incident light in their environment (camouflage). These proteins were first identified by Crookes *et al.* (2004) in the reflective tissues of the squid *Euprymna scolopes.* They have a

very unsual composition with four relatively rare residues (tyrosine, methionine, arginine and tryptophan) comprising ~ 57% of a reflectin, and several common residues (alanine, isoleucine, leucine and lysine) (Kramer *et al.,* 2007). These protein based reflectors in cephalopods are a remarkable example of nanofabrication in animal systems. The high content of amino acids in cuttlefish skin may also be ascribed to the uptake of amino acids by the epithelial cells of the integument as explained by Eguileor *et al.* (2000).

2.4.3 Isolation of glycosaminoglycans

Glycosaminoglycans occur in the connective tissue matrix covalently linked to protein through *O*-serine or *O*-threonine linkages. Glycosaminoglycans can be removed from tissues as proteoglycans by concentrated salt solutions or as free polysaccharide chains by extraction with proteolytic enzyme or alkali followed by treatment with organic solvents and precipitation with quaternary ammonium salts and/or barium salts.

The preparation of acetone dry powder (ADP) using chilled acetone resulted in considerable amounts of dry defatted tissue (Table 2.4). The yield of ADP was depended upon the relative amount of moisture or fat present in the different tissues. Since all the tissues analyzed contained relatively lower fat content, the yield of ADP was mainly depened on the mositure content, as observed for the gladius/pen of squid and cuttlefish with higher yield of acetone dry powder due to the lower moisture content. Defatted tissues were further subjected to proteolytic digestion using papain. Papain (E.C. 3.4.22.1, molecular weight 38.9 KDa, optimal pH: 6.0 -7.0) is a cysteine hydrolase that is active under a wide range of conditions. It is very stable even at elevated temperatures. To exclude the possibility of partial proteolysis of the glycans, the solution of extracted glycans were incubated with fresh papain every 24 hour. The supernatant obtained on protease digestion was subjected to precipitation using 95% (v/v) ethanol to obtain crude GAGs. Since the GAGs are soluble in water, they could be separated from the papain digests by resuspending in water followed by centrifugation and lyophilization or vacuum drying. Ethanol, 95 % (v/v) in water

is an azeotropic mixture with a boiling point of 78.15°C and aids in the non – destructive precipitation of GAGs, especially of the chondroitin sulphate type.

Isolation of glycosaminoglycans from various sources by using different methods has been discussed by many workers. Mankin and Lippiello (1971) studied the glycosaminoglycan content and distribution in cartilages of osteoarthritic humans by subjecting the ethanol defatted tissues to successive pronase and papain digestion in phosphate buffer. Albano and Mourao (1986) isolated GAGs from ascidian tunic by a similar protocol of papain digestion, from the dry defatted tissue as well as from the proteoglycan isolates obtained by using guanidine hydrochloride. They reported that the sulphated polysaccharides can be extracted from the ascidian tunic by proteolytic enzyme or by guanidine hydrochloride solutions, in spite of the fact that a high molecular weight compound could not be extracted by guanidine hydrochloride solutions compared to the proteolytic digestion method. Nakano *et al.* (1996) isolated sulphated GAGs from porcine skeletal muscle by papain digestion followed by precipitation using CPC (cetylpyridinium chloride). Cesaretti *et al.* (2004) isolated heparin with high anticoagulant activity from the clam *Tapes phylippinarum* and demonstrated the evidence for the presence of a high content of antithrombin III binding site. Muthusamy *et al*. (2004) studied on the structural characterization of chondroitin sulphate proteoglycans (CSPG) from bovine trachea by using 4 M guanidine HCl (GdnHCl) extraction, followed by caesium chloride (CsCl) density gradient centrifugation. A similar method of isolation was followed by Pedersen *et al.* (1999) for the studies on GAGs from bovine semitendinosus (ST) and psoas major (PM) muscles. Nandini *et al.* (2005) and Li *et al.* (2007) extracted GAGs from the livers of blue shark (*Prionace glauca),* by a similar method followed by dehydration and delipidation with acetone. The enzyme used was actinase E, followed by trichloroacetic acid precipitation of residual proteins and peptides. The crude GAG fraction was recovered from the combined extract by precipitation with 80% ethanol and cetylpyirdinium chloride was used to precipitate the dermatan sulphate GAGs. Lauder *et al.* (2000) extracted CS-GAGs from fresh diced cartilages by papain digestion using sodium acetate buffer for 24 hours at 65°C.

The GAGs were further recovered by ethanol precipitation at 6°C. Liu *et al.* (2002) isolated glycosaminoglycans from the body wall of sea cucumber using a similar method of papain digestion followed by precipitation in 95% ethanol and studied the hypolipidemic effect of these GAGs in rats. Santos *et al.* (2002) adopted a unique method for the isolation of heparin from the mollusc, *Anomalocardia brasiliana* which involved the complexation of the GAGs obtained by digestion using maxatase, with an ion-exchange resin, Amberlite IRA 900, followed by filtering of the complex on cheese cloth and further recovery of the GAGs by a linear gradient of NaCl and final precipitation of the GAGs using methanol.

Chondroitin sulphate GAGs can also be liberated by activation of endogenous enzymes (autolysis), without using the expensive exogenous proteinases commonly used to extract GAG from tissues. Jibril (1967) incubated calf scapula cartilage in 0.1 M sodium acetate, ranging in pH from 2 to 9, at three different temperatures (3°C, 25°C, and 37°C) and found that the amount of uronic acid released by tissue autolysis is highest at pH 4.2 and 37°C. Nakano *et al.* (1998) extracted GAG from bovine cartilaginous tissues including nasal cartilage, occipital articular cartilage, and temporomandibular joint disk using 0.1 M sodium acetate, pH 4.5, at 37°C. Tissue autolysis was suggested to occur under the incubation condition to release GAG-peptide. Even though the above mentioned extraction method with 0.1 M sodium acetate is useful for the preparation of GAG-peptide at a low cost, the isolates occur as a GAG-peptide which requires further purification.

In the present study histochemical analyses proves that glycosaminoglycans occur at different concentrations in the different tissues analyzed and that the GAGs are prevalent in the cranial cartilages of both the cephalopod species studied (Plates 2.1(f) and 2.2 (f)), followed by the fins (Plates 2.1(c) and 2.2 (c)).The results obtained from the histochemical studies supports the data obtained on the percentage yield of GAGs from the different tissues (Fig. 2.4). Histochemical localization of the glycosaminoglycans in the tissues of different organisms was demonstrated by several workers. Gandra *et al.* (2000) demonstrated the presence

of sulphated glycosaminoglycans in the tissues of *Styela plicata* (Tunicata) using 1, 9 – DMMB method and reported the presence of various sulpahted GAGs in the intestine, heart, pharynx and cloak in this primitive chordate in different concentrations. Coleman *et al.* (1998) studied the proteoglycans and glycosaminoglycan chains of rabbit synovium using immunohistochemical methods and reported that the disruption of GAGs could be an important factor in joint disease. Xiaodong *et al.* (2007) employed histochemical techniques to study the structure and secretion function of the cuttlebone sac of the golden cuttlefish, *Sepia esculenta* in order to provide some histological basis for the mechanism of shell formation. Thirugnanasambandam and Pandian (2007) demonstrated the presence of GAGs in various tissues of the clam *Katelysia opima* using different histological staining procedures like alcian blue, aldehyde fuschin and periodic acid – Schiff's (PAS) reagent.

The results obtained in this study revealed that unusually high amount of glycosaminoglycans were found in the cranial cartilages of both squid and cuttlefish, among all tissues analyzed and the results are comparable to the amount of glycosaminoglycans in cartilages from other sources as well (Rodrigues *et al.,* 2005; Garnjanagoonchorn *et al.,* 2007). The presence of GAGs in the fins can be attributed to the long strip of cartilage that is present in the fins and aids as a supportive structure to the animal while swimming. The presence of GAGs in other edible tissues such as mantle and tentacle are of great importance from a nutritional point of view. The total GAG yield as a function of dry defatted weight differed little between the two cephalopod species.

The results reveal that the cranial cartilages of these cephalopods that can be excised with much ease during processing can be used commercially as a feasible source of GAGs. The isolated glycosaminoglycans were further analyzed for purity using various techniques including electrophoresis, ion-exchange HPLC, and susceptibility to enzymatic digestion in the succeeding chapters.

2.5 Conclusion

Seafood is a rich source of a variety of structurally novel and biologically active metabolites that have the prospect of utilization as pharmaceuticals and bioactive substances. Glycosaminoglycans (GAGs) from various sources have been shown to have several physiological functions, but very little is known of the functions of the GAGs from cephalopod species, especially in the Indian waters. The present study on the distribution and localization of sulphated glycosaminoglycans in the tissues of squid (*Loligo duvauceli*) and cuttlefish (*Sepia pharaonis*) may bring new insights about the function of this class of polysaccharides in invertebrates.

At present glycosaminoglycans are produced by chemical companies from slaughter house discards and also from potentially endangered species like whales, shark, rays etc. The currently available methods for GAG preparation are expensive and also the biotechnological production processes for glycosaminoglycans is in the infantile stage. Thus in the present situation the development of techniques by more reliable methods for the production of GAGs from readily available sources is needed. The protocol developed in this study employs the liberation of GAGs directly from the defatted tissues by digestion with an exogenous proteinase (papain) and further precipitation using ethanol. The present study reveals that among all the tissues analyzed, the cranial cartilage of both the cephalopod species contained appreciable amount of glycosaminoglycans. Cartilage is a nonedible byproduct with little saleable value, but its rich presence of glycosaminoglycan (GAG) can revolutionize it to a material, which can be converted to a highly profitable product. In this context the present protocol developed for the isolation of GAGs from the selected tissues of squid and cuttlefish and the analytical techniques employed for the identification of GAGs will provide great insights into a much reliable method for the extraction and identification of glycosaminoglycans from other potential sources as well.

Chapter 3: Qualitative and Quantitative Analysis of the Isolated Glycosaminoglycans

Contents

3.1 Introduction

The analysis of isolated GAGs includes both the determination of macroscopic properties and the study of the GAG composition. This is done firstly by the evaluation of the purity of the isolated polysaccharides and second by the characterization of these compounds. Chromatographic, electrophoretic and spectroscopic (NMR, FT-IR) methods are commonly used in carrying out these two purposes. The strategies used to recover this analytical information focus on the study of either the GAGs or their oligosaccharide and disaccharide fragments obtained after enzymatic or chemical depolymerisation.

Electrophoresis is the process of migration of charged molecules through solutions in an applied electric field. In gel electrophoresis, molecules are separated in aqueous buffers supported within a polymeric gel matrix. A variety of matrices including starch, agarose, acrylamide and cellulose acetate are used for electrophoresis. The high resolution capacity of polyacrylamide and agarose gel electrophoresis makes them the method of choice for most applications. Agarose (a polygalactose polymer) gels are particularly used when applied to very large macromolecules such as nucleic acids, lipoproteins and others. Polyacrylamide (formed as a result of the polymerization of acrylamide (monomer) and N, N^1-methylene bis acrylamide (cross-linker) gels are among the most useful and most versatile in gel electrophoresis separations, because they can readily resolve a wide array of proteins and nucleic acids.

Agarose gels are generally used for the electrophoresis of proteins, nucleic acids and other macromolecules as agarose gels are capable of forming gels with large pore sizes. Agarose gels are also used for the electrophoresis of polyanionic glycans such as the glycosaminoglycans and proteoglycans (Cassaro and Dietrich, 1977; Bjornsson, 1993; Dietrich *et al*., 1989; Mulloy *et al.,* 1994; Theocharis *et al.*, 1999; Volpi and Maccari, 2002). A simple and reliable agarose gel electrophoretic method has been employed in this study for the identification and preliminary characterization of the GAGs isolates from the respective tissues of squid (*Loligo duvauceli*) and cuttlefish (*Sepia pharaonis*).

Anion exchange chromatography is a type of adsorption chromatography in which retention of a solute (negatively charged) occurs due to its reversible electrostatic interaction with the oppositely (positively) charged groups on an ion exchange resin. DEAE-cellulose is an example of a weak anion exchanger, where the amino group can be positively charged below pH ~ 9 and gradually loses its charge at higher pH values. A strong anion exchanger, on the other hand, contains a strong base, which remains positively charged throughout the pH range normally used for ion exchange chromatography (pH 1-14). The glycosaminoglycans, in general are highly (negatively) charged molecules and can thus be separated easily by anion exchange techniques.

The development of a number of high performance liquid chromatography (HPLC) techniques for the analysis of carbohydrates allows for a much more detailed study of polymeric sugars including GAGs (Imanari *et al*., 1996; Karamanos *et al.,* 1997; Falshaw *et al.*, 2000). By manipulating the chemistry of the stationary phase and/or composition of the mobile phase, along with other operating conditions (*ie.* elution pattern), it is possible to obtain very subtle separations of the compound of interest. Therefore the isolated glycosaminoglycans were also analyzed by means of HPLC techniques.

3.2 Materials and methods

The following commercial chemicals/biochemicals (numbers 1-4) used for the study were obtained from Sigma-Aldrich, Saint Louis, USA.

1. Chondroitin sulphate A (C9819, molecular weight (MW) 20-30 kD, 70% pure, 30% CS C, 15.1% sulphate).
2. Chondroitin sulphate B (C3788, MW 40-45 kD, 90% pure, 18.5% sulphate).
3. Chondroitin sulphate C (C4384, MW 50-58.8 kD, 90% pure, 18.0% sulphate). Hyaluronic acid (H1504)
4. Chondroitinase ABC (EC 4.2.2.4) isolated from *Proteus vulgaris* (Product No C2902).
5. Papain, 2 x crystalline (EC 3.4.22.2) and CTAB (cetyltrimethylammonium bromide) were from E Merck, A.G, (Darmstedt, Germany).
6. Toluidine blue was from Fisher Scientific (USA).
7. DEAE – cellulose (DE52) was from Whatman International (Kent, Great Britain).
8. Agarose (standard medium EEO) and Proteinase K were from SRL, India Pvt. Ltd.
9. 1, 9 – dimethyl methylene blue (DMMB) chloride (catalogue No 03610), was from Polysciences Inc, Warington, PA.

   All other chemicals used were of analytical grade.

3.2.1 Chemical composition analysis

The content of hexuronic acid, hexosamines, sialic acid, hydroxyproline, fucose, protein and sulphated GAG were estimated using the standardized procedures by subtracting the values obtained for control papain from that of the crude GAG precipitates of each of the sample. All OD (optical density) measurements were taken using a Hitachi (Model No U 2800) UV/VIS Spectrophotometer. The details of various methods adopted are described below.

3.2.1.1 Estimation of hexosamines

The hexosamines were determined after hydrolysis of the samples (crude GAGs) with 6 M HCl at 100°C for 4 hours according to the method of Rondle and Morgan (1955). To 1.0 ml of the hydrolysed samples added 1.0 ml acetyl acetone reagent. Washed down the tubes with 1.0 ml distilled water, capped using glass stoppers and kept in a boiling water bath for 20 minutes. Cooled to room

temperature and added 5.0 ml of redistilled ethanol. Finally added 1.0 ml Ehrlich's reagent and made up the volume to 10.0 ml with ethanol. The tubes were warmed in a water bath at 65 to 70°C for 10 minutes. Cooled to 18°C and the OD were measured at 530nm. Glucosamine/galactosamine standards were also run for calculation.

3.2.1.2 Estimation of uronic Acid

The estimation of hexuronic acid in the samples was carried out by the carbazole reaction of Dische (1947), using glucuronolactone as standard. To the tubes placed in an ice bath added 5 ml of borax solution (0.025% in Conc. H_2SO_4. 1.0 ml each of samples were layered over this and mixed thoroughly. It was then brought to room temperature and heated in a boiling water bath for 10 minutes. The tubes were again cooled to room temperature and added 0.2 ml carbazole reagent to each tube. Sample tubes were then placed in a boiling water bath for 15 minutes cooled to room temperature and OD was measured at 530 nm.

3.2.1.3 Estimation of sialic acid

Sialic acid was determined by the thiobarbituric acid (TBA) method of Aminoff (1961) after hydrolysis of the samples with 0.05 M H_2SO_4 at 80°C for one hour. N-acetyl neuraminic acid was used as standard. To 0.5 ml of aqueous samples added 0.25 ml of periodic acid reagent and kept in a water bath at 37°C for 30 minutes. Added 0.2 ml of sodium arsenite reagent to each tube to remove excess periodate and kept aside for 2 minutes. Mixture was heated in a boiling water bath for 7 minutes after the addition of 2ml of thiobarbituric acid reagent and cooled immediately in an ice bath. Added 5 ml of n-butanol acid mixture and the contents were mixed well and centrifuged at 500xg for 10 minutes. Optical density of the supernatants was measured at 580nm.

3.2.1.4 Estimation of hydroxyproline

Hydroxyproline (as a measure of collagen), was determined by the method of Woessner (1961) after hydrolysis of the samples in 7N H_2SO_4 at 105°C for 3 hours. The hydrolyzed samples were filtered using Whatman No. 1 and diluted to 2.0 ml with distilled water before estimation. To the samples thus prepared, added

1.0 ml of Oxidant solution (Chloramine T reagent). Mixed well and kept undisturbed for 20 minutes at room temperature. Added 1.0 ml of colour reagent (p–dimethylamino benzaldehyde), mixed gently and capped each tube using glass stoppers and immediately palced in a water bath at 60 $\pm$ 5°C for 15 minutes. Cooled under running tap water and measured the OD at 588 $\pm$ 2nm. Freshly prepared hydroxyproline solution was used as standard.

3.2.1.5 Estimation of fucose

Fucose was estimated by the method of Dische and Shettles (1948). Samples for analysis were made up to 1 ml with distilled water, followed by the addition of 4.5 ml cold sulphuric acid reagent to the tubes cooled in an ice bath. Vortex mixed immediately to ensure mixing. Tubes were brought to room temperature (20-22°C). Tubes were capped and placed in a boiling water bath for 3 minutes and cooled to room temperature in a water bath. To each tube added 0.1 ml of cysteine reagent. Separate aliquots of each sample heated with H_2SO_4 reagent but without the addition of cysteine reagent were also set. The absorption due to methyl pentoses were determined by subtracting the $OD_{396} - OD_{427}$ of the samples analysed without cysteine reagent from the $OD_{396} - OD_{427}$ of the samples analysed with cysteine reagent.

3.2.1.6. Estimation of protein

Protein in the samples was estimated according to Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard. Pipetted out 0.1 to 1.0 ml of standard BSA solution as well as samples into test tubes and made up the volume to 1.0 ml in each tube. To all tubes added 5.0 ml of alkaline copper reagent and kept for 10 minutes at room temperature. Added 0.5 ml of Folin-ciocalteau phenol reagent to all tubes and mixed well. Absorbance was measured at 670 nm.

3.2.1.7 Estimation of sulphated glycosaminoglycans

Sulphated GAGs was determined according to the 1, 9 - dimethyl methylene blue (DMMB) dye-binding assay of Barbosa *et al.* (2003). Materials (crude GAG samples) were digested in a solution of 50µg/ml Proteinase K in 100mM K_2HPO_4, pH 8.0 at 56°C overnight. Proteinase K was inactivated by

heating the preparation for 10 minutes at 90°C (Calabro *et al.*, 2000). The samples were centrifuged and filtered through ultra free filters to eliminate DNA and tissue debris from the extract and used for the estimation of sulphated GAG.

Added 1.0 ml DMMB complexation solution to the above Proteinase K treated samples and vortex mixed for 30 minutes to promote complete complexation of the GAG with DMMB. Insoluble GAG – DMMB complex were separated from the soluble materials including DMMB excess by centrifugation (12,000 x g, 10 minutes). Supernatants were discarded and the pellets were dissolved in 1.0 ml of decomplexation solution and mixed well for 30 minutes. Chondroitin sulphate standards were also subjected to DMMB complexation and decomplexation and the absorbance was measured at 656 nm.

3.2.2 Electrophoretic methods

3.2.2.1 Agarose gel electrophoresis

Glycosaminoglycans obtained by papain digestion and subsequent ethanol precipitation (Section 2.2.4) were analyzed using agarose gel electrophoresis by the method of Bjornsson (1993), with modifications. The crude GAG precipitates were electrophoresed on a agarose gel electrophoresis using 10 mM Tris/acetate buffer, pH 8.3. Chondroitin-4-sulphate (CS-A), dermatan sulphate (CS-B), chondroitin-6-sulphate (CS-C), heparin and hyaluronic acid (HA) were used as standards. The gels were run on a horizontal gel electrophoretic system at 110V for 3 hours. After electrophoresis the macromolecules were fixed with 0.1% Cetavlon (CTAB or N-cetyl-N,N,N-trimethylammonium bromide) according to the method of Pavao *et al*. (1995), and stained with 0.02% (w/v) toluidine blue in 3% acetic acid containing 0.5 % (v/v) triton x 100. The gels were finally destained using 3% acetic acid and the results were documented. Gel concentrations containing 2.0 % agarose gels were most suitable for the characterization of the glycosaminoglycan isolates from squid and cuttlefish.

3.2.2.2 Susceptibility of GAGs to enzyme activity

Glycosaminoglycans (~50 µg each) obtained from the cranial cartilages of squid and cuttlefish (Section 2.2.4) were incubated with 0.01 U chondroitinase

ABC (EC 4.2.2.4) at 37ºC for 12 h in 50 mM Tris/HCl buffer (pH 8.0). Process was repeated by adding 0.01U of fresh enzyme and the reaction continued for another 12 hours (Gandra *et al*., 2000). The susceptibility of the glycosaminoglycans to chondroitinase ABC digestion was checked by comparing the gel pattern obtained with that of intact glycosaminoglycans using agarose gel electrophoresis method (Section 3.2.2.1).

3.2.2.3 SDS - Polyacrylamide gel electrophoresis.

Single dimension SDS/PAGE was performed on a vertical slab gel electrophoresis system according to Laemmli (1970) with and without prior treatment with 5% (v/v) β- mercaptoethanol (β-ME) in order to analyze the purity of the GAG isolates. The running gels were 12% and the sample wells were made using a 20 well template. Sample digestions were performed by incubating the 0.1N NaOH solubilized samples with an equal volume of SDS sample buffer at 100°C for 5 to 10 minutes. The gels were stained with CBB R-250 (coomassie brilliant blue R-250). A mixture of 7% acetic acid and 20% methanol in water was used as destain.

3.2.3. Chromatographic methods

3.2.3.1 DEAE - Cellulose anion exchange chromatography

DEAE – cellulose anion exchange chromatography was performed according to the method of Vieira *et al.* (1991). Briefly, 200 mg crude GAG extract was applied to a DEAE –cellulose column (7 x 2 cm) equilibrated with 0.05 M sodium acetate buffer (pH 5.5). The column was eluted first with 0.05 M Sodium acetate buffer (pH 5.5) followed by 0.05 M sodium acetate buffer (pH 5.5) containing 1:1 (v/v) 1.2 M NaCl and finally upto 2.0 M NaCl. The flow rate of the column was 12ml/hour and fractions of 3.0 ml were collected and glycosaminoglycans were analyzed by the DMMB dye binding assay (Section 3.2.1.7). The column was finally washed with four bed volumes of 2.0 M NaCl.

3.2.3.2 Glycosaminoglycan analysis by SAX – HPLC

Analytical strong anion exchange (SAX) HPLC was performed to identify the constitutive disaccharide obtained upon digestion with chondroitinase ABC.

Identification and quantification of the squid as well as cuttlefish GAG samples were performed as described by Falshaw *et al*. (2000).

3.2.3.2. (a) Sample preparation

The glycosaminoglycans obtained from the respective tissues of squid and cuttlefish were incubated with chondroitinase ABC as mentioned in section 3.2.2.2. The samples were dissolved in Milli-Q grade water and filtered using 0.45 µm Nylon filters (Millipore, USA). For chromatographic analysis 20µl each of the above samples were injected into the HPLC (Water's HPLC Model No 2487).

3.2.3.2. (b) Apparatus

The HPLC apparatus is the same as mentioned in section 2.2.3.3 except that the column used for identification of the constitutive disaccharide units was a Spherisorb 5µm, 250mm length (£), 4.6 mm i.d., SAX (strong anion exchange) column. The detection was monitored at 232nm using a dual λ absorbance detector (UV/VIS Model 484). Data analysis was performed using EMPOWER 2 chromatography software.

3.2.3.2. (c) Chromatographic conditions

Chromatographic separation made use of continuous gradient elution with Solvent A (NaH_2PO_4 buffer 0.001 M, pH 6.00 containing 0.15 M NaCl) and Solvent B (NaH_2PO_4 buffer 0.001 M, pH 6.00 containing 2.0 M NaCl). From 0 to 4 min the column was eluted isocratically with a NaH_2PO_4 buffer (0.001 M, pH 6.00 containing 0.15 M NaCl). From 4 to 15 min the NaCl concentration was linearly increased to 0.47 M (keeping the pH and phosphate concentration constant), and then from 15 to 18 min the NaCl concentration was linearly increased to 2.0 M. The column was then eluted isocratically for 12 min with NaH_2PO_4 buffer (0.001 M, pH 6.00 containing 2.0 M NaCl). The flow rate of the column was 1 ml/min. The disaccharides were identified by their retention times using standards obtained from Sigma chemical company. The concentrations of all the standards were 10µg/µl. The peak areas of all individual samples were

compared with standard disaccharides. HPLC gradient profile designed for separation of GAG - disaccharides is given in Table 3.1.

Table 3.1: HPLC gradient profile developed for the analysis of GAG – disaccharides

Sl. No.	Flow (ml/ min)	Time (min)	% Solvent A	% Solvent B	%C	Curve
1	1.00	0	100.0	0.0	0.0	6
2	1.00	4	100.0	0.0	0.0	6
3	1.00	15	83.0	17.0	0.0	6
4	1.00	18	0.0	100.0	0.0	6
5	1.00	30	0.0	100.0	0.0	6

3.3 Results

3.3.1. Chemical composition analysis of the GAG isolates

The chemical composition analyses of the ethanol precipitated fractions were determined after subtracting the values obtained for control papain from that of the samples. The results (Table 3.2 and 3.3 respectively of squid and cuttlefish) indicated that polyanionic glycans are widely distributed in both the sample species, constituting not only supporting structures, but also specialized tissues and organs. The relative amounts of the sulphated GAGs as obtained by the 1, 9 – DMMB method and the content of hexosamine and uronic acid of the GAGs extracted from the different tissues of squid and cuttlefish showed that large variations in the amounts of sulphated mucopolysaccharides were observed among the tissues analyzed. The protein and hydroxyproline estimations showed that the samples were free of collagen or any other residual proteins. Sialic acid was absent in all of the samples analyzed. Squid cranical cartilage glycans showed exceptionally positive results for fucose.

Table 3.2: Composition of the GAG isolates in the edible and non-edible tissues of squid.

Chemical composition analysis of squid crude GAGs (mg/g dry tissue)							
		Edible Tissue			Non Edible Tissue		
Sl. No.	Compound	Mantle	Tentacle	Fin	Skin	Gladius	Cranial cartilage
1	Hexosamines	3.25 ± 0.65	3.5 ± 0.11	6.59 ± 0.67	3.09 ± 0.87	0.46 ± .01	41.8 ± 0.55
2	Uronic acid	4.27 ± 0.55	1.98 ± 0.28	8.42 ± 1.03	3.22 ± 0.98	0.018 ± 0.00	38.03 ± 0.92
3	Sialic acid	*ND	ND	ND	ND	ND	ND
4	Hydroxyproline	ND	ND	ND	ND	ND	ND
5	Fucose **	ND	ND	ND	ND	ND	0.36 ± 0 .05
6	Protein	ND	ND	ND	ND	ND	ND
7	Sulphated GAG	7.65 ± 0.24	4.23 ± 0.07	12.73± 0.52	5.28 ± 0.02	ND	78.15 ± 0.62

*ND: not detected

** µg/g

Table 3.3: Composition of the GAG isolates in the edible and non-edible tissues of cuttlefish.

Chemical composition analysis of cuttlefish crude GAGs (mg/g dry tissue)							
		Edible Tissue			Non Edible Tissue		
Sl. No.	Compound	Mantle	Tentacle	Fin	Skin	Pen	Cranial cartilage
1	Hexosamines	4.11 ± 0.88	0.79 ± 0.11	3.88 ± 0.94	0.84 ± 0.15	0.18 ± 0.01	36.8 ± 0.45
2	Uronic acid	2.98 ± 0. 35	1.02 ± 0.66	5.21 ± 0.39	0.59 ± 0.008	0.042 ± 0.00	32.08 ± 0.95
3	Sialic acid	*ND	ND	ND	ND	ND	ND
4	Hydroxyproline	ND	ND	ND	ND	ND	ND
5	Fucose	ND	ND	ND	ND	ND	ND
6	Protein	ND	ND	ND	ND	ND	ND
7	Sulphated GAG	6.11± 0.98	1.91 ± 0.01	8.49 ± 1.21	1.47 ± 0.09	ND	69.19 ± 1.36

*ND: not detected

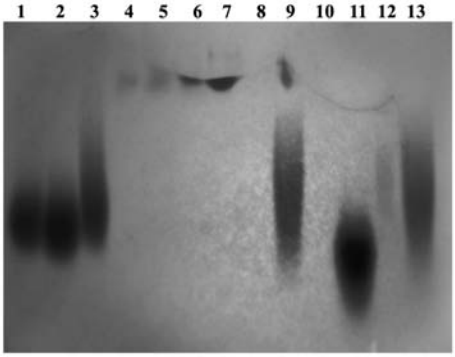
3.3.2 Electrophoretic methods

3.3.2.1 Agarose gel electrophoresis

Agarose gel electrophoresis of the glycans was done according the method of Bjornsson (1993) with modifications by employing a 2% gel concentration in 10mM Tris acetate buffer using a horizontal electrophoresis system. In order to determine the tissue distribution of sulphated glycosaminoglycans in the cephalopod specimens, the respective tissues from the edible portions (mantle, tentacle and fin) and non-edible portions (skin, gladius and cranial cartilage) were subjected to papain digestion and precipitation of the GAGs using ethanol as depicted in section 2.2.4, and the extracted glycans were analyzed by agarose gel electrophoresis. Electrophoretic mobility of the samples on agarose gel was visualized with toluidine blue staining.

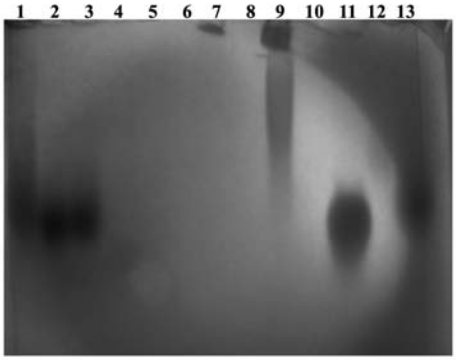
Agarose gels with a 2.0% concentration were found to be most appropriate for the electrophoresis of the glycans isolated from the tissues of *Loligo duvauceli* and *Sepia pharaonis*. The isolated glycans were eluted off the gel with a lower concentration (< 2 %), whereas gels with higher agarose concentrations (> 2%) did not permit the entry of glycans into the gel matrix. Plates 3.1 and 3.2 respectively shows the toluidine blue stained agarose gel electophoretograms of the GAGs extracted from the tissues of squid and cuttlefish. The concentrations of all the standard GAGs were ~10µg/µl in each lane, except for hyaluronic acid (~ 5µg/µl).

Toluidine blue staining of the agarose gels showed that, there was great variation in electrophoretic mobilities among the GAG isolates from various tissues. Electrophoretic separation of the crude papain digests of the respective tissues of squid and cuttlefish revealed the presence of glycosaminoglycans with mobility slightly lower than that of standard GAGs. The toluidine blue was bound only to the sulphated polysaccharide, but was not found to bind with other compounds as evidenced by the absence of bands in the gel lanes loaded with control papain (Plate 3.1 and 3.2, lane numbers 10). The cranial cartilage glycans (~20µg/µl) in both the cephalopod species migrated as the main metachromatic band in each of the gels (Plate 3.1 and 3.2, lane numbers 9).



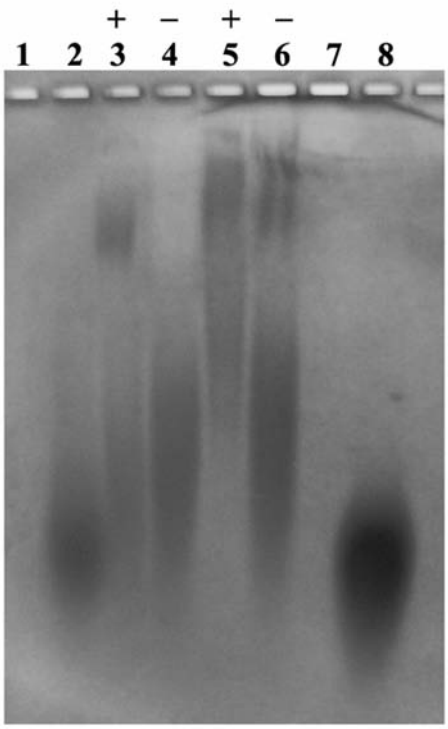
Lanes
1: standard marker Chondroitin sulphate A
2: Chondroitin sulphate B
3: Chondroitin sulphate C
4: mantle GAGs
5: skin GAGs
6: tentacle GAGs
7: fin GAGs
8: glycans from gladius
9: cranial cartilage GAGs
10: control papain
11: standard heparin
12: hyaluronic acid
13: mixed marker of chondroitin sulphate A, B, & C.

Plate 3.1: Agarose gel electrophoretic pattern of the glycosaminoglycans isolated from the selected tissues of squid.



Lanes
1: standard marker Chondroitin sulphate A
2: Chondroitin sulphate B
3: Chondroitin sulphate C
4: mantle GAGs,
5: skin GAGs
6: tentacle GAGs
7: fin GAGs
8: glycans from pen
9: cranial cartilage GAGs
10: control papain
11: standard heparin
12: hyaluronic acid
13: mixed marker of chondroitin sulphate A, B, & C.

Plate 3.2: Agarose gel electrophoretic pattern of the glycosaminoglycans isolated from selected tissues of cuttlefish.



Lanes
1: blank
2: standard heparin
3: chondroitinase ABC treated (+) cuttlefish cranial cartilage GAGs
4: chondroitinase ABC untreated (-) cuttlefish cranial cartilage GAGs
5: chondroitinase ABC treated (+) squid cranial cartilage GAGs
6: chondroitinase ABC untreated (-) squid cranial cartilage GAGs
7: control papain
8: mixed marker of chondroitin sulphate A, B, & C

Plate 3.3: Agarose gel electrophoretic pattern of the Chondroitinase ABC treated (+) as well as untreated (-) glycosaminoglycans isolated from cranial cartilages of squid and cuttle fish.

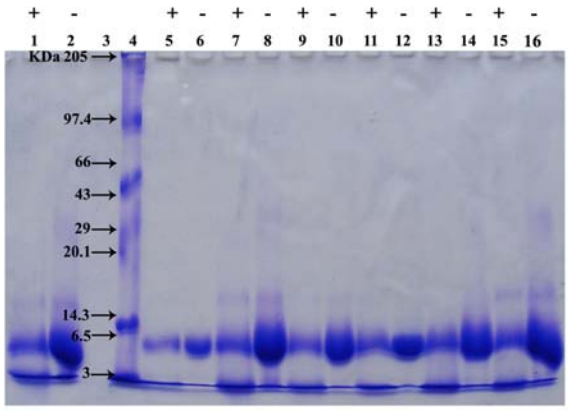


Plate 3.4: SDS - PAGE of the crude glycosaminoglycan fraction obtained from the different tissues of squid

Lane 1: control papain (+), Lane 2: control papain (-), Lane 3: blank, Lane 4 : molecular weight marker, Lane 5: crude GAGs of cranial cartilage (+), Lane 6: cranial cartilage (-), Lane 7: crude glycans of gladius (+), Lane 8: gladius (-), Lane 9: fin (+), Lane 10: fin (-), Lane 11: tentacle (+), Lane 12: tentacle (-), Lane 13: skin (+), Lane 14: skin (-), Lane 15: mantle (+), Lane 16: mantle(-)

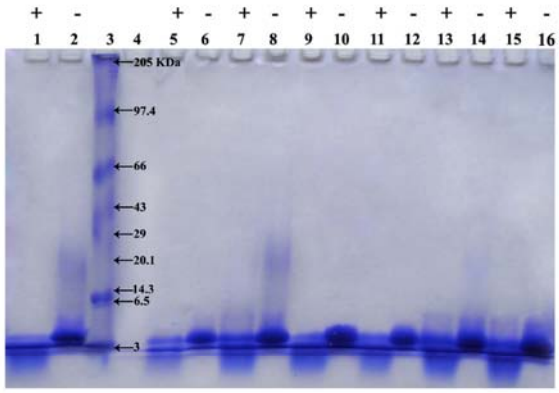


Plate 3.5: SDS - PAGE of the crude glycosaminoglycan fraction obtained from the different tissues of cuttlefish

Lane 1: control papain (+), Lane 2: control papain (-), Lane 3: molecular weight marker, Lane 4: blank, Lane 5: crude GAGs of cranial cartilage (+), Lane 6: cranial cartilage (-), Lane 7: crude glycans of pen (+), Lane 8: pen (-), Lane 9: fin (+), Lane 10: fin (-), Lane 11: tentacle (+), Lane 12: tentacle (-), Lane 13: skin (+), Lane 14: skin (-), Lane 15: mantle (+), Lane 16: mantle(-)

The glycosaminoglycans from the fin in both the species (Plate 3.1 and 3.2, lane numbers 7) displayed almost similar electrophoretic pattern. The glycans from the mantle, skin and tentacle in the case of cuttlefish displayed metachromatic bands with low mobility and less defined migration and was obtained as faint bands on completion of the destaining procedure (Plate 3.2, lane numbers 4, 5 and 6 respectively). The mantle, skin and tentacle glycosaminoglycans in the case of squid displayed metachromatic bands with low mobility and expected to be of high molecular weight compared to the cranial cartilage glycans (Plate 3.1, lane numbers 4, 5 and 6 respectively). Most strikingly, the gladius/pen in both the cephalopod species did not show any metachromatic bands (Plate 3.1 and 3.2, lane numbers 8) indicating that these tissues contained no detectable amounts of sulphated glycosaminoglycans. The results, although preliminary, and based upon a relatively small number of randomly selected tissues, suggested that these compounds have indeed different structures and occur in different proportions depending on the species and type of tissue analyzed. The lanes with control papain ((Plate 3.1 and 3.2, lane numbers 10) did not show any metachromatic bands indicating that residual proteins in the papain control are not retained in the gel matrix and gets eluted off during electrophoresis due to their low molecular weights. The above results thus reveals that the GAGs could be successfully isolated using the protocol (Section 2.2.4) designed in this study.

3.3.2.2 Susceptibility to enzyme activity

In order to assess the susceptibility of the GAGs to the action of chondroitinases, a qualitative analysis using agarose gel electrophoresis was performed after digestion of the glycosaminoglycans with chondroitinase ABC. The crude GAGs obtained from the cranial cartilages of both the cephalopod specimens were used as substrates. The results (Plate 3.3) showed that the GAGs from the cranial cartilages of both the species of cephalopods were almost resistant to degradation with chondroitin ABC lyase under the stipulated conditions.

3.3.2.3. Qualitative SDS –PAGE

Qualitative SDS-polyacrylamide gel electrophoresis was performed for

crude glycosaminoglycan isolates of the respective squid and cuttlefish tissues, in order to assess the purity of these GAGs. The gels were visualized after staining with commassie brilliant blue. The broad range molecular weight markers (HMWM 205 KDa to 3 KDa) consisted of the following proteins: Myosin (205 KDa), Phosphorylase B (97.4 KDa), Bovine serum albulin (66 KDa), Ovalbumin (43 KDa), Carbonic anhydrase (29 KDa), Soyabean trypsin inhibitor (20.1 KDa), Lysozyme (14.3), Aprotinin (6.5 KDa) and Insulin (3 KDa). The Plates 3.4 and 3.5 show the electrophoretogram in 12% SDS-PAGE gels of the crude glycosaminoglycans obtained from the different tissues of squid and cuttlefish respectively. Comparison of the protein bands in the gels with their R_f values showed a similar pattern and hence the bands were compared visually. The protein bands in the gels showed the presence of only papain residues on comparsion of the sample lanes with that of control papain (Lane numbers 1 and 2 respectively, the β ME untreated (-) as well as β ME treated (+) in plates 3.4 and 3.5). The results revealed that the crude GAG isolates of all the samples were free of any connective tissue proteins, proteoglycans, collagen or link proteins and comprised only papain that gets precipitated along with the GAGs during ethanol (95% v/v) precipitation which can be easily removed from the preparation as mentioned in Section 2.2.4.

3.3.3 Chromatographic methods

3.3.3.1. DEAE - Cellulose anion exchange chromatography

The glycosaminoglycans of the cranial cartilage were separated by ion exchange chromatography on a DEAE–cellulose column in order to purify and further fractionate the GAGs. Typical chromatograms of squid and cuttlefish glycosaminoglycans are shown in Fig. 3.1 and 3.2 respectively. Glycosaminoglycans were eluted in a broad peak, from 0.6M NaCl up to 2 M NaCl, with recovery over 98%, which would represent different glycosaminoglycans regarding their structure or charge density or both. The majority of the material was observed between 0.6 and 1.0 M NaCl. In the case of cuttlefish GAGs there were two prominent peaks in high ionic strength. This again indicates the presence of an unsulphated species along with a more highly charged

one. The elution profile of squid and cuttlefish cranial cartilage GAGs can be compared to that of the agarose gel electrophoretograms (lane 9, Plate 3.1 and 3.2 respectively). The chondroitin sulphate species migrated on electrophoresis slightly slower than reference chondroitin sulphate. The pattern of elution would represent high-molecular-weight and charge heterogeneity of glycosaminoglycan chains based on their structure or charge density or both. Other glycosaminoglycans, if present, were in very small amounts or retained in the column and were not detected.

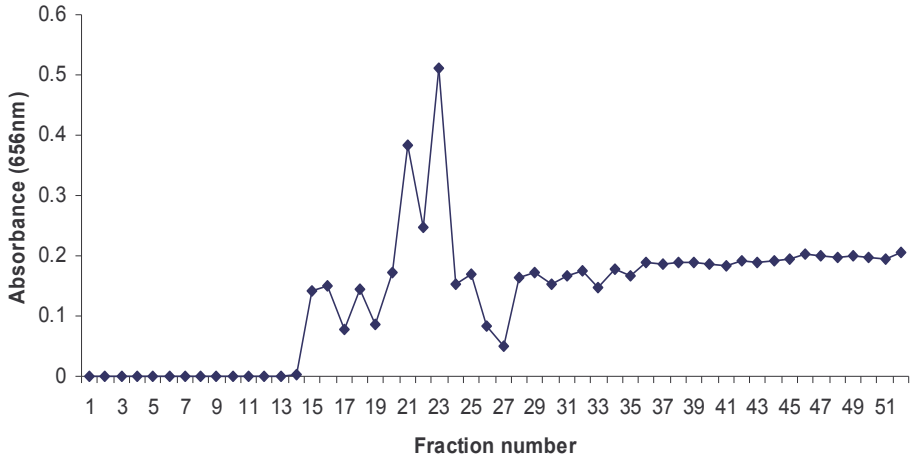


Fig 3.1 Anion–exchange chromatogram of cranial cartilage glycosaminoglycans of squid.

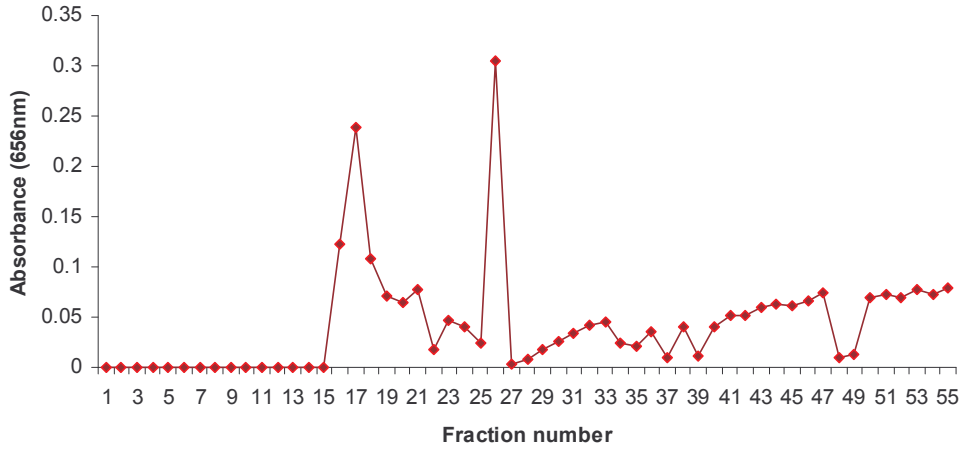


Fig 3.2 Anion–exchange chromatogram of cranial cartilage glycosaminoglycans of cuttlefish.

3.3.3.2 Glycosaminoglycan analysis by SAX – HPLC

The isolated glycosaminoglycans from the selected tissues of squid and cuttlefish were subjected to disaccharide analysis by HPLC after digestion with chondroitinase ABC and the results are shown in Fig. 3.3 (a-f) and 3.4 (a-f).

The analytical anion exchange HPLC results indicated similar pattern in the case of mantle (3.4(a)) and tentacle (Fig 3.3 (b) and 3.4(b)) glycosaminoglycans in both the cephalopods. The mantle GAGs of squid showed some unassigned peaks in addition to the GAG disaccharides (Fig 3.3(a)).Similar pattern was observed for GAGs from the fin of squid (Fig 3.3(c)).The SAX-HPLC of the fin GAGs of cuttlefish ((Fig 3.4(c)) showed several unidentified peaks. The skin GAGs in both cephalopods (Fig 3.3 (d) and 3.4(d)) showed almost similar pattern of peaks at low ionic strength but there was no visible peak eluting at high ionic strength. The gladius/pen glycans did not produce any characteristic peak and were observed below the baseline indicating the absence of any specific kind of GAGs (Fig 3.3 (e) and 3.4(e)). The cranial cartilage of cuttlefish GAGs showed a broad peak with a number of separate maxima eluting at high ionic strength (Fig 3.3 (f) and 3.4(f)) as that of standards (Appendix 3) indicating the presence of GAG disaccharides. Since the GAGs were not completely susceptible to digestion with chondroitinase ABC the exact disaccharide composition of the GAGs could not be determined.

3.4 Discussion

There has been tremendous progress in the development of various technologies to analyze GAGs. Most methods of analyzing the purified oligosaccharides involve degradation of these species using the GAG-degrading enzymes or other chemical methods (Ernst *et al*., 1995). Each technique provides a distinct attribute (mass, charge or composition) that pertains to a single GAG oligosaccharide or a set of attributes that characterize mixtures of GAG oligosaccharides. On the other hand, the validity of these methods is markedly influenced by the heterogeneity of glycosaminoglycans.

3.4.1 Chemical composition analysis

The chemical composition analyses of the glycosaminoglycans provide

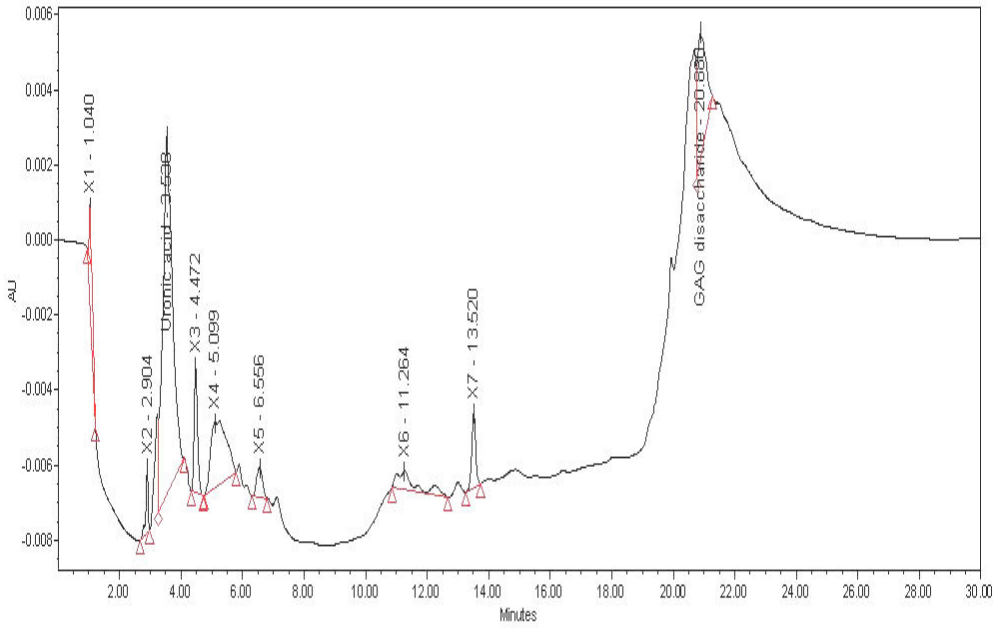


Figure 3.3(a): SAX – HPLC Chromatogram of the mantle GAGs of squid

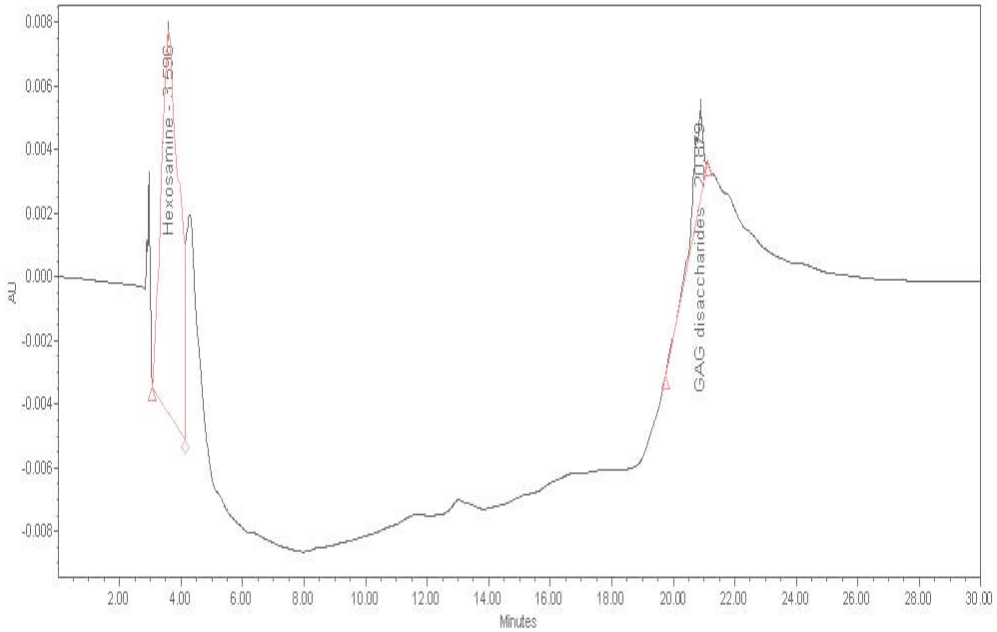


Figure 3.4(a): SAX – HPLC Chromatogram of the mantle GAGs of cuttlefish

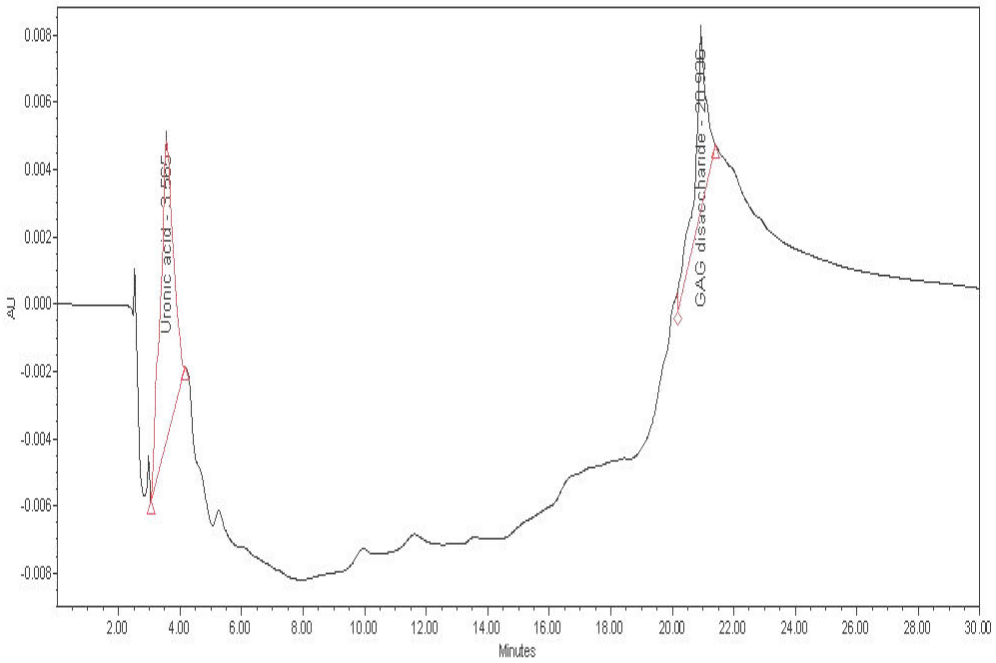


Figure 3.3(b): SAX – HPLC Chromatogram of the tentacle GAGs of squid

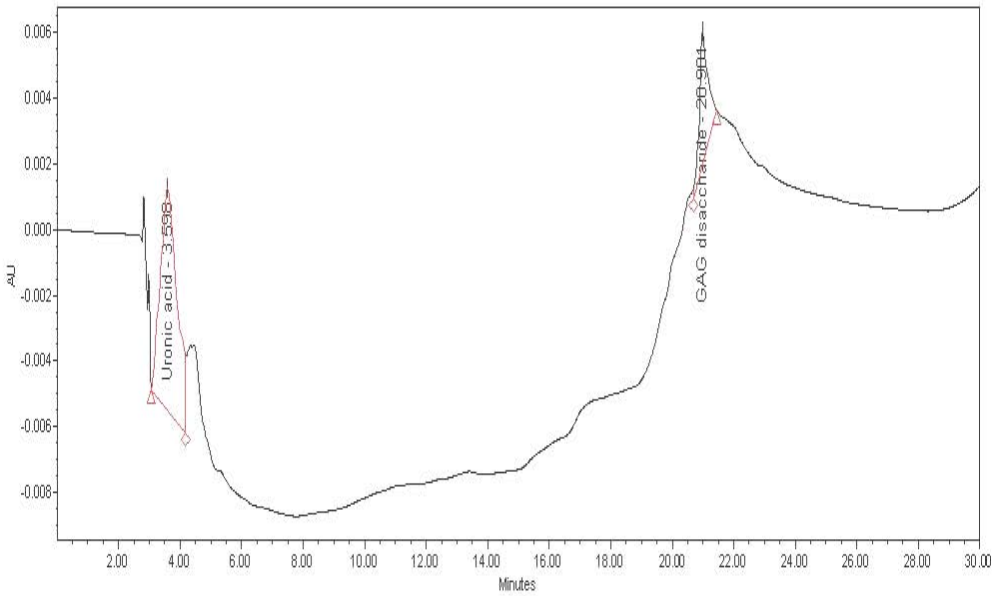


Figure 3.4(b): SAX – HPLC Chromatogram of the tentacle GAGs of cuttlefish

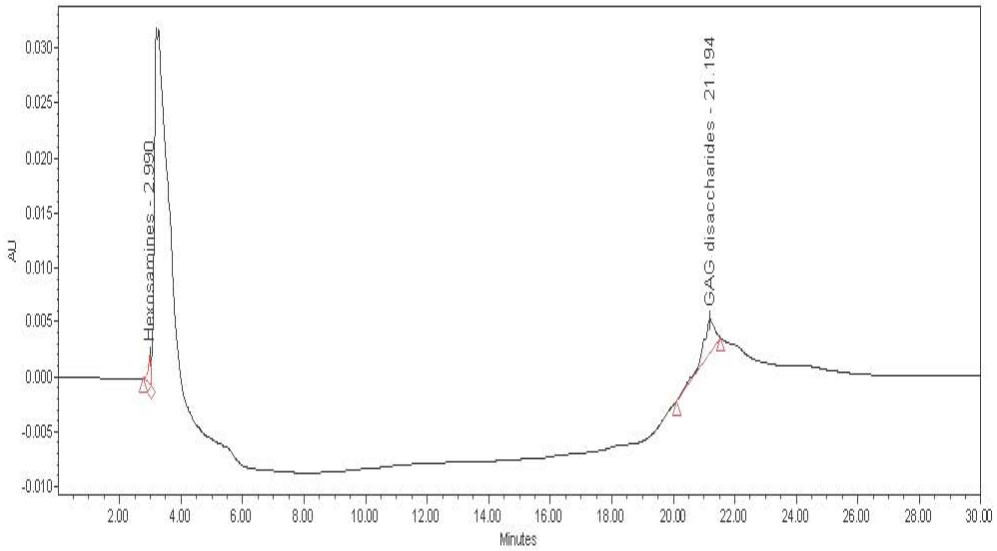


Figure 3.3(c): SAX – HPLC Chromatogram of the fin GAGs of squid

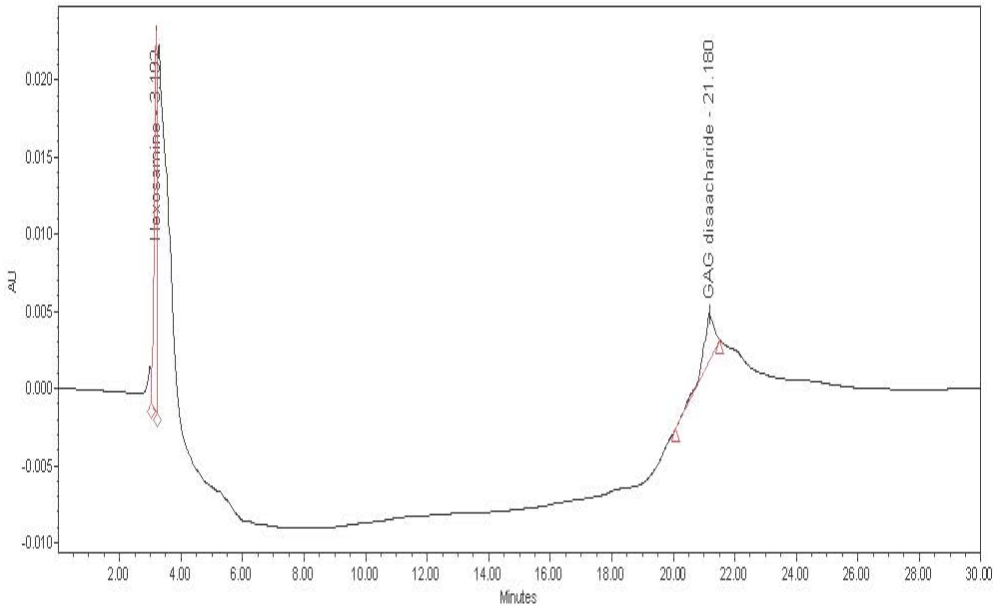


Figure 3.4(c): SAX – HPLC Chromatogram of the fin GAGs of cuttlefish

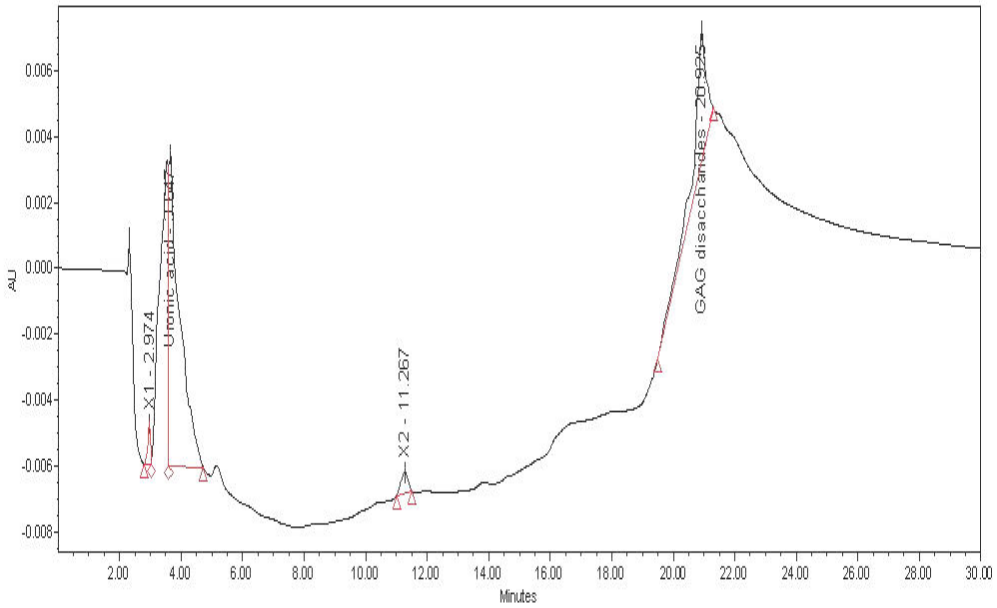


Figure 3.3(d): SAX – HPLC Chromatogram of the skin GAGs of squid

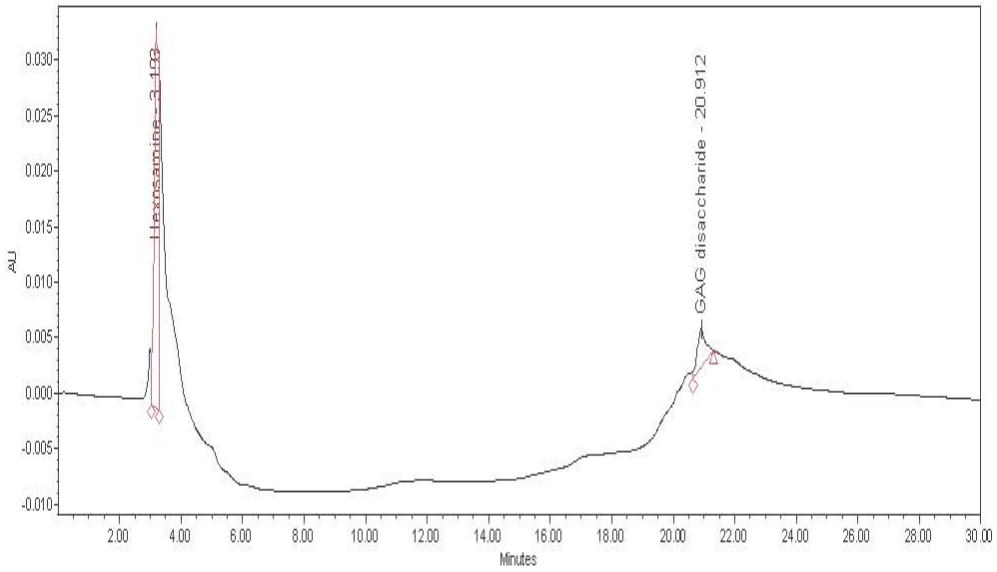


Figure 3.4(d): SAX – HPLC Chromatogram of the skin GAGs of cuttlefish

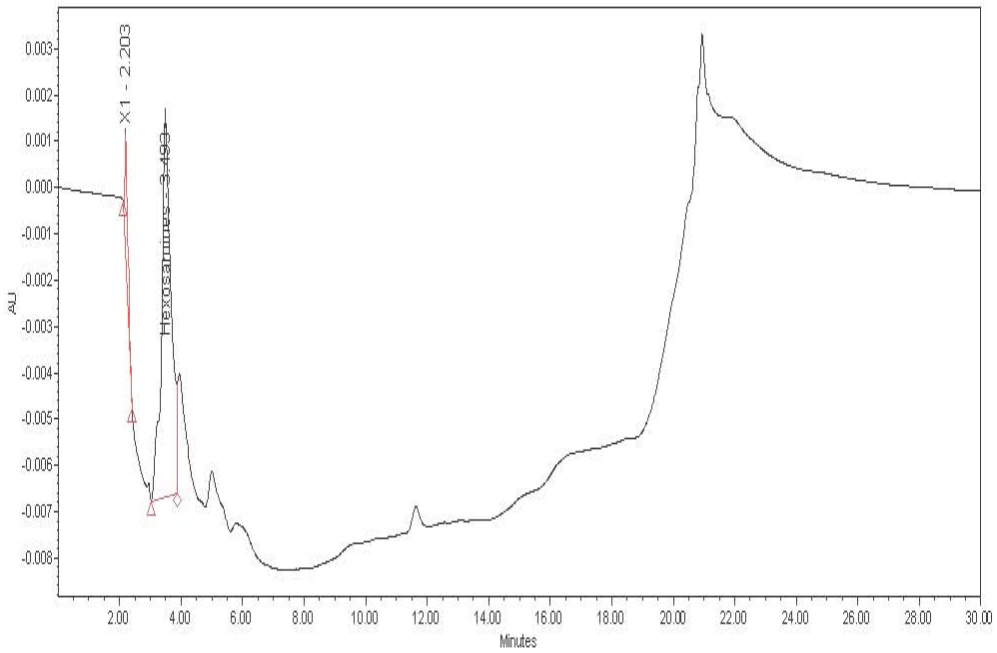


Figure 3.3(e): SAX – HPLC Chromatogram of the glycans of squid gladius

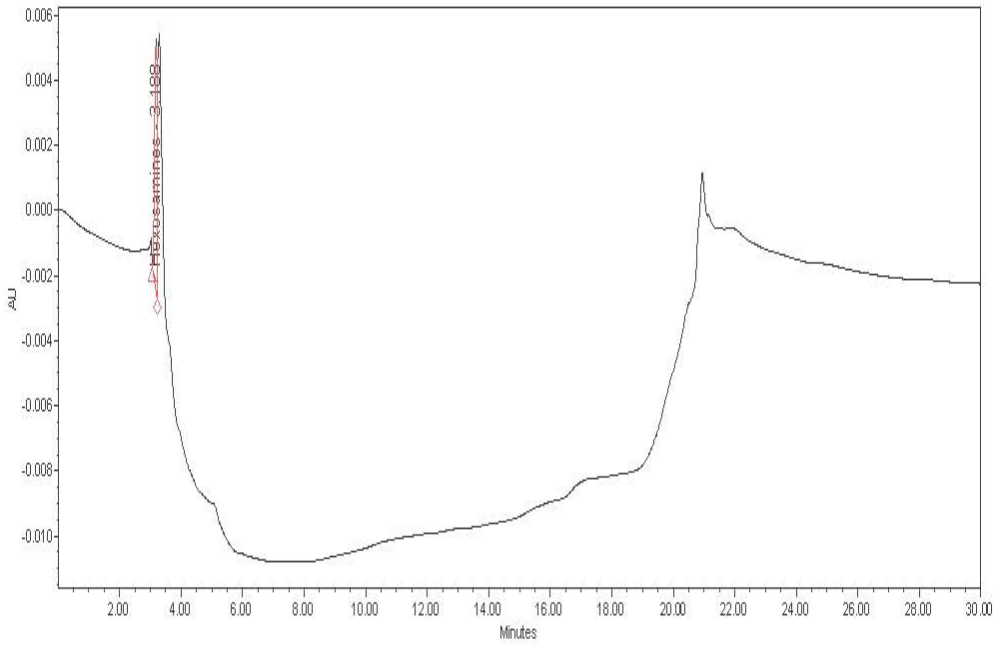


Figure 3.4(e): SAX – HPLC Chromatogram of the glycans of cuttlefish pen

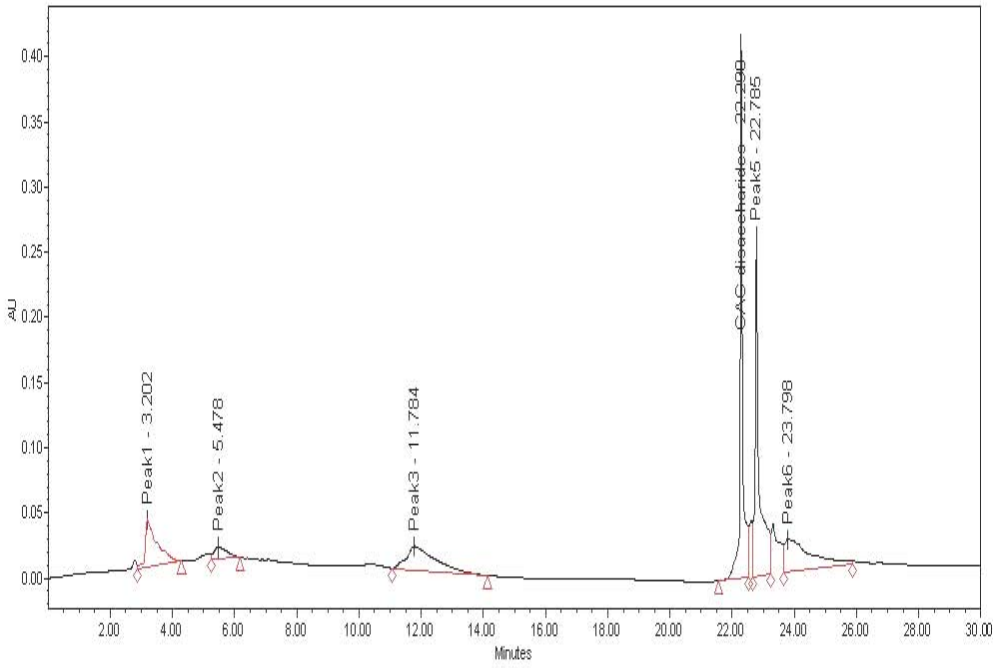


Figure 3.3(f): SAX – HPLC Chromatogram of the cranial cartilage GAGs of squid

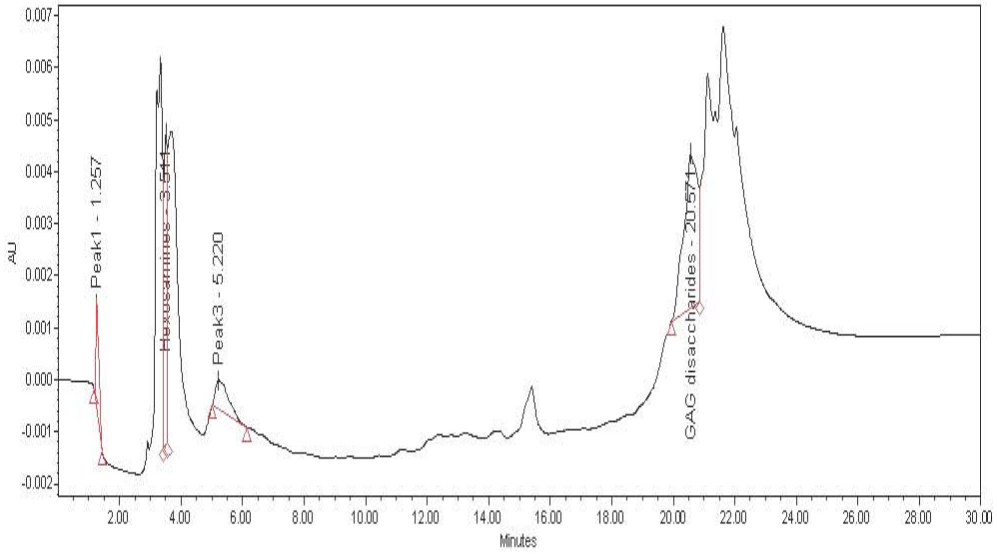


Figure 3.4(f): SAX – HPLC Chromatogram of the cranial cartilage GAGs of cuttlefish.

data about the relative amounts and type of GAGs present in the different tissues analyzed. The 1, 9 –DMMB dye binding assay proposed by Barbosa *et al*. (2003) is a versatile method to detect the relative amount of sulphated GAGs within a preparation. Individual glycosaminoglycans species in mixtures cannot feasibly be determined by spectrophotometric analysis. The relative proportion of the different GAGs could not be determined since it requires the treatment with specific mucoploysacchridases and hence not included in this study. However the relative propotions of the uronic acid and the total hexosamines in the different tissues after acid hydrolysis could be determined by the analytical methods employed. The traces of hexosamines in the gladius/pen samples can be attributed to the amino sugars in the chitin residues that might have precipitated during extraction. The presence of fucose in the cranial cartilage samples of squid requires further investigation. Fucose containg glycosaminoglycans in invertebrates have been reported by several authors (Kariya *et al*., 1990; Vieira *et al*., 1991; Kariya *et al*., 1997). Neutral monosaccharides have also been found as branches in oversulphated chondroitin sulphate E from squid (*Illex illecebrosus coidentii)* cranial cartilage (Habuchi *et al*., 1977; Vynios and Tsiganos, 1990), in chondroitin sulphate from sea cucumber (Vieira *et al*., 1991) and in chondroitin from squid skin (Karamanos *et al*., 1990), while they have not been found in galactosaminoglycans from mammalian tissues (Karamanos *et al*., 1992). None of the GAG isolates contained sialic acid in contrast to those from vertebrates as reported by Karamanos *et al.* (1990).

The glycosaminoglycans were further characterized by electrophoretic and anion exchange chromatographic methods.

3.4.2 Electrophoretic methods

Agarose gel electrophoresis is a technique to analyze GAGs in mixtures (Theocharis *et al.*, 1999; Volpi and Maccari, 2002). The most common methods for the visualization of sulphated GAGs after their separation by electrophoresis are based on the metachromatic activity of polysaccharides and their complexation with cationic dyes such as azure A, toluidine blue, alcian blue and methylene blue.

A horizontal gel running apparatus was used in the present study, in contrast to the vertical gel running system mentioned by Bjornsson (1993), for better results. A similar technique employing horizontal agarose gel system for the characterization of acidic poysaccharides was also explained by Theocharis *et al.* (1999) by employing the Tris-acetate buffer system of Bjornsson (1993). In contrast to the methods of Theocharis *et al.* (1999) that employed a 1.2 % gel concentration, the medium EEO (electroendo-osmosis) agarose gels with 2% gel concentrations gave better results for the squid and cuttlefish GAGs isolated in this study. The GAG polymers showed a retarded electrophoretic migration on agarose gel, when compared to the chondroitin sulphate standards obtained from vertebrate sources. This pattern was probably due to the higher charge density of the invertebrate glycan allowing stronger interaction between the sulphate groups on the polysaccharide and the buffer.

Separation of GAGs using agarose gel electrophoresis and staining with toluidine blue is a good and sensitive way to identify and quantify the contaminations of other polysaccharides within GAG preparations. In the present study visualization of the isolated glycosaminoglycans was performed by a procedure involving staining with the cationic dye toluidine blue, according to the method of Bjornsson (1993). The detection limit for the determination of GAGs with this method is from > 0.1 to 1.0 μg. The method can be used to stain complex sulphated GAGs like heparin, heparan sulphate, chondroitin sulphate and dermatan sulphate. Non sulphated polysacchrides such as hyaluronic acid gave little sensitivity with toluidine blue staining. The selective staining of sulphated polysaccharides with toluidine blue was explained by Pandain and Thirugnanasambandan (2008) in their studies on glycosaminoglycans from backwater clam *Marcia opima.* Pavao *et al.* (1998) also showed similar results in highly sulphated dermatan sulphates from ascidians. Theocharis *et al*. (1999) reported the absence of any known glycosaminoglycans structures on the polysaccharide isolates of the pen case of *Sepia officinalis* obtained by papain digestion. The results obtained in this study were well in accordance to the above findings.

The electrophoretic migration of the sulphated polysaccharides in acetate buffers and 1, 3-diaminopropane buffers depends on the structure of the polysaccharide backbone; whereas in sodium barbital buffer depends more on their charge density (Dietrich *et al*., 1989; Mulloy *et al.,* 1994). The relative molecular weight of the standard chondroitin sulpahte A and chondrotin sulphate C were 40,000 Da and 60,000 Da respectively. The investigation of molecular weight of the glycosaminoglycans by agarose gel electroporesis revealed the presence of glycans with mobility slightly smaller than that of standard GAGs can be attributed to the high molecular weights of these cephalopod glycosaminoglycans. Thus the difference in electrophoretic mobility of the various GAGs is a first indication of distinctive structure of these polysaccharides.

Agarose gel electrophoresis has a number of advantages over cellulose acetate electrophoresis in that, it can be used for preparative purposes by scaling up the load of mucopolysaccharide samples and the thickness of the agarose blocks. It accepts heavily contaminated mixtures without change of the pattern of fractionation and hence can be used for purification purposes also. The low molecular weight residues of papain controls are expected to have eluted off from the 2% agarose gels (Plate 3.1 and 3.2, lane numbers 10). The present protocol developed can in fact be employed for the purification of GAGs from crude papain digests. Compared to other agarose gel electrophoretic methods and PAGE, the protocol developed has an added advantage that the results can be obtained in 3 hours of operation as in the case of cellulose acetate electrophoresis. However the time factor is compensated for by leaving the gels overnight for fixation in cetavlon (CTAB).

The intact and enzyme degraded glycosaminoglycans in agarose gels were stained with toluidine blue in order to visualize the susceptibility of the isolated glycosaminoglycans to the action of chondroitinase ABC. Electrophoresis of control and enzyme-incubated glycans revealed that the glycans were almost resistant to the activity of bacterial chondroitinases unlike that of the GAGs from other vertebrate sources. The susceptibility of GAGs from different sources to

various mucopolysaccharidases has been investigated by several scientists (Habuchi *et al*., 1977; Tsegenidis, 1992; Sugahara *et al.,* 1996a; Falshaw *et al*., 2000). Chondroitinase ABC is a bacterial eliminase that obliterates the stereochemical difference at C-5 between GlcA and IdoA by introducing unsaturation between C-4 and C-5 of the non-reducing terminal uronates of resultant disaccharides. The horizontal agarose gel electrophoresis (Plate 3.3) of control and the enzyme (Chondroitinase ABC) incubated glycans from the cranial cartilages of both the cephalopod species indicated that these compounds are relatively resistant to the mucopolysaccharidases under stipulated conditions and hence differs from all known vertebrate glycosaminoglycans. Whether these glycosaminoglycans are completly degraded by the bacterial chondroitinases under modified conditions or on prolonged incubation requires further investigation. Habuchi *et al*. (1977) and Tsegenidis (1992) have suggested that the limited digestion of specific chondroitin sulphates by chondroitinases may probably be due to the enzyme not being able to digest disaccharides with neutral sugar substitutions. A similar possibility cannot be ruled out in the resistance of CS–GAGs; especially in the case of GAGs from cranial cartilage of squid (*Loligo duvauceli*) and cuttlefish (*Sepia pharaonis*) to chondroitinase ABC digeston.

3.4.3 Chromatographic methods

DEAE cellulose anion-exchange chromatography has the advantage that the polysaccharide solution need not be concentrated before application on the column, and it is useful for isolating low-molecular-weight glycosaminoglycans. The technique also allows identifying the charge distribution of the GAG isolates and can also be applied for the purification of GAGs from the papain residues, which gets eluted off at the beginning of the salt gradient. Gel filtration columns can separate glycosaminoglycans species based on their molecular weight, but this is of little value for preparative fractionation due to high-molecular-weight heterogeneity of glycosaminoglycan chains. The separating problems due to variations in molecular weight might essentially be overcome by using electrophoretic methods.

HPLC techniques involve charge-based separation of GAG fragments using an amine-based or a strong anion exchange (SAX) column coupled to a high-performance liquid chromatography (HPLC) instrument. Over the last few years, high performance liquid chromatography (HPLC) (Karamanos *et al.,* 1997) and capillary zone electrophoresis (CZE) methods (Karamanos and Hjerpe, 1998; Ruiz-Calero *et al.,* 2001) have been used to determine the oligosaccharide composition of GAG hydrolysate mixtures. Using the corresponding lyases (heparinases, chondroitinases and hyaluronidases), each GAG can be totally or partially depolymerised by enzymatic hydrolysis, (Lindhardt and Gunay, 1999; Imanari *et al.,* 1996; Karamanos and Hjerpe, 1998; Ruiz-Calero *et al.,* 2001) yielding a mixture of oligosaccharide fragments, composed of unsaturated uronic acid residues at their non reducing termini. The resulting characterizations of disaccharide composition are particularly important as they may reveal subtle changes in the structure of GAGs from different sources.

Various GAG oligosaccharides have been sequenced using HPLC methods. Sim *et al*. (2005) successfully employed SAX-HPLC to quantify CS-GAG content in biological samples and medicinal preparations. Over sulphated chondroitin sulphate (CSS) has been isolated and characterized from the skin of squid, *Illex illecebrosus coidentii* by Karamanos *et al*. (1992), with appreciable amounts of disulphated and tri sulphated disaccharides. On the contrary, studies by Falshaw *et al.* (2000) on the glycosaminoglycans isolated from the skin and cranial cartilage of Gould's arrow squid (*Nototodarus gouldi*) showed disulphated chondroitin sulphates with varying levels of glycosylation, but neither of the tissues exhibited the presence of trisulphated disaccharides. Disulphated chondroitin sulphate has also been detected in shark cartilage (Hjerpe *et al*., 1979), squid cornea (Karamanos *et al*., 1991b) and king crab cartilage (Seno and Murakami, 1982). Seno *et al*. (1972) reported the presence of trisulphated dermatan sulphate from hag fish skin.

Detection of GAGs during HPLC is usually accomplished by end-labellling, using either a radiolabel (3H) or a fluorescent marker. Both these

methodologies relied on end-labeling of saccharides require the introduction of expensive labeling reagent. The methodology devised in this study does not require end labeling, since it relies on UV detection of the GAGs at 232nm. Another greatest advantage of the technique is that the results are obtained within a run time 30 min. The technique of SAX –HPLC developed in the present study permits the characterization of GAGs of different origin and can be used in the quality control of both pharmaceutical preparations and raw materials. The exact proportion of the cephalopod glycosaminoglycans could not be accurately estimated since the GAG chains were not completely digested by chondroitinase ABC. Similar complexities were also reported by Karamanos *et al*. (1992), in their studies on over sulphated chondroitin sulphate (CSS) from squid skin.

Chromatographic and electrophoretic approaches share several limitations. The enzymatic cleavage is characteristically based purely on gel mobility or HPLC elution, which are not completely predictable. The major limitation of glycosaminglycan elution from SAX or amine columns using salt is that the elution is biased and may not be sensitive to the separation of species with low sulphation. The ladder of peaks or gel shifts caused by treatment with exolytic enzymes is not fully comprehensible, as their substrate specificities have not been thoroughly characterized. Sensitive methodologies based on capillary electrophoresis (CE) (Rhomberg *et al*., 1998) and mass spectrometry (MS) have been developed to detect the femto/picomolar amounts of GAG. Various MS techniques have been applied to GAGs, including liquid secondary ion MS, matrix-assisted laser desorption/ionization (MALDI-MS), and electrospray ionization (ESI-MS) (Juhasz and Biemann, 1995; Desaire and Leary, 2000; Behr *et al*., 2005). There are reports that MS analysis has been complicated owing to inefficient complexation of oligosaccharides with metal ions (Na+) during separation and labile sulphate groups that may dissociate during ionization. These factors result in complex spectra for longer oligosaccharides that are difficult to interpret and also the techniques are relatively very expensive.

The study of intact polymers by chromatographic and electrophoretic

techniques are comparatively less expensive and they provide preliminary information regarding molecular mass, polydispersity or charge density (Toida and Lindhardt, 1996), whereas little information is achieved about fine structure of these compounds due to the high microheterogeneity of these compounds. To date, electrophoretic and chromatographic methods for the separation/analysis of intact GAGs have not given entirely successful results because of their complex intrinsic structural characteristics. Hence further characterization of the GAG isolates was done by means of spectroscopic techniques like FT-IR and NMR in order to obtain details on the infrared spectra and characterize these GAG isolates as explained in the succeeding chapter.

3.5 Conclusion

The isolated GAGs showed strong metachromatic bands on agarose gel electrophoresis and the chemical analyses indicated the presence of amino sugars in a molar proportion that varied between species and from among the tissues selected. Among all the tissues examined for *Loligo duvauceli* and *Sepia pharaonis*, the cranial cartilages of both the cephalopod species contained appreciable amounts of sulphated GAGs. The endoskeletal portions such as the gladius/pen of both cephalopods species did not show the presence of any sulphated GAGs. The preponderant polysaccharide in the cranial cartilage GAGs of both the cephalopods species were proposed to be of the chondroitin sulphate type as evidenced by the agarose gel electrophoresis and HPLC techniques employed. The cranial cartilage GAGs of both the cephalopods species were almost resistant to chondroitinase ABC digestion under the stipulated conditions. The different electophoretic mobilities of the GAG isolates are attributed to the difference in backbone structure of these polysaccharides. Even though the chromatographic and electrophoretic approaches possess certain limitations, the qualitative agarose gel electrophoretic method devised in this study and the SAX-HPLC methods of GAG analysis are iterative and can be employed for the preliminary identification, analysis and quantification of GAGs from other potential sources as well.

Chapter 4: Physico - Chemical characterization of the isolated Glycosaminoglycans

Contents

4.1 Introduction

Every atom, ion or molecule has a unique and characteristic relationship with electromagnetic radiation which shows changes in the rotational, vibrational and electronic energies. Spectroscopy is the measurement of electromagnetic radiation absorbed or emitted when molecules, or atoms, or ions of a sample move from one allowed energy state to another. Spectroscopic methods, particularly CD (circular dichroism) and FT-IR (Fourier transform infrared) spectroscopy, are important tools for the characterization of biomolecules including complex polysaccharides and measurement of secondary structural changes in protein–ligand interactions in solution (Middaugh *et al*., 1992). In addition, energies resulting from energy differences that arise when a sample is placed in a magnetic or electric fields are susceptible to spectroscopic studies. Nuclear magnetic resonance (NMR) and electron spin resonance are two such studies.

4.1.1 Fourier transform infrared (FT-IR) spectroscopy

FT-IR spectroscopy is a well established and constantly developing analytical technique which allows for simple, reliable and rapid analysis of a wide range of sample types (Naumann *et al.,* 1988). It is a complementary technique arising from the absorption of infrared (IR) radiation through resonance of non-centrosymmetric (IR active) modes of vibration (Oberg *et al.,* 2004). It is based on the principle that when a sample is interrogated with an IR beam, the functional groups within the sample will absorb the infrared radiation and vibrate in one of a number of ways, either stretching, bending, deformation or combination vibrations. These absorptions/vibrations can then be correlated directly to (bio) chemical

species and the resultant infrared absorption spectrum can be described as an infrared 'fingerprint' characteristic of any chemical or biochemical substance.

The IR region of an electromagnetic spectrum extends from 2.5 to 400 μm. The range of 2.5-25 μm (4000-400 cm^{-1}) is known as the midrange region, and from 25 to 400 μm (from 400 to 25 cm^{-1}), it is known as the far- IR region. The IR spectrum is generally recorded in the range of 1200-4000 cm^{-1}.

The infrared spectrum of a compound is essentially the superposition of absorption bands of specific functional groups, in addition to subtle interactions with the surrounding atoms of the molecule and thus imposes the stamp of individuality on the spectrum of each compound. For qualitative analysis, one of the best features of an infrared spectrum is that the absorption or 'the lack of absorption' in specific frequency regions can be correlated with specific stretching and bending motions and, in some cases, with the relationship of these groups to the remainder of the molecule. Thus, by interpretation of the spectrum, it is possible to state that certain functional groups are present in the material and that certain others are absent. With this one datum, the possibilities for the unknown can be sometimes narrowed so that comparison with a library of pure spectra permits identification.

The applications of FT-IR have been numerous and diverse in biosciences. During the last decade it has been recognized that FT-IR, in combination with the appropriate multivariate analysis strategies, has considerable potential as a metabolic fingerprinting tool for the rapid detection and diagnosis of disease or dysfunction, and indeed, a significant number of studies have been undertaken on tissues, cell and biofluids in an emergent area of research termed 'infrared pathology' by Diem and co-workers (1999) and now more commonly referred to as metabolic fingerprinting (Goodacre *et al*., 2004). FT-IR spectroscopy has been used to acquire metabolic fingerprints from a wide range of biological fluids for the diagnosis of a variety of diseases including cancer, diabetes, arthritis and degenerative brain diseases like bovine spongiform encephalopathy (BSE) and transmissible spongiform encephalopathy (TSE). This is achieved by linking the

infrared spectra of normal and diseased subjects to a disease related interpretation in a process described as 'disease pattern recognition (DPR). In the field of reproductive biology, FT-IR has been used to analyze follicular fluids. The difference observed in the biochemical nature of the fluids may be reflective of the developmental capacity of the oocyte, suggesting that FT-IR could provide a biomarker related to oocyte quality (Thomas *et al.,* 2000). FT-IR has also been used in the field of microbiology, for the rapid and accurate identification of bacteria to the sub-species level (Naumann *et al.,* 1991), differentiation of clinically relevant species (Goodacre *et al*., 2004), rapid enumeration of food spoilage bacteria *in situ* (Ellis *et al*., 2002), metabolic footprinting of tryptophan-metabolism mutants (Kaderbhai *et al*., 2003) and discrimination or identification of a range of bacterial genera (Winder and Goodacre, 2004).

4.1.2 Nuclear magnetic resonance (NMR) spectroscopy

NMR has eventually become an indispensable analytical technique in biology, medicine, physics, chemistry and food science. It is an extremely powerful technique for determining the structure of molecules. NMR based methods have several inherent advantages. The NMR instrument communicates with the investigated object by electromagnetic waves in the radiofrequency range. This makes most NMR techniques rapid, non – invasive and non – destructive for the sample, not harmful for the operator and non – polluting for the environment. The applications of NMR in biological research may be divided into three main groups according to the type of equipment used, as they can provide versatile information about the chemical composition and structure of biological systems at various levels. These are: magnetic resonance imaging (MRI), low field (LF) NMR and high resolution (HR) NMR.

High resolution NMR has been shown to be particularly well – suited in the profiling of biological material (Nicholson and Foxall*,* 1995; Fan, 1996), and it has emerged as a popular technique for the analysis of foodstuff including fish and fish products. HR NMR gives a 'finger print' of the composition of the sample analyzed and allows identification/quantification of metabolites with relevance for

the quality and nutritional value. The most common magnetic nuclei for analysis of biological materials are ^{1}H, ^{13}C and ^{31}P. Nuclei in different chemical environment have slightly different resonance frequencies. The resonances obtained from NMR are expressed as chemical shift values (δ) in units of parts per million (p.p.m.) relative to a reference compound (example: tetramethylsilane; δTMS = 0.00 p.p.m.) for ^{1}H. The NMR spectra are presented as signals at different chemical shift values and are dependent on the molecular structure (Hunter *et al.,* 2005), type of the solvent, its concentration and pH (Fan, 1996). NMR in chemistry has traditionally been limited to analysis of liquid samples. High – resolution magic angle spinning (MAS) NMR is a strong tool for studying heterogeneous systems (Bollard *et al.,* 2000; Rooney *et al.,* 2003) and it enables studies of a wide range of chemical components in intact materials. ^{1}H NMR spectroscopy has been proposed for detecting variations in the chemical composition of intact GAGs (Toida *et al.,* 1993; Toida *et al.,* 1997a,1997b) and GAG-derived oligosaccharides (Sugahara *et al.,* 1992a; Sugahara *et al.,* 1995) allowing the differentiation of GAGs from various sources.

Hence an attempt has been made in this chapter to characterize the isolated GAGs by spectroscopic techniques like FT-IR and NMR. The GAGs isolated from the different tissues of squid and cuttlefish were analyzed by employing attenuated total reflectance (ATR) Fourier transform infrared spectroscopy and compared with that of authentic samples. Based on these and the previous results on the yield, the samples of the GAG isolates from the cranial cartilages of both the cephalopod species were subjected to further characterization using NMR.

4.2 Materials and methods

4.2.1 ATR – FTIR spectroscopy

The GAGs obtained from the selected tissues (mantle, tentacle, fin, skin, pen and cranial cartilage) of squid and cuttlefish were prepared as described in Section 2.2.4. These were then filtered using 0.45µm nylon filters and dried under vacuum at room temperature and finally subjected to FT-IR spectroscopy analysis. The authentic samples analyzed include chondroitin sulphate C (C4384, MW 50-

58.8 kD, 90% pure, 18.0% sulphate, obtained from Sigma-Aldrich, Saint Louis, USA), Heparin, Bovine serum albumin (BSA), N-acetyl glucosamine and fucose were from SRL, India Pvt. LTd. The FT-IR spectra of the samples were compared with the authentic standards.

FT-IR spectroscopy of purified GAG samples were performed by attenuated total reflectance (ATR) spectroscopy on a Thermo Nicolet, Avatar 370 instrument according to the method of Toida *et al.* (1999), which helped to analyze different amino and hydroxyl group of these sample molecules. The samples were analyzed from 450 to 4000 cm^{-1}.

4.2.2 Nuclear magnetic resonance (NMR) spectroscopy

The GAGs from the cranial cartilages of squid and cuttlefish were subjected to NMR analysis in order to assess the purity of the isolates. Samples (100 mg each) were dissolved in 0.5 ml 99.8% deuterium oxide (D_2O) after micro-filtration through 0.45 μm nylon filters. Further the samples were lyophilized twice from 99.8% D_2O, and dissolved in the same solvent (0.8 ml) in a 5 mm NMR tube for analysis. Before spectrum acquisition, samples were sonicated for 60 s to remove air bubbles. The ^{1}H and ^{13}C NMR spectra were recorded in a fully automated manner as described by Yates *et al.* (1996) with delays of 8 s and 1.5 s for the ^{1}H and ^{13}C spectra, respectively. The authentic samples of chondroitin sulphate C (C4384, MW 50-58.8 kD, 90% pure, 18.0% sulphate, Sigma-Aldrich, Saint Louis, USA), and N-acetyl glucosamine (as a component of non – chondroitin GAG disaccharides) were also subjected to NMR analysis and compared with that of the GAGs isolated from the cephalopod samples.

4.2.2.1 Apparatus

The ^{1}H spectra were obtained at 400 MHz with a Bruker AVANCE III 400 spectrometer using a 5 mm ^{1}H /X inverse probe and the ^{13}C spectra were obtained at 100 MHz with a 10 mm probe using a Bruker AVANCE III 400 instrument equipped with a processing and plotting software. All spectra were recorded in D_2O at 30°C and chemical shift values are expressed in parts per million (ppm).

The spectral width was 8000 Hz and the pulse was 45°. The acquisition time was 3.984 s and the number of scans was 256. No water suppression procedure was applied.

4.2.2.2 Data pre-processing

Free induction decay (FID) data were Fourier transformed (FT), pre-processed and converted into ASCII files for further analysis using the Bruker TOPSPIN software (Version 2.1). No filter functions or zero-filling procedures were applied to the raw FID data for resolution enhancement and increase of the signal-to-noise ratio. The phase correction was performed using an automatic (zero and first-order) phase correction procedure after fourier transform (FT).

4.3 Results

4.3.1 ATR – FTIR spectroscopy

The IR spectra represents a quick (in minutes) and simple (no derivatization required) method of obtaining information on the composition of the GAGs isolated. The absorption spectra were read between 450 and 4000^{cm-1}. The IR spectra obtained for the GAGs isolated from the different tissues of squid and cuttlefish are shown in Fig. 4.1 (a-f) and 4.2 (a-f) respectively. The IR spectra of the samples were compared with the standard chondroitin sulphate C (Fig. 4.3a). The IR spectra of standard heparin (Fig. 4.3b), albumin (Fig. 4.4a), N-acetyl glucosamine (Fig. 4.4b), and fucose (Fig. 4.4c) were also analyzed for comparison.

The IR spectra of the all the GAG samples were characterized by a broad band above 3000 $^{cm-1}$ and intense absorption around 1650 and 1050^{cm-1}. Among all the tissues analyzed the results of IR spectra of the cranial cartilage GAG isolates of both the squid and cuttlefish species showed closer peak intensities of similar configuration to the chondroitin sulphate standard on comparison of Fig. 4.1(f), 4.2(f) and 4.3(a). In the case of standard chondroitin sulphate C, the stretching and deformation vibrations of –C-O-H- bands at 1637.63^{cm-1} and 1420.03^{cm-1} corresponds to the presence of combined carboxylate with amine and sulphate. The peak intensities at 1629.80^{cm-1} and 1411.75^{cm-1} in the cranial cartilage GAG isolates

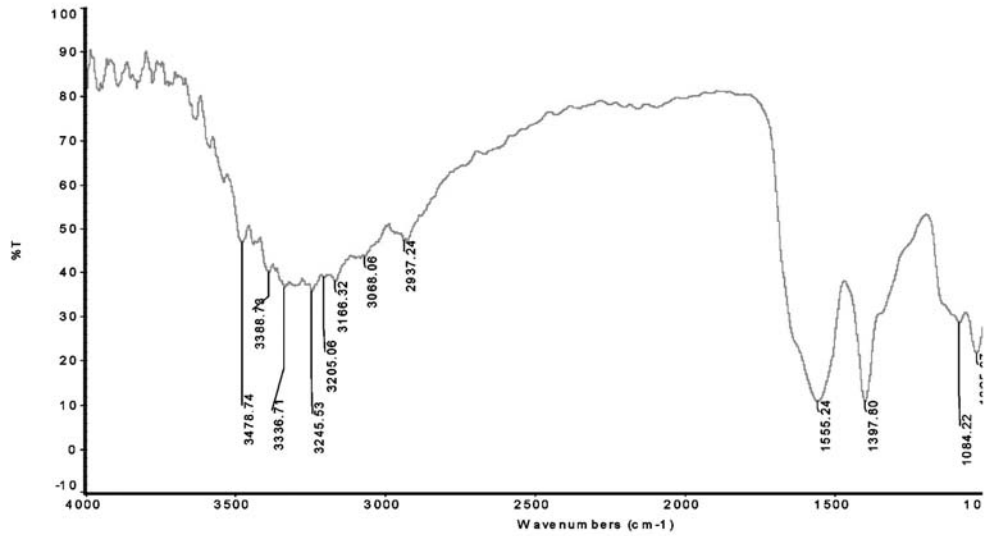


Figure 4.1 (a): FT-IR spectra of GAG isolates from squid mantle

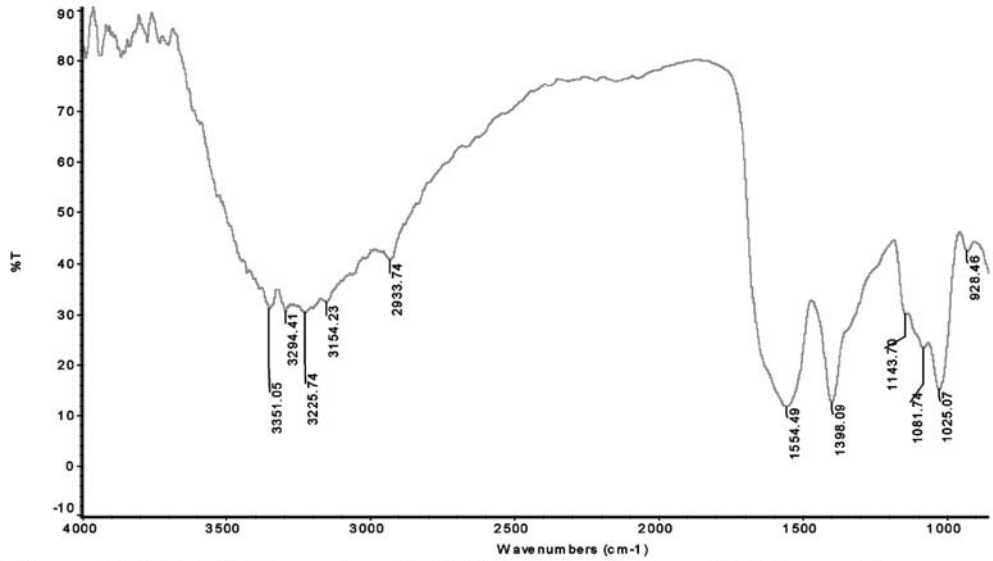


Figure 4.2 (a): FT-IR spectra of GAG isolates from cuttlefish mantle

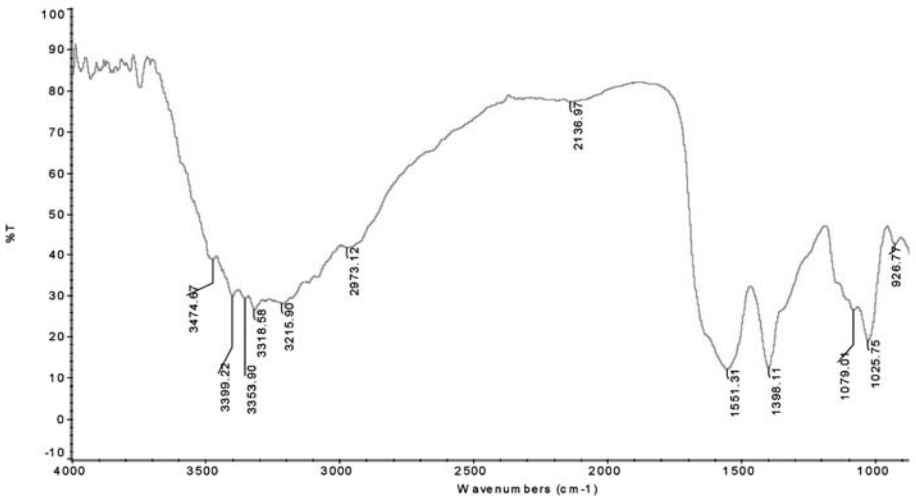


Figure 4.1 (b): FT-IR spectra of GAG isolates from squid tentacle

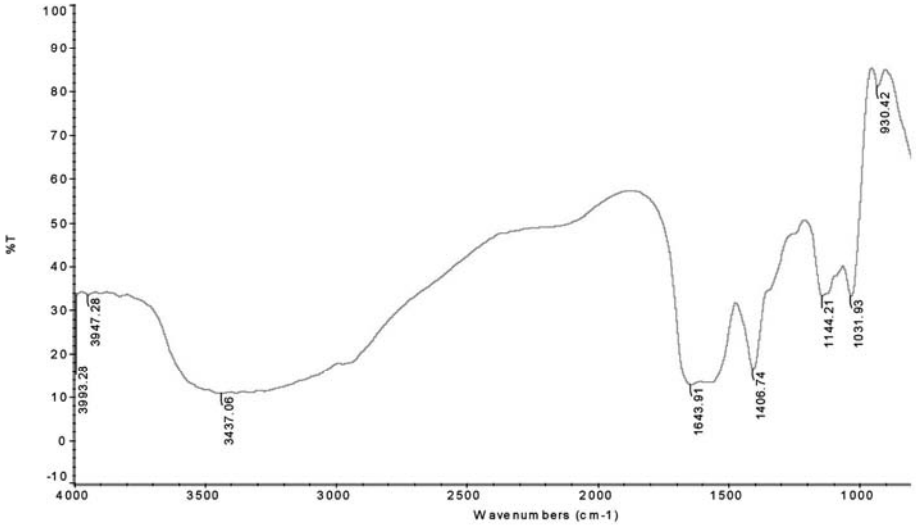


Figure 4.2 (b): FT-IR spectra of GAG isolates from cuttlefish tentacle

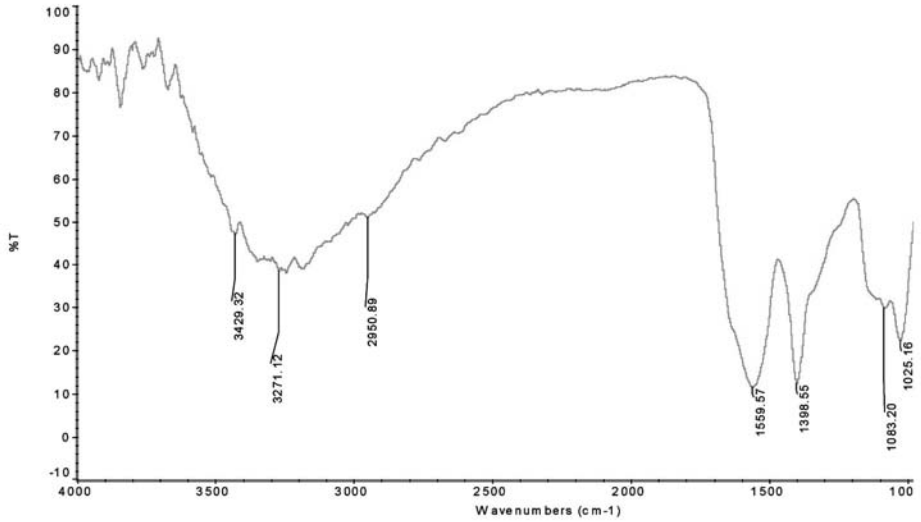


Figure 4.1 (c): FT-IR spectra of GAG isolates from squid fin

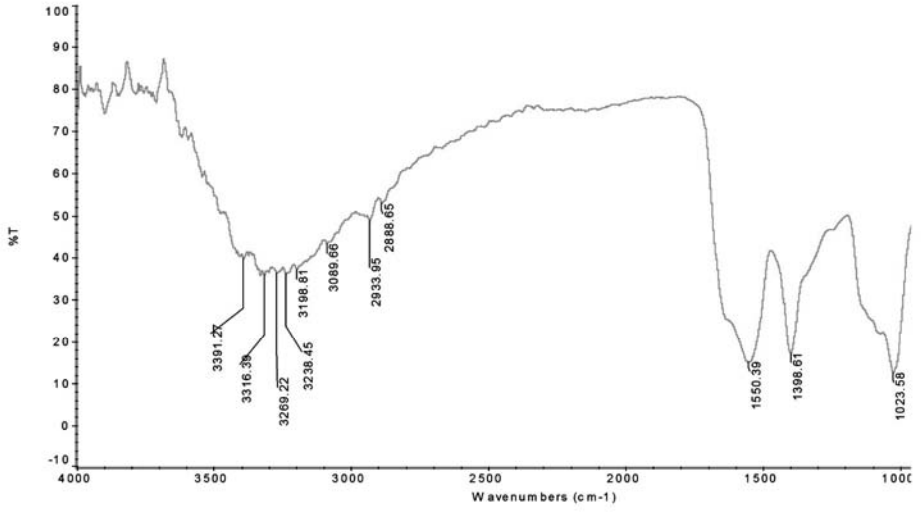


Figure 4.2 (c): FT-IR spectra of GAG isolates from cuttlefish fin

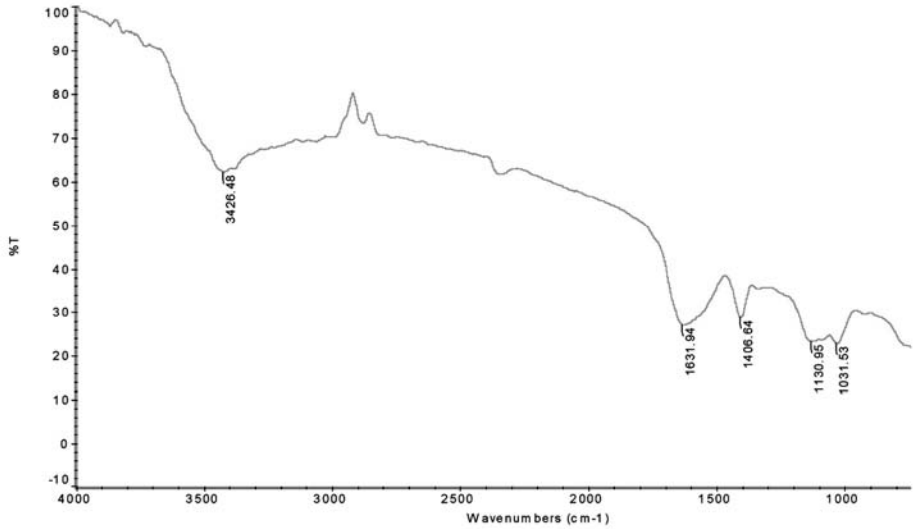


Figure 4.1 (d): FT-IR spectra of GAG isolates from squid skin

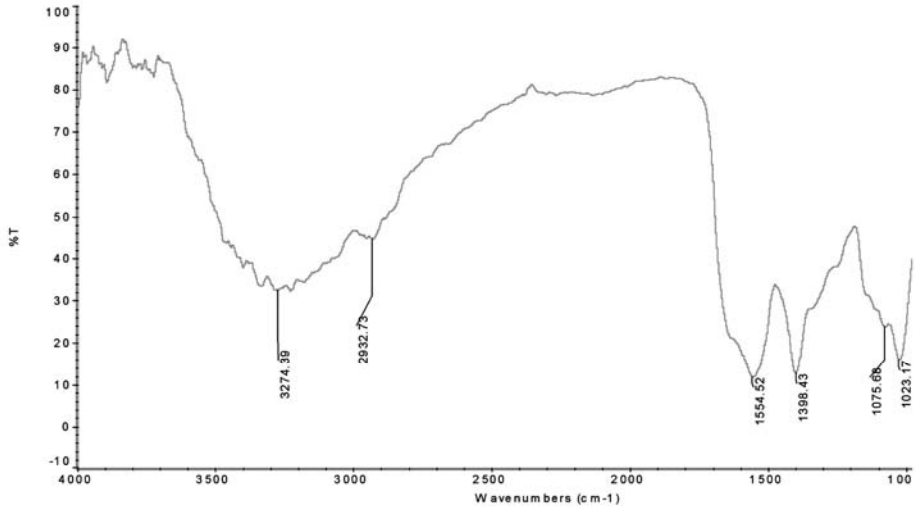


Figure 4.2 (d): FT-IR spectra of GAG isolates from cuttlefish skin

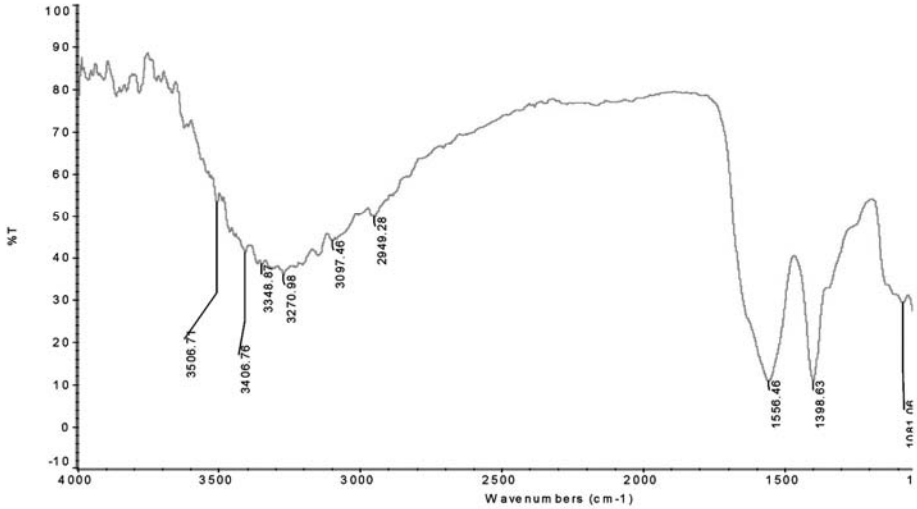


Figure 4.1 (e): FT-IR spectra of GAG isolates from squid gladius

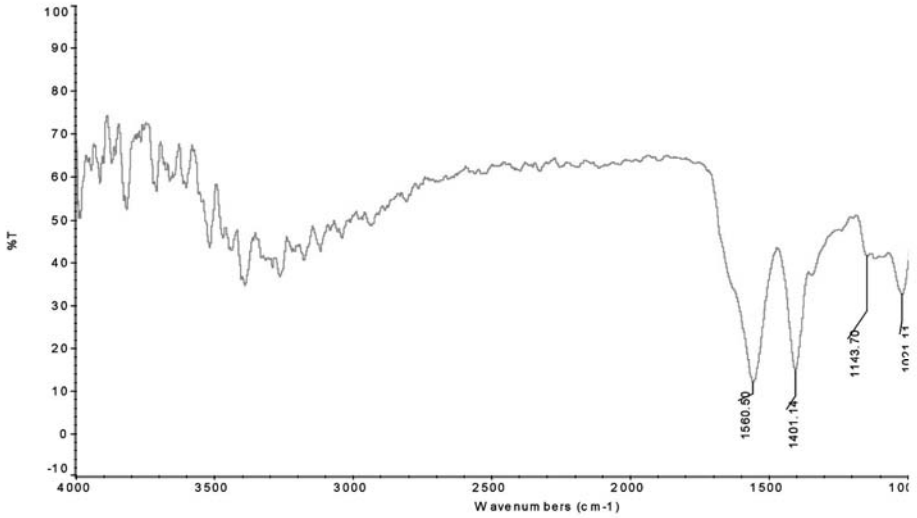


Figure 4.2 (e): FT-IR spectra of GAG isolates from cuttlefish pen

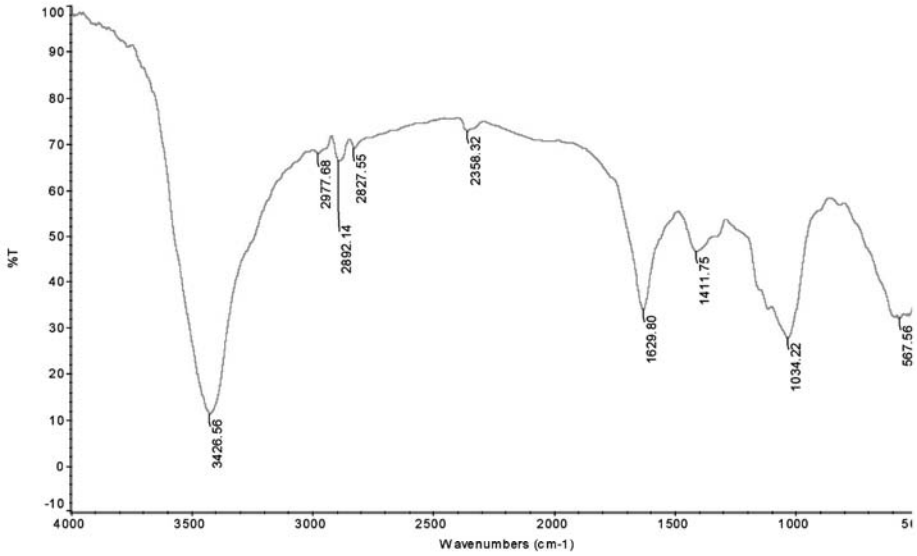


Figure 4.1 (f): FT-IR spectra of GAG isolates from squid cranial cartilage

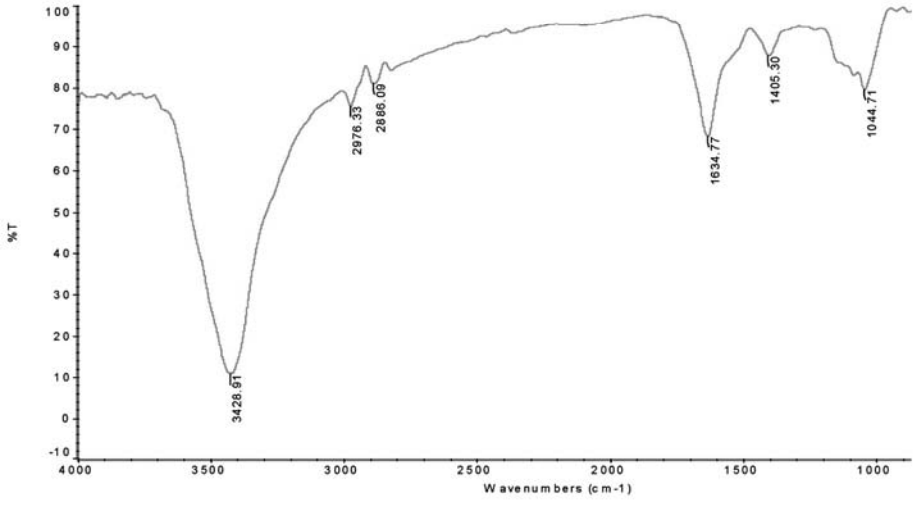


Figure 4.2 (f): FT-IR spectra of GAG isolates from cuttlefish cranial cartilage

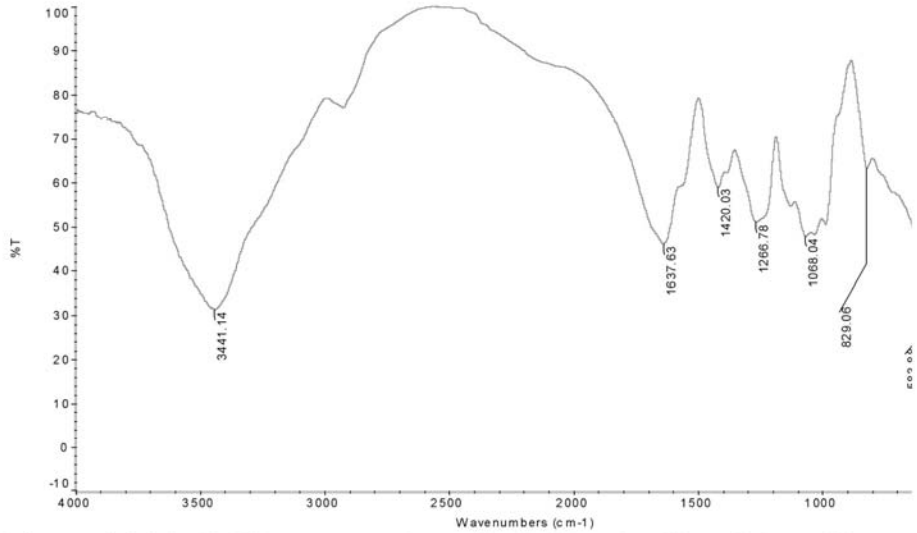


Figure 4.3 (a): FT-IR spectra of standard Chondroitin sulphate C

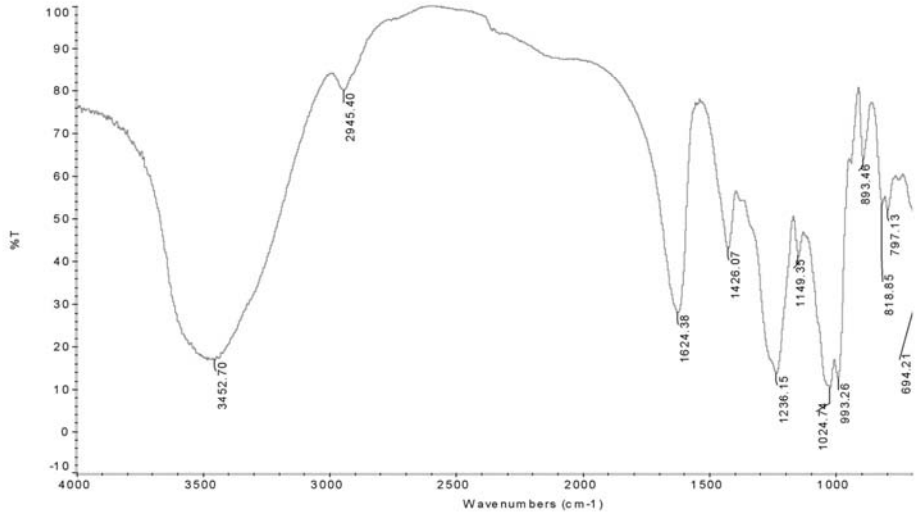
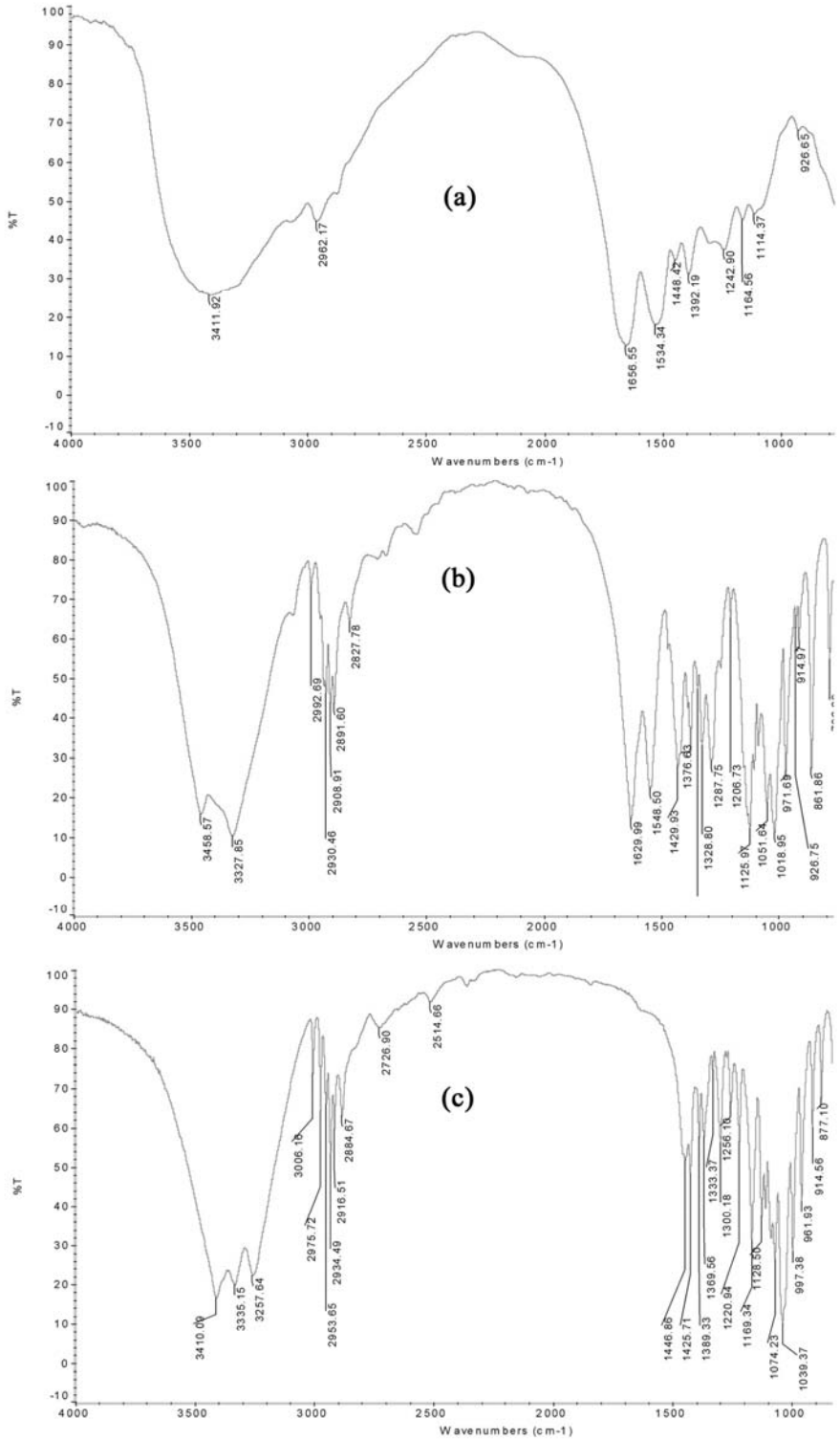


Figure 4.3 (b): FT-IR spectra of standard Heparin



Figures 4.4 (a,b &c): FT-IR spectra of standard (a) Albumin (b) N-acetyl glucosamine & (c) Fucose

of squid as well as prominent peaks at 1643.77^{cm-1} and 1405.30^{cm-1} in the case of cuttlefish GAGs corresponds to the of –C-O-H- bands indicating the presence of combined carboxylate with amine and sulphate. The broad band above 3000^{cm-1} was assigned to hydroxyl-stretching vibrations of polysaccharide and water involved in hydrogen bonding. The sulphate band in the case of standard chondroitin sulphate (Fig 4.3(a)) started from 1266.78^{cm-1} and down to 829.06^{cm-1}. Similar peak intensities were found at the 1034.22^{cm-1} and at 1044.71^{cm-1} in the cranial cartilage GAGs of squid and cuttlefish respectively, indicating heavily sulphated groups. The IR spectral data confirms that the prominent GAG types in the cranial cartilages of both cephalopods are of the chondroitin sulphate type.

The IR spectra of the mantle GAGs of squid ((Fig. 4.1a) and cuttlefish (Fig. 4.2a), as well as the tentacle of squid (Fig. 4.1b), showed almost similar pattern. The intense band at 1555.24^{cm-1}, 1555.49^{cm-1}, and 1551.31^{cm-1} respectively, of the above mentioned samples represents the absorption intensities of the carboxylic group. The acetyl amino group is represented by band intensities at 1397.80^{cm-1}, 1398.09^{cm-1} and 1398.11^{cm-1} for squid mantle, cuttlefish mantle and squid tentacle respectively.

In the case of squid skin (Fig. 4.1d) –C-O-H- bands indicating the presence of combined carboxylate with amine were obtained at 1631.94^{cm-1} and 1406.84^{cm-1}. In the case of cuttlefish skin (Fig. 4.2d) –C-O-H- stretching vibrations was identified at 1554.52^{cm-1} and 1398.43^{cm-1} and for cuttlefish tentacle these were obtained at the 1643.91^{cm-1} and 1406.74^{cm-1}. The fin GAGs of squid (Fig. 4.1c) showed peak intensities corresponding to combined carboxylate with amine and sulphate at 1559.57^{cm-1} and 1398.53^{cm-1}, whereas in cuttlefish fin (Fig. 4.2c), these intensities were at 1550.39^{cm-1} and 1398.61^{cm-1}.

Because no absorption band at 1540^{cm-1} was detected in each IR spectrum, it was confirmed that neither the purified GAGs from the cranial cartilages of squid and cuttlefish nor the GAGs from all other representative tissues in both the species contained residual protein contaminants. The absorption bands at 1556.46^{cm-1}, 1398.63^{cm-1} obtained for squid gladius (Fig. 4.1e) and at 1560.50^{cm-1}

and 1401.14^{cm-1} cuttlefish pen (Fig. 4.2e) can be attributed to the –C-O-H vibrations of the N –acetyl groups of chitin glucosamines that might have released from these chitinous tissues during digestion with papain and subsequent precipitation with ethanol. The broad band above 3000^{cm-1} can be assigned to hydroxyl-stretching vibrations of N –acetylglucosamine units of chitin in the squid gladius, and in the case of cuttlefish pen the diffuse peaks obtained 3000^{cm-1} prominently due to water present in the sample.

4.3.2 Proton NMR spectroscopy

Nuclear magnetic resonance (NMR) spectroscopic analysis was successfully carried out particularly for the characterization of heterogeneous mixtures of GAGs obtained from the cranial cartilages of both the cephalopod species. The isolated GAGs, each obtained from the cranial cartilages of squid and cuttlefish were characterized using ^{1}H NMR spectroscopy. The characteristic signals (^{1}H) for the commonly occurring sugar residues have been identified (Fig. 4.5 and Fig. 4.6). Authentic samples of chondroitin sulphate C (Fig 4.7(d)) and the monosaccharide N-acetylglucosamine (Fig 4.7(e)) were also analyzed and compared with the sample spectra for confirmation.

^{1}H NMR spectra are represented in Fig 4.5 and 4.6 for squid and cuttlefish respectively are comparable with the authentic chondroitin sulphate C (Fig 4.7(d)). Although the resulting ^{1}H NMR spectra of all the samples analyzed were in the range 15.04 to 1.620 ppm acquired in steps of 0.001 ppm (Fig 4.5 and 4.6 inset), the δ range was limited from 6.31 to 0.41 ppm since the ^{1}H NMR peaks of GAGs fell within this range. Fig. 4.7 (a), Fig. 4.7 (b), Fig. 4.7 (c) and Fig. 4.7 (f) shows the comparison ^{1}H NMR spectra of the samples and reference standards of each GAG type plotted in the range δ = 0.5 to 3.0 ppm and δ = 4.0 to 6.0 ppm respectively.

Owing to the presence of hydrogens in different environments, the spectra of these GAGs presented overlapping signals. Despite their broad signals, it can be seen that each compound showed a characteristic spectrum, the shape of which was distinguishable from the others. The spectra were divided into three ranges, from

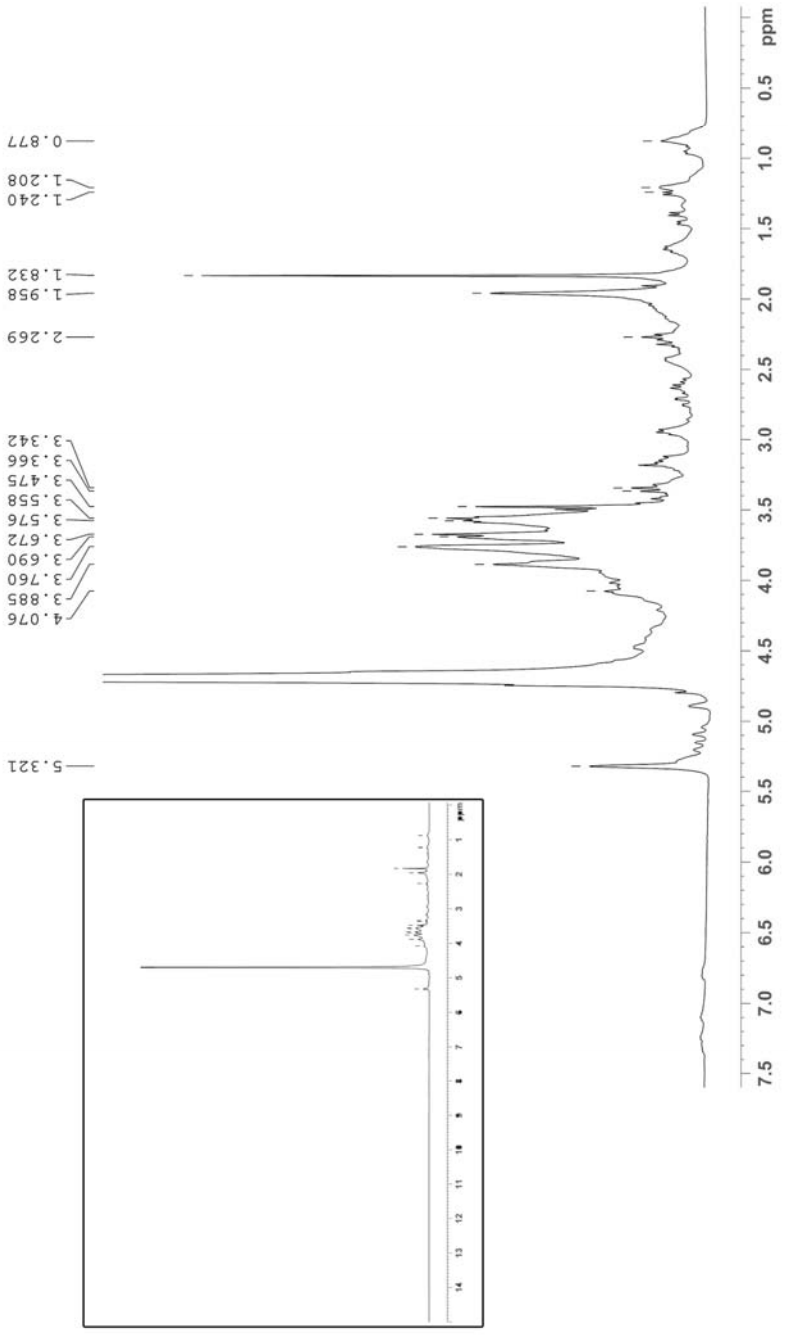


Figure 4.5: ^{1}H NMR spectra of CS - GAGs from squid cranial cartilage

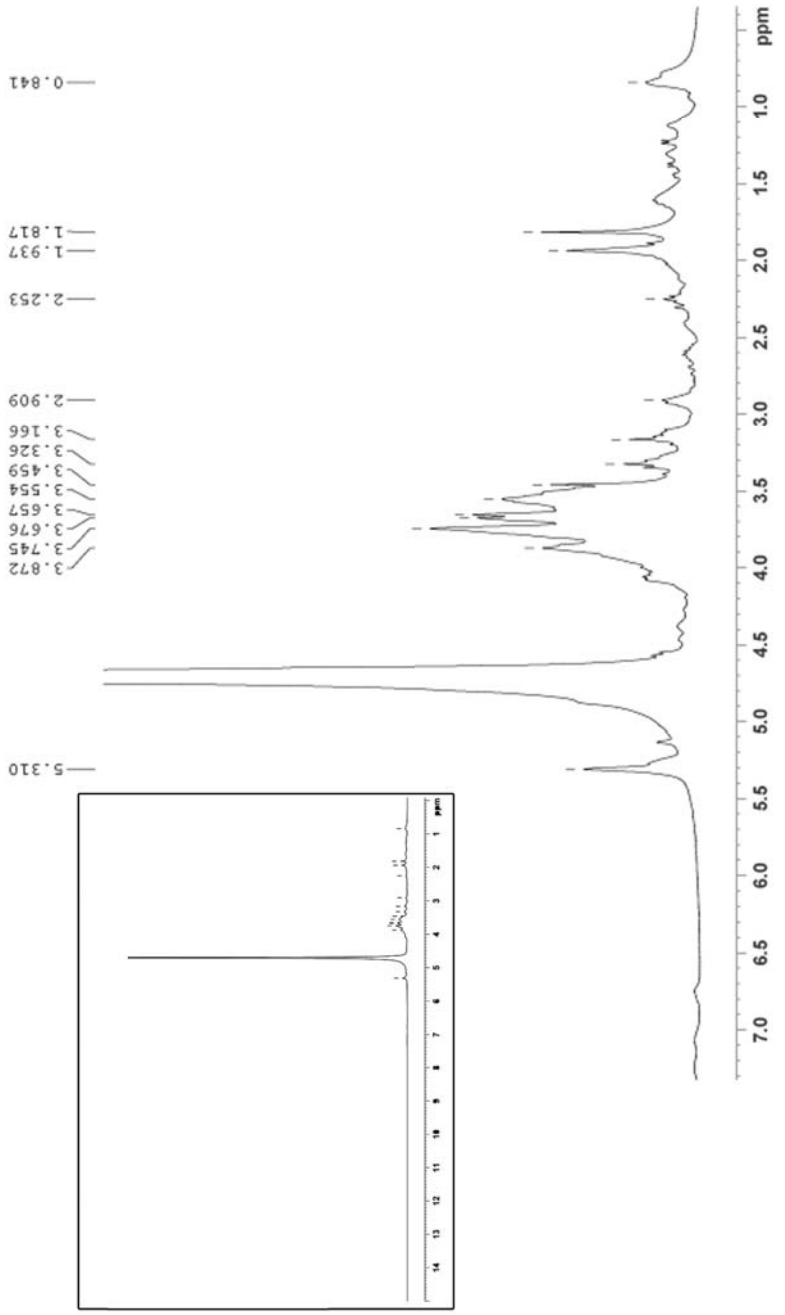


Figure 4.6: ^{1}H NMR spectra of CS - GAGs from cuttlefish cranial cartilage

1.9 to 2.2 ppm (range A), from 2.9 to 4.5 (range B) and from 5.0 to 5.7 ppm (range C), representing the methyl protons and the sugar ring protons; relevant for the characterization of GAG samples. The water range was from 4.50 to 5.00 ppm. The anomeric signals of the *N*-acetyl-D-galactosamine, CS-C and the glucuronic acid of chondroitin sulphates, shifted upfield (4.67 and 4.46 ppm, respectively) so that the water signal interfered with them. In the central range (from 2.9 to 4.5 ppm) differences in the spectra of the GAGs were difficult to correlate to defined structures since signals of several ring hydrogens, H-2, H-3, H-4, H-5 and H-6 of both hexuronic acids and hexosamine, overlapped within a narrow spectral range. However, the resulting evolvents were significantly dissimilar, so that this range was expected to contain relevant information for GAGs characterization. ^{1}H NMR spectra also provides information about characteristic sulphation sites. *O*-sulphation causes downfiled shifts of protons bound to *O*-sulphated carbon atoms by approximately 0.4 – 0.7 ppm. Thus the sulphation positions can be determined by comparison with proton signals of nonsulphated saccharide residues. The data obtained was compared with those reported previously for the authentic ΔHexAα1-3GalNAc, unsulphated chondroitin sulphate reported by Yamada *et al*. (1992) (Appendix 4, Table 4.1). In the spectra of both the cephalopod samples there were downfield shifts indicating presence of sulphation sites. Finally, in the range 1.9–2.2 ppm, the differences in the spectra were mainly due to the relative intensity and the chemical shift of the methyl hydrogens of the acetamido group of GalNAc and GlcNAc, which appeared respectively at 2.08 ppm and 2.05 ppm.

The data were virtually identical with the authentic ΔHexAα1-3GalNAc, chondroitin sulphate spectra, indicating that the disaccharide is derived from chondroitin, but not from hyaluronic acid and that structural modifications by any uncharged groups or sulphate are also present. The GAGs especially from squid cranial cartilage showed the presence of fucose as evidenced by the colorimetric estimation by cysteine-sulphuric acid method (Chapter 3, Table 3.2). But the characteristic chemical shifts of methyl group of fucose around δ 1.5 ppm could not be assigned from the ^{1}H NMR spectra and hence require further characterization.

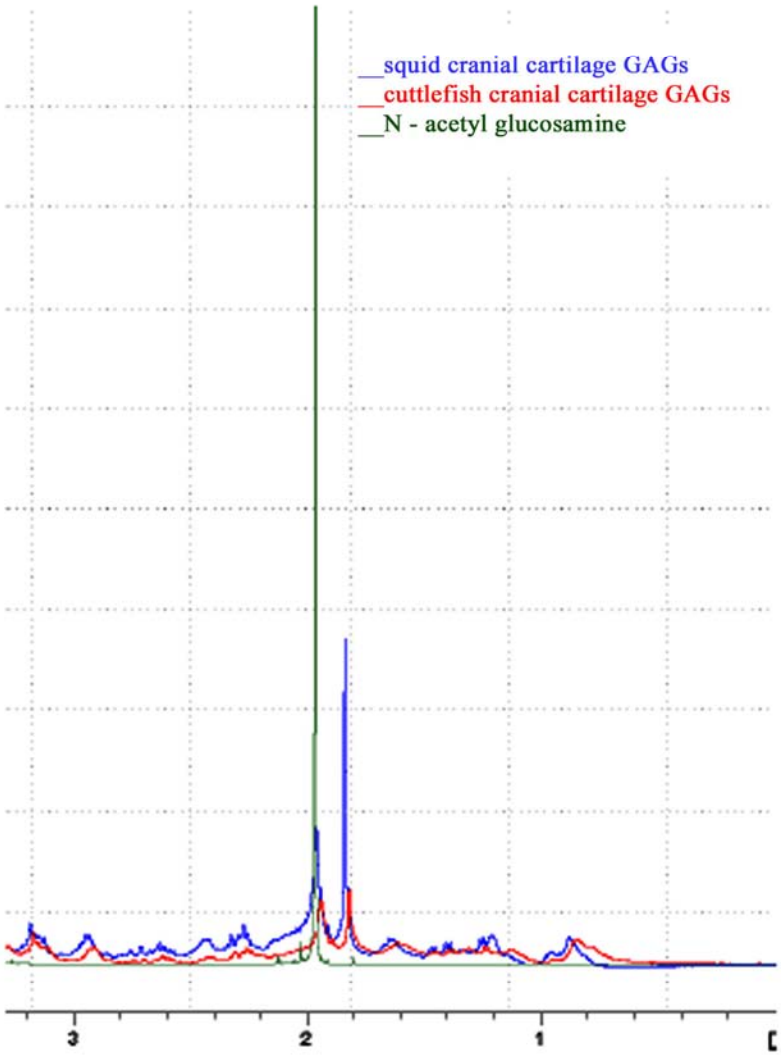


Figure 4.7 (a): Comparison ^{1}H NMR spectra of CS - GAGs and standard N -acetyl glucosamine (1-3 ppm)

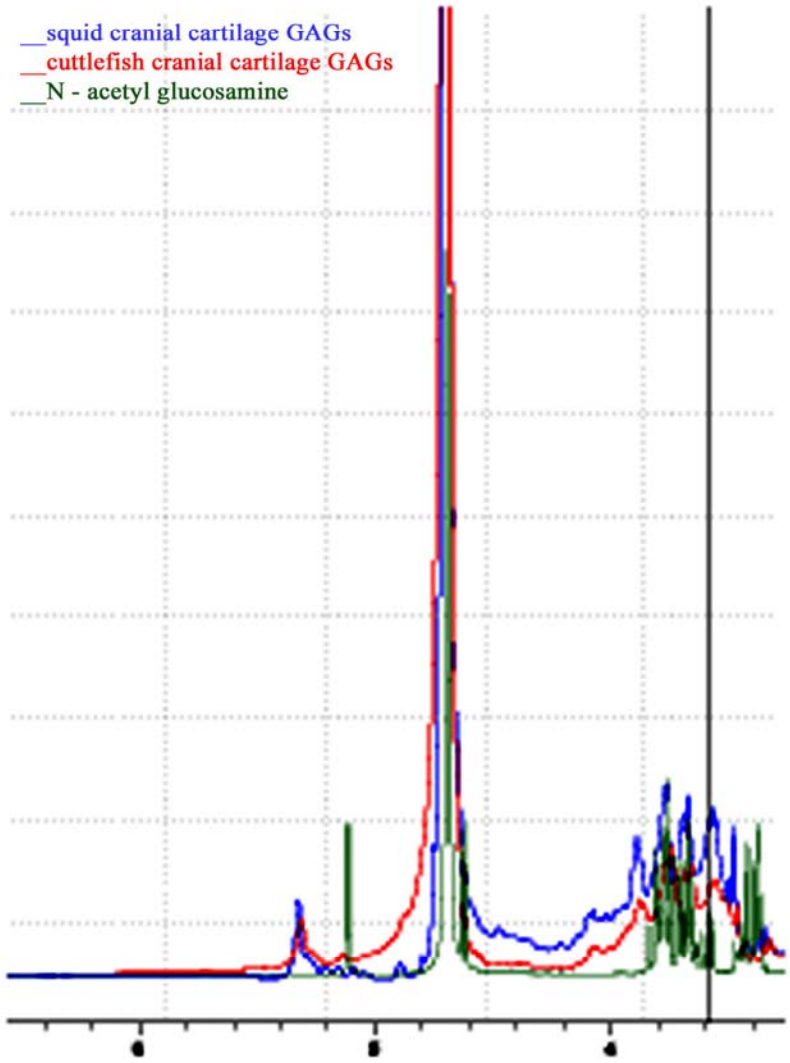


Figure 4.7(b): Comparison ^{1}H NMR spectra of CS - GAGs and standard N -acetyl glucosamine (4-6 ppm)

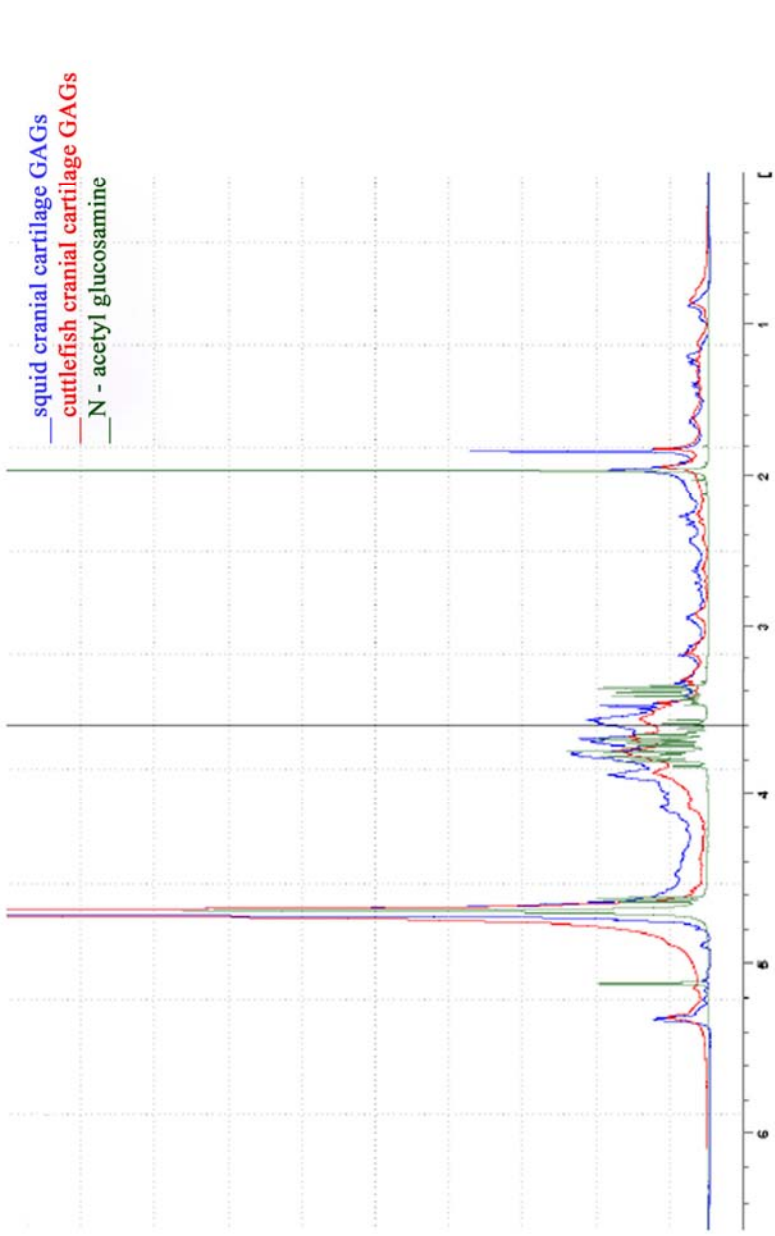


Figure 4.7(c): Comparison ^{1}H NMR spectra of CS - GAGs and standard N -acetyl glucosamine (1-6 ppm)

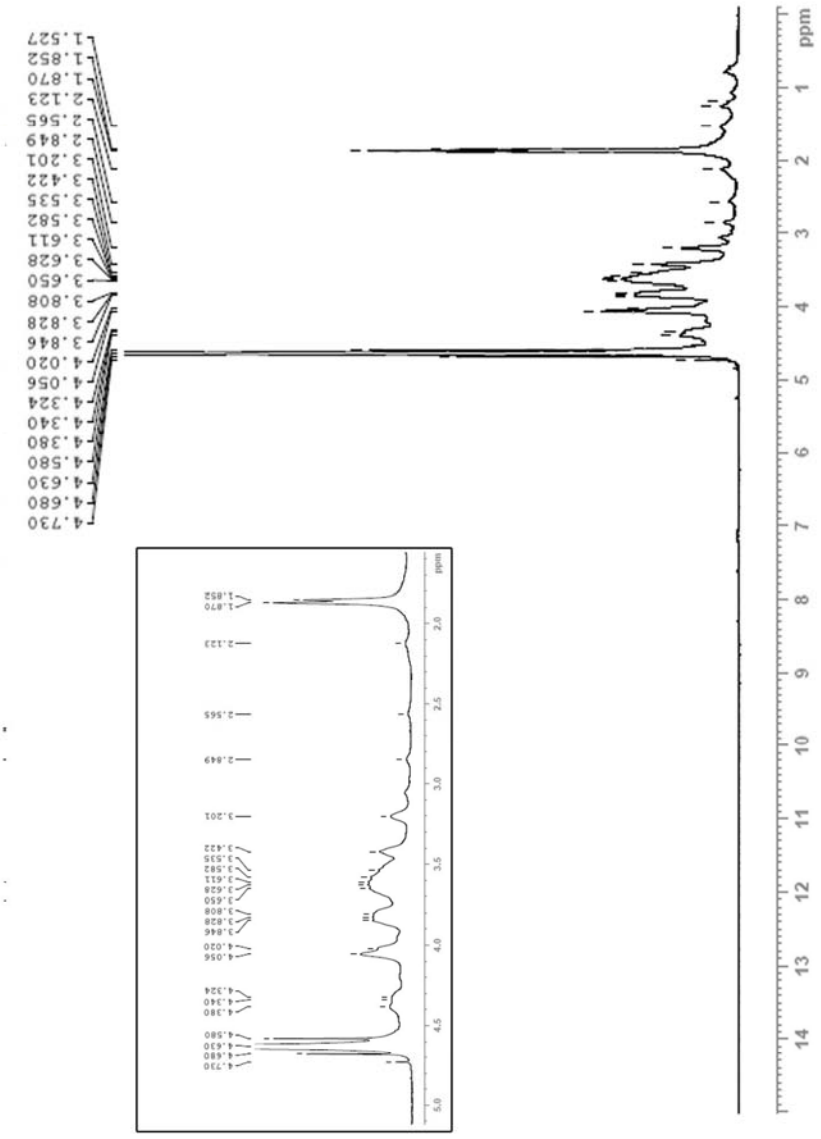


Figure 4.7 (d): ^{1}H NMR spectra of standard Chondroitin sulphate C

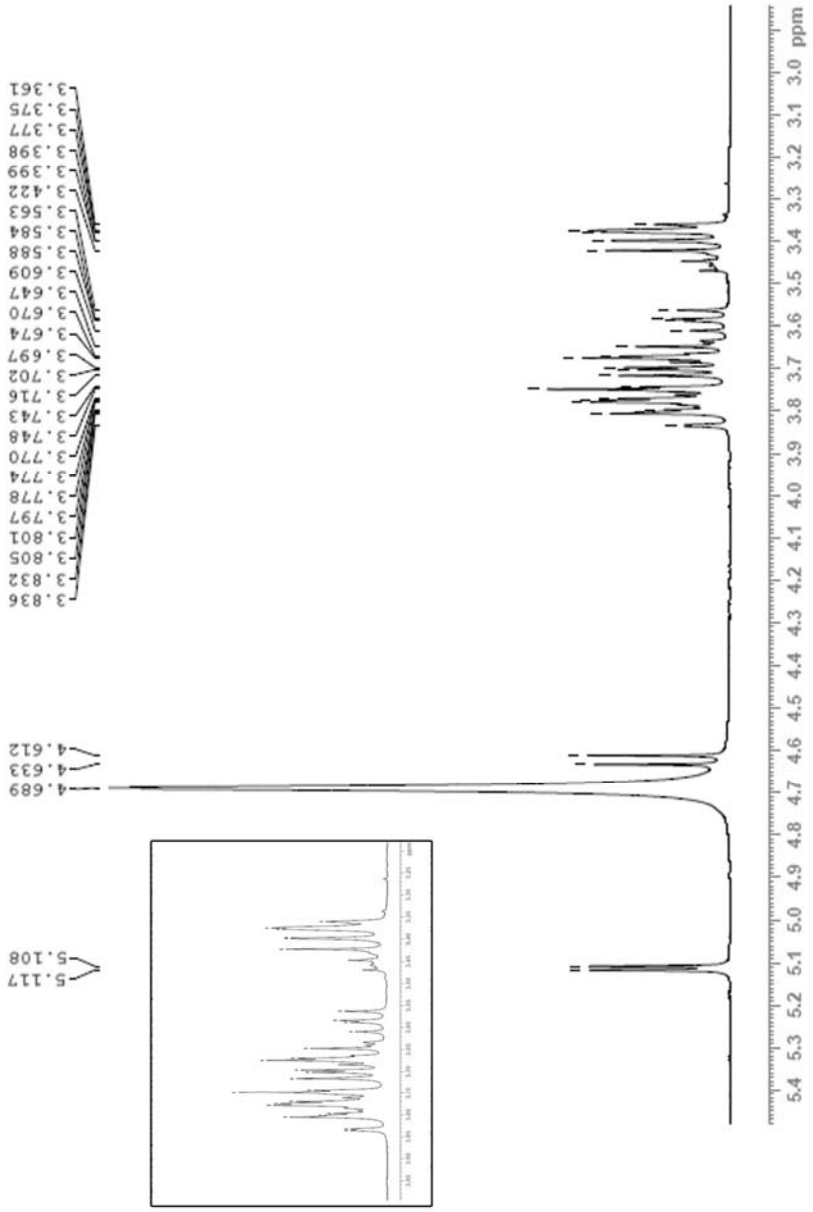


Figure 4.7 (e): ^{1}H NMR spectra of standard N - acetyl glucosamine

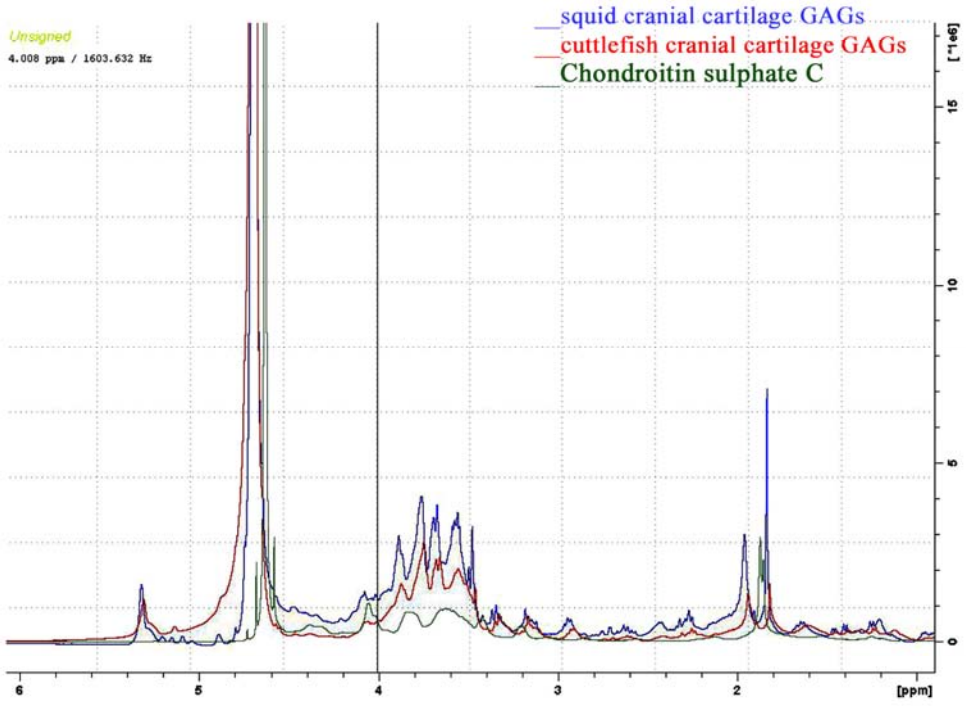


Figure 4.7 (f): Comparison ^{1}H NMR spectra of sample CS – GAGs and standard Chondroitin sulphate C

4.4 Discussion

4.4.1 ATR – FTIR spectroscopy

IR spectroscopy is an analytical tool able to detect characteristic vibrational modes of individual chemical groups and bonds. It has been used to examine and provide important data on a wide variety of biological molecules. Due to its holistic nature, FT-IR has been recognised as a valuable tool for metabolic fingerprinting/footprinting, owing to its ability to analyse carbohydrates, amino acids, fatty acids, lipids, proteins and polysaccharides simultaneously (Ellis *et al.,* 2002; Kaderbhai *et al.,* 2003; Harrigan *et al*., 2004). The extracted and purified GAGs produced spectra indicating the presence of methyl groups, carboxyl groups and sulphate. Whilst not as specific and sensitive as some techniques, such as GC-ToF-MS (gas chromatography – time of flight- mass spectrometry) (O'Hagan *et al.,* 2005), the rapidity and reproducibility of FT-IR cannot be overstressed. One potential disadvantage of FT-IR in the mid-IR is that the absorption of water is very intense, but this problem can be overcome in one of several ways such as dehydration of samples, subtraction of the water signal, or by application of attenuated total reflectance (ATR) (Ellis *et al.,* 2002; Winder and Goodacre, 2004). Since the GAGs obtained from the respective tissues of squid and cuttlefish were hygroscopic in nature the IR spectra was taken by applying ATR-FT-IR. In addition, another perceived disadvantage is that as a holistic measurement is made with biochemical information spread across the whole of the IR spectrum, validated and robust chemometrics must be used in order to turn data into information (Ruiz-Calero., 2002).

4.4.2 Proton NMR spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is a non-destructive analytical method for the determination of natural products. NMR methods are suitable for determining the proton chemical shifts, which indicate the presence, type and location of substituents, such as acetyl, methyl, phosphate and sulphate groups (Vliegenthart *et al*., 1983). NMR spectroscopy can be applied successfully, particularly for the characterization of heterogeneous mixtures of GAGs (Casu and Torri, 1999; Yamada *et al.,* 1999; Guerrini *et al.,* 2002; Huckerby *et al.,* 2005).

The characteristic signals (proton) for the commonly occurring saccharide species have been identified. The differences between GAGs are due to three main factors of variation: the identities of the hexosamine unit (substituted galactosamine or glucosamine) and the hexuronic acid (mainly GlcA or L-IdoA2S or nonsulphated L-IdoA) and the sulphation of both residues. These factors are responsible for the spectra and, thus, information about GAG composition may be estimated from them. In this study, the identification of the spectral signals was based on the assignment of the chemical shifts obtained from one dimensional ^{1}H NMR characterization.

Owing to the complexity of GAGs, ^{1}H NMR spectra of intact GAGs reveal broad peaks of overlapped signals. Despite the difficulty involved in interpreting them, characteristic signals can be assigned to different groups of substituted uronic and hexosamine units with similar chemical environments. In this way, several authors have employed ^{1}H NMR spectroscopy for the characterization of GAGs. The estimation of dermatan sulphate contamination in heparins by means of the quantitation of the corresponding ^{1}H NMR signals have been reported (Ruiz-Calero *et al.,* 2002). Kinoshita *et al.* (1997) characterized novel tetrasaccharides isolated from squid cartilage chondroitin sulphate E by ^{1}H NMR spectroscopy and reported on the sulphation pattern of these saccharides. Characterization of dermatan sulphate from the skin of the eel *Anguilla japonica* using one dimensial ^{1}H NMR was reported by Sakai *et al.* (2003). Sulphated oligosaccharide characterization of chondroitin sulphate D from shark cartilage (Sugahara *et al.,* 1996c; Nadanaka and Sugahara, 1997) and chondroitin sulphate K from king crab cartilage (Sugahara *et al.,* 1996b) were performed using 500MHz ^{1}H NMR spectroscopy. Sequence determination of a non-sulphated glycosaminoglycan like polysaccharides from melanin-free ink of the squid, *Ommastrephes bartrami* by using ^{1}H NMR spectroscopy was reported by Chen *et al.* (2008). ^{1}H NMR spectroscopy has been also used as a tool to provide characteristic fingerprints of complex carbohydrates for quality assessment and purity control (Abeygunawardana *et al.,* 2000).

NMR also provides the most accurate quantitative estimate for IdoA versus GlcA in a GAG preparation (Guerrini *et al.,* 2002). The 1-D proton NMR spectrum along with the 2-D spectra affords information on the chemical shifts and coupling constants of most of the ring protons of the constituent monosaccharides. This data is used to identify and quantify the uronic acid and hexosamine constituents of a given oligosaccharide, as well as provide direct linkage information for adjacent monosaccharides. Despite these clear advantages of NMR, there are some important limitations in applying NMR to characterize tissue-derived GAGs. NMR requires milligram quantities of material (particularly for the ^{13}C spectra), which is not available from tissue samples. In the present study the ^{13}C spectra of the samples could not be assigned due to insufficient sample quantities (Appendix 4, Fig 4.1 and 4.2). The sensitivity of this technique is lower than that based on detection of chromatographic effluents and MS. Specifically, NOESY and ROESY spectra usually require more sample and high-field instruments for reasonable sensitivity and signal resolution.

^{1}H NMR spectra are used as multivariate data for chemometric analysis. Principal component analysis (PCA) or artificial neural network (ANN) analysis have proved to be efficient for specific applications mainly in the fields of food characterisation and authentication, (Sacchi *et al.,* 1998) clinical diagnoses, (Kaartinen *et al.,* 1998) and carbohydrate characterization (Thomsen and Meyer 1989). In this way, the information extracted by principal component analysis (PCA) from the ^{1}H NMR spectra has been applied in establishing methods for detecting typical patterns in heparins from bovine, porcine and ovine intestinal mucosa and bovine lungs, as well as for differentiating between GAG classes (Heparin, CSB, CSA, CSC and HA) (Yamada *et al.,* 1992; Yates *et al.,* 1996; Chuang *et al.,* 2000; Huckerby *et al.,* 2005).

4.5 Conclusion

Spectroscopic techniques involving FT-IR and NMR are exploited in very diverse fields including chemical and biochemical analysis, environmental monitoring, field applications, process control monitoring and imaging amongst

many others. The manifold advantages of such approaches based on vibrational spectroscopy include low cost, small size, compactness, robustness, lightweight, as well as mass reproducibility.

The significance of the NMR method proposed lies in the fact that the study and characterization of these compounds (GAGs), which in general involves chromatographic and electrophoretic techniques, is often complex owing to their macromolecular, polydispersive and heterogeneous structures. Although nuclear magnetic resonance spectroscopy has rarely been used in control and routine analyses, recent studies demonstrate the usefulness of this technique when analysing complex macromolecular samples. In addition, nuclear magnetic resonance spectra of unfractionated compounds can be correlated to their disaccharide composition; thus, the tedious and time consuming enzymatic or chemical hydrolysis and further analysis of fragments released can be avoided.

The results obtained from the FT-IR spectra and proton NMR reveals that the cranial cartilage GAGs of both the cephalopod species are of the chondroitin sulphate type with characteristic modifications. Thus the present investigation showed that the cranial cartilages of both these cephalopods could be used as a possible source of chondroitin sulphate in future.

Chapter 5: Bioactive Potential of the isolated Glycosaminoglycans - Role in Biomineralization

Contents

5.1 Introduction

Biomineralization is a widespread phenomenon in nature leading to the formation of a variety of solid inorganic structures by living organisms, such as intracellular crystals in prokaryotes; exoskeletons in protozoa, algae, and invertebrates; spicules and lenses; bone, teeth, statoliths, and otoliths; eggshells; plant mineral structures; oyster pearls and also pathological biomineralization such as gall stones and kidney stones. Calcifications, particularly those involving the formation of $CaCO_3$ crystals, are among the most common normal and pathological biomineralizations (Addadi *et al*., 2006).

Three crystal phases of anhydrous calcium carbonate are encountered in nature. The most stable phase thermodynamically is calcite followed by aragonite and vaterite. Under ambient conditions $CaCO_3$ crystal commonly exists in the stable form, calcite; aragonite is the high pressure polymorph, whereas vaterite is thermodynamically unstable (Zhou and Zheng, 1998). Vaterite acts as a precursor in the formation of aragonite or calcite and is a very unstable phase of calcium carbonate rarely occurring in nature. The assembly of these biominerals consists of a four-stage process. It starts with the fabrication of a hydrophobic solid organic substrate or scaffolding onto which nucleation of the crystalline phase takes place closely associated with some polyanionic macromolecules. Crystal growth is then controlled by the addition of gel-structuring polyanionic macromolecules, and finally mineralization arrest is accompanied by the secretion of a new inert scaffolding of the same type or the deposition of other hydrophobic inhibitory

macromolecules (Arias and Fernandez, 2008).

Aragonite's crystal lattice differs from that of calcite, resulting in a different crystal shape, an orthorhombic system with acicular crystals. It is formed by biological and physical processes, including precipitation from marine and freshwater environments. Aragonite forms naturally in almost all mollusc shells, and as the calcareous endoskeleton of warm- and cold-water corals (Scleractinia). Because the mineral deposition in mollusc shells is strongly biologically controlled, some crystal forms are distinctively different from those of inorganic aragonite. In some molluscs, the entire shell is aragonite; in others, aragonite forms only discrete parts of a bimineralic shell (aragonite plus calcite). Aragonite also forms in the ocean and in caves as inorganic precipitates called marine cements and speleothems, respectively. The nacreous layer of the aragonite fossil shells of some extinct ammonites forms an iridescent material called ammolite. Ammolite is primarily aragonite with impurities that make it iridescent and valuable as a gemstone. Aragonite is metastable and is thus commonly replaced by calcite in fossils.

The mineral vaterite, also known as μ-$CaCO_3$, is another phase of calcium carbonate that is metastable at ambient conditions typical of Earth's surface, and decomposes even more readily than aragonite. Biovaterite was first reported to occur in the repair tissues of shells of certain gastropods. In biological systems, vaterite exists in a variety of organisms controlled by gene or environment, such as gallstone in humans (Palchik and Moroz, 2005) or otolith in diversified freshwater fish (Oliveira and Farina, 1996; Tomas *et al.,* 2004). The existence of the vaterite is one of the key factors influencing the quality of freshwater cultured pearls (Qiao *et al*., 2007).

During the past decade, there has been remarkable progress in the development of bioinspired procedures for controlling inorganic crystal nucleation and growth. A great variety of aminoacids, proteins and polysaccharides have been shown to be involved in controlling biomineralization (Xie *et al*., 2005). A large number of proteins have been described which are involved in the control of

biomineralization (Michenfelder *et al*., 2003). These proteins are usually highly negatively charged and contain carboxylate, sulphate, or phosphate as functional groups, which may bind Ca^{2+} ions and could control crystal nucleation and growth by lowering the interfacial energy between the crystal and the macromolecular substrate. Since the works of Abolins-Krogis (1958) a slow but increasing interest has been developed to explore the role of polysaccharides in biomineralization, despite the fact that their involvement in biomineralization seems to appear very early in evolution (Benzerara *et al*., 2006). A mixture of hydroxylated, carboxylated, or sulphated functional moieties of polysaccharide may also be associated with biominerals. Although chitin occurs in many calcium carbonate-based biominerals, its role is almost passive. In fact it has been demonstrated that neither chitin nor chitosan *per se* modify *in vitro* calcium carbonate nucleation and growth (Neira-Carrillo *et al.,* 2005). However, in biominerals chitin occurs not by itself but constitutes an insoluble two-dimensional scaffolding or three dimensional compartment wherein chitin-associated polyanionic polysaccharides or proteins control crystallization in a confined space (Levi-Kalisman *et al.,* 2001; Arias and Fernandez, 2008).

Growth control of calcium carbonate crystal by organic chemicals contributes an important part to its mineralizing mechanism and biomimetic preparation. $CaCO_3$ is a common biomineral and extensive attention has been paid to its mineralization mechanism and biomimetic synthesis (Yu *et al*., 2004). $CaCO_3$ usually exists in calcite crystal; however, in the presence of various polymers such as biomacromolecules, dendritic polymers/double-hydrophilic block copolymer, it will exist in other or a mixed type of crystal, with different morphologies and sizes (Manoli *et al*., 2002). These organic polymers control $CaCO_3$ crystal growth, interacting with inorganic molecules via supermolecule fore assembly, interfacial molecular recognition and vector adjustment (Mann, 1993). Even though many macromolecules of various types have been described to be involved in biomineralization; the first indication about the possible role of glycosaminoglycans in calcium carbonate-based biominerals came from the studies on the occurrence of sulphated GAGs in the calcifying granules formed on glass

cover slips inserted either between the shell and the shell-forming cells of the mantle or at the initial site of shell regeneration (Saleuddin and Chan, 1969). With regard to sulphated GAG and glycoproteins as a nucleation factor, Wada (1980) emphasized the potential importance of sulphated sugars for mollusc shell formation. Dauphin (2003) conducted XANES (X-ray absorption near edge structure) analyses on Pinna and Pinctada prism and found a specific distribution pattern of sulphate and proteins. In principle, sulphate groups for calcification mostly involve sulphated GAG (and some sulphated glycoproteins), and these are certainly water soluble. It has been shown by Manoli and Dalas (2000), that the glycosaminoglycans C4S, C6S, and DS in solution stabilize the spontaneous precipitation of vaterite and influence the crystal size distribution. Marie *et al.* (2007) also found that sulphate rather than insoluble shell organics dominated in the freshwater mussel *Unio pictorum*, soluble shell organics. Nudelman *et al.* (2006) and Bezares *et al.* (2008), on the other hand, used cetylpyridinium chloride (CPC) for sulphate precipitation and stained decalcified insoluble shell fragments with colloidal irons. Sulphate presence in insoluble shell organics might be due to sulphated sugars attached to insoluble proteins as well. A case in point is the protein (Pearlin) extracted from insoluble organics in *Pinctada fucata* nacre that contains sulphate, which was confirmed by Alcian Blue (pH1) staining (Miyashita *et al.,* 2001). Compared with acidic shell proteins, there is still no structural information available about sulphated GAGs or insoluble sulphated glycoproteins for establishing a nucleation model as related to shell formation and thus require further investigation.

The adsorption capacity of different glycosaminoglycans onto coral were reported by Volpi (1999), with heparin having maximum adsorption followed by highly sulphated chondroitin sulphate, dermatan sulphate, and heparan sulphate; whereas hyaluronic acid showed no adsorption on to granules of coral. Volpi (1999) concluded that the percentage adsorption of polyanions onto coral depends mainly on their charge density, with sulphate groups being more important than carboxyl groups. It was hypothesized by Liu *et al.* (2010) that, anion groups in the organic molecule, HEP (heparin) might interact with calcium ion, resulting in

alteration of the crystal growth process. Based on this hypothesis, the influence of the highly sulphated chondroitin sulphate GAGs isolated from the cranial cartilages of squid and cuttlefish on the morphology and structure of $CaCO_3$ crystal was investigated in this study. The present chapter reports on the effect of glycosaminoglycans isolated from the cranial cartilages of squid and cuttlefish, on $CaCO_3$ (calcite) crystallization in defined conditions *in vitro*. Even though the study does not establish a direct role of these sulphated GAGs in mollusc shell formation, the results obtained in the present study obviously reflects the bioactive potential of these cephalopod GAGs in the crystal morphology of calcium carbonate.

5.2 Materials and methods

5.2.1 $CaCO_3$ crystal growth *in vitro*

The *in vitro* syntheis of $CaCO_3$ crystals were performed according to the method of Liu *et al.* (2010) as outlined in Fig 5.1. Briefly, 5.0 ml solutions each of the isolated GAGs from the cranial cartilages of squid as well as cuttlefish; each at a concentration of ~100mg/ml respectively were individually added to a 25.0 ml flask containing equal volume of 0.4 M Na_2CO_3 with agitation at 1200 rpm for 10 min. Then 10.0 ml of 0.2 M $CaCl_2$ was introduced and a white colored suspension was formed immediately. After agitating for 30 min, the suspension was placed for 10 min, followed by addition of an appropriate volume of deionised water. The suspension was centrifuged at 2000 rpm for 4 min, and the pellets were washed with deionised water twice to obtain $CaCO_3$ crystal. Appropriate controls with deionised water (5.0 ml) without the presence of GAGs were also used for $CaCO_3$ crystal growth formation. The crystal morphology and structure was examined by a scanning electron microscope (SEM), an X-ray diffraction (XRD) machine and a Fourier transform infrared spectrophotometer (FTIR). The crystal samples used for XRD and FTIR analysis were prepared by freeze drying.

5.2.2 SEM – EDAX

Control $CaCO_3$ crystals as well as $CaCO_3$ crystals prepared in the presence of squid and cuttlefish cranial cartilage GAGs were subjected to lyophilization and

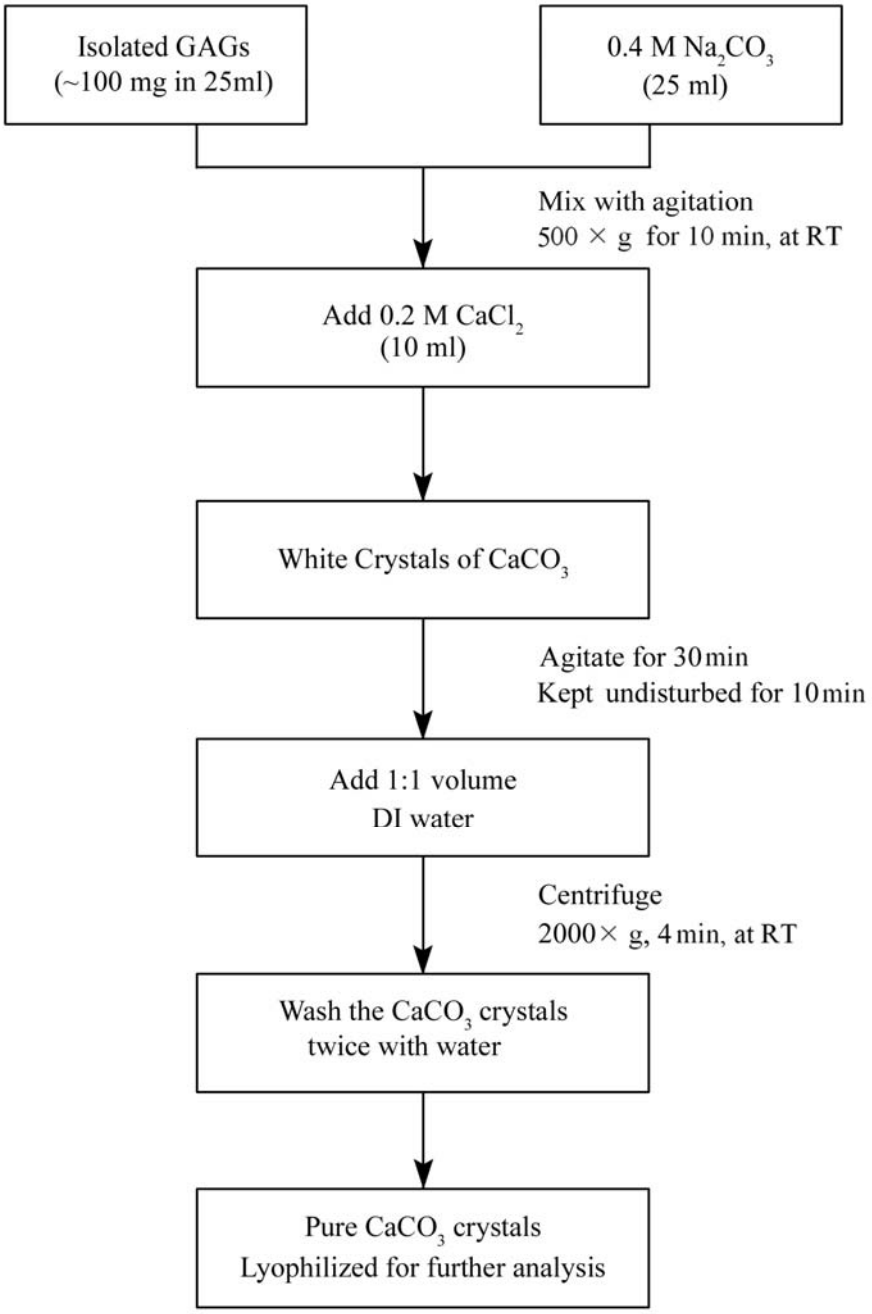


Fig. 5.1 Flow chart for *in vitro* synthesis of $CaCO_3$ crystals in presence of GAGs isolated from squid and cuttlefish

analysed using Scanning electron microscopy - energy dispersive X ray spectra (SEM-EDAX). The samples were coated with gold ions using an ion sputter coater, under the following conditions: 0.1 Torr pressure, 20mA current and 70 s coating time. Surface structure of the crystals formed were visualized by a scanning electron microscope JEOL Model JSM - 6390LV using a 15 KV accelerating voltage.

Electron microscopic element analyses (measuring energy dispersive X-ray spectra) were performed using a JEOL Model JED – 2300 EDS make. All measurements were performed at room temperature.

5.2.3 X - Ray diffraction analysis

X-ray powder diffraction (XRD) analysis was performed with a Bruker AXS D 8 Advanced X-ray powder diffractometer on finely powdered samples using Cu Ka radiation (40 kV and 30 mA) and a Ni filter with a scanning speed of 0.005° 2q s^{-1}. The time constant was set at 2 s.

5.2.4 FT- IR spectroscopy

IR spectroscopy of solid sample ($CaCO_3$ crystals formed *in vitro* in presence of the isolated GAGs) was tested using Thermo Nicolet, Avatar 370 instrument. Sample were mixed with 500 mg of dried potassium bromide (KBr) and then compressed to prepare a salt-disc (3 mm diameter). The disc was analyzed from 450 to 4000 cm^{-1}.

5.3 Results

5.3.1 Role of GAGs in $CaCO_3$ crystal growth

The crystallization of $CaCO_3$ (calcite) in defined conditions *in vitro* was performed as mentioned in section 5.2.1. The $CaCO_3$ crystals were obtained as a white powder that gets precipitated according to the chemical equation shown below.

$$Na_2CO_3 + CaCl_2 \longrightarrow CaCO_3 + 2NaCl$$

Organic additives cause complicated effects on the nucleation, growth and morphology of $CaCO_3$ crystals, and various environmental factors may also contribute to crystallization, such as pH, temperature, solution supersaturation and inorganic ion. While these environmental conditions remained constant, the morphology of $CaCO_3$ crystals was only determined by the absence or presence of sulphated GAGs. The $CaCO_3$ crystals were synthesized in the presence glycosaminoglycans (CS-GAGs) isolated from the cranial cartilages of squid and cuttlefish, as well as commercial chondroitin sulphate A. The $CaCO_3$ crystals synthesized in the absence of GAGs served as appropriate controls. The crystals obtained were characterized by scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FT-IR) and X-ray diffraction (XRD). The results show that, $CaCO_3$ crystals with various morphologies were formed in system in the presence of different types of GAGs whose ability to induce the formation of vaterite was enhanced in turn. This indicated that the GAGs played an important role in the process of crystal growth of $CaCO_3$.

At pH 7.0 GAGs are negatively charged due to their sulphate and carboxyl groups. Adsorption is therefore predominantly to calcium sites due to electrostatic binding. Two competitive reactions were involved in the present system: one was the interaction between Ca^{2+} and CO_3^{2-} the other was the interaction between Ca^{2+} and $COOH^-$ or HSO_3^- in the GAGs. The presence of CS-GAGs in the system, results in more obvious electrostatic interaction between Ca^{2+} and CO_3^{2-} or Ca^{2+} and HSO_3^-, a large number of Ca^{2+} were enriched on the interface of CS-GAG/water. With the increase of local Ca^{2+} concentration, nucleation of $CaCO_3$ in the chemical micro-environment nearby GAG molecules provided much more nucleation sites for $CaCO_3$ crystallization and produced more and smaller crystallization nuclei. These small crystallization nuclei enhanced greatly the surface energy of crystallization, resulting in the production of a lot of $CaCO_3$ crystals with morphologies differing from that of the control $CaCO_3$ crystals.

5.3.2 SEM – EDAX

SEM images of control $CaCO_3$ crystals (Plate 5.1 (a)) and $CaCO_3$ crystals

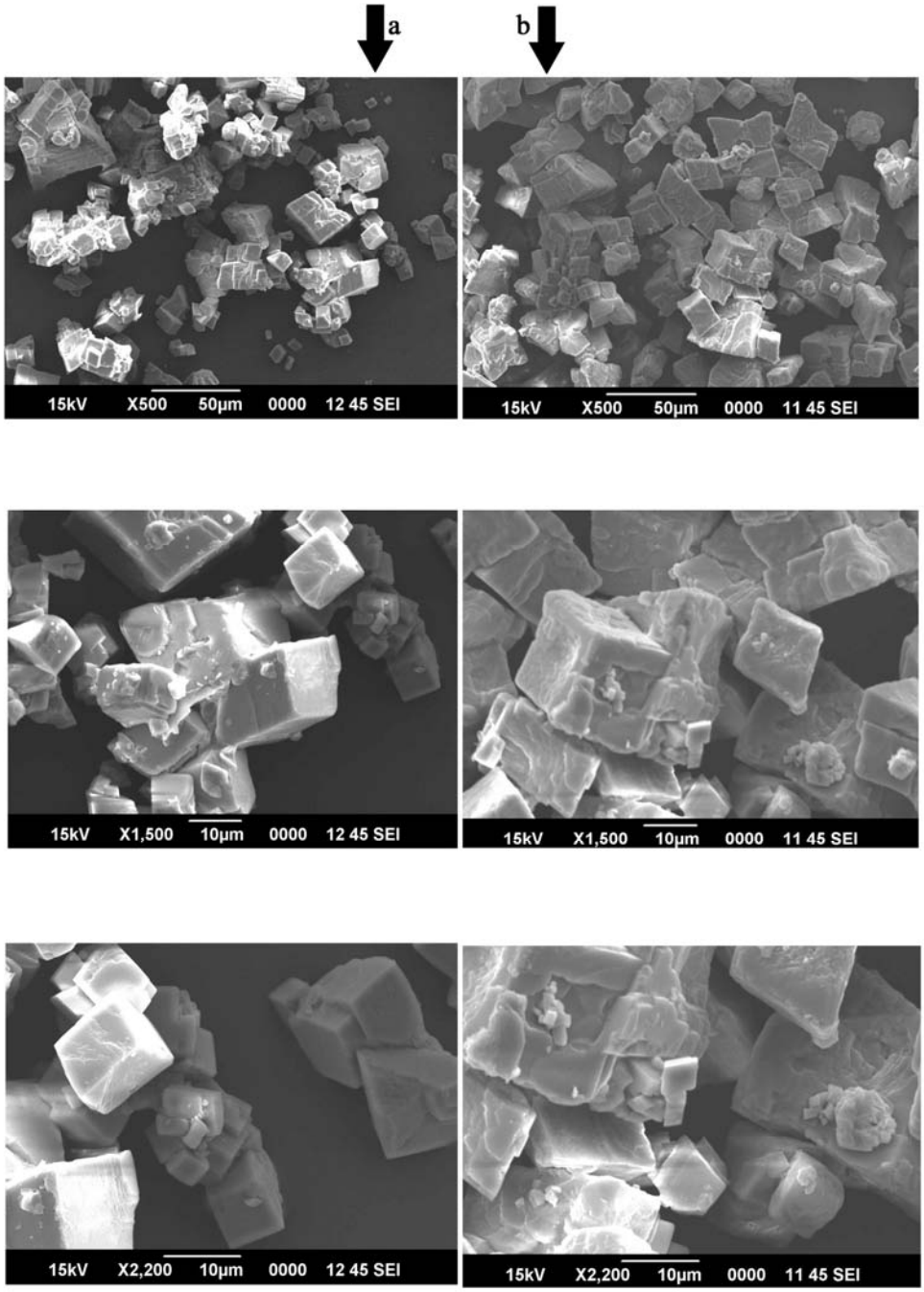


Plate 5.1: SEM images of (a) control $CaCO_3$ crystals & (b) crystals synthesized in the presence of standard Chondroitin sulphate A at different magnifications

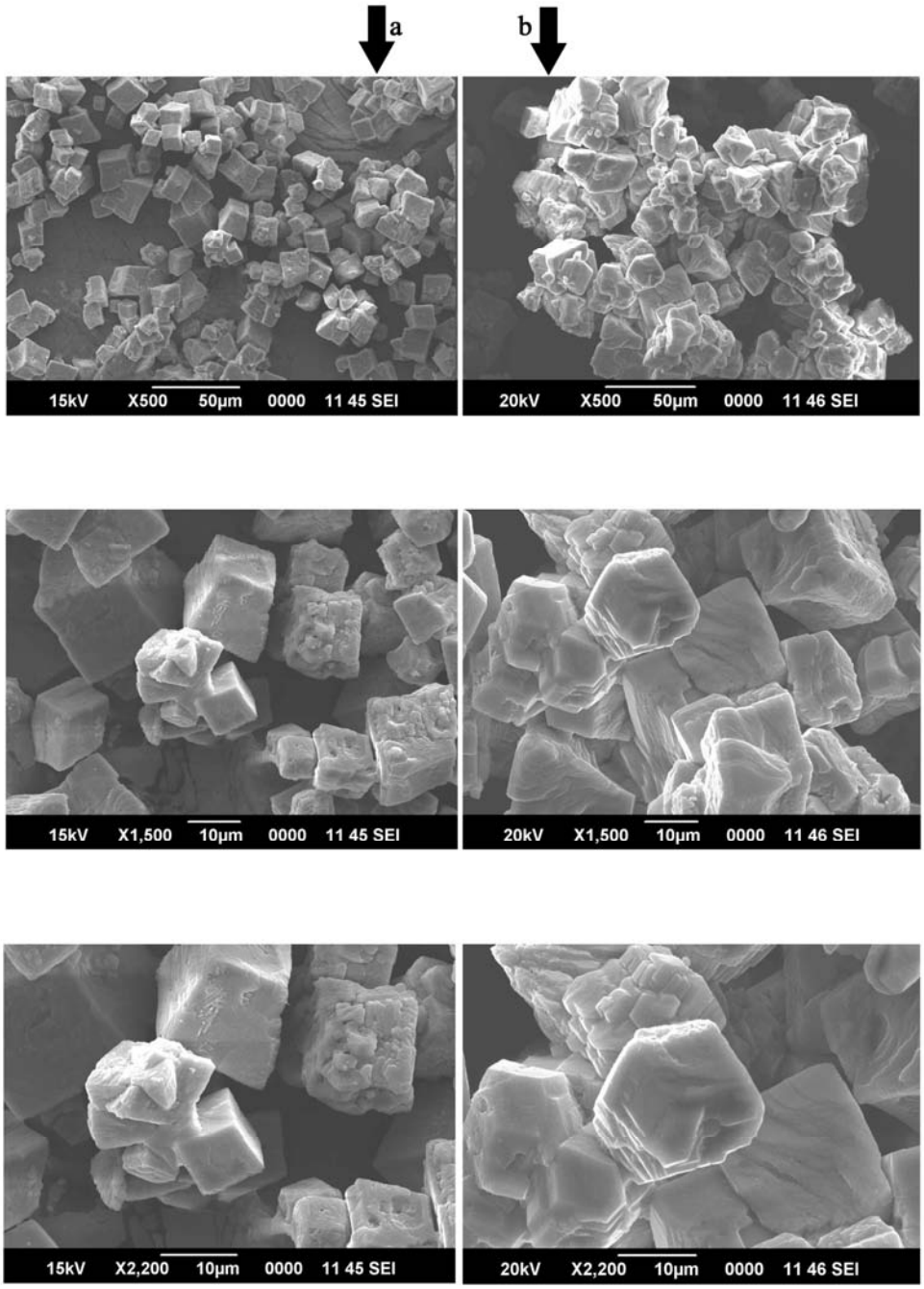


Plate 5.2: SEM images of $CaCO_3$ crystals synthesized in the presence of cranial cartilage GAGs from (a) squid & (b) cuttlefish at different magnifications

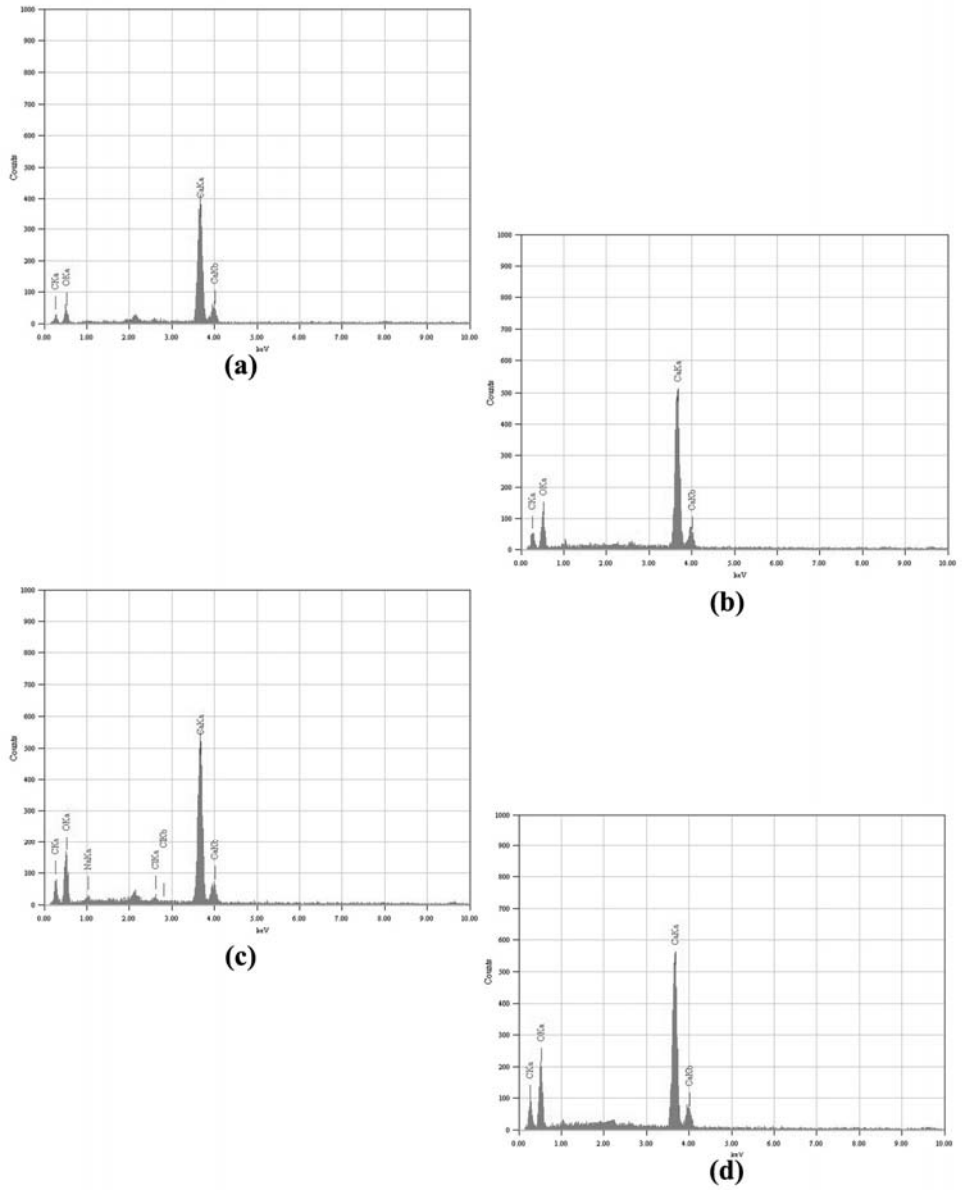


Plate 5.3: SEM-EDAX of $CaCO_3$ crystals synthesized *in vitro*

(a) control $CaCO_3$ crystals, (b) $CaCO_3$ crystals in presence of standard Chondroitin sulphate A, (c) $CaCO_3$ crystals in presence of squid cranial cartilage GAGs, (d) $CaCO_3$ crystals in presence of cuttlefish cranial cartilage GAGs

synthesized in the presence of different GAGs, ie., standard Chondroitin sulphate A, GAGs isolated from the cranial cartilages of squid and cuttlefish (Plates 5.1(b), 5.2 (a) and 5.2 (b) respectively) depicted the role of GAGs in the crystal morphology of calcium carbonate. Typical calcite crystals were formed in the control samples synthesized in the absence of any GAGs (Plate 5.1 (a)). $CaCO_3$ crystal gradually changed from an irregular rhombus to a subsphaeroidal shape, in the case of samples synthesized in the presence of different GAGs, confirming the gradual transition from calcites to vaterites. Therefore, it is concluded that the GAGs especially of the cuttlefish samples remarkably affected the morphology and size of the $CaCO_3$ crystal (Plate 5.2 (b)). At a higher magnification, the surface appeared not a smooth but a stripe-piled structure, indicating that the $CaCO_3$ crystals might be formed by a gradual block accumulation (Plates 5.1 (b), 5.2 (a) and 5.2 (b)). These results implied that the squid and cuttlefish GAGs were a good modifier of $CaCO_3$ crystallization (Plates 5.2 (a) and 5.2 (b)). Plate 5.3 (a-d) shows the main elements (increasing energy values from left to right) contained in the crystals synthesized *in vitro*, as obtained by the energy-dispersive-X-ray (EDAX) analysis (Appendix 5). Plate 5.3 (a) shows the main elements in the control $CaCO_3$ crystals. Plate 5.3 (b), (c) and (d) shows the main elements in the $CaCO_3$ crystals synthesized in the presence of standard Chondroitin sulphate A, squid cranial cartilage GAGs and cuttlefish cranial cartilages respectively.

5.3.3 X - Ray diffraction analysis

Fig 5.2 (a – d) depicted the XRD spectra of $CaCO_3$ with GAGs (Fig 5.2 (b, c, d)) and without GAGs (Fig 5.2(a)). In the absence of GAG, diffraction peaks at (012), (104), (110), (113), (202), (018) and (116) came from a typical calcite of $CaCO_3$ crystal. Peak intensities corresponding to vatrerite such as (004), (110), (112), (114), (300) and (118) were not present indicating that the formed crystral was typical of calcite. Even if a pure production of spherical vaterites was not achieved and a strong (104) diffraction peak was still found in XRD spectra, the morphological analysis showed that the crystals are attaining a gradual transition.

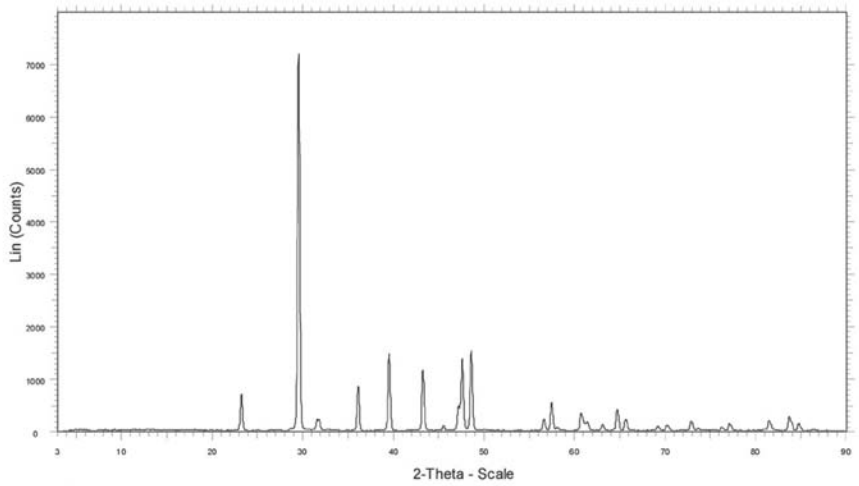


Figure 5.2 (a): XRD pattern of control $CaCO_3$ crystals

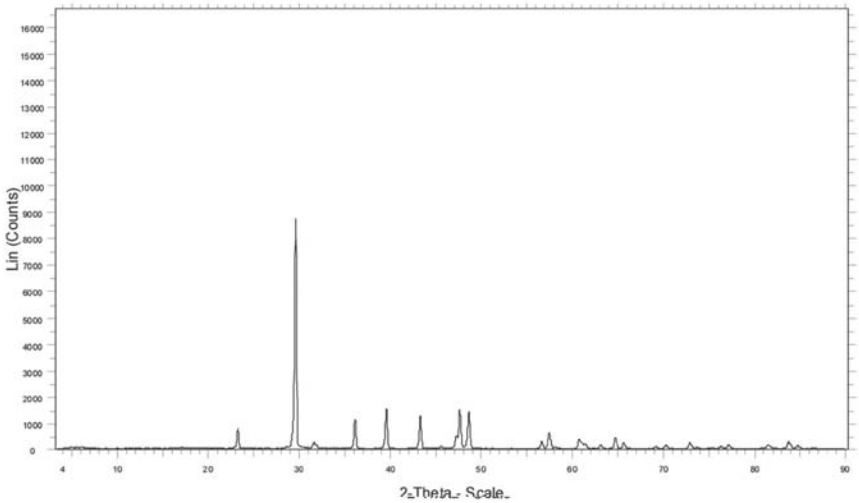


Figure 5.2 (b): XRD pattern of $CaCO_3$ crystals synthesized in the presence of standard Chondroitin sulphate A

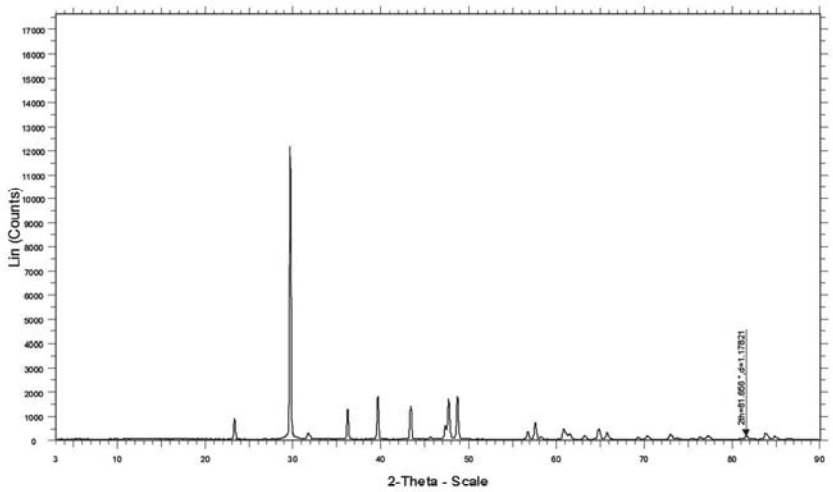


Figure 5.2 (c): XRD pattern of CaCO3 crystals synthesized in the presence of GAGs from squid cranial cartilage

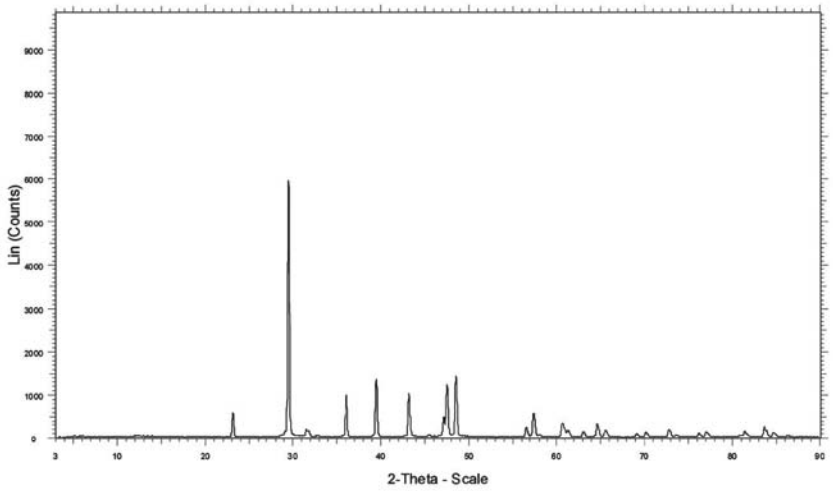


Figure 5.2 (d): XRD pattern of $CaCO_3$ crystals synthesized in the presence of GAGs from cuttlefish cranial cartilage

5.3.4 FT- IR spectroscopy

FTIR spectra of control $CaCO_3$ crystals and the crystals synthesized in the presence of different GAGs - standard Chondroitin sulphate A, GAGs isolated from the cranial cartilages of squid and cuttlefish are given in Fig 5.3 a – d respectively. The presence of peaks at 873 cm^{-1} and 711 cm^{-1} revealed the formation of calcite crystals in the absence of GAGs. The slight peak shifts can be seen in the samples synthesized in the presence of sulphated GAGs (704.47 cm^{-1} and 706.45 cm^{-1}). These results implied that different types of $CaCO_3$ crystals could be generated through the adjustment of GAG type and concentration contained in the system.

5.4 Discussion

Biomineralization research has mostly focused on the role of protein, acidic macromolecules and a variety of hydroxylated, polycarboxylated and sulphated polysaccharides. Although the principle concept behind biomineralization was developed many years ago, the individual components have not been well scrutinized. The present chapter focuses on the role of squid and cuttlefish GAGs (isolated from the cranial cartilage) in the morphology of crystal structures synthesized *in vitro*. The analysis of the polymorphic forms of the $CaCO_3$ crystals structure synthesized in the stipulated conditions *in vitro* have been demonstrated by various techniques including scanning electron microscopy – elemental X ray dispersion analysis (SEM-EDAX), spectroscopic analysis of the crystals using FT-IR and identification of the crystalline phases of the $CaCO_3$ crystals were performed using XRD. Whether or not, the formation of organic mineralized endoskeletal structures of cephalopods is influenced by GAGs or any acidic macromolecules present especially in the mantle epithelial cells, requires the analysis and characterization of these macromolecules in these sites. Hence the present study does not establish a direct role of the sulphated GAGs in the cranial cartilages of squid and cuttlefish on mollusc shell formation. Instead, the results obtained in the present study illustrate the bioactive potential that the GAGs elicit in the morphology of calcium carbonate crystals *in vitro*.

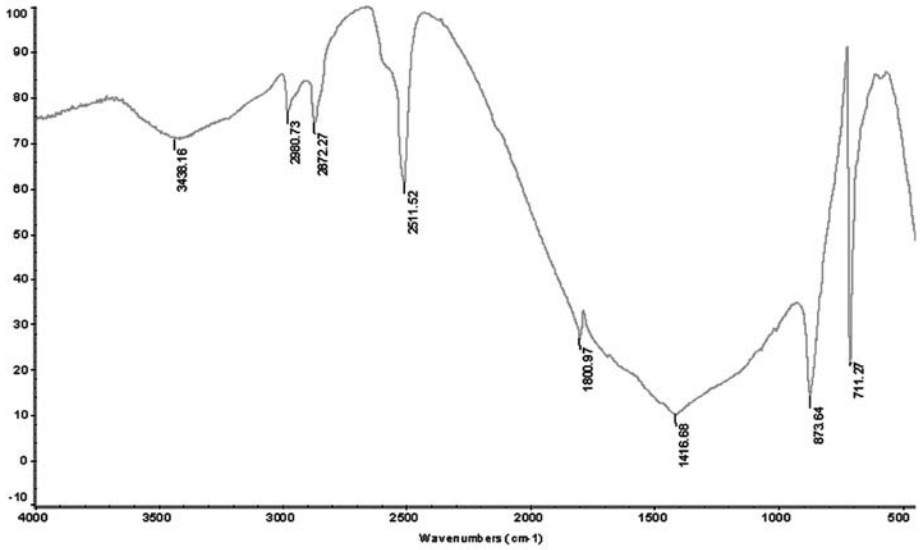


Figure 5.3(a): FT-IR spectrum of control $CaCO_3$ crystals

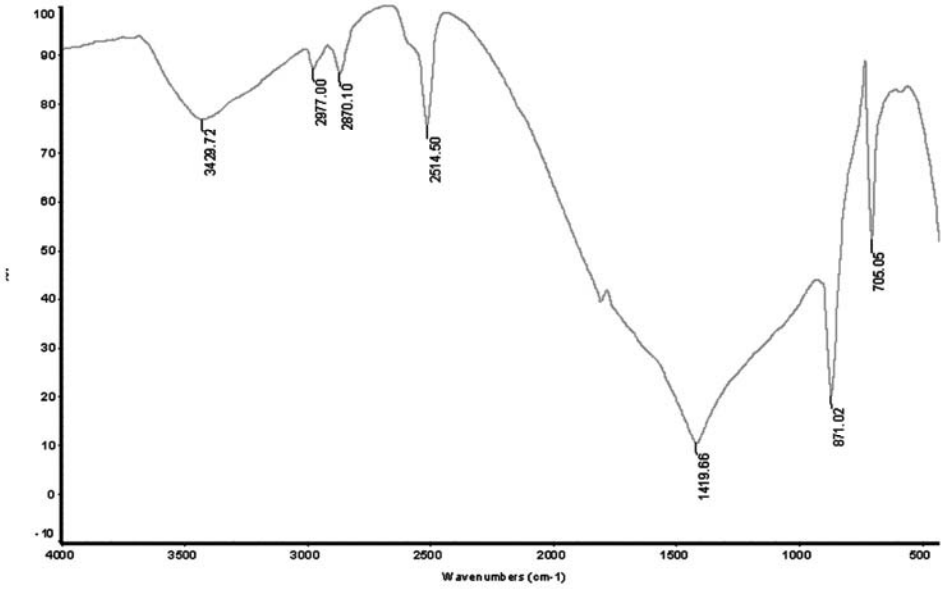


Figure 5.3(b): FT-IR spectrum of $CaCO_3$ crystals synthesized in the presence of standard Chondroitin sulphate A

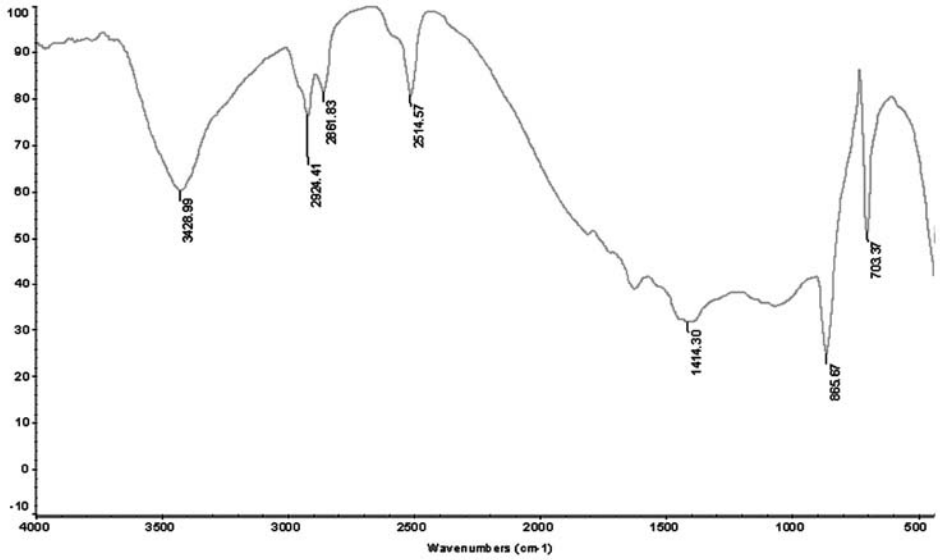


Figure 5.3(c): FT-IR spectrum of $CaCO_3$ crystals synthesized in the presence of CS-GAGs from squid cranial cartilage GAGs

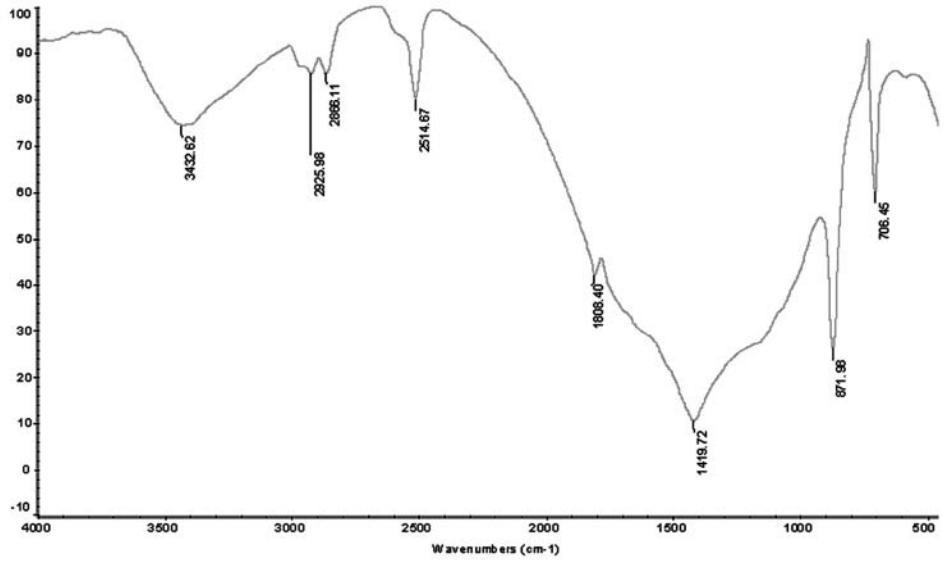


Figure 5.3(d): FT-IR spectrum of $CaCO_3$ crystals synthesized in the presence of CS-GAGs from cuttlefish cranial cartilage GAGs

5.4.1 Role of GAGs in $CaCO_3$ crystal growth

The calcium carbonate crystals were synthesized *in vitro* as depicted in Fig 5.1, in the presence of GAGs isolated from the cranial cartilages of squid and cuttlefish; as well as in the absence of GAGs which served as the appropriate controls. It has been suggested in the earlier works of Kobayashi (1971) that all or most tissues are potentially calcifiable and that many endogenous factors including glycosaminoglycans may be among these modulatory factors that normally preclude the process. The removal or degradation of these might be a prerequisite for biological calcifications. In addition, amorphous biological deposits of calcium salts, consisting of continuous random networks of small structural units related to the crystalline state or of microcrystalline/cluster networks have been described (Taylor *et al*., 1986). The association of various glycosaminoglycans with calcifications occurring in the lesions of progressive massive fibrosis and pleural plaques (Wusteman *et al.,* 1972), in the aortic ground substance during arteriosclerosis (Wexler and Thomas, 1967), from asbestos-dust nuclei (Davis, 1970), in cartilage (Hunter *et al*., 1985) and of glycosaminoglycan-like sulphated carboxylated poly-saccharides with calcification in marine algal coccoliths (Borman *et al*., 1982) have also been discussed.

The mechanism by which glycosaminoglycans inhibit $CaCO_3$ crystallization is not clear. It has been suggested that chondroitin sulphate inhibits skeletal calcification by complexing calcium and sulphate ions (Bowness *et al*., 1967). Manoli and Dalas (2000) investigated the kinetics of spontaneous precipitation of vaterite ($CaCO_3$) from an aqueous solution in the presence of chondroitin sulphates (CSA, CSB, CSC) by the constant composition method. They found that chondroitin sulphate influences the particle size distribution of the vaterite crystals formed and stabilized this mineral phase by preventing the transformation to calcite. Role of DS and KS proteoglycans has been reported to be closely associated with calcite crystals (statoconia) in the chicken ear, and non characterized proteoglycans have also been found to be involved in the calcification process of fish otolith (Borelli *et al*., 2001). The occurrence of a

mucopolysaccharide layer at the outer surface of the mineralizing ectoderm of the polyp suggested its role in coral mineralizations (Goldberg, 2001). The occurrence of proteoglycans in crustacean carapaces has not been fully documented.

Wassel and Embery (1997) studied the adsorption of heparin and chondroitin 4-sulphate onto hydroxyapatite. The presence of iduronic acid in the polysaccharide backbone is not important for adsorption of GAGs onto coral. In fact, dermatan sulphate and heparan sulphate are composed of a higher percentage of iduronic acid, in contrast with chondroitin sulphate species that are very rich in glucuronic acid. Therefore, the presence of iduronic acid is not a prerequisite of the polyanion to be adsorbed onto granules of coral, even if it is very important for binding several proteins (Jackson *et al*., 1991). The greater adsorption specificities were shown by heparin and shark chondroitin sulphate due to their larger negative charge (2.13 and 1.21, respectively) than beef chondroitin sulphate, heparan sulphate and dermatan sulphate (0.91, 1.06 and 1.09, respectively). It is noteworthy that hyaluronan is not adsorbed onto coral, indicating that the sulphate groups may be of paramount importance in the adsorption process (Wassel and Embery, 1997).

$CaCO_3$ and $Ca_3 (PO_4)_2$ biomineralization are involved in the formation of choleic and pancreatic stones and tooth and bone, respectively. Calcifications, particularly those involving the formation of $CaCO_3$ crystals, are associated with other pathological biomineralizations also (Addadi *et al*., 1987). Studies have shown that changes in cardiac valve glycosaminoglycan metabolism precede degenerative valve calcification (Hallgrimsson *et al*., 1970), and deficiencies in urinary glycosaminoglycans may lead to calcifications resulting in renal-stone formation (Gjaldbaek, 1982). Exogenous heparin restores injured bladder urothelium and its functional crystal anti-adhesion properties (Gill *et al*., 1982). Calcium-containing crystals are mitogenic (Cheung *et al*., 1986), and calcifications commonly occur in breast carcinomas also which results from disarrangement of the crystal inhibitory function of epithelial-stroma-junction glycosaminoglycans (Winters *et al*., 1986). Therefore, the roles of certain sulphated polysaccharides like heparin and CS in these biomineralization-involving physiological processes deserves further investigation.

5.4.2 SEM – EDAX

The results obtained in the present study were in accordance to the studies by Manoli and Dalas (2000). Their results showed that the glycosaminoglycans C4S, C6S, and DS in solution stabilize the spontaneous precipitation of vaterite and influence the crystal size distribution. When calcium carbonate precipitation was assayed in solutions of GAGs, a clear effect of the sulphate groups has been observed by Addadi *et al.* (1987). When hyaluronic acid, (a non-sulphated but carboxylated GAG) was added, 20-µm long piles of unmodified calcite crystals were observed as compared with calcite crystallization in the absence of the polymer. The edges of the crystals in the pile are parallel to each other, probably indicating that hyaluronic acid (HA) induced a monocrystalline aggregate. In the presence of desulphated DS, which is an epimeric form of HA but a shorter polymer, which has the carboxylate groups in an inverted configuration, isolated rhombohedral (Fermin *et al.,* 1990) calcite crystals showing rounded corners with planes oriented parallel to the axis were observed (Arias *et al.,* 2004). When DS, itself, was added, isolated calcite crystals were formed in a columnar morphology as a cylinder with three faces forming a cap at both ends (Fermin *et al.,* 1990). The addition of heparin, a highly sulphated helicoidal polymer, induces the formation of large rosette-like aggregates of calcite crystals, where the majority of the (Fermin *et al.,* 1990) faces appear not to be lost. In the presence of KS, cuboctahedral calcite crystal aggregates are formed (Arias *et al.,* 2004).

Mao *et al.* (2004) opines that, although the described cooperative role of the carboxylate and sulphate groups could also betting in GAGs, the pattern of sulphation appears to be crucial for the interactions established between the GAG and the mineral. It has also been shown that functionalized self-assembled monolayers with sulphate groups are more active than other negatively charged groups in inducing calcium carbonate nucleation, and the sulphate groups induce a face-selective nucleation (Aizenberg *et al.,* 1999).

5.4.3 X - Ray diffraction analysis

X –Ray diffraction furnishes a rapid and accurate method for the identification of the crystalline phases present in a material. Sometimes it is the only method available for determining the possible polymorphic forms of a substance – for example the detection of the three crystal phases of anhydrous calcium carbonate namely calcite, aragonite and vaterite. Studies conducted by Liu *et al.* (2010) revealed that in the presence of varying concentrations of heparin, calcium carbonate was stimulated to grow orientationally, forming a spherical vaterite form. According to their studies, in the absence of GAG, diffraction peaks at (012), (104), (110), (113), (202), (018) and (116) came from a typical calcite of $CaCO_3$ crystal. Peak intensities corresponding to vatrerite such as (004), (110), (112), (114), (300) and (118) indicated that the crystals were attaining a gradual transition from calcite to vaterite. The XRD spectra of the control and the test groups analyzed in this study revealed the prominence of diffraction peaks intensities mainly of calcite type calcium carbonate crystals; the morphological analysis proved that the crystals were attaining a gradual transition in crystal morphology.

5.4.4 FT- IR spectroscopy

Even though FT- IR spectroscopy provides a valuable tool for evaluating the infrared spectral characteristic of any chemical or biochemical substance, application of infrared spectroscopy proved to be inadequate due to band overlapping. There is a need for the quantitative determination of the ternary mixtures of calcium carbonate polymorphs (calcite, aragonite and vaterite), which are present in a number of materials including limestones, industrial scale formation and several pathological cases. The use of XRD has been reported only for the calcite – aragonite mixture. In order to address the problem, the use of Fourier transform Raman spectroscopy (FT-RS) can be applied which is infact a comprehensive non-destructive methodology reported for the simultaneous quantitative determination of the calcium carbonate crystal phases in their ternary mixtures.

The results obtained in this study are in accordance to those reported by Volpi (1999) and Liu *et al.* (2010). Eventhough the influence of chondroitin sulphate GAGs of squid and cuttlefish were not as prominent as that of heparin, the SEM images prove that the chondroitin sulphate GAGs are capable of altering the morphology of calcium carbonate crystals. According to Volpi (1999) the adsorption capacities of different glycosaminoglycans onto coral were dependend mainly on their charge density, with sulphate groups being more important than carboxyl groups. In the present study the influence of the cranial cartilage GAGs of squid and cuttlefish on crystal morphology of calcium carbonate can thus be attributed to their higher sulphation. The SEM images showed that the cuttlefish GAGs showed a much more prominent influence on $CaCO_3$ crystal morphology than that of squid GAGs, and whether this difference is due to modification such as any neutral monosaccharide attachment, requires further structural characterization of these GAGs.

Vopli (1999) opines that due to the peculiar adsorption properties of the different glycosaminoglycans, the combination of granules of natural coral with glycosaminoglycans makes this material potentially useful in osseointegration in bone metabolism and periodontal therapy. During the last decade, an increased understanding of biomineralization has initiated improvements in biomimetic synthesis methods and production of new generation of biomaterials. The use of natural biogenic structures and materials such as corals, seashells, animal bones, etc., for medical purposes has been motivated by limitations in generating synthetic materials with the requisite structure and mechanical integrity.

5.5 Conclusion

The formation of biominerals provides a unique guide for the design of materials, especially those that need to be fabricated at ambient temperatures. In biominerals, the small amount of organic component not only reinforces the mechanical properties of the resulting composite but also exerts a crucial control on the mineralization process, contributing to the determination of the size, crystal morphology, specific crystallographic orientation, and superb properties of the

particles formed. Therefore, biological routes of structuring biominerals are becoming valuable approaches for novel materials synthesis.

It has been shown that functionalized self-assembled monolayers with sulphate groups are more active than other negatively charged groups in inducing calcium carbonate nucleation, and the sulphate groups induce a face-selective nucleation. In order to understand the precise role of these sulphated macromolecules (GAGs) in calcium carbonate nucleation and growth, not only their structure but also their supramolecular assembly must be considered. In the present study the influence of the cranial cartilage GAGs of squid and cuttlefish on crystal morphology of calcium carbonate due to their higher sulphation can be attributed to their greater bioactive potential. Even though the precise mechanism involved in controlling crystal nucleation, growth, and morphology is far from being understood, calcium carbonate crystallization on solid functionalized substrates depends on the spacing, ordering, and orientation of the terminal group.

Biologically produced biominerals are inorganic-organic hybrid composites formed by self-assembled processes under mild conditions, showing interesting properties, controlled hierarchical structures, and remodeling or repair mechanisms which still remain to be developed into a practical engineering process. Questions such as how mineralization is controlled in a micro- or nanosized confined space or how is it possible to regulate the spatiotemporal deposition of particular solid interfaces or macromolecules in solution to produce self assembled mineralized structures will require new interdisciplinary approaches to be answered.

Chapter 6: Glycosaminoglycan impregnated Fish collagen sheet – Potential Role as Biomaterials

Contents

6.1 Introduction

The design, development and evaluation of biomaterials that can sustain life or restore a certain body function, is a very important and rapidly expanding area in the field of materials science. During the last few decades, regenerative medicine and tissue engineering have gained extensive attention. A frequently applied approach is the stimulation of the body 'to heal itself'. Therefore, materials need to be developed which can function as cell carriers *in vitro* or which can be implanted *in vivo* with the aim to induce cell migration, adhesion and proliferation. In order to fulfill these requirements, the materials developed should meet some criteria. The structural requirements include small fiber diameters (<250 nm), high-matrix porosity (pores > 10 μm), large void volume, large surface-to-volume ratio, mechanical strength, and a three-dimensional structure to allow for cell infiltration (Matthews *et al*., 2002). It must be biocompatible (O'brien *et al.,* 2007) and, depending on the application, also biodegradable (Kang *et al.,* 1999) and should also serve as a reservoir for growth factors which must be released on time scales supporting tissue regeneration (Lieberman *et al*., 2002).

Since the early 1980s, numerous extracellular matrix derived wound dressings have been developed to promote wound healing (Paddle-Ledinek *et al.,* 2006). The ideal dressing needs to ensure that the wound remains moist with exudates, but not macerated, and free of infection while fulfilling prerequisites concerning structure and biocompatibility (Purna and Babu, 2000). Furthermore, they should be non-cytotoxic, non-antigeneic and should induce migration and proliferation of epithelial cells, fibroblasts and endothelial cells, as well as the

synthesis of extracellular matrix components required for wound repair (Balakrishnan *et al.,* 2005). In addition, wound dressings should exhibit ease of application and removal, and proper adherence, in order to ensure that there will be no areas of non-adherence left to create fluid-filled pockets for bacterial proliferation (Quinn *et al.,* 1985).

A key issue in the development of biomaterials is the design of a material that mimics the natural environment of cells i.e. in mimicking the native extracellular matrix (as much as possible, both in terms of chemical composition and physical structure). Collagen is one of the most commonly used biopolymer for creating cellular scaffolds due to its innocuous nature.

6.1.1 Collagen

Collagen is the most abundant and ubiquitous protein in the body of almost all metazoan organisms, as a major component of extracellular matrix (Balian and Bowes, 1977). Collagen family of proteins is more diversified and includes a series of related, yet chemically distinct, macromolecular species. Most characteristic feature of this family of proteins is the capacity to form supramolecular aggregates in extracellular spaces (Piez, 1984). Being a major protein of the connective tissue, it plays an important part in imparting tensile strength and integrity of the muscle and also has a significant influence on the functional and rheological properties of the meat. Collagen molecule comprises of three long left handed α – helix polypeptide chains, with each polypeptide intertwined around each other towards the right, to form a structure called as the collagen triple helix. The triple helical structure of collagen was coined by Dr. G. N. Ramachandran, for which he was considered for Nobel Prize. Each polypeptide in the collagen triple helix is characterized by the presence of a glycine–X–Y sequence, in which X and Y are usually imino acids, proline and hydroxyproline involved in interchain hydrogen bonding and thus conferring very significantly towards the stability of the polypeptide (Piez, 1984). The collagens that form fibrils are Type I, II, III, V and XL while Type IV, VIII and X form network like structures (Gathercole and Keller, 1991).

Traditional sources of collagen for industrial use have been limited to land-based animals, such as bovine or porcine skin and bone. But these are subject to major constraints and skepticism among consumers due to socio-cultural and health-related concerns. The outbreak of bovine spongiform encephalopathy (BSE) and foot-and-mouth disease (FMD) crisis in recent decades have raised concerns among consumers over collagen and collagen-derived products of land-animal origin (Yamauchi, 2002). Researchers have found that fish offal such as bones, skin, scales, fins and airbladder can serve as alternative source of collagen. A number of studies have focused on the extraction of collagen from different species of fish including Antartic ice fish (Rigby, 1968), carp (Kimura *et al*., 1991), plaice (Montero *et al*., 1995), hake (*Merluccius hubbsi*) (Ciarlo *et al*., 1997; Fernandez-Diaz *et al*., 2001), Baltic cod (*Gadus morhua*) (Fernandez-Diaz *et al*., 2001; Sadowska *et al.,* 2003; Sadowska and Kolodziejska, 2005; Zelechowska *et al.*, 2010), puffer fish (Nagai and Suzuki, 2002a), megrim (Montero and Gomez-Guillen, 2000), sea bass (Nagai, 2004a), bone and scales of the subtropical fish black drum (*Pogonia cromis*) and sheepscranial seabream (*Archosargus probatocephalus*) (Ogawa *et al.,* 2004), Nile perch (*Lates nilotics*) (Muyonga *et al*., 2004), big eye snapper (*Priacanthus tayenus*) (Kittiphattanabawon *et al.*, 2005), brownstripe red snapper (*Lutjanus vita*) (Jongjareonrak *et al.*, 2005), skin of brown backed toadfish (*Lagocephalus gloveri*) (Senaratne *et al.,* 2006), catfish (*Ictalurus punctaus*) (Liu *et al.*, 2007a), grass carp (*Ctenopharyngodon idella*) (Zhang *et al.*, 2007), deep-sea redfish (*Sebastes mentella*) (Wang *et al*., 2007), skate (*Raja kenojei*) (Hwang *et al.,* 2007), walleye pollock (*Theragra chalcogramma*) (Yan *et al.*, 2008), pipefish (*Syngnathus Schlegeli*) (Khan *et al.*, 2009), as well as flatfish (Heu *et al.*, 2010), surf smelt (*Hypomesus pretiosus japonicas*) (Nagai *et al.*, 2010) and unicorn leatherjacket (*Aluterus monoceros*) (Ahmad *et al.*, 2010).

Collagen has also been isolated and characterized in starfish, (*Asterias amurenis*) (Kimura *et al.,* 1993), octopus skin (Kimura *et al.,* 1981); octopus arm and mantle (Nagai *et al*., 2002; Mizuta *et al.,* 2003), paper nautilus (Nagai and Suzuki, 2002b), marine sponge (Swatschek *et al*., 2002), kuruma prawn (Mizuta *et*

al., 1997), rhizostomous jellyfish (*Rhopilema asamushi*) (Nagai *et al.,* 1999, 2000), purple sea urchin (Nagai and Suzuki, 2000a), pearl oyster (*Pinctada fucata*) (Mizuta *et al.,* 2002), sea cucumber (*Stichopus japonicus)* (Cui *et al.,* 2007), scallop mantle (Shen *et al.,* 2007), cuttlefish (Nagai *et al*., 2001), as well as in different species of squid (Sadowska and Sikorski, 1987; Mizuta *et al.,* 1994*,* 1996; Hassan and Mathew, 1999; Raman and Mathew, 2005; Ando *et al*., 2001; Nagai, 2004b; Mingyan *et al.,* 2009).

6.1.2 Collagen based biomaterials

Collagen has wide applications in the fields of medicine, cosmetics and breweries due to its properties like abundance, biocompatibility and biodegradability, as well as non toxic and non antigenic nature (Lee *et al*., 2001). Collagen, in its purified form, has been incorporated into many pharmaceutical and cosmetic preparations. It is also used extensively for tissue engineering applications, because its signature biological and physico-chemical properties are retained in *in vitro* preparations (Landsman *et al*., 2009). For scaffold materials in tissue engineering (TE) applications and materials for implants, surface properties which encourage cell attachment, proliferation, and differentiation along the preferred lineage are desirable. Collagen has found use as a scaffold material for tissue engineering as well as a coating material for implants (Lee *et al*., 2001). Collagen in fibrillar form has been used to coat titanium surfaces, improving osteoblast spreading, attachment, proliferation, and differentiation *in vitro* (Park *et al.,* 2005). Since collagen is unique in its ability to form insoluble fibers that have high tensile strength collagen based biomaterials are widely in use as burn cover dressings, antithrombogenic surfaces, dental implants, for facial upliftment in cosmetic surgery and in controlled drug delivery systems (Boateng *et al*., 2008; Brett, 2008).

Scientists have also attempted to insert a number of different therapeutic compounds into collagens to facilitate tissue regeneration (Purna and Babu, 2000; Landsman *et al*., 2009). Two dimensional and 3-D collagen constructs have been widely used as tissue scaffolds in a variety of applications, including skin

replacement, bone substitution, and artificial blood vessels and valves. The prevalence of collagen in the majority of human tissue underlies its ability to support the growth of a wide variety of tissues, while its structure imparts favorable properties such as mechanical strength. Collagens are weakly immunogenic compared with other proteins; however, collagen expresses antigenicity in physiological condition. Studies show that the antigenicity of a collagen biomaterial can be reduced by the process of cross-linking (O'brein *et al.,* 2004). Collagen crosslinking, using physical and/or chemical methods, is often applied to prevent a rapid degradation of collagen-based biomaterials during *in vivo* application, to suppress its antigenicity and to improve mechanical properties. The ability of crosslinked CS to bind anti-chondroitin sulphate antibodies, and its susceptibility to chondroitinase ABC digestion, demonstrate its conformational mobility and bioavailability. Bio-characteristics of GAGs include the binding and modulation of growth factors and cytokines, the inhibition of proteases, and the involvement in adhesion, migration, proliferation and differentiation of cells. This suggests that the GAG moiety of collagen/GAG matrices, like in proteoglycans, may act as natural biostimulators and remain functional and display its biocharacteristics.

6.1.3 Gelatin based biomaterials

Gelatin is a substantially pure protein food ingredient obtained by the irreversible denaturation of collagen. In addition to its central role in food industry, gelatin has also been used widely for the preparation of biomaterial scaffolds. Crosslinked gelatin-chondroitin sulphate-hyaluronan scaffolds were applied as biomimetic material for natural cartilage (Chang *et al.,* 2003). Articular cartilage is limited to have self-repair capacity due to its avascular characteristic and inactive mitotic activity of chondrocytes. Tissue engineering approach is currently used to prepare transplantable hyaline-like tissue which may provide a more suitable alternative to repair defects (Banu and Tsuchiya, 2007; Zhao *et al.,* 2009).

Materials consisting of a gelatin-chondroitin sulphate-hyaluronan matrix have also been studied for wound treatment (Wang *et al.,* 2006). Liu *et al.* (2007b)

demonstrated the efficacy of chitosan-gelatin scaffolds containing bFGF (Basic Fibroblast Growth Factor) as a tissue engineering scaffold to improve skin regeneration. Dainiak *et al*. (2010) have also advocated a gluteraldehyde (0.1% optimum) cross linked gelatin–fibrinogen cryogel dermal matrix for wound repair. Murakami *et al.* (2010) developed a functional wound dressing by using a hydrogel blend of alginate, chitin/chitosan, and fucoidan (60:20:2:4 w/w); ACF-HS (alginate – chitin/chitosan – fucoidan hydrogel sheets) for the treatment of healing - impaired wounds.

6.1.4 Blended collagen-GAG scaffolds

Collagen and GAGs, as constituents of native tissues, are widely utilized to fabricate scaffolds serving as an active analogue of native ECM (extracellular matrix). Glycosaminoglycans (GAGs) are the components of connective tissues that take an active part both in the tissue metabolism and in the stimulation of reparative processes during traumatic and burn damage. Furthermore, since GAGs are practically non-immunogenic, composite matrices consisting of collagen and GAGs would be well-suited for wound healing, tissue engineering and drug delivery applications. Collagen-GAG scaffolds have been used extensively for *in vitro* studies of cell-ECM interactions and as a platform for tissue biosynthesis including *in vivo* studies of induced tissue or organ regeneration.

The development of blended collagen and glycosaminoglycan (GAG) scaffolds can potentially be used in many soft tissue engineering applications since the scaffolds mimic the structure and biological function of native extracellular matrix (ECM) (Zhong *et al*., 2005; Keogh *et al*., 2010). The extracellular matrix of cartilage is composed primarily of type II collagen and large, networks of proteoglycans (PGs) that contain glycosaminoglycans such as hyaluronic acid (HA) and chondroitin sulphate (CS). Inferior fibro-cartilaginous tissue which may induce osteoarthritis generally forms after arthroplasty, mosaicplasty, or periosteal autografts. Cartilage is limited to have self-repair capacity due to its avascular characteristic and inactive mitotic activity of chondrocytes. Tissue engineering is now showing promise as a possible method for cartilage repair, and the cell-based

strategies for cartilage tissue engineering are considered to have great potential in preserving physiological hyaline like cartilage tissue (Lee *et al*., 2006). Tissue engineering approach is currently used to prepare transplantable hyaline-like tissue which may provide a more suitable alternative to repair such defects (Banu and Tsuchiya, 2007; Zhao *et al*., 2009). Thus in the present work an attempt has been made to explore the possibility of incorporating the glycosaminoglycans isolated from the cranial cartilages of squid as well as cuttlefish, into fish collagen sheet (FCS) – a typical biomaterial scaffold developed from fish scales.

6.2 Materials and methods

Fish scales from the fish, *Lates calcarifer* of fresh sea catch was used as the source of fish collagen. The scales of fresh fish were carefully removed from the skin and washed thoroughly in running water and dried at 40ºC in a hot air oven. The dried scales were stored in polythene bag at room temperature until used. The chondroitin sulphate GAGs, isolated from the cranial cartilages of squid and cuttlefish (prepared and purified as mentioned in Section 2.2.4) served as the source of glycosaminoglycans.

6.2.1 Preparation of fish collagen sheets (FCS)

6.2.1. (a) Control fish collagen sheet (cFCS)

Fish collagen sheet was prepared according to the method of Sankar *et al*. (2008) with slight modifications. Briefly, dry fish scales were treated with different concentrations (20%, 25% and 30% v/v) of HCl solutions (2:1, v/w, HCl solution/fish scales) for 24 h at room temperature. Later, acid solution was decanted and the scales were washed thoroughly. Then water was added to scales (2:1, v/w) and pH of the scales was adjusted to 7 using 0.1N NaOH solution. To check the pH a sample scale was cut vertically, pH paper was inserted and the pH was noted. Further, scales were washed and pulverized with a float of 1:1 (v/w, water/fish scales) using a domestic blender for 15 min at 12,000 rpm. The fish scales treated with 25% HCl solution could be pulverized and made into a paste. The resulting paste was cast into sheet in a polythene tray and dried at room temperature. The dried sheets were stored in polythene covers for further studies.

6.2.1. (b) Glycosaminoglycan impregnated fish collagen sheet (GAG – FCS)

The glycosaminoglycans were isolated from the cranial cartilages of squid as well as cuttlefish as mentioned in Section 2.2.4. These glycosaminoglycans were resuspended in minimum amount of deionised double distilled (Milli Q) water and mixed with the fish scale collagen paste in the ratio 1: 50 (GAG: collagen) by simple mechanical blending followed by casting and drying. The details of the preparation of GAG impregnated fish collagen sheet is depicted in Fig. 6.1. The control fish collagen sheet was designated as cFCS. The fish collagen sheet impregnated with glycosaminoglycans from the cranial cartilages of squid and cuttlefish were designated as SQ-FCS and CF-FCS respectively.

6.2.2 Scanning electron microscopy

The samples were coated with gold ions using an ion sputter coater under the following conditions: 0.1 Torr pressure, 20mA current and 70 s coating time. Surface structures as well as cross section of the different collagen sheets were visualized by a scanning electron microscope JEOL Model JSM - 6390LV. The SEM images were acquired at an accelerating voltage of 15 KV.

6.2.3 FT-IR spectroscopy

IR spectrums of the samples were taken using a Thermo Nicolet, Avatar 370 instrument by preparing 500 mg KBr pellets containing 2 – 5 mg of samples. The samples were analyzed from 450 to 4000 cm^{-1}.

6.2.4 Differential scanning calorimetry

Differential scanning calorimetry (DSC) was employed to investigate the endothermic peak temperature shift among the scaffolds. DSC was performed on a Mettler Toledo DSC 822e calorimeter fitted with air cooling compressor and liquid nitrogen cooler at ambient temperature. The temperature was calibrated using indium as standard. The fish collagen sheets (cFCS, SQ-FCS and CF-FCS) were and sealed in aluminium pans. Samples were heated from 30 to 350^0C at a controlled heating rate of 20^0C /min, with an empty sealed pan as a reference. The shrinkage temperature was taken at the peak of the plotted thermal transition curve.

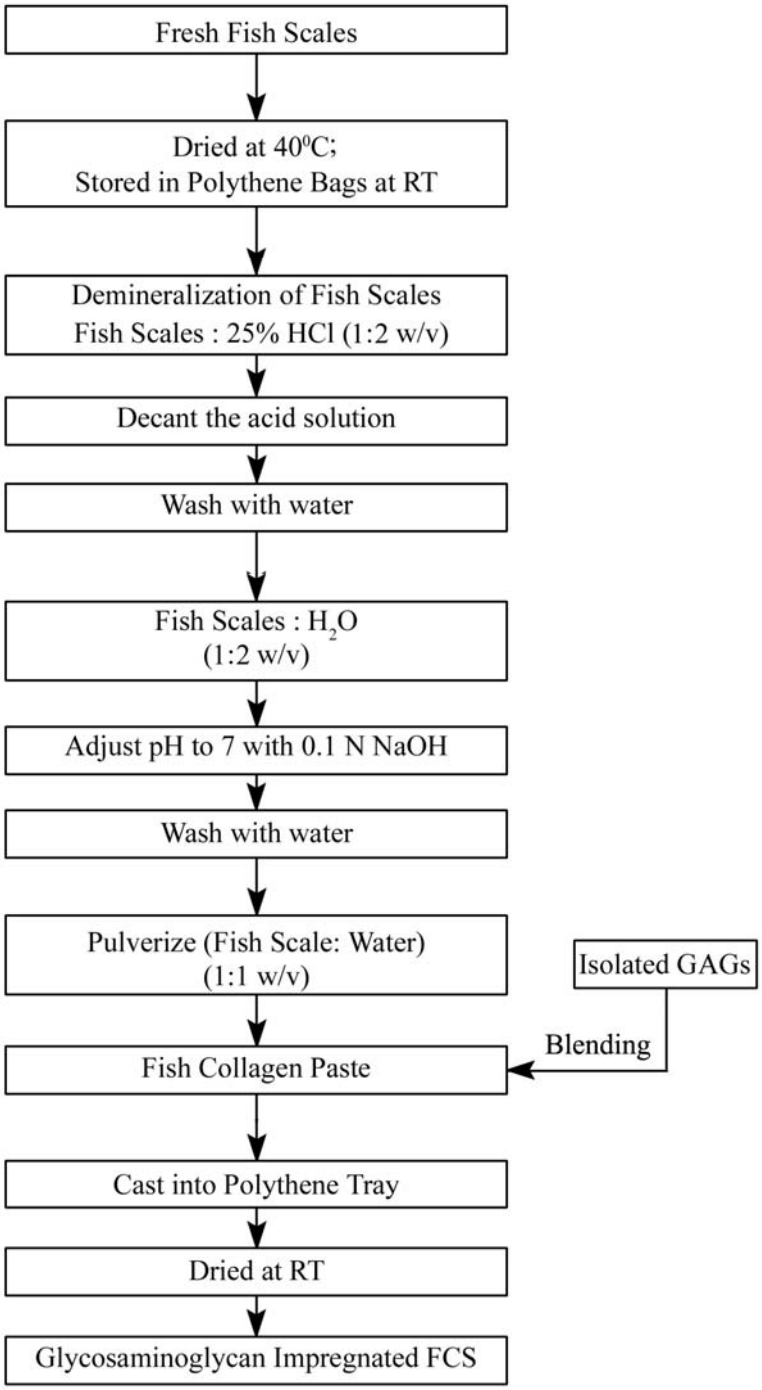


Fig. 6.1 Flow chart for the preparation of glycosaminoglycan impregnated fish collagen sheet (GAG - FCS)

6.2.5 Percentage swelling ratio

Percentage swelling ratios of the scaffolds were determined according to the method of Vlierberghe *et al.* (2008). Pre-weighed samples were immersed in phosphate buffered saline (PBS) pH 7.4 for known interval of time. The medium was carefully withdrawn at intervals and the scaffolds were blotted with tissue paper uniformly to remove the excess fluid. The wet weights of the scaffolds were measured until equilibrium weight was attained. The swelling ratio was calculated using the formula.

$$\text{Percentage swelling ratio} = \frac{\text{Final weight - initial weight}}{\text{Initial weight}} \times 100$$

All data points are the mean ± standard deviation of three separate measurements.

6.2.6 Blood compatibility evaluation

The haemolytic potential of the material is the measure of the extent of haemolysis that may be caused by the material when it comes in contact with blood. Haemolytic potential of hydrogels was determined according to O'Leary and Guess (1968). Fresh blood was obtained from healthy human volunteers. Human blood (0.1 mL) anticoagulated with citrate was added to 7.5 mL of PBS containing gel (~ 0.15 g) in different test tubes. A separate positive control (100% haemolysis induced by replacing the PBS with 7.5 mL of 0.1% Na_2CO_3 solution) and a negative control (0% haemolysis, PBS with no material added) were also set up. Each set of experiments was done in triplicate. All the test tubes containing samples and the control were incubated for 1 h at 37°C. After incubation, the tubes were centrifuged at 500 x g for 5 min. The percentage haemolysis was calculated by measuring the optical density (OD) of the supernatant solution at 545 nm in a UV-vis spectrophotometer (Model: Hitachi U2800) as per the following formula:

$$\text{Haemolyis (\%)} = \frac{\text{OD of the test sample - OD of negative control}}{\text{OD of positive control}} \times 100$$

Data are expressed as mean ± standard deviation of three separate measurements.

6.2.7. Statistical analysis

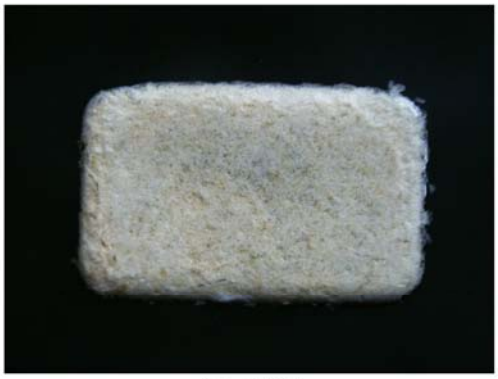
Statistical calculations for the haemolytic potential were performed in the SPSS 11.0 software package (SPSS, Chicago, IL). Data analysis was perfomed using one-way analysis of variance (ANOVA) with post-hoc multiple comparision analysis performed using Tukey's HSD test. *P* values less than or equal to 0.05 were considered statistically significant. Data are presented as mean $\pm$ standard deviation (SD).

6.3 Results

6.3.1 Glycosaminoglycan impregnated fish collagen sheet

The non crosslinked collagen – GAG scaffolds were successfully developed and have the prospect of being used as biomaterials (Plates 6.1 a, b, and c). The source of collagen was the demineralized scales of *Lates calcarifer*, while the chondroitin sulphate rich GAGs isolated from the cranial cartilages of squid as well as cuttlefish formed the source of glycosaminoglycans. The scales of *Lates calcarifer*, were hydrolyzed under controlled acidic conditions, neutralized and made into a sheet, the fish collagen sheet (FCS) as shown in Fig. 6.1. The scales were first treated with different concentrations of HCl for 24 h at room temperature. The 25% HCl was found to be most suitable in order to pulverize the scales (Table 6.1). The fish scales (25% HCl treated) when subjected to repeated washing following the demineralization step, a creamish white softened material was obtained and was free of all traces of melanin, usually found in the rim of the *Lates calcarifer* scales. Pure collagen was not isolated in this study. The moderately demineralized scales thus obtained could easily be pulverized into a smooth paste. Each of the respective GAGs isolated from the cranial cartilages of squid and cuttlefish were blended with this paste by gentle mixing and cast into a plastic tray to obtain GAG impregnated fish collagen sheets.

Preparation of composite gelatin-GAG scaffolds without chemical crosslinking were also attempted in this study according to the method of Dawlee *et al.* (2005), excepting the use of an oxidizing agent. Production of non-cross linked gelatin/GAG hydrogels were not successful since the scaffolds could not be cast into a sheet and rather remained as a viscous gel in the mold.



(a) cFCS

(b) SQ-FCS

(c)CF –FCS

Plate 6.1: Fish collagen sheets (FCS) prepared from the scales of *Lates calcarifer*

(a) Control - Fish collagen sheet, (b) Squid glycosaminoglycan impregnated fish collagen sheet, (c) Cuttlefish glycosaminoglycan impregnated fish collagen sheet.

Table 6.1: Effect of different concentations of HCl solution on fish scales

Concentration	Observation
20% HCl solution	No effect on scales
25% HCl solution	Scales could be made into a paste and cast into a sheet
30% HCl solution	Scales were dissolved

6.3.2 Scanning electron microscopy

SEM images of the collagen sheets (Plates 6.2 a - f) have shown fibrous and porous nature. As the mineral present in the scale was partially dissolved by acid, porous nature of the sheet is clearly seen. The fibrilar structure of collagen present in the sheet and its organization are clearly viewed.

6.3.3 FT-IR spectroscopy

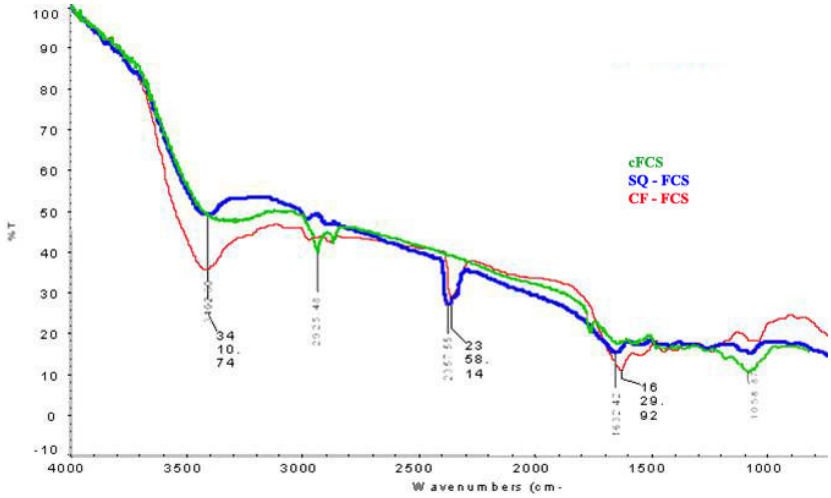
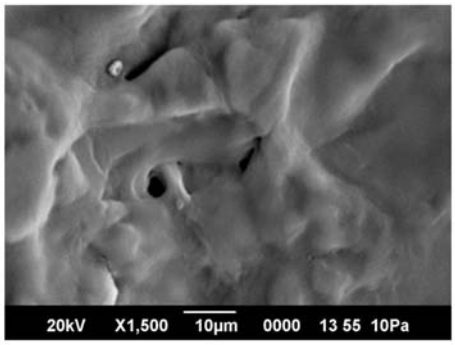
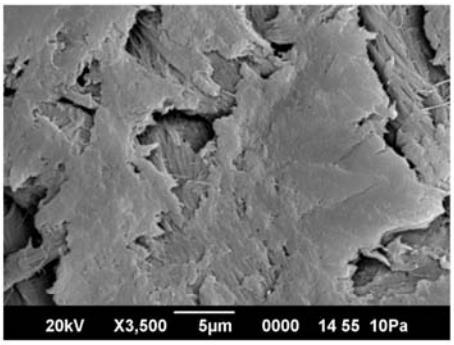


Fig. 6.2 FT-IR spectra of the glycosaminoglycan impregnated fish collagen sheets.

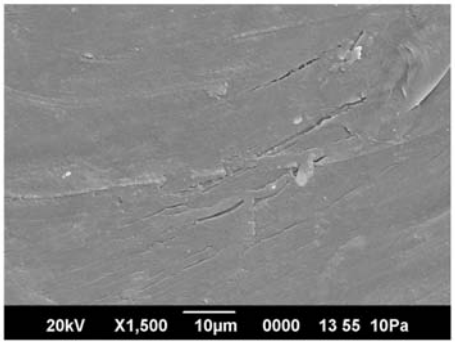
Analysis of FT-IR spectra revealed the functional groups in the scaffolds. FTIR spectra of fish scale collagen sheets are shown in Fig. 6.2. The regions of amide I, amide II, and amide III are known to be directly related with the configuration of a polypeptide. Amide A band (3,400- 3,440 cm^{-1}) is related to N-H stretching vibrations Amide I band (1,600-1,660 cm^{-1}) is associated with stretching vibrations of carbonyl groups (C=O bond) in peptides, being the most important factor in investigating the secondary structure of a protein. Amide II



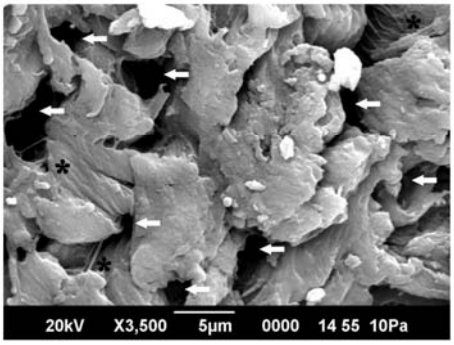
(a) Surface view of cFCS



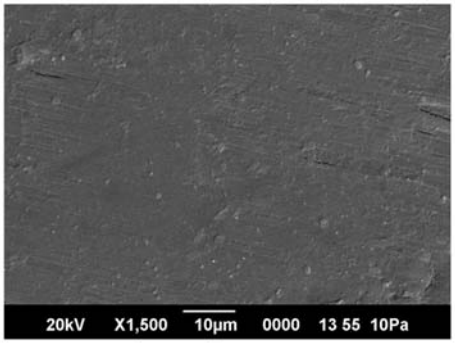
(b) Cross section of cFCS



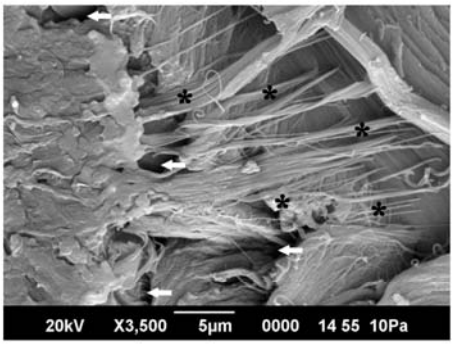
(c) Surface view of SQ - FCS



(d) Cross section of SQ-FCS



(e) Surface view of CF- FCS



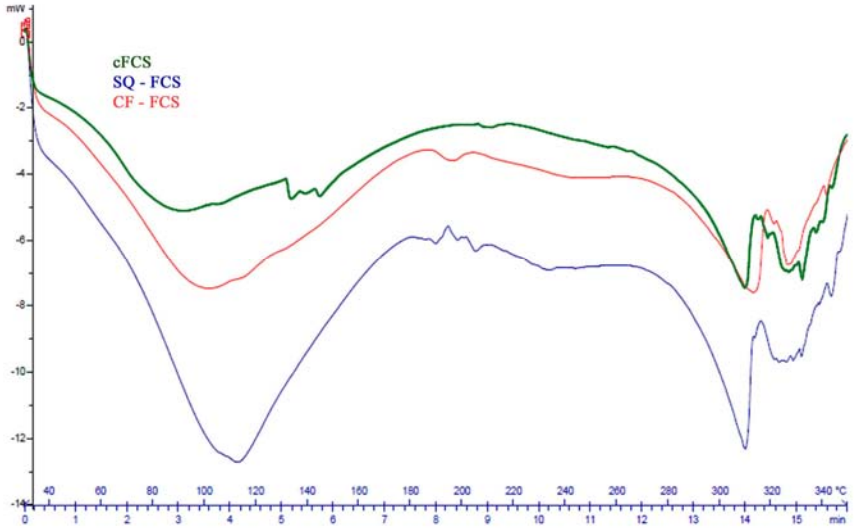
(f) Cross section of CF-FCS

Plate 6.2: Scanning electron micrographs of the fish collagen sheets.

* collagen fibres
⇦ pores

(approximately 1,550 cm^{-1}) is associated with NH bending and CN stretching. Amide III (1,220- 1,320 cm^{-1}) is related to CN stretching and NH, and is involved with the triple helical structure of collagen. The IR spectrum of the fish collagen sheets (Fig. 6.2) shows amide I peak at 1678, 1629 and 1632 cm^{-1} in the case of cFCS, SQ-FCS and CF-FCS respectively. The amide II peak is weak. Peaks at 1058 and 1176 cm^{-1} represents the P-H bending and phosphate stretching respectively, indicating the presence of calcium salts. The prominent peak at 2925 cm^{-1} and a shoulder at 2862 cm^{-1} represent the CH_2-CH_3 stretching vibrations, which are characteristic of collagen. A free N-H stretching vibration occurred in the range of 3400−3440 cm^{-1}, and when the NH group of a peptide was involved in hydrogen bond, the position was shifted to lower frequencies.

6.3.4 Differential scanning calorimetry

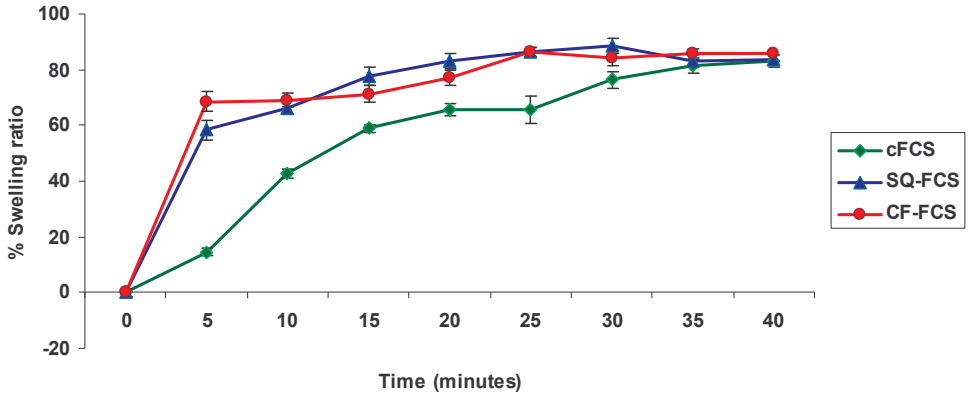


The thermal properties of the scaffolds were analyzed by DSC as shown in Fig. 6.3. There was an endothermic peak around 92^0C for the control fish collagen sheet (cFCS), 102^0C for the SQ-FCS and 112^0C for the CF-FCS scaffold. The glycosaminoglycan impregnated fish collagen sheets (SQ-FCS and CF-FCS) showed higher peak temperatures than did control fish collagen sheets. Each scaffold had only one obvious endothermic peak, indicating that there was no noticeable phase separation in the blended scaffolds. The endothermic peak could result from loss of bound water retained within the scaffold. The results

demonstrate that the addition of GAGs onto the fish collagen sheets caused the peak temperature to shift to a larger temperature, indicating an increase in the denaturation temperature.

6.3.5 Percentage swelling ratio

The SQ-FCS and CF-FCS scaffolds showed a very rapid and high percentage swelling ratio (58.47 % and 68.5%) than the control fish collagen sheets (cFCS: 14.5%) in the first five minutes itself (Fig. 6.3). The high uptake of PBS (phosphate buffered saline) is due to free diffusion through open porous structure of the scaffolds by a wicking action of the porous scaffold and did not result in extensive change in dimensions of the scaffolds. The scaffolds remained stable in medium (PBS) with a good handling property which is due to inherent properties of the collagen scaffolds.



All values expressed as mean n = 3. Error bars indicate standard deviation one standard deviation above and below (±) the averaged curve.

Fig 6.3: Swelling ratio (%) of the fish collagen sheets.

6.3.6 Blood compatibility evaluation

The haemolysis assay showed that the scaffolds were non-haemolytic in nature. The haemolytic potential of a material is defined as the measure of the extent of haemolysis that may be caused by the material when it comes in contact with blood. Table 6.2 shows the percentage haemolysis of blood in contact with different samples at 37°C for 60 minutes. All the samples were found to be nonhaemolytic, the extent of haemolysis being lower than the permissible level of

5%. The ANOVA results (Appendix 6.1) for percentage of haemolysis shows that there was no significant differences ($p>0.05$; $n=3$) among the samples of collagen sheets (FCS) tested.

Table 6.2 Haemolytic potential of the fish collagen sheets

Sample	Haemolysis (%)
Control FCS (cFCS)	4.22 $\pm$ 0.91
Squid GAG – FCS (SQ-FCS)	4.53 $\pm$ 0.53
Cuttlefish GAG – FCS (CF-FCS)	3.78 $\pm$ 0.59

All values expressed as mean ± standard deviation, n = 3; (P > 0.05).

6.4 Discussion

The extracellular matrix (ECM), such as collagen, elastin, and proteoglycans, fills the extracellular space in living tissues. These biopolymers have been utilized for many years and have recently attracted great interest as basic materials for scaffolds used in cell-incorporated, tissue-engineered devices or hybrid tissues. Since scaffold must mimic the ECM in both structure and function, biologic scaffolds derived from extracellular matrix (ECM) can be implanted in their natural forms to achieve the desired results. The challenge in preparing these biologic scaffold materials for clinical use, however, lies in retaining the natural structure and composition of the ECM while ensuring its safety for clinical implantation. To mimic the high proportion of collagen present in most native tissue, collagen scaffolds are widely used in tissue engineering.

The main sources of industrial collagen are limited to those from bovine and pig skin and bones. Mammalian collagen differs from fish collagen in that fish collagens have lower gelling and melting temperatures but relatively higher viscosities than equivalent bovine forms (Leuenberger, 1991). However, frequent occurrences of bovine spongiform encephalopathy (BSE) (Yamauchi, 2002), and foot and mouth diseases of cattle, has limited the utility of mammalian collagens and gelatins. One alternative is to replace bovine collagen with collagens from

aquatic organisms, mainly marine vertebrates and invertebrates (Nagai *et al.*, 1999, 2000, 2001, 2002; Nagai and Suzuki, 2000a, b, c, 2002a, b). Therefore, replacement of mammalian sources with fish and seafood byproducts is gaining significance nowadays despite the limitations of fish gelatins and collagens, with low melting and gelling temperatures and high deterioration rate compared to mammalian sources.

The fish scales are example of composite structure and composed of mainly type I collagen fibers and calcium deficient hydroxyapatite, which together form a highly ordered three dimensional structure. The fish collagen sheet (FCS) developed by Sankar *et al.* (2008) from the scales of *Lates calcarifer* was proposed to be used as wound dressing material on leprosy, diabetis and other chronic ulcers. It is possible for fish scales to have potential as an important alternate source of collagen because they contain a large quantity of collagen. Kimura *et al.* (1991) reported that collagen from carp scale could be extracted with 0.5 M acetic acid and the yield was about 7% on dry weight basis. On the contrary, Nomura *et al.* (1996) reported that the extraction of collagen from the scales of sardine with a different solvent system (0.05 M Tris–HCl, pH 7.5, containing 0.5 M EDTA) gave a comparatively lower yield (5%).

In the present study, both the GAG impregnated fish collagen sheets (SQ-FCS and CF-FCS) could be cast properly into a sheet like material, as in the case of control fish collagen sheets (cFCS); and hence a further chemical cross linking strategy which might otherwise cause some deleterious effects was omitted. Attempts to prepare non-cross linked gelatin-GAG scaffolds using the isolated CS-GAGs and commercial bacteriological grade gelatin according to the method of Dawlee *et al.* (2005) was not successful since it could not be cast into a sheet like material and remained as a rather viscous gel in the mold. Native chondroitin sulphate-GAGs does not form crosslinks with gelatin, since the cross linking may occur only in the presence of periodate oxidized GAGs (aldehyde derivative of the GAGs) and hence could not be successfully cast into a sheet like scaffold. Chemically cross linked gelatin-chondroitin hydrogels were successfully

developed by Dawlee *et al.* (2005), by converting chondroitin-6-sulphate to its aldehyde derivative by periodate oxidation using sodium *m*-periodate.

Morphological analysis of the Fish collagen sheets (FCS) by SEM revealed cofibrils, which are heterogeneous in size, shape, and width-to-length ratio (Plate 6.2). Inner matrix sections contained porous lattice- like lamellae (Plate 6.2 (d), (f)), which are suitable for usage in biomedical applications. CS-GAG was distributed throughout the matrix, as evidenced using 1, 9 – DMMB dye binding method. The topographic analysis of the big cofibrils revealed that the microfibrils are aligned parallel to each other. The presence of GAG resulted in the formation of straight cofibrils (Plate 6.2(c), (e)) in comparison to the more twisted fibrils of pure collagen (Plate 6.2(a)). This suggests that the GAG moiety of collagen/GAG matrices, like in proteoglycans, may remain functional and display its biocharacteristics. A similar mechanism was already proposed after comparable observations in earlier reports. *In vitro* collagen fibrillogenesis in the presence of GAGs has already been studied for quite a long time (Obrink, 1973; Guidry and Grinnell, 1987; McPherson *et al*., 1988, Milan *et al*., 2005). Douglas *et al*., (2007) studied on the effect of CS-GAGs on the morphology of collagen fibrils during fibrillogenesis *in vitro*, and demonstrated that the CS-GAGs caused fibrils of collagen type I and II to become thinner during binding. These studies have revealed many issues concerning the impact of GAG composition, concentration, pH value and ionic strength on the kind and kinetics of growing fibrils. Besides this it was pointed out that the electrostatic interaction between GAGs and collagen molecules is a key element in the assembly process. The interactions between CS and collagen are believed to be ionic since increasing ionic strength has diminished or abolished CS binding (Obrink, 1973). Regarding differences in molecular weight, it has been suggested that GAGs might "bridge" basic, cationic regions in neighboring collagen molecules and thus exert a stabilizing effect and that a higher GAG chain length would result in a more stable interaction (McPherson *et al*., 1988). The results of SEM (Plate 6.2) and FT-IR (Fig. 6.2) analysis revealed that the characteristics of the glycosaminoglycans impregnated fish collagen sheets were well in accordance to the above findings.

Data on physico-chemical properties reveals that the scaffold is suitable to serve as a matrix material for *in vitro* cell culture with its high porosity, open interconnected porous structure and adequate swelling. The scaffolds are hence envisaged with the capacity to absorb large amount of nutrients and uniformly distribute it throughout the matrix without significant change in dimension or loss of mechanical stability when used as an *in vitro* cell culture scaffold. The scaffold is highly porous with open interconnected pores as evidenced from the scanning electron micrograph (Plates 6.2) and medium uptake ability (percentage swelling ratio). The open pores in the 3D scaffold are continuous allowing uniform distribution of cells and can also permit the free diffusion of nutrients and waste materials in and out of the construct. The FT-IR analysis revealed the functional groups within the scaffolds, and the data on DSC analysis suggests that covalent bonds are formed among the GAG blended scaffolds. The presence of covalent bonds in fact, can alter the microstructure and porosity of the scaffolds that in turn may influence the amount of bound water retained within the scaffold.

Collagen-GAG scaffolds in gel or sponge form have been extensively manufactured as constructs with high porosity, high surface area, and specific three-dimensional shape for tissue engineering applications (Freyman *et al.,* 2001). Traditional scaffolds may exhibit poor mechanical properties and unacceptable physical structure for many specific organ or tissue regenerations (Xu *et al.,* 2004). This is due at least in part to the great differences existing between native ECM and these manufactured scaffolds. In native connective tissue, the collagen structure is organized in a three-dimensional network of nanoscale diameter fibers (Kadler *et al.,* 1996). Stronger interaction between cells and fibers has been reported when the fiber diameter is smaller than the size of the cells (Xu *et al.,* 2004).

Electro spinning is a very useful technique for producing polymeric nanofibers by applying electrostatic forces to draw a positively charged polymer solution into a fine filament in nanoscale (Huang *et al.,* 2003). Zhong *et al.* (2005) adopted electrospinning to fabricate a novel nanofibrous collagen-GAG scaffold

based on collagen and chondroitin sulphate (CS) by cross linking with gluteraldehyde. Traditional collagen gels can self-assemble into nanoscale fiber like super structure, (Yunoki *et al.,* 2004) but their application is limited due to poor physical strength. Collagen sponges manufactured by freeze-drying are mechanically stronger, but they only possess a microporous structure with a magnitude larger than the nanoscale fibrillar network of native ECM (Sheu *et al.,* 2001). On contrary, Zeugolis *et al.* (2008) proved that the electrospinning of collagen is simply an expensive method to produce gelatin and that its signature characteristics are even lost in the process. Studies in the field of stem cell research during the last years have shown that such ECM microenvironments provide not only adhesion sites and mechanical stability to cellular structures, but also allow for the presentation of important matrix-bound growth factors. Cellular functions seem to be triggered both by the kind of immobilization and by the interactions of these molecules with their soluble counterparts. It has been particularly demonstrated that both the growth factor binding properties and the degree of sulphation of the GAGs are essential for the homing, proliferation, and degree of differentiation of hematopoietic stem cells (Stamov *et al*., 2008).

Collagen/CS matrices may be suitable scaffolds for culturing chondrocytes and repair of articular cartilage (Grande *et al.,* 1997). Favorable characteristics of scaffolds from these natural materials encourage host cells to repopulate and form new tissues that closely simulate the native organization. Additionally, they enhance biological interactions with cells and speed up tissue regeneration by introducing cell-specific ligands or extracellular signaling molecules, such as peptides and oligosaccharides. The combination of collagen and GAG is unique in skin regeneration since collagen or GAG alone cannot heal full thickness wounds. A collagen-glycosaminoglycan (GAG) analogue was successfully used for nerve and dermal regeneration as well as promoting angiogenesis *in vivo* (Pieper *et al.,* 1999).

Douglas *et al*. (2007) have shown that the addition of chondroitin sulphate (CS) to collagen fibrils has enhanced osteoblast adhesion and differentiation

further and has promoted the differentiation of human mesenchymal stem cells toward the osteoblastic lineage. TE scaffolds consisting of collagen with immobilized CS have stimulated the proliferation of fibroblasts and chondrocytes *in vitro* while promoting cellular in growth and reducing foreign body response *in vivo* (Pieper *et al.,* 1999; Zhong *et al.,* 2005). In addition, growth factors (GFs) have maintained their bioactivity when immobilized on scaffolds containing CS; it has been speculated that CS may exert a stabilizing influence on growth factors (Mi *et al.,* 2006). The blended collagen – GAG scaffold developed in the present study, using the GAGs isolated from the cranial cartilages of squid and cuttlefish is therefore, appended with the characteristics of the respective native GAGs as well. The development of blended collagen and glycosaminoglycan (GAG) scaffolds can hence be used potentially in many soft tissue engineering applications since the scaffolds have the potential to mimic the structure and biological function of native extracellular matrix (ECM).

Since each component in a scaffold has to be controlled, when constructed with components which mimic nature's extracellular matrices; it is important that the type (and amount) of GAGs is crucial for a tissue engineering scaffold. For example, in cartilage CS is the main glycosaminoglycan making up 20% of the dry weight; in skin, dermatan sulphate is most abundant (about 1% of the dry weight), whereas in the vitrous body of the eye it is hyaluronate (Iozzo, 2005). The chondroitin sulphate type GAGs are the important glycosaminoglycans found in the proteoglycans of bone and cartilage extra cellular matrices. Hence the collagen-GAG scaffolds prepared in this study (by the blending of collagen type I from fish scales and the chondroitin sulphate rich GAGs from the cranial cartilage of the cephalopod specimens) can be a promising entity towards the tissue engineering scaffolds especially of the osteoblastic lineage. Unlike chitosan or alginate, chondroitin sulphate is an endogenous substance in the skin, cartilage etc of higher vertebrates and collagen is a major protein of the connective tissue. They are both biocompatible and completely biodegradable. Therefore, a composite matrix consisting of collagen and CS-GAGs would be well-suited for wound healing, tissue engineering and drug delivery applications.

6.5 Conclusion

Novel non-cross linked collagen-GAG scaffolds could be obtained by simple blending of the glycosaminoglycans isolated from the cranial cartilages of squid as well as cuttlefish, with fish collagen sheet (FCS) – a typical biomaterial scaffold from fish scales, developed with the prospect of utilization of fish processing waste. A pure, colorless and odorless preparation of collagen from fish scales, free from other components of connective tissue would be most desirable for use as biomaterials. The results of this investigation revealed that the attachment of glycosaminoglycans (GAGs) offers the opportunity to exploit the bio-characteristics of these polysaccharides as well as valorization of the collagen biomaterial. The biomaterials developed showed good equilibrium swelling properties, porosity and blood compatibility. Even though attempts to develop non crosslinked gelatin – GAG scaffolds were futile; a blended collagen – GAG scaffold using the chondroitin sulphate-GAGs isolated from the cranial cartilages of both squid and cuttlefish have been developed successfully and has the prospect of being used as a biomaterial. The blended collagen-GAG biomaterial prepared in this study can most suitably, be a promising entity towards the tissue engineering scaffolds especially of the osteoblastic lineage. In recent times, there is increasing worldwide concern about the use and over exploitation of natural seafood resources and hence maximum utilization of fish landings as well as the production of commercially viable products from fish waste has also emerged. In this context, the biomaterials developed in this study are a promising entity for greater utilization of fish waste/processing discards that has become an important issue for the seafood industry.

Chapter 7: Summary and Conclusion

Glycosaminoglycans (GAGs) are a fascinating group of molecules owing to their outstanding properties and wide therapeutic potential. The structure and organization of glycosaminoglycans from tissues of several vertebrates have been extensively studied. However, detailed studies on glycosaminoglycans from tissues of invertebrates, including marine cephalopods are rather limited. Glycosaminoglycans (GAGs) from various sources have been shown to have several physiological functions (Chapter 1), but very little is known of the functions of the GAGs from cephalopod species, especially in the Indian waters. The present study was aimed at the isolation and characterization of glycosaminoglycans from selected tissues of the edible as well as non-edible parts of the two commercially important species of cephalopods, squid (*Loligo duvauceli*) and cuttlefish (*Sepia pharaonis*) in view of their nutritional significance as well as benefits on the utilization of waste generated during processing. The study also envisaged the prospect of the utilization of these GAGs for their bioactive properties.

To investigate the existence of GAGs in the selected cephalopod tissues, the extraction protocol was developed based on a three step process in which modifications of three combined procedures were employed. The protocol developed was based on the stability of the GAGs at temperatures beyond ambient conditions, as well as their property as water soluble polysaccharides. The protocol developed in this study employs the liberation of GAGs directly from the defatted tissues by digestion with an exogenous proteinase (papain) and recovery of the product using ethanol precipitation. The GAGs could be easily separated from the co-precipitating papain residues by simple resuspension in water and recovered by centrifugation.

The protocol of GAG isolation developed was successfully employed with good reproducibility and did not require harsh chemicals such as guanidine hydrochloride or cetyl pyridinium chloride which may otherwise require further purification from these toxic chemicals. The yield of extractable GAGs varied

between species, tissues, different anatomical location etc which are important for preparation of GAGs. In both squid and cuttlefish the yield of crude GAGs in the respective tissues of squid and cuttlefish were in the order, cranial cartilage > fin > mantle > skin > tentacle. The gladius/pen of both the species showed no evidence of the presence of GAGs (Chapter 2). The data were also supported by histochemical staining of the respective tissues by staining using 1, 9 dimethyl methylene blue. The study revealed that among all the tissues analyzed, the cranial cartilage of both the cephalopod species contained appreciable amount of glycosaminoglycans with a yield of ~80g/Kg dry defatted tissue. The cranial cartilage is a tissue with little saleable value, and is usually discarded during processing, but its rich presence of glycosaminoglycan (GAG) can revolutionize it to a material, which can be converted to a highly profitable product. The presence of GAGs in the edible portions such as the mantle, fin etc, highlights the nutritional significance of these comparatively low fat seafood delicacies.

The objective of characterization of the cephalopod glycosaminoglycans was accomplished by employing various electrophoretic and chromatographic methods (Chapter 3) and spectroscopic techniques like FT- IR and NMR (Chapter 4). The quantitative analysis of the various components like uronic acid, hexosamines, sialic acid, fucose, hydroxyproline and proteins were performed using standard methods. The amounts of sulphated GAGs were estimated by metachromatic activity assay using 1, 9 dimethyl methylene blue. The glycans isolated were free of any extraneous proteins as evidenced by electrophoretic techniques (PAGE). The toluidine blue staining revealed the presence of sulphated glycosaminoglycans in the different tissues with different mobilities on agarose gels using acetate buffer systems. The enzyme digestion studies also revealed that the glycans were almost resistant to the activity of mucopolysaccharidases unlike vertebrate glycosaminoglycans. An attempt to elucidate the constituent disaccharide composition of the different glycans was also performed using SAX – HPLC (Strong anion exchange- High performance liquid chromatography). The gradient elution pattern developed was suitable for identification of the glycans within a maximum run time of 30 minutes. The preponderant GAG in the cranial

cartilage of both the cephalopods species were proposed to be of the chondroitin sulphate type as evidenced by the agarose gel electrophoresis and HPLC techniques employed.

The results from the present study confirm that, the spectroscopic techniques involving FT-IR (Fourier transform–Infrared) and NMR (Nuclear magnetic resonance) which is exploited in diverse fields like chemical and biochemical analysis, environmental monitoring, field applications and process control monitoring can be applied to the characterization of these cephalopod glycosaminoglycans as well. The manifold advantages of such approaches based on vibrational spectroscopy include low cost, small sample size, as well as mass reproducibility. Analysis of FT-IR spectra revealed the functional groups in the glycosaminoglycans from the different sources. The IR spectra of all the GAG samples were characterized by intense absorption around 1650^{cm-1} and 1050^{cm-1} that corresponds to the presence combined carboxylate, amine and sulphate present in the glycosaminoglycans. The NMR spectra were acquired in steps of 0.001 ppm, and the δ range of the GAGs fell within 6.31 to 0.41 ppm. The significance of the NMR method proposed lies in the fact that the study and characterization of these compounds (GAGs), which in general involves chromatographic and electrophoretic techniques, is often complex owing to their macromolecular, polydispersive and heterogeneous structures. Although nuclear magnetic resonance spectroscopy has rarely been used in control and routine analyses, recent studies demonstrate the usefulness of this technique when analysing complex macromolecular samples. In addition, nuclear magnetic resonance spectra of unfractionated compounds can be correlated to their disaccharide composition; thus, the tedious and time consuming enzymatic or chemical hydrolysis and further analysis of fragments released could be avoided.

In this context, the present protocol developed for the isolation of GAGs from the selected tissues of squid and cuttlefish and the analytical techniques employed for the preliminary identification of GAGs will provide great insights into a much reliable method for the production and identification of

glycosaminoglycans from other potential sources as well. The results obtained from the IR spectra and proton NMR confirms that the cranial cartilage GAGs of both the cephalopod species are of the chondroitin sulphate type with characteristic modifications such as higher degree of sulphation. Thus the present investigation showed that the cranial cartilages of both these cephalopods could be used as possible source of chondroitin sulphate in future. Results of the FT-IR, ^{1}H NMR spectroscopy, agarose gel electrophoresis and PAGE showed that the isolated GAGs are free from impurities, highlighting the suitability of the extraction protocol employed.

The bioactive potential as exemplified by the role in biomineralization is discussed in chapters 5. In order to understand the bioactive role of these sulphated macromolecules (GAGs) the influence of the cranial cartilage GAGs of squid and cuttlefish on crystal morphology of calcium carbonate was studied. The morphological evaluation of the calcium carbonate crystals synthesized *in vitro* were greatly influenced due to presence of the highly sulphated GAGs of squid and cuttlefish as evidenced by scanning electron microscopic (SEM) analysis. Compared to control $CaCO_3$ crystals, the test $CaCO_3$ crystals gradually changed from an irregular rhombus to a subsphaeroidal shape, especially in the case of samples synthesized in the presence of cuttlefish cranial cartilage GAGs, indicating the gradual transition from calcites to vaterite form of the crystal type. The higher bioactive potential was attributed to the greater sulphation characteristic of the cephalopod GAGs. The formation of biominerals provides a unique guide for the design of materials, especially those that need to be fabricated at ambient temperatures. Therefore, biological routes of structuring biominerals are becoming valuable approaches for novel material synthesis. Questions such as how mineralization is controlled in a micro- or nanosized confined space or how is it possible to regulate the spatiotemporal deposition of particular solid interfaces or macromolecules in solution to produce self assembled mineralized structures will require new interdisciplinary approaches to be answered.

Chapter 6 demonstrates the potential role of the cephalopod glycosaminoglycans as biomaterials, illustrated by its utilization for the valorization of fish collagen sheets (FCS) – a typical biomaterial scaffold from fish scales, developed with the prospect of utilization of fish processing waste. The chondroitin sulphate rich cephalopod GAGs were successfully impregnated into the fish collagen sheets developed from the scales of sea bass (*Lates calcarifer*) by a single step simple mechanical blending, without cross-linking by chemical or other physical methods. The blended collagen-GAG biomaterials (named as SQ-FCS and CF-FCS, of squid and cuttlefish GAGs respectively) were highly porous and showed good equilibrium swelling properties compared to control (cFCS). The SQ-FCS and CF-FCS scaffolds showed a very rapid and high percentage swelling ratio (58.47% and 68.5%) than the control fish collagen sheets (cFCS: 14.5%) attaining equilibrium swelling in the first five minutes. These results infact can be attributed to the high porosity of the GAG impregnated fish collagen sheets. Blood compatibility evaluation showed that the GAG impregnated sheets were biocompatible as evidenced by their haemolytic potential within permissible limits (< 5%). Analysis of FT-IR spectra revealed the functional groups in the scaffolds with amide I (1,600-1,660 cm^{-1}) and amide III (1,220-1,320 cm^{-1}) peaks indicating the carbonyl groups (C=O bond) and the CN stretching and NH vibrations characteristic of the triple helical structure of collagen. The differential scanning calorimetry (DSC) analysis showed that there were considerable peaks shifts in the samples compared to control fish collagen sheet (cFCS) having an endothermic peak around 92^0C, at 102^0C for the SQ-FCS and at 112^0C for the CF-FCS scaffold. The results of FT-IR and DSC analysis revealed that the ionic interactions were established between the collagen and the GAGs which attributed to the higher porosity and swelling properties of the glycosaminoglycan impregnated scaffolds. The attachment of glycosaminoglycans (GAGs) thus offers the opportunity to exploit the bio-characteristics of these polysaccharides as well as valorization of the collagen biomaterial.

The marine ecosystem can be considered a rather unexplored source of biological material with therapeutic activity. Most of the commercially available

glycosaminoglycans have so far been extracted from slaughter house discards, which often points to the recent health concern issues on interspecies viral infections, and also from potentially endangered species like whales, shark, rays etc. In recent times, there is increasing worldwide concern about the use and over exploitation of seafood resources and hence the need for maximum utilization of fish landings as well as the production of commercially viable products from fish waste has also emerged. In this context, the biomaterials developed in this study are a promising entity for greater utilization of fish waste/processing discards that has become an important issue for the seafood industry. This study also indicates that squid and cuttlefish cranial cartilages have potential in supplementing those of land vertebrates as a source of chondroitin sulphate type GAGs and thus can be a promising account so as to fulfill the needs without the overexploitation of other endangered resources.

Future perspectives:

- Screening of the glycosaminoglycans from other potential sources (eg: fish gills, scales, skeleton etc) which are hitherto considered as waste.
- Process optimization of glycosaminoglycan isolation using Response Surface Methodology.
- Further characterization of the modifications of CS-GAGs of squid and cuttlefish using 2D NMR spectroscopy.
- Optimisation of the Collagen: GAG ratio for improving the suitability of the collagen- GAGs scaffold.
- Cytotoxicity evaluation and biocompatibility studies on the suitability of the blended collagen- GAGs scaffolds as biomaterials in *in vivo* model systems.
- Scope of extending to large scale production processes of the glycosaminoglycan isolation protocol developed.

******

References

Abeygunawardana, C., Williams, T.C., Sumner, J.S., and Hennessey, J. P. (2000) Development and validation of an NMR-based identity assay for bacterial polysaccharides. *Anal. Biochem.,* **279**, 226-240.

Abolins-Krogis, A. (1958) The morphological and chemical characteristics of organic crystals in the regenerating shell of *Helix pomatia L. Acta Zool.,* **39**, 19-38.

Ackley, B.D., Crew, J.R., Elamaa, H., Pihlajaniemi, T., Kuo, C.J., and Kramer, J.M. (2001) The NC1/endostatin domain of *Caenorhabditis elegans* type XVIII collagen affects cell migration and axon guidance. *J. Cell. Biol.,* **152**, 1219–1232.

Addadi, L., Joester, D., Nudelman, F., and Weiner, S. (2006) Mollusk shell formation: a source of new concepts for biomineralization processes. *Chem. Eur. J.,* **12**, 980-987.

Addadi, L., Moradian, J., Shay, E., Maroudas, N. G., and Weiner, S. (1987) A chemical model for the cooperation of sulfates and carboxylates in calcite crystal nucleation: Relevance to biomineralization. *Proc. Natl. Acad. Sci.,* **84**, 2732-2736.

Adebowale, A., Du, J., Liang, Z., Leslie, J.L., and Eddington, N.D. (2002) The bioavailability and pharmacokinetics of glucosamine hydrochloride and low molecular weight chondroitin sulfate after single and multiple doses to beagle dogs. *Biopharm. Drug. Dispos.,* **23**, 217–225.

Agnelli, G., Piovella, F., Buoncristiani, P., Severi, P., Pini, M., D'Angelo, A., Beltrametti, C., Damiani, M., Andrioli, G.C., Pugliese, R., Iorio, A., and Brambilla, G. (1998) Enoxaparin plus compression stockings compared with compression stockings alone in the prevention of venous thromboembolism after elective neurosurgery. *N. Engl. J. Med.,* **339**, 80–85.

Ahmad, M., Benjakul, S., and Nalinanon, S. (2010) Compositional and physicochemical characteristics of acid solubilized collagen extracted from the skin of unicorn leatherjacket (*Aluterus monoceros*). *Food Hydrocolloids.,* **24**, 588-594.

Aizenberg, J., Black, A. J., and Whitesides, G. M. (1999) Control of crystal nucleation by patterned self-assembled monolayers. *Nature.*, **398**, 495-498.

Albano, R. M. and Maurao, P. A. S. (1986) Isolation, fractionation and preliminary characterization of a novel class of sulfated glycans from the tunic of *Styela plicata* (Chordata tunicate). *J. Biol. Chem*., **261**, 758 – 765.

Alexopoulou, A. N., Multhaupt, H. A., and Couchman, J. R. (2007) Syndecans in wound healing, inflammation and vascular biology. *Int. J. Biochem. Cell Biol.* **39**, 505−528.

Alkhalil, A., Achur, R.N., Valiyaveettil, M., Ockenhouse, C.F., and Gowda, D.C. (2000) Structural requirements for the adherence of *Plasmodium falciparum*-infected erythrocytes to chondroitin sulphate proteoglycans of human placenta. *J. Biol. Chem.,* **277**, 8882–8889.

Amado, M., Almeida, R., Schwientek, T., and Clausen, H. (1999) Identification and characterization of large galactosyltransferase gene families: Galacto-syltransferases for all functions. *Biochim. Biophys. Acta.,* **1473**, 35-53.

Aminoff, D. (1961) Methods for the quantitative estimation of N –acetylneuraminic acid and their application to hydrolysates of sialomucoids. *Biochem. J*., **81**, 384 – 392.

Ando, M., Ando, M., Makino, M., Tsukamasa, Y., Makinodan, Y., and Miyoshi, Y. (2001) Interdependence between heat solubility and pyridinoline contents of squid mantle collagen. *Journal of Food Science*., **66**, 265-269.

Andres, J L., DeFalcis, D., Noda, M., and Massague, J. (1992) Binding of two growth factor families to separate domains of the proteoglycan betaglycan. *J. Biol. Chem.,* **267**, 5927–5930.

Anno, K., Kawai, Y., and Seno, N. (1964) Isolation of chondroitin from squid skin. *Specialized Section on Mucoproteins and Mucopolysaccharides. Biochim. Biophys. Acta.,* **83**, 348 - 349.

Anno, K., Seno, N., Mathews, M. B., Yamagata, T., and Suzuki, S (1971) A new dermatan polysulfate, chondroitin sulfate H, from hagfish notochord. *Biochimica et Biophysica Acta.,* **237**, 173-177.

AOAC (1995) Official Methods of the Analysis of the Association of the Official Analytical Chemists, 16 th Edition. Association of Official Analytical Chemists, Arlington, Virginia.

Arias, J. L. and Fernandez, M. S. (2008) Polysaccharides and proteins in calcium carbonate based biomineralization. *Chem Rev.,* **108**, 4475-4482.

Arias, J. L., Neira, A. C., Arias, J. I., Escobar, C., Bodero, M., David, M., and Fernandez, M. S. (2004) Sulfated polymers in biological mineralization: A plausible source for bio-inspired engineering. *J. Mater. Chem.,* **14**, 2154 - 2160.

Astrid, Z. and Jean-Pierre, A. (2005) Hepatitis C virus and hepatitis B virus bind to heparin: Purification of largely IgG-free virions from infected plasma by heparin chromatography. *Gen Virol.,* **86**, 677-685.

Averbeck, M., Gebhardt, C.A., Voigt, S., Beilharz, S., Anderegg, U., Termeer, C.C., Sleeman, J.P., and Simon, J.C. (2007) Differential regulation of hyaluronan metabolism in the epidermal and dermal compartments of human skin by UVB irradiation. *Journal of Investigative Dermatology.,* **127**, 687– 697.

Baeurle, S.A., Kiselev, M.G., Makarova, E.S., and Nogovitsin, E.A. (2009) Effect of the counterion behavior on the frictional–compressive properties of chondroitin sulfate solutions. *Polymer.,* **50**, 1805–1813.

Balakrishnan, B., Mohanty, M., Umashankar, P.R., and Jayakrishnan, A. (2005) Evaluation of an *in situ* forming hydrogel wound dressing based on oxidized alginate and gelatin. *Biomaterials.,* **26**, 6335–6342.

Balian, G. and Bowes, J.H. (1977) The structure and properties of collagen. In: *The science and technology of gelatin* (Eds. Ward, A and Courts, A) Academic Press., London pp 1- 27.

Banu, N. and Tsuchiya, T. (2007) Markedly different effects of hyaluronic acid, chondroitin sulfate-A on the differentiation of human articular chondrocytes in micromass, 3-D honeycomb rotation cultures. *J. Biomed. Mater. Res.,* **80A,** 257–267.

Banyard, J., Bao, L., and. Zetter, B.R. (2003) Type XXIII collagen, a new transmembrane collagen identified in metastatic tumor cells. *J. Biol. Chem.,* **278**, 20989-20994.

Bao, X., Mikami, T., Yamada, S., Faissner, A., Muramatsu, T., and Sugahara, K. (2005) Heparin-binding growth factor, pleiotrophin, mediates neuritogenic activity of embryonic pig brain-derived chondroitin sulfate/dermatan sulfate hybrid chains. *J. Biol. Chem.,* **280**, 9180–9191.

Barbosa, I., Garcia, S., Barbier-Chassefiere, V., Caruelle, J., Martelly, I., and Garcia, P. (2003) Improved and simple micro assay for sulfated glycosaminoglycans quantification in biological extracts and its use in skin and muscle tissue studies. *Glycobiology.,* **13**, 647-653.

Barnhill, J.G., Fye, C.L., Williams, D.W., Reda, D.J., Harris, C.L., and Clegg, D.O. (2006) Chondroitin product selection for the glucosamine/chondroitin arthritis intervention trial. *J. Am. Pharm. Assoc.,* **46**, 14–24.

Bathe, M., Rutledge, G.C., Grodzinsky. A.J., and Tidor, B. (2005) A coarse-grained molecular model for glycosaminoglycans: Application to chondroitin, chondroitin sulfate, and hyaluronic acid. *Biophys. J.,* **88**, 3870–3887.

Behr, J.R., Matsumoto, Y., White, F.M., and Sasisekharan R. (2005) Quantification of isomers from a mixture of twelve heparin and heparan sulfate disaccharides using tandem mass spectrometry. *Rapid Commun. Mass Spectrom.,* **19**, 2553–2562

Belcher, C., Yaqub, R., Fawthrop, F., Bayliss, M., and Doherty, M. (1997) Synovial fluid chondroitin and keratan sulphate epitopes, glycosaminoglycans, and hyaluronan in arthritic and normal knees. *Ann. Rheum. Dis.,* **56**, 299–307.

Benzerara, K., Menguy, N., Lopez-Garcia, P., Yoon, T. H., Kazmierczak, J., Tyliszczak, T., Guyot, F., and Brown, G. E. (2006) Nanoscale detection of organic signatures in carbonate microbialites. *Proc. Natl. Acad. Sci.*, **103**, 9440 -9445.

Bergqvist, D., Agnelli, G., Cohen, A.T., Eldor, A., Nilsson, P.E., Moigne-Amrani, A.L., and Dietrich-Neto, F. (2002) Duration of prophylaxis against venous thromboembolism with enoxaparin after surgery for cancer. *N. Engl. J. Med.,* **346**, 975–980.

Bernhardt, R.R. and Schachner, M. (2000) Chondroitin sulfates affect the formation of the segmental motor nerves in zebrafish embryos. *Developmental Biology*., **221**, 206-219.

Bezares, J., Robert, J.A., and Hawley, M. (2008) Macromolecular structure of the organic framework of nacre in *Haliotis rufescens*: Implications for growth and mechanical behavior. *J. Struct. Biol*., **163**, 61–75.

Bink, R.J., Habuchi, H., Lele, Z., Dolk, E., Joore, J., Rauch, G.J., Geisler, R., Wilson, S.W., Hertog, J., Kimata, K., and Zivkovic*a,* D. (2003) Heparan Sulfate 6-*O*-sulfotransferase is essential for muscle development in zebrafish. *J.Biol.Chem*., **278**, 31118–31127.

Bjork, I. and Lindahl, U. (1982) Mechanism of the anticoagulant action of heparin. *Mol. Cell Biochem.,* **48**, 161–182.

Bjornsson, S. (1993) Size-dependant separation of proteoglycans by electrophoresis in gels of pure agarose. *Anal. Biochem.,* **210**, 292 -298.

Bland, C.E., Ginsburg, H., Silbert, J. E., and Metcalfe, D.D. (1982) Mouse heparin proteoglycan. Synthesis by mast cell-fibroblast monolayers during lymphocyte-dependent mast cell proliferation. *J. Biol. Chem.* **257**, 8661–8666.

Boateng, J. S., Matthews, K. H., Stevens, H. N. E., and Eccleston, G. M. (2008) Wound healing dressings and drug delivery systems: A review. *J.Pharm. Sci.,* **97**, 2892-2923.

Bollard, M. E. S., Garrod, E., Holmes, J. C., Lindon, E., Humpfer, M., Spraul, M., and Nicholson, J. K. (2000) High resolution ^{1}H and $^{1}H - ^{13}C$ magic angle spinning spectroscopy of rat liver. *Magnetic Resonance in Medicine.,* **44**, 201 -207.

Borelli, G., Mayer-Gostan, N., DePontual, H., Boeuf, G., and Payan P. (2001) Biochemical relationships between endolymph and otolith matrix in the trout (*Oncorhynchus mykiss*) and turbot (*Psetta maxima*). *Calcif. Tissue Int*., **69**, 356–364.

Borman, A. H., de Jong, E. W., Huizinga, M., Kok, D. J., Westbroek, P., and Bosch, L. (1982) The role in CaCO3 crystallization of an acid Ca2+-binding polysaccharide associated with coccoliths of *Emiliania huxleyi*. *Eur. J. Biochem.,* **129**, 179-183.

Borsig, L., Wang, L., Cavalcante, M. C. M., Cardilo-Reis, L., Ferreira, P.L., Mourao, P.A. S., Esko, J. D., and Pavao, M. S. G. (2007) Selectin blocking activity of a fucosylated chondroitin sulfate glycosaminoglycan from sea cucumber: Effect on tumor metastasis and neutrophil recruitment. *J. Biol. Chem*., **282**, 14984–14991.

Bowness, J. A. T. and Lee, K. M. (1967) Effects of chondroitin sulphates on mineralization *in vitro. Biochem J.,* **103**, 382-390.

Bradbury, E.J., Moon, L.D.F., Popat, R.J., King, V.R., Bennett, G.S., Patel, P.N., Fawcett, J. W., and Stephen B. McMahon. (2002) Chondroitinase ABC promotes functional recovery after spinal cord injury. *Nature.,* **416**, 636–640.

Brett, D. (2008) A review of collagen and collagen-based wound dressings. *Wounds.,* **20**, 347-356.

Bruyere, O. and Reginster, J.Y. (2007) Glucosamine and chondroitin sulfate as therapeutic agents for knee and hip osteoarthritis. *Drugs Aging.,* **24**, 573-580.

Bulow, H. E. and Hobert, O. (2006) The molecular diversity of glycosaminoglycans shapes animal development. *Annu. Rev. Cell Dev. Biol..,* **22**, 375–407.

Burson, S.L., Fahrenbach, M.J., Frommhagen, L.H., Riccardi, B.A., Brown, R.A., Brockman, J.A., Lewry, H.V., and Stockstad, E.L.R. (1956) Isolation and purification of mactins, heparin-like anticoagulants from mollusca. *J. Amer. Chem. Soc.,* **78**, 5874–5878.

Calabro, A., Benavides, M., Tammi, M., Hascall, V.C., and Midura, R.J. (2000) Microanalysis of enzyme digests of hyaluronan and chondroitin/dermatan sulphate by fluorophore-assisted carbohydrate electrophoresis (FACE). *Glycobiology*., **10**, 273-281.

Cambiazo, V. and Inestrosa, N.C. (1990) Proteoglycan production in Drosophila egg development: Effect of beta-D-xyloside on proteoglycans synthesis and larvae motility. *Comp. Biochem. Physiol. B.*, **97**, 307-314.

Carulli, D., Laabs, T., Geller, H.M., and Fawcett, J.W. (2005) Chondroitin sulfate proteoglycans in neural development and regeneration. *Curr. Opin. Neurobiol.,* **15**, 116–120.

Cassaro, C. M. F. and Dietrich, C. P. (1977) Distribution of sulfated mucopolysaccharides in invertebrates. *J. Biol. Chem*., **252**, 2254 – 2261.

Casu, B. and Torri, G. (1999) Structural characterization of low molecular weight heparins. *Semin. Thromb. Hemost.,* **25**, 17–25.

Cesaretti, M., Luppi, E., Maccari, F., and Volpi, N. (2004) Isolation and characterization of a heparin with high anticoagulant activity from the clam *Tapes phylippinarum*: Evidence for the presence of a high content of antithrombin III binding site. *Glycobiology.,* **14**, 1275–1284.

Chang, C. H., Liu, H. C., Lin, C. C., Chou, C. H., and Lin, F. H. (2003) Gelatin chondroitin-hyaluronan tri-copolymer scaffold for cartilage tissue engineering. *Biomaterials.*, **24**, 4853-4858.

Chatziioannidis, C.C., Karamanos, N.K., and Tsegenidis, T (1999) Isolation and characterisation of a small dermatan sulphate proteoglycan from ray skin (*Raja clavata*). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*., **124**, 15-24.

Chaussard, G. and Domard, A. (2004) New aspects of the extraction of chitin from squid pens. *Biomacromolecules.,* **5**, 559-564.

Chen, S., Xu, J., Xue, C., Dong, P.,Sheng, W., Yu, G., and Chai, W. (2008) Sequence determination of a non-sulfated glycosaminoglycan-like polysaccharide from melanin-free ink of the squid *Ommastrephes bartrami* by negative-ion electrospray tandem mass spectrometry and NMR spectroscopy. *Glycoconj J.,* **25**, 481 – 492.

Cherr, G.N., Meyers, S.A., Yudin, A.I., VandeVoort, C.A., Myles, D.G., Primakoff, P., and Overstreet, J.W. (1996) The PH-20 protein in cynomolgus macaque spermatozoa: Identification of two different forms exhibiting hyaluronidase activity. *Dev. Biol,* **175**,142–153.

Cheung, H. S., Van Wyk, J. J., Russell, W. E., and McCarty, D. J. (1986) Mitogenic activity of hydroxyapatite: Requirement for somatomedin C.*J. Cell Physiol*. **128**, 143-148.

Chuang, W., Christ, M.D., Peng, J., and Rabenstein, D.L. (2000) An NMR and molecular modeling study of the site-specific binding of histamine by heparin, chemically-modified heparin, and heparin-derived oligosacchrides. *Biochemistry.*, **39**, 542–3555.

Ciarlo, A.S., Paredi, M.E., and Fraga, A.N. (1997) Isolation of soluble collagen from hake skin (*Merluccius hubbsi*). *Journal of Aquatic Food Product Technology.,* **6**, 65-77.

Coleman, P., Kavanagh, E., Mason, R.M., Levick, J.R., and Ashhurst, D.E. (1998) The proteoglycans and glycosaminoglycan chains of rabbit synovium. *Histochemical Journal..,* **30**, 519-524.

Conte, A., Palmieri, L., Segnini, D., and Ronca, G. (1991) Metabolic fate of partially depolymerized chondroitin sulfate administered to the rat. *Drugs. Exp. Clin. Res.,* **17**, 27-33.

Cool, S. M. and Nurcombe, V. (2006) Heparan sulfate regulation of progenitor cell fate. *J. Cell Biochem.,* **99**, 1040–1051.

Copeland, C.E. and Six, C.K. (2009) A tale of two anticoagulants; Warfarin and heparin. *Journal of surgical education.,* **66**, 176 – 181.

Crookes, W.J., Ding, L., Huang, Q.L., Kimbell, J.R., Hortwitz, J., and McFall-Ngai, M. J. (2004) Reflectins: The unusual proteins of squid reflective tissues. *Science.,* **303**, 235 – 238.

Cui, F., Xue, C.H., Li, Z.J., Zhang, Y.Q., Dong, P., Fu, X.Y., and Gao, X. (2007) Characterzation and subunit composition of collagen from the body wall of sea cucumber *Stichopus japonicas. Food Chem.,* **100**, 1120 -125.

Dainiak, M.B., Allan, I. U., Savina, I.N., Cornelio, L., James, E.S., James, S.L., Mikhalovsky, S. V., Jungvid, H., and Galaev, I. Y. (2010) Gelatin–fibrinogen cryogel dermal matrices for wound repair: Preparation, optimisation and *in vitro* study. *Biomaterials.,* **31**, 67–76.

Dar, A. and Calabro, A. (2009) Synthesis and characterization of tyramine-based hyaluronan hydrogels. *J. Mater. Sci: Mater. Med..,* **20**, 33-44.

Dauphin, Y. (2003) Soluble organic matrices of the calcitic prismatic shell layers of two pteriomorphid bivalves. *J. Biol. Chem.,* **278**, 15168–15177.

David, G. and Bernfield, M. (1998) The emerging roles of cell surface heparan sulfate proteoglycans. *Matrix Biol.,* **17**, 461-463.

Davies, Y., Lewis, D., Fullwood, N.J., Nieduszynski, l.A., Marcyniuk, B., AIhon, J., and Tullo, A. (1999) Proteoglycans on normal and migrating human corneal endothelium. *Exp. Eye Res.,* **68**, 303-311.

Davis, J. M. G. (1970) Asbestos dust as a nucleation center in the calcification of old fibrous tissue lesions, and the possible association of this process to the formation of asbestos bodies. *Exp. Mol. Pathol,.* **12**, 133-147.

Dawlee, S., Sugandhi, A., Balakrishnan, B., Labarre, D., and Jayakrishnan, A. (2005) Oxidized chondroitin sulfate-cross-linked gelatin matrixes: A new class of hydrogels. *Biomacromolecules.,* **6**, 2040-2048.

Deepa, S. S., Umehara, Y., Higashiyama, S., Itoh, N., and Sugahara, K. (2002) Specific molecular interactions of oversulfated chondroitin sulfate E with various heparin-binding growth factors: Implications as a physiological binding partner in the brain and other tissues. *J. Biol. Chem*., **277**, 43707–43716.

Deepa, S.S., Kalayanamitra, K., Ito, Y., Kongtawelert, P., Fukui, S., Yamada, S., Mikami, S., and Sugahara, K. (2007) Novel sulfated octa and decasaccharides from squid cartilage chondroitin sulfate E: Sequencing and application for determination of the epitope structure of the monoclonal antibody MO-225. *Biochemistry.,* **46**, 2453-2465.

DeLoia, J. A., Krasnow, J. S., Brekosky, J., Babaknia, A., Julian, J., and Carson, D.D. (1998) Regional specialization of the cell membrane-associated, polymorphic mucin (MUCI) in human uterine epithelia. *Hum. Reprod.,* **13**, 2902-2909.

Denholm, E.M., Cauchon, E., Poulin, C., and Silver, P.J. (2000) Inhibition of human dermal fihroblast proliferation by removal of dermatan sulfate. *J. Pharmacol.,* **400**, 145-153.

Desaire, H. and Leary, J.A. (2000) Detection and quantification of the sulfated disaccharides in chondroitin sulfate by electrospray tandem mass spectrometry. *J.Am. Soc. Mass Spectrom.,* **11**, 916–920.

Dey, S. and Varman, A. R. (1983) Acid mucopolysaccharided in the lens cuticle of the fresh water prawn, *Macrobrachium birmanicum*. *Current Science.,* **52**, p219.

Diem, M., Boydston-White, S., and Chiriboga, L. (2005) Infrared spectroscopy of cells and tissues: Shining light onto a novel subject. *Appl. Spectrosc.,* **53**, 148A–161A.

Dietrich, C.P., Nader, H.B., Paiva, J.F., Tersariol, I.L.S., Santos, E.A., Holme, K.R., and Perlin, A.S. (1989) Heparin in molluscs: Chemical, enzymatic degradation and ^{13}C, ^{1}H n.m.r. spectroscopical evidences for the maintenance of the structure through evolution. *Int. J. Biol Macromol.,* **11**, 361–366.

Dietrich, C.P., Paiva, J.F., Castro, R.A.B., Chavante, S.F., Jeske, W., Fareed, J., Gorin, P.A.J., Mendes, A., and Nader, H.B. (1999) Structural features and anticoagulant activities of a novel natural low molecular weight heparin from the shrimp *Penaeus brasiliensis*. *Biochim.Biophys. Acta.,* **1428**, 273–283.

Dietrich, C.P., Paiva, J.F., Moraes, C.T., Takahashi, H.K., Porcionatto, M.A., and Nader, H.B. (1985) Isolation and characterization of a heparin with high anticoagulant activity from *Anomalocardia brasiliana*. *Biochim. Biophys. Acta.,* **843**, 1–7.

Dinglasan, R. R. and Jacobs-Lorena, M. (2005) Insight into a conserved lifestyle: Protein–carbohydrate adhesion strategies of vector-borne pathogens. *Infect. Immun.,***73**, 7797– 7807.

Dische, Z. (1947) A new specific colour reaction of hexuronic acids. *J. Biol. Chem.,* **167**, 189 -198.

Dische, Z. and Shettles, L. B. (1948) A specific color reaction of methyl pentoses and a spectrophotometric micromethod for their determination. *J. Biol. Chem*., **175**, 595 – 603.

Doege, K., Sasaki, M., Horigan, E., Hassell, J. R., and Yamada, Y. (1987) Complete primary structure of the rat cartilage proteoglycans core protein deduced from cDNA clones. *J. Biol. Chem*., **262**, 17757-17767.

Douglas, T., Heinemann, S., Mietrach, C., Hempel, U., Bierbaum, S., Scharnweber, D., and Worch, H. (2007) Interactions of collagen types I and II with chondroitin sulfates A-C and their effect on osteoblast adhesion. *Biomacromolecules.,* **8**, 1085-1092.

Du, J., White, N. and Eddingto, N.D. (2004) The bioavailability and pharmacokinetics of glucosamine hydrochloride and chondroitin sulfate after oral and intravenous single dose administration in the horse. *Biopharm. Drug Dispos.,* **25**, 109 –116.

Dupont, E., Savard, P.E., Jourdain, C., Juneau, C., Thibodeau, A., Ross, N., Marenus, K., Maes, D.H., Pelletier, G., and Sauder, D.N. **(**1998) Antiangiogenic properties of a novel shark cartilage extract: Potential role in the treatment of psoriasis. *Journal of Cutaneous Medicine and Surgery.,* **2**, 146-152.

Edmunds, L.H., (2004) Cardiopulmonary bypass after 50 years. *N Engl J Med*., **351,** 1604-1606.

Eguileor, M., Leonardi, M. G., Grimaldi, A., Tettamanti, G., Fiandra, L., Giordana, B., Valvassori, R., and Lanzavecchia, G. (2000) Integumental aminoacid uptake in a carnivorous predator mollusk (*Sepia officinalis,* Cephalopoda). *Tissue and Cell.,* **32**, 389 -398.

Ellis, D. I., Broadhurst, D., Kell, D. B., Rowland, J. J., and Goodacre, R. (2002) Rapid and quantitative detection of the microbial spoilage of meat by fourier transform infrared spectroscopy and machine learning. *Appl. Environ. Microbiol.,***68**, 2822–2828.

Ely, R., Supriya, T., and Naik, C.G. (2004). Antimicrobial activity of marine organisms collected off the coast of South East India. *J. Exp. Mar. Biol. Ecol.,* **309,** 121-127.

Ernst, S., Langer, R., Cooney, C. L., and Sasisekharan, R. (1995) Enzymatic degradation of glycosaminoglycans. *Crit.Rev.Biochem.Mol.Biol..,* **30**, 387 – 444.

Esko, J.D. and Lindahl U. (2001). Molecular diversity of heparan sulfate. *J. Clin. Invest.,* **108**, 169–173.

Esko, J.D. and Selleck, S.B. (2002) Order out of chaos: Assembly of ligand binding sites in heparin sulfate. *Annu. Rev. Biochem.,* **71**, 435–471.

Esko, J.D. and Zhang, L. (1996) Influence of core protein sequence on glycosaminoglycan assembly. *Curr. Opin. Struct. Biol.,* **6**, 663–670.

Falshaw, R., Hubl, U., Ofman, D., Slim, G.C., Tariq, M.A., Watt, D.K., and Yorke, S.C. (2000) Comparison of the glycosaminoglycans isolated from the skin and head cartilage of Gould's arrow squid (*Nototodarus gouldi*). *Carbohydrate Polymers.,* **41**, 357–364

Fan, T. W. M. (1996) Metabolite profiling by one-and two-dimensional NMR analysis of complex mixtures. *Progress in Nuclear Magnetic Resonance Spectroscopy.,* **28**, 161 – 219.

Feizi, T. (2000) Progress in deciphering the information content of the 'glycome' – a crescendo in the closing years of the millennium.*Glycoconjugate J.,* **17**, 553–565.

Fermin, C. D., Lovett, A. E., Igarashi, M., and Dunner, K. (1990) Immunohistochemistry and histochemistry of the inner ear gelatinous membranes and statoconia of the chick (*Gallus domesticus*). *Acta Anatomica* ., **138**, 75-83.

Fernandez, J. A., Petaja, J., and Griffin, J.H. (1999) Dermatan sulfate and LMW heparin enhance the anticoagulant action of activated protein C. *Thromb. Haemost.,* **82**, 1462-1468.

Fernandez-Diaz, M.D., Montero, P., and Gomez-Guillen, M.C. (2001) Gel properties of collagens from skins of cod (*Gadus morhua*) and hake (*Merluccius merluccius*) and their modification by the coenhancers magnesium sulphate, glycerol and transglutaminase. *Food Chem*., **74**, 161-167.

Folkman, J., Langer, R., Linhardt, R.J., Haudenschild, C., and Taylor, S. (1983) Angiogenesis inhibition and tumor regression caused by heparin or a heparin fragment in the presence of cortisone. *Science.,* **221**, 719–725.

Forsyth, R., Brigden, C., and Northrop, A. (2006) Double blind investigation of the effects of oral supplementation of combined glucosamine hydrochloride (GHCL) and chondroitin sulfate (CS) on stride characteristics of veteran horses. *Equine Veterinary Journal. Supplement.,* **36,** 622–625.

Fraser, J.R, Laurent, T.C., and Laurent, U.B. (1997) Hyaluronan: Its nature, distribution, functions and turnover. *Journal of Internal Medicine.,* **242**, 27–33.

Fraser, P.E., Darabie, A.A., and McLaurin, J.A. (2001) Amyloid-beta interactions with chondroitin sulphate-derived monosaccharides and disaccharides. Implications for drug development. *J. Biol. Chem.,* **273**, 6412–6419.

Freyman, T. M., Yannas, I. V., Yokoo, R., and Gibson, L. J. (2001) Fibroblast contraction of a collagen–GAG matrix. *Biomaterials.,* **22**, 2883-2891.

Fukuta, M., Inasawa, J., Torii, T., Tsuzuki, K., Shimada, E., and Habuchi, O. (1997) Molecular cloning and characterization of human keratan sulfate Gal-6-sullotransferase. *J. Biol. Chem.,* **272**, 32321-32328.

Fukutomi, M., Kobayashi, S., Niwaya, K., Hamada, Y., and Kitamura, S. (1996) Changes in platelet, granulocyte and complement activation during cardiopulmonary bypass using heparin-coated equipment. *Artif. Organs.,* **20**, 767–776.

Fullwood, N.J., Davies, Y., Nieduszynski, I.A., Marcyniuk, B., Ridgway, A.E., and Quantock, A.J. (1996) Cell surface-associated keratan sulfate on normal and migrating corneal endothelium. *Invest. Ophthalmol. Vis. Sci.,* **37**, 1256-1270.

Funderburgh, J.L. (2000) Keratan sulfate: Structure, biosynthesis, and function. *Glycobiology.,* **10**, 951–958.

Funderburgh, J.L. (2002) Keratan sulfate biosynthesis. *IUBMB Life.,* **54**, 187–194.

Funderburgh, J.L., Caterson, B., and Conrad, G.W. (1987) Distribution of proteoglycans antigenically related to corneal keratan sulfate proteoglycan. *J. Biol. Chem.,* **262**, 11634–11640.

Funderburgh, J.L., Mann, M.M., and Funderburgh, M.L. (2003) Keratocyte phenotype mediates proteoglycan structure: A role for fibroblasts in corneal fibrosis. *J. Biol. Chem.*, **278**, 45629–45637.

Fuster, M. M. and Esko, J. D. (2005) The sweet and sour of cancer: Glycans as novel therapeutic targets. *Nature Rev. Cancer.,* **5**, 526–542.

Gallo, R.L., Ono, M., Povsic, T., Page, C., Eriksson, E., Klagsbrun, M., and Bernfield, M. (1994) Syndecans, cell surface heparan sulfate proteoglycans, are induced by a proline-rich antimicrobial peptide from wounds. *Proc. Natl.Acad. Sci.,* **91**, 11035-11039.

Gandra, M.M, Cavalcante, C. M., and Pavao, M. S. G. (2000) Anticoagulant sulfated glycosaminoglycans in the primitive chordate *Styela plicata* (Tunicata). *Glycobiology.,* **10**, 1333 – 1340.

Garnjanagoonchorn, W., Wongekalak, L., and Engkagul, A. (2007) Determination of chondroitin sulfate from different sources of cartilage. *Chemical Engineering and Processing.,* **46**, 465-471.

Gathercole, L. J. and Keller, A. (1991) Crimp morphology in the fibre-forming collagens. *Matrix.,* **11**, 214-234.

Gerecht, S., Burdick, J.A., Ferreira, L.S., Townsend, S.A., Langer, R., and Vunjak-Novakovic, G. (2007) Hyaluronic acid hydrogel for controlled self-renewal and differentiation of human embryonic stem cells. *Proc. Natl. Acad. Sci.,* **104**, 11298-11303.

Gilbert, M.E., Kirker, K.R., Gray, S.D., Ward, P.D., Szakacs, J.G., Prestwich, G.D., and Orlandi, R.R. (2004) Chondroitin sulfate hydrogel and wound healing in rabbit maxillary sinus mucosa. *Laryngoscope.,* **114**, 1406–1409.

Gill, W. B., Jones, K. W., and Ruggiero, K. J. (1982) Protective effects of heparin and other sulfated glycosaminoglycans on crystal adhesion to injured urothelium. *J. Urol*. **127**, 152-154.

Gjaldbaek, J. C. (1982) Inhibition of chondroitin sulphate and heparin on the growth and agglomeration of calcium oxalate monohydrate crystals *in vitro. Clin. Chim. Acta.,* **120**, 363-365.

Glossl, J., Beck, M., and Kresse, H. (1984) Biosynthesis of proteodermatan sulfate in cultured human fibroblasts. *J. Biol. Chem.,* **259**, 14144-14150.

Goldberg, W. M. (2001) Acid polysaccharides in the skeletal matrix and calicoblastic epithelium of the stony coral *Mycetophyllia reesi. Tissue Cell.*, **33**, 376 – 387.

Gomez, A.M., Kozlowski, E.O., Pomin, V.H., de Barros, C.M., Zaganeli, J.L., and Pavao, M.S.G (2010) Unique extracellular matrix heparan sulfate from the bivalve *Nodipecten nodosus* (Linnaeus, 1758) safely inhibits arterial thrombosis after photochemically induced endothelial lesion. *J. Biol. Chem.*, **285**, 7312-7323.

Goodacre, R. Vaidyanathan, S., Dunn, W.B., Harrigan, G.G. and Kell, D.B. (2004) Metabolomics by numbers - acquiring and understanding global metabolite data. *Trends in Biotechnology.,* **22**, 245-252.

Grande, D.A., Halberstadt, C., Naughton, G., Schwartz, R., and Manji, R. (1997) Evaluation of matrix scaffolds for tissue engineering of articular cartilage grafts. *J Biomed Mater Res.,* **34**, 211-220.

Greenwood, J.A. and Murphy-UIIrich, J.E. (1998) Signaling of de-adhesion in cellular regulation and motility. *Microsc. Res. Tech..,* **43**, 420-432.

Guerassimov, A., Zhang, Y., Cartman, A., Rosenberg, L.C., Esdaile, J., Fitzcharles, M.A., and Poole, A.R. (1999) Immune responses to cartilage link protein and the G I domain of proteoglycan aggrecan in patients with osteoarthritis. *Arthritis Rheum.,* **42**, 527-533.

Guerrini, M., Raman, R., Venkataraman, G., Torri, G., Sasisekharan, R., and Casu, B. (2002) A novel computational approach to integrate NMR spectroscopy and capillary electrophoresis for structure assignment of heparin and heparan sulfate oligosaccharides. *Glycobiology.,* **12**, 713–719.

Guidry, C. and Grinnell, F. (1987) Heparin modulates the organization of hydrated collagen gels and inhibits gel contraction by fibroblasts. *J.Cell Biol.,* **104**, 1097–1103.

Habuchi, O., Hirahara, Y., Uchimura, K., and Fukuta, M. (1996) Enzymatic sulfation of galactose residue of keratan sulfate by chondroitin 6-sulfotransferase. *Glycobiology.,* **6**, 51-57.

Habuchi, O., Sugiura, K., and Kawai, N. (1977) Glucose branches in chondroitin sulphate from squid cartilage. *J. Biol.Chem*., **252**, 4570 –5476.

Hacker, U., Nybakken, K., and Perrimon, N. (2005) Heparan sulphate proteoglycans: The sweet side of development. *Nat. Rev. Mol. Cell Biol.,* **6**, 530– 541.

Haerry, T.E., Heslip, T.R., Marsh, J.L., and O'Connor, M.B. (1997) Defects in glucuronate biosynthesis disrupt wingless signaling in drosophila. *Development*., **124**, 3055-3064.

Hallak, L.K., Spillmann, D., Collins, P.L., and Peeples, M. E. (2000) Glycosaminoglycan sulfation requirements for respiratory syncytial virus infection. *Journal of Virology.,* **74,** 10508-10513.

Hallgrimsson, J., Friberg, U., and Burke, J. F. (1970) Changes with age in the metabolism of sulfated glycosaminoglycans in guinea pig cardiac valves. *Exp. Mol. Pathol*., **12**, 70-83.

Harrigan, G. G., LaPlante, R. H., Cosma, G. N., Cockerell, G., Goodacre, R., Maddox, J. F., Luyendyk, J. P.,. Ganey, P. E, and Roth, R. A. (2004) Application of high-throughput Fourier-transform infrared spectroscopy in toxicology studies: Contribution to a study on the development of an animal model for idiosyncratic toxicity. *Toxicol. Lett*., **146**, 197–205.

Hasegawa, N., Torii, T., Nagaoka, I., Nakayasu, K., Miyajima, H. and Habuchi, O. (1999) Measurement of activities of human serum sulfotransferases which transfer sulfate to the galactose residues of keratan sulfate and to the nonreducing end N-acetylglucosamine residues of N-acetyllactosamine trisaccharide: Comparison between normal controls and patients with macular corneal dystrophy. *J. Biochem.,* **125**, 245-252.

Hassan, F. and Mathew, S. (1999) Distribution of collagen in the muscle tissue of commercially important tropical fishes *J. Food. Sci. Technol.,* **33**, 121 – 123.

Hassell, J.R., Newsome, D.A., Krachrner, J.H. and Rodrigues. M.M. (1980) Macular corneal dystrophy: Failure to synthesize a mature keratan sulfate proteoglycan. *Proc. Natl. Acad. Sci.,* **77**, 3705-3709.

Heu, M.S., Lee, J.H., Kim, H.J., Jee, S.J., Lee, J.S., Jeon, Y., Shahidi, F. and Kim, J. (2010) Characterization of acid- and pepsin-soluble collagens from flatfish skin. *Food Sci. Biotechnol.,* **19**, 27-33.

Hirabayashi, J., Arata, Y., and Kasai, K. (2001) Glycome project: Concept, strategy and preliminary application to *Caenorhabditis elegans. Proteomics.*, **1**, 295–303.

Hjerpe, A., Antonopoulos, C. A., and Engfeldt, B. (1979) Determination of sulphated disaccharides from chondroitin suphates by high-performance liquid chromatography. *J. Chromatogr.* **171**, 339 – 344.

Hjerpe, A., Engfeldt, B., Tsegenidis, T., Antonopoulos, C. A. Vynios, D. H., and Tsiganos, C. P. (1983) Analysis of the acid polysaccharides from squid cranial cartilage and examination of a novel polysaccharide. *Biochim. Biophys. Acta*., **757**, 85 – 91.

Hoglund, L. (1976) Changes in acid mucopolysaccharides during the development of the blowfly, *Calliphora erythrocephala. Journal of Insect Physiology*., **22**, 917-924

Hovingh, P. and Linker A. (1993) Glycosaminoglycans in *Anodonta californiensis*, a freshwater mussel. *Biol. Bull.,* **185**, 263–276.

Hovingh, P. and Linker, A. (1982) An unusual heparan sulfate isolated from lobsters (*Homarus americanus*). *J. Biol. Chem.,* **257**, 9840–9844.

Huang, Z. M., Zhang, Y. Z., Kotaki, M., and Ramakrishna, S. (2003) A review on polymer nanofibers by electrospinning and their applications in nanocomposites. *Compos. Sci. Technol*., **63**, 2223 – 2253.

Huckerby, T.N., Nieduszynski, I.A., Giannopoulos, M., Weeks, S.D., Sadler, I.H., and Lauder, R.M. (2005) Characterization of oligosaccharides from the chondroitin/ dermatan sulfates. ^{1}H-NMR and ^{13}C-NMR studies of reduced trisaccharides and hexasaccharides. *FEBS J.,* **272**, 6276–6286.

Hunter, C. A., Packer, M. J., and Zonta, C. (2005) From structure to chemical shift and *vice – versa. Progress in Nuclear Magnetic Resonance Spectroscop.,* **47**, 27 – 39.

Hunter, G. K., Allen, B. L., Grynpas, M. D., and Cheng, P.T. (1985) Inhibition of hydroxyapatite formation in collagen gels by chondroitin sulphate. *Biochem.J*., **228**, 463- 469.

Hwang, H. Y., Olson, S. K., Esko, J. D., and Horvitz, H. R. (2003) *Caenorhabditis elegans* early embryogenesis and vulval morphogenesis require chondroitin biosynthesis. *Nature.,* **423**, 439–443.

Hwang, J. H., Miuta, S., Yokoyama, Y., and Yoshinaka, R. (2007) Purification and characterization of molecular species of collagen in the skin of skate (*Raja kenojei*). *Food Chem.,* **100**, 921-925

Ichijo, H. and Kawabata, I. (2001) Roles of the telencephalic cells and their chondroitin sulfate proteoglycans in delimiting an anterior border of the retinal pathway. *J. Neurosci.,* **21**, 9304–9314.

Imanari, T., Toida, T., Koshiishi, I. and Toyoda, H. (1996) High-performance liquid chromatography analysis of glycosaminoglycan-derived oligosaccharides. *J. Chromatogr A.,* **720**, 275 - 293.

Inatani, M. and Tanihara, H. (2002) Proteoglycans in retina. *Prog. Retin. Eye Res.,* **21**, 429–447.

Iozzo, R. V. (1998) Matrix proteoglycans: From molecular design to cellular function. *Annu. Rev. Biochem.,* **67**, 609–652.

Iozzo, R. V. (2001) Series introduction: Heparin sulphate proteoglycans; intricate molecules with intriguing functions. *J. Clin. Invest.,* **108**, 165 – 167.

Iozzo, R. V. (2005) Basement membrane proteoglycans: From cellar to ceiling. *Nature: Rev. Mol. Cell Biol.,* **6**, 646–656.

Iozzo, R.V. (1997) The family of the small leucine-rich proteoglycans: Key regulators of matrix assembly and cellular growth. *Crit. Rev. Biochem. Mol. Biol.,* **32**, 141-174.

Isobe, N. and Seno, N. (1971) Chondroitin-protein complex from squid skin. *Biochim. Biophys. Acta*., **252**, 612 – 615.

Itano, N. and Kimata, K. (2002) Mammalian hyaluronan synthases. *IUBMB Life.,* **54**, 195–199.

Ito, M., Morinaga, T., and Kitomikado, M. (1982) Occurrence of keratan sulfate in connective tissues of teleost. *Bulletin of Japaenese Society of Scientific Fisheries.,* **48**, 1445 – 1452.

Ito, N., Yokota, M., Nagaike, C., Morimura, Y., Hatake, K., Tanaka, O., and Matsunaga, T. (1996) Simultaneous expression of keratan sulphate epitope (a sulphated poly-N- acetyllactosamine) and blood group ABH antigens in papillary carcinomas of the human thyroid gland. *Histochem. J.,* **28**, 613-623.

Jackson, R.J., Busch, S.J., and Cardin, A.D. (1991) Glycosaminoglycans: Molecular properties, protein interactions, and role in physiological processes. *Physiol. Rev.*, **71**, 481-539.

Jibril, A. O. (1967) Proteolytic degradation of ossifying cartilage matrix and the removal of acid mucopolysaccharides prior to bone formation. *Biochim. Biophys. Acta.*, **136**, 162- 165.

Jongjareonrak, A., Benjakul, S., Visesanguan, W., Nagai, T., and Tanaka, M. (2005) Isolation and characterization of acid and pepsin-solubilised collagens from the skin of Brownstripe red snapper (*Lutjanus vitta*). *Food Chem*., **93**, 475-484.

Jordan, K.M. and Arden, N.K. (2003) EULAR Recommendations: An evidence based approach to the management of knee osteoarthritis: Report of a task force of the standing committee for international clinical studies including therapeutic trials (ESCISIT). *Ann. Rheum. Dis.*, **62**, 1145–1155.

Jordan, R.E. and Marcum, J.A. (1987) Anticoagulantly active heparin from clam (*Mercenaria mercenaria*). *Arch. Biochem. Biophys.,* **248**, 690–695.

Juhasz, P. and Biemann, K. (1995) Utility of non-covalent complexes in the matrix assisted laser desorption ionization mass spectrometry of heparin-derived oligosaccharides. *Carbohydr. Res.,* **270**, 131– 147.

Kaartinen, J., Mierisova, S., Oja, J.M.E., Usenius, J., Kauppinen, R.A., and Hiltunen, Y. (1998) Automated quantification of human brain metabolites by artificial neural network analysis from *in vivo* single-voxel ^{1}H NMR spectra. *J. Magn. Reson.,* **134,**176 – 179.

Kaderbhai, N. N., Broadhurst, D. I., Ellis, D. I., Goodacre, R., and Kell, D. B. (2003) Functional genomics via metabolic footprinting: monitoring metabolite secretion by *Escherichia coli* tryptophan metabolism mutants using FT–IR and direct injection electrospray mass spectrometry. *Comp. Funct. Genom.* **4**, 376–391.

Kadler, K. E., Holmes, D. F., Trotter, J. A., and Chapman, J. A. (1996) Collagen fibril formation. *Biochem. J.*, **316**, 1-11.

Kakizaki, I., Kojima, K., Takagaki, K., Endo, M., Kannagi, R., Ito, M., Maruo, Y., Sato, H., Yasuda, T., Mita, S., Kimata, K., and Itano, N. (2004) A novel mechanism for the inhibition of hyaluronan biosynthesis by 4-methylumbelliferone. *J. Biol. Chem*., **279**, 33281–33289.

Kang, H.W. Tabata, Y., and Ikada, Y. (1999) Fabrication of porous gelatin scaffolds for tissue engineering. *Biomaterials.,* **20**, 1339- 1344.

Karamanos, N. K., Vanky, P., Tzanakakis, G.N., Tsegenidis, T., and Hjerpe, A. (1997) Ion-pair high-performance liquid chromatography for determining disaccharide composition in heparin and heparan sulphate. *J. Chromatogr. A.,* **765**, 169.

Karamanos, N.K. and Hjerpe, A. (1998) A survey of methodological challenges for glycosaminoglycan/proteoglycan analysis and structural characterization by capillary electrophoresis. *Electrophoresis*., **19,** 2561- 2571.

Karamanos, N.K., Aletras, A. J., Tsegenids, T., Tsiganos, C.P., and Antonopoulos, C. A. (1992) Isolation, characterization and properties of the oversulphated chondroitin sulphate proteoglycan from squid skin with peculiar glycosaminoglycan sulphation pattern. *Eur. J. Biochem.,* **204**, 553 – 560.

Karamanos, N.K., Aletras, A. J., Antonopoulos, C. A., Hjerpe, A., and Tsiganos, C.P. (1990) Chondroitin proteoglycans from squid skin. Isolation, characterization and immunological studies. *Eur. J. Biochem.,* **192**., 33 – 38.

Karamanos, N.K., Manouras, A., Politou, D., Gritsoni, P., and Tsegenidis, T. (1991a) Isolation and high performance liquid chromatographic analysis of ray (*Raja clavata)* skin glycosaminoglycans. *Comparative Biochemistry and Physiology. Part B: Biochemistry and Molecular Biology*., **100**, 827 – 832.

Karamanos, N.K., Manouras, A., Tsegenids, T., and Antonopoulos, C. A. (1991b) Isolation and chemical study of the glycosaminoglycans from squid cornea. *International Journal of Biochemistry.,* **23**, 67 – 72.

Karamanos, N.K., Tsegenids, T., and Antonopoulos, C. A. (1986) Study of the glycosaminoglycans from squid skin. *Comparative Biochemistry and Physiology. Part B: Biochemistry and Molecular Biology.*, **85**, 865 – 868.

Kariya, Y., Watabe, S., Hashimoto, K., and Yoshida, K. (1990) Occurrence of chondroitin sulfate E in glycosaminoglycan isolated from the body wall of sea cucumber *Stichopus japonicus J. Biol. Chem.,* **265**, 5081-5085.

Kariya, Y., Watabe, S., Kyogashima, M., Ishihara, M., and Ishii, T. (1997) Structure of fucose branches in the glycosaminoglycan from the body wall of the sea cucumber *Stichopus japonicus. Carbohydr. Res*., **297**, 273-279.

Keogh, M.B., O'Brien, F.J., and Daly, J.S. (2010) Substrate stiffness and contractile behaviour modulate the functional maturation of osteoblasts on a collagen–GAG scaffold. *Acta Biomaterialia*., **6**, 4305 - 4313.

Khan, S. B., Qian, Z., Ryu, B. M., and Kim, S. (2009) Isolation and biochemical characterization of collagens from seaweed pipefish, *Syngnathus schlegeli. Biotechnology and Bioprocess Engineering.,* **14**, 436-442.

Kim**,** Y. S., Jo**,** Y.Y., Chang**,** M., Toida**,** T., Park**,** Y., and Linhardt, R. J. (1996) A new glycosaminoglycan from the giant African snail *Achatina fulica***.** *J. Biol.Chem.,* **271**, 11750 – 11755.

Kimura, S., Miyauchi, Y., and Uchida, N. (1991) Scale and bone type I collagens of carp (*Cyprinus carpio*). *Comp. Biochem. Phys.,* **99B**, 473–476.

Kimura, S., Omura, Y., Ishida, M., and Shirai, S. (1993) Molecular characterization of fibrillar collagen from the body wall of starfish *Asterias amurenis*. *Comp. Biochem. Phys.,* **104B**, 663-668.

Kimura, S., Takema, Y., and Kubota, M. (1981) Octopus skin collagen. Isolation and characterization of collagen comprising two distinct α – chains. *J. Biol. Chem.,* **256**, 13230 -13234.

Kinoshita, A., Yamada, S., Haslam, S. M., Morris, H. R., Dell, A., and Sugahara, K. (1997) Novel tetrasaccharides isolated from squid cartilage chondroitin sulfate E contain unusual sulfated disaccharide units GlcA(3- O- sulfate)β1-3GalNAc(6 – O – sulfate) or GlcA (3 –O – sulfate) β 1-3GalNAc(4,6 – O- disulfate). *J. Biol. Chem.,* **272**, 19656 – 19665.

Kitagawa, H., Tanaka, Y., Yamada, S., Seno, N., Haslam, S. M., Morris, H. R., Dell, A., and Sugahara, K. (1997) A novel pentasaccharide sequence GlcA(3 – sulfate)(β1 - 3)GalNAc(4- sulfate)(β1 -4)(Fucα -3) – GlcA(β 1-3)GalNAc(4 – sulfate) in the oligosaccharides isolated from King Crab cartilage chondroitin sulfate K and its differential susceptibility to chondroitinases and hyaluronidase. *Biochemistry.,* **36**, 3998 – 4008.

Kittiphattanabawon, P., Benjakul, S., Visesanguan, W., Nagai, T., and Tanaka, M., (2005) Characterization of acid-soluble collagen from skin and bone of bigeye snapper (*Priacanthustayenus*). *Food Chem*., **89**, 363-372.

Knudson, C.B. and Knudson, W. (1993) Hyaluronan-binding proteins in development, tissue homeostasis, and disease. *FASEB J*., **7**, 1233-1241.

Kobayashi, S. (1971) Acid mucopolysaccharides in calcified tissues. *Int. Rev. Cytol*. **30**, 257-371.

Koeller, K. M. and Wong, C. H. (2000) Emerging themes in medicinal glycoscience. *Nat. Biotechnol.*, **18**, 835–841.

Kojima, T., Leone, C.W., Marchildon, G.A., Marcum, J.A., and Rosenberg, R.D. (1992) Isolation and characterization of heparan sulfate proteoglycans produced by cloned rat microvascular endothelial cells. *J. Biol. Chem.*, **267**, 4859-4869.

Kramer, K. L. and Yost, H. J. (2003) Heparan sulphate core proteins in cell – cell signaling. *Annu. Rev. Genet*., **37**, 461–484.

Kramer, R.N., Crookes, W. J., and Naik, R. R. (2007) The self organizing properties of squid reflectin protein. *Nature Materials.,* **6**, 533 -538.

Krusius, T., Finne, J., Margolis, R.K., and Margolis, R.U. (1986) Identification of an O-glycosidic mannose-linked sialylated tetrasaccharide and keratan sulfate oligosaccharides in the chondroitin sulfate proteoglycan of brain. *J. Biol. Chem.,* **261**, 8237–8242.

Kurita, K., Tomita, K., Tada, T., Ishii, S., Nishimura, S., and Shimoda, K. (1993) Squid chitin as a potential alternative chitin source: Deacetylation behavior and characteristic properties *Journal of Polymer Science Part A: Polymer Chemistry.,* **31**, 485 – 491.

Kusche-Gullberg, M. and Kjellen, L. (2003) Sulfotransferases in glycosaminoglycan biosynthesis. *Curr. Opin. Struct. Biol*., **13**, 605–611.

Kwan, T.S., Pelletier, J.P., Verges, J., Lajeunesse, D., Montell, E., Fahmi, H., Lavigne, M., and Martel-Pelletier, J. (2007) Chondroitin and glucosamine sulfate in combination decrease the pro-resorptive properties of human osteoarthritis subchondral bone osteoblasts: A basic science study. *Arthritis Res.Ther*., **9**(6):R117.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*., **227**, 680 - 685.

Landsman, A., Taft, D., and Riemer, K. (2009) The role of collagen bioscaffolds, foamed collagen, and living skin equivalents in wound healing. *Clin. Paediatr. Med Surg*., **26**, 525-533.

Laparra, J. M., Tako, E., Glahn, R. P., and Miller, D. D. (2008) Isolated glycosaminoglycans from cooked haddock enhance nonheme iron uptake by Caco-2 cells. *J. Agric. Food Chem.,* **56**, 10346–10351.

Lauder, R.M., Huckerby, T.N., and Nieduszynski, I.A. (2000) A finger printing method for chondroitin/dermatan sulfate and hyaluronan oligosaccharides. *Glycobiology.,* **10**, 393-401.

Laurent, T.C. and Fraser, J.R.E. (1992) Hyaluronan. *FASEB J.,* **6**, 2397–2404.

Lee, A. and Langer, R. (1983). Shark cartilage contains inhibitors of tumour angiogenesis. *Science.,* **221**, 1185–1187.

Lee, C. H., Singla, A., and Lee, Y. (2001) Biomedical applications of collagen. *Int. J. Pharm*., **221**, 1-22.

Lee, C., Huang, C. and Lee, Y. (2006) Biomimetic porous scaffolds made from poly (L-lactide) -g-chondroitin sulfate blend with poly (L-lactide) for cartilage tissue engineering. *Biomacromolecules.,* **7**, 2200-2209.

Lee, J.K., Bhakta, S., Rosen, S.D., and Hemmerich, S. (1999) Cloning and characterisation of a mammalian N-acetylglucosamine-6- sulfotransferase that is highly restricted to intestinal tissue. *Biochem. Biophys. Res. Commum.,* **263**, 543-549.

Leuenberger, B.H. (1991) Investigation of viscosity and gelation properties of different mammalian and fish gelatins. *Food Hydrocolloids.,* **5**, 353-361.

Levi-Kalisman, Y., Falini, G., Addadi, L., and Weiner, S. (2001) Structure of nacreous organic matrix of a bivalve mollusk shell examined in the hydrated state using Cyto-TEM. *J. Struct. Biol.* **135**, 8–17.

Leygue, E., Snell, L., Dotz1aw, H., Hole, K., Hiller-Hitchcock, T., Roughley, P.J., Watson, P.H., and Murphy, L.C. (1998) Expression of lumican in human breast carcinoma. *Cancer Res.,* **58**, 1348-1352.

Li, F., Shetty, A.K., and Sugahara, k. (2007) Neuritogenic activity of chondroitin/dermatan Sulfate hybrid chains of embryonic pig brain and their mimicry from shark liver: Involvement of the pleiotrophin and hepatocyte growth factor signaling pathways. *J. Biol. Chem.,* **282**, 2956–2966.

Liang, J.H. and Wong, K.P (2000) The characterization of angiogenesis inhibitor from shark cartilage. *Advances in Experimental Medicine and Biology*., **476**, 209-223.

Liaw, P.C., Becker, D.L., Stafford, A.R., Fredenburgh, J.C., and Weitz, J.L. (2001) Molecular basis for the susceptibility of fibrin-bound thrombin to inactivation by heparin cofactor II in the presence of dermatan sulfate but not heparin. *J. Biol. Chem.,* **276**, 20959-20965.

Lieberman, J. R., Daluiski, A., and Einhorn, T. A. (2002) The role of growth factors in the repair of bone: biology and clinical applications. *J. Bone Joint Surg. Am.* **84A**, 1032-1044.

Lin, X. (2004) Functions of heparan sulfate proteoglycans in cell signaling during development. *Development.,* **131**, 6009–6021.

Lindahl, U., Kusche-Gullberg, M. and Kjellen, L. (1998) Regulated diversity of heparan sulfate. *J. Biol. Chem.,* **273**, 24979–24982.

Lindblom. A., Bengtsson – Olivecrona, G., and Fransson, LA. (1991) Domain structure of endothelial heparan sulphate. *Biochem J.,* **279**, 821-829.

Linhardt, R.J. (1991) Heparin: An important drug enters its seventh decade. *Chem. Indust.* **2**, 45-50.

Linhardt, R.J. and Gunay, N.S. (1999) Production and chemical processing of low molecular weight heparins. *Sem. Thromb. Hem.,* **3**, 5-16.

Linhardt, R.J., Ampofo, S.A., Fareed, J., Hoppensteadt, D., Folkman, J., and Mulliken, J.B. (1992) Isolation and characterization of human heparin. *Biochemistry.,* **31**, 12441–12445.

Liu, H., Fan, H., Cui, Y., Chen, Y., Yao, K., and Goh, J.C.H. (2007b) Effects of the controlled-released basic fibroblast growth factor from chitosan-gelatin microspheres on human fibroblasts cultured on a chitosan-gelatin scaffold. *Biomacromolecules.,* **8**, 1446-1455.

Liu, H.H., Ko, W.C., and Hu, M. L. (2002) Hypolipidemic effect of glycosaminoglycans from the sea cucumber *Metriatyla scabra* in rats fed a cholesterol-supplemented diet. *J. Agric. Food Chem.,* **50**, 3602-3606.

Liu, H.Y., Li, D., and Guo, S.D. (2007a) Studies on collagen from the skin of channel catfish (*Ictalurus punctaus*). *Food Chem.,* **101**, 621 – 625.

Liu, X.R., Zhu, B.Q., Shao, Y.Y., and Yang, X.L. (2010) Control of morphology and structure of calcium carbonate crystals by heparin. *Chinese Sci. Bull.,* **55**, 1107 – 1111.

Lowry, O.H., Rosegrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem.,* **193**, 265 -175.

Maimone, M.M. and Tollefsen, D.M. (1990) Structure of a dermatan sulfate hexasaccharide that binds to heparin cofactor II with high affinity. *J. Biol. Chem.,* **265**, 18263-18271.

Malavaki, C., Mizumoto, S., Karamanos, N., and Sugahara, K. (2008) Recent advances in the structural study of functional chondroitin sulfate and dermatan sulfate in health and disease. *Connect. Tissue Res.,* **49**, 133–139.

Mankin, H.J. and Lippiello, L. (1971) The glycosaminoglycans of normal and arthritic cartilage. *J. Clin. Invest.*, **50**, 1712 -1719.

Mann S. (1993) Molecular tectonics in biomineralization and biomimetic materials chemistry. *Nature.,* **365**, 499-505.

Manoli, F. and Dalas, E. (2000) Spontaneous precipitation of calcium carbonate in the presence of chondroitin sulfate. *J. Cryst. Growth.,* **217**, 416 - 421.

Manoli, F., Kanakis, J., and Malkaj, P. (2002) The effect of aminoacids on the crystal growth of calcium carbonates. *J. Crystal Growth.*, **236,** 363-370.

Mao, C., Solis, D.J., Reiss, B. D., Kottman, S. T., Sweeney, R. Y., Hayhurst, A., Georgiou, G., Iverson, B., and Belcher, A. M. (2004) Virus-based toolkit for the directed synthesis of magnetic and semiconducting nanowires. *Science.,* **303**, 213-217.

Marie, B., Luquet, G., Barros, J.P.D., Guichard, Z., Morel, S., Alcaraz, G., Bollache, L., and Marin, F. (2007) The shell matrix of the freshwater mussel *Unio pictorum* (Paleo heterodonta, Unionoida). *FEBS J.,* **274**, 2933–2945.

Mathew, S., Menon, P. V. G., and Kurup, P.A. (1982) Changes in glycoproteins in isoproterenol induced myocardial infarction in rats. *Ind. J. Biochem. Biophys.,* **19**, 41 -43.

Matthews, J. A., Wnek, G. E., Simpson, D. G., and Bowlin, G. L. (2002) Electrospinning of collagen nanofibers. *Biomacromolecules.*, **3**, 232-238.

McAlindon, T.E., LaValley, M.P., Gulin, J.P, and Felson, D.T (2000) Glucosamine and chondroitin for treatment of osteoarthritis: a systematic quality assessment and meta-analysis. *JAMA.,* **283**, 1469–1475.

McCarty, M.F., Russel, A.L., and Seed, M.P. (2000) Sulphated glycosaminoglycans and glucosamine may sinergize in promoting synovial hyaluronic acid synthesis. *Med. Hypotheses.,* **54**, 798–802.

McPherson, J.M., Sawamura, S.J., Condell, R.A., Rhee, W., and Wallace, D.G. (1988) The effects of heparin on the physicochemical properties of reconstituted collagen. *Coll. Relat. Res.,* **8**, 65–82.

Medeiros, G.F., Mendes, A., Castro, R.A.B., Bau, E.C., Nader, H.B., and Dietrich, C.P. (2000) Distribution of sulfated glycosaminoglycans in the animal kingdom: Widespread occurrence of heparin-like compounds in invertebrates. *Biochim. Biophys. Acta.,* **1475**, 287–294.

Meyer, K. and Palmer, J.W. (1934) The polysaccharide of the vitreous humor. *J.Biol. Chem.,* **107**, 629–634.

Meyer, K., and Chaffee, E. (1941) The mucopolysaccharides of skin *J. Biol. Chem.,* **138**, 491-499.

Meyer, K., Hoffman, P. and Linker, A. (1958) Mucopolysaccharides of costal cartilage. *Science.,* **128**, 896.

Meyer, K., Linker, A., Davidson, F.A. and Weissman, B. (1953) The mucopoly-saccharides of bovine cornea. *J. Biol.Chem.*, **205**, 611-616.

Mi, F. L., Shyu, S. S., Peng, C. K., Wu, Y. B., Sung, H. W., Wang, P. S., and Huang, C. C. (2006) Fabrication of chondroitin sulfate-chitosan composite artificial extracellular matrix for stabilization of fibroblast growth factor. *J. Biomed. Mater. Res., Part A.*, **76**, 1-15.

Michel, B.A., Stucki, G., Frey, D., DeVathaire, F., Vignon, E., Bruehlmann, P. and Uebelhart, D. (2005) Chondroitins 4 and 6 sulfate in osteoarthritis of the knee: a randomized, controlled trial. *Arthritis Rheum.,* **52**, 779–786.

Michenfelder, M., Fu, G., Lawrence, C., Weaver, J. C., Wustman, B. A., Taranto, L., Evans, J. S. and Morse, D. E. (2003) Characterization of two molluscan crystal-modulating biomineralization proteins and identification of putative mineral binding domains. *Biopolymers.*, **70**, 522 -533.

Middaugh, C.R., Mach, H., Burke, C.J., Volkin, D.B., Dabora, J.H., Tsai, P.K., Bruner, M.W., Ryan, J.A., and Marfia, K.E. (1992) Nature of the interaction of growth factors with suramin. *Biochemistry.,* **31**, 9016–9024.

Milan, A.M., Sugars, R.V., Embery, G., and Waddington, R.J. (2005) Modulation of collagen fibrillogenesis by dentinal proteoglycans. *Calcif. Tissue Int.,* **76,** 127–135.

Miller, B., Sheppard, A.M. and Pearlman, A.L. (1997) Developmental expression of keratan sulfate-like immunoreactivity distinguishes thalamic nuclei and cortical domains. *J. Comp. Neurol.*, **380**, 533–552.

Mingyan, Y., Bafang, L., and Xue, Z. (2009) Isolation and characterization of collagen from squid (*Ommastrephes bartrami*) skin. *J. Ocean Univ. China.,* **8**, 191 – 196.

Minniti, A.N., Labarca, M., Hurtado, C., and Brandan, E. (2004) *Caenorhabditis elegans* syndecan (SDN) is required for normal egg laying and associates with the nervous system and the vulva. *J. Cell Sci.,* **117**, 5179–5190.

Mitchell, C., Nivison, M., Jackson, L.F., Fox, R., Lee, D.C., Campbell, J.S., and Fausto, N. (2005) Heparin-binding epidermalgrowth factor-like growth factor links hepatocyte priming with cell cycle progression during liver regeneration. *J.Biol. Chem.,* **280,** 2562–2568.

Miyashita, T., Takagi, R., Okushima, M., Nakano, S., Miyamoto, H., Nishikawa, E., and Matsushiro, A. (2001) Complementary DNA cloning and characterization of Pearlin, a new class of matrix protein in the nacreous layer of oyster pearls. *Mar. Biotechnol.,* **2**, 409–418.

Mizuguchi, S., Uyama, T., Kitagawa, H., Nomura, K. H., Dejima, K., Gengyo-Ando, K., Mitani, S., Sugahara, K., and Nomura, K. (2003) Chondroitin proteoglycans are involved in cell division of *Caenorhabditis elegans. Nature.,* **423**, 443–448.

Mizuta, S., Miyagi, T., Nishimiya, T., and Yoshinaka, R. (2002) Partial characterization of collagen in mantle and adductor of pearl oyster (*Pinctada fucata). Food Chem.***, 79**, 319 – 325.

Mizuta, S., Tanaka, Y., and Yoshinaka, R. (2003) Comparision of collagen types of arm and mantle muscle of the common octopus (*Octopus vulgaris*). *Food Chem.,* **81**, 527 – 552.

Mizuta, S., Yoshinaka, R., Sato, M., and Sakaguchi, M. (1994) Isolation and partial characterization of two distinct types of collagen in the squid *Todarodes pacifus. Fisheries Science.,* **60**, 467 – 471.

Mizuta, S., Yoshinaka, R., Sato, M., and Sakaguchi, M. (1996) Effect of pepsin digestion on two distinct types of collagen in the muscle and skin of squid *Todarodes pacifus. Fisheries Science.,* **62**, 965 -969.

Mizuta, S., Yoshinaka, R., Sato, M., and Sakaguchi, M. (1997) Histological and biochemical changes of muscle collagen during chilled storage of the Kuruma prawn *Peneaus japonicus. Fisheries Science.,* **63**, 784 – 793.

Montero, P. and Gomez-Guillen, M.C.(2000) Extracting conditions for megrim (*Lepidorhombus boscii*) skin collagen affect functional properties of the resulting gelatin. *J. Food Sci.,* **65**, 434-438.

Montero, P., Alvarez, C., Marti, M.A., and Borderias, A.J. (1995) Plaice skin collagen extraction and functional properties. *J. Food Sci*., **60**, 1-3.

Mourao, P. A. S. and Perlin, A. S. (1987) Structural features of sulfated glycans from the tunic of *Styela plicate* (Chordata-Tunicata). *Eur. J. Biochem.*, **166**, 431-436.

Mourao, P.A.S., Boisson-Vidal, C., Tapon-Bretaudiere, J., Drouet, B., Bros, A., and Fischer, A.M. (2001) Inactivation of thrombin by a fucosylated chondroitin sulfate from echinoderm. *Thrombosis Research.*, **102,** 167-176.

Mourao, P.A.S., Pavao, M.S.G., Mulloy, B., and Wait, R. (1997) Chondroitin ABC lyase digestion of an ascidian dermatan sulfate. Occurrence of unusual 6 –O- sulfo-2-acetamido -2- deoxy -3-O- (2-O-sulfo-α-L-idopyranosyluronic acid)β –D-galactose units. *Carbohydrate Research.,* **300**, 315- 321.

Mulloy, B., Ribeiro, A.C., Alves, A.P., Vieira, R. P., and Mourao, P. A. S. (1994) Sulfated fucans from echinoderms have a regular tetrasaccharide repeating unit defined by specific patterns of sulfation at the o-2 and o-4 positions. *J. Biol. Chem*., **269**, 22113-22123.

Murakami, K., Aoki, H., Nakamura, S., Nakamura, S.I., Takikawa, M., Hanzawa, M., Kishimoto, S., Hattori, H., Tanaka, Y., Kiyosawa, T., Sato, Y., and Ishihara, M. (2010) Hydrogel blends of chitin/chitosan, fucoidan and alginate as healing-impaired wound dressings. *Biomaterials.,* **31**, 83–90

Muthusamy, A., Achur, R.N., Valiyaveettil, M., Madhunapantula, S.V., Kakizaki, I., Bhavanandan, V.P., and Gowda, C.D. (2004) Structural characterization of the bovine tracheal chondroitin sulfate chains and binding of *Plasmodium falciparum* - infected erythrocytes. *Glycobiology.,* **14**, 635-645.

Muyonga, J.H., Cole, C.G.B., and Duodu, K.G. (2004) Characterisation of acid soluble collagen from skins of young and adult Nile perch (*Lates niloticus*). *Food Chem.*, **85**, 81 -89.

Na, K., Kim, S., Park, K., Kim, K., Woo, D.G., Kwon, I.C., Chung, H., and Park, K. (2007) Heparin/Poly (l-lysine) nanoparticle-coated polymeric microspheres for stem-cell therapy. *J. Am. Chem. Soc.,* **129**, 5788-5789.

Nadanaka, S. and Sugahara, K. (1997) The unusual tetersaccharide sequence GlcAβ1-3GalNAc(4 – sulfate) β1-4GlcA(2-sulfate)β1-3GalNAc(6–sulfate) found in the hexasaccharides prepared by testicular hyaluronidase digestion of shark cartilage chondroitin sulfate D. *Glycobiology.,* **7**, 253 – 263.

Nader, H.B., Chavante, S.F., Santos, E.A., Oliveira, F.W., de-Paiva, J.F., Jeronimo, S.M.B., Medeiros, G.F., de-Abreu, L.R.D., Leite, E.L., de-Sousa-Filho, J.F., Castro, R.A.B., Toma, L., Tersariol, .L.S., Porcionatto, M.A., and Dietrich, C.P. (1999). Heparan sulfates and heparins: similar compounds performing the same functions in vertebrates and invertebrates? *Braz. J. Med. Biol. Res.,* **32**, 529–538.

Nader, H.B., Ferreira, T.M.P.C, Paiva, J.F., Medeiros, M.G.L., Jeronimo, S.M.B., Paiva, V.M.P., and Dietrich, C.P. (1984) Isolation and structural studies of heparin sulfates and chondroitin sulfates from three species of molluscs. *J Biol Chem.,* **259**, 1431–1435.

Nagai, T. (2004a) Characterization of collagen from Japanese sea bass caudal fin as waste material. *European Food Research and Technology.,* **218**, 424-427.

*Nagai, T. (2004b) Collagen from diamondback squid (*Thysanoteuthis rhombus*) outer skin. *Zeitschrift für Naturforschung.,* **59**, 271-275.

Nagai, T. and Suzuki, N. (2000b) Isolation of collagen from fish waste material-skin, bone and fins. *Food Chem.,* **68**, 277-281.

Nagai, T. and Suzuki, N. (2000c). Preparation and characterization of several fish bone collagens. *Journal of Food Biochemistry.*, **24**, 427–436.

Nagai, T. and Suzuki, N. (2002a) Collagen of the skin of ocellate puffer fish (*Takifugu rubripes*). *Food Chemistry.,* **78**, 173–177.

Nagai, T. and Suzuki, N. (2002b) Preparation and partial characterization of collagen from paper nautilus (*Argonauta argo*, Linnaeus) outer skin. *Food Chemistry.*, **76**, 149–153.

Nagai, T. and Suzuki, T. (2000a) Partial characterization of collagen from purple sea urchin (*Acanthocidaris crassipina*) test. *Intl J. Food. Sci. Technol.,* **35**, 497-501.

Nagai, T., Nagamori, K., Yamashita, E., and Suzuki, N. (2002) Collagen of octopus *Callistoctopus arakawai* arm. *International Journal of Food Science and Technology.*, **37**, 285–289.

Nagai, T., Ogawa, T., and Nakamura, T. (1999) Collagen from edible jellyfish exumbrella. *J. Sci. Food. Agri.,* **79**, 855 -858.

Nagai, T., Suzuki, N., Tanoue, Y., Kai, N., and Nagashima, T. (2010) Characterization of acid-soluble collagen from skins of surf smelt (*Hypomesus pretiosus japonicas* Brevoort). *Food and Nutrition Sciences.,* **1**, 59-66.

Nagai, T., Worawattanamateekul, W., Suzuki, N., Nakamura, T., Ito, T., Fujiki, K., Nakao, M., and Yano, T. (2000) Isolation and characterization of collagen from rhizostomous jellyfish (*Rhopilema asamushi*). *Food Chemistry.*,**70**, 205–208.

Nagai, T., Yamashita, E., Taniguchi, K., Kanamori, N., and Suzuki, N. (2001) Isolation and characterisation of collagen from the outer skin waste of cuttlefish *(Sepia lycidas)*. *Food Chemistry.,* **72**, 425-429.

Nakagawa, H., Hama, Y., Sumi, T., Li, S., Maskos, K., Kalayanamitra, K., Mizumoto, S., Sugahara, K., and Li, Y. (2007) Occurrence of a nonsulfated chondroitin proteoglycan in the dried saliva of *Collocalia* swiftlets (edible bird's-nest).*Glycobiology.,* **17**,157–164.

Nakano, T., Nakano, K., and Sim, J.S. (1998) Extraction of glycosaminoglycan peptide from bovine nasal cartilage with 0.1 M sodium acetate. *J. Agric. Food Chem.,* **46**, 772-778

Nakano, T., Sunwoo, H.H., Li, X., Price, M.A., and Sim, J.S. (1996) Study of sulfated glycosaminoglycans from porcine skeletal muscle epimysium including analysis of iduronosyl and glucuronosyl residues in galactosaminoglycan fractions. *J. Agric. Food Chem.*, **44**, 1424-1434.

Nakato, H., Futch, T.A., and Selleck, S.B. (1995) The division abnormally delayed (dally) gene: a putative integral membrane proteoglycans required for cell division patterning during postembryonic development of the nervous system in drosophila. *Development.,* **121**, 3687-3702.

Nakazawa, K., Takahashi, I., and Yamamoto, Y. (1998) Glycosyltransferase and sulfotransferase activities in chick corneal stromal cells before and after *in vitro* culture. *Arch. Biochem. Biophys.,* **359**, 269-282.

Nandini, C.D., Itoh, N., and Sugahara, K. (2005) Novel 70-kDa chondroitin sulfate/dermatan sulfate hybrid chains with a unique heterogenous sulfation pattern from shark skin, which exhibit neuritogenic activity and binding activities for growth factors and neurotrophic factors. *J. Biol. Chem.,* **280**, 4058–4069.

Naumann, D., Fijala, V., Labischinski, H., and Giesbrecht, P. (1988) The rapid differentiation and identification of pathogenic bacteria using Fourier transform infrared spectroscopic and multivariate statistical analysis. *J. Mol. Struct*., **74**, 165–170.

Naumann, D., Helm, D., and Labischinski, H. (1991) Microbiological characterizations by FT-IR spectroscopy. *Nature.*, **351**, 81–82.

Neira-Carrillo, A.; Yazdani-Pedram, M.; Retuert, J.; Diaz-Dosque, M.; Gallois, S.; Arias, J. L. (2005) Selective crystallization of calcium salts by poly(acrylate)-grafted chitosan. *J. Colloid Interface Sci.*, **286**, 134 - 141.

Nicholson, J. K. and Foxall, P. J. D. (1995) 750 MHz ^{1}H and ^{1}H – ^{13}C NMR spectroscopy of human blood plasma. *Analytical Chemistry.,* **67**, 793 -811.

Nieduszynski, I. A., Huckerby, T. N., Dickenson, J.M., Brown, G.M., Tai, G.H., Morris, H.G., and Eady, S. (1990) There are two major types of skeletal keratan sulphates. *Biochem. J.,* **271**, 243–245.

Nizet, V., Ohtake, T., Lauth, X., Trowbridge, J., Rudisill, J., Dorschner, R.A., Pestonjamasp, V., Piraino, J., Huttner, K., and Gallo, R.L. (2001) Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature.,* **414**, 454 - 457.

Noble, P.W. (2002) Hyaluronan and its catabolic products in tissue injury and repair. *Matrix Biol.,* **21**, 25–29.

Nomura, Y., Sakai, H., Ishii, Y., and Shirai, K. (1996). Preparation and some properties of type I collagen from fish scales. *Bioscience, Biotechnology, and Biochemistry.*, **60**, 2092–2094.

Nudelman, F., Gotliv, B.A., Addadi, L., and Weiner, S. (2006) Mollusk shell formation: mapping the distribution of organic matrix components underlying a single aragonitic tablet in nacre. *J. Struct. Biol.,* **153**, 176–187.

Nunes, M.L. and Batista, N.B.I. (2003) FISH products: contribution for a healthy food. *Electron. J. Environ. Agric. Food Chem*., **2**, 453-457.

Nurnadia, A.A., Azrina, A., and Amin, I. (2011) Proximate composition and energetic value of selected marine fish and shellfish from the West coast of Peninsular Malaysia. *International Food Research Journal.,* **18**, 137-148.

*O'Brien, F. J., Harley, B. A., Waller, M. A., Yannas, I. V., Gibson, L. J., and Prendergast, P. J. (2007) The effect of pore size on permeability and cell attachment in collagen scaffolds for tissue engineering. *Technol. Health Care*., **15**, 3–17.

O'Hagan, S., Dunn, W. B., Brown, M., Knowles, J. D, and Kell, D. B. (2005) Closed-loop, multiobjective optimization of analytical instrumentation: Gas Chromatography/Time-of-Flight Mass Spectrometry of the metabolomes of human serum and of yeast fermentations. *Anal. Chem*.,**77**, 290–303.

O'Leary, R. K. and Guess, W. L. (1968) Toxicological studies on certain medical grade plastics sterilized by ethylene oxide. *J. Pharm. Sci*., **57**, 12-17.

Oberg, K.A., Ruysschaert, J.M., and Goormaghtigh, E. (2004) The optimization of protein secondary structure determination with infrared and circular dichroism spectra. *Eur. J. Biochem*., **271**, 2937–2948.

Obrink, B. (1973) The influence of glycosaminoglycans on the formation of fibers from monomeric tropocollagen *in vitro. Eur. J. Biochem.,* **34**, 129–137.

Ogawa, M., Portier, R.J., Moody, M.W., Bell, J., Schexnayder, M.A., and Losso, J.N. (2004) Biochemical properties of bone and scale collagens isolated from the subtropical fish black drum (*Pogonia cromis*) and sheepshead seabream (*Archosargus probatocephalus*). *Food Chem.,* **88**, 495-501.

Oikawa, T., Ashino-Fuse, H., Shimamura, M., Koide, U., and Iwaguchi, T. (1990) A novel angiogenic inhibitor derived from Japanese shark cartilage (I). Extraction and estimation of inhibitory activities toward tumour and embryonic angiogenesis. *Cancer Lett.,* **51**, 181–186.

*Oliveira, A. M. and Farina, M. (1996) Vaterite, calcite, and aragonite in the otoliths of three species of piranha. *Naturwissenschaften.,* **83**, 133-135.

Olsson, L., Stigson, M., Perris, R., Sorrell, J.M., and Lofberg, J. (1996) Distribution of keratan sulphate and chondroitin sulphate in wild type and white mutant axolotl embryos during neural crest cell migration. *Pigment Cell Res.,* **9**, 5-17.

Ototani, N., Kikuchi, M., and Yosizawa, Z. (1981) Comparative studies on the structures of highly-active and relatively-inactive forms of whale heparin. *J. Biochem.,* **90**, 241–246.

Ototani, N., Kikuchi, M., and Yosizawa, Z. (1982) Structure and biological activity of finback- whale (*Balanoptera physalua* L.) heparin octasaccharide: chemical, carbon -13 nuclear magnetic resonance, enzymic and biological studies. *Biochem. J.,* **205**, 23 -30.

Pacheco, R. G., Vicente, C. P., Zancan, P., and Mourao, P. A. S. (2000) Different antithrombotic mechanisms among glycosaminoglycans revealed with a new fucosylated chondroitin sulfate from an echinoderm. *Blood Coagulation & Fibrinolysis.,* **11**, 563-573.

Paddle-Ledinek, J.E., Nasa, Z., and Cleland, H.J. (2006) Effect of different wound dressings on cell viability and proliferation. *Plast. Reconstr. Surg*., **117**(Suppl), 110S–118S.

Palchik, N. A. and Moroz, T. N. (2005) Polymorph modifications of calcium carbonate in gallstones. *J. Cryst. Growth.*, **283**, 450-456.

Pandian, V. and Thirugnanasambandan, S. (2008) Glycosamimoglycans (GAG) from backwater clam *Marcia opima* (Gmelin). *Iranian Journal of Pharmacology and Therapeutics.,* **7**, 147 -151.

Papakonstantinou, E., Karakiulakis, G., Eickelberg, O., Perruchoud, A.P., Block, L.H., and Roth, M. (1998) A 340 kDa hyaluronic acid secreted by human vascular smooth muscle cells regulates their proliferation and migration. *Glycobiology.,* **8**, 821-830.

Park, B. S., Heo, S. J., Kim, C. S., Oh, J. E., Kim, J. M., Lee, G., Park, W. H., Chung, C. P., and Min, B. M. (2005) Effects of adhesion molecules on the behavior of osteoblast-like cells and normal human fibroblasts on different titanium surfaces. *J. Biomed. Mater. Res. (Part A).,* **74***,* 640-651.

Park, Y., Zhang, Z., Laremore, T.N., Li, B., Sim, J., Im, R., Ahn, M.I., Kim, Y, S., and Linhardt, R. J. (2008) Variation of acharan sulfate and monosaccharide composition and analysis of neutral N -glycans in African giant snail (*Achatina fulica*). *Glycoconjugate J*., **25**, 863-877.

Pavao, M.S.G., Aiello, K. R. M., Werneck, C. C., Silva, L. C., Valente, A. P., Mulloy, B., Colwell, M.S., Tollefsen, D. M., and Mourao, P. A. S (1998) Highly sulfated dermatan sulfate from ascidians. Structure versus anticoagulant activity of these glycosaminoglycans. *J. Biol.Chem.,* **273**, 27848 – 27857.

Pavao, M.S.G., Mourao, P. A. S., Mulloy, B., and Tollefsen, D. M. (1995) A unique dermatan sulfate like glycosaminoglycans from ascidian: its structure and the effect of its unusual sulfation pattern on anticoagulant activity. *J. Biol.Chem.,* **270**, 31027 – 31036.

Pedersen, M.E., Kolset, S.O., Sorensen, T., and Eggen, K.H. (1999) Sulfated glycosaminoglycans and collagen in two bovine muscles (M. semitendinosus and M. psoas major) differing in texture. *J. Agric. Food Chem.,* **47**, 1445-1452.

Pejler, G., Danielsson, A., Bjork, I., Lindahl, U., Nader, H.B., and Dietrich, C.P. (1987) Structure and antithrombin-binding properties of heparin isolated from the clams *Anomalocardia brasiliana* and *Tivela mactroides. J. Biol. Chem.,* **262**, 1413–1421.

Penc, S.F., Pomahac. B., Eriksson, E., Detmar, M., and Gallo, R.L. (1999) Dermatan sulfate activates nuclear factor-kappab and induces endothelial and circulating intercellular adhesion molecule. *J.Clin.Invest.,* **103**, 1329-1335.

Petaja, J., Fernandez, J.A., Gruber, A., and Griffin, J.H. (1997) Anticoagulant synergism of heparin and activated protein C *in vitro*. Role of a novel anticoagulant mechanism of heparin, enhancement of inactivation of factor V by activated protein *C. J. Clin. Invest.,* **99**, 2655-2663.

Pfeiler, E., Toyoda, H., Williams, M. D., and Nieman, R. A. (2002) Identification, structural analysis and function of hyaluronan in developing fish larvae (leptocephali). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology.,* **132**, 443-451.

Pieper, J. S., Oosterhof, A., Dijkstra, P. J., Veerkamp, J. H., and vanKuppevelt, T. H. (1999) Preparation and characterization of porous cross-linked collagenous matrices containing bioavailable chondroitin sulphate. *Biomaterials*., **20**, 847–858.

Piez, K. A. (1984) Molecular and aggregate structure of the collagens *in Extracellular Matrix Biochemistry*, K. A. Piez and A. H. Reddi, Eds. New York: Elsevier, pp. 1-39.

Pinto, D. O., Ferreira, P. L., Andrade, L. R., Petrs-Silva, H., Linden, R., Abdelhay, E., Araujo, H.M.M., Alonso, C.V., and Pavao, M. S.G. (2004) Biosynthesis and metabolism of sulfated glycosaminoglycans during *Drosophila melanogaster* development. *Glycobiology.,* **14**, 529 -536.

Plaas, A.H., West, L.A., Wong-Palms, S., and Nelson, F.R. (1998) Glycosaminoglycan sulfation in human osteoarthritis. Disease-related alterations at the non-reducing termini of chondroitin and dermatan sulfate. *J. Biol. Chem.,* **273**, 12642–12649.

Pothacharoen, P., Siriaunkgul, S., Ong-Chai, S., Supabandhu, J., Kumja, P., Wanaphirak, C., Sugahara, K., Hardingham, T., and Kongtawelert, P. (2006) Raised serum chondroitin sulfate epitope level in ovarian epithelial cancer. *J.Biochem.,* **140**, 517–524.

Prame, G. (1967) Glycosaminoglycans of retrobulbar tissue of the crucian carp (*Carassius carassius* L.) *Experimental Eye Research.,* **6**, 171-178

Prevo, R., Banerji, S., Ferguson, D.J.P., Clasper, S., and Jackson, D.G. (2001) Mouse LYVE-1 is an endocytic receptor for hyaluronan in lymphatic endothelium. *J. Biol. Chem.,* **276,** 19420-19430.

Price, R. D., Berry, M. G., and Navsaria, H. A. (2007) Hyaluronic acid: the scientific and clinical evidence. *Journal of Plastic, Reconstructive and Aesthetic Surgery.,* **60**, 1110 -1119.

Puhl, W. and Scharf, P. (1997) Intra-articular hyaluronan treatment for osteoarthritis. *Ann. Rheum. Dis*., **56**, 637–40.

Purna, S.K. and Babu, M. (2000) Collagen based dressings–a review. *Burns*., **26**, 54–62.

Qiao, L., Feng, Q., and Li, Z. (2007) Special vaterite found in freshwater lackluster pearls. *Crystal growth and design.,* **7**, 275 – 279.

Quinn, K.J., Courtney, J.M., Evans, J.H., Gaylor, J.D.S., and Reid, W.H. (1985) Principles of burn dressings. *Biomaterials.,* **6**, 369–377.

Raats, C. J. I., Van en Born, J., and Berden, J. H. M. (2000) Glomerular heparan sulfate alterations: mechanisms and relevance for proteinuria. *Kidney Int.,* **57**, 385–400.

Radhakrishnamurthy, B., Srinivasan, S. R.., Dalfers, E. R., and Berenson, G. S. (1970) Composition of glycopeptides from chondroitin sulfate- protein complex from squid skin. *Comparative Biochemistry and Physiology.,* **36**, 107 – 117.

Ragan, P.M., Chin, V.I., Hung, H.K., Koichi Masuda, Thonar, E. J. M. A., Arner, E.C., Grodzinsky, A.J., and Sandy, J.D. (2000) Chondrocyte extracellular matrix synthesis and turnover are influenced by static compression in a new alginate disk culture system. *Arch. Biochem. Biophys.,* **383**, 256–264.

Raman, M. and Mathew, S. (2005) Effect of structural proteins on the textural characteristics of rohu and squid at various cook temperatures. *J. Food. Sci. Technol.,* **42**, 430-434.

Rhiner, C., Gysi, S., Frohli, E., Hengartner, M.O. and Hajnal, A. (2005) Syndecan regulates cell migration and axon guidance in *C. elegans*. *Development.,* **132**, 4621–4633.

Rhomberg, A.J., Ernst, S., Sasisekharan, R. and Biemann, K. (1998) Mass spectrometric and capillary electrophoretic investigation of the enzymatic degradation of heparin-like glycosaminoglycans. *Proc. Natl. Acad. Sci.,* **95**, 4176– 4181.

Rigby, B. J. (1968) Aminoacid composition and thermal stability of the skin collagen of the Antartic ice fish. *Nature.,* **219**, 166 -167.

Roden, L., Baker, J. R., Cifonelli, J. A. and Mathews, M. B. (1972) Isolation and characterization of connective tissue polysaccharides. In: *Methods in Enzymology.* (Eds. S. P. Colowick and N. O. Kaplan). Academic Press, New York. **28**, 73 – 140.

Roden, L., Koerner, T., Olson, C., and Schwartz N. B. (1985) Mechanisms of chain initiation in the biosynthesis of connective tissue polysaccharides. *Fed. Proc.*, **44**, 373-380.

Rodrigues, E.D., Pimentel, E.R., Mourao, P.A.S., and Gomes, L. (2005) Distribution of small proteoglycans and glycosaminoglycans in humerus-related articular cartilage of chickens. *Braz. J. Med. Biol. Res.,* **38**, 381-390.

Rolls, A., Avidan, H., Cahalon, L., Shori, H., Bakalash, S., Litvak, V., Lev, S., Lider, O., and Schwartz, M. (2004) A disaccharide derived from chondroitin sulphate proteoglycan promotes central nervous system repair in rats and mice. *Eur.J Neurosci.,* **20**, 1973– 1983.

Ronca, G. and Conte, A. (1993) Metabolic fate of partially depolymerized shark chondroitin sulphate in man. *Int. J. Clin. Pharm. Res.,* **13**, 27–34.

Rondle, C. J. M. and Morgan, W. T. J. (1955) The determination of glucosamine and galactosamine. *Biochem J.*, **61**, 586–589.

Rooney, O. M., Troke, J., Nicholson, J. K., and Griffin, J. L. (2003) High-resolution diffusion and relaxation-edited magic angle spinning ^{1}H NMR spectroscopy of intact liver tissue. *Magnetic Resonance in Medicine.,* **50**, 925 -930.

Roper, C. F. E., Sweeny, M. J., and Naven, C. E. (1984) FAO *Species catalogue.* Cephalopods of the world. An annotated and illustrated catalogue of species of interest to fisheries. *FAO Fish. Synop*., **125,** pp**.** 3-277.

Ruiz-Calero, V., Moyano, E., Puignou, L., and Galceran, M.T. (2001) Pressure-assisted capillary electrophoresis-electrospray ion trap mass spectrometry for the analysis of heparin depolymerised disaccharides. *J. Chromatogr. A.,* **914**, 277-291.

Ruiz-Calero, V., Saurina, J., Hernandez-Cassou, S., Galceran, M. T., and Puignou, L. (2002) Proton nuclear magnetic resonance characterisation of glycosaminoglycans using chemometric techniques. *Analyst.,* **127,** 407– 415.

Saari, H., Konttinen, Y.T., Friman, C., and Sorsa, T. (1993) Differential effects of reactive oxygen species on native synovial fluid and purified human umbilical cord hyaluronate. *Inflammation..,* **17**, 403-415.

Sacchi, R., Mannina, L., Fiordiponti, P., Barone, P., Paolillo, L., Patumi, M., and Segre, A. (1998) Characterization of Italian extra virgin olive oils using ^{1}H-NMR spectroscopy. *J. Agric. Food Chem.*, **46**, 3947–3951.

Sadowska, M. and Kolodziejska, I. (2005) Optimisation of conditions for precipitation of collagen from solution using k-carrageenan. Studies on collagen from skin of Baltic cod (*Gadus morhua*). *Food Chem*., **91**, 45-49.

Sadowska, M. and Sikorski, Z. E. (1987) Collagen in tissues of squid *Illex argentius* and *Loligo patagonica* –contents and solubility. *J. Food Biochem.,* **11**, 109 – 120.

Sadowska, M., Kolodziejska, I., and Niecikowska, C. (2003) Isolation of collagen from the skins of Baltic cod (*Gadus morhua*). *Food Chem*., **81**, 257-262.

Sakai, S., Kim, W.S., Lee, I.S., Kim, Y.S., Nakamura, A., Toida, T., and Imanari, T (2003) Purification and characterization of dermatan sulfate from the skin of the eel, *Anguilla japonica*. *Carbohydrate Research.,* **338**, 263-269.

Sakko, A.J., Butler, M.S., Byers, S., Reinboth, B.J., Stahl, J., Kench, J.G., Horvath, L.G., Sutherland, R.L., Stricker, P.D., Henshall, S.M., Marshall, V.R., Tilley, W.D., Horsfall., D.J., and Ricciardelli, C. (2008) Immunohistochemical level of unsulfated Chondroitin disaccharides in the cancer stroma is an independent predictor of prostate cancer relapse. *Cancer Epidemiol. Biomark. Prev.,* **17**, 2488–2497.

Saleuddin, A. S. M. and Chan, W. (1969) Shell regeneration in *Helix*: shell matrix composition and crystal formation. *Can. J. Zool*. 47, p1107.

Sankar, S., Sekar, S., Mohan, R., Rani, S., Sundaraseelan, J. and Sastry, T. P. (2008) Preparation and partial characterization of collagen sheet from fish (*Lates calcarifer*) scales. *International Journal of Biological Macromolecules.*, **42**, 6-9.

Santos, A.M.I., Velasco-Martín, A., Hernández-Velasco, E., Martín-Gil, J., and Martín-Gil F.J. (1994) Thermal behaviour of aqueous solutions of sodium hyaluronate from different commercial sources. *Thermochim. Acta.,* **242**, 153–160.

Santos, E. A., Rocha, L. R.M., Pereira, N.M.L., Andrade, G. P.V., Nader, H. B., and Dietrich, C. P. (2002) Mast cells are present in epithelial layers of different tissues of the mollusk *Anomalocardia brasiliana*. *In situ* characterization of heparin and a correlation of heparin and histamine concentration. *The Histochemical Journal.,* **34**, 553– 558.

Santos, J. A., Mulloy, B., and Mourao, P. A. S. (1992) Structural diversity among sulfated α – L – galactans from ascidians (tunicates). Studies on the species *Ciona intestinalis* and *Herdmania monus. Eur. J. Biochem.,* **204**, 669 – 677.

Saravanan, R. and Shanmugam, A. (2010) Isolation and characterisation of low molecular weight glycosaminoglycans from marine mollusc *Amussium pleuronectus* (Linne) using chromatography. *Appl. Biochem.Biotechnol.,* **160**, 791 – 799.

Sasaki, K., Kurata-Miura, K., Ujita, M., Angata, K., Nakagawa, S., Sekine, S., Nishi, T., and Fukuda, M. (1997) Expression cloning of cDNA encoding a human beta-1,3-N- acetylglucosaminyltransferase that is essential for poly-N-acetyllactosamine synthesis. *Proc. Natl. Acad. Sci.*, **94**, 14294-14299.

Sauerland, K., Plaas, A.H., Raiss, R.X., and Steinmeyer, J. (2003) The sulfation pattern of chondroitin sulfate from articular cartilage explants in response to mechanical loading. *Biochim. Biophys. Acta.,* **1638**, 241–248.

Schiraldi, C., Cimini, D., and Rosa, M. D. (2010) Production of chondroitin sulfate and chondroitin. *Appl. Microbiol. Biotechnol.,* **87**, 1209–1220.

Selleck, S. B. (2000) Proteoglycans and pattern formation: sugar biochemistry meets developmental genetics. *Trends Genet.,* **16**, 206 -212.

Sen, J., Goltz, J.S., Stevens, L., and Stein, D. (1998) Spatially restricted expression of pipe in the Drosophila egg chamber defines embryonic dorsal-ventral polarity. *Cell*., **95**, 471-481.

Senaratne, L. S., Park, P. J., and Kim, S. K (2006) Isolation and characterization of collagen from brown backed toadfish (*Lagocephalus gloveri*) skin. *Biores. Technol*., **97**, 191-197.

Seno, N. and Murakami, K. (1982) Structure of disulfated disaccharides from chondroitin polysulfates, chondroitin sulfate D and K. *Carbohydrate Res*., **103**, 190 -194.

Seno, N., Akiyama, F., and Anno, K. (1972) A novel dermatan polysulfate from hagfish skin, containing trisulfated disaccharide residues. *Biochim. Biophy. Acta.,* **264**, 229 – 233.

Seyfried, N.T., Blundell, C.D., Day, A.J., and Almond, A. (2005) Preparation and application of biologically active fluorescent hyaluronan oligosaccharides. *Glycobiology*., **15**, 303-312.

Shang, S. F. and Wang, H. (1996) Sensitive determination of aminoacids in kelp by reverse phase high performance liquid chromatography with precolumn derivatization using phenylisothiocyanate. *Chromatographia.,* **43**, 309 -312.

Shen, X. R., Kurihara, H., and Takahashi, K. (2007) Characterization of molecular species of collagen in scallop mantle. *Food Chem.,* **102**, 1187 -1191.

Sher, I., Zisman-Rozen, S., Eliahu, L., Whitelock, J.M., Maas-Szabowski, N., Yamada, Y., Breitkreutz, D., Fusenig, N.E., Arikawa-Hirasawa, E., Iozzo, R.V., Bergman, R., and Ron, D. (2006) Targeting perlecan in human keratinocytes reveals novel roles for perlecan in epidermal formation. *J. Biol. Chem.,* **281**, 5178–5187.

Shetty, A.K., Kobayashi, T., Mizumoto, S., Narumi, M., Kudo, Y., Yamada, S., and Sugahara, K. (2009) Isolation and characterization of a novel chondroitin sulfate from squid liver integument rich in N- acetylgalactosamine(4,6-disulfate) and glucuronate(3-sulfate) residues. *Carbohydrate Research.,* **344**, 1526–1532

Sheu, M. T., Huang, J. C., Yeh, G. C., and Ho, H. O. (2001) Characterization of collagen gel solutions and collagen matrices for cell culture. *Biomaterials.,* **22**, 1713-1719.

Shu, X,Z., Ghosh, K., Liu, Y., Palumbo, F.S., Luo, Y., Clark, R.A.F., and Prestwich, G.D. (2004a) Attachment and spreading of fibroblast on an RGD peptide-modified injectable hyaluronan hydrogel. *J. Biomed. Materials Res.*, **68**, 365-75.

Shu, X.Z., Liu, Y., Palumbo, F.S., Luo, Y., and Prestwich, G.D. (2004b) *In situ* crosslinkable hyaluronan hydrogels for tissue engineering. *Biomaterials*., **25**, 1339-1348.

Sikder, S.K. and Das, A. (1979) Isolation and characterization of glycosaminoglycans (mucopolysaccharides) from the skin of the fish *Labeo rohita. Carbohydr. Res.,* **71**, 273 - 285.

Sikder, S.K. and Das, A. (1980) Investigation on fish *Mastacembalus armatus* skin: Part II – Isolation and characterization of glycosaminoglycans (mucopolysaccharides). *Indian Journal of Chemistry.,* **19B**, 423 – 424.

Silas, E. G., Prabhkaran N.K., Meiyappan, M.M., Satyanarayana, R.K., Sarvesan, R., and Sreenivasan, P.V. (1985) Cephalopod bionomics, fisheries and resources of the Exclusive Economic Zone of India –Utilization and export of cephalopods. *Bull. Centr. Mar. Fish. Res. Inst.,* **37**, 165 – 195.

Silbert, J.E. and Sugumaran, G. (2002) Biosynthesis of chondroitin/dermatan sulfate. *IUBMB Life.,* **54**, 177–186

Sim, J., Jun, G., Toida, T., Cho, S.Y., Choi, D.W., Chang, S., Linhardt, R.J. and Kim, Y.S. (2005) Quantitative analysis of chondroitin sulfate in raw materials, ophthalmic solutions, soft capsules and liquid preparations. *J. Chromatogr. B.,* **818**, 133–139.

Simone, C.B., Simone, N.L., Simone, C.B. (1998) Shark cartilage for cancer. *Lancet.,* **351**, p1440.

Souza, A. R. C., Kozlowski, E. O., Cerqueira, V.R., Castelo-Branco, M. T. L., Costa, M. L., and Pavao, M. S. G. (2007) Chondroitin sulfate and keratan sulfate are the major glycosaminoglycans present in the adult zebrafish *Danio rerio* (Chordata-Cyprinidae). *Glycoconj J*., **24**, 521–530.

Spicer, A.P., Tien, J.L., Joo, A., and Bowling, R.A. (2002) Investigation of hyaluronan function in the mouse through targeted mutagenesis. *Glycoconj. J.,* **19,** 341–345

Spring, J., Paine-Saunders, S.E., Hynes, R.O., and Bernfield, M. (1994) Drosophila syndecan: conservation of a cell-surface heparan sulfate proteoglycan. *Proc. Natl Acad. Sci.,* **91**, 3334-3338.

Srinivasan, S. R., Radhakrishnamurthy, B., Dalfers, E. R., and Berenson, G. S. (1969) Glycosaminoglycans from squid skin. *Comparative Biochemistry and Physiology.,* **28**, 169 – 172.

Stamov, D., Grimmer, M., Salchert, K., Pompe, T. and Werner, C. (2008) Heparin intercalation into reconstituted collagen I fibrils: Impact on growth kinetics and morphology. *Biomaterials*., **29**, 1-14.

Stern, R. (2004) Hyaluronan catabolism: a new metabolic pathway. *Eur. J. Cell Biol*., **83**, 317–325.

Stern, R. (2007) Complicated hyaluronan patterns in skin: Enlightenment by UVB? *J.Investigative Derm.,* **127**, 512–513.

Stern, R., Asari, A.A. and Sugahara, K.N. (2006) Hyaluronan fragments: An information-rich system *Eur. J. Cell Biol.,* **85,** 699 -715.

Stringer, S.E., Mayer-Proschel, M., Kalyani, A., Rao, M., and Gallagher, J.T. (1999) Heparin is a unique marker of progenitors in the glial cell lineage. *J. Biol. Chem.*, **274**, 25455-25460.

Sugahara, K. and Kitagawa, H. (2002) Heparin and heparan sulfate biosynthesis. *IUBMB Life.,* **54**, 163–175

Sugahara, K. and Mikami, T. (2007) Chondroitin/dermatan sulfate in the central nervous system. *Curr. Opin. Struct. Biol.,* **17**, 536–545.

Sugahara, K., Masuda, M., Harada, T., Yamashina, I., DeWaard, P., and Vligenthart, J.F.G (1991) Structural studies on sulphated oligosaccharides derived from the carbohydrate – protein linkage region of chondroitin sulphate proteoglycans of whale cartilage. *Eur. J. Biochem.,* **202**, 805 -811.

Sugahara, K., Nadanaka, S., Takeda, K., and Kojima, T. (1996a) Structural analysis of unsaturated hexasaccharides isolated from shark cartilage chondroitin sulfate D that are substrates for the exolytic action of chondroitin ABC lyase. *Eur. J. Biochem.,* **239**, 871-880.

Sugahara, K., Ohi, Y., Harada, T., de Waard, P., and Vliegenthart, J. F. G. (1992b) Structural studies on sulfated oligosaccharides derived from the carbohydrate-protein linkage region of chondroitin6–sulfate proteoglycans of shark cartilage: Six compounds containing 0 or 1 sulfate and/or phosphate residue *J. Biol. Chem.,* **267**, 6027 -6035.

Sugahara, K., Tanaka, Y. and Yamada, S. (1996c) Preparation of a series of sulfated tetrasaccharides from shark cartilage chondroitin sulphate D using testicular hyaluronidase and structural determination by 500MHz ^{1}H NMR spectroscopy. *Glyconj.J.,* **13**, 609 -612.

Sugahara, K., Tanaka, Y., Yamada, S., Seno, N., and Kitagawa, K. (1996b) Novel sulfated oligosaccharides containing 3-*O*-sulfated glucuronic acid from king crab chondroitin sulphate K. *J. Biol. Chem.,* **271**, 26745-26754.

Sugahara, K., Tsuda, H., Yoshida, K., Yamada, S., deBeer, T., and Vliegenthart, J.F.G. (1995) Structure determination of the octa- and decasaccharide sequences isolated from the carbohydrate-protein linkage region of porcine intestinal heparin. *J. Biol. Chem*., **270**, 22914 -22923.

Sugahara, K., Yamada, S., Yoshida, K., deWaard, P., and Vliegenthart, J.F.G. (1992a) A novel sulfated structure in the carbohydrate-protein linkage region isolated from porcine intestinal heparin. *J. Biol. Chem.,* **267**, 1528 -1533.

Sugahara, K.N., Murai, T., Nishinakamura, H., Kawashima, H., Saya, H., and Miyasaka, M. (2003) Hyaluronan oligosaccharides induce CD44 cleavage and promote cell migration in CD44- expressing tumor cells. *J. Biol. Chem.,* **278**, 32259–32265.

Summers, A.P., Koob-Emunds, M.M., Kajiura, S.M., and Koob, T.J. (2003) A novel fibrocartilaginous tendon from an elasmobranch fish (*Rhinoptera bonasus*). *Cell Tissue Res.,* **312**, 221–227.

Suzuki, M. (1939) Prosthetic group of cornea mucoid. *J. Biochem.,* **311**, 185-191.

Swatschek, D., Schatton, W., Kellermann, J., Muller, W.E.G., and Kreuter, J. (2002) Marine sponge collagen: isolation, characterization and effects on the skin parameters surface-pH, moisture, and sebum. *Eur. J. Pharm. Biopharm.,* **53**, 107-113.

Sylvie, L., Eric, C., Anne, W., Herve, M., Christine, R., Jeanne-Marie, B., Jean-Louis, R., and Jacques, C. (2003) Optimal anticoagulation strategy in haemodialysis with heparin-coated polyacrylonitrile membrane. *Nephrol. Dial. Transplant.,* **18**, 2097–2104.

Tai, G.H., Huckerby, T.N., and Nieduszynski, I.A. (1996) Multiple non-reducing chain termini isolated from bovine corneal keratan sulfates. *J. Biol. Chem.,* **271**, 23535–23546.

Tammi, R., Ripellino, J.A., Margolis, R.U., Maibach, H. I., and Tammi, M. (1989) Hyaluronate accumulation in human epidermis treated with retinoic acid in skin organ culture. *Journal of Investigative Dermatology.*, **92**, 326-332.

Taylor, M., Simkiss, K. and Greaves, G. N. (1986) Extended X-ray absorption fine structure studies of calcification. *Biochem. Soc. Trans*., **14**, 549-552.

Termeer, C.C., Hennies, J., Voith, U., Ahrens, T., Weiss, J.M., Prehm, P., and Simon, J.C. (2000) Oligosaccharides of hyaluronan are potent activators of dendritic cells. *J. Immunol.,* **165**, 1863–1870.

Thanonkaew, A., Benjakul, S., and Visessanguan, W. (2006) Chemical composition and thermal property of cuttlefish (*Sepia pharaonis*) muscle. *Journal of Food Composition and Analysis.,* **19**, 127–133.

Theocharis, A. D., Karamanos, N. K., and Tsegenidis, T. (1999) Isolation and analysis of a novel acidic polysaccharide from the case of squid pen. *International Journal of Biological Macromolecules.*, **26**, 83 -88.

Thirugnanasambandan, S. and Pandian, V. (2007) Histological and analytical evaluation of glycosaminoglycan from the clam *Katelysia opima. Trends in Medical Research.,* **2**, 167 -175.

Thomas, N., Goodacre, R., Timmins, E.M., Gaudoin, M., and Fleming, R. (2000) Fourier transform infrared spectroscopy of follicular fluids from large and small antral follicles. *Hum. Reprod.,* **15**, 1667–1671.

*Thomsen, J. U. and Meyer, B. (1989) Pattern recognition of the NMR spectra of sugar alditols using a neural network. *J. Magn. Reson.*, **84**, 212-217.

Toida, T. and Lindhardt, R. J. (1996) Detection of glycosaminoglycans as a copper (II) complex in capillary electrophoresis. *Electrophoresis.,* **17**, 341-346.

Toida, T., Huang, Y., Washio, Y., Maruyama, T., Toyoda, H., Imanari, T., and Lindhardt, R.J. (1997a) Chemical microdetermination of heparin in plasma. *Anal. Biochem.*, **251**, 219 -226.

Toida, T., Maruyama, T., Ogta, Y., Suzuki, A., Toyoda, H., Imanari, T., and Linhardt, R. J. (1999) Preparation and anticoagulant activity of fully O- sulphonated glycosaminoglycans. *Int. J. Biol. Macromol.,* **26**, 233 – 241.

Toida, T., Toyoda, H., and Imanari, T. (1993) High-resolution proton nuclear magnetic resonance studies on chondroitin sulfates. *Anal. Sci.*, **9**, 53 -58.

Toida, T., Yoshida, H., Toyoda, H., Koshiishi, T., Imanari, T., Hileman, R.E., Fromm, J.R., and Linhardt, R.J. (1997b) Structural differences and the presence of unsubstituted amino groups in heparin sulfates from different tissues and species. *Biochem. J*., **322**, 499–506.

Tomas, J., Geffen, A. J., Allen, I. S., and Berges, J. (2004) Analysis of the soluble matrix of vaterite otoliths of juvenile herring (*Clupea harengus*): Do crystalline otoliths have less protein. *Comp. Biochem. Physiol.,* **139**, 301-308.

Toole, B.P. (1990) Hyaluronan and its binding proteins, the hyaladherins. *Curr. Opin. Cell Biol.,* **2**, 839–844.

Toole, B.P. (2004) Hyaluronan: from extracellular glue to pericellular cue. *Nat. Rev. Cancer.,* **4**, 528–539.

Toyoda, H., Kinoshita-Toyoda, A., and Selleck, S.B. (2000a) Structural analysis of glycosaminoglycans in drosophila and *Caenorhabditis elegans* and demonstration that tout-velu, a drosophila gene related to EXT tumor suppressors, affects heparan sulfate *in vivo. J. Biol. Chem.,* **275**, 2269-2275.

Toyoda, H., Kinoshita-Toyoda, A., and Selleck, S.B. (2000b) Structural analysis of glycosaminoglycans in animals bearing mutations in sugarless, sulfateless, and tout-velu. Drosophila homologues of vertebrate genes encoding glycosaminoglycan biosynthetic enzymes. *J. Biol. Chem.,* **275**, 21856-21861.

Toyoki, Y., Yoshihara, S., Sasaki, M., and Konn, M. (1997) Characterization of glycosaminoglycans in regenerating canine liver. *J. Hepatol*., **26**, 1135–1140.

Trowbridge, J.M. and Gallo, R.L. (2002) Dermatan sulfate: new functions from an old glycosaminoglycan. *Glycobiology.,* **12**, 117R–125R.

Tsegenidis, T. (1992) Influence of oversulphation and neutral sugar presence on the chondroitanase AC and ABC actions towards glycosaminoglycans from ray (*Raja clavata*) and squid (*Illex illecebrosus coidentii*) skin. *Comparative Biochemistry and Physiology B - Comparative Biochemistry.*, **103**, 275 – 279.

Turnbull, J., Powell, A., and Guimond, S. (2001) Heperan sulfate: decoding a dynamic multifunctional cell regulator. *Trends Cell Biol.,* **11,** 75 -82.

Uchimura, K., Muramatsu, H., Kadomatsu, K., Fan, Q.W., Kurosawa, N., Mitsuoka, C., Kannagi, R., Habuchi, O., and Muramatsu, T. (1998) Molecular cloning and characterization of an N-acetylglucosamine-6-O-sulfotransferase. *J. Biol. Chem.,* **273**, 22577-22583.

Uchisawa, H., Okuzaki, B., Ichita, J., and Matsue, H. (2001) Binding between calcium ions and chondroitin sulfate chains of salmon nasal cartilage glycosaminoglycan. *International Congress Series*., **1223**, 205-220.

Uchiyama, H., Kikuchi, K., Ogamo, A., and Nagasawa, K. (1987) Determination of the distribution of constituent disaccharide units within the chain near the linkage region of shark-cartilage chondroitin sulfate C. *Biochimica et Biophysica Acta.,* **926**, 239-248.

Uebelhart, D., Thonar, E.J.M.A., Delmas, P.D., Chantraine, A., and Vignon, E. (1998) Effects of oral chondroitin sulfate on the progression of knee osteoarthritis: a pilot study. *Osteoarth. Cart.,* **6**, 39-46.

Ueoka, C., Kaneda, N., Okazaki, I., Nadanaka, S., Muramatsu, T., and Sugahara, K. (2000) Neuronal cell adhesion, mediated by the heparin-binding neuroregulatory factor midkine, is specifically inhibited by chondroitin sulfate E: Structural and functional implications of the over-sulfated chondroitin sulfate. *J. Biol. Chem.* **275,** 37407-37413.

Ulrich, P.N. and Boon, J.K, (2001) The histological localization of heparin in the northern quahog clam, *Mercenaria mercenaria*. *J. Invert. Pathol.,* **78**, 155–159.

Vieira, R. P., Mulloy, B., and Mourao, P. A. S. (1991) Structure of a fucose- branched chondroitin sulfate from sea cucumber. Evidence for the presence of 3-O-sulfo-beta-D-glucuronosyl residues. *J. Biol. Chem.,* **266**, 13530 -13536.

Vilela – Silva, A. E. S., Werneck, C.C., Valente, A. P., Vacquier, V. D., and Mourao, P.A. S. (2001) Embryos of the sea urchin *Strongylocenttrotus purpuratus* synthesize a dermatan sulphate enriched in 4 – O – and 6 – O- disulphated galactosamine units. *Glycobiology*., **11**, 433 - 440.

Vliegenthart, J.F.G., Dorland, L., and Van Halbeek, H. (1983) High-Resolution, ^{1}H-nuclear magnetic resonance spectroscopy as a tool in the structural analysis of carbohydrates related to glycoproteins. *Adv. Carbohydr. Chem. Biochem.* **41**, 209-374.

Vlierberghe, S.V., Dubruel, P., Lippens, E., Masschaele, B., VanHoorebeke, L., Cornelissen, M., Unger, R., Kirkpatrick, C. J., and Schacht, E. (2008) Toward modulating the architecture of hydrogel scaffolds: curtains versus channels. *J. Mater. Sci: Mater. Med.,* **19**, 1459–1466.

Volpi, N. (1999) Adsorption of glycosamioglycans onto coral – a new possible implant biomaterials for regeneration therapy. *Biomaterials.,* **20**, 1359 -1363.

Volpi, N. (2002) Oral bioavailability of chondroitin sulfate (Condrosulf) and its constituents in healthy male volunteers. *Osteoarthritis Cartilage.,* **10**, 768–777.

Volpi, N. and Maccari, F. (2002) Detection of submicrogram quantities of glycosaminoglycans on agarose gels by sequential staining with toluidine blue and stains-all. *Electrophoresis.,* **23**, 4060-4066.

Volpi, N. and Mucci, A. (1998) Characterization of a low-sulfated chondroitin sulfate from the body of *Viviparus ater* (mollusca gastropoda). Modification of its structure by lead pollution. *Glycoconjugate Journal.,* **15**, 1071–1078.

Vynios, D. H. and Tsiganos, C. P. (1990) Squid proteoglycans: Isolation and characterization of three populations from cranial cartilage. *Biochimica Biophysica Acta.,* **1033**, 139 -147.

Wada, K. (1980) Initiation of mineralization in bivalve molluscs. In: Omori, M., Watanabe, N. (Eds.), *The Mechanism of Biomineralization in Animals and Plants*. Tokai University Press, Tokyo, pp. 79–92.

Wang, L., An, X., Xin, Z., Zaho, L., and Hu, Q. (2007) Isolation and characterization of collagen from the skin of deep-sea redfish (*Sebastes mentella*). *J. Food Sci.,* **72**, E450-E455.

Wang, L., Fuster, M., Sriramarao, P., and Esko, J.D. (2005) Endothelial heparan sulfate deficiency impairs L-selectin- and chemokine-mediated neutrophil trafficking during inflammatory responses. *Nat. Immunol.,* **6**, 902–910.

Wang, T.W., Sun, J.S., Wu, H.C., Tsuang. Y.H., Wang. W., and Lin, F.H. (2006) The effect of gelatin–chondroitin sulfate–hyaluronic acid skin substitute on wound healing in SCID mice. *Biomaterials.,* **27**, 5689– 5697.

Warda, M., Gouda, E.M., Toida, T., Chi, L., and Linhardt, R.J. (2003b) Isolation and characterization of raw heparin from dromedary camel intestine: evaluation of a new source of pharmaceutical heparin. *Comp. Biochem. Physiol. C Toxicol. Pharmacol*., **136**, 357–365.

Warda, M., Mao, W., Toida, T., and Linhardt, R.J. (2003a) Turkey intestine as a commercial source of heparin? Comparative structural studies of intestinal avian and mammalian glycosaminoglycans. *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.,* **134**, 189–197.

Wassel, D.T.H. and Embery, G. (1997) Adsorption of chondroitin-4-sulphate and heparin onto hydroxyapatite - Effect of bovine serum albumin. *Biomaterials.,* **18**, 1001-1007.

Wasserman, L., Ber, A., and Allalouf, D. (1972) Acidic glycosaminoglycan composition of the gills of *Cyprinus carpio*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology.,* **42**, 669-677.

Weiler, J.M., Edens, R.E., Linhardt, R.J. and Kapelanski, D.P. (1992) Heparin and modified heparin inhibit complement activation *in vivo*. *J. Immunol.,* **148**, 3210–3215.

Weissman, B. and Meyer, K. (1954) The structure of hyalobiuronic acid and of hyaluronic acid from umbilical cord. *J. Am. Chem. Soc.,* **76**, 1753–1757.

West, L.A., Roughley, P., Nelson, F.R., and Plaas, A.H. (1999) Sulphation heterogeneity in the trisaccharide (GalNAcSbeta1, 4GlcAbeta1, 3GalNAcS) isolated from the non-reducing terminal of human aggrecan chondroitin sulphate. *Biochem. J.,* **342**, 223–229.

Westergren-Thorsson, G., Onnervik, P.O., Fransson, L.A. and Malmstromr, A. (1991) Proliferation of cultured fibroblasts is inhibited by L-iduronate containing glycosaminoglycans. *J. Cell Physiol.,* **147**, 523-530.

Wexler, B. C. and Thomas, L. L. (1967) Growth of aortic explants and arteriosclerosis. *Nature*., **214**, 243-245.

Winder, C. L. and Goodacre, R. (2004) Comparision of diffuse reflectance absorbance and attenuated total reflectance FT-IR for the discrimination of bacteria. *Analyst*., **129**, 1118–1122.

Winters, C., Davies, R.L.I., Morgan, A. J., and Gravelle, I. H. (1986) Human breast microcalcification: Comparative study involving contact microradiography and backscattered-electron-imaging plus x-ray microanalysis. *Micron. Microsc. Acta*., **17**, 11-23.

Woessner, J. F. (1961) The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Arch. Biochim. Biophys.,* **93**, 440 – 447.

Wusteman, F. S., Gold, C., and Wagner, J. C. (1972) Glycosaminoglycans and calcification in the lesions of progressive massive fibrosis and in pleural plaques. *Am.Rev. Respir. Dis.* **106**, 116-118.

Xiao, S., Zheng, X. D., Wang, Z. P., and Wang, R. C. (2005) A study of the organic matrix of cuttlebone: molecular weights, characterized infrared spectrum and aminoacid composition. *Phuket. Mar. Bio.Cent. Res. Bull.,* **66**, 235 – 241.

Xiaodong, Z., Shu, X., Zhaoping, W., and Rucai, W (2007) Histological and histochemical analyses of the cuttlebone sac of the golden cuttlefish *Sepia esculenta*. *Journal of Ocean University of China.,* **6**, 393-397.

Xie, A., Shen, Y., Zhang, C., Yuan, Z., Zhu, X., and Yang, Y. (2005) Crystal growth of calcium carbonate with various morphologies in different amino acid systems. *Journal of Crystal Growth.,* **285,** 436 - 443.

Xu, C. Y., Inai, R., Kotaki, M., and Ramakrishna, S. (2004) Electrospun nanofiber fabrication as synthetic extracellular matrix and its potential for vascular tissue engineering. *Tissue Eng.*, **10**, 1160-1168.

Xu, C.X., Jin, H., Chung, Y.S., Shin, J.Y., Woo, M.A., Lee, K.H., Palmos, G.N., Choi, B.D. and Cho, M.H. (2008) Chondroitin sulfate extracted from the *Styela clava* tunic suppresses TNF-alpha-induced expression of inflammatory factors, VCAM-1 and iNOS by blocking Akt/NF-kappaB signal in JB6 cells. *Cancer Lett.,* **264**, 93-100.

Yamada, S. and Sugahara, K. (2008) Potential therapeutic application of chondroitin sulfate/dermatan sulphate. *Curr. Drug Discov. Technol.,* **5**, 289–301.

Yamada, S., Morimoto, H., Fujisawa, T., and Sugahara, K. (2007) Glycosaminoglycans in *Hydra magnipapillata* (Hydrozoa, Cnidaria): Demonstration of chondroitin in the developing nematocyst, the sting organelle, and structural characterization of glycosaminoglycans. *Glycobiology.,* **17**, 886–894.

Yamada, S., Onishi, M., Fujinawa, R., Tadokoro, Y., Okabayashi, K., Asashima, M., and Sugahara, K. (2009) Structural and functional changes of sulfated glycosaminoglycans in *Xenopus laevis* during embryogenesis. *Glycobiology.,* **19**, 488–498.

Yamada, S., VanDie, I., VanDen, E, D. H., Yokota, A., Kitagawa, H., and Sugahara, K. (1999) Demonstration if glycosaminoglycans in *Caenorhabditis elegans. FEBS Lett.,* **459**, 327–331.

Yamada, S., Yoshida, K., Sugiura, M., and Sugahara, K. (1992) One- and two-dimensional ^{1}H-NMR characterization of two series of sulfated disaccharides prepared from chondroitin sulfate and heparan sulfate/heparin by bacterial eliminase digestion. *J. Biochem*., **112**, 440-447.

Yamaguchi, Y. (1996) Brevican - a major proteoglycan in adult brain. *Perspect. Dev. Neurobiol.,* **3**, 307–317.

Yamauchi, K. (2002) *Bovine Spongiform Encephalopathy and People*. Iwanami Press, Tokyo, Japan.

Yan, M., Li, B., Zhao, X., Ren, G., Zhuang, Y., Hou, H., Zhang, X., Chen, L., and Fan, Y. (2008) Characterization of acid- soluable collagen from the skin of walleye pollock (*Theragra chalcogramma*). *Food Chem.,* **107**, 1581 – 1586.

Yates, E. A., Santini, F., Guerrini, M., Naggi, A., Torri, G., and Casu, B. (1996) ^{1}H and ^{13}C NMR spectral assignments of the major sequences of twelve systematically modified heparin derivatives. *Carbohydr. Res.,* **294**, 15-27.

Yoshihara, S., Kon, A., Kudo, D., Nakazawa, H., Kakizaki, I., Sasaki, M., Endo, M. and Takagaki, K. (2005) A hyaluronan synthase suppressor, 4-methylumbelliferone, inhibits liver metastasis of melanoma cells. *FEBS Lett*., **579**, 2722–2726.

Yu, J., Lei, M., Cheng, B., and Zhao, X. (2004) Facile preparation of calcium carbonate particles with unusual morphologies by precipitation reaction. *J. Cryst. Growth.*, **261,** 566-570.

Yunoki, S., Nagai, N., Suzuki, T., and Munekata, M. (2004) Novel biomaterial from reinforced salmon collagen gel prepared by fibril formation and cross-linking. *J. Biosci. Bioeng*. **98**, 40-47.

Zelechowska, E., Sadowska, M., and Turk, M. (2010) Isolation and some properties of collagen from the backbone of Baltic cod (*Gadus morhua*). *Food Hydrocolloids.,* **24**,325-329.

Zeugolis, D.I., Khew, S.T., Yew, E.S. Y., Ekaputra, A.K., Tong, Y. L.L., Hutmacher, D.W., Sheppard, C. and Raghunath, M. (2008) Electro-spinning of pure collagen nano-fibres – Just an expensive way to make gelatin? *Biomaterials*., **29**, 2293-2305.

Zhang, H., Uchimura, K. and Kadomatsu, K. (2006) Brain keratan sulfate and glial scar formation. *Ann. N. Y. Acad. Sci.*, **1086**, 81–90.

Zhang, W. and Moskowitz, R.W. (2007) OARSI recommendations for the management of hip and knee osteoarthritis, Part I: Critical appraisal of existing treatment guidelines and systematic review of current research evidence activity. *Osteoarthritis and Cartilage.,* **15**, 981- 999.

Zhang, Y., Liu, W. T., Li, G. Y., Shi, B., Miao, Y. Q. and Wu, X. H. (2007) Isolation and partial characterization of pepsin- soluble collagen from the skin of grass carp (*Ctenopharyngodon idella*). *Food Chem*., **103,** 906-912.

Zhao, H.G., Ma, L., Gong, Y.H., Gao, C.Y. and Shen, J.C. (2009) A polylactide/ fibrin gel composite scaffold for cartilage tissue engineering: fabrication and an *in vitro* evaluation. *J. Mater. Sci: Mater. Med*., **20**, 135–143.

Zhao, J., Liao, D. and Gregersen, H. (2007) Biomechanical and histomorphometric esophageal remodeling in type 2 diabetic GK rats. *J. Diabetes Complicat.,* **21**, 34 - 40.

Zhong, S., Teo, W.E., Zhu, X., Beuerman, R., Ramakrishna, S. and Yung, L.Y.L (2005) Formation of collagen – glycosaminoglycan blended nanafibrous scaffolds and their biological properties. *Biomacromolecules.,* **6**, 2998 -3004.

Zhou, D., Dinter, A., Gallego, R .G., Kamerling, J. P., Vliegenthart,. F. G., Berger, E.G. and Hennet, T. (1999) A β-1, 3-N-acetylglucosaminyltransferase with poly-N-acetyllactosamine synthase activity is structurally related to β -1, 3-galactosyltransferases. *Proc. Natl. Acad. Sci*., **96**, 406-411.

Zhou, G.T. and Zheng, Y.F. (1998) Synthesis of aragonite-type calcium carbonate by overgrowth technique at atmospheric pressure. *Journal of Materials Science Letters.,* **17**, 905- 908.

Zou, X.H., Foong, W.C., Cao, T., Bay, B.H., Wand, O.H. and Yip, G.W. (2004) Chondroitin sulfate in palatal wound healing. *J. Dent. Res.,* **83**, 880–885.

* Not referred in original

Appendix 1

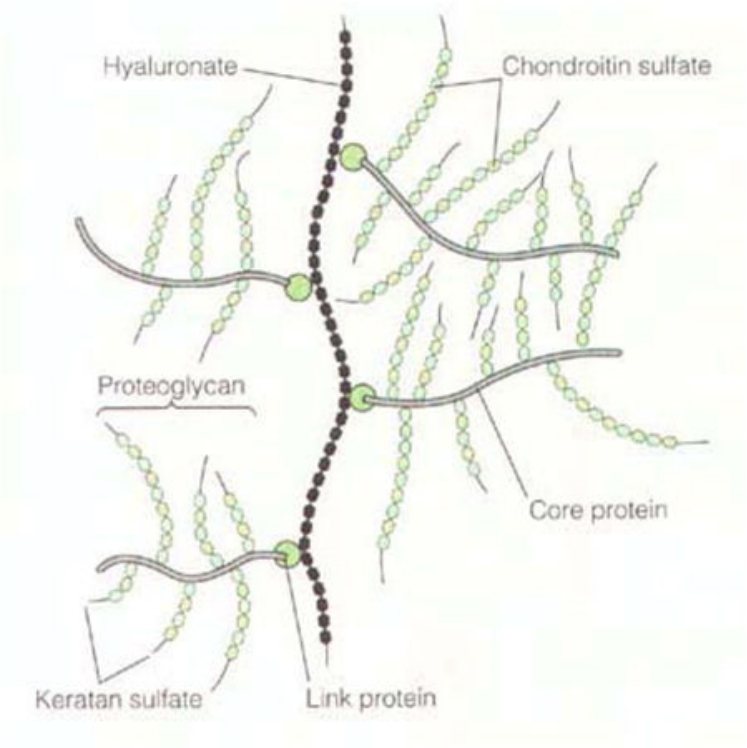


Figure 1.1 Schematic representation of a proteoglycan

[*Source*: www.pro.ovh.net]

Appendix 2

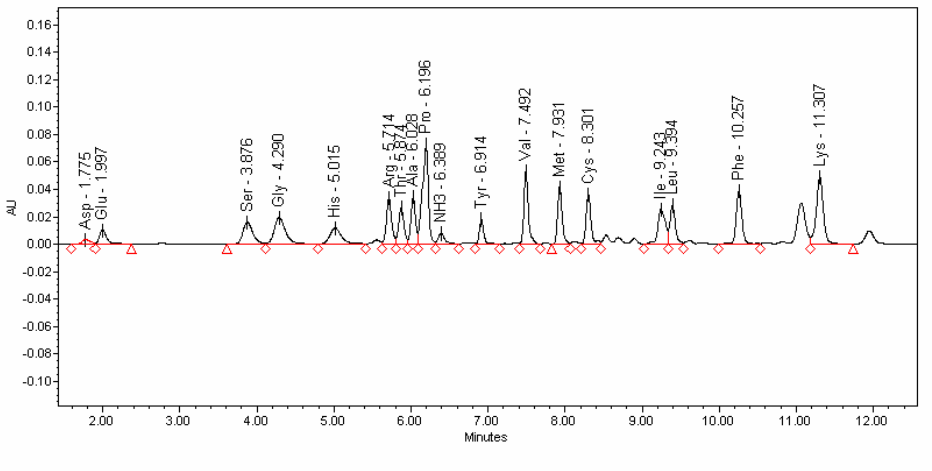


Fig 2.1: Amino acid profile of standard amino acids.

Table 2.1: ANOVA for proximate composition of squid tissues

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
moisture	Between Groups	13652.993	5	2730.599	1052.520	.000
	Within Groups	46.698	18	2.594		
	Total	13699.691	23			
protein	Between Groups	2551.939	5	510.388	641.310	.000
	Within Groups	14.325	18	.796		
	Total	2566.264	23			
fat	Between Groups	9.328	5	1.866	7.769	.000
	Within Groups	4.323	18	.240		
	Total	13.651	23			
ash	Between Groups	35.462	5	7.092	40.681	.000
	Within Groups	3.138	18	.174		
	Total	38.600	23			

2.1 (a): Homogenous subsets for moisture

MOISTURE

	TISSUE	N	Subset for alpha = .05		
			1	2	3
Tukey HSD[a]	Gladius	4	18.3375		
	Head cartilage	4		77.1000	
	Tentacle	4			81.8425
	Skin	4			83.0150
	Mantle	4			83.8275
	Fin	4			84.3125
	Sig.		1.000	1.000	.299

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

2.1 (b): Homogenous subsets for protein

PROTEIN

	TISSUE	N	Subset for alpha = .05		
			1	2	3
Tukey HSD[a]	Tentacle	4	13.0475		
	Skin	4	13.9200	13.9200	
	Mantle	4	14.2000	14.2000	
	Fin	4	14.2275	14.2275	
	Head cartilage	4		15.7650	
	Gladius	4			41.8175
	Sig.		.450	.082	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

2.1 (c): Homogenous subsets for fat

FAT

	TISSUE	N	Subset for alpha = .05		
			1	2	3
Tukey HSD[a]	Gladius	4	.0300		
	Mantle	4	.4650	.4650	
	Head cartilage	4	.8450	.8450	
	Fin	4	1.0700	1.0700	1.0700
	Skin	4		1.3000	1.3000
	Tentacle	4			2.0000
	Sig.		.071	.205	.127

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

2.1 (d): Homogenous subsets for ash

ash

	tissue	N	Subset for alpha = .05	
			1	2
Tukey HSD[a]	Fin	4	.3650	
	Mantle	4	.4600	
	Skin	4	.7500	
	Tentacle	4	.8500	
	Head cartilage	4	1.2900	
	Gladius	4		3.9050
	Sig.		.055	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

Table 2.2 ANOVA for proximate composition of cuttlefish tissues

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
moisture	Between Groups	10493.905	5	2098.781	419.450	.000
	Within Groups	90.066	18	5.004		
	Total	10583.970	23			
protein	Between Groups	111.624	5	22.325	12.848	.000
	Within Groups	31.278	18	1.738		
	Total	142.902	23			
fat	Between Groups	28.335	5	5.667	31.287	.000
	Within Groups	3.260	18	.181		
	Total	31.595	23			
ash	Between Groups	866.043	5	173.209	649.403	.000
	Within Groups	4.801	18	.267		
	Total	870.844	23			

2.2 (a): Homogenous subsets for moisture

MOISTURE

			Subset for alpha = .05		
	TISSUE	N	1	2	3
Tukey HSD[a]	CF Pen	4	23.4475		
	CF Head cartilage	4		75.6275	
	CF Skin	4		78.0300	78.0300
	CF Tentacle	4		79.1850	79.1850
	CF Mantle	4		80.3900	80.3900
	CF Fin	4			82.9575
	Sig.		1.000	.069	.057

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

2.2 (b): Homogenous subsets for protein

PROTEIN

			Subset for alpha = .05	
	TISSUE	N	1	2
Tukey HSD[a]	CF Tentacle	4	13.9650	
	CF Fin	4	14.5000	
	CF Mantle	4		17.7125
	CF Pen	4		18.2975
	CF Head cartilage	4		18.9225
	CF Skin	4		19.5925
	Sig.		.992	.371

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

2.2 (c): Homogenous subsets for fat

FAT

	TISSUE	N	Subset for alpha = .05		
			1	2	3
Tukey HSD[a]	CF Pen	4	.0200		
	CF Mantle	4	.1410		
	CF Fin	4	.3995		
	CF Head cartilage	4	.7613	.7613	
	CF Skin	4		1.4525	
	CF Tentacle	4			3.1825
	Sig.		.187	.245	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

2.2 (d): Homogenous subsets for ash

ASH

	TISSUE	N	Subset for alpha = .05			
			1	2	3	4
Tukey HSD[a]	CF Mantle	4	.5400			
	CF Fin	4	.8900	.8900		
	CF Skin	4	.9500	.9500		
	CF Tentacle	4		1.8225		
	CF Head cartilage	4			3.2000	
	CF Pen	4				17.4275
	Sig.		.866	.160	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

Table 2.3: Amino acid content of the edible and non-edible tissues of squid (*Loligo duvauceli)*.

*Name of amino acids	Edible Tissues (µ moles/g.)			Non edible Tissues (µ moles/g.)		
	Mantle	Tentacle	Fin	Gladius	Skin	HC
Aspartic acid	29.66	94.70	218.90	604.12	58.97	128.02
Glutamic acid	177.27	54.39	189.02	192.15	24.19	85.24
Serine	41.32	74.23	144.50	838.11	95.59	259.75
Glycine	166.61	31.13	8.09	244.93	5.14	64.88
Histidine	16.42	23.38	108.68	366.28	15.19	32.88
Arginine	216.52	64.04	68.09	802.46	46.53	105.67
Threonine	34.88	52.72	207.54	195.31	43.13	39.91
Alanine	86.41	50.27	26.49	138.08	13.49	30.64
Proline	137.17	15.45	3.58	171.11	1.83	4.39
Tyrosine	66.95	417.67	47.16	835.99	192.92	183.83
Valine	56.97	0.00	38.06	8.01	0.00	3.68
Methionine	17.10	109.04	8.04	51.85	10.64	13.38
Cysteine	1.52	0.00	2.01	145.45	0.00	4.92
Isoleucine	69.03	361.34	11.05	127.62	33.38	29.34
Leucine	87.72	126.51	65.56	132.29	6.17	21.73
Phenylalanine	33.70	253.29	38.95	82.28	0.67	15.03
Lysine	111.84	244.32	284.49	195.64	190.63	159.08

*Not corrected for degradation during acid hydrolysis
Tryptophan: Not analyzed

Table 2.4: Amino acid content of the edible and non-edible tissues of cuttlefish (*Sepia pharaonis*)

*Name of amino acids	Edible Tissues (µ moles/g.)			Non edible Tissues (µ moles/g.)		
	Mantle	Tentacle	Fin	Pen	Skin	HC
Aspartic acid	244.50	177.47	179.47	129.85	2917.15	109.07
Glutamic acid	289.58	150.86	127.47	47.39	2410.79	88.49
Serine	219.57	192.40	204.58	27.53	1436.76	350.65
Glycine	30.69	13.62	8.05	113.14	2505.93	3.61
Histidine	309.05	156.98	172.06	94.07	621.69	176.19
Arginine	49.75	25.00	24.28	99.25	916.46	19.09
Threonine	164.86	74.16	95.60	57.00	755.06	114.66
Alanine	424.35	201.27	235.77	188.15	1915.95	248.02
Proline	23.62	12.29	27.70	72.73	750.54	28.00
Tyrosine	291.85	78.92	80.44	277.49	1004.89	126.09
Valine	55.68	9.64	29.24	312.03	341.17	56.31
Methionine	204.79	42.20	54.97	356.41	637.92	36.39
Cysteine	55.30	7.13	21.33	128.14	249.19	20.82
Isoleucine	171.51	28.03	68.30	298.83	686.29	118.64
Leucine	249.97	48.74	90.12	456.68	1048.66	144.90
Phenylalanine	477.93	129.66	250.35	314.12	496.14	391.10
Lysine	146.20	28.38	145.56	98.23	630.62	109.91

*Not corrected for degradation during acid hydrolysis
Tryptophan: Not analyzed

Table 2.5: ANOVA for ADP yield of squid tissues

ANOVA

YIELD

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	14516.666	5	2903.333	1578.648	.000
Within Groups	33.104	18	1.839		
Total	14549.770	23			

2.5(a): Homogenous subsets for ADP yield

YIELD

	TISSUE	N	Subset for alpha = .05	
			1	2
Tukey HSD [a]	Head cartilage	4	14.8150	
	Skin	4	16.0750	
	Fin	4	16.4725	
	Mantle	4	17.5400	
	Tentacle	4	17.6300	
	Gladius	4		82.4500
	Sig.		.080	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

Table 2.6: ANOVA for ADP yield of cuttlefish tissues

ANOVA

YIELD

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11382.163	5	2276.433	1251.035	.000
Within Groups	32.754	18	1.820		
Total	11414.916	23			

2.6(a) Homogenous subsets for ADP yield

YIELD

	TISSUE	N	Subset for alpha = .05			
			1	2	3	4
Tukey HSD[a]	CF Head cartilage	4	15.6425			
	CF Fin	4	17.5550	17.5550		
	CF Skin	4		19.2825	19.2825	
	CF Tentacle	4		20.2050	20.2050	
	CF Mantle	4			20.7825	
	CF Pen	4				76.9475
	Sig.		.377	.107	.625	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

Table 2.7: ANOVA for GAG yield of squid tissues

ANOVA

gag

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	190.008	5	38.002	406.920	.000
Within Groups	1.681	18	.093		
Total	191.689	23			

2.7 (a) Homogenous subsets for GAG yield

gag

	tissue	N	Subset for alpha = .05		
			1	2	3
Tukey HSD[a]	SQ Gladius	4	.0560		
	SQ Tentacle	4	.4573		
	SQ Skin	4	.5725		
	SQ Mantle	4	.6313	.6313	
	SQ Fin	4		1.3125	
	SQ Head cartilage	4			8.0900
	Sig.		.133	.053	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

Table 2.8: ANOVA for GAG yield of cuttlefish tissues

ANOVA

gag

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	198.899	5	39.780	556.513	.000
Within Groups	1.287	18	.071		
Total	200.185	23			

2.8 (a) Homogenous subsets for GAG yield

gag

	tissue	N	Subset for alpha = .05			
			1	2	3	4
Tukey HSD[a,b]	CF Pen	4	.0843			
	CF Tentacle	4	.2333	.2333		
	CF Skin	4	.2870	.2870		
	CF Mantle	4		.8033	.8033	
	CF Fin	4			.9825	
	CF Head cartilag	4				8.1550
	Sig.		.886	.069	.928	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

Table 2.9: Two way ANOVA for GAG yield

Descriptive Statistics

Dependent Variable: gag

species	tissues	Mean	Std. Deviation	N
squid	SQM	.6313	.04246	4
	SQT	.4573	.04535	4
	SQF	1.3125	.36854	4
	SQS	.5725	.02361	4
	SQG	.0560	.00942	4
	SQHC	8.0900	.64807	4
	Total	1.8533	2.88692	24
cuttfi	CUM	.8033	.01828	4
	CUT	.2333	.06576	4
	CUF	.9825	.19517	4
	CUS	.2870	.03071	4
	CUP	.0843	.00943	4
	CUHC	8.1550	.62056	4
	Total	1.7575	2.95021	24
Total	SQM	.6313	.04246	4
	SQT	.4573	.04535	4
	SQF	1.3125	.36854	4
	SQS	.5725	.02361	4
	SQG	.0560	.00942	4
	SQHC	8.0900	.64807	4
	CUM	.8033	.01828	4
	CUT	.2333	.06576	4
	CUF	.9825	.19517	4
	CUS	.2870	.03071	4
	CUP	.0843	.00943	4
	CUHC	8.1550	.62056	4
	Total	1.8054	2.88792	48

2.9 (a) Homogenous subsets

gag

			Subset				
	tissues	N	1	2	3	4	5
Tukey HSD[a,b]	SQG	4	.0560				
	CUP	4	.0843				
	CUT	4	.2333	.2333			
	CUS	4	.2870	.2870	.2870		
	SQT	4	.4573	.4573	.4573		
	SQS	4	.5725	.5725	.5725		
	SQM	4	.6313	.6313	.6313	.6313	
	CUM	4		.8033	.8033	.8033	
	CUF	4			.9825	.9825	
	SQF	4				1.3125	
	SQHC	4					8.0900
	CUHC	4					8.1550
	Sig.		.209	.219	.058	.069	1.000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .082.

a. Uses Harmonic Mean Sample Size = 4.000.

b. Alpha = .05.

Appendix 3

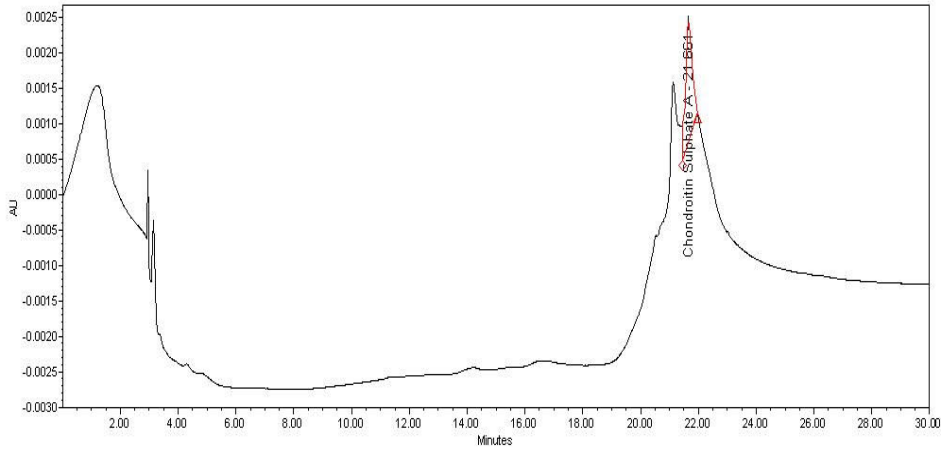


Fig 3.1: SAX-HPLC chromatogram of standard chondroitin sulphate A

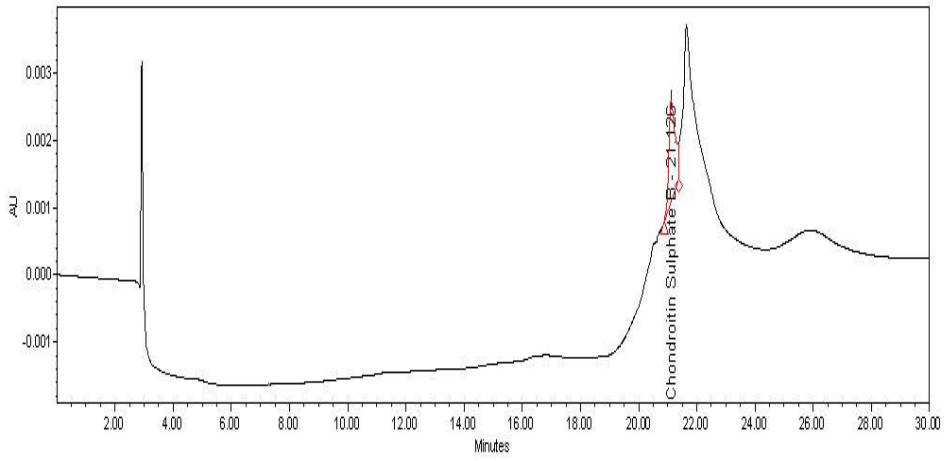


Fig 3.2: SAX-HPLC chromatogram of standard chondroitin sulphate B

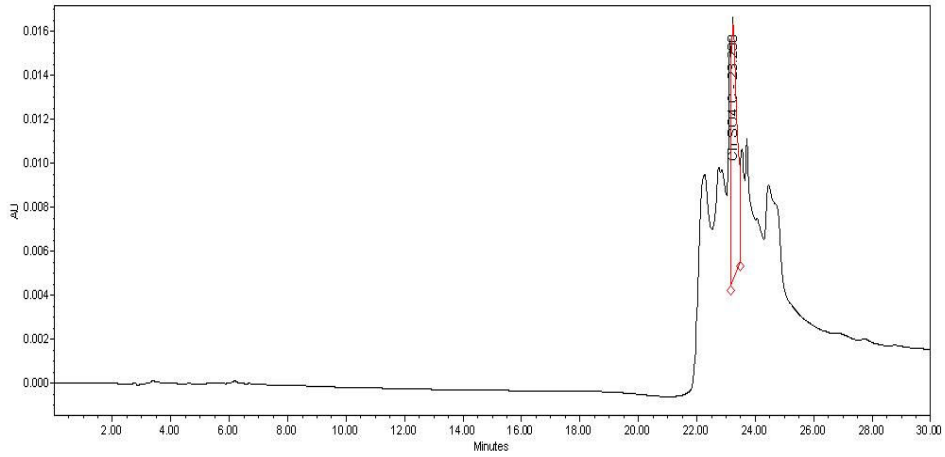


Fig 3.3: SAX-HPLC chromatogram of standard chondroitin sulphate C

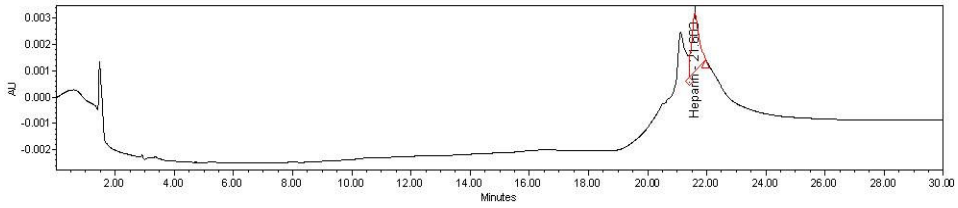


Fig 3.4: SAX-HPLC chromatogram of standard heparin

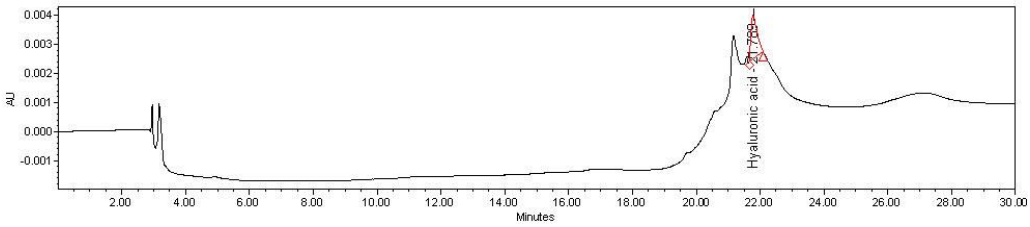


Fig 3.5: SAX-HPLC chromatogram of standard hyaluronic acid

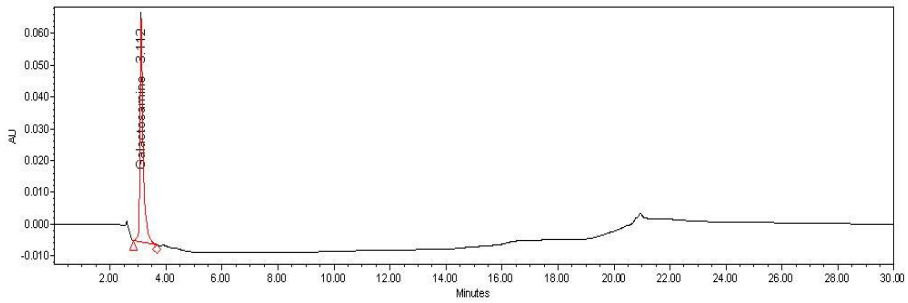


Fig 3.6: SAX-HPLC chromatogram of standard galactosamine

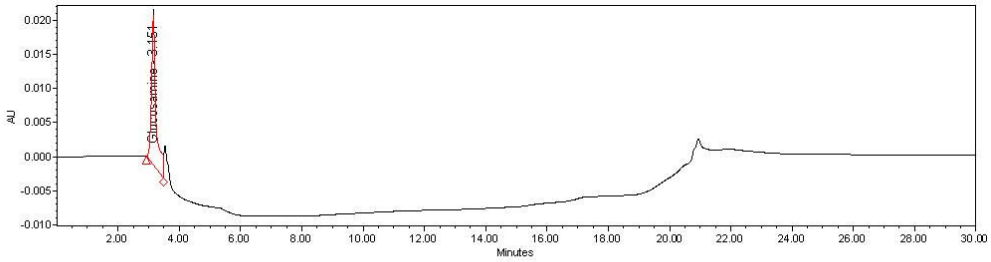


Fig 3.7: SAX-HPLC chromatogram of standard glucosamine

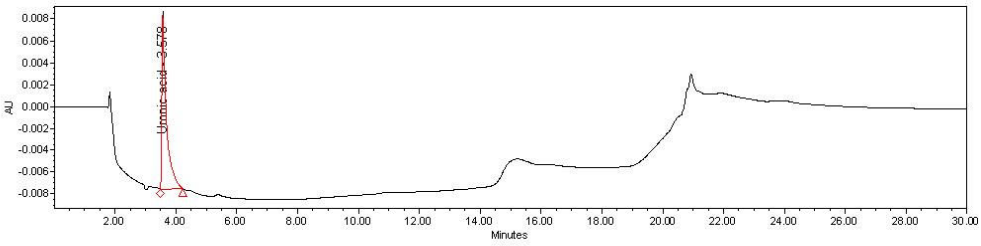


Fig 3.8: SAX-HPLC chromatogram of standard glucuronic acid

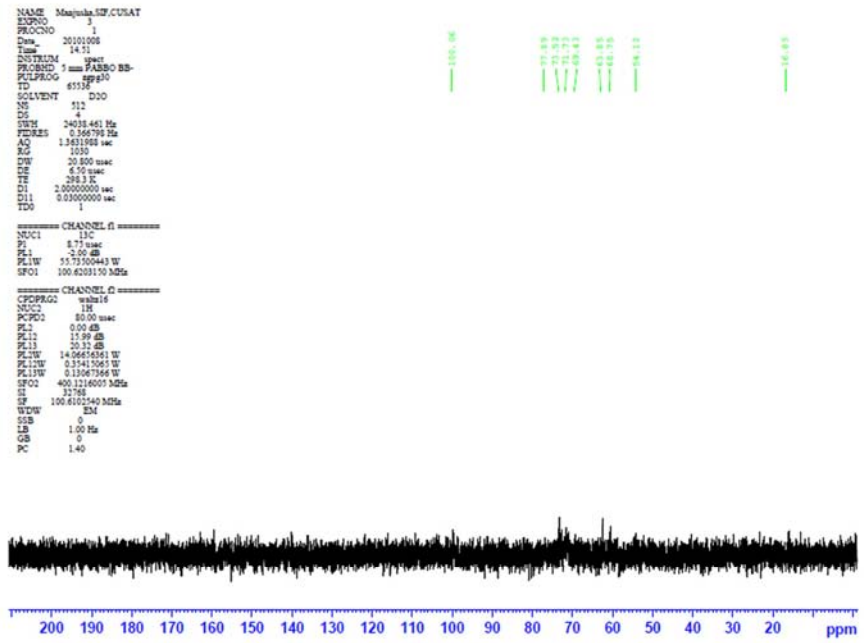
m

Appendix 4

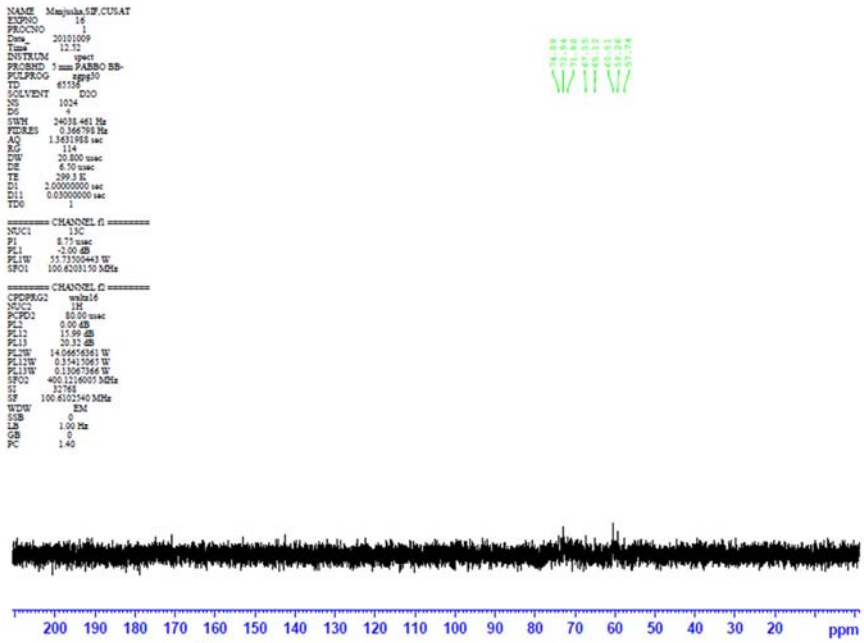
Table 4.1: ^{1}H chemical shifts of the reference compound: unsulphated chondroitin sulphate

^{1}H chemical shifts of the reference compound: unsulphated chondroitin sulphate as described by Yamada *et al*. (1992)

	Reporter groups	α	β
Gal NAc	H -1	5.215	4.71
	H- 2	4.285	3.989
	H -3	4.103	3.927
	H- 4	4.176	4.106
	H -5	4.142	ND
	H - 6	ND	ND
	H – 6’	ND	ND
	NAc	2.055	2.055
Δ HexA	H -1	5.245	5.197
	H- 2	3.81	3.807
	H -3	4.099	4.099
	H- 4	5.904	5.904



Appendix 4.1: ^{13}C NMR spectra of CS-GAGs from squid cranial cartilage



Appendix 4.2: ^{13}C NMR spectra of CS-GAGs from cuttlefish cranial cartilage

Figure 4.1 & 4.2: ^{13}C NMR spectra of the sample GAGs.

Appendix 5

Table 5.1: Values of SEM -EDAX (Elemental composition)

Control $CaCO_3$ crystals				
Element	(keV)	Mass%	Atom%	K
C K	0.277	12.12	19.53	7.6916
O K	0.525	52.41	63.36	29.2934
Ca K	3.69	35.47	17.12	56.496
Total		100	100	

Table 5.2: CaCO3 Crystals synthesized in presence of standard Chondroitin SO_4 A

Element	(keV)	Mass%	Atom%	K
C K	0.277	16.25	24.21	10.6116
O K	0.525	57.12	63.9	38.4367
Ca K	3.69	26.63	11.89	42.9254
Total		100	100	

Table 5.3: $CaCO_3$ Crystals synthesized in presence of Squid GAG

Element	(keV)	Mass%	Atom%	K
C K	0.277	19.69	28.25	12.1738
O K	0.525	56.93	61.32	42.0754
Na K	1.041	1.07	0.8	0.8342
Cl K	2.621	0.59	0.29	0.9266
Ca K	3.69	21.71	9.34	35.2529
Total		100	100	

Table 5.4: $CaCO_3$ Crystals synthesized in presence of cuttlefish GAGs

Element	(keV)	Mass%	Atom%	K
C K	0.277	19.29	27.33	12.5761
O K	0.525	60.1	63.92	45.5815
Ca K	3.69	20.61	8.75	32.6697
Total		100	100	

Appendix 6

Table 6.1: ANOVA for haemocompatibility

ANOVA

HAEMO

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.844	2	.422	.865	.468
Within Groups	2.929	6	.488		
Total	3.774	8			

6.1 (a): Homogenous subsets

HAEMO

	FCS	N	Subset for alpha = .05
			1
Tukey HSD[a]	CF FCS	3	3.7833
	c FCS	3	4.2200
	SQ FCS	3	4.5300
	Sig.		.441

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.