Biochemical Characterization and Bio-evaluation of Collagen and Collagen Peptides Extracted and Purified from Fish Skin: in vitro and in vivo Studies on Antiarthritic and Wound Healing Properties

Thesis submitted to
Cochin University of Science and Technology
In partial fulfillment of the requirements for degree of

Doctor of Philosophy

in

Biochemistry

Under the faculty of Marine Sciences

Cochin University of Science and Technology
Cochin – 682022, India

by

Hema.G.S
(Reg. No. 4199)

Biochemistry and Nutrition Division
Central Institute of Fisheries Technology
CIFT Junction, Matsyapuri P.O.
(Indian Council of Agricultural Research)
Cochin – 682 029, India

July 2015

Declaration

I, Hema.G.S do hereby declare that the thesis entitled **"Biochemical Characterization and Bio-evaluation of Collagen and Collagen Peptides Extracted and Purified from Fish Skin: *In vitro* and *In vivo* Studies on Antiarthritic and Wound Healing Properties"** is a genuine record of bonafide research carried out by me under the supervision of Dr. Suseela Mathew, Principal Scientist, Biochemistry & Nutrition Division, Central Institute of Fisheries Technology, Cochin and has not previously formed the basis of award of any degree, diploma, associateship, fellowship or any other similar titles of this or any other university or Institution.

Cochin
July 2015

Hema.G.S.
(Reg. No.4199)

Dedicated to

. . . My Acha & Amma

Acknowledgements

First and foremost I thank the lord Almighty for guiding and protecting me in every walk of my personal and professional life.

I take this opportunity to express my profound gratitude and deep regards to my supervising guide and mentor Dr.Suseela Mathew, HOD, Department of Biochemistry and Nutrition Division, CIFT 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 express my deep gratitude to Dr. George Ninan, Senior Scientist, Fish Processing Division, CIFT for his valuable advices, kind support, motivation and critical comments during this study.

I thankfully acknowledge Dr.Sreenivasa Gopal, Red. Director, CIFT for the entire prospect, encouragement, support and the facilities provided in CIFT for the smooth conduct of my research.

I wish to express my gratitude to Dr. Ravisankar, Director, and CIFT for the prospectus and encouragement.

I gratefully acknowledge the Department of Biotechnology, Govt. of India, and New Delhi for awarding the financial support as junior and senior fellowship for the successful completion of the work.

I am most grateful to Scientists of Biochemistry and Nutrition Division Dr. Anandan, Dr. Asha, Dr. Niladri for their valuable advices, supports enabling the successful completion of the work. My collective and individual acknowledgement is also for the technical supports, Dr. Usha Rani, Ms. Ramani, Mr. Mathai, Ms. Jaya, Ms Lekha, Mr. Suresh, Mr. Sivan at Biochemistry Nutrition Division. Special thanks to Mr. Reghu for helping me to complete the official procedures of the work.

I am immensely thankful to Dr. Ganesan, technical officer, CIFT for the valuable supports for conducting in vivo studies. It is his constant involvement even sundays helped the successful completion of the animal studies.

I take this opportunity to thank Dr. Asha Nair, Scientist, RGCB, for providing facilities and motivating me into the fascinating world of cell lines and cell culture studies. I sincerely thank the staff of RGCB for helping me to get expertise in cell culture works.

I am immensely thankful to scientists of Microbiology and biotechnology division, CIFT, for providing the necessary facilities at various stages of my work.

I wish to express my hearty thanks to Sri. Joshy George, Scientist, CIFT for providing valuable suggestions and guidance in the statistical analysis of the wealth of data generated.

I extend my sincere thanks to Librarian and staffs of libraries of CIFT and RGCB for their kind support and consideration during my literature survey.

Thanks to all administrative staff of CIFT for their great help throughout my research period.

I fondly cherish the love, care and support of my dear friends Shyni, Remya, Jones, Nabajyothy, Texin, Navaneeth, Ajeesh, Vishnu, Biji, Anju, Ananthanarayanan, Nithin, Meenu, Pradeep, Dhiju and I remember all my friends for their support and enjoyable moments we had together during this couple of years.

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

I express my deep sense of gratitude and regard, to my parents and my brothers for their prayers, affection and love. My Acha and Amma have provided me with the best of all in my life, and the encouragement and support smoothly paved my path towards the successful completion of the work. I express my heartfelt gratitude to my father in law and mother in law for the encouragement, support and the patience extended towards me.

Last but not the least, thanks are due to my beloved husband Vipin Raj for his unreserved and unconditional love, prayers, moral support and motivation at all stages of my work and without his generous support this work would not have been successfully completed.

My heartfelt apologies to my son Devaprayag, for unknowingly sacrificing the beautiful moments we could 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.

Hema.G.S

CONTENTS

Chapter 1

Introduction and Review of Literature

Chapter 5

List of Tables

List of Figures

Abbreviations

µ moles	Micromoles
µg	Microgram
µl	Microlitre
°C	Degree celsius
ACP	Acid phosphatase
ACTH	Adrenocorticotropic hormone
ALP	Alkaline phosphatase
ANOVA	Analysis of Variance
AOAC	Association of the official analytical chemists
ASC	Acid soluble collagen
BSA	Bovine serum albumin
BSE	Bovine spongiform encephalopathy
CFA	Complete Freunds Adjuvant
CHCA	α-cyano-4-hydroxycinnamic acid
cm	Centimeter
CM-Cellulose	Carboxy methyl-Cellulose
COX	Cyclooxygenases
cP	Centipoise
CRP	Creatinine phosphatase
CuSO4	Copper sulphate
CuSO4	Copper sulphate
DAPI	4',6-diamidino-2-phenylindole
DH	Degree of hydrolysis
DMSO	Dimethyl sulphoxide
DNA	Deoxy ribo nucleic acid
ECM	Extra cellular matrix
EDTA	Ethelene diamine tetra acetic acid
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
ERKs	Extracellular signal-regulated kinases

FBS	Foetal bovine serum
FCP	Fish collagen peptide
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FMD	Foot-and-mouth disease
FTIR	Fourier Transform Infrared Spectroscopy
FU	Fluorophore Units
g	Grams
GAG	Glycosaminoglycans
Gy	Gray (unit)
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
HPLC	High performance liquid chromatography
K_2SO_4	Di potassium sulphate
KBr	Potassium bromide
kDa	Kilo Dalton
Kg	Kilogram
KOH	Potassium hydroxide
L	Litre
M	Molar
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry
mg	Milligram
min	Minutes
mL	Millilitre
mm	Millimeter
MMPs	Mitogenactivated protein Kinases
MTT	3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
Na3VO4	Sodium ortho vandate
NaCl	Sodium chloride
NaF	Sodium fluoride

NaOH	Sodium hydroxide
NP-40	Nonyl phenoxypolyethoxylethanol
ns	Nano seconds
NSAIDS	Non-steroidal anti-inflammatory drugs
OA	Osteoarthritis
OD	Optical density
PBS	Phosphate buffered saline
PDC	Pepsin digestible collagen
PDGF	Platelet-derived growth factor
PFA	Para formaldehyde
PGE2	Prostaglandin E2
PGHS	prostaglandin G/H synthase 1
PMNL	Polymorpho nuclear leukocytes
PP	Protease from bovine pancreas
rpm	Revolution per minute
RA	Rheumatoid arthritis
RMSE	Root mean square error
RNA	Ribo nucleic acid
ROS	Reactive oxygen species
RSM	Response Surface Methodology
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SGOT	Serum glutamate oxaloacetate transaminase
SGPT	Serum glutamate pyruvate transaminase
TEMED	Tetramethylethylenediamine
TGF	Transforming growth factor
TNBS	2, 4, 6-trinitrobenzenesulfonic acid
TNF	Tumor necrotic factor
TSE	Transmissible spongiform encephalopathy
UV	Ultraviolet

Chapter 1

INTRODUCTION AND REVIEW OF LITERATURE

Contents

1.1 General Introduction

The utilization of waste from fish processing industry for the production of value added products has attracted substantial attention. In fish processing industry, large amount of waste is generated. These wastes are a mixture of heads, viscera, skin and bone (Morrissey *et al.*, 2005). About 30% of such waste consists of skin and bone with high content of collagen (Gomez-Guillen *et al.*, 2002). Fish skin, which is a major byproduct of the fish-processing industry, could provide a valuable source of collagen (Badii and Howell, 2006). The solid waste from surimi processing, which may range from 50–70% of the original raw material (Morrissey *et al.*,2005), could also be the initial material for obtaining collagen from under-utilized fish resources.

Collagen is the most abundant protein of animal origin, comprising approximately 30% of total animal protein (Birk and Bruckner, 2005). Being a major constituent of the connective tissues, collagen plays an important part in increasing mechanical strength, integrity and rheological properties of the muscles and fillets. Collagen extracted from fish skin, a polymer that is a by-product of food manufacture, has various industrial applications in cosmetic technology and medicine. Collagens of fish skins studied in recent

years were mainly from marine species, such as black drum (*Pogonia cromis*) (Ogawa *et al.,* 2003), brown stripe red snapper (*Lutjanus vitta*) (Nagai *and Susuki,* 2000a), and ocellate puffer fish (*Takifugu rubripes*) (Nagai, Araki & Suzuki, 2002a). Isolation and characterization of collagen from fresh water fish, however, was rarely reported, except for the Nile perch (*Lates niloticus*) (Muyonga *et al.,* 2004a), grass carp (*Ctenopharyngodon idella*) (Zhang *et al.,* 2007) and channel catfish (*Ictalurus punctaus*) (Liu, Li, & Guo, 2007).

Collagen has a wide range of applications in leather and film industries, pharmaceutical, cosmetic and biomedical materials and food (Slade and Levine, 1987; Stainsby, 1987; Bailey and Light, 1989; Hassan and Sherief, 1994). Generally, pig and cow skins and bones are the main sources of collagen isolation. Fish offal, such as skins, scales, as well as bones is the tissues that are mainly structured by collagen. So far, skin and bone collagen from several fish species have been isolated and characterised (Kimura *et al.,* 1991; Nagai *et al.,* 2002; Nagai and Suzuki, 2000b).

Collagen could be extracted and further enzymatically hydrolysed by a process employing commercially available proteolytic enzymes to liberate physiologically active peptides. By selection of suitable enzymes and controlling the conditions, the properties of the end product can be selected. Specifically, some collagen derived peptides may exhibit interesting antioxidant activity, potent anti hypertensive activity, anti microbial activity against different strains of bacteria, protective effect on cartilage, or capacity to stimulate bone formation. Collagen hydrolysates from fish disposals may also exhibit other interesting activities (e.g., satiety, calciotropic, or opioid). The bioactive properties of collagen derived peptides, and also their resistance to protein digestion, make them potential ingredients of health promoting foods (Bailey and Light, 1989; Hassan and Sherief, 1994; Slade and Levine, 1987; Stainsby, 1987).

1.2 Significance of the study

In fish processing plants, there is huge amount of skin that is left as the waste. When this skin is taken and processed into fish collagen, it will save large amount of money that is used for extraction of collagen from other animals. Disposal of waste can cause pollution to environment. The waste not only causes pollution but also emits offensive odour (Takeshi and Nobutaka, 2000).

Fish collagen can be used as an alternative to replace mammalian collagen, especially collagen extracted from bovine, when we consider the outbreak of bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE) and the foot-and-mouth disease (FMD) issues. BSE and TSE are progressive neurological disorders affecting cattles caused by proteinacious infectious particles called prions. FMD is viral disease causing fever and blisters inside foot and mouth of cattles (Shen *et al.*, 2007).

As a consequence, the alternative sources of collagen, especially from aquatic animals including fresh water and marine fish have received increasing attention. The use of fish collagen may contribute to the recycling of an unutilized resource, with consequent highly value added production.

The study aims in producing collagen that has been extracted from fish skin to replace other animal collagen so as to overcome the problem of other animal collagen issues. Also the study utilized the abandoned fish waste produced by fish processing industry since bone, skin, fin and scales of fish can be a useful source of collagen.

The study develops wound healing hydrogel and anti arthritic formulations from the purified fish collagen which are high value products of pharmaceutical importance. The products from the study can be effectively utilized in the wound care management and diseases associated with bone and joint degeneration.

1.3 Objectives of the study

The present study aims to

1. To isolate and characterize collagen from five different species of fishes.
2. To evaluate tissue regenerative potential of collagen.
3. To develop fish collagen hydrolysate - optimization of the process parameters and characterization of collagen peptide.
4. To evaluate the anti arthritic activity of fish collagen peptide in Complete Freunds Adjuvant (CFA) induced rat model systems.
5. To evaluate the stimulating effect of collagen hydrolysate on collagen synthesis in osteoblast cell lines.

1.4 Review of literature

Fish solid waste constitutes 50-70% of the original raw material, depending upon the method of meat extraction from the carcass (Morrisserry and Park, 2000). About 30% of such waste consists of skin and bone with high collagen content (Gomez-Guillen *et al.*, 2002). High value products can be developed with this fish waste, besides helping to minimise harmful environmental pollution.

As far as fish collagen is concerned, the huge number of species having very different intrinsic characteristics has aroused the interest of the scientific community in optimising the extracting conditions as well as characterising the yields, and physio-chemical and functional properties of the resulting collagens.

Fish collagen has lower denaturation temperatures compared to vertebrates' collagen. The denaturation temperature of mammalian collagen was higher than 30°C while most fish collagens denature at temperatures below 30°C (Ogawa *et al.*, 2003). Marine collagen had a lower denaturation temperature by about 10°C than that of the porcine skin collagen (Nagai *et al.*, 2008). This indicates that fish

collagen is generally less stable than mammalian counterparts (Ogawa *et al.*, 2003).

1.4.1 The Collagen Molecule

1.4.1.1 Distribution and molecular structure

Collagen is one of the most abundant biological macromolecules of extracellular matrix where it provides the major structural and mechanical support to tissues. The presence of collagen in all connective tissue makes it one of the most studied biomolecules of the extracellular matrix. This fibrous protein species is the major component of skin and bone and represents approximately 25% of the total dry weight of mammals (Alberts *et al.*, 2002).

Collagen molecules are comprised of three chains (two α and one β chains) that assemble together due to their molecular structure. Every α chain is composed of more than a thousand amino acids based on the sequence -Gly-X-Y-. X and Y positions are mostly filled by proline and 4-hydroxyproline (Whitford, 2005).

There are approximately twenty-five different chain conformations, each produced by their unique gene. The combination of these chains, in sets of three, assembles to form the twenty-nine different types of collagen currently known. Although many types of collagen have been described, only a few types are used to produce collagen based biomaterials (Brodsky and Persikov, 2005). Type I collagen is currently the gold standard in the field of tissue-engineering. The fibroblast is responsible for the majority of the collagen production in connective tissue. Collagen pro-α chain is synthesized from a unique mRNA within the rough endoplasmic reticulum and is then transferred to the Golgi apparatus of the cell. During this transfer, some proline and lysine residues are hydroxylated by the lysyloxydase enzyme. Specific lysine residues are glycosylated and then pro-α chain self-assemble into procollagen prior to their encapsulation in excretory vesicles. Following their

passage through the plasma membrane, the propeptides are cleaved outside the cell to allow for the auto-polymerisation by telopeptides. This step marks the initiation of tropocollagen self-assembly into 10 to 300 nm sized fibril and the agglomeration of fibril into 0.5 to 3 μm collagen fibers. Fibril-forming collagens are the most commonly used in the production of collagen-based biomaterials (Van der Rest and Garrone, 1991; Prockop and Kivirikko, 1995)

It is a unique protein, able to form insoluble fibers with a high tensile strength and contains right-handed triple super helical rod consisting of three polypeptide chains (Gelse *et al.*, 2003).

1.4.1.2 Collagen types

There are at least 27 different types of collagen, named type I–XXVII (Birk and Bruckner, 2005). The collagen variants vary in their macromolecular structure (Baily, 1998). Type I collagen is commonly found in connective tissues, including tendons, bones and skins (Muyonga *et al.*, 2004a). Type I collagen is predominant in higher order animals and especially in the skin, tendon and bone where extreme forces are transmitted. It is a compound of three chains, two of which are identical, termed α1, and one α2 chain with different amino acid composition. Type II collagen is essentially unique to hyaline cartilage. Type III is found in limited quantities (~ 10%) in association with type I. Thus, type III can be a minor contaminant of type I collagen prepared from skin (Piez, 1985). In addition, blood vessels predominantly contain type III. Collagen types I, II, and III have large sections of homologous sequences, independent of the species (Timpl, 1984). Type III collagen is mainly found in embryonic tissue, scar tissue, arteries and intra organ connections (Baily and Light, 1989). It is composed of identical α1 chains and contains intra and possibly intermolecular disulphide bonds.

Figure 1.1 *Schematization of a collagen α chain triple helix segment. (b) Assembled tropocollagen molecules. (c) Collagen fibril ranging from 10 to 300 nm in diameter. (d) Aggregated collagen fibrils forming a collagen fiber with a diameter ranging from0.5 to 3 μm (Alberts et al., 2002).*

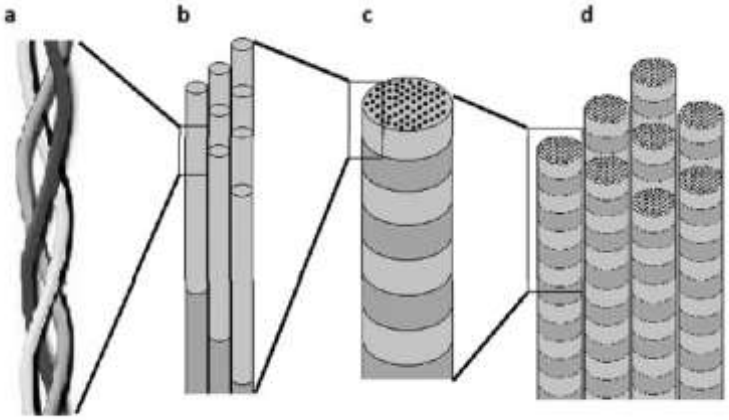
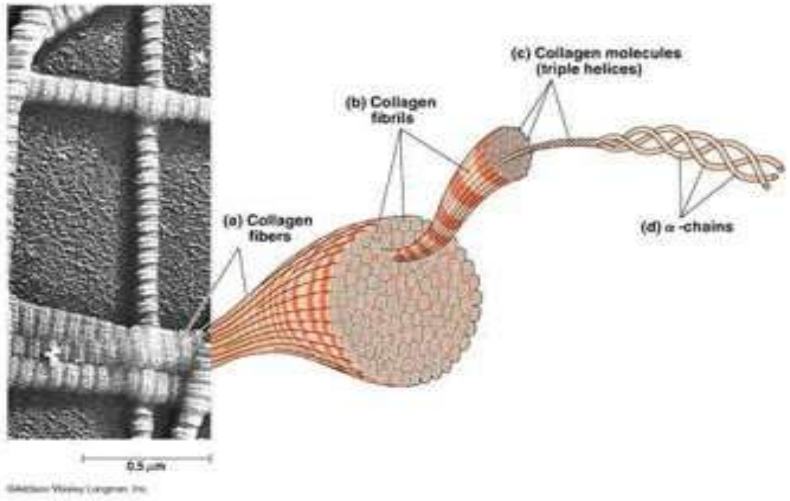


Figure 1.2 The ultra structure of collagen type I *Transmission electron microscopy and diagrammatic cross section of (a) collagen fibres. (b) Collagen fibres consist of collagen fibrils. (c) Collagen molecules make up the collagen fibril. (d) Collagen molecules are, in turn, triple helices of 3 α chains.*



Type IV collagen is a highly specialized form found only as a loose fibrillar network in the basement membrane. Type IV collagen is high in hydroxyproline and hydroxylysine. In addition to the usual 4-hydroxyproline, it also contains 3 hydroxyproline. Type V collagen contains α1 and α2 chains in the ratio of 1:2 as well as α3 chains. The α3 chains contain more cysteine than α1 and α2 chains (Kuhn, 1987).

Table 1.1 Different types of collagen

Type	Molecular formula	Polymerized form	Tissue distribution
I	[α1(I)]2α2(I)	fibril	bone, skin, tendons, ligaments, and cornea.
II	[α1(II)]3	fibril	cartilage, intervertebrate disc, notochord, vitreous humor in the eye.
III	[α1(III)]3	fibril	skin,blood vessels
V	[α1(V)]2α2(V) and α1(V)α2(V)α3(V)	fibril (assemble with type I)	idem as type I
XI	α1(XI)α2(XI)α3(XI)	fibril (assemble with type II)	idem as type II
IX	α1(IX)α2(IX)α3(IX)	lateral association with type II fibril	cartilage
XII	[α1(XII)]3	lateral association with type I fibril	tendons, ligaments
IV	[α1(IV)]2α2(IV)	Sheet-like Network	basal lamina
VII	[α1(VII)]3	anchoring fibrils	beneath stratified squamous epithelia

(RémiParenteau-Bareil *et al.,* 2010)

Collagen can be extracted from various sources considering that it is one of the most abundant proteins on earth. It can be extracted from almost every living animal, even including alligators (Wood *et al.,* 2008). Nonetheless, common sources of collagen for tissue engineering applications include bovine skin and tendons, porcine skin and rat tail among others. Marine life forms are also a considerable source of collagen.These collagens are widely used in the industry, but less for research and clinical usage. All these collagen sources are worth investigating considering that collagen properties differ from one animal to another (Lin and Liu, 2006].

1.4.1.3 Fish Collagen

Fish collagen is a complex structural protein that is mainly concentrated in skin, cartilage, airbladder and scales. Collagen is a unique protein compared to other fish muscle proteins and this uniqueness of fish lies in the amino acid content and they are rich in non-polar amino acids (above 80%) such as Gly, Ala, Val and Pro.

In fish, collagen is a major fraction of skin, scales and airbladder (Foegeding *et al.,* 1996). Collagen is the fibrous protein that contributes to the unique physiological functions of connective tissues in skin, tendons, bones, cartilage and others (Wong, 1989). Collagen contents vary considerably with fish species, age and season (Nagai *et al.,* 2002a). Collagen obtained from different species and habitats might be different in terms of molecular compositions and properties (Foegeding *et al.,* 1996).

Most fish collagens have been found to consist of two α - chain variants, which are normally designated as α1 and α2 (Nagai *et al.,* 2001; Gomez-Guillen *et al.,* 2002). The different collagen variants also vary in the nature of the constituent α chain. Different α chain types vary slightly in amino acid composition and as a result have small differences in hydrophobicity (Nagai and Suzuki, 2002). These chain variants, though having approximately the same molecular weight (95,000 Da), can be separated by SDS - PAGE due

to their different affinity for SDS. The α2 have a higher affinity for SDS and consequently exhibit a higher mobility than α1 (Hayashi and Nagai, 1980)

1.4.2 Isolation and purification of collagen from fishes

The major impediment to dissolution of collagen type I from tissue is the presence of covalent cross links between molecules. Collagen is insoluble in organic solvents. Water soluble collagen represents only a small fraction of total collagen and the amount depends on the age of the animal and type of tissue extracted. In some tissues, notably skin from young animals, cross linking is sufficiently low to extract a few percent under appropriate conditions. Furthermore, collagen molecules present within fibrillar aggregates can be dissociated and brought into aqueous solution. However, the nature of the cross links prevalent in different tissues determines the particular solvent to be used and the corresponding yields.

1.4.2.1 Acid soluble collagen

Dilute acidic solvents, e.g. 0.5 M acetic acid, citrate buffer are efficient to extract collagen from the tissues. The intermolecular cross links of the aldimine type are dissociated by the dilute acids and the repelling charges on the triple-helices lead to swelling of fibrillar structures (Trelstad and Birk, 1984)

Dilute acids will not disassociate less labile cross links such as keto-imine bonds. Therefore collagen from tissues containing higher percentages of keto-imine bonds, i.e. bone, cartilage, or tissues from older animals has a lower solubility in dilute acid solvents. In order to acid extract collagen, generally, tissue is ground in the cold, washed with neutral saline to remove soluble proteins and polysaccharides, and the collagen is extracted with a low ionic strength, acidic solution (Bazin and Delaumay, 1976). It is possible to solubilize ~ 2% of tissue collagen with dilute salt or acid

solutions. These collagen molecules can be reconstituted into large fibrils with similar properties as native fibrils by adjusting the pH or temperature of the solution (Piez, 1984). The remaining 98% is referred to as insoluble collagen although this dominant collagen material is not absolutely insoluble and can be further disintegrated without major damage to the triple-helical structures. The two most common approaches are the use of strong alkali or enzymes to cleave additional cross links and suspend or dissolve at first acid-insoluble structures.

1.4.2.2 Enzyme treated collagen

Collagen material can be solubilized by treating connective tissue with an aqueous solution comprising of alkali hydroxide and alkali sulfate, e.g. approximately 10% sodium hydroxide and 10% sodium sulfate for ~ 48 h (Cioca, 1981; Roreger, 1995). Thus, fat associated with the insoluble collagen is saponified, non-helical telopeptide regions are truncated and the collagen fibers disintegrated. The size and molecular weight of the resulting collagen material depend on the time of treatment and alkali concentration (Roreger, 1995). The presence of alkali sulfate controls the swelling of the collagen structures and protects the native triple-helical characteristics. It has to be noted that similar to gelatin, the isoelectric point of the resulting material is shifted to lower pH as asparagine and glutamine are converted into aspartic and glutamic acid. Much higher yields compared with acidic extraction can be achieved by taking advantage of the fact that the collagen triple-helix is relatively resistant to proteases, i.e. pepsin or chymotrypsin below ~20°C (Piez, 1984).

The efficacy of enzymatic treatment arises from selective cleavage in the terminal non-helical regions breaking peptide bonds near cross links and releasing molecules which dissolve. Some cross links presumably remain, attaching small peptide remnants to the solubilized molecules (Miller *et al,* 1984). Thus, the telopeptide ends of the polymer chains are dissected but under appropriate conditions

the helices remain essentially intact. The resulting material, so-called atelocollagen, benefits from the removal of the antigenic determinant located on the non-helical protein sections and provokes milder immune response (Knapp *et al.,* 1977). Pepsin at a 1:10 weight ratio of enzyme to dry weight tissue in dilute organic acid (0.5 M acetic acid) provides a propitious medium in which collagen can be swollen and solubilized (Piez, 1985).

Soluble collagen is purified mainly by precipitation after pH, salt concentration or temperature adjustment (Li, 1995). The high viscosity of even dilute solutions interferes with purification methods such as chromatography, electrophoresis and differential sedimentation. Collagen solutions contain varying proportions of monomer and higher molecular weight covalently linked aggregates, depending on the source and method of preparation. Truly monomeric solutions are difficult if not impossible to obtain (Piez, 1985). Pepsin solubilized collagen usually contains higher proportions of monomer than salt- or acid extracted material (Piez, 1984). Soluble collagen can be stored frozen or lyophilized. In the course of drying, denaturation or non-specific cross linking can occur and the degree of association upon reconstitution can change (Lee, 1983).

1.4.2.3 Insoluble collagen

Instead of disintegration and transfer into soluble material, extensively cross linked collagen can be dispersed as opalescent, fine fibrillar suspensions by the use of mild denaturation agents and mechanical fragmentation usually at an acidic pH. Fibrillar collagen is more resistant to proteolysis than most other non-collageneous tissue constituents, which are removed during processing by selective proteolysis and washing (Li, 1995). In additional steps collagen material can be subjected to chemical modifications such as succinylation (Singh *et al.,* 1995) acetylation (Srivastava *et al.,* 1990), methylation (Wang *et al.,* 1978) or attachment to other polymers (Panduranga and Rao, 1995).

Due to their high biocompatibility, collagens extracted from land-based animal skins have been widely used in the pharmaceutical, food, healthcare, and cosmetic industries (Ogawa *et al.*, 2004). Commonly isolated from by-products of land-based animals, such as cows, pigs and poultry, collagen has been widely used in food, pharmaceutical, and cosmetic industries because of its excellent biocompatibility and biodegradability, and weak antigenicity (Liu *et al.*, 2009). However, the outbreaks of bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE), foot-and-mouth disease (FMD) and avian influenza have raised anxiety among some consumers of collagen and collagen-derived products from these land-based animals.

Therefore, the global demand for collagen from alternative sources such as aquatic animals has been increasing over the years. With the rapid development of the fish processing industry in China, large quantities of by-products are generated, accounting for 50–70% of the original raw material (Kittiphattanabawon *et al.*, 2005). Collagens from fish skin or swim bladders (a waste product in fish processing), may be good substitutes, because of their safety and solubility in neutral salt solutions and dilute acids. Also, the development of fish swim bladder-based collagens would add significant value to the fish processing industry (Trevitt and Singh, 2003). Consequently, optimal use of these by-products is a promising way to protect the environment, to produce value-added products, to increase the revenue to the fish processors, and to create new job/business opportunities.

1.4.3 Characteristics of Fish Collagen

The physical and chemical properties of collagen differ depending on the tissues such as skin, swim bladder and the myocommata in muscle. Fish collagen is heat sensitive due to labile cross links as compared to mammals. Different fish species contain varying amounts of collagen in the body tissue that reflect the swimming behaviour and it influences the textural characteristics of

fish muscle (Montero and Borderias, 1989). Collagen is unique in its ability to form insoluble fibres that have high tensile strength (Gelse *et al.*, 2003).

In addition to differences in molecular species, fish collagens have been shown to vary widely in their amino acid composition. In particular, the levels of imino acids (proline and hydroxyproline) vary significantly among fish species (Balian and Bowes, 1977; Poppe, 1997; Gudmundsson and Hafsteinsson, 1997). The amount of imino acids, especially hydroxyproline, depends on the environmental temperature in which the fish lives and it affects the thermal stability of the collagens (Rigby, 1968; Balian and Bowes, 1977). Collagens derived from fish species living in cold environments have lower contents of hydroxyproline and they exhibit lower thermal stability than those from fish living in warm environments. This is because hydroxyproline is involved in inter-chain hydrogen bonding, which stabilizes the triple helical structure of collagen (Darby and Creighton, 1993). In the absence of proline hydroxylation, the essential triple helical conformation of collagen is thermally unstable at well below physiological temperatures (Berg and Prockop, 1973).

1.4.3.1 Amino Acid Composition of collagen

For amino acid analysis, the strict condition for sample preservation is important and indispensable before collagen extraction. This means that the hydroxyproline content in relation to collagen stability strongly depends on these sampling procedures (Swatschek *et al.*, 2002). Several works showed that amino acid composition of fish collagens was almost similar to that of mammalian collagens (Nagai *et al.*, 2000a; Bae *et al.*, 2008). Furthermore, the degree of hydroxylation of proline was calculated to be 40-48%, which was also similar level to that of the mammalian (about 45%). A linear relationship between the stability of collagen and the hydroxyproline content has been reported. The difference in hydroxyproline amount might relate to the species, environment and

the fish body temperature (Zhang and Webster, 2009). It is very interesting that the degree of hydroxylation of proline of fishes in cold sea, for example chum salmon, was reported to be low (35-37%) (Matsui *et al.*, 1991) compared to that of fishes in relatively warm sea, from similar environments. Difference in collagen denaturation temperatures is also associated to the proline and hydroxyproline content. This is because proline and hydroxyproline can stabilize the triple helix due to the non-covalent bonding of their pyrrolidine ring. Greater the value of proline and hydroxyproline, greater is the thermal stability of the collagen (Lin and Liu, 2006).

Collagen contains two uncommon derivative amino acids not directly inserted during translation. These amino acids are found at specific locations relative to glycine and are modified post-translationally by different enzymes, both of which require vitamin C as a cofactor. One is hydroxyproline derived from proline and the other is hydroxylysine derived from lysine. Depending on the type of collagen, varying numbers of hydroxylysine are glycosylated (mostly having disaccharides attached). Glycine is the most abundant amino acid and accounted for more that 30% of all amino acids (Nagai *et al.*, 2000b).

1.4.3.2 Viscosity of collagen

High viscosity is one of the physico-chemical characteristics of collagen. Fish collagen may have a range of viscosity about 12 to 19 dL/g (Ogawa *et al.*, 2004). As collagen is made up by structured systems it is characterized by a high degree of viscosity due to greater electrostatic repulsion among the collagen molecular chains in solution even at low concentrations. The study showed that the relative viscosity of collagen decreased continuously on heating up to 30°C. Rate of decrease was retarded in the temperature range of 35-50°C. This is due to the breaking of hydrogen bonds during the high temperature which stabilize the collagen structure. As collagen a of protein, it can be denatured at above 40°C. This collagen would be denatured to a mixture of random-coil single, double and triple

strands (Kittiphattanabawon *et al.*, 2005). The triple helix structure of collagen stabilized by hydrogen bonds was converted into the random coil arrangement by the process of thermal depolymerization which accompanied by variations in physical properties like viscosity, sedimentation, diffusion, light scattering and optical activity (Ahmad and Benjakul, 2010).

Viscosity measurement is commonly used to determine the thermal stability of collagen. This tool is used to measure the transitions in polymers and to learn about the loss of viscosity with heating which is attributed to denaturation of collagen (Zhang *et al.*, 2007). Thermal denaturation measurement of collagen provided useful signs to the thermal stability of collagen in relation to environment and amino acid content (Li *et al.*, 2008). The temperature of denaturation of collagen solution from grass carp was 28.4°C (Zhang *et al.*, 2007) and 32°C (Li *et al.*, 2008), chub mackerel was 25 to 28°C (Kittiphattanabawon *et al.*, 2005) and nile perch was 36-36.5°C (Muyonga *et al.*, 2004b). Higher denaturation temperature for collagen of Nile perch may be attributed to the higher amino acid content than that of cold-water fish collagens (Muyonga *et al.*, 2004b). The denaturation temperature is proportional to the content of hydroxypoline. Hydroxypoline is believed to play an important role in the stabilization of the triple-stranded collagen helix due to its hydrogen bonding ability through its hydroxyl group (Li *et al.*, 2008).

1.4.3.3 Solubility of collagen

In general, fish collagen would be more soluble in the acidic pH ranges while at neutral pH it will show sharp decrease in solubility. On the other hand, solubility slightly decreased at extremely acidic pH. According to Ahmad *et al.* (2010) collagen was soluble in the pH range from 1 to 4 with the highest solubility at pH 2 and the lowest solubility at pH 6 to 7. When pH values are above and below isoelectric point (pI), a protein has a net negative or positive charge, respectively. Therefore, more water interacts with

the charged proteins (Kittiphattanabawon *et al.,* 2005). At pH near the pI, a collagen molecule is unstable and tends to coagulate. This is because of the increase in hydrophobic interaction among the collagen molecules. The higher solubility at lower pH would increase from the greater repulsive force between collagen molecules. In alkaline condition, the slight increase in solubility was observed (Ahmad *et al.,* 2010).

The solubility of collagen in 0.5M acetic acid can be maintained in the absence of NaCl. Increasing the NaCl concentration will reduce the solubility of collagen (Kittiphattanabawon *et al.,* 2005). The slight decrease in solubility was determined in the presence of 1 to 2% NaCl. The sharp decrease was observed as the salt concentration rise up to 6%. At 6% of NaCl, the solubility of 36.91% was determined. The lower solubility of collagen was mainly due to the salting out effect (Ahmad *et al.,* 2010). Higher concentration of NaCl might result in decreasing protein solubility by increasing hydrophobic interaction and aggregation. As a result, the proteins start to precipitate (Kittiphattanabawon *et al.,* 2005).

Fish skin collagens have been reported to develop minimal amounts of mature cross-links. By measuring hydrothermal isometric tensions that fish collagen cross-links do not mature to thermally stable bonds. As a result of its low content of stable cross-links, fish collagen can easily be solubilised (Muyonga *et al.,* 2004b).

1.4.4 Characteristics of fish collagen to be used as biomaterial

Biomaterials made of collagen offer several advantages: they are biocompatible and non-toxic and have well-documented structural, physical, chemical, biological and immunological properties (Chvapil, 1979; Ramshaw *et al.,* 1995). It has to be stressed that collagen properties like mechanical strength, fluid absorption volume or haemostatic activity differ depending on the

animal source and anatomical location of the raw material. For local antibiotic delivery, the goal should be able to maintain the highest possible, but not toxic, local drug concentration without achieving systemic effects. This can be achieved by physical and possibly also chemical incorporation of the drug into a collagen matrix in the course of the manufacturing process to assure drug immobilization. Drugs may be complexed to collagen through direct binding of the drug to free amino or carboxylic groups of the collagen molecule (Chvapil, 1979). Drug release occurs by diffusion from a collagen matrix implanted or injected as such or polymerized after intra-tissue injection (Stemberger *et al.*, 1997). For example, a tetracycline solution injected subcutaneously reached a maximum serum concentration after 3 h which slowly decreased within the next 20 h. When the same amount of tetracycline solution was soaked into a collagen sponge and inserted into a natural body cavity, the drug release was detected over a period of 14 days resulting in a relatively constant serum concentration of the drug (Chvapil, 1979).

1.4.4.1 Biocompatibility

The primary reason for using collagen as biomaterial is its excellent biocompatibility, low antigenicity (Pati *et al.*, 2012), high level of direct cell adhesion, and high degree of biodegradability (Lee *et al.*,2001). An immune response against collagen mainly targets epitopes in the telopeptide region at each end of the tropocollagen molecule (Steffen *et al.*, 1968). The application of fish collagen as a scaffold for tissue engineering has been attempted (Nagai *et al.*, 2008 ; Sugiura *et al.*, 2009). Atelocollagen is a processed natural biomaterial produced from bovine type I collagen. It inherits useful biomaterial characteristics from collagen, including a low rate of inflammatory responses, high level of biocompatibility, and high degree of biodegradability (Miyata *et al.*, 1992; Hanai *et al.*, 2006). The components of collagen that are attributed to its immunogenicity, namely, telopeptides, are eliminated during atelocollagen production. Therefore, atelocollagen exhibits little

immunogenicity (Sano *et al.*, 2003). The ability to obtain a substantial amount of collagen from fish waste (scales, skin, and bone) would result in the development of an alternative to bovine collagen for use in food, cosmetics, and biomedical materials. Elastic salmon collagen (SC) vascular grafts have been prepared by incubating a mixture of acidic SC solution and fibrillogenesis-inducing buffer containing a cross-linking agent, water-soluble carbodiimide. Upon subcutaneous placement in rat tissues, the SC grafts induced little inflammatory reactions (Nagai *et al.*, 2008). Tests of pellet implantation into the para vertebral muscle in rabbits have demonstrated that tilapia collagen rarely induces inflammatory responses at one or four weeks after implantation, a finding that is statistically similar to that of porcine collagen and high-density polyethylene as a negative control (Sugiura *et al.*, 2009).

1.4.4.2 Biodegradability

Biodegradability is a valuable aspect for most collagen-based biomaterials. Collagen biocompatibility and possible degradation by human collagenases are responsible for the widespread use of this material in many biomedical applications. Collagenases such as matrix metalloproteinase (MMP) are responsible for most collagen degradation *in vivo*. On the other hand, the rate of the degradation process often needs to be regulated using diverse methods such as crosslinking techniques (Weadock *et al.*, 1996). *In vitro* degradation studies (using collagenase solution) have demonstrated a higher level of stability among cross linked scaffolds derived from tropical fresh water fish scale collagen, with only a 50% reduction in mass after 30 days, whereas the uncross linked scaffold has been shown to degrade completely within four days (Pati *et al.*,2012). Upon placement in subcutaneous tissues in rats, grafts gradually biodegrade. One month after implantation, fibroblasts and macrophages begin to penetrate the surface of the graft, without signs of necrosis (Nagai *et al.*, 2008).

1.4.5 Collagen-Based Biomaterials

1.4.5.1 Types of collagen-based biomaterials

Collagen-based biomaterials can originate from two fundamental techniques. The first one is a decellularized collagen matrix preserving the original tissue shape and ECM structure, while the other relies on extraction, purification and polymerization of collagen and its diverse components to form a functional scaffold. Physical methods include snap freezing that disrupt cells by forming ice crystals, high pressure that burst cells and agitation, that induce cell lysis and used most often in combination with chemical methods to facilitate penetration of active molecules in the tissue. Chemical methods of decellularization include a variety of reagents that can be used to remove the cellular content of ECM. These substances range from acid to alkaline treatments, as well as chelating agents such as EDTA, ionic or non-ionic detergents and solutions of extreme osmolarity. Enzymatic treatments such as trypsin, which specifically cleaves proteins and nucleases that remove DNA and RNA, are also commonly used to produce acellular scaffold. However, none of these methods can produce an ECM completely free of cellular debris and a combination of techniques is often required to obtain a material free of any cell remnant.

A plethora of biomolecules can also be added to collagen solution to produce collagen-based biomaterials. These biomolecules, typically glycosaminoglycans, elastin and chitosan are added to the compound to potentially enhance the properties of collagen (Zhong and Young, 2009; Caissie *et al.*, 2006). The other type of collagen-based biomaterial is made by processing a collagen solution with other biomolecules like GAG (Chen *et al.*, 2005)

1.4.6 wound healing

Wound healing is a dynamic process and the performance requirements of a dressing can change as healing progresses. However, it is widely accepted that a warm, moist environment encourages rapid healing and most modern wound care products are designed to provide these conditions (Barnett and Irving, 1991). Fluid balance in burn injury is very important since heavy loss of water from the body by exudation and evaporation may lead to a fall in body temperature and increase in the metabolite. Besides this, dressing should have certain other properties like ease of application and removal, and proper adherence so that there will not be any area of non-adherence left to create fluid-filled pockets for the proliferation of bacteria (Quinn *et al.*,1985)

Wounds that exhibit impaired healing, including delayed acute wounds and chronic wounds, generally have failed to progress through the normal stages of healing. Such wounds frequently enter a state of pathologic inflammation due to a postponed, incomplete, or uncoordinated healing process. Most chronic wounds are ulcers that are associated with ischemia, diabetes mellitus, venous stasis disease, or pressure (Mathieu *et al.*, 2006; Menke *et al.*, 2007).

1.4.6.1 Biochemical processes in wound healing

The wound-healing process consists of four highly integrated and overlapping phases: hemostasis, inflammation, proliferation, and tissue remodeling or resolution (Gosain and DiPietro, 2004). These phases and their biophysiological functions must occur in the proper sequence, at a specific time, and continue for a specific duration at an optimal intensity (Mathieu *et al.*, 2006). There are many factors that can affect wound healing which interfere with one or more phases in this process, thus causing improper or impaired tissue repair.

In adult humans, optimal wound healing involves the following events: (1) rapid hemostasis; (2) appropriate inflammation; (3) mesenchymal cell differentiation, proliferation, and migration to the wound site; (4) suitable angiogenesis; (5) prompt re-epithelialization (re-growth of epithelial tissue over the wound surface); and (6) proper synthesis, cross-linking, and alignment of collagen to provide strength to the healing tissue (Gosain and DiPietro, 2004; Mathieu *et al.*, 2006).

The first phase of hemostasis begins immediately after wounding, with vascular constriction and fibrin clot formation. The clot and surrounding wound tissue release pro-inflammatory cytokines and growth factors such as transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF). Once bleeding is controlled, inflammatory cells migrate into the wound (chemotaxis) and promote the inflammatory phase, which is characterized by the sequential infiltration of neutrophils, macrophages, and lymphocytes (Gosain and DiPietro, 2004; Broughton *et al.*, 2006; Campos *et al.*, 2008). A critical function of neutrophils is the clearance of invading microbes and cellular debris in the wound area, although these cells also produce substances such as proteases and reactive oxygen species (ROS), which cause some additional bystander damage. Macrophages play multiple roles in wound healing. In the early wound, macrophages release cytokines that promote the inflammatory response by recruiting and activating additional leukocytes. Macrophages are also responsible for inducing and clearing apoptotic cells (including neutrophils), thus paving the way for the resolution of inflammation. As macrophages clear these apoptotic cells, they undergo a phenotypic transition to a reparative state that stimulates keratinocytes, fibroblasts, and angiogenesis to promote tissue regeneration (Meszaros *et al.*, 2000; Mosser and Edwards, 2008). In this way, macrophages promote the transition to the proliferative phase of healing.

Table1.2 Normal wound healing process

Phase	Cellular and Bio-physiologic Events
Hemostasis	1.Vascular constriction
	2.Platelet aggregation, degranulation and fibril formation
Inflammation	1.Neutrophil infiltration
	2.Monocyte infiltration and differentiation to macrophage
	3.Lymphocyte infiltration
Proliferation	1.Re-epithelialization
	2.Angiogenesis
	3.Collagen synthesis
	4.Extra cellular matrix formation
Remodeling	1.Collagen remodelling
	2.Vascular maturation and regression

(Guo and Di Pietro, 2010)

Inflammation is a normal part of the wound-healing process, and is important to the removal of contaminating micro-organisms. In the absence of effective decontamination, however, inflammation may be prolonged, since microbial clearance is incomplete. Both bacteria and endotoxins can lead to the prolonged elevation of pro-inflammatory cytokines such as interleukin-1 (IL-1) and TNF-α and elongate the inflammatory phase. If this continues, the wound may enter a chronic state and fail to heal. This prolonged inflammation also leads to an increased level of matrix metalloproteases (MMPs), a family of proteases that can degrade the extra cellular matrix. In tandem with the increased protease content, a decreased level of the naturally occurring protease inhibitors occurs. This shift in protease balance can cause growth factors that appear in chronic wounds to be rapidly degraded (Edwards and Harding, 2004; Menke *et al.*, 2007).

1.4.6.2 Moist wound healing theory

The most significant advancement in wound care came with Winter's (Winter, 1962) study in 60's, which showed that occluded wounds healed much faster than dry wounds and moist wound healing environment optimized the healing rates. He demonstrated that when wounds on pigs are kept moist, epithelialisation is twice as rapid as on wounds allowed to dry by exposure to air. Later Hinman and Maibach (2000) confirmed Winter's work on human beings in 1963. An open wound, which is directly exposed to air, will dehydrate and a scab or escar is formed. This forms a mechanical barrier to migrating epidermal cells and is then forced to move in a deeper level of tissue, which prolongs the healing process. Moist healing prevents the formation of scab as the dressing absorbs wound exudate secreted from the ulcer (Winter and Scales, 1963).

1.4.6.3 Collagen hydrogel

The term "hydrogel" refers to a broad class of polymeric materials that are swollen extensively in water but that do not dissolve in water. They have been used in a wide variety of biomedical applications and may be synthesized from monomers or monomers mixed with polymers. Hydrogels are attractive as biomaterials; they are highly permeable to water, ions, and small molecules (Peppas and Khare, 1993). Hydrogels comprised of naturally derived macromolecules and have potential advantages of biocompatibility, cell-controlled degradability, and intrinsic cellular interaction.

Hydrogels have structural similarity to the macromolecular-based components in the body and are considered biocompatible (Jhon and Andrade, 1973). Gels are formed when the network is covalently crosslinked (Hoffman, 2001). Biocompatible hydrogels are currently used in cartilage wound healing, bone regeneration, wound dress, and as carriers for drug delivery. Hydrogels are often favorable for promoting cell migration, angiogenesis, high water

content, and rapid nutrient diffusion (Bryant and Anseth, 2001). Some of the examples of hydrogel forming polymers of natural origin are collagen (Wallace and Rosenblatt, 2003), gelatin (Kim *et al.*, 2004) and chitosan (Francis and Mathew, 2000)

1.4.6.4 Collagen as wound healing biopolymer

Wound dressings are generally classified as

1. Passive products,
2. Interactive products and
3. Bioactive products, based on its nature of action.

Traditional dressings like gauze and tulle dressings that account for the largest market segment are passive products. Interactive products comprise of polymeric films and forms, which are mostly transparent, permeable to water vapour and oxygen but impermeable to bacteria. These films are recommended for low exuding wounds. (Schoof *et al*., 2001; Kuberka *et al*., 2002) Bioactive dressing is one which delivers substances active in wound healing; either by delivery of bioactive compounds or dressings constructed from material having endogenous activity. These materials include proteoglycans, collagen, non-collagenous proteins, alginates or chitosan. (Ruszczak, 2000; Hansen *et al*., 2001; Froget *et al*., 2003; Gomathi *et al*., 2003). In November 1999, Food and Drug Administration of the United States of America (FDA) reclassified the dressing categories as,

1. Non-resorbable gauze/sponge dressing for external use,
2. Hydrophilic wound dressing,
3. Occlusive wound dressing,
4. Hydrogel wound and burn dressing and
5. Interactive wound and burn dressings.

Both artificial and natural polymers have been used to reconstitute dermis. Collagen is a natural substrate for cellular attachment, growth and differentiation, and promotes cellular

proliferation and differentiation. Natural polymers such as fibrin (Keiser *et al.*, 1994; Siedler and Schuller, 2000), hyaluronic acid (King *et al.*, 1991; Murashita *et al.*, 1996), fibrinogen (Vacanti and Langer, 1998) and collagen (Ruszczak, 2000; Hansen *et al.*, 2001; Froget *et al.*, 2003; Gomathi *et al.*, 2003) have been recently tested in different matrix systems for local drug delivery and wound healing.

Collagen is unique in possessing different levels of structural order: primary, secondary, tertiary and quaternary (Ho *et al.*, 2001). In vivo, collagen molecules form fibers having a specific internal and structural orientation and strengthened together by two types of covalent crosslinking: intramolecular and intermolecular. Intermolecular cross-linking is essential to form macromolecular fibers and, consequently, for its mechanical stability and other physical properties.

Collagen is a natural substrate for cellular attachment, growth and differentiation in its native state. In addition to its desirable structural properties, collagen has functional properties. Certain sequences of the collagen fibrils are chemotactic and promote cellular proliferation and differentiation. Collagen provides considerable strength in its natural polymeric state. The source of collagen either purified from animal sources or as an integral component of a more complex extracellular matrix, and its treatment prior to use are important variables in the design of tissue-engineered devices. Biomaterials made of collagen offers several different advantages: they are biocompatible and nontoxic to tissues (including neural and brain tissue) and have well-documented structural, physical, chemical, biological and immunological properties. Additionally, mechanical and to some extent immunologic properties of collagen scaffolds can be influenced by modification of matrix properties (porosity, density) or by different chemical treatment affecting its degradation rate. Collagen contains a number of biological functional groups and has been clinically

used as a wound dressing. Its potential as artificial skin, bone grafts and pharmaceutics has been intensively investigated (Schoof *et al.*, 2001; Kuberka *et al.*, 2002).

Several methods of cross-linking and sterilization can be utilized to alter the rate of *in vivo* degradation or to change the mechanical properties of collagen (Friess and Lee, 1966). These methods include glutaraldehyde treatment, carbodiimide treatment, dye-mediated photooxidation, exposure to polyepoxy compounds and glycerol treatment.

Different approaches to utilize animal-derived collagen for tissue substitution have been developed in the past 20 years: (a) the collagen gel, made of a mixture of fibroblasts and bovine collagen, (b) the collagen sponge based upon the production of a lyophilized collagen matrix in which fibroblasts are cultured and migrate, (c) the synthetic mesh composed of a nylon or a polyglactic acid mesh on which fibroblasts are cultured, (d) the collagen membrane used alone or with reconstructed epidermal sheet, and (e) the *in vitro* reconstructed skin-like products based on collagen matrix (Ruszczak, 2000).

The use of collagen sponges or pads either plain or containing antimicrobial drug has been reported in successful regeneration of dermal component and acceleration of wound healing. Especially, the use of drug containing collagen sponges was found beneficial in both partial-thickness and full-thickness burn wounds.

1.4.6.5 Immunology and biocompatibility of xenogenic collagen material

The presence of an immune response to collagen or any other biomaterial must be viewed in the context of its clinical performance. The immune response to xenotransplants includes both natural and induced humoral components, while a humoral response to allo transplants is generally seen only after sensitization.

The level of natural antibodies that react with organ xenotransplants increases proportionally with the phylogenic distance between the xenogenic species involved. In organ transplantation, the presence of such antibodies leads to hyper acute rejection, which occurs within minutes to hours after revascularization, and, consequently, to the loss of the transplanted tissue. This negative phenomenon can be avoided if an acellular and avascular tissue or a purified connective tissue matrix made from a natural biologic polymer such as collagen is used (Sachs, 1998; Timpl, 1984; De Lustro *et al*., 1990)

Collagen is a naturally occurring, highly conserved protein that is ubiquitous among mammalian species and accounts for approximately 30% of all body proteins. Since it is one of the first proteins synthesized during embryogenesis and then during organogenesis, its homology between species is very high.

Bovine and porcine type I collagen provide a readily available source of scaffold material for numerous applications and have been shown to be very compatible with human systems. The traditional and still widely used method of collagen extraction from tissues such as skin, tendons and ligaments is solubilization, and then reconstitution into injectable low-osmotic gels, fibrils and pads (Schoof *et al*., 2001).

Empiric observation based on the widespread use of xenogenic collagen and collagen-derived products for more than 50 years indicates that, in the case of these highly purified or native xenogenic collagens, no danger of acute or latent immunologic reaction occurs. Thus, appropriately purified xenogenic collagen has little or no significant immunogenicity and no discernible threat of inducing a systemic autoimmune disease.

Experimental and clinical studies have shown that both a sponge and a film consisting of xenogenic collagen, which was applied to the injured surface of the skin, did not cause any foreign-

body reaction, nor any immune rejection reaction or sensitization (Vacanti and Langer, 1998; Soo *et al.*, 1993). The xenogenous collagen did not promote any extensive inflammatory reactions or immunologic rejection. The take of collagen implants and tissue remodelling was complete and quick, allowing them to be even immediately combined with autologous thin split-skin grafting as well as with full-skin grafting.

The implanted collagen sheet enhanced the initial adhesion of keratinocytes allotransplants, supporting biological activity of the cells. Moreover, collagen membranes have been successfully used to speed-up the healing and re-epithelialization of split-skin donor sites showing a benefit over other currently used methods (Horch and Stark, 1998; Ruszczak and Schwartz, 1999). The implantation of collagen-based dermis substitutes protect against the contracture of wound borders and against typical scar formation.

1.4.7 Fish collagen hydrolysate

Fish processing waste, which otherwise cause serious environmental pollution, is a promising cost effective collagen source (Arnesen and Gilberg, 2007). Fish collagen from skins, bones, fins and scales could be extracted and hydrolyzed by chemical pre-treatment and subsequent heating at temperatures higher than 45ºC (Najafian and Babji, 2012).

Collagen contains bioactive peptides inactive within their sequence which can be released during gastrointestinal digestion or by controlled enzymatic hydrolysis. These collagen and gelatin derived peptides may exert a wide variety of physiological activities in the body, and could have potential applications in functional foods. Interest in nutraceuticals is growing rapidly worldwide, as they are a safe alternative to pharmaceutical drugs, which use is sometimes limited by toxicity or intolerance reactions.

Collagen and collagen hydrolysates could be attractive nutraceuticals for their interesting bioactive properties. The beneficial effect of collagen or gelatin hydrolysates on different diseases has been reported in animal or clinical studies, and actually several supplements including collagen-derived peptides have been patented and are currently commercialized in the USA, Japan and many European countries. Moreover, hydrolyzed collagen products have received GRAS status (Generally Recognized as Safe) by the US Food and Drug Administration (FDA). Although mammalian collagen are widely used in the field of nutraceuticals, the use of collagen from marine-discarded sources for preparing protein hydrolysates is nowadays increasing, as they are not associated with the risk of outbreaks of bovine spongiform encephalopathy.

The resistance of some collagen-derived peptides to protein digestion is one of the most interesting properties of collagen hydrolysates. Several studies focused on the effect of oral intake in both animal and human models have revealed the excellent absorption and metabolism of Hyp-containing peptides. Some of these collagen-derived peptides have revealed biological activity *in vivo* after absorption from the digestive tract (Moskowitz *et al.*, 2000).

Extensive researches have reported that the collagen peptides derived from variety of fish have various bioactivities (Fahmi *et al.*,2004; Mendis *et al.*,2005; Jung *et al.*,2006; Huo *et al.*,2009; Gómez *et al.*,2010; Ngo *et al.*,2010).

1.4.7.1 Collagen hydrolysates production

The main source of collagen peptides are bovine hide, bone, pig skin or fish bones and fish skin. Marine sources are an alternative to bovine or porcine and they are not associated with the prions related to risk of Bovine Spongiform Encephalopathy (BSE) (Karim and Bhat, 2009). Collagen hydrolysates are manufactured in controlled hydrolysis process to obtain soluble peptides. The raw

material is washed, homogenized and demineralized with diluted mineral acid or alkaline. The raw material is extracted in several stages with warm water. Further enzymatic degradation of gelatin results in a final product which is collagen hydrolysate (Moskowitz, 2000). Clemente (2000) has presented enzymatic hydrolysis as the most appropriate method for preparation of tailor-made peptides.

Enzymatic hydrolyzing process can produce small fragments of collagen peptides. Furthermore, some of its bioactivity increased obviously (Huo and Zhao, 2009) and its antigenicity decreased (Fujita and Yoshikawa, 1999) after hydroysis.

A number of commercial proteases have been used for the production of these hydrolysates, including trypsin, chymotrypsin, pepsin, alcalase, properase E, pronase, collagenase, bromelain and papain. Besides commercial proteases, enzymatic extracts from fish viscera have been used to obtain bioactive hydrolysates from skin and bones of different fish. Protease specificity affects size, amount, free amino acid composition and, peptides and their amino acid sequences, which in turn influences the biological activity of the hydrolysates (Nam *et al.*, 2008)

1.4.7.2 Optimization of hydrolysate production

The functional properties of peptides are highly influenced by their molecular structure and weight, which are greatly affected by processing conditions. Enzymatic hydrolysis has become a valuable tool for modifying the functionality of proteins (Korhonen *et al.*, 1998). Several factors, like pH, time, enzyme activity, and temperature influence enzyme function, offering possibilities to control the process. Depending on the specificity of the enzyme, conditions applied in hydrolysis process, and the extent of hydrolysis, wide variety of peptides will be generated. The resultant protein hydrolysate will possess particular properties according to the new peptides generated (Zhang and Webster, 2009).

Response surface methodology has been a quite effective method of statistical and mathematical analysis for experiment data since it was first proposed by Box and Wilson (1951). It can evaluate the influence of all the variables in the multiple factor experiment design. The mutual interaction among factors can also be estimated simultaneously (Myers *et al.*, 2008). Hong *et al.*,2013; Emna *et al.*,2013; Yong *et al.*,2009, in their studies successfully used RSM methods for optimizing enzyme hydrolysis conditions in the production of collagen hydrolysate.

1.4.7.3 Purification and identification of bioactive peptides

Enzymatically hydrolyzed fish peptides exhibit different physicochemical properties and biological activities depending on their molecular weight and amino acid sequence. Therefore the molecular weight of the bioactive peptide is one of the most important factors in producing bioactive peptides with the desired biological activities (Kim and Mendis, 2006; Kim and Wijesekara, 2010). An ultrafiltration membrane system can separate the peptides that have the desired molecular weights and functional properties from fish protein hydrolysates (Je *et al.*, 2005). Such a system can also control the molecular weight distribution of the appropriate peptide. Small peptides with different bioactivities are concentrated from the higher molecular weight fractions and remaining enzymes using a membrane with a low-molecular mass cut off such as 500, 1000 or 3000 Da. Nanofiltration, ion exchange membranes, and column chromatography can also be used (Pihlanto and Korhonen, 2003)

Often the most useful method for peptide separation is HPLC. Commercially available reversed-phase columns allow rapid separation and detection of their hydrophilic and hydrophobic characteristics (Shahidi and Zhong, 2008). Ferreira *et al.* (2007) recommended that peptides with different surface hydrophobicities can be separated by reversed-phase columns with a polystyrene divinyl benzene copolymer based packing. Choosing the right pore

size to achieve optimal separation of peptides is important, as the wrong pore size will result in poor resolution. In addition to pore size, the ligand on the gel also plays an important role in obtaining effective separation. Another essential factor for effective separation is determining the appropriate hydrophobicity of the gel. The appropriate pore size, hydrophobicity, particle size and column size should be combined to achieve high recovery and resolution in the isolation of peptides and proteins (Kuriyama *et al.*, 2005). HPLC is usually used in conjunction with other analyzing equipments including a UV detector or mass spectrometer.

Liquid chromatography followed by tandem mass spectrometry detection (LC–MS/MS) is commonly used to identify peptide sequences (Perkins *et al.*, 1999). Matrix assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometric analysis is also useful for generating peptide profiles of protein hydrolysates or semipurified fractions.

Collagen hydrolysates vary from each other in respect of peptides molecular weight, ranging from 2 to 6 kDa (Moskowitz, 2000; Zague, 2008). Its molecular weight is less than the average molecular weight of peptones. After purification, the product is concentrated and dried. The most common post-dried procedures are related to the control of molecular size and the elimination or reduction of bitterness in the resulting hydrolysates. The most efficient procedure to remove residual high molecular weight peptides and proteins or to reduce the antigen content of hypoallergenic formulas, is ultrafiltration (Clemente, 2000)

Several analyses may be done for the quality control of these products: the osmolarity, analysis of the hydrolysis degree, the molecular weight distribution, the total nitrogen, amino acid composition and the presence of toxic compounds (e.g. biogenic amines or pathogens). Protein hydrolysate qualitative analysis use different techniques based on spectrophotometric, chromatographic and electrophoretic methods (Emna *et al.*, 2013; Yong *et al.*, 2009)

1.4.7.4 Bioactive properties and applications of collagen hydrolysates

Collagen hydrolysates have been reported to have beneficial biological functions. Despite the fact that collagen hydrolysate has been generally regarded as having a low biological value, because it does not contain all of the essential amino acids, it's a reputable nutritional component often used to supplement other proteins because of its superb digestibility and high consumer tolerance (Zague, 2008). Its excellent properties are result of their amino acid composition and molecular structure. Peptide fractions from protein hydrolysates may vary in their effectiveness for a given biological activity. The average molecular weight of protein hydrolysates is one of the most important factors which determine their biological properties. Bioactive peptides usually contain 2–20 amino acid residues per molecule; the lower their molecular weights, the higher their chances of crossing the intestinal barrier and exerting a biological effect (Kim and Wijesekara, 2010)

According to the opinion of many researchers, beneficial effects of oral administration of collagen hydrolysates results by crossing the intestinal barrier, by dietary bioactive peptides, which reach the blood circulation and become available for metabolic processes (Zague, 2008). Collagen hydrolysates are used in medical applications, such as high-energy supplements, geriatric products and enteric, therapeutic or weight-control diets. Applications of protein hydrolysates are in treatment of patients with specific disorders of digestion, absorption and amino acid metabolism. Tests also included clinical cure of patients with malnutrition attached with trauma, burns, cancer and hepatic encephalopaties (Clemente, 2000). Collagen hydrolysates are good source of amino acids for people suffering from anorexia, anaemia and for vegetarians (because of absence of meat in their diet). Diet supplements containing collagen hydrolysates are considered as improvement

agents in tendon or joint regeneration in physically active athlets with activity related joint pain (Moskowitz, 2000 and Zague, 2008)

Orally consumed collagen hydrolysate has been shown to be absorbed intestinally and to accumulate in cartilage. Specifically, collagen hydrolysate ingestion stimulates a significant increase in the synthesis of extracellular matrix macromolecules by chondrocytes (Bello and Oesser, 2006). According to medical data clinical investigations suggest that ingestion of collagen hydrolysates reduces pain in patients suffering from osteoarthritis and osteoporosis. It is considered that about 15% of world populations suffer from joint pain-related diseases.

Increasing risk agents are senility (over 50% of elderly people suffer from rheumatism), gender (a high amount of patients are women, particularlly after menopause), body weight (huge body weight is a reason of joint overload and results in joint pain), constantly excessive sport activity, joint injury (e.g. dislocations), metabolic diseases (e.g. diabetes). Collagen hydrolysates are involved in cartilage matrix synthesis (Zague, 2008). For the last two decades scientists have studied a relationship between therapeutic trials in joint diseases and collagen, gelatin or collagen hydrolysates. In numerous studies researchers accepted dose of 10g of collagen hydrolysates daily as a safe and well tolerated by patients. Additionally clinical tests have proved that this level of daily ingested proteins can reduce the pain in comparison with placebo group patients (Moskowitz, 2000).

Several scientific reports have presented good bioavailability of hydrolyzed collagen, by animals and human beings after oral administration. Oesser *et al.* (1999) discovered that about 95% of orally applied collagen hydrolysate was absorbed within the first 12h. Zague, (2008) described the high safety of eating collagen hydrolysates in an animal model (1.66 g/kg of body weight per day). Studies related with preparations consisting gelatin derivated peptides showed good tolerance and little side effects including a

sensation of unpleasant taste, a feeling of heaviness in the stomach, and a bloated feeling or pyrosis after oral administration (Oesser *et al.*, 1999)

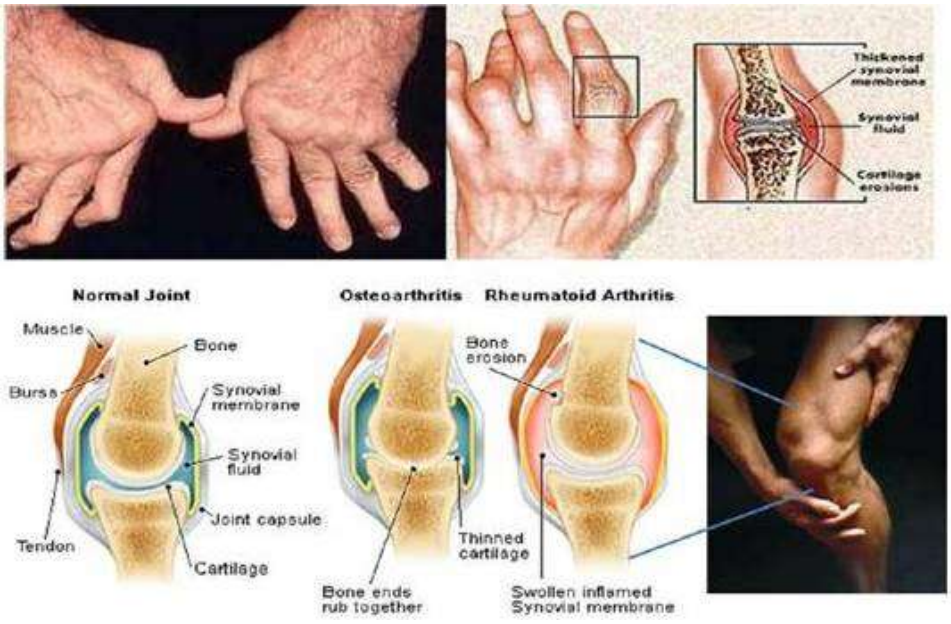
1.4.8 Role in bone and joint disease – Arthritis

Arthritis is a form of joint disorder that involves inflammation of one or more joints. There are different forms of arthritis. The most common form, osteoarthritis is a result of trauma to the joint, infection of the joint, or age (Felson *et al.*, 2000; Leyland *et al.*, 2012). Osteoarthritis is characterized by progressive destruction of joint cartilage and its associated structures (bone, synovial and fibrous joint capsules), remodeling of the periarticular bone, and inflammation of the synovial membrane (Blagojevic *et al.*, 2010). This disorder is basically produced by an imbalance between the synthesis and degradation of the articular cartilage. This imbalance leads to the classic pathologic changes of wearing away and destruction of cartilage. (Kuptniratsaikul *et al.*, 2002; Loeser *et al.*, 2012)

Rheumatoid arthritis (RA) is characterized by inflammation of the synovial membrane of diarthrodial joints. Early indications of RA are swelling and pain of the proximal inter-phalangeal and later, the larger joints become affected, especially those of the knee, elbow and ankle. (Mc Innes and O'Dell, 2010) Hyperplasia or thickening of the synovial membrane is promoted by cytokines and growth factors released from migrating cells. The synovial membrane becomes revascularised making it redder than normal. The cytokine enriched environment produced by pro-inflammatory cytokines (IL-1α, IL-6 and TNF-α) results in the aberrant growth of complex vessels known as pannus which invades the cartilage resulting in the degradation of the articular surfaces (Astusi *et al.*, 2005; McInnes and Schett, 2011).

Rheumatoid arthritis progresses in three stages. The first stage is the swelling of the synovial lining, causing pain, warmth, stiffness, redness and swelling around the joints. Second is the rapid division and growth of cell, or pannus, which causes the synovium to thicken. In the third stage, the inflamed cell releases enzyme that may digest the bone and cartilage, often causing the joints to lose its shape and alignments, more pain and loss of movements (Scott *et al.*, 1998; Mc Innes and Schett, 2011).

Figure 1.3 *showing deformities in small joints of rheumatoid arthritis patient (top). Schematic diagram showing the comparison among normal and the joints affected with osteoarthritis and rheumatoid arthritis (below)*



1.4.8.1 Autoantibodies and anti CCP assay

The rheumatoid factor (RF) and anti-cyclic citrullinated peptide (CCP) antibodies are considered clinically useful as disease markers (Mewar and Wilson, 2006). The rheumatoid factor is probably the most studied autoantibody in RA. It binds to the Fc region of IgG and contributes to the formation of immune complexes. Antibodies specific to the post-translational modification (citrulline) on proteins like, keratin, filaggrin, fibrinogen, vimentin,

fibronectin, α-enolase, CII etc, referred as anti-citrullinated protein antibodies (ACPAs) also monitored in the arthritic conditions. ACPAs are rarely found in healthy individuals but in RA patients they seem to be 70–90% and have high disease specificity. Proteins are citrullinated during apoptosis and inflammatory process in RA, they are found years before disease onset in the plasma and levels seem to be elevated in the synovial fluid (Schellekens *et al.*, 2000).

Citrullination is the post-translational conversion of positively charged peptidyl arginine to neutral peptidyl citrulline. The conversion is catalyzed by Peptidyl Arginine Deamiminase (PAD) enzyme in the presence of calcium ions. In humans, five PAD isotypes (PAD1,PAD2, PAD3, PAD4 and PAD6) are described with varied tissue expression and only PAD2 and PAD4 have been found to express in inflamed synovial tissue of RA and in other inflammatory arthritides (Foulquier *et al.*,2007).

An ELISA was developed to detect antibodies directed against filaggrin derived from human skin and has high specificity and sensitivity for the diagnosis of RA (Palosuo *et al.*, 1998). The target amino acid in filaggrin is citrulline, a post-translationally modified arginine residue (Schellekens *et al.*, 1998). Subsequently, an ELISA assay for the detection of antibodies to a cyclic peptide containing citrulline was made comercially available, which was easier to standardize, and also had high sensitivity and specificity for the diagnosis of RA. This became the assay for the detection of anti-cyclic citrullinated peptide (anti-CCP) antibodies. Anti-citrullinated protein antibodies are highly specific for RA (De Rycke *et al.*, 2004). The citrullination is catalyzed by peptidyl arginine deiminase; arginine residues on fibrin and fibrinogen may be favored sites for deimination within rheumatoid joints (Kinloch *et al.*, 2008). Intracellular citrullinated proteins colocalized with the deimidase in 59 percent of RA synovial samples versus 17 percent of control samples. However, citrullinated proteins may also be found in the synovium of other forms of arthritis, in nonsynovial tissue from

patients with RA (e.g. pulmonary rheumatoid nodules), in the lungs of patients with interstitial pneumonitis, in brain from patients with multiple sclerosis, and in normal brain (Bongartz *et al.*, 2007).

1.4.8.2 Cycloxygenases

Cyclooxygenase (COX, also called Prostaglandin H Synthase or PGHS) enzymes contain both cyclooxygenase and peroxidase activities. COX catalyzes the first step in the biosynthesis of prostaglandins (PGs), thromboxanes, and prostacyclins; the conversion of arachidonic acid to PGH_2. It is now well established that there are two distinct isoforms of COX.

Cyclooxygenase-1 (COX-1) is constitutively expressed in variety of cell types and is involved in normal cellular homeostasis. A variety of mitogenic stimuli such as phorbol esters, lipopolysaccharides, and cytokines lead to the induced expression of a second isoform of COX, cyclooxygenase-2 (COX-2). COX-2 is responsible for the biosynthesis of PGs under acute inflammatory conditions. This inducible COX-2 is the target enzyme for the anti-inflammatory activity of nonsteroidal anti-inflammatory drugs. Pharmacological inhibition of COX can provide relief from the symptoms of inflammation and pain. Non-steroidal anti-inflammatory drugs, such as aspirin and ibuprofen, exert their effects through inhibition of COX. However these NSAIDS inhibit the activities of both COXs (COX I and COX ll). COX I is involved in the normal homeostasis mechanisms in the body and its inhibition can lead to the development of dyspepsia, erosions, gastric ulcers and renal dysfunction (Smith *et al.*, 2000).

1.4.8.3 Chondroprotectives

There are different classes of anti-arthritic drugs available like non-steroidal anti-inflammatory drugs (NSAIDS), Monoclonal antibodies, uricosuric agents, gold compounds, anti-cytokinine immunosuppressant like glucocorticoids, etc. Though the goal of

these drugs has been to relieve pain and to decrease joint inflammation, these drugs are known to produce various side effects including gastrointestinal disorders, organ damages, immunodeficiency and humoral disturbances. (Roth, 2005; Harirforoosh and Jamali, 2009) Selective COX-2 inhibitors make alternative approach to arthritic treatment with reduced GI side effects, but on long term treatment leads to serious cardiovascular and thrombotic side effects. Accordingly, reducing side effects should be considered while designing improved therapeutics for arthritis, besides enhancing medicinal effectiveness (Moore, 2007).

The extracellular framework and two-thirds of the dry mass of adult articular cartilage are polymeric collagen. Treatment with chondroprotectives, such as glucosamine sulfate, chondroitin sulfate, hyaluronic acid, collagen hydrolysate, or nutrients, such as antioxidants and omega-3 fatty acids are being increasingly recognized as an alternate approach to arthritic treatment (Jerosch, 2011; Henrotin *et al.,* 2012). Numerous clinical studies have demonstrated that the targeted administration of selected micronutrients leads to a more effective reduction of OA symptoms, with less adverse events. Their chondroprotective action can be explained by a dual mechanism: (1) as basic components of cartilage and synovial fluid, they stimulate the anabolic process of the cartilage metabolism; (2) their anti-inflammatory action can delay many inflammation-induced catabolic processes in the cartilage. These two mechanisms are able to slow the progression of cartilage destruction and may help to regenerate the joint structure, leading to reduced pain and increased mobility of the affected joint (Sawitzke *et al.,* 2010).

Treatment of arthritis includes analgesics and anti-inflammatory agents, lubricating, cushioning agents and nutritional supplements. Treatment for osteoporosis includes oestrogenic hormone replacement, bisphosphonates, calcitonin, selective oestrogen receptor agonists, fluorides, and parathormone derivatives.

Nonetheless, therapeutic responses are limited in many patients and it has adverse effects affecting health and organ systems of the body. Advances in treatment of osteoarthritis and osteoporosis include new and safer compounds (e.g., glucosamine, chondroitin sulphate, or methyl-sulfonyl-methane) capable of repairing damaged articular cartilage or at least decelerating its progressive degradation (Brief *et al.*, 2001).

Oral administration of collagen hydrolysate from shark skin has increased production of newly synthesized type I collagen and proteoglycan in the bone matrix of ovariectomized rats (Nomura *et al.*, 2005). Guillermin *et al.* (2010) administered a diet enriched with a collagen hydrolysate too variectomized mice during 12 weeks, and observed that osteoblast activity was increased at the end of the experiment, while differentiation and maturation of osteoclasts was lowered. These effects on osteoblasts and osteoclasts led to a significant stimulation of bone formation and mineralization.

Experimental studies have suggested that some collagen-derived peptides orally administered are absorbed intact in the intestine. Subsequently, these peptides would accumulate preferably in cartilage, where finally may stimulate cartilage metabolism (Oesser *et al.*, 1999). Some evidences exist on the ability of collagen hydrolysates to stimulate biosynthesis of type II collagen and proteoglycans in chondrocytes (Oesser *et al.*, 2003). Raabe *et al* (2010) have reported the marked effect of a fish collagen hydrolysate on chondrogenic differentiation of equine adipose tissue-derived stromal cells. These studies suggest that effectiveness of collagen hydrolysates on biosynthesis of macromolecules would be based on their unique amino acid composition, very similar to that of type II collagen. Oral administration of collagen hydrolysates would provide high levels of glycine and proline, two amino acids essentials for the stability and regeneration of cartilage (Walrand *et al.*, 2008). The therapeutic effect of collagen hydrolysates on osteoarthritis could also be mediated by the effect of specific

peptides on gene expression and function of chondrocytes. The effect of bioactive peptides on chondrocytes metabolism could be mediated by interaction with specific receptors on cell membranes (Nakatani *et al.*, 2009). To conclude, collagen hydrolysates are safer compounds that could provide, with less overall toxicity, a greater symptomatic relief than pharmaceutical drugs.

Chapter 2

ISOLATION & CHARACTERIZATION OF COLLAGEN FROM DIFFERENT SPECIES OF FISHES

Contents

2.1 Introduction

In recent years the utilization of waste from fish processing industry for the production of value added products has attracted substantial attention. The fish processing industry generates a large amount of waste. These wastes are a mixture of heads, viscera, skin and bone (Morrissey and Park, 2000). About 30% of such waste consists of skin and bone with high content of collagen (Gomez-Guillen *et al.*, 2002). Fish skin, which is a major byproduct of the fish-processing industry, could provide a valuable source of collagen (Badii and Howell, 2006). The solid waste from surimi processing, which may range from 50–70% of the original raw material (Morrissey *et al.*, 2005), could also be the initial material for obtaining collagen from under-utilized fish resources.

Collagen is one of the most abundant biological macromolecules of extracellular matrix where it provides the major structural and mechanical support to tissues. Native collagens from different sources find applications in biomedical and pharmaceutical industries as well as cosmetics. Denatured collagen, known as gelatin, finds applications in the food and biomedical industries. Biomedical and pharmaceutical applications of collagen include the

treatment of hypertension, urinary incontinence and pain associated with osteoarthritis, use in tissue engineering for implants in humans, inhibition of angiogenic diseases, such as diabetes complications, obesity, and arthritis (Rehn *et al.*, 2001).

Commonly, the main sources for collagen production are pig skin, cattle skin and bone. The outbreak of BSE, TSE and foot and mouth diseases has resulted in justified anxiety amongst users of cattle collagen (Kittiphattanabawon *et al.*, 2005). As a consequence, much attention has been paid to alternative sources of collagen, especially from fish skin and fish bone from the seafood processing industries. Gudmundsson and Hafsteinsson (1997); Choi and Regenstein (2000) have suggested that, the commercial use of fish skin and bones, which are normally discarded, is a good waste management practice leading to additional economic benefit.

So far, skin collagen from several fish species have been isolated and characterized such as big eye snapper skin (Jongjareonrak *et al.*, 2010), Nile perch skin (Muyonga *et al.*, 2004), Baltic cod skin (Sadowska *et al.*, 2003) and deep-sea redfish skin (Wang *et al.*, 2008). The major impediment to dissolution of collagen type I from tissue is the presence of covalent cross links between molecules. Collagen is insoluble in organic solvents. Water soluble collagen represents only a small fraction of total collagen and the amount depends on the age of the animal and type of tissue extracted. Collagen molecules present within fibrillar aggregates can be dissociated and brought into aqueous solution. However, the nature of the crosslinks prevalent in different tissues determines the particular extractant to be used and the corresponding yields.

Many protocols were tried for the extraction of collagen from fish processing discards, particularly fish skin. For isolation and characterization of collagen extracted from the skin of stripped catfish, the skin is washed with cold water at 5 to 8°C (Prabjeet *et al.*, 2011). Collagen is extracted at appropriate temperatures to obtain good quality collagen. However, according to Nagai and susuki (2000b) in isolation of collagen from fish waste material-skin, bone,

and fin, all the preparative procedures are to be done at 4°C. For extraction using pepsin-digestion, collagen extraction was done at 4°C for 24 hours before being salted with 0.5M NaCl (Nagai and susuki, 2000b). They extracted collagen from fish waste materials such as skin, bone, and fins, for three days using 0.5M acetic acid. However, the skin was not completely solubilized with 0.5M acetic acid for three days. So the residues were re-extracted with the same solution for a further two days. All residues were then solubilized and highly viscous solutions were obtained. Senar atne *et al.* (2006) who used 0.5M acetic acid combined with 10% (w/v) pepsin found that the fish skin completely got solubilized. The addition of 0.9M NaCl into collagen extract before centrifugation helped to induce salting out of collagen with better purification (Falguni *et al.*, 2010).

The physical, chemical and rheological properties of collagen depend upon their source. The properties of collagen markedly vary with the habitat, species, and part of fish being isolated (Falguni *et al.,* 2010). A number of studies have addressed properties of fish skin Collagen (Arnesen and Gildberg, 2007; Choi and Regenstein, 2000; Gomez- Guillen & Montero, 2001; Grossman and Bergman, 1992; Gudmundsson, 2002; Gudmundsson and Hafsteinsson, 1997; Holzer, 1996; Jamilah and Harvinder, 2002; Jongjareonrak *et al.*, 2006; Zhou and Regenstein, 2005) showing that their properties differ from those of mammalian collagens.

Fish collagen has lower denaturation temperatures compared to vertebrate's collagen. The denaturation temperature of mammalian collagen was higher than 30°C while most fish collagens denature at temperatures below 30°C (Ogawa *et al.,* 2003). Marine collagen had a lower denaturation temperature by about 10°C than that of the porcine skin collagen (Nagai *et al.,* 2008). This indicates that fish collagen is generally less stable than mammalian counterparts (Ogawa *et al.,* 2003). Bae *et al.* (2008) observed that the amino acid composition of collagens from cartilaginous fish differ from those of bony fish.

Collagen is composed of three triple helix polypeptide chains (2α and 2β chains). Each chain contains about 1000 amino acid residues in size and has an average length of 300 nm and diameter of 1.4 nm. Collagen triple helix is stabilized mainly by inter and intra chain water mediated hydrogen bonding as well as directs inter-chain hydrogen bonding (Brodsky and Persikov, 2005). Collagen has a repetitive primary sequence, of which every third residue is glycine (Whitford, 2005). Most fish collagens have been found to consist of two α-chain variants, which are normally denoted as α1 and α2 (Nagai *et al.*, 2001; Gomez-Guillen *et al.*, 2002). These alpha chain variants though having approximately the same molecular weight (~95,000Da) can be separated by SDS PAGE due to their different affinity for SDS (Kubo and Takagi, 1984).

Cho *et al.* (2005) reported that collagen extracted from yellow fin tuna skin showed better functional properties than those from other fish sources. Some studies have ascertained that freshwater fish can have a high collagen yield (Grossman and Bergman, 1992; Jamilah and Harvinder, 2002; Muyonga *et al.*, 2004a). Only a few studies have been conducted on warm-water fish collagen and these showed that collagen from these species had better functional properties than those from cold-water fish species (Gilsenan and Ross-Murphy, 2000; Grossman and Bergman, 1992)

Dog shark and skipjack tuna are commercially important species available along the south west coast of India. Rohu is one of the major carp species, a natural inhabitant of the freshwater sections of the rivers of India and contributes to the inland catch. At present, the fishery is sustainable for all the above species.

The objective of the present study was to isolate and characterize collagen from the skin of five different species of fishes viz. Albacore tuna (*Thunnus alalunga*), Dog shark (*Scoliodon sorrakowah*), and one among Indian Major Carps ie, Rohu (*Labeo rohita*), Queen Fish (*Scomberoides lysan*) and Grouper (*Epinephelus malabaricus*).

2.2 Materials and methods

2.2.1 Raw materials

The skins of the selected species in the iced condition were procured from Fort Cochin (9.9680°N, 76.2449°E), Kerala, India. Skin were stored in ice separately with a skin/ ice ratio of 1:2 (w/w) and transported within 1 h to the laboratory. The skin was washed with cold water (5-8°C) and cut into small pieces (2 ± 0.5 cm^2). The prepared skin samples were packed in polyethylene bags, added glaze water and kept at -20°C prior to collagen extraction.

2.2.2 Collagen extraction

Acid Soluble Collagen (ASC) and Pepsin Digestible Collagen (PDC) were extracted from the skin of five species of fishes. All the extraction procedures were carried out at 4°C.

2.2.2.1 Pretreatment of the skin

The source material was minced and mixed with 30 volumes of 0.1N sodium hydroxide and kept stirred for 24h over a magnetic stirrer to remove non collagenous protein. The treated mass was strained through a coarse sieve. The process was repeated twice and the residue was washed twice with 30 volumes of chilled distilled water.

2.2.2.2 Acid extraction

The residue was homogenized in a polytron homogenizer with 30 volumes 0.5M acetic acid for one minute and the same was stirred over a magnetic stirrer for 24 h. The supernatant after centrifugation (3000 rpm, 20 min) was collected. The residue was once again extracted with acid as above and the combined supernatant was taken as acid soluble collagen (ASC). The residue from the previous step was homogenized with 30 volumes of 0.5M formic acid for 1 min and stirred for 24 h. A solution of pepsin (enzyme / tissue ratio

1:100) was added to this and kept stirring for another 24h. The supernatant after centrifuging was taken as pepsin digestible collagen (PDC).

2.2.2.3 Salt precipitation and Dialysis

Crystalline sodium chloride was added to both supernatants to the level of 10% and stirred for 24h to precipitate the collagen. The precipitate was suspended in Tris-glycine buffer (50 mM containing 0.2M NaCl, pH 7.4) and dialyzed against the same buffer for 24h and then centrifuged. The collagen obtained was spray dried to get fine powder.

2.2.2.4 Percentage yield calculation

Collagen yield (dry basis) from the skin was calculated by the following formula:

(Weight of final collagen sample in g) / (weight of skin sample in g) × 100

2.2.2.5 Solubility

The collagen solubility was determined by the method of Montero *et al.,* 1991 with a slight modification. The collagens were dissolved in 0.5 M acetic acid to obtain a final collagen concentration of 3mg/ml and the mixture was stirred at 4°C until collagen was completely solubilized.

2.2.2.6 Effect of pH on collagen solubility

Solubility at different pH and NaCl concentration was determined by the method of Kittiphattanabawon *et al.* (2005). Collagen solution (8 ml) was added to a centrifuge tube and the pH was adjusted with either 6 N NaOH or 6 N HCl to obtain a final pH ranging from 1 to 10. The volume of solution was made up to 10 ml by distilled water previously adjusted to the same pH as the collagen solution. The solution was centrifuged at 20,000g at 4°C for 30 min.

Protein content in the supernatant was determined by Lowry's method, using bovine serum albumin as a standard. Relative solubility was calculated in comparison with that obtained at the pH rendering highest solubility.

2.2.3 Proximate composition analysis

The selected skin and the collagen extract were analyzed for the proximate composition. The content of moisture, protein, fat and ash were analyzed by the method of AOAC (2000).

2.2.3.1 Determination of moisture

The moisture content was determined according to the AOAC (2000) by drying 10.0 g sample at 105°C in a thermostatically controlled hot air oven. The sample were taken in a pre-weighed petri dish and kept in oven and the reduction in weight was checked by repeatedly weighing and then cooling the sample in desiccators till the weight become constant. Moisture content was expressed as percentage.

2.2.3.2 Determination of crude protein

Micro- Kjeldahl distillation method for crude protein

One gram of the homogenized sample was accurately weighed into digestion tube. About 2.0 g of digestion mixture ($CuSO_4$ & K_2SO_4) as in the ratio of (1:8) and 10ml of H_2SO_4 (AR) were added to the sample taken in the digestion tube. The sample was digested to a clear solution in digestion unit and the solution thus obtained was made up with 100 ml of distilled water by intermittent cooling. 5ml of this sample solution was pipetted out into the Kjeldhal micro distillation apparatus, followed by the addition of 10 ml of 40% NaOH. The ammonia produced on steam distillation was absorbed into 2% boric acid solution with Tashiro's 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 of 6.25 and expressed as percentage.

2.2.3.3 Determination of crude fat

The estimation of crude fat content was done by continuous extraction of fat with petroleum ether, by soxhlet extraction method (AOAC, 2000). About 2 g of 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 solvent in the receiving flask was evaporated completely and weighed for fat content. The result was expressed as percentage of crude fat.

2.2.3.4 Determination of ash

The ash content was measured by incineration of the sample according to AOAC, 2000). About 2.0g of moisture free sample taken in a pre-weighed clean dry silica crucible was charred on a low heat, followed by incineration in a muffle furnace at 550°C to get a white ash. Silica crucible were finally cooled in desiccators and weighed. Ash content was expressed as percentage.

2.2.4 Characterization of extracted protein

2.2.4.1 Amino acid composition analysis by HPLC

Collagen samples were hydrolyzed in 6N HCl at 120°C for 24h. After cooling the test tubes the contents were filtered using Whatman No 1 filter paper. The tubes were rinsed with distilled water and filtered. The filtrate was evaporated in a vacuum flash evaporator. Then deionized water was added into the tubes and continued evaporation until the contents were acid free. The process was repeated for three times and the free amino acids were dissolved in 0.05M HCl and filtered using 0.45 micro syringe, then injected in to Shimadzu HPLC using the method of Ishida *et al*. (1981).

2.2.4.2 SDS-poly acryl amide gel electrophoresis

Electrophoretic patterns of different species of collagens were analysed according to the method of Laemmli (1970). The samples were dissolved in 50 g/L SDS solution. The mixtures were then heated at 85°C for 1h, followed by centrifugation at 8500g for 5 min to remove undissolved debris. Solubilized samples were mixed with the sample buffer (0.5 mol/L Tris–HCl, pH 6.8 containing 40 g/L SDS, 200 mL/L glycerol in the presence or absence of 100 mL/L βmercaptoethanol) with the ratio of 1:1 (volume ratio).

The mixtures were loaded onto a polyacrylamide gel made of 75 g/L separating gel and 40 g/L stacking gel and subjected to electrophoresis at a constant current of 20mA/gel. After electrophoresis, gels were fixed with a mixture of 500 mL/L methanol and 100 mL/L acetic acid for 30 min, followed by staining with 0.5 mL/L Coomassie blue R-250 in 150 mL/L methanol and 50 mL/L acetic acid for 1 h. Finally, they were destained with a mixture of 300 mL/L methanol and 100 mL/L acetic acid for 1h and destained again with the same solution for 30 min. High molecular weight protein markers were used to estimate the molecular weight of proteins. Type I collagen from calf skin was used as standard collagen.

2.2.4.3 UV spectrophotometric analysis

Collagen was dissolved in 0.5 M acetic acid to obtain a concentration of 1mg/ml. The solution was then subjected to UV–Vis measurement. Prior to measurement, the base line was set with 0.5 M acetic acid. The spectrum was obtained by scanning the wavelength in the range of 220–600 nm.

2.2.4.4 FTIR analysis

FTIR spectra of collagens were carried out as per the method described by Muyonga *et al.* (2004b) using a Nicolet Avatar 360 ESP Infrared Spectrophotometer at a scanning range from 400 to 4000

cm^{-1} at data acquisition rate of 4 cm^{-1} per point. Spectra were obtained from tablets containing 2mg collagen samples in approximately 100 mg potassium bromide (KBr). All spectra were obtained after background substraction using the Omnic software.

2.2.5 Statistical analysis

All experiments were done in triplicates. Mean values with standard deviations (SD) were reported. Statistical calculations were performed in the SPSS 20 software package (IBM SPSS statistics, Version 20). Data analyses were performed using one way analysis of variance (ANOVA) with post-hoc multiple comparison analysis performed using Tukey's HSD test. P values ≤ 0.05 were considered statistically significant.

2.2 Result and Discussion

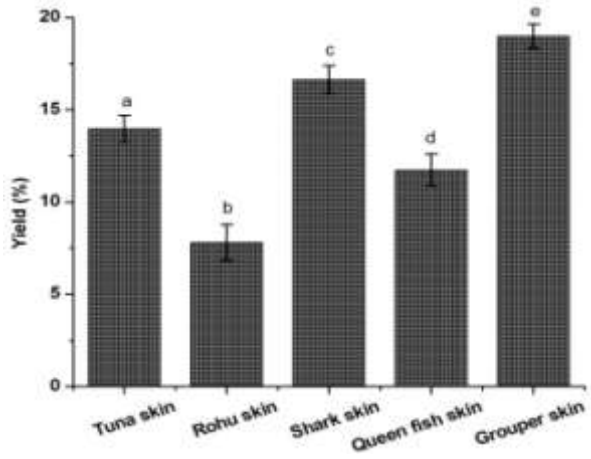
2.3.1 Collagen extraction

Acid Soluble Collagen (ASC) and Pepsin Digestible Collagen (PSC) were extracted from all the five species of fishes. The total collagen yields were 13.97% (±0.73%SD), 7.81% (±0.98% SD), 16.64% (±0.76% SD), 11.74% (±0.88% SD) and 18.99% (±0.65% SD) on dry weight basis for tuna skin, rohu skin, shark skin, queen fish skin and grouper skin respectively. Highest yield among the five species was obtained from grouper skin. One way ANOVA showed that there is significant difference in the yield of collagen from different species of fishes.

Tuna skin collagen has got completely dissolved in 0.5M acetic acid. In the case of other fishes, skin collagen was not completely solubilized with 0.5 M acetic acid, but further solubilization of the remaining residue was achieved by limited pepsin proteolysis. This result might suggest that there were lots of cross-links in fish skin collagens other than tuna at the telopeptide region, as well as at the inter-molecular cross-links, leading to a low solubility of collagen in acid (Foegeding *et al.,* 1996; Zhang *et al.,*

2007). With further limited pepsin treatment, the cross-links at the telopeptide region were cleaved without damaging the integrity of the triple helix. Therefore, a high solubility of collagen in acid was obtained after adding pepsin. The yields of ASC and PSC from different fish skins have been reported for black drum (2.3% and15.8%, respectively), for channel catfish (25.8% and 38.4% respectively), for paper nautlius (5.2% and 50% respectively) and for ocellate puffer fish (10.7% and 44.7%, respectively) (Nagai and Suzuki, 2002; Nagai *et al.*, 2002a; Ogawa *et al.*, 2003; Liu *et al.*, 2007)

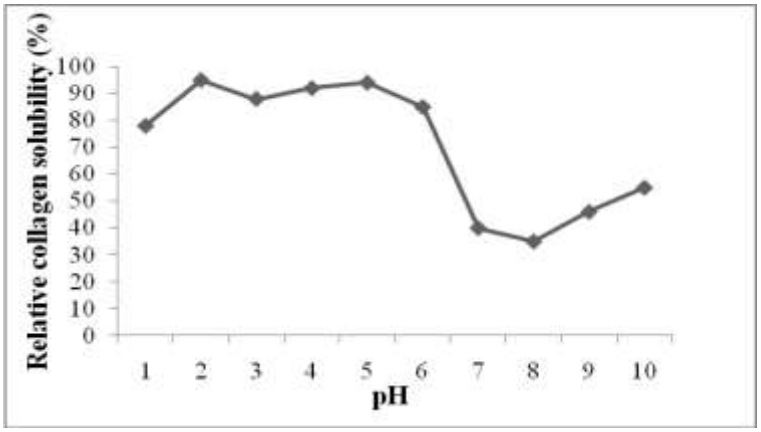
Figure 2.1 Yield of total collagen (%) from the skin of five species of fishes on dry weight basis.



2.3.2 Solubility of collagens

Highest solubility of collagens from the skin of the selected species was found at pH 2 and 5, respectively. Generally, both collagens could be more soluble in the acidic pH ranges (Foegeding *et al.*, 1996). Sharp decrease in solubility was observed at neutral pH. However, solubility was slightly decreased at very acidic pH. When pH values are above and below pI, a protein has a net negative or positive charge, respectively (Vojdani, 1996). As a consequence, more water interacts with the charged protein for water, thereby causing the protein to precipitate (Vojdani, 1996).

Figure 2.2 Relative solubility of grouper skin collagen at different pH



2.3.3 Proximate composition analysis

Table 2.1 shows the protein, moisture and ash content of the skin of the selected species of fishes and table 2.2 shows the same that of the extracted collagens. Generally skin of cartilaginous fishes which include shark and rays are low in lipid content. This lean species store majority of their fat in liver whereas skin of clupeid and scombroid species (sardines, mackerels and tuna) is rich in lipid.

One way ANOVA test was used to compare difference in the means of the moisture content, crude protein, fat and ash contents of the skins of different species of fishes. This was followed by tukey's post hoc analysis to determine in more detail how the proximate composition varies in the skin of different species of fishes. Analysis shows that there were significant differences in the moisture content of all the species. But there were no significant difference in the protein contents of queen fish and tuna skin and also in the case of grouper and rohu skin. Also there were no significant difference in the ash content of tuna and shark skin and fat content of queen fish and grouper skin. All other values were significantly showing differences in the protein, fat and ash content in different fish varieties.

Extracted collagens from skin had low contents of ash and fat, indicating the efficacy of removal of both inorganic matter and fat.

Collagen samples had low moisture contents, with protein content ranging from 88.8% to 91.72% (table 2.2).

Table 2.1 Proximate composition analysis of skin of the selected fish species

Sample	*Moisture*	*Protein*	*Ash*	*Fat*
Queen fish skin	64.65 ± 0.63^{a}	21.10 ± 0.17^{a}	7.02 ± 0.14^{b}	05.07 ± 0.16^{a}
Grouper skin	61.71 ± 0.36^{b}	18.47 ± 0.24^{b}	7.56 ± 0.58^{c}	$05.4\ 0 \pm 0.11^{a}$
Tuna skin	56.54 ± 0.09^{c}	20.54 ± 0.26^{a}	4.39 ± 0.03^{a}	08.32 ± 0.11^{b}
Rohu skin	76.54 ± 0.43^{d}	18.84 ± 0.06^{b}	2.03 ± 0.04^{d}	02.93 ± 0.05^{c}
Shark skin	68.38 ± 0.43^{e}	27.73 ± 0.36^{c}	4.19 ± 0.03^{a}	00.16 ± 0.02^{d}

All values are expressed as mean ± standard deviation, n = 3. Different superscripts in the same column indicate significant differences ($p < 0.05$)

Table 2.2 Proximate composition analysis of extracted collagen

Sample	*Moisture*	*Protein*	*Ash*	*Fat*
Tuna ASC	7.53 ± 0.30	91.08 ± 0.71	0.74 ± 0.02	0.64 ± 0.01
Rohu ASC	8.78 ± 0.06	89.94 ± 0.75	0.43 ± 0.05	0.33 ± 0.05
Rohu PDC	6.66 ± 0.58	91.72 ± 0.59	0.5 ± 0.02	0.45 ± 0.02
Shark ASC	9.13 ± 0.14	88.8 ± 0.53	0.76 ± 0.03	0.37 ± 0.08
Shark PDC	8.32 ± 0.17	90.8 ± 0.12	0.8 ± 0.02	0.42 ± 0.05
Queen fish ASC	7.53 ± 0.3	91.08 ± 0.71	0.64 ± 0.04	0.74 ± 0.04
Queen fish PDC	8.78 ± 0.06	89.94 ± 0.75	0.33 ± 0.04	0.43 ± 0.05
Grouper ASC	6.66 ± 0.52	91.72 ± 0.53	0.45 ± 0.02	0.5 ± 0.02
Grouper PDC	9.13 ± 0.14	88.8 ± 0.59	0.37 ± 0.02	0.76 ± 0.06

All values are expressed as mean ± standard deviation, n = 3.

2.3.4 Amino acid composition analysis

The amino acid compositions of collagen extracted from the various species are shown in Table 2.3. It is expressed as amino acids g/100g protein. The collagens were found to contain no tryptophan or cysteine. They were also very low in methionine, tyrosine and histidine contents, like in all other collagens.

Fish collagens have been shown to vary widely in their amino acid composition. In particular, the levels of imino acids (proline and hydroxyproline) varied significantly among fish species (Balian and Bowes, 1977; Gudmundsson and Hafsteinsson, 1997). The amount of imino acids, especially hydroxyproline, depends on the environmental temperature in which the fish lives and it affects the thermal stability of the collagens (Balian and Bowes, 1977; Kimura *et al.*, 1991; Rigby, 1968). When considering the imino acid (proline and hydroxyproline) of collagen extracted from the five species in the present study, they had a higher content when compared to, Sole (17.4%) Megrim (17.5%), Cod (15.6%), Hake (17.3%) and Squid (17.5%) (Gomez-Guillen *et al.*, 2002)

Glycine is the major amino acid in all species of collagens as reported by Senaratne *et al.* (2006) followed by proline, alanine, and hydroxyproline. In the collagen of ocellate puffer fish, glycine is the most abundant followed by alanine, proline, and glutamic acid (Takeshi *et al.*, 2002).

Table 2.3 Amino acid composition of Shark Skin Collagen (SSC), Rohu skin Collagen (RSC), Tuna skin Collagen (TSC), Queen Fish Skin Collagen (QSC) and Grouper Skin Collagen (GSC)

Amino acid	*Amino acids g/100g protein*				
	SSC	***RSC***	***TSC***	***QSC***	***GSC***
Aspartic	3.77 ± 0.38	4.43 ± 0.42	4.07 ± 0.31	3.47 ± 0.31	4.03 ± 0.42
Threonine	1.98 ± 0.17	2.30 ± 0.36	2.33 ± 0.32	2.67 ± 0.38	2.17 ± 0.25
Serine	3.53 ± 0.21	4.17 ± 0.40	4.37 ± 0.38	3.23 ± 0.35	3.80 ± 0.36
Glutamic	7.67 ± 0.21	6.27 ± 0.40	7.33 ± 0.40	6.43 ±0.32	4.90 ± 0.26
Proline	9.88 ± 0.27	11.77 ±0.85	10.23 ± 1.10	10.97 ± 0.55	12.27 ± 0.60
Glycine	32.53 ± 1.11	33.07 ± 1.24	33.43 ± 1.19	31.41 ± 1.98	31.23 ± 1.33
Alanine	11.23 ± 0.61	13.10 ±0.82	12.07 ± 0.60	12.57 ± 0.86	11.37 ± 0.47
Cystine	ND	ND	ND	ND	ND
Valine	2.60 ± 0.30	3.03 ± 0.42	2.90 ± 0.36	2.40 ± 0.36	2.73 ± 0.31
Methionine	1.03 ± 0.25	1.20 ± 0.36	1.27 ± 0.32	0.77 ± 0.15	0.90 ± 0.10
Isoleucine	1.57 ± 0.35	0.83 ± 0.06	1.10 ± 0.26	0.77 ± 0.12	1.03 ± 0.21

Leucine	2.23 ± 0.42	2.23 ± 0.38	2.03 ± 0.35	2.47 ± 0.38	2.33 ± 0.32
Tyrosine	0.13 ± 0.06	0.23 ± 0.06	0.30 ± 0.00	0.80 ± 0.10	0.37 ± 0.06
Phenylalanine	1.50 ± 0.36	1.90 ± 0.44	1.43 ± 0.23	1.37 ± 0.31	1.47 ± 0.21
Histidine	0.87 ± 0.21	0.70 ±0.10	0.87 ± 0.06	1.00 ± 0.20	1.43 ± 0.42
Lysine	2.77 ± 0.25	2.47 ± 0.38	2.57 ± 0.35	3.17 ±0.23	3.80 ± 0.44
Arginine	5.20 ± 0.46	5.53 ± 0.35	4.83 ± 0.31	4.57 ± 0.31	5.13 ± 0.45
H. Proline	9.83 ± 0.84	6.83 ± 0.67	7.90 ± 0.30	10.13 ± 1.39	9.77 ± 0.35
TOTAL	98.33	100.07	99.03	98.18	98.73
Imino acid (Pro + Hyp)	19.71	18.60	18.13	21.10	22.04

2.3.5 SDS-poly acrylamide gel electrophoresis

The protein patterns of ASC & PSC were analyzed by 7.5% resolving gel and it was found that the major constituents of both ASC & PDC consisted of α chains (α1 α2), β, γ chains. These patterns were similar to the type 1 collagen from calf skin (lane 7), and also in accordance with those of collagens from most other fish species previously reported (Muyonga *et al.*, 2004a; Nagai *et al.*, 2001).

Type I collagen consists of two identical α1 chains and one α2 chain (Burghagen, 1999; Foegeding *et al.*, 1996; Wong, 1989). Fish skin and bone have been reported to contain type I collagen as the major collagen (Ciarlo *et al.*, 1997; Montero *et al.*, 1991; Nagai and Suzuki, 2000b).

The skin collagens of bigeye snapper (Kittiphattanabawon *et al.*, 2005), brownbanded bamboo shark (Kittiphattanabawon *et al.*, 2010), Nile perch (Muyonga *et al.*, 2004a), ocellate puffer fish (Nagai *et al.*, 2002), back drum seabream, sheepshead seabream (Ogawa *et al.*, 2003), brown backed toadfish (Senaratne *et al.*, 2006), and largefin longbarbel catfish (Zhang *et al.*, 2007) all consisted of two α chains (α1& α2), β and γ components.

Figure 2.4 SDS PAGE Analysis: lane 1. High molecular weight markers, lane 2. Shark ASC, lane 3. Shark PDC, lane 4. Tuna ASC lane 5. Rohu ASC, lane 6. Rohu PDC, lane 7. Type 1 collagen from calf skin.

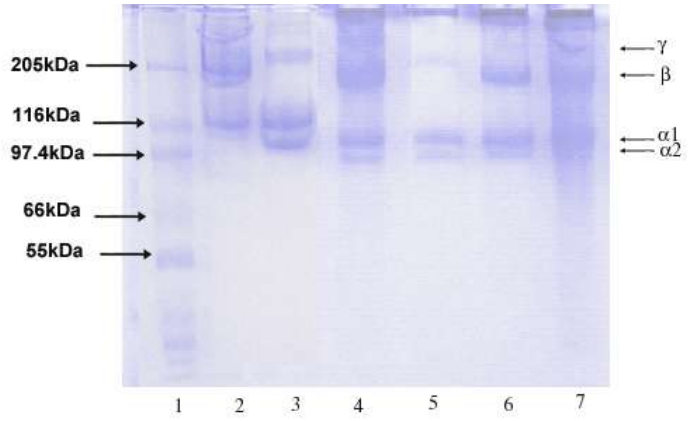
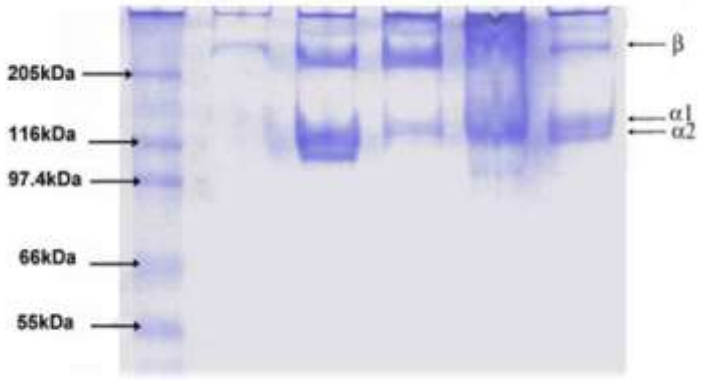


Figure 2.5 SDS PAGE Analysis: lane 1. High molecular weight marker, lane 2. Queenfish skin ASC, lane 3. Queen fish skin PDC, lane 4.Grouper skin ASC lane 5.Grouper skin PDC, lane 6. Type 1 collagen from calf skin.



2.3.6 UV spectrophotometric analysis

From UV–Vis spectra of the extracted collagens, an absorbance near 200-240 nm with high intensity was observed with no absorption peak at 280 nm. The results indicated high efficacy of non-collagenous protein removal. Collagen commonly has a low amount of tyrosine, which could absorb UV-light at 280 nm. The absorbance in this region is similar to those of collagens from channel catfish skin, walleye Pollock, and large fin long barbel

catfish (Prabjeet Singh *et al.*, 2011). Peptide bonds found in the protein also absorb at 205-230nm. The absorbance at 280nm is mainly because of tryptophan, tyrosine and phenyl alanine. Tryptophan was completely absent in the extracted collagen. Also there was negligible amount of tyrosine detected. Previous researchers indicated that collagen commonly have a low amount of tyrosine which can absorb UV-light at 280 nm. Fig.2.6 to fig 2.11 depicts various UV spectra analysis plots for the samples.

Figure 2.6 UV analysis of pure collagen from calf skin

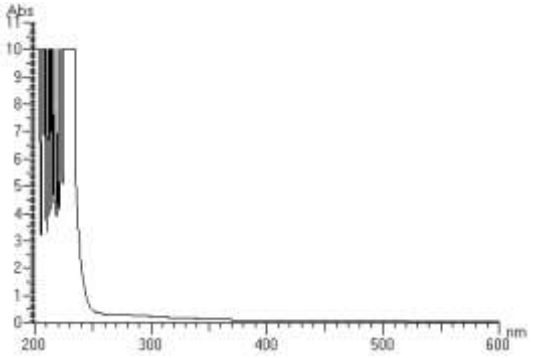


Figure 2.7 UV analysis of tuna skin collagen

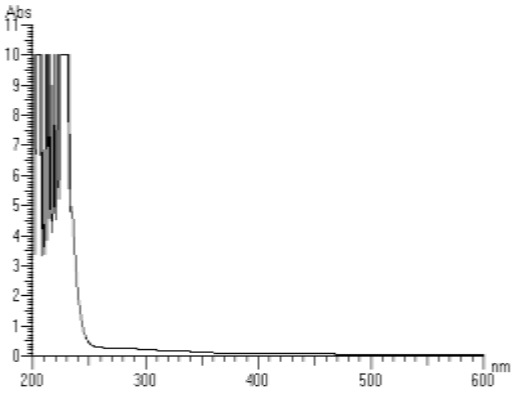


Figure 2.8 UV analysis of Rohu skin collagen

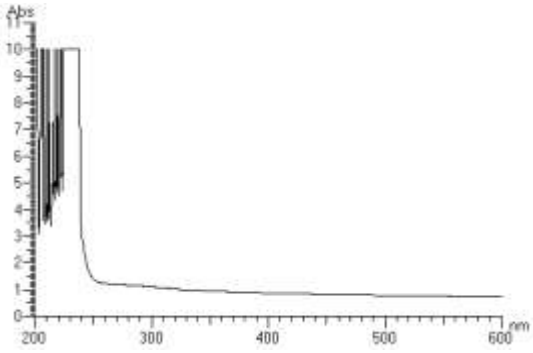


Figure 2.9 UV analysis of shark skin collagen

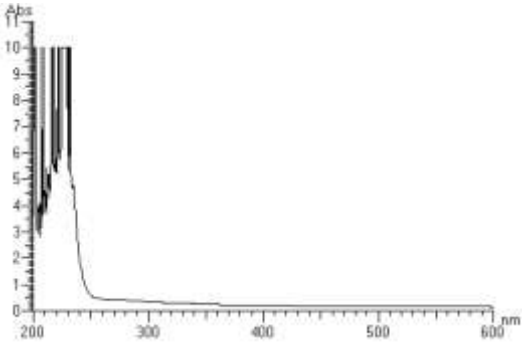
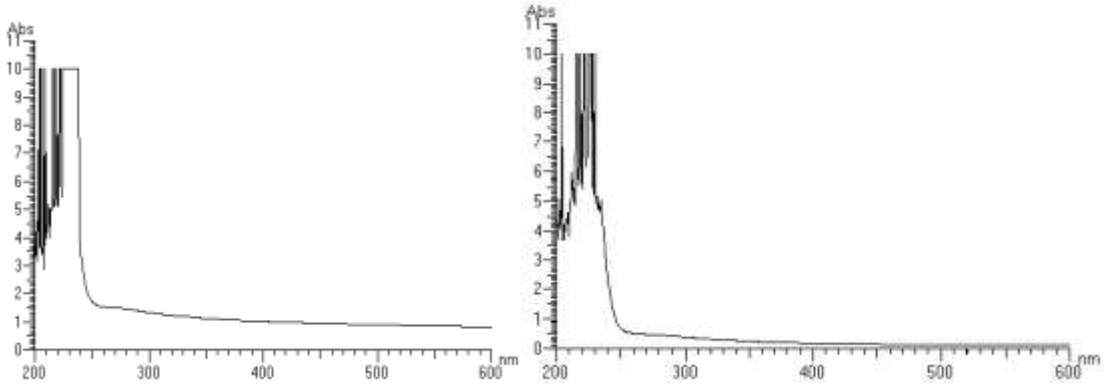


Figure 2.10 UV analysis of queen fish skin collagen

Figure 2.11 UV analysis of grouper skin collagen



2.3.7 FTIR analysis

The IR spectra obtained from the collagen extracted from the skin of different species of fishes are shown in fig. 2.12 – 2.14. The absorption spectra were obtained in the range between the wave number 400 – 4000cm^{-1}.

The analysis was used for detecting vibrational modes of individual chemical groups and bonds in the purified collagen. The spectra showed five characteristic amide bands representing amide A, amide B, amide I, amide II and amide III which are characteristic of the peptide bonds at the wavenumbers of 3413.39, 2926.45, 1644.98, 1245.79 and 1022.09 cm^{-1} respectively for shark skin. For grouper and croaker skin collagen the corresponding bands were observed in the wave numbers of 3419.17 and 3403.74 cm^{-1} for amide A band, 2916.64 and 2900.41 cm^{-1} for amide B band, 1629.55 and 1644.93 cm^{-1} for amide I band, 1205.5 and 1157.08cm^{-1} for amide II band and 1089.56 and 1063.55cm^{-1} for amide III band.

The amide B band positions found at wavenumbers in the range of 2900 to 2928 cm^{-1}, respectively, representing the asymmetrical stretch of CH_2. The amide I band with characteristic frequencies in the range from 1600 to 1700 cm^{-1} was mainly associated with the stretching vibrations of the carbonyl group along the polypeptide backbone, and was a sensitive marker of the peptide secondary structure. Amide II peak to N–H and C–N torsional vibrations, while amide III peak is associated to CH_2 residual groups from glycine and proline (Sionkowska *et al.,* 2006; Petibois *et al.,*

2006). Amide I band, amide II band and amide III band, which were known to be related to the degree of molecular order and to be involved with the triple helical structure of collagen, resulted from carbonyl group stretching, amino group bending and CH_2 stretching, respectively (Muyonga *et al.*, 2004). The FTIR spectra of skin collagen showed that the collagens extracted were of high purity.

Figure 2.12 FTIR spectra of queen fish skin collagen

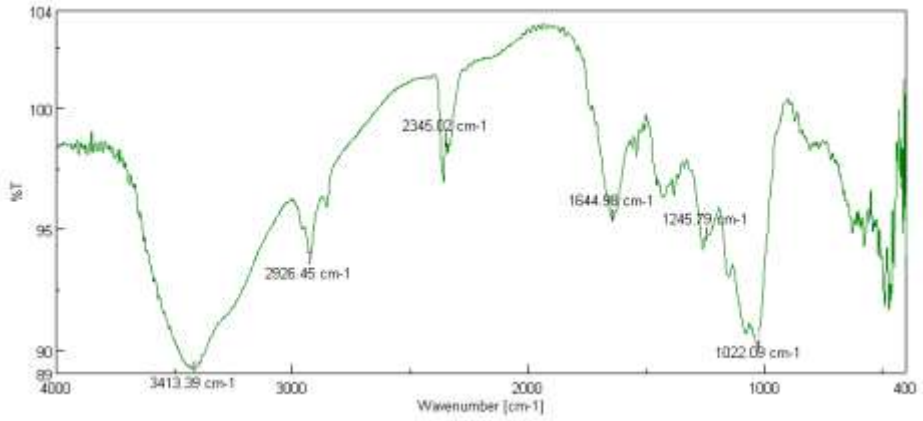


Figure 2.13 FTIR spectra of grouper skin collagen

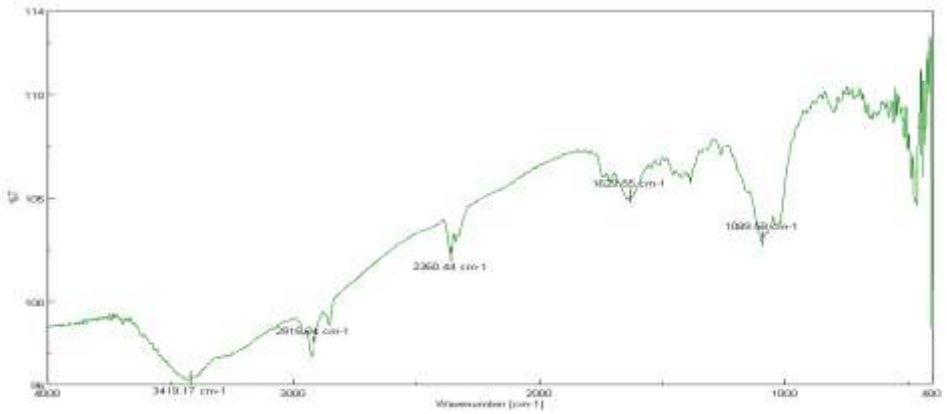
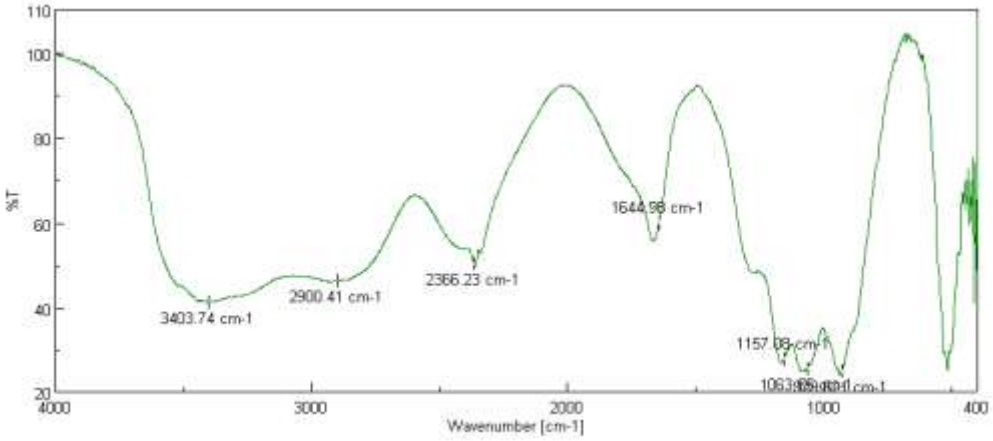


Figure 2.14 FTIR spectra of Shark skin collagen



2.4 Conclusion

A considerable quantity of acid soluble and pepsin digestible collagen were successfully extracted and characterized from the skin of five species of fishes. Pepsin aided extraction can serve as a tool for obtaining the greater yield without a marked effect on the triple-helical structure.

Extracted collagens from skin had low contents of ash and fat, indicating the efficacy of removal of both inorganic matter and fat and the protein content ranging from 88.8% to 91.72%. Further characterization studies with SDS PAGE, FTIR and UV Spectral analysis concludes that the extracted protein is collagen in pure form. HPLC analysis also confirms that the amino acid profile of extracted protein is characteristic to the protein collagen. The electrophoretic pattern reveals that the extracted skin collagen were type I nature and it consisted of two α-chains (α1 and α2).

Collagens showed high solubility at acidic pH (2–5) and the solubility markedly decreased in presence of NaCl (above 3%). High solubility of collagen is an attribute that makes it attractive as a commercial source. The results suggest that the extraction process yields collagen in pure form and could be used for wide applications in food, pharmaceutical, cosmetic and biomaterials.

Chapter 3

COLLAGEN HYDROGEL AS BIO INTERACTIVE DRESSING FOR WOUND HEALING

Contents

3.1. Introduction

A wound may be defined as disruption of the cellular or anatomical continuity of the normal organ structure. Wound healing is a complex process requiring coordination of cascade of cellular responses to injury including inflammation, epithelialization, proliferation, angiogenesis and remodeling. Healing involves migration, infiltration, proliferation, and differentiation of several cell types like keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets which culminate an inflammatory response, the formation of new tissue and wound closure (Barrientos *et al.*, 2008)

It is widely accepted that a warm, moist environment encourages rapid healing and most modern wound care products are designed to provide these conditions. Fluid balance in burn injury is very important since heavy loss of water from the body by exudation and evaporation may lead to a fall in body temperature and increase in the metabolic rate. Besides this, dressing should have certain other properties like ease of application and removal, and proper adherence so that there will not be any area of non-adherence left to create fluid-filled pockets for the proliferation of bacteria (Conti *et al.*, 2000)

The wound-healing process consists of four highly integrated and overlapping phases: hemostasis, inflammation, proliferation, and tissue remodeling or resolution (Gosain and DiPietro, 2004). These phases and their biophysiological functions must occur in the proper sequence, at a specific time, and continue for a specific duration at an optimal intensity. There are many factors that can affect wound healing which interfere with one or more phases in this process, thus causing improper or impaired tissue repair. The factors include oxygenation, infection, age and sex hormones, stress, diabetes, obesity, medications, alcoholism, smoking, and nutrition (Mathieu *et al.*, 2006).

Since the early 1980s, numerous wound dressings have been developed to promote wound healing (Balakrishnan *et al.*, 2005). 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 and non-antigenic, guarantee uniform cell distribution, maintain cell viability and phenotype, 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. 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 (Paddle-Ledinek *et al.*, 2006)

The most significant advancement in wound care came with Winter's (1962) study in 60's, which showed that occluded wounds healed much faster than dry wounds and moist wound healing environment optimized the healing rates. He demonstrated that when wounds on pigs are kept moist, epithelialisation is twice as rapid as on wounds allowed to dry by exposure to air. Later Hinman and Maibach (1963) confirmed Winter's work on human beings in 1963. An open wound, which is directly exposed to air, will dehydrate and a scab or escar is formed. This forms a mechanical barrier to

migrating epidermal cells and is then forced to move in a deeper level of tissue, which prolongs the healing process. Moist healing prevents the formation of scab as the dressing absorbs wound exudate secreted from the ulcer (Winter and Scales, 1963).

Hydrogels have been frequently utilized as scaffolds for soft tissue due to their excellent biocompatibility, biomimic microstructure and mechanical properties (Drury and Mooney, 2003; Jeon *et al.,* 2007). Natural polymers have similar components with native extra cellular matrix and are widely used for biomedical applications. Collagen and chitosan derivatives are among the most frequently used biomaterials due to their biocompatibility.

Hydrogels are attractive as biomaterials; they are highly permeable to water, ions, and small molecules (Peppas and Khare, 1993). Hydrogels comprised of naturally derived macromolecules have potential advantages of biocompatibility, cell-controlled degradability, and intrinsic cellular interaction. Hydrogels have structural similarity to the macromolecular based components in the body and are considered biocompatible (Jhon and Andrade, 1973).

Biocompatible hydrogels are currently used in cartilage wound healing, bone regeneration, wound dress, and as carriers for drug delivery. Hydrogels are often favorable for promoting cell migration, angiogenesis, high water content, and rapid nutrient diffusion (Bryant and Anseth, 2001). Some of the examples of hydrogel forming polymers of natural origin are collagen (Wallace and Rosenblatt, 2003), gelatin (Kim and Park, 2004) and chitosan (Francis Suh and Matthew, 2000)

Both artificial and natural polymers have been used to constitute hydrogels. Collagen is a natural substrate for cellular attachment, growth and differentiation, and promotes cellular proliferation and differentiation. Natural polymers such as fibrin (Keiser *et al*., 1994; Siedler and Schuller-Petrovic, 2000), hyaluronic acid (King *et al*., 1991; Murashita *et al*., 1996), fibrinogen (Vacanti and Langer, 1998) and collagen (Ruszczak, 2000; Hansen *et al*.,

2001; Froget *et al*., 2003; Gomathi *et al*., 2003; Ruszczak, 2003) have been recently tested in different matrix systems for local drug delivery and wound healing. Collagen is unique in possessing different levels of structural order: primary, secondary, tertiary and quaternary (Ho *et al*., 2001). In vivo, collagen molecules form fibers having a specific internal and structural orientation and are strengthened together by two types of covalent crosslinking: intramolecular and intermolecular. Intermolecular cross-linking is essential to form macromolecular fibers and consequently, for its mechanical stability and other physical properties.

Collagen acts as a natural substrate for cellular attachment, growth and differentiation in its native state. In addition to its desirable structural properties, collagen has functional properties. Certain sequences of the collagen fibrils are chemotactic and promote cellular proliferation and differentiation. Collagen provides considerable strength in its natural polymeric state. The source of collagen either purified from animal sources or as an integral component of a more complex extracellular matrix, and its treatment prior to use are important variables in the design of tissue-engineered devices.

Biomaterials made of collagen offers several different advantages: They are biocompatible and nontoxic to tissues (including neural and brain tissue) and have well-documented structural, physical, chemical, biological and immunological properties. Additionally, mechanical and immunologic properties of collagen scaffolds can be influenced by modification of matrix properties (porosity, density) or by different chemical treatment affecting its degradation rate (Schoof *et al*., 2001). Collagen contains a number of biological functional groups and has been clinically used as a wound dressing. Its potential as artificial skin, bone grafts and pharmaceutics has been intensively investigated (Kuberka *et al*., 2002).

Chitin and chitosan are regarded as appropriate biomaterials due to their physicochemical and biological properties. However,

acetic acid or organic solvents should be applied for material preparation, which would impart certain cytotoxicity to the final product (Shanmuga sundaram *et al.*, 2001). Carboxymethyl chitosan (CM chitosan), a water-soluble derivative of chitosan, has the merits of chitosan and has improved biocompatibility over chitosan (Zhu & Fang, 2005). Therefore, CM-chitosan has been extensively utilized in biomedical materials including moisture-retention agents, bactericides, wound dressings, artificial skin, blood anticoagulants and so on (Zhang *et al.*, 2000). Furthermore, CM-chitosan was capable of stimulating the extracellular lysozyme activity of fibroblasts, promoting the proliferation of normal skin fibroblasts and inhibiting the proliferation of keloid fibroblasts (Chen *et al.*, 2002).

An ideal wound dressing should have several key attributes. The dressing should protect the wound from bacterial infection, control evaporative water loss and prevent dehydration, control permeability of oxygen and carbon dioxide, absorb wound exudate, and enhance the healing. Additionally, it should be composed of materials that are non-toxic, non-immunogenic, flexible, durable, and comfortable when worn. Synthetic materials such as poly(urethane) (Hinrich *et al.*, 1963; Wright *et al.*, 1998) poly(vinyl alcohol) (Suzuki *et al.*, 1997), poly hydroxy ethyl methacrylate (Dressler *et al.*, 1980), and copolymers (Kim *et al.*, 2000), as well as biological materials like bovine collagen (Kim *et al.*, 2000; Boyce *et al.*, 1988b; Yannas and Burke, 1980 and Yannas *et al.*, 1982), chitin (Conti *et al.*, 2000 and Su *et al.*, 1997), and alginate (Choi *et al.*, 1999 and Choi *et al.*, 2001) have been investigated. However, until now there have been few reports of fish collagen based materials for wound dressings.

The present study aims to examine the wound healing activity of hydrogel prepared from queen fish skin collagen by conducting in vivo studies in albino rats. Circular incision wound model was used to screen the wound healing activity. Percentage closure of original wound area was calculated on various days and results indicated that

the percentage wound closure and re-epithelialization for the gel formulation treated group was comparable with those of standard group treated with megaheal. The percentage of re-epithelialization was examined by histopathological studies and biochemical analysis of reformed skin.

3.2 Materials and methods

3.2.1 Preparation of the hydrogels

Collagen gel and CM-chitosan powder, and distilled water at different ratios were mixed by a hybrid mixer for 10 min to form a homogenous gel. The total polymer concentration was fixed to 50% by weight with collagen to CM-chitosan at the ratio 1:0 and 4:1 respectively. The gels were filled into tubes (inner diameter 10mm) and subjected for γ irradiation using a ^{60}Co radiation facility, which was performed at room temperature with a dose rate of 20 Gy/min at a desired absorbed dose.

3.2.2 In vivo wound healing

The wound healing characteristics of the collagen hydrogel was evaluated in subcutaneous circular incision wound model on albino rats. The study was conducted with the approval from the Institutional Animal Ethical Committee (IAEC) of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals). Total 24 adult Wistar male rats (150-200gm) were divided into four groups with 6 animals in each group. Animals were housed under standard environmental conditions of temperature and 12 hours light and dark cycle. All the animals were provided with food and water *ad libitum*.

Before making a wound, the rats were anesthetized with 2.5% isoflurane. The surgical area was shaved with an electric razor, the mouse was strapped to a surgical board, and additional anesthesia was provided via a nose cone. After a deep surgical plane of general anesthesia had been reached, a wound, approximately 1cm in diameter, was created on the dorsal side of the mouse using curved

blade surgical scissors. Both the epidermal and dermal layers were removed.

3.2.3 Study design and Dosing schedule

Collagen hydrogel and megaheal ointment were applied topically, twice daily from day zero to day of complete healing or the 15th postoperative day whichever occurred earlier. There were four groups in the study viz. control, standard (Megaheal cream) and test groups.

Treatment Protocols: The animals were numbered, weighed and then divided into four groups with six animals in each as follows:

Group I: control group without any treatment.
Group II: standard ointment (Megaheal) applied.
Group III: Collagen hydrogel (test 1)
Group IV:Collagen chitosan hydrogel (test 2)

3.2.4 Wound contraction measurement

Wound contraction was noted by following the progressive changes in wound area planimetrically, excluding the day of wounding. The progressive changes in excision wound area were measured in mm^2 by tracing the wound boundaries on transparent paper on each 2 days interval until complete wound healing was achieved. The wound areas in all groups were recorded on graph paper. Wound contraction was expressed as reduction in percentage of the original wound formula

$$\% \text{ wound contraction} = \frac{(Ao - At)}{Ao} \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

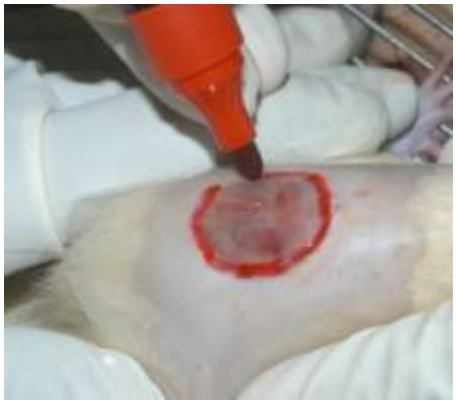
Where *Ao* is the original wound area and A_t , the area of wound at end of treatment.

Wound area was measured by tracing the wound margin using a transparent paper in each 2 days interval and healed area calculated by subtracting from the original area.

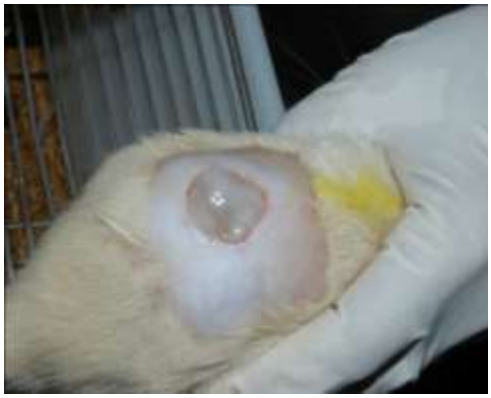
Figure 3.1 showing Mega heal ointment and the hydrogel prepared

Figure 3.2*(A) Photographic representation of measurement of wound area in excised rat(B)Collagen application in wounded area.*

(A)



(B)



3.2.5 Epithelialization period

Epithelialization time was noted as the number of days after wounding required for the scar to fall off leaving no raw wound behind. From the healed wound, a specimen sample of tissue was isolated from each group of rats for histopathological examination.

The un-epithelialized wound diameter was measured using an eyepiece micrometer. This measurement, together with the original wound diameter, was used in Eq. (2) to determine the percent re-epithelialization. The average of all six sections from each wound site was calculated and determined to be the average percent re-epithelialization for that wound.

$$\%\text{re-epithelialization} = \frac{(Do - Db)}{Do} \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

Where *Do* is the original wound diameter, *Db* is the length of un-epithelialized tissues at the times of biopsy.

Histopathological examination and biochemical parameters were carried out by using tissue specimen isolated from the healed skin of each groups of rat.

3.2.6 Histopathology

Formalin (10%) was used to fix the tissue and was embedded in paraffin wax. Serial sections of paraffin embedded tissues of 4µm were made. Sections were stained with hematoxyline eosin (H&E). All sections were analyzed using light microscopy (Olympus BX 45, Olympus, Hamburg Germany) by two pathologists in a blinded manner. The microscopic slides were photographed. Congestion, edema, PMNL, mononuclear cells, fibroblasts and vascularization were qualitatively evaluated as well as ulceration, necrosis and epithelialization were examined in the skin tissues.

3.2.7 Biochemical parameters

Circular wound area was excised and evaluated for various biochemical parameters at the end of the study. Especially collagen content, hydroxyl proline and hexosamine was estimated for evaluating the healing properties of collagen.

3.2.7.1 Estimation of hydroxyproline & collagen from reformed wound tissue

Hydroxyproline content was determined by Ehrlich's hydroxyproline assay (Reddy *et al*, 1996). Repaired wound skin tissues were dried in a hot air oven at 60–70°C to constant weight and were hydrolyzed in 6 N HCl at 130°C for 4 h in sealed tubes. The hydrolysate was neutralized to pH 7.0 and was subjected to Chloramine-T oxidation for 20 min. The reaction was terminated by addition of 0.4 M perchloric acid, colour was developed with the help of Ehrlich reagent at 60°C and the absorbance was measured at 557 nm using spectrophotometer.

Hydroxyproline content was converted to collagen content using the following equation (Ignateva *et al*, 2007):

Collagen (μg) = Hydroxyproline (μg) × dilution factor × 7.57

3.2.7.2 Estimation of hexosamine from reformed wound tissue

The hexosamine content was determined by the method of Wagner (Wagner *et al*, 1979). An aliquot of de-fatted sample was hydrolyzed with 3N HCl in a boiling water bath for four hours and neutralized. To 0.8 mL of neutral hydrolysate added 0.6 mL of acetyl acetone reagent and heated in a boiling water bath for 30 minutes. The hydrolysate was cooled and 2 mL of Ehrlich's reagent was added to it and mixed well. Absorbance was measured at 535nm. Glucosamine standards of concentrations 20mg to 80mg were similarly processed and absorbance values were recorded. From the standard graph, concentration of hexosamine in the test sample was calculated.

3.2.8 Statistical analysis

The results are expressed as Mean ± SE from n=6 observations. The findings were also analyzed for determining significance of difference by ANOVA test followed by pair-wise comparison of various group by LSD. The differences among groups were considered to be significant at $p<0.001$. The analysis was carried out by using SAS system version 9.3 (SAS Institute Inc., Cary, NC, USA)

3.3 Result and Discussion

3.3.1 Changes in wound area

Wound healing was assessed by monitoring wound contraction and re-epithelialization. Wounds supplemented with the hydrogel, had improved wound healing results compared to those wounds without any treatment (control group). The changes in the wound area in the course of experimental period are shown in table 3.1. Fig. 3.4 and 3.5 shows the results for both wound contraction and re-epithelialization, respectively. No significant difference in wound contraction was observed between any of the four experimental groups after 15 days of experimental period.

On the other hand, by day 15, wounds treated with test 1 and test 2 had significantly more re-epithelialization ($p<0.001$) than the controls group. At the end of experiment, the percentage of re-epithelialization was found to be 85±0.65 % for test treated group; whereas for control wounds this was 77.08±0.83 %.

Table 3.1*Changes in wound area (in mm^2) for in vivo wound healing experiments*

Group	0^{th} day	2^{nd} day	5^{th}day	8^{th} day	10^{th} day	12^{th} day	15^{th}day
Control	452.6±05.6	314.5±9.8	267.5±11.5	181.0±18.0	53.3±23.6	26.67±12.6	$23.3±09.3^{b}$
Std drug	455.5±14.3	336.1±12.4	213.6±8.5	141.8±6.4	38.5±8.7	12.6±2.04	$11.8±03.8^{a}$
Test 1	453.8±13.5	319.7±25.2	263.3±15.9	165.2±17.5	53.5±16.2	28.6±12.6	$12.2±17.9^{a}$
Test 2	458.3±13.6	320.6±22.6	256.12±11	177.66±13.9	55.8±8.0	26.3±15.8	$11.6±14.2^{a}$

Figure 3.3*Changes in wound area during the course of experimental period*

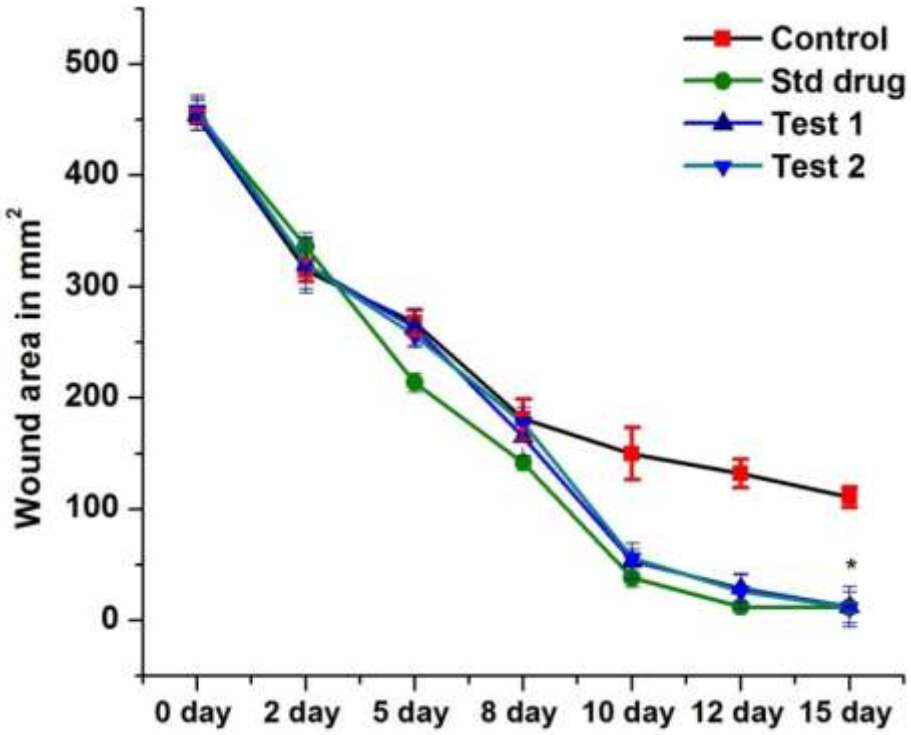


Figure 3.4 *Percent wound contraction for the in vivo wound healing experiments. Results are shown for the four experimental groups, treated with megaheal (group 2), test 1 (group 3) and test 2 (group 4) and compared against control group.(mean±SD, n = 6).*

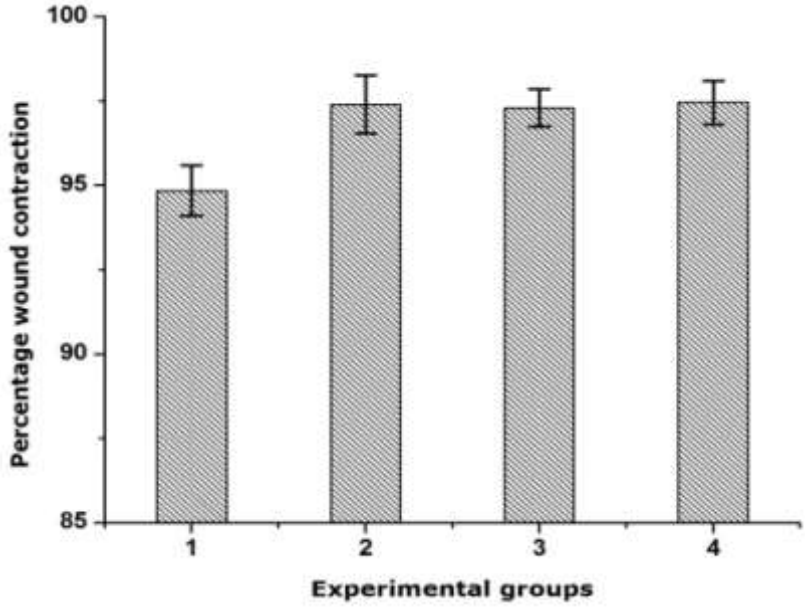
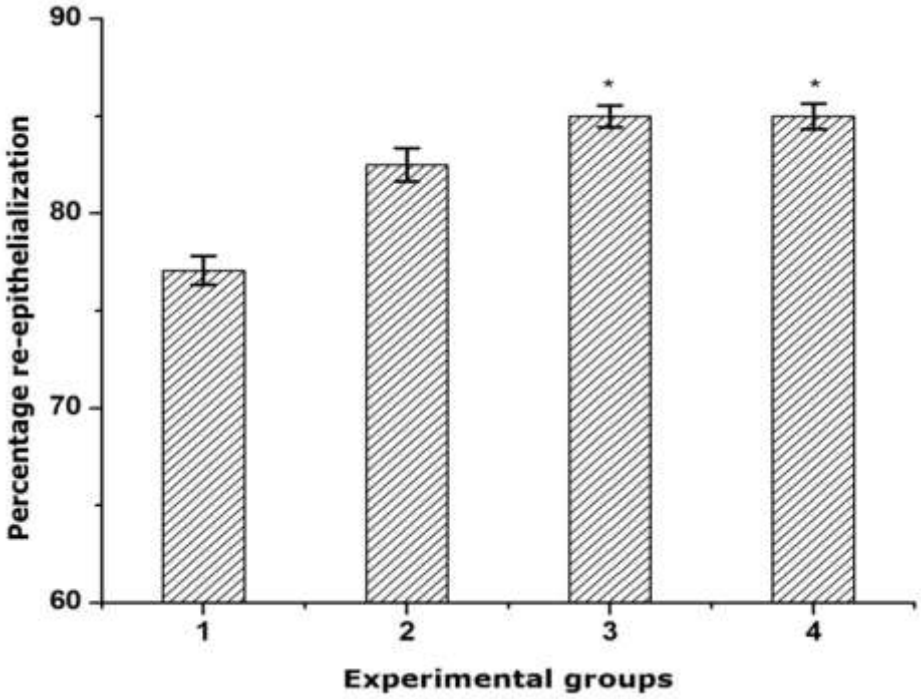


Figure 3.5 *Percent re-epithelialization for the in vivo wound healing experiments. Results are shown for the four experimental groups, treated with megaheal (group 2), test1 (group 3) and test 2 (group 4) and compared against control group. (mean±SD, n = 6). (* shows significance at p<0.001)*



3.3.2 Histopathological observations

Treatment of rat wounds with hydrogel and standard drug treated animals led to reduced macrophages, oedema, necrosis and increased collagen fibril and blood vessel formation. It can be seen that wounds treated with hydrogel were fully re-epithelialized with a well-structured layer of epidermis. Collagen was present in dermis. On the contrary, in control group increased number of macrophages, oedema, necrosis and less collagen fibril formation were observed. For some control wounds, moderate number of inflammatory cells was still present in the upper dermis. And the surface of the defect was not completely covered with new epithelium.

Figure 3.6 Wound healing profile of control group without any treatment. *Changes in the wound on 1st day, 3rd day, 5th day, 8th day, 12th day & 14th day shown in picture A- F respectively*

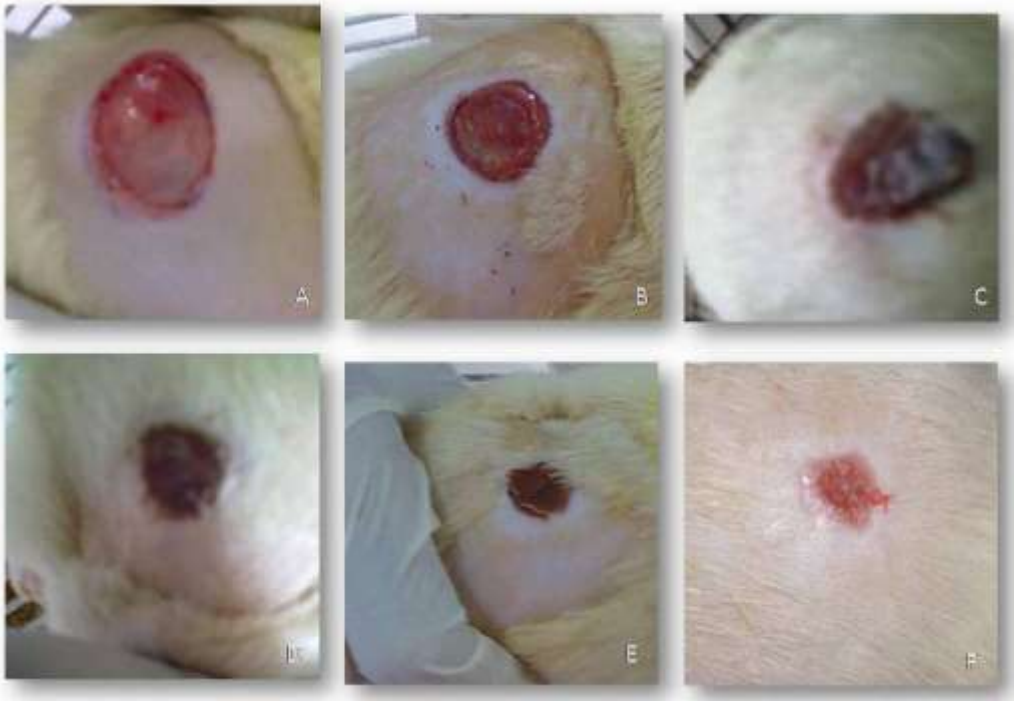
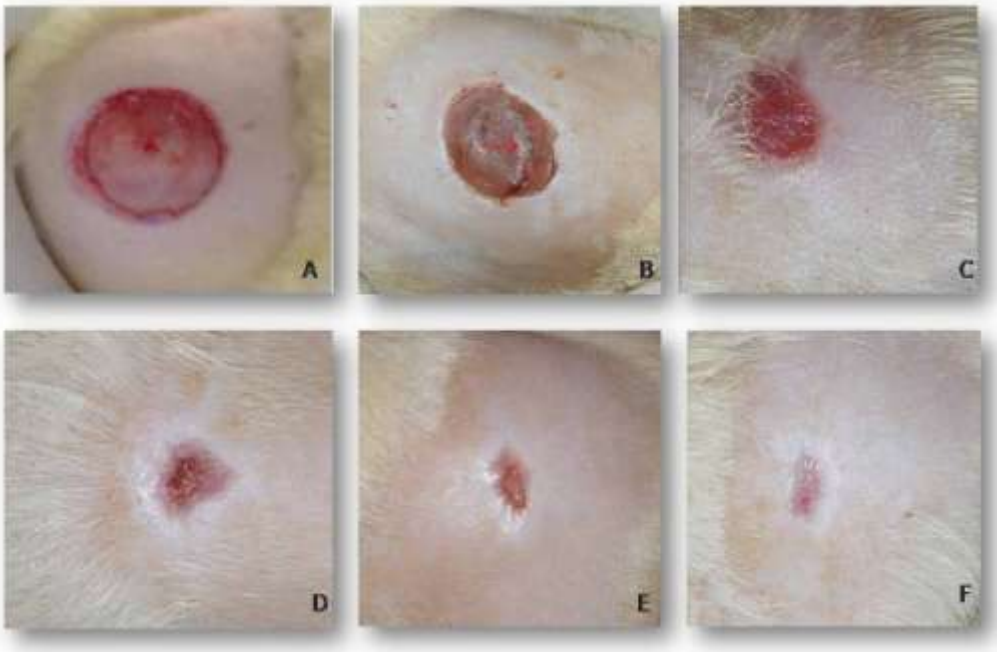


Figure 3.7 Wound healing profile of collagen hydrogel treated group (test 2). *Treatment effects in the wound on 1st day, 3rd day, 5th day, 8th day, 12th day & 14th day shown in picture A- F respectively*



3.3.3 Biochemical evaluation of reformed skin

Biochemical parameters of wound healing was evaluated and presented in figure 3.9 (A) and (B). There was a significant increase in the hydroxyproline content that is 74.93±2.214 and 74.00±2.729 µg/gm in test 1 and test 2 treated group respectively which was much more higher than control and standard drug treated group which showed the values of 46.13±0.675 and 62.15±3.935µg/gm. Increased hydroxyproline content is a reflection of increased cellular proliferation and therefore increased collagen synthesis (Ignateva *et al*., 2007). Generally an increase in hydroxyproline content is ultimately responsible for increase in collagen levels. In the present study control and standard drug treated animals showed much lesser collagen content which was 369.066±5.401 and 497.173±31.481 µg/gm as compared to test 1 and test 2 groups which showed 599.466±16.99 and 592.00±21.839 µg/gm concentration of collagen respectively.

For assessing wound healing property, the hexosamine content was evaluated in the reformed animal tissues. The hexosamine content was 22.91±10.55 and 25.15±7.96 mg/gm in the test 1 and 2 treated groups and the values for control and standard drug treated group were 6.15±13.25 and 16.31±12.50 mg/gm respectively. The values were statistically significant at $P<0.001$ when compared to control group. Hexosamine content increases in the early stages of wound healing and that the fibroblasts actively synthesize the ground substances on which the collagen can be laid on (Chitra *et al*., 1998).

Figure 3.8 Histopathological examination of newly formed 15th day wound tissue.

A: Control group B: Ointment (Mega heal) C: test 1 D: test 2 (x) Area of ulceration and (y) mixed type inflammatory cells.

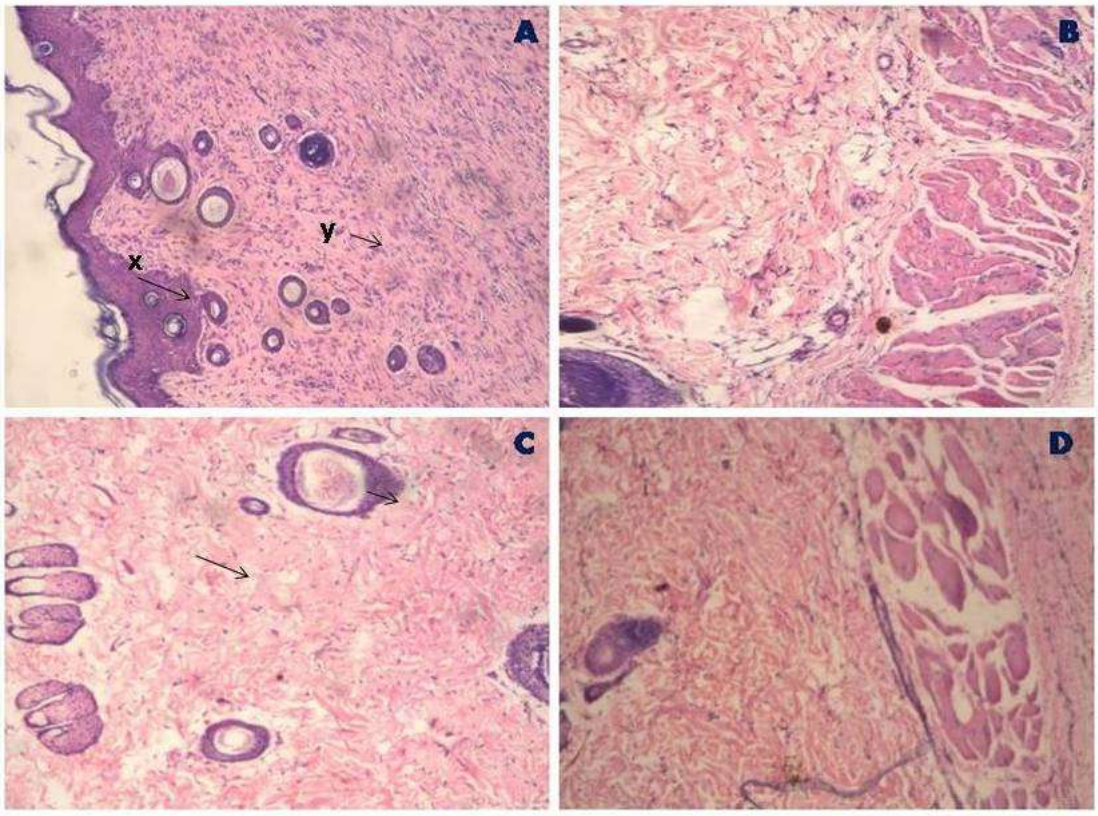


Figure 3.9 (A) Hydroxyproline and Hexosamine content in different experimental groups. *The graph shows that the increase in hexosamine content is obvious in the test rats when compared to control. (mean ± SD, n = 6)(p< 0.001). This indicates that the fibroblasts are actively synthesized, the ground substance on which the collagen can be laid on.*

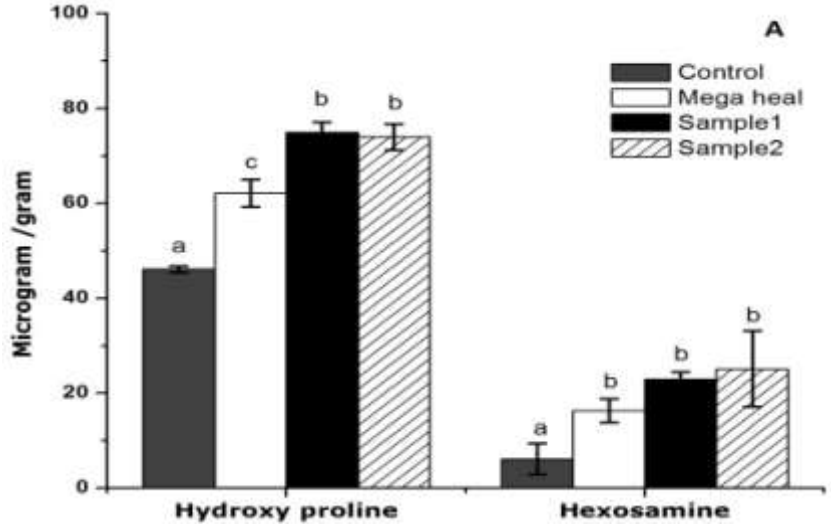
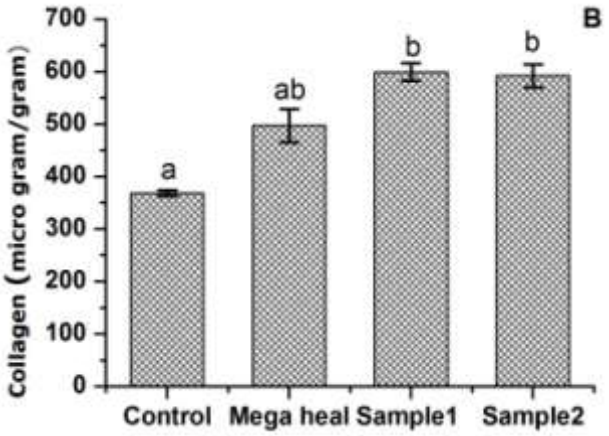


Figure 3.9 (B) Collagen content in different experimental groups. *It is evident from the graph that the amount of collagen has been increased in the test rats compared to control. (mean ± SD, n = 6)(p< 0.001).It has been stated that collagen provides tensile strength to tissues especially of healing wounds.*



3.5 Conclusion

In the present study, the wound healing efficacy of hydrogel was evaluated in experimental full thickness wounds using a rat model which demonstrated that within 2 weeks, the wound covered with gel was completely filled with new epithelium without any significant adverse reactions. There is significant increase in angiogenesis, collagen deposition, hexosamine content, epithelialization and wound contraction in hydrogel treated rats without inflammatory cells compared to the control group. Wound healing effects may be due to regulation of collagen expression and an increase in tensile strength of the wounds. Enhanced healing activity has been attributed to increased collagen formation and angiogenesis. Angiogenesis in granulation tissues improves blood supplementation to the wound site, thus providing nutrients and oxygen essential for the healing process.

Also the hydrogel provided moist environments which facilitate the smooth healing process. Winter showed that epithelialization can be accelerated if the wound is kept moist (Winter, 1962). Keratinocytes migrated more easily over a moist wound surface than underneath a scab (Winter and Scales, 1963). Epidermal cells can migrate at a speed of about 0.5 mm/day over a moist wound surface which is twice as fast as under a scab in dry wounds (Winter, 1972).

Wound contracture is a process that occurs throughout the healing process, commencing in the fibroblastic stage whereby the area of the wound undergoes shrinkage. It has 3 phases, inflammatory, proliferative, and maturational and is dependent upon the type and extent of damage, the general state of the host's health, and the ability of the tissue to repair. The inflammatory phase is characterized by hemostasis and inflammation, followed by epithelization, angiogenesis, and collagen deposition in the proliferative phase. In the maturational phase; the final phase of wound healing, the wound undergoes contraction resulting in a smaller amount of apparent scar tissue.

During wound healing, the edges of the wound pull inwards to reduce the overall wound area. Wound fibroblasts begin to assume a myofibroblast phenotype characterized by large bundles of actin-containing microfilaments and the establishment of cell–cell and cell–matrix linkages (Clark, 1996). The fibroblasts link to extracellular fibronectin and collagen and to each other through adherens junctions. Collagen bundles at the wound edge and the underlying dermis crosslink to forma collagen network. These cell–cell, cell–matrix, and matrix–matrix links provide a network through which the traction of the fibroblasts can be transmitted across the wound, leading to wound contraction.

Re-epithelialization is the process by which new cutaneous tissue covers the wound defect. This process requires the uninjured keratinocytes along the wound edges to migrate laterally to cover the wound bed. Both wound contraction (Noormohamed and Ray, 1998;

Rennekampff *et al.*, 1997) and re-epithelialization (Davis *et al.*, 1999; Singer, 1999) have been used as measures for monitoring wound closure and healing. In this study, wound contraction was monitored by measuring the area within the wound's full-thickness margins, and re-epithelialization was determined by biochemical composition analysis and histopathology studies.

Combining the collagen films with chitosan created a wound-healing environment that appeared to meet the criteria set forth above for ideal wound dressing. No signs of bacterial infection were apparent during gross examination of the wounds or histologically, suggesting that the hydrogel effectively protected the wound from bacterial infection. The wounds exposed to a collagen film were moist and hydrated, thus demonstrating that evaporative water loss and wound dehydration had been prevented. Abundant cellular proliferation suggested that oxygen and carbon dioxide permeability had been maintained. The absorption of wound exudates was clearly visible in those wounds supplemented with a collagen hydrogel. The hydrogel enhances re-epithelialization rather than a repair which was clear from the histopathological and biochemical analysis. Finally, the acceleration of re-epithelialization with the collagen treatments indicated an enhancement of the overall healing process.

Chapter 4

COLLAGEN PEPTIDE DEVELOPMENT, OPTIMIZATION AND CHARACTERIZATION

Contents

4.1 Introduction

Collagen peptide is the hydrolyzed form of collagen. After hydrolysis the product loses its gelling ability and makes it soluble in cold water. It differs greatly from other proteins as it contains amino acids glycine, proline and hydroxyl proline in a free form at a concentration that is around 10-20 times higher than in other proteins (Whitford, 2005). These amino acids play an important role in building fibrous tissues. An insufficient supply of these amino acids results in painful joints, brittle fingernails and hair. Many studies indicate that Collagen Peptide has a preventive and regenerative effect on bones, cartilage, and tendon etc. Collagen Peptide also contains all the essential amino acids except tryptophan (Nam *et al.*, 2008).

Skins, scales and bones are the major by-products of the fish processing industry. These by products are not regarded as ordinary saleable products and are usually discarded causing a heavy environmental impact. However, they are a good source of collagen. This collagen could be extracted and further enzymatically hydrolyzed to liberate physiologically active peptides. Specifically, some fish collagen derived peptides may exhibit interesting

antioxidant activity, potent antihypertensive activity, anti microbial activity against different strains of bacteria, protective effect on cartilage, or capacity to stimulate bone` formation. The bioactive properties of collagen-derived peptides, and also their resistance to protein digestion, make them potential ingredients of health promoting foods. (Nagai and Suzuki, 2000a)

Enzymatic hydrolyzing process can produce small fragments of collagen peptides. Furthermore, some of its bioactivity increased and the antigenicity decreased after hydrolysis (Suetsuna *et al.*, 2000). The functional properties of fish proteins may be improved by the use of specific enzymes and by choosing a defined set of hydrolysis conditions.

Collagen hydrolysate varies from each other with respect of peptides molecular weight, mostly their molecular weight range from 2 to 6 kDa. Its molecular weight is less than the average molecular weight of peptones. After purification, the product is concentrated and dried. The most common post dried procedures are related to the control of molecular size and the elimination or reduction of bitterness in the resulting hydrolysate. (Katarzyna and Piotr, 2009). The most efficient procedure to remove residual high molecular weight peptides and proteins or to reduce the antigen content of hypoallergenic formulas is ultrafiltration.

Bioactive peptides usually contain 2–20 amino acid residues per molecule (Pihlanto, 2000); the lower their molecular weights, the higher their chances of crossing the intestinal barrier and exerting a biological effect (Roberts, 1999). So, a three-step hydrolysis reaction was adopted, and orthogonal experiments were designed to optimize processing conditions which might result in high degree of hydrolysis. Depending on the specificity of the enzyme, environmental conditions, and the extent of hydrolysis, wide variety of peptides will be generated. The resultant protein hydrolysate will possess particular properties according to the new peptides generated (Zhang *et al.*, 2010). Several factors, like pH, time, enzyme activity,

and temperature, influence enzyme function, offering possibilities to control the process.

Present study aims to optimise the enzymatic process of collagen hydrolysate production using response surface methodology (RSM) and characterize collagen peptides from the skin of Malabar grouper with enzymatic hydrolysis methods using three different enzymes.

RSM has been a quite effective method of statistical and mathematical analysis for experiment data ever since it was first proposed by Box and Wilson (1951). It can evaluate the influence of all the variables in the multiple factor experiment design. The mutual interaction among factors can also be estimated simultaneously (Myers *et al.,* 2008). A polynomial regression equation can be given to predict the optimal condition of factors on the response. Here, we employed four main factors (pH, temperature, the ratio of enzyme to substrate (E/S) and time) as variables and five levels to optimize the hydrolysis of fish skin. The Degree of Hydrolysis (DH) was set as response to evaluate the efficiency of hydrolysis. Protein hydrolysate qualitative analysis used different techniques based on spectrophotometric, chromatographic and electrophoretic methods.

4.2 Materials and Methods

4.2.1 Raw materials

The species used for the study was Malabar grouper (*Epinephelus malabaricus*). The skin in iced condition was procured from Fort Cochin (9.9680°N, 76.2449°E), Kerala, India. Enzymes used for the hydrolyzation process were pepsin (624 U/mg), papain (3.0 U/mg), and protease from bovine pancreas (1.1 U/mg) from Sigma Chemical (St. Louis, MO, USA).

4.2.2 Optimization of enzymatic processing conditions by RSM

4.2.2.1 Experimental design

Box-Behnken design was applied to evaluate and optimize the effects of four controlled input parameters viz: pH (X_1), temperature (X_2), time (X_3), ES ratio (X_4) and their interaction on the measured response, DH (Y) for three different enzymes viz: pepsin, papain and protease. The input variables were coded at three levels (-1, 0, +1,) and the complete design consisted of 27 experimental points including 3 replications of the centre for each enzyme. The original and coded levels of the independent variables used in the RSM design for each enzyme are listed in Table 4.1.

Table 4.1 Uncoded and coded levels of independent variables used in the RSM design

		Pepsin			***Papain***			***Protease***		
Independent variable	***Coded variables***	***-1***	***0***	***1***	***-1***	***0***	***1***	***-1***	***0***	***1***
pH	X1	1.6	2	2.4	5	6	7	6	7	8
Temperature	X2	30	40	50	20	30	40	30	40	50
Time	X3	2	4	6	2	4	6	2	4	6
E/S	X4	0.5	2	8	0.5	2	8	0.05	0.2	0.8

4.2.2.2 Statistical analysis

The second-order polynomial regression model fitted to the DH (Y) as a function of input variables and the adequacy of the fitted model was assessed by using R^2 for each enzyme viz: pepsin, papain and protease. The functional form of the fitted model for each enzyme is given in the equation

$$Y = \beta_o + \sum_i \beta_i x_i + \sum_{ii} \beta_{ii} x_{ii}^2 + \sum_i \sum_{j,i<j} \beta_i \beta_j x_i x_j + e, i \neq j$$

Where, "Y" is response variable, "β_o" is intercept, "β_i" is linear regression coefficients, "β_{ii}" is quadratic regression coefficients, "$\beta_i\beta_j$" is interaction regression coefficients and "e" is error term. Ridge analysis was carried out to predict the response variable at different radius of the design region. The optimisation of response variables was done based on the ridge score and response surface plot of the response variables. The fitting of second-order polynomial regression model and numerical optimization was done by writing a SAS code in SAS 9.3.

Once the condition of pH (X_1), temperature (X_2), time(X_3), ES ratio (X_4) for maximum DH optimized based on the fitted model, the validation of the optimized condition was done for each enzyme with three replications for each enzyme.

4.2.3 Preparation of Collagen hydrolysate

The selected fish skin was thoroughly washed, minced and mixed with sodium hydroxide solution and kept stirred for 24 h over a magnetic stirrer. The treated mass was strained through a coarse sieve. The process was repeated twice and the residue was washed twice with 30 volumes of chilled distilled water. The residue was homogenized with 30 volumes of dilute acetic acid and the same was stirred over a magnetic stirrer for 24 h. The acid solution containing collagen was centrifuged. To the supernatant crystalline sodium chloride was added to the level of 5% and stirred for appropriate time to precipitate collagen. The precipitated collagen was separated and suspended in Tris-glycine buffer and dialysed against the same buffer for 24 h to get pure fish skin collagen.

For hydrolysate preparation the extracted fish skin collagen in double distilled water was preheated to 60°C for 5 min, and then hydrolyzed enzymatically. For hydrolysis three different proteolytic enzymes, via pepsin, papain and protease from bovine pancreas (PP) were used. Pepsin was dissolved in 10mM HCl solution (pH 2), papain was dissolved in deionised water and the PP dissolved in 10

mM sodium acetate buffer, pH 7.5. The hydrolysis conditions of time, temperature, E/S ratio and pH were optimised for the three enzymes by RSM. After hydrolysis, inactivation of enzymes was accomplished by heating for 3 minutes in boiling water bath.

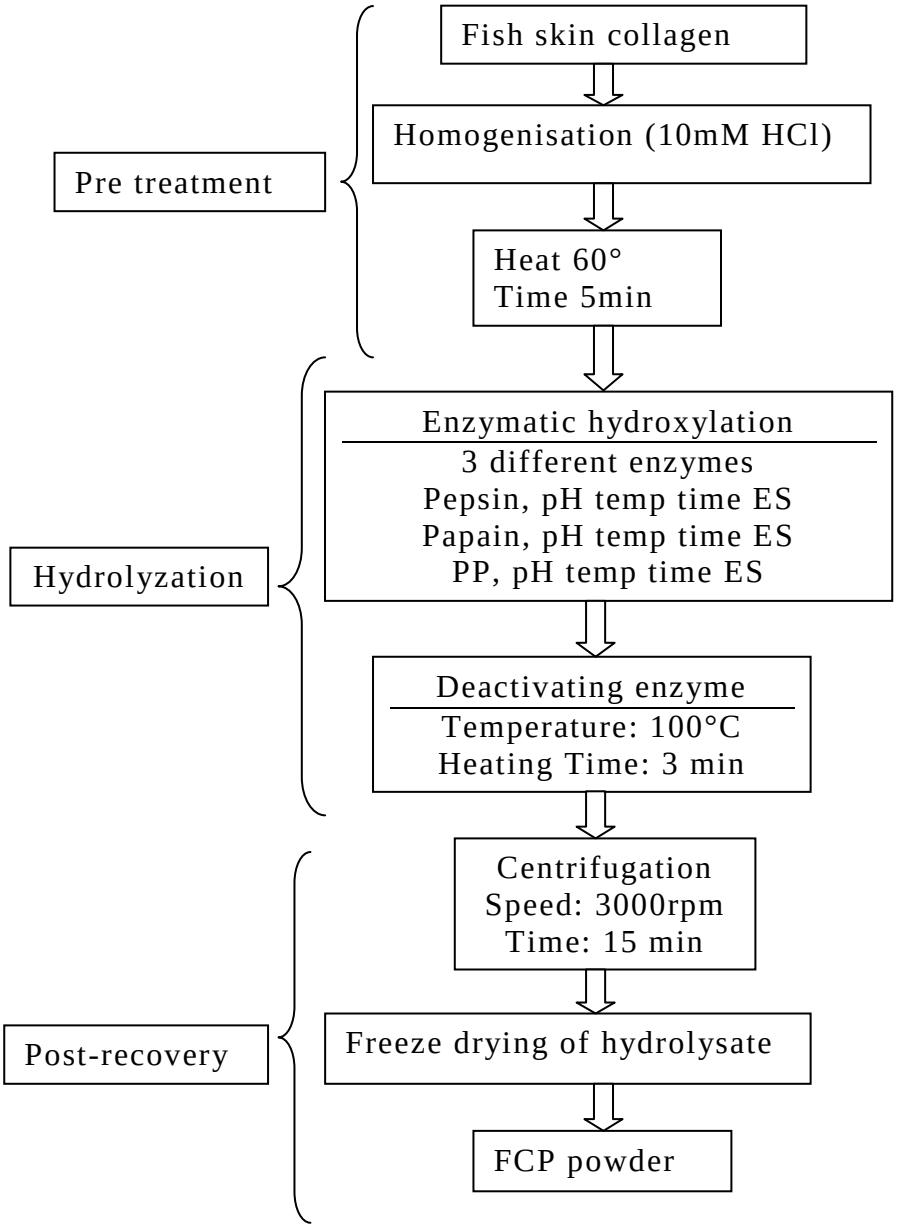
Filtration and lyophilisation: The extract was filtered to remove skin residues. Then fine residues were removed by centrifugation at 10,000 × g for 30 min at 4°C. The resulting solution was adjusted to pH 7 with saturated NaOH or HCl. Finally, the neutralised solution was filtered through Whatman No. 4 filter paper. The filtered solution was then freeze-dried in a lyophilizer. The freeze dried product was stored at 4°C until use.

4.2.4 Determination of Degree of Hydrolysis

Degree of hydrolysis (DH) is the percentage ratio between the numbers of peptide bonds cleaved to the total number of peptide bonds in the substrate studied. It was calculated by the determination of free amino group reaction with TNBS (according to TNBS method Alder-Nissen, 1979) with some modifications. Basically, this method is a spectrophotometric assay of the chromophore formed by the reaction of TNBS with primary amines. The reaction takes place under slightly alkaline conditions and is terminated by lowering the pH. TNBS also reacts slowly with hydroxyl ions, whereby the blank reading increases; this increase is stimulated by light.

Properly diluted samples (125 μL) were mixed with 2.0 mL of 0.2125 M phosphate buffer, pH 8.2, followed by the addition of 1.0 mL of 0.01% TNBS solution. The mixtures were then placed in a water bath at 50 °C for 30 min in the dark. During incubation the test tubes must be covered with aluminum foil because the blank reaction is accelerated by exposure to light. The reaction was terminated by adding 2.0 mL of 0.1 M sodium sulphite. The mixtures were cooled down at ambient temperature for 15 min and the absorbance was measured at 340 nm.

Flow diagram of enzymatic processing method to produce FCP



A TNBS standard calibration curve was prepared using amino acid glycine (11.25 mg/ml) and was found to be linear (R^2 = 0.996). The amount of free amino group liberated was expressed in terms of glycine.

The DH was evaluated as

$$\text{DH}\,(\%) = \frac{C_0 - C_t}{C_0} \times 100\%$$

Where C_0 is glycine equivalent of sample at time = 0, C_t is glycine equivalent of sample at time t.

4.2.5 Characterization

4.2.5.1 Amino acid composition of hydrolysate

Spray dried collagen peptide was hydrolyzed in 6N HCl at 120°C for 24h. After cooling the test tubes the contents were filtered using Whatman No 1 filter paper. The tubes were rinsed with distilled water and filtered. The filtrate was evaporated in a vacuum flash evaporator. Then deionized water was added into the tubes and continued evaporation until the contents were acid free. The process was repeated for three times and the free amino acids were dissolved in 0.05M HCl and filtered using 0.45 micro syringe, then injected in to Shimadzu HPLC using the method (Ishida *et al.*, 1981).

4.2.5.2 SDS PAGE

The fish collagen peptides were analyzed by Tricine-SDS-PAGE according to Schagger *et al.* (1987) with slight modifications using 16.5% separating gel, 10% spacer gel and 4% stacking gel. The lyophilized hydrolysate was dissolved (20 mg/mL) in loading buffer (12% SDS, 6% β- mercaptoethanol, 30% glycerol, 0.05% Coomassie blue G-250, and 150 mmol/L Tris–HCl at pH 7.0), heated at 100°C for 5 min, and centrifuged at 12,000 g for 2 min.

The electrophoresis was performed in a mini electrophoresis apparatus (Bio-Rad, California, USA) at room temperature using the following procedure. The voltage was kept constant at 30 V until the samples completely left the stacking gel, then the voltage was kept constant at 90V - 100V until the tracking dye was close to the bottom of the gel. The loading volume of the samples and the standards was 5 μL-10 μL. After electrophoresis, gels were fixed with a mixture of 500 mL/L methanol and 100 mL/L acetic acid for 30 min, followed by staining with 0.5 mL/L Coomassie blue R-250 in 150 mL/L methanol and 50 mL/L acetic acid for 1 h. Finally, they were destained with a mixture of 300 mL/L methanol and 100 mL/L acetic acid for 1 h and destained again with the same solution for 30 min. The approximate molecular weights of the hydrolysate were determined using appropriate prestained protein molecular weight marker 2 kDa-71 kDa (SBS Genetech Co., Ltd, Beijing, China).

4.2.5.3 MALDI-TOF Mass Spectrometric Analysis

Using MALDI-TOF mass spectrometry, the molecular weight distributions of peptides from collagen hydrolysate was estimated. The analysis was done on positive ion mode MALDI TOF mass spectrometer. The mass spectrometer used for detection of analytes, was AB SCIEX TOF/TOF 5800, AB SCIEX Co., USA with Nd: YAG 1000-Hz laser with 355 nm wavelengths, 2.0m long TOF tube and a sample target plate with 96 wells. CentriVap -50° C cold trap, LABCONCO Ltd., USA was used for concentrating sample under speed vacuum.

Standard MALDI matrices like CHCA, Sinapinic acid were obtained from Sigma-Aldrich. Acetonitrile of LC-MS Grade was bought from Sigma-Aldrich. Trifluoroacetic acid obtained from Fluka. Deionized Water (18.2 MΩ/cm, 4ppb, and 25°C), Bradykinin, Angiotensin II, P14R and ACTH combinedly called Calmix, were procured from sigma as calibration mixture for mass spectrometer.

Operating mode – Reflector positive ion, linear positive ion
Laser intensity – 3600, 4400 (Reflector positive ion mode), 6000 (Linear positive ion mode) units
Laser type – Nd: YAG 1000-Hz laser with 355 nm wavelength
Mass range – 300 to 10000 m/z (Reflector positive ion mode), 300 to 20000 m/z (Linear positive ion mode)
Delayed extraction time – 400 ns
MALDI Plate velocity – 900, 1100 µm/sec
Laser shots: 4000 per spectrum

Sample preparation: A solution containing acetonitrile: 0.1% trifluoro acetic acid in deionised water in 1:1 v/v (diluent) was prepared for reconstituting these aliquots. Five aliquotes from the collagen peptide were evaporated to dryness by using CentriVap -50° C cold trap and reconstituted in diluent. These samples were further diluted to 10X and 100X concentration followed by aliquoting in 10µL volume each. There were two spotting methods used for mass spectrometric analysis as sandwich and dried droplet. For sandwich method, reconstituted samples spotted on MALDI target plate, sandwiched with matrices (CHCA, sinapinic acid) followed by drying. Each recontituted sample was mixed with each of matrices in 1:1 v/v, separately and vortexed. These resulted samples were spotted on MALDI target plate and dried. All the spotted samples were analyzed by AB SCIEX TOF/TOF 5800 system.Standard calmix peptide mixture was used for validating method for data acquisition.

4.2.6 Functional properties of fish collagen peptide

4.2.6.1 Solubility

To determine protein solubility, 250 mg of protein hydrolysate was dispersed in 5 mL of deionized water. The mixture was stirred at room temperature for 2 h and centrifuged at 2000g for 10 min. Protein content of the supernatant was determined by AOAC (2005) method. Solubility was calculated as protein content in supernatant over total protein content in the initial dispersion. Protein solubility was calculated as follows

% Solubility = protein content in the supernatant/ total protein content in the hydrolysate x 100

4.2.6.2 Change in viscosity

Viscosity of collagen solution and collagen hydrolysate solution was determined by rotary viscometer test method (Brookfield Digital Viscometer, Model DV-E). In this test method, 3mg/ml of test solution is placed in a glass tube, housed in an insulated block at a fixed temperature (37°C). A metal spindle is then rotated in the solution at 100 rpm, and the torque required to rotate the spindle is measured. Based on the internal resistance to rotation provided by the shear stress of the solution, the solution's absolute viscosity can be determined. Absolute viscosity is represented in centipoise (cP).

4.3 Results and Discussion

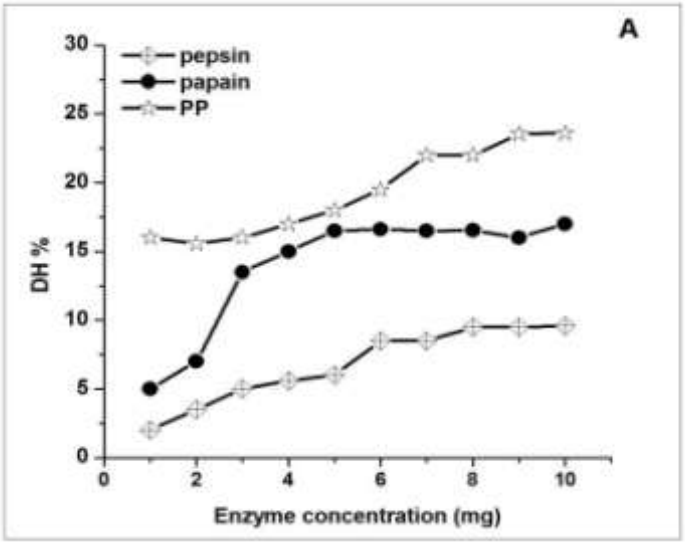
Bioactive peptides usually contain 2–20 amino acid residues per molecule (Pihlanto-Leppala, 2000); the lower their molecular weights, the higher their chances of crossing the intestinal barrier and exerting a biological effect (Roberts *et al.,* 1999). In the study, a three step hydrolysis reaction was adopted, and experimental points were designed to optimize processing conditions to obtain smaller peptides, which might possess potent bio activity.

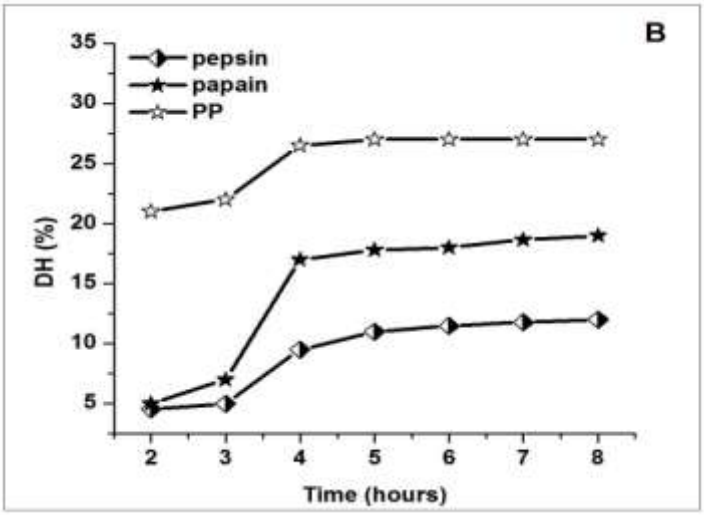
The degree of hydrolysis, DH (i.e. percentage of peptide bond cleaved) is a true reflection of the progress of hydrolysis and thus it is selected as the primary indicator for the control of hydrolysis. This study used RSM to evaluate the effects of pH, temperature, enzyme concentration and time on the DH of the skin of grouper with three different enzymes pepsin, papain and protease from bovine pancreas. A second-order polynomial regression equation was given to describe the model and predicted an optimal condition of factors to maximize the DH.

4.3.1. Single factor experiments

In single factor experiments, three of four factors (pH, temperature, and time and enzyme substrate concentration) were fixed while the other factor was ranged to evaluate its effect on hydrolysis. This was done to fix the range of time and enzyme substrate ratio in the hydrolysis process. The DH of the hydrolysate could be measured by TNBS method and the results were shown in Fig. 4.1 A and 4.1 B.

Figure 4.1 The effect of enzyme concentration (4.1 A) and reaction time (4.1 B) on the degree of hydrolysis.





As can be seen from Fig.4.1 A, there was an increase in the DH with the increase of enzyme concentration. Despite of the aim to maximise the DH, the cost and the economy should be taken into consideration as well. It is a waste to pursue higher DH with excessive enzyme. Thus, 6.0mg of enzyme concentration was adequate in the case of pepsin and papain. In the case of protease from bovine pancreas, even at low concentrations itself it gives high degree of hydrolysis and so its concentration controlled at 2.0mg.

It can be seen from Fig.4.1B that the tendency for the DH increases with increasing incubation time. Too long time means too much expenditure of energy and so on. For the same reason, time of hydrolysis should be restricted to 6.0 h.

4.3.2 Optimization of enzymatic hydrolysis using RSM

4.3.2.1 Response surface analysis

The experiment was carried out based on the Box-Behnken design of experiment set up. The results of different combinations of input variables along with the response variable on degree of hydrolysis is given in the table 4.2, table 4.3, and table 4.4 for pepsin, papain and PP respectively.

Table 4.2 Factors and levels in the RSM and experimental results for the enzyme pepsin

Pattern	*pH*	*Temperature*	*Time*	*E/S Ratio*	*DH*
0−0+	2	30	4	8	5.5
+0−0	2.4	40	2	4.25	5.6
−00+	1.6	40	4	8	7.6
+00+	2.4	40	4	8	8.2
0000	2	40	4	4.25	8.5
−0+0	1.6	40	6	4.25	2.6
−00−	1.6	40	4	0.5	3.25
0++0	2	50	6	4.25	2
00−−	2	40	2	0.5	3.25
+0+0	2.4	40	6	4.25	9.55
0000	2	40	4	4.25	8.36
00−+	2	40	2	8	7.65
0—0	2	30	2	4.25	3.25

0−0−	2	30	4	0.5	3.15
0+0+	2	50	4	8	1.95
+00−	2.4	40	4	0.5	1.14
−+00	1.6	50	4	4.25	1.17
0+−0	2	50	2	4.25	1.66
0−+0	2	30	6	4.25	6.5
00++	2	40	6	8	9.86
+−00	2.4	30	4	4.25	1.86
−−00	1.6	30	4	4.25	3.14
0+0−	2	50	4	0.5	2.65
0000	2	40	4	4.25	8.75
00+−	2	40	6	0.5	4.15
−0−0	1.6	40	2	4.25	5.76
++00	2.4	50	4	4.25	1.55

Table 4.3 Factors and levels in the RSM and experimental results for the enzyme papain

Pattern	*pH*	*Temperature*	*Time*	*E/S ratio*	*DH*
0000	6	30	4	4.25	17
0+0−	6	40	4	0.5	11
+00−	7	30	4	0.5	7.6
0000	6	30	4	4.25	18.5
00−−	6	30	2	0.5	8.75
00−+	6	30	2	8	14.5
0−+0	6	20	6	4.25	18
+0+0	7	30	6	4.25	17
−+00	5	40	4	4.25	0
0−0−	6	20	4	0.5	6.55
−00+	5	30	4	8	0
00++	6	30	6	8	19.43
−−00	5	20	4	4.25	0
+−00	7	20	4	4.25	19
−0+0	5	30	6	4.25	0
+0−0	7	30	2	4.25	12
−00−	5	30	4	0.5	0
+00+	7	30	4	8	17.78
0−0+	6	20	4	8	16
0+−0	6	40	2	4.25	2
0+0+	6	40	4	8	2.5
00+−	6	30	6	0.5	12.65
++00	7	40	4	4.25	5.01
−0−0	5	30	2	4.25	0
0—0	6	20	2	4.25	9
0000	6	30	4	4.25	19.8
0++0	6	40	6	4.25	6.88

Table 4.4 Factors and levels in the RSM and experimental results for the enzyme PP

Pattern	*pH*	*Temperature*	*Time*	*E/S Ratio*	*DH*
0+0+	7	50	4	0.8	2
+00+	8	40	4	0.8	14.76
0000	7	40	4	0.425	27
+−00	8	30	4	0.425	0
0—0	7	30	2	0.425	0
0−+0	7	30	6	0.425	0
0++0	7	50	6	0.425	0
−0−0	6	40	2	0.425	24
++00	8	50	4	0.425	0
−−00	6	30	4	0.425	0
+0−0	8	40	2	0.425	12
−+00	6	50	4	0.425	0
00−−	7	40	2	0.05	14.55
00−+	7	40	2	0.8	25.88
0−0+	7	30	4	0.8	0
00++	7	40	6	0.8	26
−00+	6	40	4	0.8	26
00+−	7	40	6	0.05	15.56
+00−	8	40	4	0.05	14.9
0000	7	40	4	0.425	26.53
0+0−	7	50	4	0.05	0
0+−0	7	50	2	0.425	0
0−0−	7	30	4	0.05	0
0000	7	40	4	0.425	27.55
−0+0	6	40	6	0.425	27.9
+0+0	8	40	6	0.425	12
−00−	6	40	4	0.05	15

The adequacy of the model was justified through analysis of variance (ANOVA). The examination of the fitted model was necessary to ensure that it provided an adequate approximation to the true system. Regression analysis was employed to fit a full response surface model for every response investigated, including all linear (X_1,X_2,X_3,X_4) and quadratic/interaction terms (X_1^2, X_2^2, X_3^2, X_4^2, X_1X_2, X_1X_3, X_1X_4, X_2X_3, X_2X_4, X_3X_4,). The regression coefficients for the 2^{nd} order response surface models in terms of coded units are shown in Table 4.5.

Table 4.5 Linear, Quadratic and Interaction regression coefficients of independent Variables where X_1: pH, X_2: temperature, X_3: time, X_4: enzyme concentration.

	Pepsin	***Papain***	***Protease***
Intercept	-97.65**	-423.62**	-608.62**
X_1	39.26	107.48**	69.08*
X_2	3.33**	5.95	18.26**
X_3	-1.2	4.32	10.5
X_4	1.02	1.83	78.2
X_1^2	-13.3**	-7.9**	-4.8*
X_2^2	-0.04**	-0.05**	-0.23**
X_3^2	-0.2	-0.68*	-0.84
X_4^2	-0.09	-0.23**	-26.87*
X_1X_2	-0.84	-0.35*	0
X_1X_3	2.2*	0.63	-0.49
X_1X_4	0.45	0.68	-7.4
X_2X_3	-0.04	-0.05	0
X_2X_4	-0.02	-0.12**	0.13
X_3X_4	0.04	0.03	-0.3
R^2	0.86	0.95	0.95

*-Indicates regression coefficients significant at 5% level of significance

**- Indicates regression coefficients significant at 1% level of significance

The reliability of fitted second order polynomial model was assessed using coefficient of determination (R^2) and Root mean square error (RMSE). The R^2 and RMSE value of fitted model was 0.86 and 1.55 for pepsin, 0.95 and 2.39 for papain and 0.95 and 3.63 for PP respectively. Based on the evaluation of R^2 value which is close to 1 and minimum RMSE value, it is inferred second order response model fitted well to the experimental data (Chauhan and Gupta, 2004). As shown in the table 6, 7 and 8 it is suggested that this quadratic model was appropriate to represent the real relationships among the chosen hydrolysis parameter and the model can explain a high percentage of the variability in the observed data.

Also the significance of linear, quadratic and interaction effect of pH, temperature, and time and enzyme concentration on DH for each enzyme was evaluated using ANOVA. The linear, quadratic and interaction effect of input factors was found to be significant at 5%

level of significance for all three enzymes except for some two way interaction effect for papain and protease. The results of ANOVA are given in table 4.9, 4.10 and 4.11.

Table 4.9 shows the linear, quadratic and interaction effect of four factors on DH in the case of pepsin. The linear effect of temperature, quadratic effect of pH and temperature was found to be significant at 1% level of significance. The interaction effect of pH and time was found to be significant at 5 % level of significance where as other effects were insignificant.

Table 4.10 shows the linear, quadratic and interaction effect of four factors on DH in the case of papain. The linear and quadratic effect of pH and temperature was found to be significant at 1% level of significance whereas time and ES ratio was found to be non-significant. The quadratic effect of time and ES ratio was significant at 5% level of significance. The interaction effect of temperature and pH, ES ratio and pH, ES ratio and temperature were found to be significant at 5% level of significance, where as other interactions were insignificant.

Table 4.11 shows the linear, quadratic and interaction effect of four factors on DH in the case of PP. The linear and quadratic effect of temperature was found to be significant at 1% level of significance. The linear effects of pH, quadratic effect of ES ratio were found to be significant at 5% level of significance where as all other effects were insignificant.

Three-dimensional response surface plots presenting the different combinations of input factors on the DH are given in Fig. 4.2, Fig 4.3 and Fig 4.4 respectively for pepsin, papain and protease. Each of the plots was drawn to predict the value of DH by varying the levels of two input factors at a time and by fixing the levels of other two factors constant. As shown in Figure, if the selected variable's value was in the optimum range, the DH increased until combination of the time, temperature, and E/S ratio reached a maximum yield of the product. But if the conditions that selected for

the hydrolyzation were out of this range, even though it has higher value for each of the variables, the DH cannot reach the high value, and it will stay at the lower point of DH. This confirmed that extremes of pH and temperature are affecting the enzyme activity. The shape of the hydrolysis curve has been associated with enzyme inactivation, product inhibition by hydrolysis products formed at high degrees of hydrolysis, a low Km value for the soluble peptides that act as effective substrate competitors to the unhydrolyzed protein, and possibly auto digestion of the enzyme (Rebeca *et al.*, 1991; Mullally *et al.*, 1995).

Fig. 4.2A shows that at lower and higher level of pH and temperature, DH was less as compared to the centre points. Fig 4.2B and 4.2C shows that lower levels of pH, DH was less irrespective of the levels of time and ES ratio and DH was higher near pH 2 irrespective of levels of time, ES ratio. Fig 4.2D & 4.2E shows that the maximum predicted DH noticed at centre points of time and temperature, ES ratio and temperature than lower and higher levels. Fig. 4.2F shows that minimum and maximum DH was observed at lower and higher levels of ES ratio and time respectively.

As shown in Fig. 4.3A and 4.3B, the increase of pH affected the DH significantly up to a certain point irrespective of the changes in time or enzyme concentration, and the mutual interactions between pH and the other two factors were not so obvious. Fig. 4.3C showed noticeable quadratic effect of interaction between temperature and pH. Fig. 4.3E showed the degree of hydrolysis is very less at higher and lower levels of temperature and enzyme concentration.

In each of the plots in Fig.4.4B, 4.4C and 4.4F, there was a peak of the curved surface that revealed an optimal condition of the factors on the response, which mean that any level of the condition lower or higher than that would produce less influence on hydrolyzing process with the result of diminishing the DH.

These graphs met well with the results of ANOVA and described the statistical data visually. In each of the plots, there was a peak of the curved surface that revealed an optimal condition of the factors on the response, which mean that any level of the condition lower or higher than that would produce a negative influence on hydrolyzing process with the result of diminishing the DH.

4.3.2.2 Optimisation and Validation Study

To maximize the DH, the conditions of four factors in this study were optimized with Numerical optimisation of Design Expert software. According to the ridge analysis the recommended desirable values are: 2.1 of pH; 36.62°C of temperature; 3.6% of E/S ratio; and 5.47 h of time for pepsin. The recommended values for papain are 6.38 of pH; 26.22°C of temperature; 4.5% of E/S ratio; and 4.25 h of time. And the recommended values for protease are 6.3 of pH; 39.86°C of temperature; 1.8% of E/S ratio; and 4.25 h of time. Under these conditions, the predicted response of DH was estimated to be 10.0049%, 20.7234% and 28.0552% pepsin, papain and protease respectively.

Once an empirical model has been developed and optimized the process parameters using the developed model, it is very much important to validate the model by extrapolating the optimized conditions by conducting the actual experiment. Accordingly to confirm the validity of the developed model, three assays were performed under the recommended conditions given above. The experimental values of DH obtained for papsin, papain and protease were 11.5 ± 0.73% and 21 ± 1.03% and 28.6 ± 0.68% respectively. The experimental values agreed with the value predicted by the model within a 95% confidence interval. The above results confirmed that the model was powerful and suitable for the estimation for experimental values.

Figure 4.2 Pepsin: Effects of different variables on the degree of hydrolysis presented in response surface (3D) plots

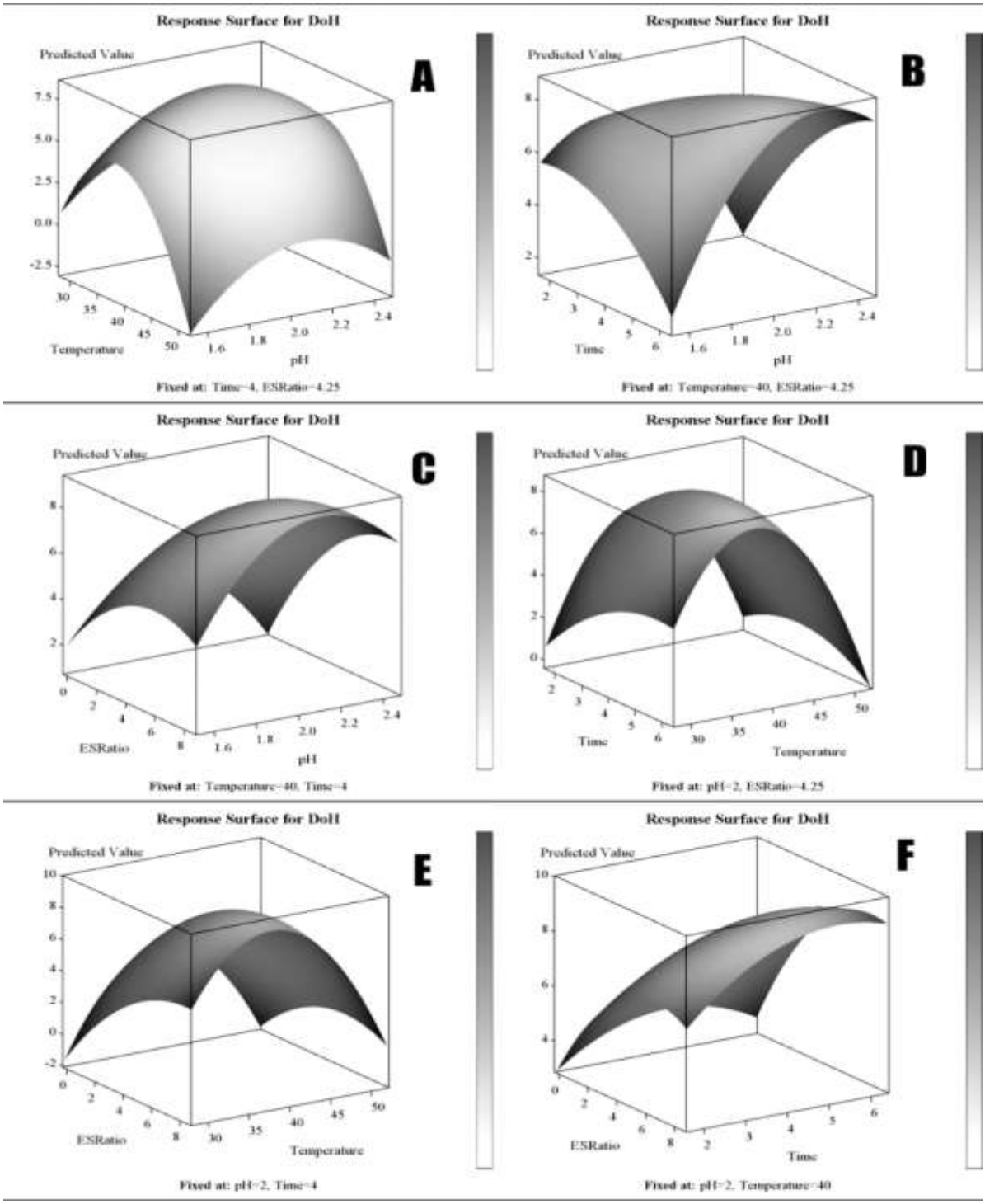


Figure 4.3 Papain: Effects of different variables on the degree of hydrolysis presented in response surface (3D) plots

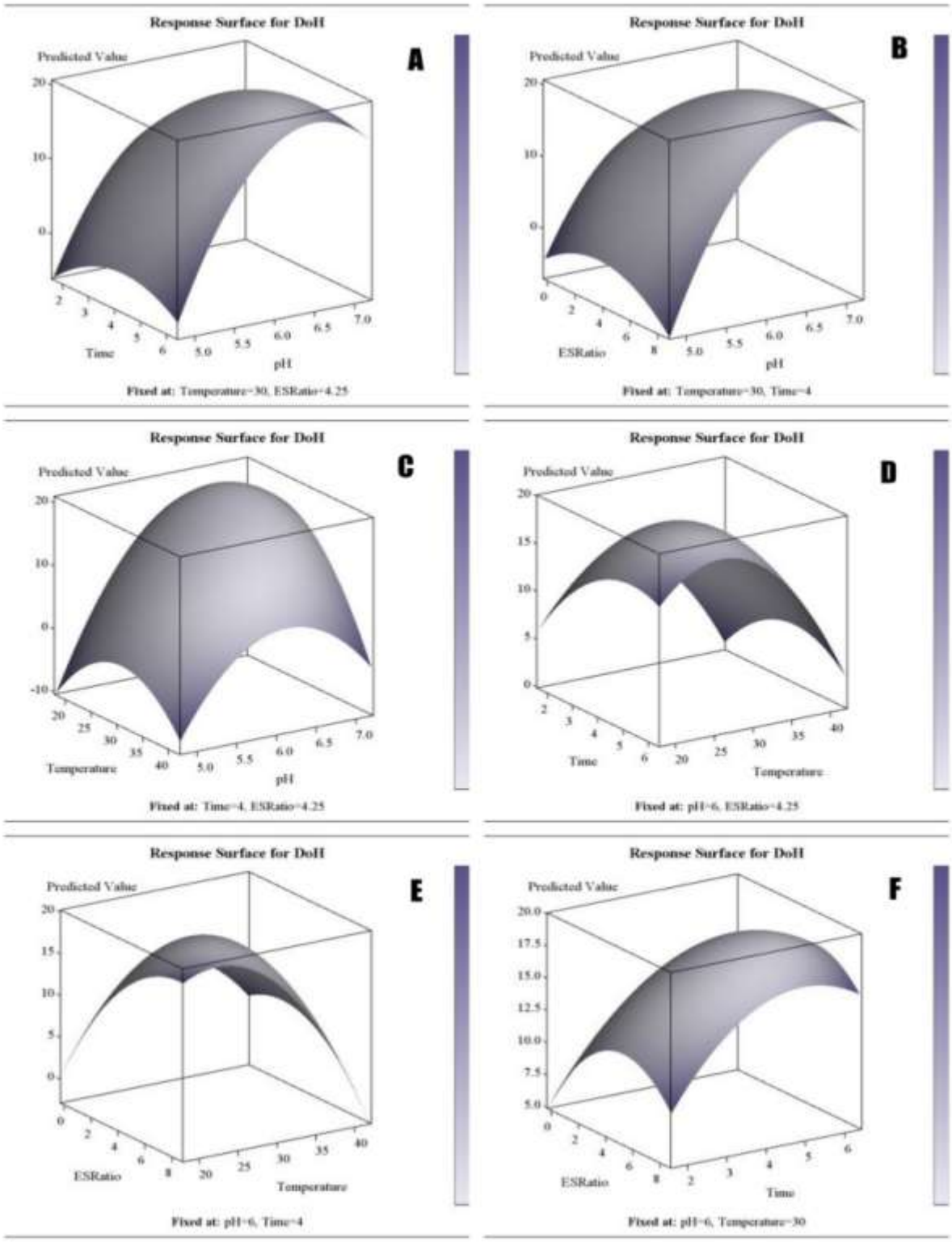
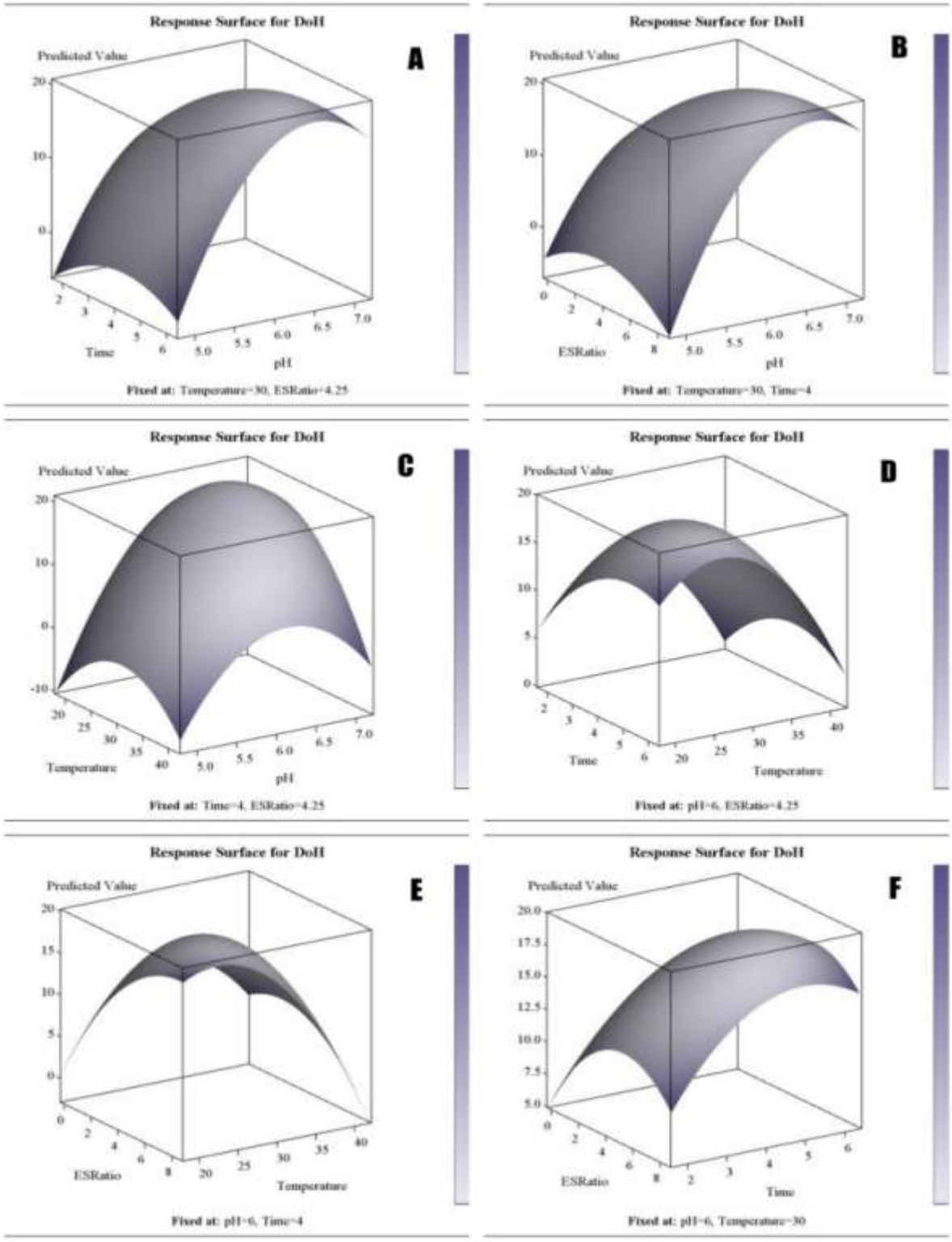


Figure 4.4 Protease: Effects of different variables on the degree of hydrolysis presented in response surface (3D) plots



4.3.3 Development of collagen hydrolysate

A three step hydrolysis of grouper skin collagen was done using the three enzymes consecutively under the optimised conditions. The process resulted in a fine powder of collagen hydrolysate (shown in fig 4.5A) having degree of hydrolysis and nitrogen recovery respectively of 56 ±0.73% and 76.11±1.03%.

4.3.3.1 Viscosity and solubility of collagen hydrolysate

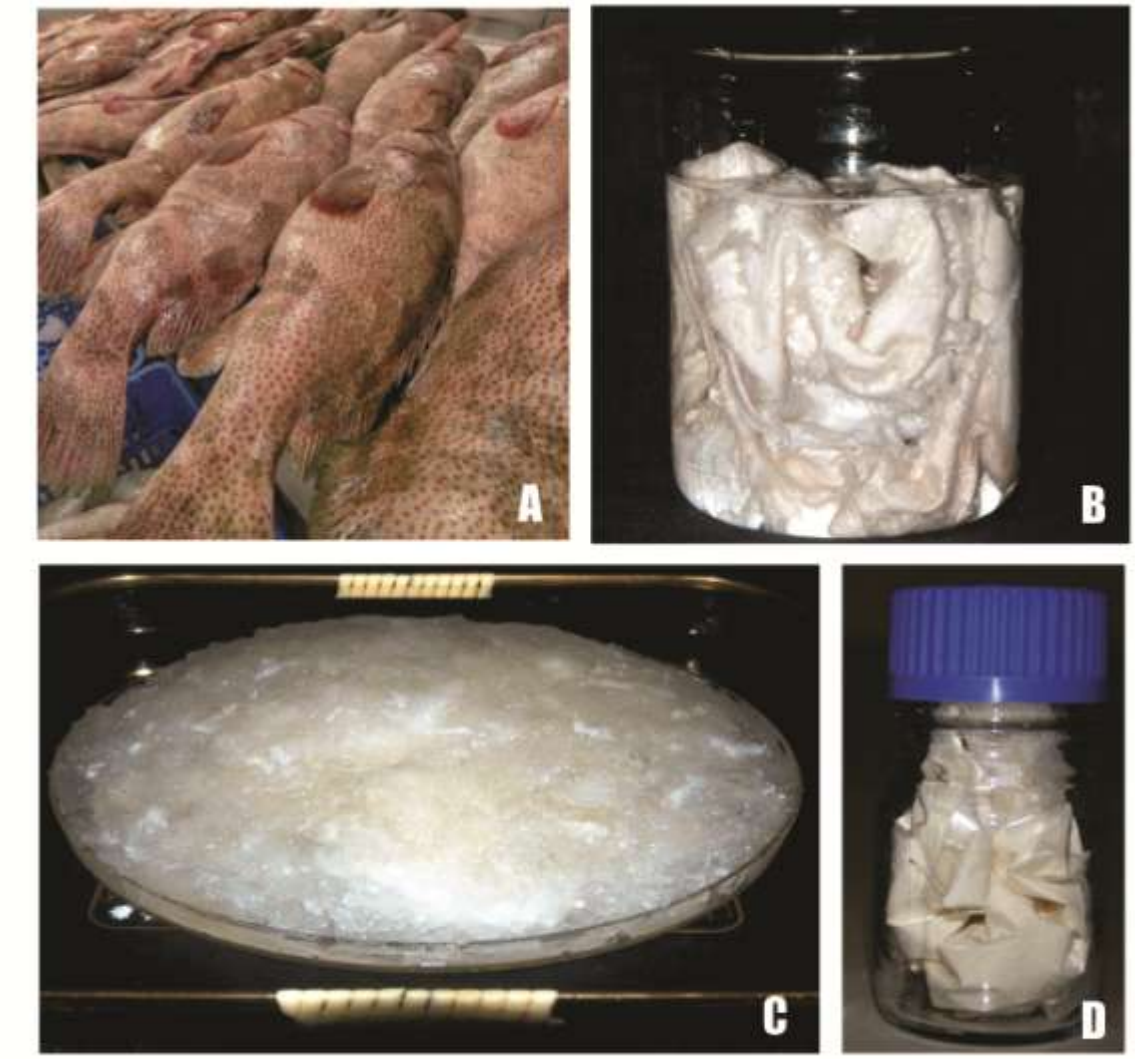
Lyophilized hydrolysate was almost 100% soluble over a wide range of pH values (3–9). The viscosity of collagen solution was 96 cP and the viscosity of collagen hydrolysate was 1.422 cP.

Hydrolysate is known to have excellent solubility at a high degree of hydrolysis (Gbogouri *et al.*, 2004). Hydrolysis potentially influenced the molecular structure, hydrophobic nature and polar groups of the hydrolysate (Kristinsson and Rasco, 2000). Higher DH means smaller peptides, which were expected to have proportionally more polar residues and the ability to form hydrogen bonds with water, thereby improving the solubility (Gbogouri *et al.*, 2004). The fish collagen hydrolysate had a DH of approximately 56 ±0.73%, and hydrolysate consisted of low molecular weight peptides that were mostly in the range of 2 kDa, which may support the findings of high solubility.

4.3.4 Characterization

Enzymatically hydrolyzed fish peptides exhibit different physicochemical properties and biological activities depending on their molecular weight and amino acid sequence. Therefore the molecular weight of the bioactive peptide is one of the most important factors in producing bioactive peptides with the desired biological activities (Kim and Mendis, 2006; Kim and Wijesekara, 2010)

Figure 4.5A Whole fish from landing centre B. Purified skin C. extracted collagen D. freeze dried collagen peptide



4.3.4.1 Amino acid composition analysis

The amino acid compositions of grouper skin collagen and its hydrolysate product are compared in table 4.12. As shown in the table, amino acid profile of the hydrolysate is comparable to the corresponding parent protein. When the amino acid composition of the collagen hydrolysate was analyzed, it was rich in proline, glycine, alanine and hydroxyproline residues and small amounts of tyrosine, histidine, and methionine, residues and complete absence

of tryptophan. The results are in agreement with those reported by Vivian Zague *et al.,* 2011; Wang *et al.,* 2008.

The amino acid composition of the porcine skin cocktail hydrolysate as reported by Moskowitz (2000) was different from that of the fish skin gelatine hydrolysate, although the major amino acid components of the two collagen hydrolysates were same. Glycine was the most abundant amino acid in all collagens and the amount was approximately 21% to 23%, which was one of the characteristics of collagens isolated from living organisms. Prabjeet *et al.* (2011) reported that the major amino acid in fish collagen is glycine, followed by proline, alanine, and hydroxyproline. Vivian Zague *et al.* (2011) reported that collagen hydrolysate had high contents of glycine (24.5%), glutamic acid (10.1%), arginine (8.1%), proline (13.8%), and hydroxyproline (7.4%) residues and small amounts of tyrosine, cysteine, histidine, and methionine residues.

Amino acid composition of the lyophilized hydrolysate was analyzed in order to determine the possible effect of the amino acid profile on biological activity. A previous report shows that the bioactivity of the lyophilized hydrolysates depended upon the amino acid sequence of the peptides. High content of hydrophobic amino acids could increase the solubility of collagen peptides in lipid and then enhance their antioxidant activities (Kim *et al.,* 2001). Rajapakse *et al.* 2005 found that fish skin gelatin peptides possessed higher antioxidant activity than peptides derived from other proteins because of the high percentage of Glycine and Proline. Chen *et al.,* (2006) reported that histidine and proline played important roles in the antioxidant activity of synthetic peptides. Therefore, we can predict one factor responsible for the bio activities of collagen hydrolysates were inherent to their characteristic amino acid sequences.

Table 4.12 Amino acid composition (g/100g protein) of grouper skin collagen (GC) and grouper skin collagen hydrolysate (GCH)

Amino acids	*GC*	*GCH*
Aspartic acid	4.03 ± 0.42	2.35± 0.75
Threonine	2.17 ± 0.25	1.43± 0.05
Serine	3.80 ± 0.36	2.65± 0.92
Glutamic acid	4.90 ± 0.26	2.21± 0.23
Proline	12.27 ± 0.60	9.59± 0.11
Glycine	31.23 ± 1.33	26.81± 0.76
Alanine	11.37 ± 0.47	10.80± 0.65
Cystine	ND	ND
Valine	2.73 ± 0.31	1.21± 0.45
Methionine	0.90 ± 0.10	0.43± 0.62
Isoleucine	1.03 ± 0.21	0.84± 0.40
Leucine	2.33 ± 0.32	1.50± 0.72
Tyrosine	0.37 ± 0.06	0.21± 0.44
Phenylalanine	1.47 ± 0.21	0.67± 0.72
Histidine	1.43 ± 0.42	0.28± 0.22
Lysine	3.80 ± 0.44	2.47± 0.55
Arginine	5.13 ± 0.45	3.55± 0.11
H. Proline	9.77 ± 0.35	5.99± 0.32

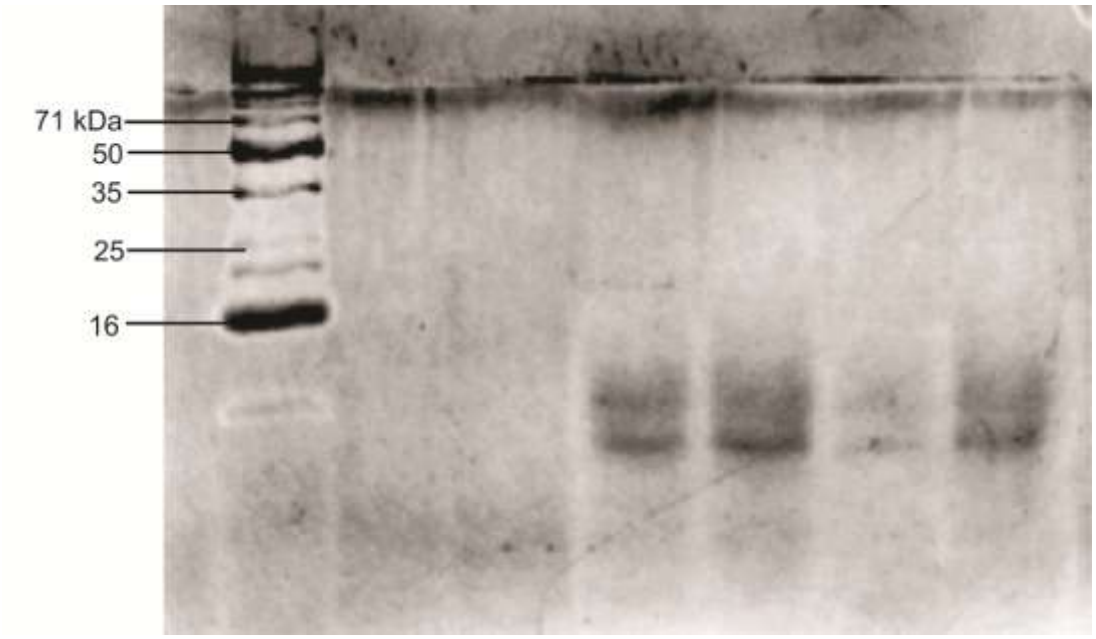
4.3.4.2 SDS PAGE

The electrophoretic pattern of the fractionated hydrolysate presented in Fig 4.6 indicated that the peptides formed due to hydrolysis were less than 16kDa in molecular weights, corresponding well with the higher DH values observed in the study.

This result indicated that the hydrolysis process had successfully cleaved the peptide bonds, resulting in lower molecular weight and higher DH. It can be predicted that most of the triple helices of collagen hydrolysate had been destroyed and parts of their peptide bonds were also broken out. Therefore, there were wide distributions and lower molecular weights for gelatin and collagen hydrolysate (Zhongkai Zhang *et al.*, 2005). In order to obtain protein hydrolysates of high nutraceutical value, the dietary protein in it

should rich in low molecular weight species, with the amounts of free amino acids as low as possible (Vijayalakshmi *et al.*, 1986).

Figure 4.6 Tricine SDS PAGE pattern of collagen hydrolysate. First lane shows molecular markers. Lane 4,5,6,7 are 1.5mg/ml, 2mg/ml, 2.5mg/ml, 3mg/ml concentrations of hydrolysate respectively.



4.3.4.3 MALDI TOF mass spectrum analysis

Molecular weight distribution of the hydrolysate was obtained from MALDI TOF mass spectrometer with resolving capabilities in the order of 400-1000 and accuracy ranging from ± 0.2% to 0.005%. The mass spectra (shown in fig.4.8) indicated the molecular weight of the most active peptides in the range below 2000 kDa. No signals were obtained above 2000m/z. Standard Calmix peptide mixture was used for calibrating the instrument (spectra shown in fig.4.7).

Figure 4.7 Mass spectra obtained for standard calmix peptide mixture (Bradykinin, Angiotensin II, P14R, ACTH 18-39 clip. This mixture was used for calibration of instrument).

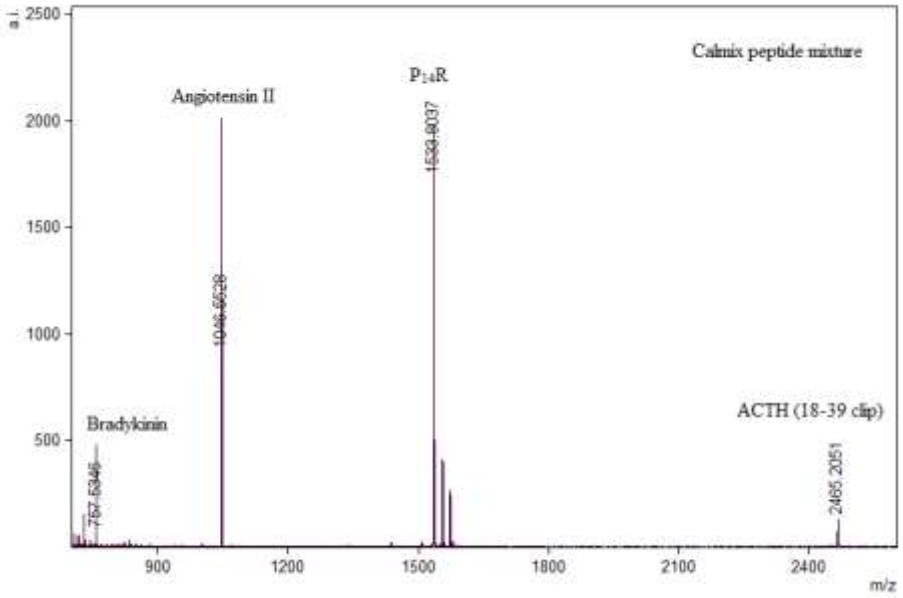
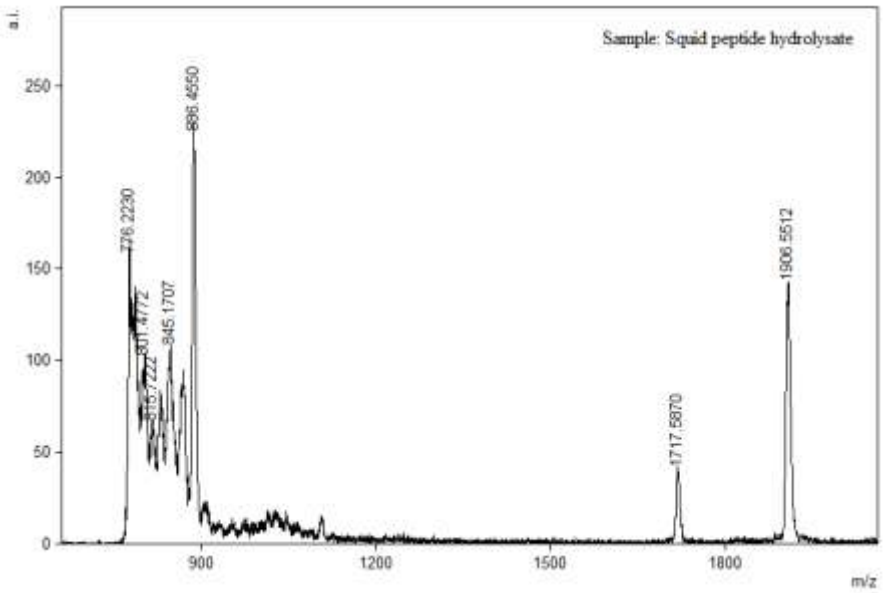


Figure 4.8 Mass spectra obtained for Collagen hydrolysate. Spectra acquired till 20000 m/z. However, no signals were obtained above 2000 m/z



4.4 Conclusion

In order to develop bioactive peptides, the grouper fish skin collagen was hydrolysed enzymatically using three different enzymes; pepsin, papain and protease consecutively. Since degree of hydrolysis depends on the bioactivity of peptides, we optimised the hydrolysis parameters of each enzyme using RSM with Box Behkenn model in order to get maximum degree of hydrolysis. The optimum hydrolysis conditions are: 2.1 of pH; 36.62°C of temperature; 3.6% of E/S ratio; and 5.47 h of time for pepsin. The optimum hydrolysis conditions for papain are 6.38 of pH; 26.22°C of temperature; 4.5% of E/S ratio; and 4.25 h of time. And the optimum hydrolysis conditions for protease are 6.3 of pH; 39.86°C of temperature; 1.8% of E/S ratio; and 4.25 h of time. Under these conditions the selected enzymes give maximum activity.

The three step hydrolysis process using the enzymes pepsin, papain and protease resulted in degree of hydrolysis and nitrogen recovery respectively of 56 ±0.73% and 76.11±1.03% under the optimised conditions. Lyophilized hydrolysate was almost 100% soluble over a wide range of pH values.

Several analyses were done for checking the quality control of the product; Filtration, analysis of hydrolysis degree, the molecular weight distribution, the total nitrogen, amino acid composition and the presence of toxic compounds (heavy metal contamination or pathogens).

SDS-PAGE combined with MALDI TOF method was successfully applied to determine the molecular weight distribution of the hydrolysate. The electrophoretic pattern indicated that the peptides formed due to hydrolysis were less than 16kDa in molecular weights. MALDI TOF spectral analysis showed that the molecular weight of most of the active peptides is in the range below 2000 kDa. The spray dried product is a pure mixture of peptides and can be recommended for further bioactivity studies.

Interest in nutraceuticals is growing rapidly worldwide, as they are a safe alternative to pharmaceutical drugs, which use is sometimes limited by toxicity or intolerance reactions. Collagen and collagen hydrolysates could be attractive nutraceuticals for their interesting bioactive properties. The beneficial effect of collagen or gelatine hydrolysates on different diseases has been reported in animal or clinical studies, and actually several supplements including collagen-derived peptides have been patented and are currently commercialised in USA, Japan and Europe. Moreover, hydrolysed collagen products have received GRAS status (Generally Recognized as Safe) from the US Food and Drug Administration (FDA).

Although mammalian gelatines are widely used in the field of nutraceuticals, the use of gelatines from marine-discarded sources for preparing protein hydrolysates is nowadays increasing, as they are not associated with the risk of outbreaks of bovine spongiform encephalopathy and also meet certain religious requirements of Jewish and Muslim markets. The resistance of some collagen-derived peptides to protein digestion is one of the most interesting properties of collagen hydrolysates. Several studies focused on the effect of oral intake in both animal and human models have revealed the excellent absorption and metabolism of Hyp-containing peptides. Some of these collagen-derived peptides have revealed biological activity in vivo after absorption from the digestive tract (Moskowitz, 2000).

4.5 Appendix

Table 4.6 ANOVA table for pepsin

Regression	DF	Type I Sum of Squares	R-Square	F Value	Pr > F
Linear	4	63.865817	0.2947	6.62	0.0047
Quadratic	4	103.904346	0.4794	10.78	0.0006
Cross product	6	20.034625	0.0924	1.39	0.2965
Total Model	14	187.804788	0.8665	5.57	0.0025

Table 4.7 ANOVA table for papain

Regression	DF	Type I Sum of Squares	R-Square	F Value	Pr > F
Linear	4	763.898117	0.5452	33.24	<.0001
Quadratic	4	402.105207	0.2870	17.50	<.0001
Cross product	6	166.147575	0.1186	4.82	0.0100
Total Model	14	1332.150899	0.9508	16.56	<.0001

Table 4.8 ANOVA table for PP

Regression	DF	Type I Sum of Squares	R-Square	F Value	Pr > F
Linear	4	230.233333	0.0680	4.35	0.0210
Quadratic	4	2959.308241	0.8745	55.93	<.0001
Cross product	6	35.807500	0.0106	0.45	0.8309
Total Model	14	3225.349074	0.9531	17.42	<.0001

Table 4.9 ANOVA for the second order polynomial model in case of pepsin

Pepsin Parameter	DF	Standard Error	t Value	Pr > |t|
Intercept	1	30.677907	-3.18	0.0079
pH	1	19.076136	2.06	0.0620
Temperature	1	0.688307	4.84	0.0004

Time	1	2.868527	-0.42	0.6829
ES Ratio	1	1.451825	0.70	0.4977
pH*pH	1	4.201772	-3.17	0.0080
Temperature*pH	1	0.194072	0.53	0.6027
Temperature*Temperature	1	0.006723	-6.34	<.0001
Time*pH	1	0.970358	2.29	0.0410
Time*Temperature	1	0.038814	-0.94	0.3672
Time*Time	1	0.168071	-1.24	0.2402
ESRatio*pH	1	0.517524	0.87	0.3999
ESRatio*Temperature	1	0.020701	-0.98	0.3454
ESRatio*Time	1	0.103505	0.42	0.6806
ESRatio*ESRatio	1	0.047807	-1.88	0.0842

Table 4.10 ANOVA for the second order polynomial model in case of papain

Papain Parameter	DF	Standard Error	t Value	Pr > |t|
Intercept	1	51.511430	-8.22	<.0001
pH	1	13.270730	8.10	<.0001
Temperature	1	0.992742	5.99	<.0001
Time	1	4.587782	0.94	0.3647
ES Ratio	1	2.330710	0.79	0.4464
pH*pH	1	1.037884	-7.70	<.0001
Temperature*pH	1	0.119844	-2.92	0.0129
Temperature*Temperature	1	0.010379	-5.58	0.0001
Time*pH	1	0.599222	1.04	0.3175
Time*Temperature	1	0.059922	-0.86	0.4069
Time*Time	1	0.259471	-2.66	0.0209
ES Ratio*pH	1	0.319585	2.12	0.0552
ES Ratio*Temperature	1	0.031959	-3.74	0.0028
ES Ratio*Time	1	0.159793	0.21	0.8335
ES Ratio*ES Ratio	1	0.073805	-3.07	0.0096

Table 4.11 ANOVA for the second order polynomial model in case of PP

Protease Parameter	DF	Standard Error	t Value	Pr > |t|
Intercept	1	109.655598	-5.55	0.0001
pH	1	23.614131	2.93	0.0127
Temperature	1	1.842178	9.91	<.0001
Time	1	8.062146	1.30	0.2170
ES Ratio	1	41.486329	1.88	0.0839
pH*pH	1	1.574893	-3.05	0.0101
Temperature*pH	1	0.181853	0.00	1.0000
Temperature*Temperature	1	0.015749	-14.53	<.0001
Time*pH	1	0.909265	-0.54	0.6017
Time*Temperature	1	0.090926	0.00	1.0000
Time*Time	1	0.393723	-2.15	0.0531
ES Ratio*pH	1	4.849411	-1.53	0.1529
ES Ratio*Temperature	1	0.484941	0.27	0.7880
ES Ratio*Time	1	2.424706	-0.12	0.9036
ES Ratio*ES Ratio	1	11.199236	-2.40	0.0335

……

Chapter 5

PREVENTIVE EFFECT OF FISH COLLAGEN PEPTIDE IN CFA INDUCED ARTHRITIC RATS

Contents

5.1 Introduction

Arthritis is a form of joint disorder that involves inflammation of one or more joints. There are different forms of arthritis. The most common form, osteoarthritis (degenerative joint disease) is a result of trauma to the joint, infection of the joint, or age (Felson *et al.*, 2000; Leyland *et al.*, 2012). Osteoarthritis (OS) is characterized by progressive destruction of joint cartilage and its associated structures (bone, synovial and fibrous joint capsules), remodeling of the periarticular bone, and inflammation of the synovial membrane (Blagojevic *et al.*, 2010). This disorder is basically produced by an imbalance between the synthesis and degradation of the articular cartilage. This imbalance leads to the classic pathologic changes of wearing away and destruction of cartilage. (Kuptniratsaikul *et al.*, 2002; Loeser *et al.*, 2012)

Rheumatoid arthritis (RA) is characterized by inflammation of the synovial membrane of diarthrodial joints. Early indications of RA are swelling and pain of the proximal inter-phalangeal and later, the larger joints become affected, especially those of the knee, elbow and ankle. (Mc Innes and O'Dell, 2010) Hyperplasia or

thickening of the synovial membrane is promoted by cytokines and growth factors released from migrating cells. The synovial membrane becomes revascularised making it redder than normal. The cytokine enriched environment produced by pro-inflammatory cytokines (IL-1α, IL-6 and TNF-α) results in the aberrant growth of complex vessels known as pannus which invades the cartilage resulting in the degradation of the articular surfaces (Astusi *et al.*, 2005; McInnes and Schett, 2011).

Rheumatoid arthritis progresses in three stages. The first stage is the swelling of the synovial lining, causing pain, warmth, stiffness, redness and swelling around the joints. Second is the rapid division and growth of cell, or pannus, which causes the synovium to thicken. In the third stage, the inflamed cell releases enzyme that may digest the bone and cartilage, often causing the joints to lose its shape and alignments, more pain and loss of movements (Scott *et al.*, 1998; Mc Innes and Schett, 2011).

There are different classes of anti-arthritic drugs available like non-steroidal anti-inflammatory drugs (NSAIDS), Monoclonal antibodies, uricosuric agents, gold compounds, anti-cytokinine immunosuppressant like glucocorticoids, etc. Though the goal of these drugs has been to relieve pain and to decrease joint inflammation, these drugs are known to produce various side effects including gastrointestinal disorders, organ damages, immunodeficiency and humoral disturbances. (Harirforoosh and Jamali, 2009; Roth, 2005) Selective COX-2 inhibitors make alternative approach to arthritic treatment with reduced GI side effects, but on long term treatment leads to serious cardiovascular and thrombotic side effects. Accordingly, reducing side effects should be considered while designing improved therapeutics for arthritis, besides enhancing medicinal effectiveness (Moore, 2007).

The extracellular framework and two-thirds of the dry mass of adult articular cartilage are polymeric collagen. Treatment with chondroprotectives, such as glucosamine sulfate, chondroitin sulfate, hyaluronic acid, collagen hydrolysate, or nutrients, such as

antioxidants and omega-3 fatty acids are being increasingly recognized as an alternate approach to arthritic treatment (Henrotin *et al.*, 2012; Jerosch, 2011). Numerous clinical studies have demonstrated that the targeted administration of selected micronutrients leads to a more effective reduction of OA symptoms, with less adverse effects. Their chondroprotective action can be explained by a dual mechanism: (1) as basic components of cartilage and synovial fluid, they stimulate the anabolic process of the cartilage metabolism; (2) their anti-inflammatory action can delay many inflammation-induced catabolic processes in the cartilage. These two mechanisms are able to slow the progression of cartilage destruction and may help to regenerate the joint structure, leading to reduced pain and increased mobility of the affected joint (Sawitzke *et al.*, 2010).

Rat adjuvant arthritis is a chronic, polyarticular, erosive type of arthritis induced by an injection of killed mycobacterium (Pearson and Wood, 1959). It is an experimental model that shares some features with human rheumatoid arthritis. One of the most important features of adjuvant arthritis is the chronic synovitis, including inflammatory cell infiltration, pannus formation, cartilage destruction and bone erosion. Adjuvant arthritis is widely used for studying the pathogenesis of rheumatoid arthritis and for searching new drugs for treatment of rheumatoid disease (Greenwald, 1991; Zheng and Wei, 2005).

The present research work was aimed at the scientific validation of the anti arthritic effect of Fish Collagen Peptide (FCP) of fish skin origin in CFA induced rat model system. Freund's complete adjuvant induced arthritis in rat model which is the best and most widely used experimental model for arthritis with clinical and laboratory features which closely mimic the clinical features of human rheumatoid disease. This model is sensitive to anti-inflammatory and immune inhibiting medicines and considers being relevant for the study of pathophysiological and pharmacological control of inflammation process as well as for the evaluation of anti-

arthritic potential of drugs (Greenwald *et al.*, 1991; Simoes *et al.*, 2005).

Various experimental parameters like changes in Paw edema, Body weight, Arthritic index evaluated during the course of treatment and at the end of the study X ray radiographs, various blood parameters relevant in the arthritic condition, bone histopathology of synovial joints analysis were performed.

5.2 Materials and methods

5.2.1 Animals used

Wistar strain male albino rats, weighing130 ± 20 g, were selected for the study. The animals were kept in polypropylene cages (with stainless steel grill top) under hygienic and standard environmental conditions (temperature of 22 ± 2°C, humidity 60-70%, and 12 hr light/dark cycle). The animals were allowed a standard diet (procured from M/s Sai feeds, Bangalore, India) and water ad libitum. The study was conducted with the approval from the Institutional Animal Ethical Committee (IAEC) of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals).

5.2.2 Toxicity study

For toxicity studies, two groups of rats with 6 animals each were taken. One group observed as control and the other group is treated with FCP.For acute oral toxicity study, FCP at a single dose of 2g/kg body weight was given orally to test group whereas an equal volume of water was given to control group. Observations were made and recorded systematically 1, 2, 4 and 6 h after FCP administration. The visual observations were noted. The number of survivors was noted after 24 h and these were then maintained for a further 14 days with once in daily observation. On day 15, all rats were fasted for 16–18 h, then anesthetized with ethyl ether and sacrificed.

For sub acute oral toxicity study, FCP at the dose of 1g/kg body weight for 14 days, whereas an equal volume of water was given to control and kept for other 14 days after treatment. During the period of administration, the animals were weighed and observed daily to detect signs of toxicity. Daily visual observations were made and recorded systematically. At the end of the period, all rats were fasted for 16–18 h, then anesthetized with ethyl ether and sacrificed.

5.2.3 Complete Freund's adjuvant induced Arthritis

Arthritis was induced in rats by the intraplantar injection of 0.1 ml of Complete Freund's Adjuvant (CFA) containing1.0 mg dry heat-killed Mycobacterium tuberculosis per milliliter sterile paraffin oil into a foot pad of the left hind paw of male rats. A glass syringe (1 ml) with the locking hubs and a 26G needle was used for injection. The rats were anesthetized with ether inhalation prior to and during adjuvant Injection, as the very viscous nature of the adjuvant exerts difficulty while injecting. The paw swellings were periodically examined up to 21 days. The diameter of each paws from the ankle were measured using screw-gauge in mm measurement.

5.2.4 Experimental setup

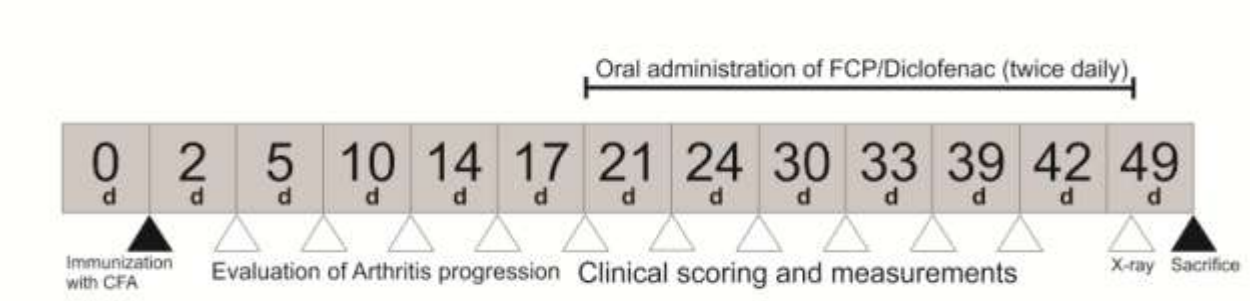
The effect of test drug for the established type of Adjuvant-induced arthritis was carried out by the method of Persico *et al.* (1988). The dosing schedule of the drug and the experimental set up are shown in table 5.1 and fig.5.1 respectively. The animals were divided into five groups of six animals each. Group I served as control (without treatment), Group II served as arthritic control (disease control), Group III was treated with diclophenac sodium (positive control), the standard drug. Group IV & V were treated with different concentrations of test sample, the FCP. The animals in all the groups (except group II) were administered with the treatment by oral route twice a day for 28 days. Animals in group I was administered with

10ml/ kg distilled water. While the animals in group III were treated with the standard drug diclophenac sodium (5mg/kg). Animals of group IV and V were fed with 0.5g/kg and 1.0g/kg FCP.

Table 5.1 Dosing schedule and treatment in different groups

Sl. No	Group	No. of Animals	Treatment	Dose
1	Group 1 Normal Control	6	Neither CFA treated nor drug treated	-----
2	Group 2 Disease Control	6	Freund's adjuvant (CFA)	0.2 ml (3mg/ml)
3	Group 3 Standard Drug Treatment	6	Diclofenac sodium + CFA	10 mg/kg body weight
4	Group 4 FCP treatment	6	Collagen peptide + CFA	0.5g/kg body weight
5	Group 5 FCP treatment	6	Collagen peptide + CFA	1 g/kg body weight

Figure 5.1 Experimental design of arthritis study



5.2.5 Evaluation of the development of arthritis

Rats were inspected daily for the onset of arthritis characterized by oedema and/or erythema in the paws. The incidence and severity of arthritis were evaluated using a system of arthritic scoring, and measurement of paw oedema every 2 days from the starting day of the experiment. Animals were observed for presence or absence of nodules in different organs like ear, fore paw, hind paw, nose and tail.

5.2.5.1 Paw oedema

Paw size of both hind limbs were recorded on the day of CFA injection, and measured every 2 days beginning on the day of starting the experiment using screw-gauge in mm measurement. The 6thday measurement is indicative of primary lesions and13thday measurement will aid in estimating secondary lesions. On the 21st day, the secondary phase of rheumatoid arthritis becomes more evident and inflammatory changes spreads systemically and becomes observable in the limb not injected with Freund's adjuvant. The mean changes in injected paw oedema with respect to initial paw size, were calculated on respective days and percentage inhibition of paw oedema with respect to untreated group was calculated using following formula.

Percentage inhibition = 1-Change in paw oedema of untreated group/ change in paw oedema of treated group x 100

5.2.5.2 Body weight

Changes in body weight have also been used to assess the course of the disease and the response to therapy of anti arthritic drugs. Body weight of each animal was measured every 3 days alternatively using electronic balance.

Percentage increase in body weight = (final body weight-initial body weight)/ initial body weight x 100

5.2.5.3 Arthritis score assessment

The incidence and severity of arthritis were evaluated using a system of arthritic scoring every 3 days beginning on the day next to adjuvant injection by two independent observers. Lesions of both hind paws of each rat were graded from 0 to 4 according to its clinical arthritic signs described by Brand *et al.*, (2007). The scoring system is in table 5.2

Table 5.2 Scoring system for subjective evaluation of arthritis severity

Severity score	*Degree of inflammation*
0	No evidence of erythema and swelling
1	Erythema and mild swelling confined to tarsals or ankle joint
2	Erythema and mild swelling extending from the ankles to the tarsals
3	Erythema and moderate swelling extending from the ankles to the tarsals
4	Erythema and severe swelling encompass the ankle, foot and digits, or ankylosis of the limb

The total arthritis scores were calculated from the sum of both hind paws, with a maximum possible score of 8 for each rat.

5.2.5.4 Biochemical Analysis

The animals were sacrificed by ether anesthesia at the end of the experiment and the blood was collected by cardiac puncture prior to the sacrifice. The spleen were rapidly removed and washed with ice-cold saline. The tissues were cut into small pieces and homogenised using tris buffer (0.01 M, pH 7.4) at 4°C to give 10% homogenate. The haemolysate was extracted. The collected blood with anti-coagulant was centrifuged to remove the plasma.

Blood samples were collected from a carotid artery into heparinized and dry non-heparinized centrifuge tubes. The heparinized blood was used for hematological study and the serum separated from the non-heparinized blood was assayed for biochemical analysis. For biochemical analysis, blood was centrifuged at 1500 g for 10 min to obtain serum and the following parameters like ALP (marker for bone destruction), ACP (the lysosomal enzyme activity), SGOT, SGPT, CRP, ceruloplasmin, urea, creatinine, were estimated by using respective kits.

5.2.5.5 Anti CCP

Anti Cyclic Citrullinated Peptide Ab has been assayed since it is a convenient immune marker for the inflammation. This test is considered as a novel arthritis detection test commonly employed in humans. The analyses anti-CCP was carried out in the Department of Clinical Immunology, DDRC Ernakulam.

Anti-CCP antibodies were detected using a commercial anti-CCP2 enzyme linked immunosorbent assay kit, following the manufacturer's instructions. Briefly, microtitre plates were incubated for 60 minutes at room temperature with serum samples diluted at 1:100 in phosphate buffered saline. Prediluted anti-CCP standards and positive and negative controls were added to each plate. All assays were done in triplicate. After three washes, plates were incubated for 30 minutes at room temperature with alkaline phosphatase labelled murine monoclonal antibody to human IgG. After three further washes, the enzyme reaction was developed for 30 minutes, stopped with sodium hydroxide-EDTA-carbonate buffer, and the plates were read at 550 nm wavelength. Anti-CCP was considered positive when the absorbance value was higher than the cutoff of the kit (5 U/ml). The concentration of anti-CCP autoantibody was estimated by interpolation from a dose–response curve based on standards. All serum samples with high concentrations of anti- CCP antibodies were further quantified at a greater sample dilution.

5.2.5.6 COX (Cycloxygenase) activity assay

The COX levels in CFA-induced rat paws were determined using Cayman's COX fluorescent activity assay kit. It provides a convenient fluorescent based method for determining COX I and COX ll activities in both crude (tissue homogenates) and purified enzyme preparations. The assay utilizes the peroxidase component of COXs. In this assay, the reaction between PGG2 and ADHP (10-acetyl-3,7-dihydroxyphenoxazine) produces the highly fluorescent compound resorufin which can be analyzed using an excitation

wavelength of 530-540 nm and an emission wavelength of 585-595 nm The kit also includes isozyme-specific inhibitors for distinguishing COX ll activity from COX l. The COX concentration is expressed as Fluorophore Units (FU) from the standard curve for resorufin. 1 FU is defined as the amount of enzyme that will cause the formation of 1 nm fluorophore per minute at 22°C.

5.2.5.7 Histological processing and assessment of arthritis damage

The histopathologic assessment was focused on the ankle joints with the most severe joint damage and each joint was evaluated separately. Hematoxylin and eosin-stained sections were observed for inflammation and pannus formation by two independent observers.

Histopathological changes were scored using the following parameters. Infiltration of cells was scored on a scale from 0 to 3, depending on the amount of inflammatory cells in the synovial tissues. Inflammatory cells in the joint cavity were graded on a scale from 0 to 3 and expressed as exudate. A characteristic parameter in Freund's complete adjuvant is the progressive loss of articular cartilage. This destruction was separately graded on a scale from 0 to 3, ranging from the appearance of dead chondrocytes (empty lacunae) to complete loss of the articular cartilage. Bone erosion was scored on a scale ranging from 0 to 3, ranging from no abnormalities to complete loss of tarsus. Cartilage and bone destruction by pannus formation was scored ranging from 0, no change; 1, mild change (pannus invasion within cartilage); 2, moderate change (pannus invasion into cartilage/subchondral bone); 3, severe change (pannus invasion into the subchondral bone); and vascularity (0, almost no blood vessels; 1, a few blood vessels; 2, some blood vessels; 3, many blood vessels).

5.2.5.8 Radiological findings

X-ray radiography analysis has been done in order to display changes in the joints such as bony erosion and variation at joints of

different experimental animals. Before sacrificing the animals; X-rays were taken at the joints of the hind paw of the animals for evaluating the bone damage. Radiographs were taken using X-ray apparatus (Siemens- 60MA, Germany) and industrial X-ray film (Fuji photo film, Japan). The X-ray apparatus was operated at 220 V with a 40 V peak, 0.2 second exposure times, and a 60cm tube-to film distance for leg projection. Radiological visual scoring was performed by two different observers and visual scoring values were calculated based on the following conditions

Erosions: 0-3 (none, mild, moderate, severe),
Joint space narrowing: 0-3(none, minimal, moderate, severe), and
Joint space destruction: 0-3(none, minimal, extensive, ankylosis)

5.2.5.9 Statistical analysis

The results are expressed as Mean ± SE from n=6 observations. The findings were also analyzed for determining significance of difference by ANOVA test followed by pair-wise comparison of various group by LSD. The differences among groups were considered to be significant at $p<0.05$.The analysis was carried out by using SAS system version 9.3 (SAS Institute Inc., Cary, NC, USA)

5.3 Results and Discussion

A considerable part of the disability caused by arthritis conditions is joint damage. Accordingly, preventing and diminishing joint damage is an important treatment goal in early arthritis. Hence, reliable predictors of joint damage are required.

The immunologically mediated complete Freund's adjuvant (CFA) arthritic model of chronic inflammation is considered as the best available experimental model of arthritis (Williams, 1998) as it has been shown to share a number of clinical and immunological features with human arthritis. Method mimics the human pathophysiological state including chronic swelling in multiple

joints due to accumulation of inflammatory cells, joint cartilage erosion, bone destruction and used to investigate the activity of various potent anti-inflammatory and anti-arthritic agents. Therefore, the findings with this model are considered to have higher clinical reproducibility in arthritis.

In CFA model, macrophages play a central role. After activation they are capable of synthesizing mediators such as PGE2 and cytokines such as TNF-α and IL-1 and they induce the production of a variety of enzymes which initiate cartilage and bone destruction (Hopkins *et al.,* 1990). It is also reported that damage to the cartilage in arthritic joint is associated with the cellular output of toxic agents such as nitric oxide and its oxidizing product (e.g. peroxynitrite),free radicals and products of hydrogen peroxide (e.g. hydroxyl radical and hypochlorous acid).

Augmentation in migration of total leukocyte, lymphocytes and monocytes/macrophages from blood into the synovial cavity influence the arthritic condition of joint (Levy *et al.,* 2006) and these mediators are responsible for the pain, destruction of cartilage and leads to severe disability.

5.3.1 Toxicity study

Acute and sub acute oral toxicity study in male Wistar albino rats shows no evidence of significant adverse effect or health risk toxic effects. According to the hematological, biochemical, and organ weight examinations, some parameters differed in both the male and female rats but none of these appeared to be of toxicological significance, and were slightly higher or lower than those of the controls. Correspondingly, these data are within the normal limits established under laboratory control as determined by Lillie *et al.* (1996). Thus, it can be concluded that FCP is virtually non-toxic. The study results provide an experimental basis for FCP to be safely used as ingredients of functional foods or pharmaceuticals.

Arthritis was induced reproducibly in all animals injected the adjuvant, with onset of injected hind paw (right paw) erythema and swelling (arthritis onset) occurring on day 9, swelling of non-injected hind paw (left paw) began on day 11 and persisted to the end of the experiment.

In the present study the arthritic rats showed a soft tissue swelling that was noticeable around the ankle joints during the acute phase of arthritis and was due to oedema of periarticular tissues such as ligaments and joint capsules (fig.5.2). The swelling has been found to be increasing in the initial phase of inflammation and then becomes constant in 2 weeks. These changes in paw volume have been found to be associated with an increase in granulocytes and monocytes (Arend and Dayer, 1990). Because, the activation of macrophages results in the production of several cytokines including IL-1, IL-6, interferon-γ (IFN-γ) and TNF-α which have been implicated in immune arthritis (Dai *et al.*, 2000).

TNF-α is mainly involved in the perpetuation of the inflammatory cascades in autoimmune diseases, which affect connective tissues where the connective tissues become hyper contracted due to inflammation (Kinne *et al.*, 2000). Furthermore, macrophage derived nitrous oxide may increase vasodialation and vascular permeability at the inflammatory site, which may aggravate the arthritic process (Nissler *et al.*, 2004). Moreover, prostaglandins greatly potentiate exudates by inducing relaxation of arteriolar smooth muscle cells and increasing the blood supply to the tissue (Simon *et al.*, 1965).

Several scientific reports have presented good bioavailability of hydrolyzed collagen, after oral administration by animals and human beings. Oesser *et al.* (1999) discovered that about 95% of orally applied collagen hydrolysate was absorbed within the first 12 h. Zague (2008) described the high safety of eating collagen hydrolysates in an animal model (1.66 g/kg of body weight per day).

5.3.2 Effect on Paw edema

The challenge with CFA (1%, 0.1ml) showed significant increase in paw oedema which has reached to peak on 3rd week and remained constant by the end of the study in arthritis control as compared to normal control. The data indicate that the intradermal injection of CFA may induce arthritis and was characterized by initial swelling of one or more limbs, which resulted in increase of paw oedema of 54.65% in the arthritic control group.

Later characteristics of the disease included gross joint deformation and total loss of joint mobility. The observations are in agreement with the study of Courtenay *et al.* (1980) who also demonstrated the incidence of arthritis in course of time by using complete freunds adjuvant.

Rats fed with FCP (0.5 & 1.0g/kg) showed significant and dose-dependent attenuation in paw oedema from day 30 onwards as compared to Disease Control rats. Rats treated with diclophenac (10mg/kg) significantly decreased ($P < 0.05$) paw volume from day 25 to 50 and the effect is comparable with the test sample, FCP 0.1 g/kg ($P < 0.05$). Also there is significant difference between the two dosages. (Significant difference is shown in table 5.3 and trend of the treatment effect in different groups is shown in fig. 5.4).

5.3.3 Effect on Body weight

As the incidence and severity of arthritis increased, there is significant decrease in the body weights of the arthritis control occurred as compared with normal control during the course of the experimental period. But the results showed the drug treated group could ameliorate the weight loss occurred during arthritis and there is no significant difference in the effect of diclophenac and FCP (1g/kg) ($P < 0.05$) treated groups.

Figure 5.2 Morphological representations of rat paw. *(A)Normal control (B)ArthritisControl.*

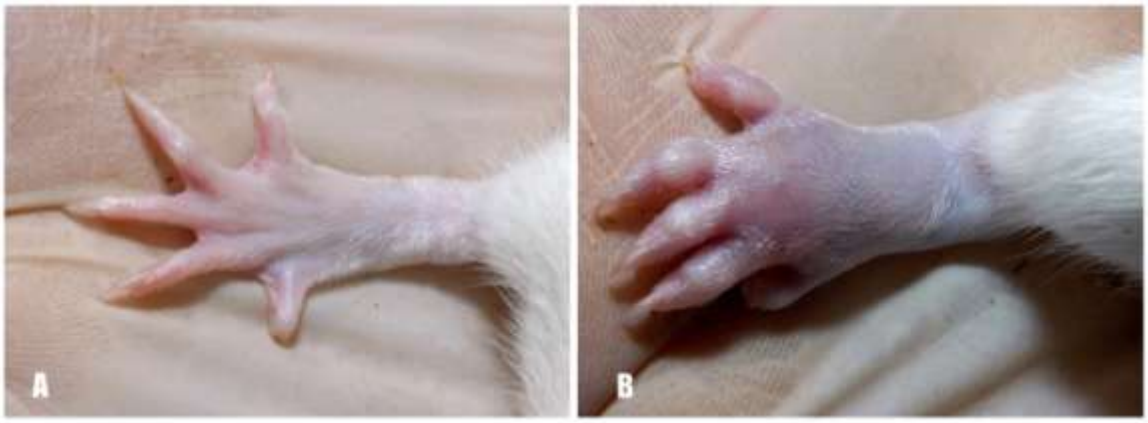
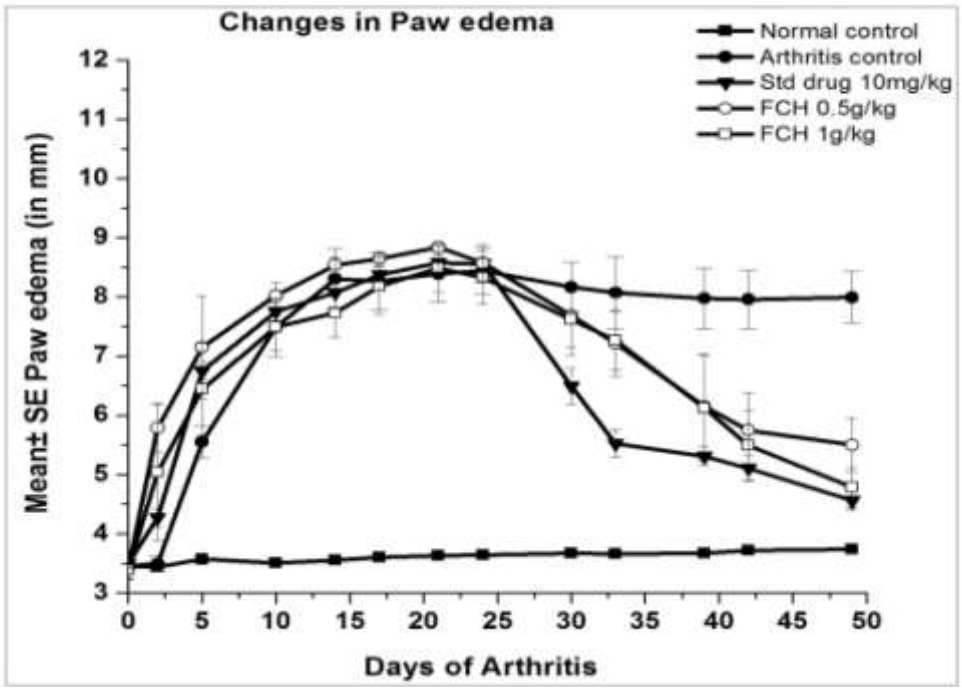


Figure 5.3 Morphological representations of rat paw after the treatment period

Figure 5.4 Mean paws oedema change over time. Values are plotted as the mean ± SE (n=6) in each group



5.3.4 Arthritic score assessment

Arthritic score is a clinical assessment of joint swelling (Funk *et al.,* 2006). In the present study as a result of CFA induced inflammation, the arthritic score was increased till the end of the study ($p<0.05$) in CFA treated rats when compared with control rats. Treatment with standard drug and FCP beginning on day 21 showed significantly decreased ($p<0.05$) arthritic score. There is no significant difference in the treatments ($p<0.05$). The significant difference in each group is shown in table 5.5. The trend of arthritic score is graphically shown in fig.5.6.

The alteration in plasma protein induces the synthesis of proinflammatory cytokines, prostaglandins, leukotrienes and matrix metallo proteinases that caused fluid accumulation in the synovium. This results in an increase in arthritis scores due to damage in joints and bones of the rats paw (Cai *et al.,* 2007).

Table 5.3 Effect of FCP on Paw edema of adjuvant arthritic rats

Treatments	*Day 21*	*Day 24*	*Day 30*	*Day 33*	*Day 39*	*Day 42*	*Day 49*	*% inhibition on 49th day*
Normal control	3.63 ± 0.02	3.64 ± 0.02	3.67 ± 0.01	3.66 ± 0.01	3.66 ± 0.02	3.72 ± 0.02	3.74 ± 0.04[a]	
Arthritis control	8.39 ±0. 48	8.44 ± 0.39	8.17 ± 0.41	8.07 ± 0.61	7.98 ± 0.51	7.96 ± 0.49	7.80 ± 0.44[b]	
Std drug	8.58 ± 0.11	8.56 ± 0.09	6.49 ± 0.30	5.53 ± 0.24	5.31 ± 0.16	5.11 ± 0.21	4.56 ± 0.16[c]	90.13
FCP 0.5mg/kg	8.83 ± 0.08	8.57 ± 0.31	7.68 ± 0.53	7.22 ± 0.56	6.15 ± 0.88	5.75 ± 0.64	5.50 ± 0.46[d]	88.11
FCP 1.0mg/kg	8.49 ±0. 40	8.33 ± 0.45	7.62 ± 0.59	7.27 ± 0.49	6.13 ± 0.89	5.49 ± 0.58	4.79 ± 0.33[c]	89.29

Values are expressed as mean ± SE (n=6). Statistical significance was calculated by ANOVA followed by tukey's studentized range (HSD) test

Table 5.4 Effect of FCP on body weight of adjuvant arthritic rats

Treatments	*Day 21*	*Day 24*	*Day 30*	*Day 33*	*Day 39*	*Day 42*	*Day 49*	*% increase in Body weight*
Normal control	157.16 ±0.60	165.00 ± 0.86	170.50 ± 0.85	173.50 ± 0.56	179.66 ± 0.95	188.17 ± 0.91	194.17 ± 1.08[a]	23.55
Arthritiscontrol	152.33 ± 0.61	153.50 ± 0.84	151.66 ± 0.41	147.83 ± 0.61	146.16 ± 0.51	143.83 ± 0.60	142.83 ± 0.83[b]	-6.24
Std drug	140.00 ± 0.85	143.00 ± 0.77	148.00 ± 0.89	149.50 ± 0.76	153.83 ± 0.52	156.67 ± 1.11	161.17 ± 0.87[c]	15.12
FCP 0.5mg/kg	149.50 ± 0.34	151.50 ± 0.42	152.66 ± 0.61	154.33 ± 0.71	156.83 ± 1.13	160.50 ± 1.23	165.00 ± 1.39[d]	10.37
FCP 1.0mg/kg	145.66 ± 0.66	148.66 ± 0.76	152.33 ± 0.84	155.50 ± 0.85	159.00 ± 0.93	163.33 ± 1.16	166.83 ± 1.01[c]	14.53

Values are expressed as mean ± SE (n=6). Statistical significance was calculated by ANOVA followed by tukey's studentized range (HSD) test

5.3.5 Effect on biochemical parameters

The biochemical profiles of the treated and control groups arepresented in Table 5.6 and 5.7. Some of the biochemical values of the collagen peptide treated rats differed slightly from those of the controlgroups, but some values were in the range of normal and are graphically shown in fig.5.7.

The biochemical estimations of ALP, ACP, and SGPT, SGOT, blood urea, creatinine and total protein were carried out to detect the toxic effect on the liver and kidney. In the present study, no significant changes were observed in biochemical parameters after 42 days of drug treatment compared with normal group.

The toxicity in liver, kidney and heart are the most common adverse effect when we administer nonsteroidal anti-inflammatory drugs, anti-rheumatic drugs and steroids. The cytoplasmic enzymes like AST and ALT serves as indicators and suggestive for disturbances of the cellular integrity induced by pathological conditions. These enzymes are used as sensitive markers for evaluation of protective activity, against persistent inflammation. The increased enzyme activity may result from one of the several mechanisms which include the release of various enzymes from leukocytes, from necrotic or inflamed synovial tissue and production and release of an increased amount of enzymes due to altered synovial tissue (Schellekens, *et al.*, 2000).

AntiCCP assay is a novel and most reliable method for detecting the prognosis of the arthritic inflammation in affected animals. Significant decrease in anti CCP level has seen in group IV and V rats. This clearly indicates the positive effect of collagen peptide against rheumatoid arthritis.

Figure 5.5 Mean body weight change over time. Values are plotted as the mean ± SE (n=6) in each group

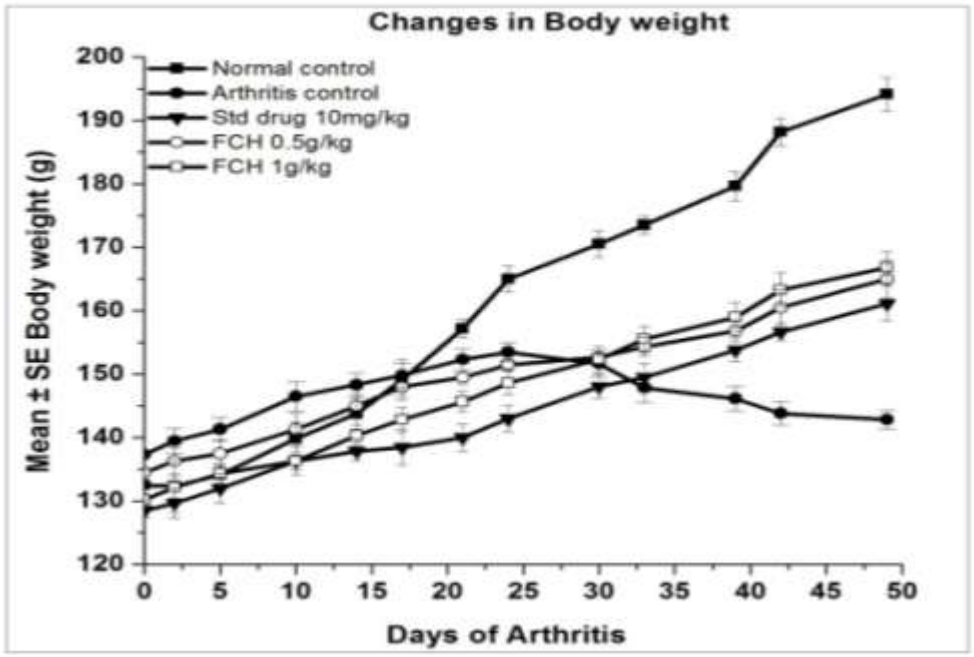
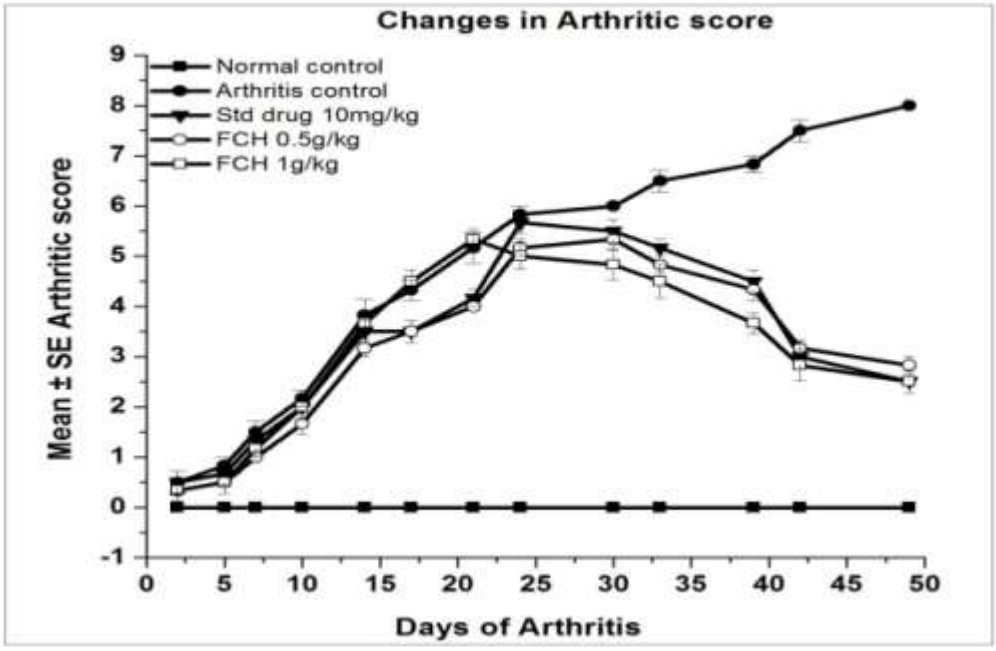


Figure 5.6 Changes in arthritic score over time. Values are plotted as the mean ± SE (n=6) in each group



5.5 Effect of FCP on Arthritic score of adjuvant arthritic rats

Treatments	***Day 21***	***Day 24***	***Day 30***	***Day 33***	***Day 39***	***Day 42***	***Day 49***
Normal control	0	0	0	0	0	0	0[a]
Arthritis control	5.17 ± 0.31	5.83 ± 0.39	6.00 ± 00	6.50 ± 0.22	6.83 ± 0.17	7.50 ± 0.22	8.00 ± 0.00 [b]
Std drug	4.17 ±0.16	5.67 ± 0.21	5.50 ± 0.22	5.17 ± 0.17	4.50 ± 0.22	3.00 ± 0.26	2.50 ± 0.22 [c]
FCP 0.5mg/kg	4.00 ± 0.00	5.17 ± 0.17	5.33 ± 0.21	4.83 ± 0.17	4.33 ± 0.21	3.17 ± 0.16	2.83 ± 0.17[c]
FCP 1.0mg/kg	5.33 ± 0.21	5.00 ± 0.26	4.83 ± 0.30	4.50 ± 0.34	3.67 ± 0.21	2.83 ± 0.31	2.50 ± 0.22 [c]

Values are expressed as mean ± SE (n=6). Statistical significance was calculated by ANOVA followed by tukey's studentized range (HSD) test

As far as enzyme markers are concerned ACP level is a convenient method to predict the prognosis of inflammation status in affected animals. Remarkably ACP level was clearly in favor to predict the anti arthritic effect of collagen peptide and it even does a slight advantage over standard drug.

Table 5. 6 Effect of FCP on biochemical parameters of adjuvant arthritic rats evaluated on 49th day

Treatments	ACCP	ALP	ACP	SGOT	SGPT
Normalcontrol	04.07±0.08^{c}	120.67±1.45^{a}	19.67±1.20^{a}	128.33±0.88^{c}	44.00±0.57^{b}
Arthritiscontrol	14.33±0.33^{a}	136.67±2.33^{a}	24.00±0.57^{a}	151.33±6.88^{ab}	28.67±2.03^{d}
Std drug	05.37±0.09^{b}	120.33±1.45^{b}	21.67±1.67^{a}	167.67±1.45^{a}	32.67±2.03^{cd}
FCP 0.5mg/kg	07.10±0.30^{b}	113.33±0.88^{b}	19.67±1.20^{a}	152.67±4.33^{ab}	38.00±0.57^{bc}
FCP 1.0mg/kg	06.53±0.15^{b}	120.67±5.36^{b}	20.67±1.45^{a}	143.00±4.16^{bc}	55.67±2.73^{a}

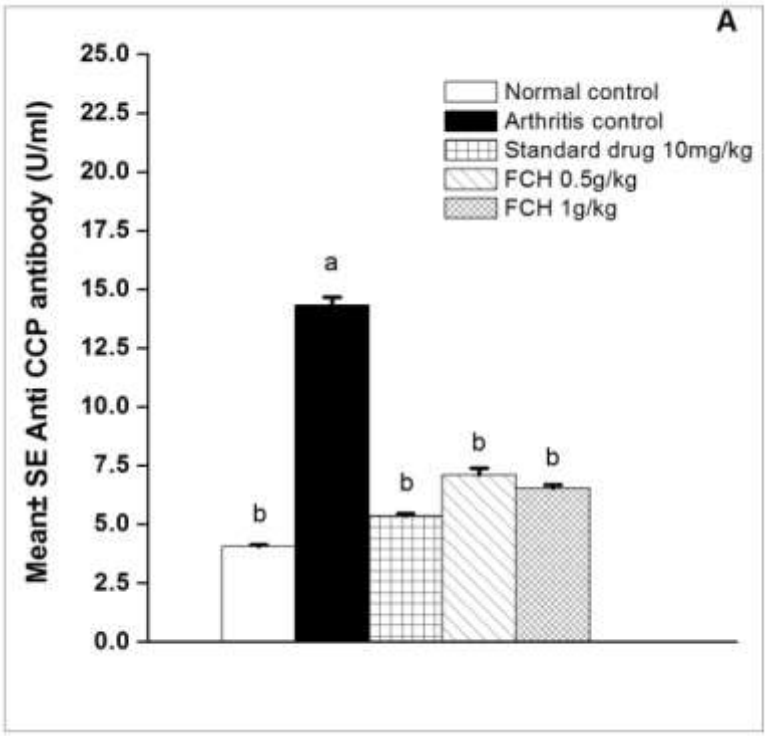
Values are expressed as mean ± SE (n=6). Statistical significance was calculated by ANOVA followed by tukey's studentized range (HSD) test

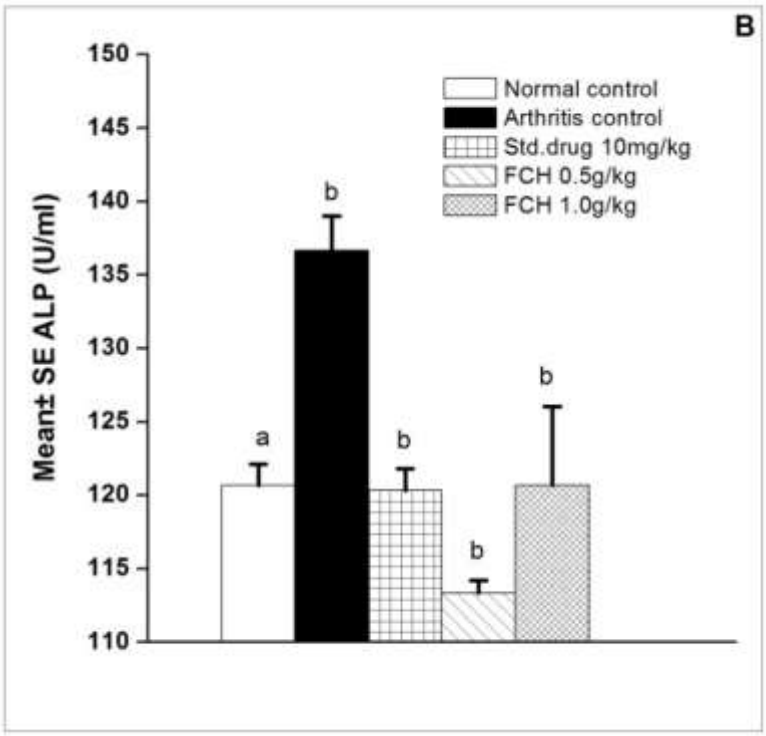
Table 5.7 Effect of FCP on biochemical parameters of adjuvant arthritic rats evaluated on 49th day

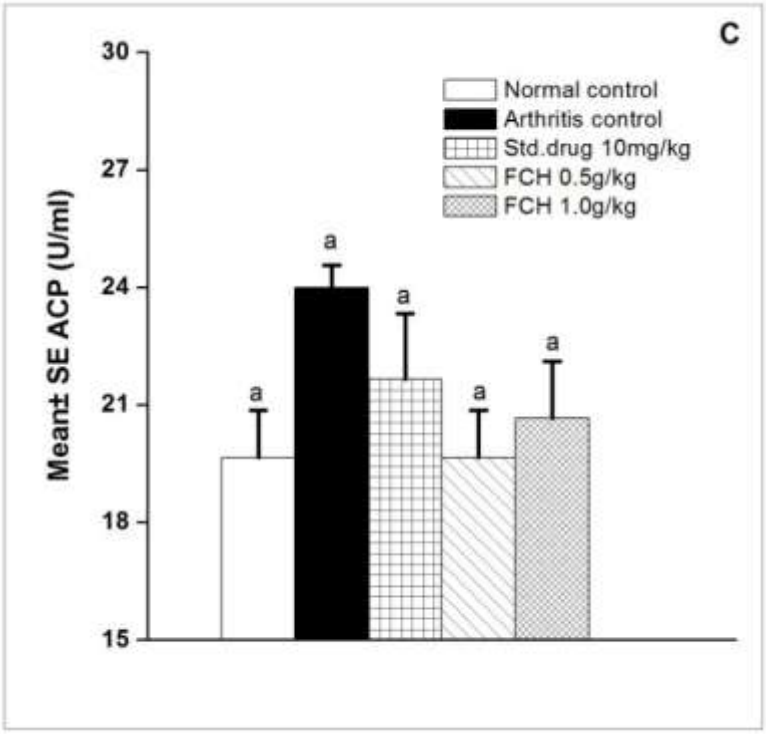
Treatments	CRP	Ceruloplasmin	Urea	Creatinine
Normal control	1.96±0.03^{a}	23.00 ± 2.52^{a}	32.00 ± 1.73^{a}	0.43±0.03^{a}
Arthritis control	2.10 ±0.15^{a}	23.00 ± 1.15^{a}	32.33± 4.81^{a}	0.43±0.03^{a}
Std drug	2.03±0.14^{a}	22.33 ± 0.33^{a}	40.67± 2.33^{a}	0.43±0.03^{a}
FCP 0.5mg/kg	2.13±0.03^{a}	20.67 ± 0.33^{a}	34.00 ± 2.88^{a}	0.33±0.09^{a}
FCP 1.0mg/kg	1.27±0.14^{b}	20.00 ± 1.15^{a}	35.33± 0.33^{a}	0.37±0.09^{a}

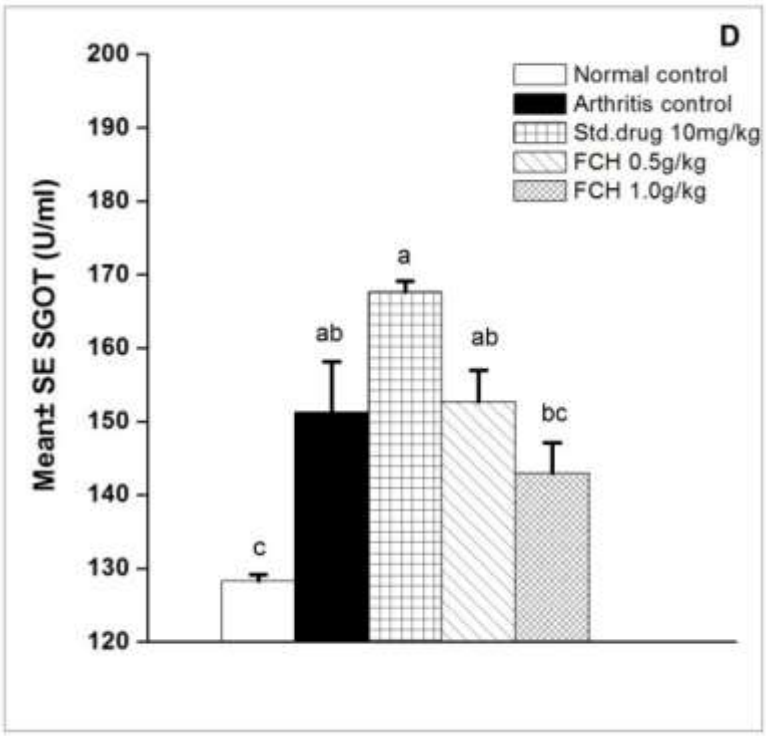
Values are expressed as mean ± SE (n=6). Statistical significance was calculated by ANOVA followed by tukey's studentized range (HSD) test

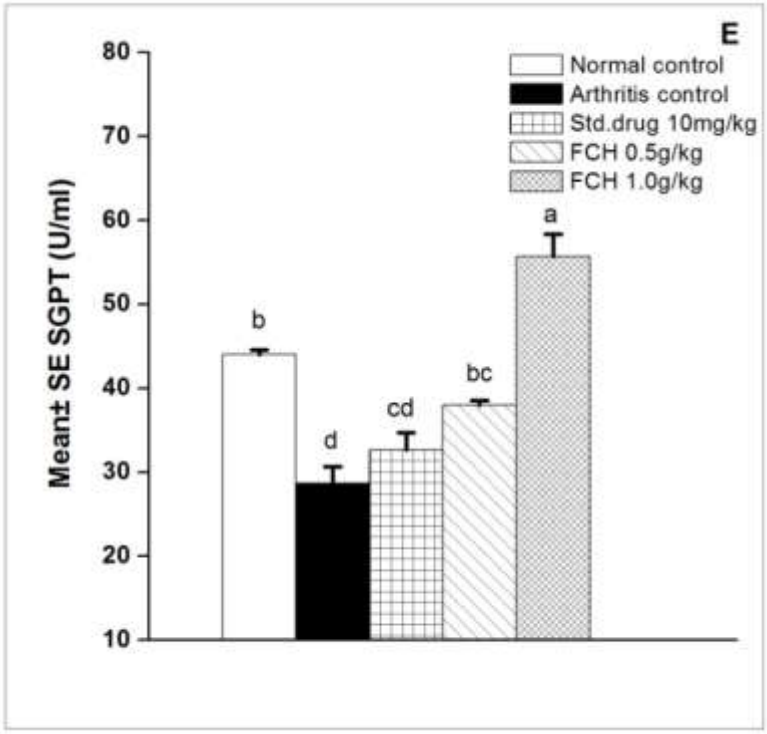
Figure 5.7 Effect of treatment on biochemical parameters of adjuvant arthritic rats (A) Anti CCP antibody (B) ALP (C) ACP (D) SGOT (E) SGPT (F) CRP (G) Ceruloplasmin (H) Urea (I) Creatinine

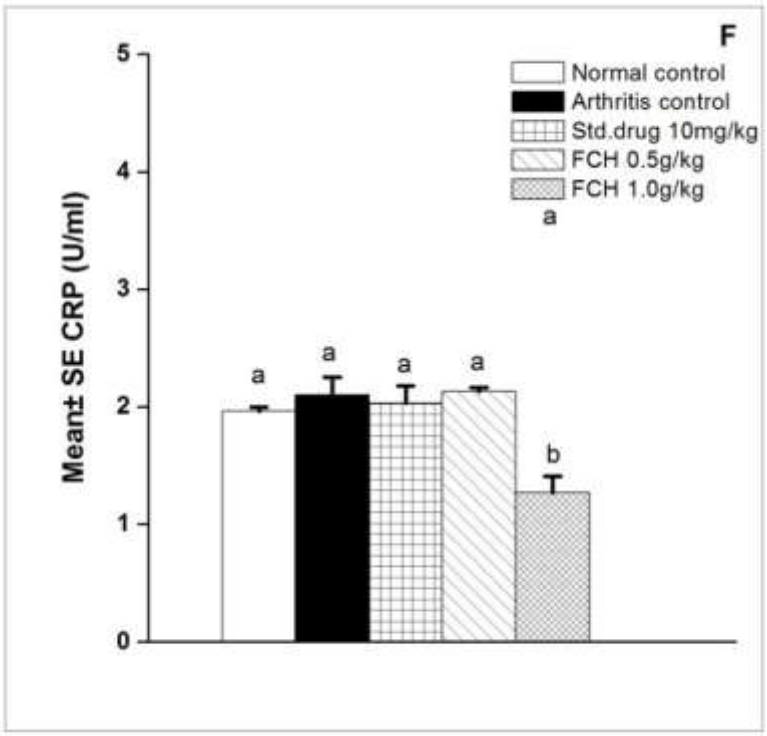

A
Mean± SE Anti CCP antibody (U/ml)
25.0
22.5
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
Normal control
Arthritis control
Standard drug 10mg/kg
FCH 0.5g/kg
FCH 1g/kg
a
b
b
b
b

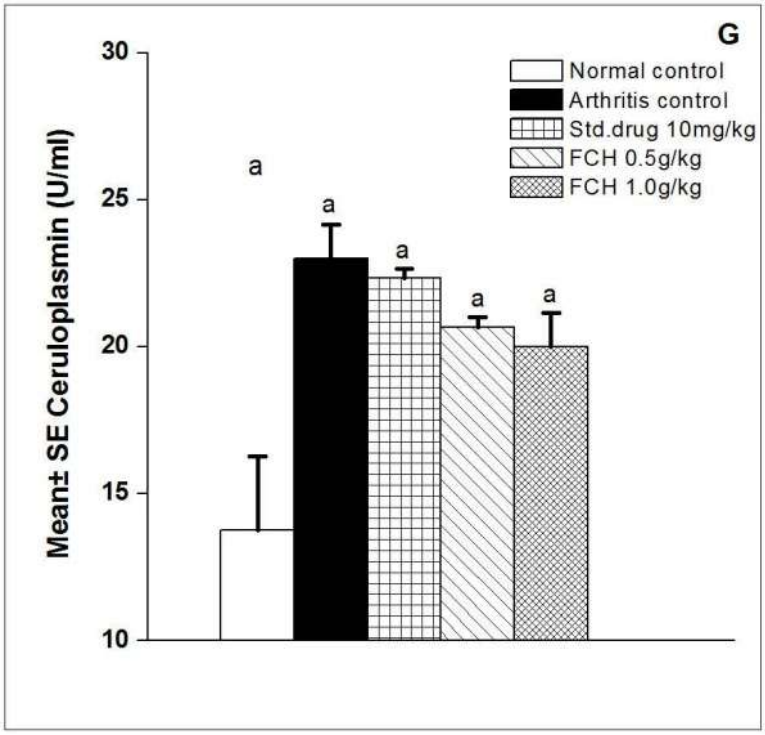

B
Mean± SE ALP (U/ml)
150
145
140
135
130
125
120
115
110
Normal control
Arthritis control
Std.drug 10mg/kg
FCH 0.5g/kg
FCH 1.0g/kg
a
b
b
b
b

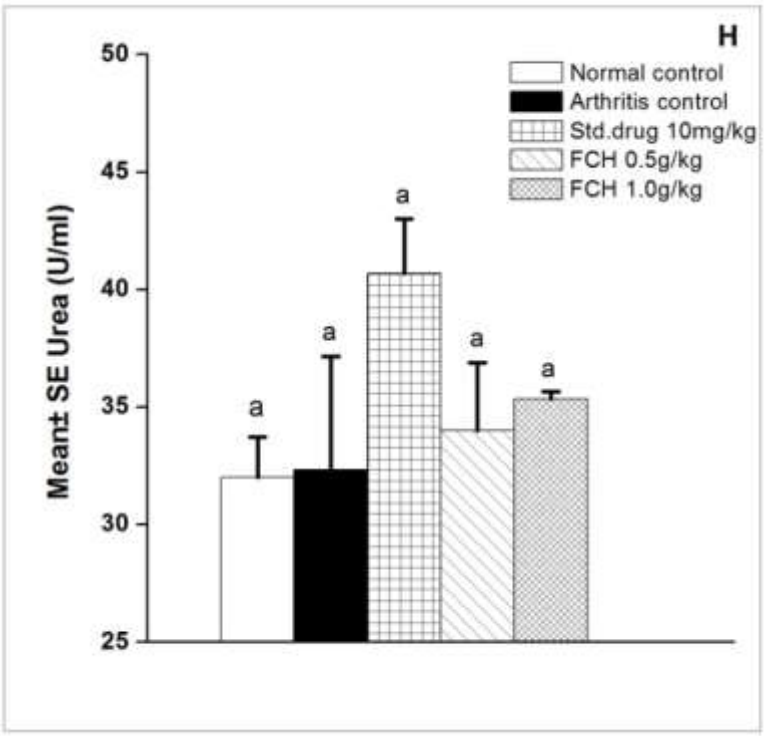

C
Normal control
Arthritis control
Std.drug 10mg/kg
FCH 0.5g/kg
FCH 1.0g/kg
Mean± SE ACP (U/ml)
30
27
24
21
18
15
a
a
a
a
a

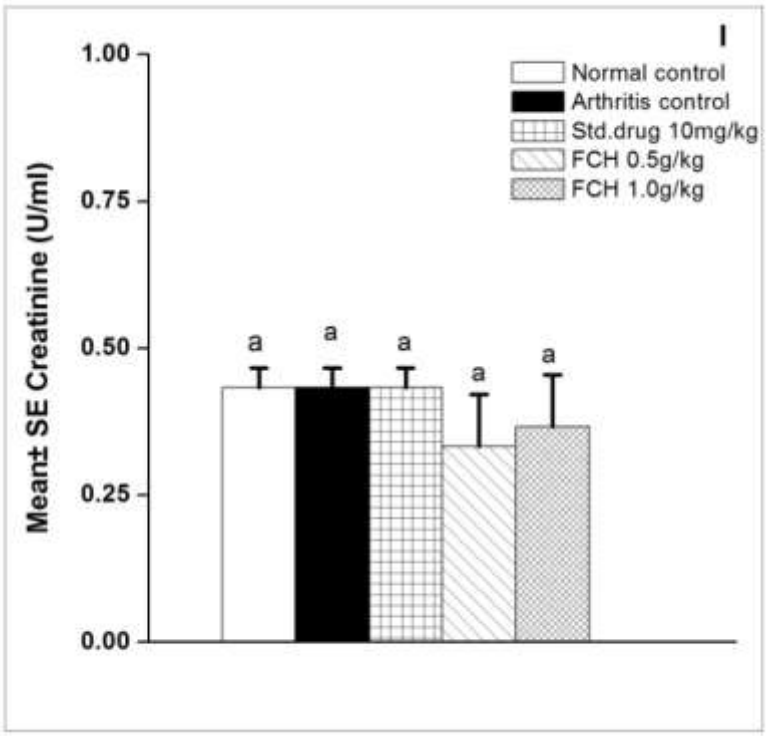

D
Normal control
Arthritis control
Std.drug 10mg/kg
FCH 0.5g/kg
FCH 1.0g/kg
Mean± SE SGOT (U/ml)
200
190
180
170
160
150
140
130
120
c
ab
a
ab
bc


E
Normal control
Arthritis control
Std.drug 10mg/kg
FCH 0.5g/kg
FCH 1.0g/kg
Mean± SE SGPT (U/ml)
80
70
60
50
40
30
20
10
b
d
cd
bc
a


F
Normal control
Arthritis control
Std.drug 10mg/kg
FCH 0.5g/kg
FCH 1.0g/kg
Mean± SE CRP (U/ml)
5
4
3
2
1
0
a
a
a
a
b
a


G
Normal control
Arthritis control
Std.drug 10mg/kg
FCH 0.5g/kg
FCH 1.0g/kg
Mean± SE Ceruloplasmin (U/ml)
30
25
20
15
10
a
a
a
a
a


H
Normal control
Arthritis control
Std.drug 10mg/kg
FCH 0.5g/kg
FCH 1.0g/kg
Mean± SE Urea (U/ml)
50
45
40
35
30
25
a
a
a
a
a



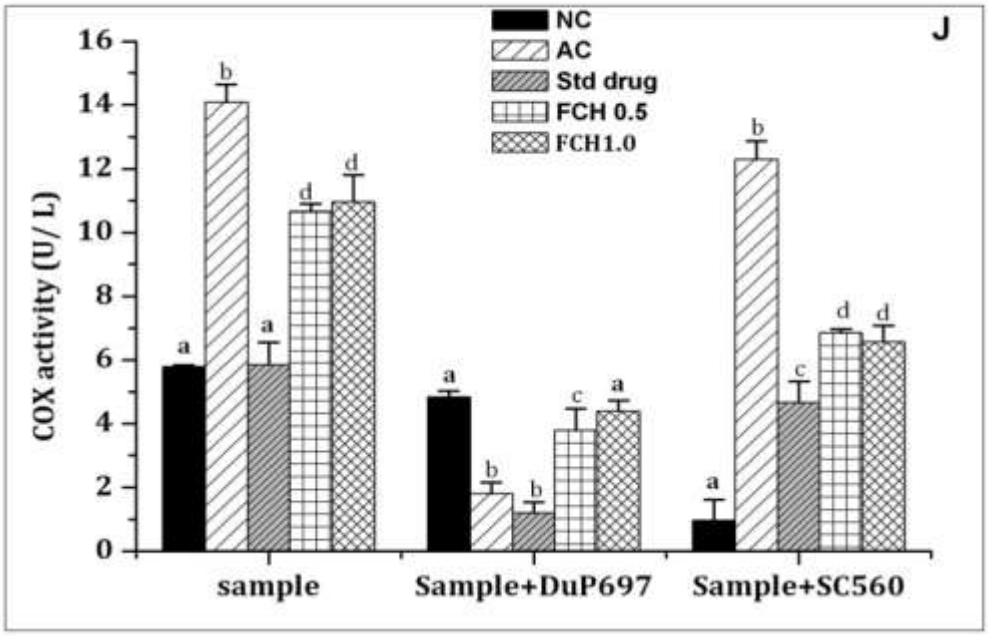
5.3.6 Effect of inhibition of COX

The total COX activity from paw exudate was significantly ($p<0.05$) increased in arthritic control (57.15%) and FCP treated group (31.25%) meanwhile in drug treated group there was significant reduction in total COX activity.The activities of COX I and COX II were individually monitored in all the groups with the help of enzyme specific inhibitors SC-560 and DUP-697 which are selective COX I and COX II inhibitors. In the treated groups there was significant reduction in the COX II activity compared to arthritic control and the inhibition is more for diclophenac treated group (61.5%). But it shows a negative effect that it not only inhibit COX II but also significantly inhibit biologically important COX I. There is no significant difference between the COX I levels of arthritic control and diclophenac treated group. COX I levels are comparable with normal control and FCP (1g/kg) treated group. The normal control group showed negligible

COX II activities thus confirming the fact that COX II is an inducible form of COX released only during conditions of acute inflammations. The effect is graphically shown in fig.5.8.

FCP is thus highly desirable since inflamed tissues could be targeted without disturbing the homeostatic functions of prostaglandins in noninflamed organs. Theoretically, then, selective COX-2 inhibition should preserve the anti-inflammatory efficacy without causing the associated toxicities of NSAIDs.

Figure 5.8 Effect of treatment on COX activity in adjuvant arthritic rats



5.3.7 Radiological findings

Bone destruction, which is a common feature of adjuvant arthritis, was examined by radiological analysis. Soft tissue swelling is the earlier radiographic sign, whereas prominent radiographic changes like bony erosions and narrowing of joint spaces can be observed only

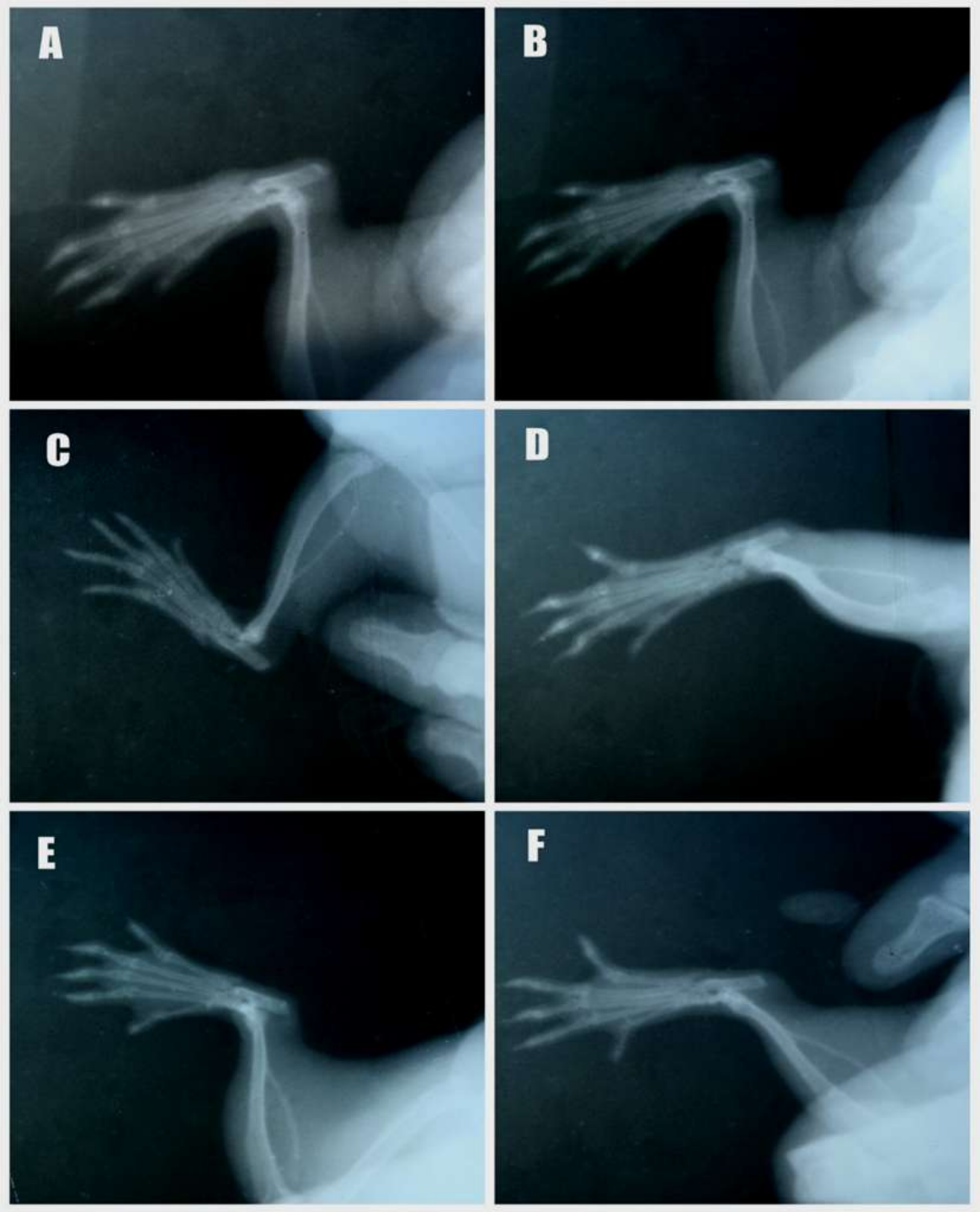
in the developed stages of arthritis (Harris, 1990). The loss of articular cartilage leads to diminished joint space, which may be brought about a variety of pathological mechanisms.

Fig. 5.9 shows radiographic changes in joints of control and treated rats. Arthritis control group had developed definite joint space narrowing of the intertarsal joints, diffuse soft tissue swelling that included the digits, diffuse demineralization of bone, marked periosteal thickening, and cystic enlargement of bone and extensive erosions produced narrowing or pseudowidening of all joint spaces. The degree of bone resorption, diminished joint space and tissue swelling was markedly reduced in test treated groups.

The standard drug diclophenac sodium treated groups, there is considerable reduction in soft tissue swelling and narrowing of the joint space as compared to arthritis control.

Test treated groups (FCP of different concentrations, 0.5mg/kg and 1mg/kg body weight) shows moderate effect on change in joint architecture and it attenuate abnormalities consisted of asymmetric soft tissue swelling and small erosions, periosteal thickening, and minimal joint space narrowing, predominantly localized to the proximal areas of the paws. But there is no observable difference in the two dosages.

Figure 5.9 Radiographic changes in joints of control and treated rats. *A- Normal control, B & C – Arthritis control, D – diclophenac treated group, E &F – FCP treated group. No evidence of pathological changes was observed in vehicle control group. Control group showing severe inflammation with diffused joint space and bone erosion. Treated group showing decreased soft tissue swelling with no evidence of bone erosion and inflammation.*


A
B
C
D
E
F

5.3.8 Histopathological changes in hind paw joints

Soft tissue swelling around ankle joint of arthritic rat was considered to be due to oedema of periarticular tissues such as ligament and capsule. Diminished joint space is the hallmark of arthritis, which is due to articular cartilage destruction brought by cytokines such as TNF- α and IL-1, which stimulate the release of proteolytic enzymes such as collagenases, glycohydrolases and neutral proteases. As a result, the pannus invades the joint and sub-chondral bones and eventually the joint is destroyed and undergoes fibrous fusion or ankylosis (Sudaroli and chatterjee, 2007).

Histopathological evaluation of the tibiotarsal joint of Arthritis Control (AC) rat showed massive influx of inflammatory cells, synovial hyperplasia with mono and polymorphonuclear cells accumulation in the joint and oedema associated with granuloma formation. It also shows the presence of higher degree of necrosis and degeneration with partial erosion of the cartilage (Fig.5.10B and 5.11B).

In the tibiotarsal joint Normal rats (NC), there was intact articular cartilage with normal synovial lining and connective tissue structure. It does not show any evidence of lymphocytic infiltration (Fig.5.10A and 5.11A). Treatment with diclophenac (10 mg/kg) showed normal connective tissue of tibiotarsal joint with the presence of lower degree of edema. There was absence of necrosis aswell as lymphocytic infiltration (Fig.5.10C and 5.11C). Tibiotarsal joint of rats treated with FCP (0.5g/kg and 1.0g/kg) showed less inflammatory signs like scanty cellular infiltrateless oedema. It does not show any sign of granuloma formation (Fig. 5.10D and 5.11D). Degeneration of the ankle joint was not observed in any of the drug treated groups when compared with the normal control.

Persistent inflammation produces swollen joints with severe synovitis, decreased nociceptive threshold, and massive subsynovial infiltration of mononuclear cells, which along with angiogenesis leads to pannus formation. Expansion of the pannus induces bone erosion and cartilage thinning, leading to the loss of joint function (Feldmann *et al.*, 1996)

Figure 5.10 Histopathological changes in tibiotarsal joints (10X magnification) A- Normal control, B- Arthritis control, C- Diclofenac treated group, D- FCP treated group.

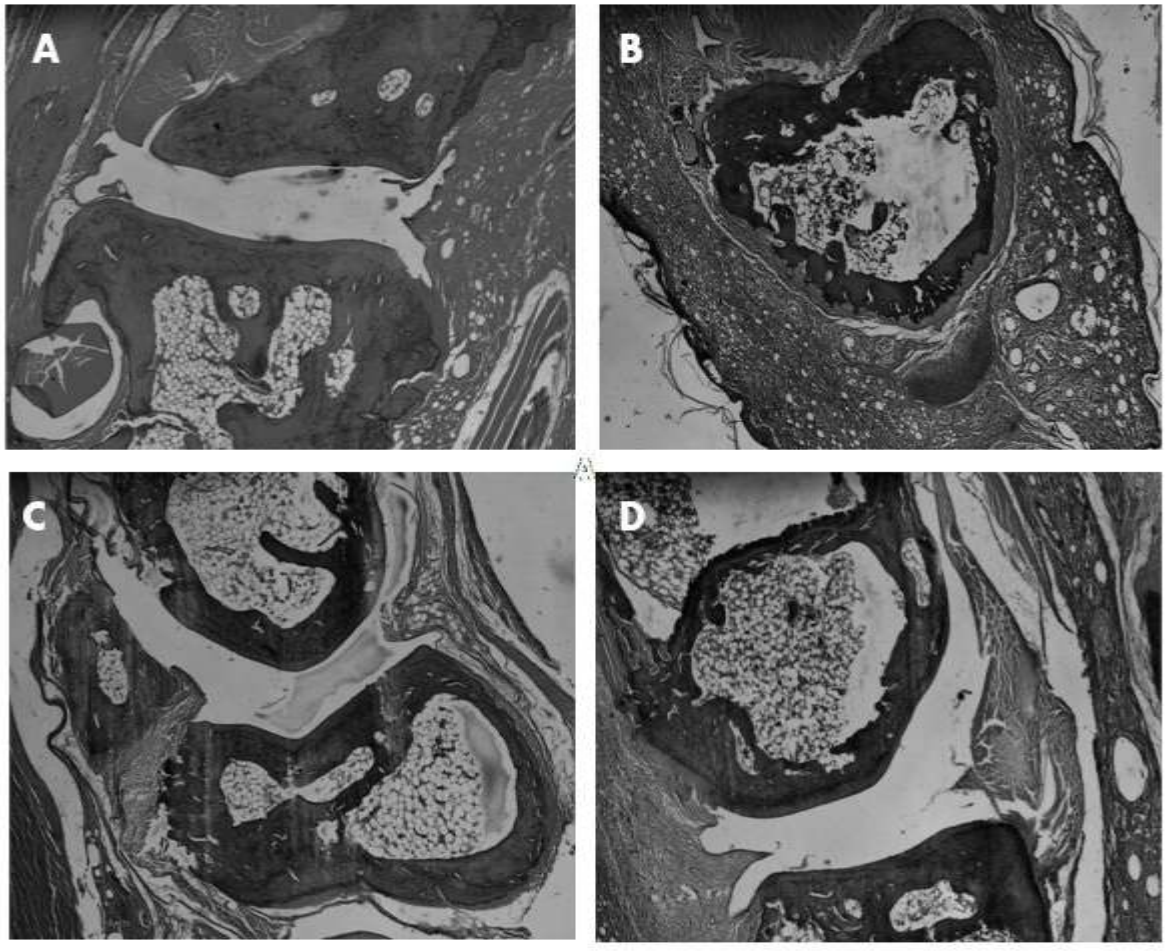
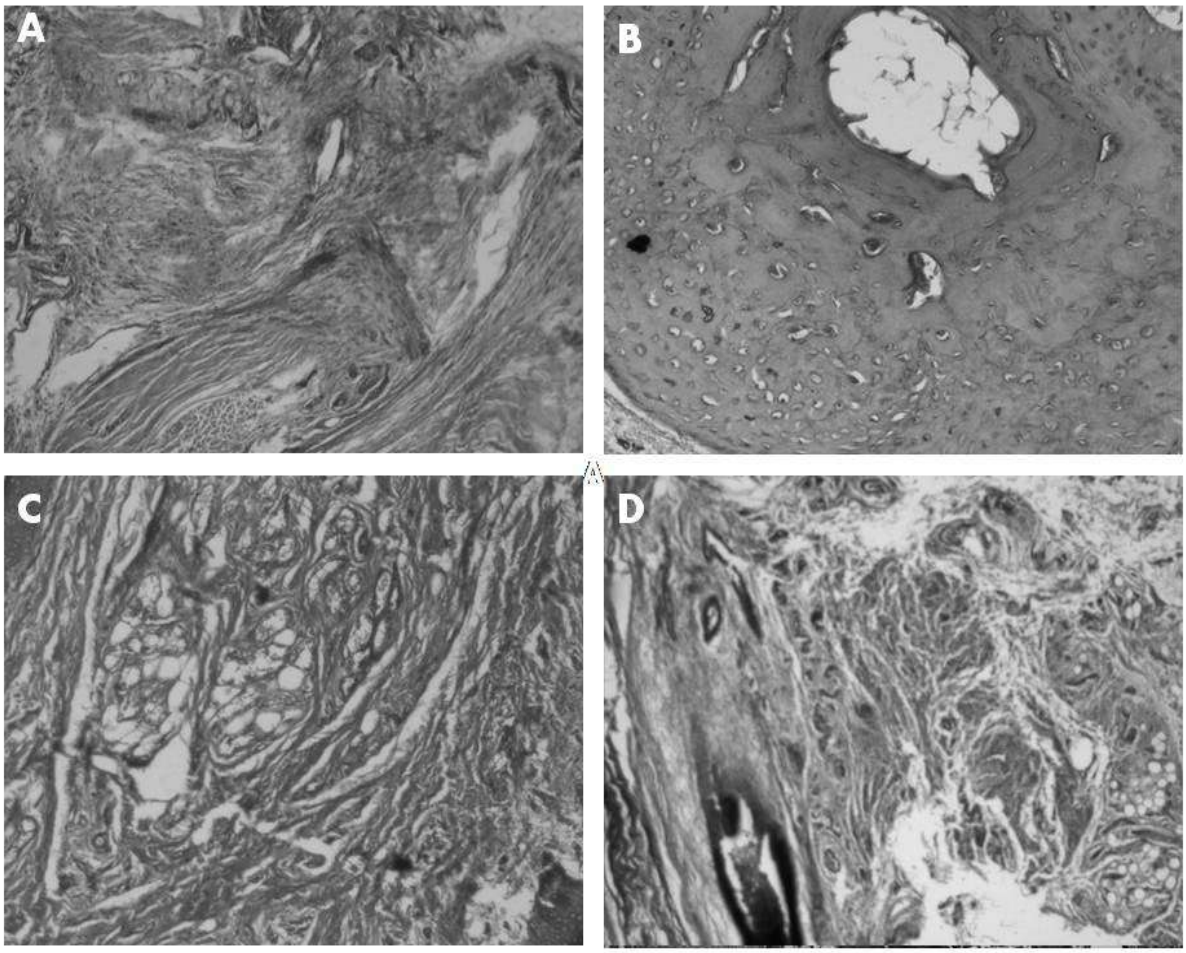


Figure 5.11 Histopathological changes in tibiotarsal joints (100 X magnification) A- Normal control, B- Arthritis control, C- Diclofenac treated group, D- FCP treated group



5.4 Conclusion

The result concluded that the collagen peptide from fish skin exerts potent anti arthritic activity significantly ($p<0.05$) altering the pathogenesis during arthritis without exerting any side effect during the chronic treatment and proved significant for the treatment of arthritis.

The mechanism by which oral administration of hydrolyzed collagen may improve bone formation in rats remain unclear, but has been suspected to be associated with the release and absorption of collagen derived peptides acting on bone metabolism, as observed in cartilage (Oesser *et al.*,1999). This effect could also be mediated by interaction of small collagen-derived peptides with the bone matrix. In this sense, the type I collagen-derived peptide Asp-Gly-Glu-Ala may bind to α1β1 integrin receptors on cell membranes and stimulate osteoblast-related gene expression of bone marrow cells (Mizuno *et al.*, 2001).

Collagen hydrolysate has been used in pharmaceuticals and food supplements for improving skin and cartilage tissues. It is digested and absorbed in the digestive tract, appears in the human blood partly in a peptide form, (Iwai *et al.*, 2005; and Ohara *et al.*, 2007) and is accumulated in skin for up to 96 hours as shown by Oesser *et al.* (1999). Recently, it has been shown that not only amino acids but also oligopeptides are absorbed by the small intestine. Moreover, Pro-Hyp, a dipeptide, was identified as the major constituent of food derived peptides to be detected in human serum and plasma. We hypothesized that in the study that some Pro-Hyp containing peptides reaches the articular cartilage and acts as a bioactive peptide, exertinga chondroprotective effect.

Oesser and Seifert, (2003) in their studies have showed some evidences exist on the ability of collagen hydrolysates to stimulate biosynthesis of type II collagen and proteoglycans in chondrocytes. Raabe *et al*, (2010) have reported the marked effect of a fish collagen hydrolysate on chondrogenic differentiation of equine adipose tissue-derived stromal cells. These studies suggest that effectiveness of collagen hydrolysates on biosynthesis of macromolecules would be based on their unique amino acid composition, very similar to that of type II collagen. Oral administration of collagen hydrolysates would provide high levels of Glycine and Proline, two amino acids essentials for the stability and regeneration of cartilage (Walrand *et al.*, 2008). The therapeutic effect of collagen hydrolysates on osteoarthritis could also be mediated by the effect of specific peptides on gene expression and function of chondrocytes (Ohara *et al.*, 2007).

.....

Chapter 6

EVALUATION OF EXPRESSION OF COLLAGEN PROTEINS IN OSTEOBLAST CELLS UPON TREATMENT WITH FISH COLLAGEN PEPTIDES (FCP)

Contents

7.1 Introduction

Osteoblasts are cells responsible for the synthesis and mineralization of bone during both initial bone formation and later bone remodeling. Osteoblasts form a closely packed sheet on the surface of the bone, from which cellular processes extend through the developing bone. They arise from the differentiation of osteogenic cells in the periosteum, the tissue that covers the outer surface of the bone, and in the endosteum of the marrow cavity (Cooper, 2000; Spreafico, 2006).

Collagen is the major component of the extracellular matrix in multicellular organisms, including humans, and is an important component of ligaments, cartilage and bone. It accounts for about 30% of the total proteins in the body. Type I collagen is the most common type of collagen, and is abundant in bone (Bello, A.E. and Oesser, S. 2006).

Arthritis is the most common musculoskeletal disorder. Treatment of arthritis includes analgesics and anti-inflammatory agents, lubricating, cushioning agents and nutritional supplements (Wu, 2004). Advances in treatment of arthritis include new and safer compounds (e.g., glucosamine, chondroitin sulphate, or methyl-

sulfonyl-methane) capable of repairing damaged articular cartilage or at least decelerating its progressive degradation (Brief *et al.*, 2001)

Collagen hydrolysates are safer compounds that could provide, with less overall toxicity, a greater symptomatic relief than pharmaceutical drugs. Hye Kyung Kim *et al.* (2013) reported osteogenic activity of bovine collagen peptide via ERK/MAPK pathway mediated boosting of collagen synthesis and its therapeutic efficacy in osteoporotic bone. Oral administration of collagen hydrolysate was also demonstrated to reduce bone loss by increasing the quantity of type 1 collagen and proteoglycan in the bone matrix of ovariectomized (OVX) rats (De Almeida Jackix *et al.*, 2010). Collagen peptides have been used therapeutically as a dietary supplement to improve conditions of joints.

Collagen hydrolysate is absorbed in its high-molecular weight form, containing peptides of 2.5-15 kDa (Oesser *et al.*, 1999). Studies reported detection of peptides such as prolyl-proline (Pro-Pro), alanyl-hydroxypropyl-glycine (Ala-Hyp-Gly), prolyl-hydroxyproline (Pro-Hyp), prolyl-hydroxypropyl-glycine (Pro-Hyp-Gly), isoleucyl-hydroxyproline (Ile-Hyp), leucyl-hydroxyproline (Leu-Hyp) and phenylalanyl-hydroxyproline (Phe-Hyp) in human venous blood after ingestion of collagen hydrolysate. Pro- Hyp was the most prevalent among those peptides (Iwai *et al.*, 2005 and Ohara *et al.*, 2007).

Many reports indicated that various peptides obtained from collagen hydrolysate shows biological activity. For example, the Asp-Gly-Glu-Ala tetrapeptide regulated the expression of osteoblast treated genes in the bone marrow (Mizuno *et al.*, 2001). Correspondingly, a hydrogel containing peptides from collagen has been used as a scaffold for a true cartilage like extracellular matrix in regenerative medicine for effective

and lasting repair of articular cartilage (Kisiday *et al.*, 2002). These findings suggest that collagen hydrolysate contains bioactive peptides that affect cartilage homeostasis.

Experimental studies have suggested that some collagen-derived peptides orally administered are absorbed intact in the intestine. Subsequently, these peptides would accumulate preferably in cartilage, where finally may stimulate cartilage metabolism (Oesser *et al.,* 1999). Some evidences exist on the ability of collagen hydrolysates to stimulate biosynthesis of type II collagen and proteoglycans in chondrocytes (Oesser *et al.,* 2003).

Oral administration of collagen hydrolysates would provide high levels of glycine and proline, two amino acids essentials for the stability and regeneration of cartilage. The therapeutic effect of collagen hydrolysates on osteoarthritis could also be mediated by the effect of specific peptides on gene expression and function of chondrocytes (Walrand, *et al.,* 2008).

In the previous chapter we have evaluated the anti arthritic effect of fish collagen peptides in CFA induced arthritic model. The results suggest that the peptide is having a positive role in alleviating the symptoms of arthritis. In this scenario we are hypothesizing that the antiarthritic effect of collagen peptide may be due to the stimulating effect on collagen biosynthesis in arthritic joints.

Accordingly, an *in vitro* study was designed to investigate the biological effects of fish collagen peptide (FCP) on human osteoblast cell lines (HOS).To elucidate this novel function regarding collagen synthesis, we treated human osteoblast cells with collagen peptide in both a time and dose dependent manner followed by measurements on biosynthesis and secretion of type 1 collagen. MTT assay was done to check the cytotoxic effect of FCP on the growth of cells. During treatment with FCP, the collagen synthesis is quantitatively screened in the culture cells. Quantitative

measurement of collagen was done by sirius red staining, western blot analysis and immune fluorescence analysis.

7.2 Materials and Methods

7.2.1 Osteoblast culture

An osteoblast cell line derived from a human osteosarcoma, (HOS) were cultured under standard conditions: Dulbecco's modified Eagles culture medium was supplemented with 10% (v/v) foetal bovine serum, antibiotics: penicillin (100 U/ml) and streptomycin (100 mg/m), and an antifongotic (0.25 mg/ml Amphotericin B).

Cells were incubated in a temperature controlled, humidified incubator with 5% CO_2 at 37°C. Cells were used between passage numbers five and 20 and grown in the complete culture medium, in 75cm^2 culture flasks and subcultured by trypsinization (0.05% trypsin). Culture medium was changed every 2-3 days.

Both serial cultures of fresh or cryopreserved cells were used for the determination of the following parameters:

(i) time to reach 50% confluency in the standard culture flasks of 75 cm^2

(ii) Number of dead cells floating on top of the culture medium expressed as percentage of total seeded cells, obtained by trypsinization. Approximately $5x10^4$ cells were seeded at every passage in each well.

(iii) the total number of cells at saturation density determined after trypsinization on the Coulter counter, at increasing passages

7.2.2 MTT assay

Cell viability was assessed using the MTT colorimetric assay. MTT was taken up into cells by endocytosis or protein-facilitated mechanism and reduced, mainly by mitochondrial enzymes, to yield a purple formazan product which is largely impermeable to cell membranes, thus resulting in its accumulation within living cells. Solubilization of the cells results in the liberation of the purple product which can be detected using a colorimetric measurement. The ability of cells to reduce MTT provides an indication of the mitochondrial integrity and activity which, in turn, may be interpreted as a measure of cell number/ proliferation/ viability/ survival/toxicity.

Operatively, 100µl of cell suspension was inoculated to each well of 96-well plates at the density of 2×10^4 cells/well (the area of each well was 0.32 cm^2). After 24 h of culture, the medium was removed by aspiration and replaced with 100µl FCP at 0.1–10 mg/mL concentrations for 1, 2, 4, and 12 h.

After incubation, cells were observed under a contrast phase microscope before adding MTT solution, prepared fresh as 5mg/ml in H_2O, filtered through a 0.22µm filter, and kept for 5 min at 37°C. MTT solution (10µl) was added to each well, and the plates were incubated in the dark for 4h at 37°C in CO_2 incubator. After suction removal of the solution, 100 ml of DMSO were added to each well, and the plate was vortexed for 10 min. Absorbance of the solution was measured at 540 nm.

7.2.3 FCP treatment and Protein extraction

The HOS cells were seeded in 60mm culture dishes at a density of 1×10^5 cells and left in the CO_2 incubator for 24 hours for the cells to adhere. Cells were incubated with an optimal concentration based on the MTT assay. The cells were treated with different concentrations of fish collagen peptide for different time periods (6, 12 and 24 hours). The cells were then scraped with

scraper in 1 mL of 1X PBS and were centrifuged at 5000rpm for 5 minutes at 4°C.The spent medium was centrifuged in an eppendorf at 5000rpm for 5 minutes at 4°C. The cells in each group were lysed in a lysis buffer (1M HEPES, 10% NP-40, 1mM NaF, 1mM Na_3VO_4, 0.5M EDTA, 100% protease inhibitor cocktail, deionized water) and then they were sonicated with a cell disrupter for 1 min in ice cold water. The cells were centrifuged at 14000rpm for 10 minutes at 4°C to get the cell lysate. The supernatant was collected and stored at -80°C for evaluation.

7.2.4 Quantification of collagen from FCP treated cells

7.2.4.1 Collagen chromogenic precipitation with Sirius Red

Sirius Red specifically binds to the [Gly-X-Y]n helical structure of fibrillar collagens such as type I to V collagen, and is used for detecting all types and species of collagen. The assay can be used in the range from 1 to 50 micrograms of protein. The method is specific, sensitive, simple, and rapid. Total amount of collagen synthesized and deposited by the cells after 72 h were measured, using the colorimetric procedure with picro-Sirius red staining. This method has the advantage to estimate not only freshly synthesized collagen but also the total amount of collagen accumulated by the cells during the culture period (Keira *et al.*, 2004).

Collagen extract from treated cells (100µl) were put in eppendorf tubes and was precipitated with 1 ml of a solution of dye sirius red in 0.5 M acetic acid. After shaking, the tubes were maintained in rest for 30 minutes at room temperature and then centrifuged for 30 minutes at 30,000g. The supernatant was disposed and the pellet then washed consecutively with distilled water and 0.01N HCl to remove unbound dye. The bound dye was solubilized by incubation in 1 mL potassium hydroxide 0.1N 15 minutes, in room temperature. Then absorbance of the solution was determined in spectrophotometer of 540 nm wavelength. Optical densities obtained were interpolated in a curve of absorbance, using collagen type I from calf skin soluble in acetic acid as standard.

7.2.4.2 Western blotting and densitometric analysis

The HOS cells were seeded in 60 mm culture dishes at a density of 1×10^5 cells and were cultured with or without FCP. Cells in the FCP group were incubated with an optimal concentration based on the MTT assay. After different time interval (6hr, 12hr and 24hr), the cells were retrieved with a rubber policeman. The cells in each group were lysed in a lysis buffer (1M HEPES, 10% NP-40, 1mM NaF, 1mM Na3VO4, 0.5M EDTA, 100% protease inhibitor cocktail, deionized water) and then they were sonicated with a cell disrupter for 1 min in icecold water. After centrifugation of the lysates at 1,000 rpm for 10 min at 4°C, the supernatants were subjected to a Western blot analysis. The protein concentrations in each group were determined by Bradford assay.

For Bradford assay a set of standards was prepared from a stock of protein (BSA) with stock concentration of 1mg/mL. Each sample was added into 96 well plates in triplicates. The sample volume was 50µL. The protein solution of unknown concentration was diluted by 100 times and added in triplicates to the well. Bradford reagent (200µL) was added to all the wells and was incubated in dark for 5 minutes and absorbance was taken at 570 nm. A standard graph was plotted with the known concentration on X-axis and the corresponding absorbance on Y axis. The concentration of the unknown protein was obtained from the graph.

The proteins were denatured by boiling in Lammeli sample buffer for 5 min at 203.00°F, separated by SDS–PAGE. The proteins were transferred onto polyvinylidene difluoride membranes. The membranes were incubated with 5% (w/v) nonfat milk powder in TBS for 1 h to prevent nonspecific binding. Following the blocking, the membranes were incubated overnight at 4°C with a primary antibody (Anti-Collagen 1 antibody).The specific antibody binding was detected with a horseradish peroxidase-conjugated secondary antibody and visualized using the enhanced chemiluminescence plus Western blotting detection reagents. To confirm equal loading conditions membranes were stripped and reprobed with β-actin

antibody. The band density of each group was quantified by a densitometric analysis using the Scion Image software program. The ratio of the densitometric value of the FCP group to the value of the control group was then calculated.

7.2.4.3 Immunocytochemistry (Confocal imaging)

Confocal Slide Preparation: Plate cells at about 50% confluence on a 4-chamber glass slide (Nunc, Lab-Tek) in 15% FBS medium for 1h at 37°C. All remaining steps are performed at room temperature with the slides being rocked during incubations. While still in growth media, remove all but 500µL medium and add collagen peptide treatment (0.3mg/mL to 1.0 mg/mL) to the appropriate chambers for 24h time.

Pour off the media, wash each chamber twice quickly with 0.5 mL PBS, and then fix the cells by adding 0.5 mL of 4% PFA to each chamber and incubating for 15 min. Wash the cells three times in 0.5 mL PBT (per chamber), 5 min each, and then incubate the cells for 30 minutes in 0.5 mL of 1% BSA. All remaining steps are performed in the dark to protect fluorescent markers. After washing, the cells were incubated with primary antibody (Anti-Collagen 1 antibody), (1/1000: in 3% BSA/ PBS) for 12h at 4°C. An FITC-conjugated goat polyclonal to rabbit IgG was used at dilution at1/160 as secondary antibody.

After incubation and washing, the cells were counterstained with DAPI for staining nucleus. After washing, allowed the slide to dry and removed the plastic chamber piece and sealer holding it in place completely. Placed one drop of mounting media (80%glycerol) on each sheet of cells, and covered with a No. 1.5 thickness cover slip. Gently pushed out if any air bubbles that formed underneath the cover slip and sealed the edges with clear nail polish. Stored slides at 4°C in the dark before and in between viewing under fluorescence.

Immunofluorescence was visualized using Nikon AIR confocal imaging system and Andor Revolution XD Spinning Disc Microscope with and orixon 897 EMCCD cameras.

7.2.5 Statistical analysis

The results are expressed as Mean ± SE from n=3 observations. The findings were also analyzed for determining significance of difference by ANOVA test followed by pair-wise comparison of various group by LSD. The differences among groups were considered to be significant at $p<0.05$.The analysis was carried out by using SAS system version 9.1 (SAS Institute Inc., Cary, NC, USA)

7.3 Result and Discussion

7.3.1 MTT assay

MTT assay measured the reduction in cell viability. For each cell type the linear relationship between cell number and signal produced is established, thus allowing an accurate quantification of changes in the rate of cell proliferation. Absorbance values that are higher than the control cells indicated a percentage inhibition of growth cells.

Results (Table 6.1) shows, there is no significant retardation in the proliferation of cells up to 1.5 mg/ml of FCP treatment and 6 hours of incubation time. But the concentrations of FCP above 2.0 mg/ml significantly affected the growth of cells.

Table 6.1 MTT assay.

Concentration of FCP (µg/mL)	% inhibition on growth of cells
0.0	09.23±0.35
0.2	09.41±0.57
0.4	09.51±0.42
0.5	10.15±0.23
0.6	12.56±0.47
0.8	12.51±0.53

1.0	14.54±0.38
1.5	21.21±0.40
2.0	30.51±0.63*
5.0	58.32±0.74*
10.0	60.51±0.29*

Values are the average of three separate experiments in quadruplicate and are expressed as mean ± SD. *$p < 0.05$.

Figure 6.1 Human osteoblast cells in culture dishes

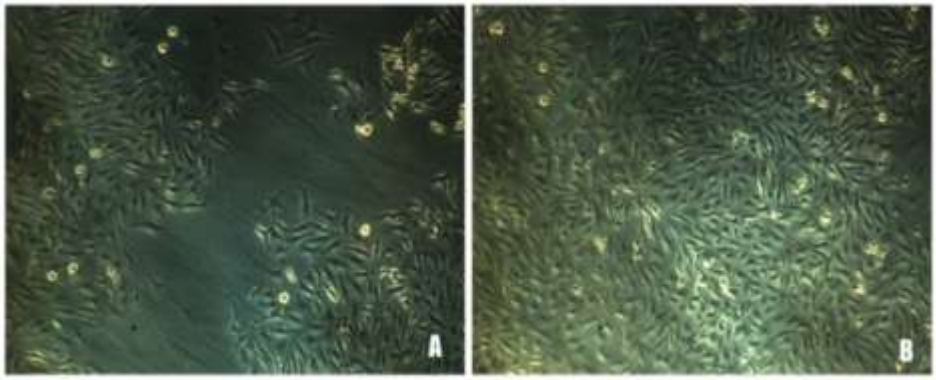
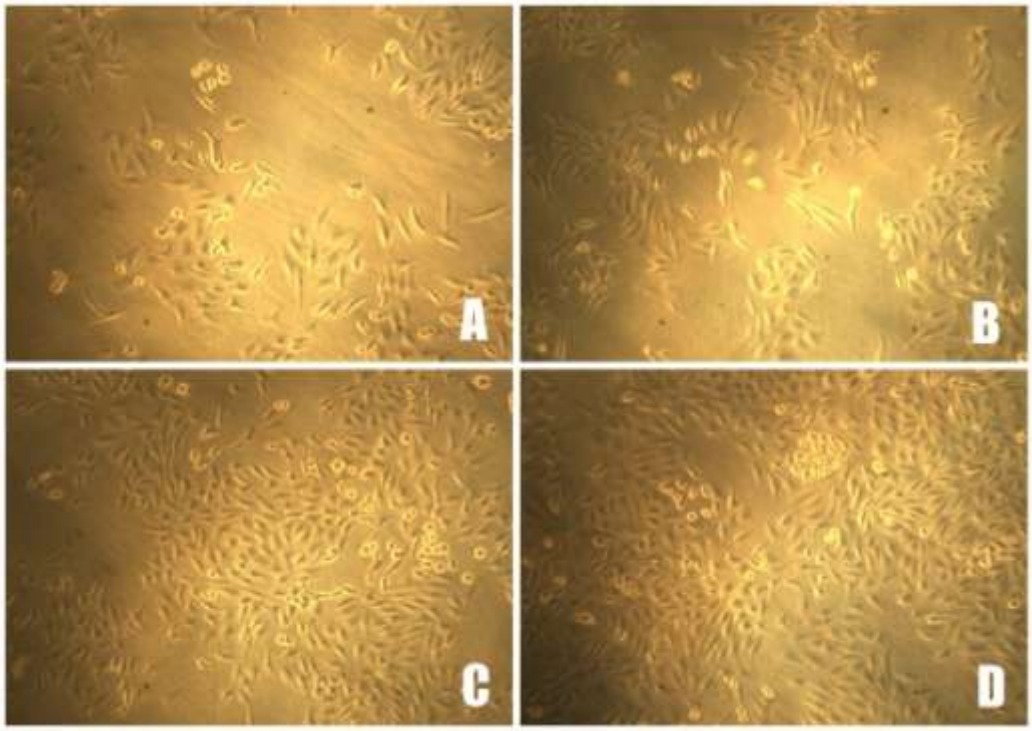


Figure 6.2 Photographs of the HOS cells after the different lengths of incubation time. *(A. HOS cells before FCP treatment B. 6 hours after FCP treatment C. 12 hours after FCP treatment D. 24 hours after FCP treatment)*



7.3.2 Quantification of Type 1 collagen from FCP stimulated cells

Osteoblast cells were treated with FCP at different concentrations ranging from 0.5 mg/mL to 1 mg/mL for different time intervals of 6 to 24 hours (Fig 6.2).To investigate whether the peptides present in the collagen hydrolysate can stimulate the synthesis of collagen upon treatment, we measured the total collagen present in the cells protein extract.

7.3.2.1 Picro Sirius red staining

Collagen content was determined at different concentrations of FCP and at different time intervals by colorimetric analysis using picro-sirius red staining (Fig.6.3). The precipitated collagen was dissolved in 0.5N acetic acid and quantitatively measured colorimetrically.

Figure 6.3 Conic tubes (1.8mL) with the steps of collagen quantification (chromogenic precipitation reaction with dye Sirius Red)*(1) Addition of dye (2) 30 minutes after addition of dye, with filaments (complex collagen-dye) in red (3) post centrifugation, with complex collagen-dye precipitated in the bottom of the tube 4) post-elution with KOH 0.1 N solution.*

The treatment of cultured osteoblast cells with 0.6 mg/ml FCP over a culture period of 24 hours induced a marked increase in collagen secretion into the culture medium with significantly high when compared to the control cells receiving no FCP supplement (Fig. 6.4, A). At the end of the experimental period (24 hours), collagen secretion was almost 1.5 fold higher in FCP stimulated cultures in comparison with the control cells.

Fig. 6.4, B shows increased concentration s of FCP in the culture medium induced a dose dependent stimulation of collagen secretion in 24 hours of time. From concentrations above 0.5 mg/mL of FCP, there is a significant enhancement of collagen secretion could be observed compared to untreated cells. Oesser *et al.* (1999) reported that chondrocytes treated with collagen hydrolysate at concentrations ranging from 0.5 mg/mL up to 5mg/mL secreted a maximum of 2.2 fold more collagen than the untreated cells.

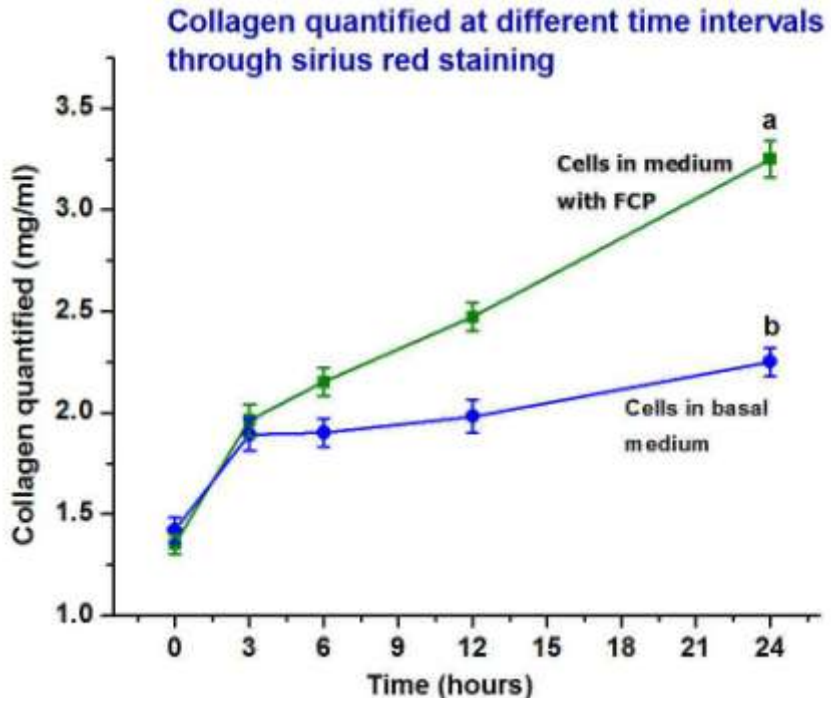
7.3.3.2 Western blotting

Western blotwas done to confirm the result of stimulatory effect of FCP on HOS cells analysed through Sirius red staining. The immune reaction was done by using rabbit polyclonal antibodies against collagen type 1 antibody. Yoshihito Ishida *et al.* (2006) used the same antibodies to detect type 1 collagen from fibroblast cells.

Two immunoreactive bands were detected by using anti collagen antibody (Fig.6.5A). The two bands obtained corresponds to the α and β chains of type 1 collagen.Expression levels of type 1 collagen, from cell lysate of FCP treated cells, showed that there is dose dependent increase in type 1 collagen synthesis.

Figure 6.4 Collagen quantified (A) at different time intervals & and (B) at different FCP concentrations through Sirius red staining *The results were expressed as mean ± SE . Data were analyzed by one way ANOVA followed by post-hoc procedure using SAS 9.3. There is significant changes (p<0.05) in collagen content observed between cells treated with FCP and cells in basal medium with collagen free protein hydrolysate.*

(A)



(B)

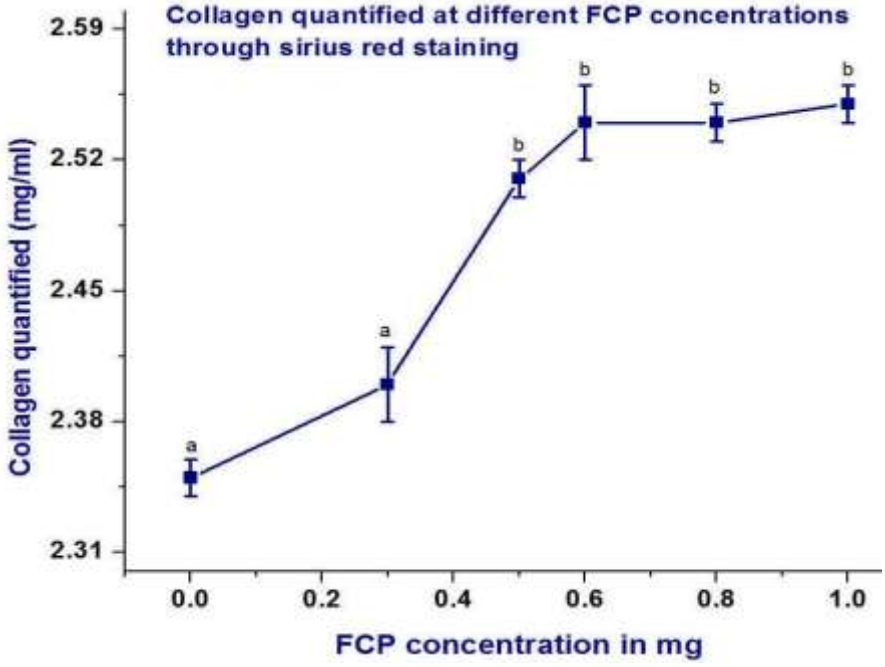
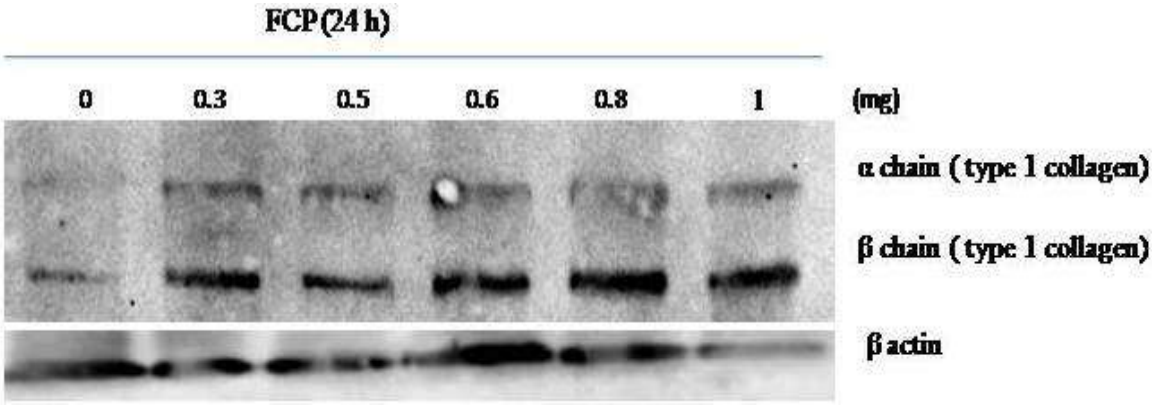
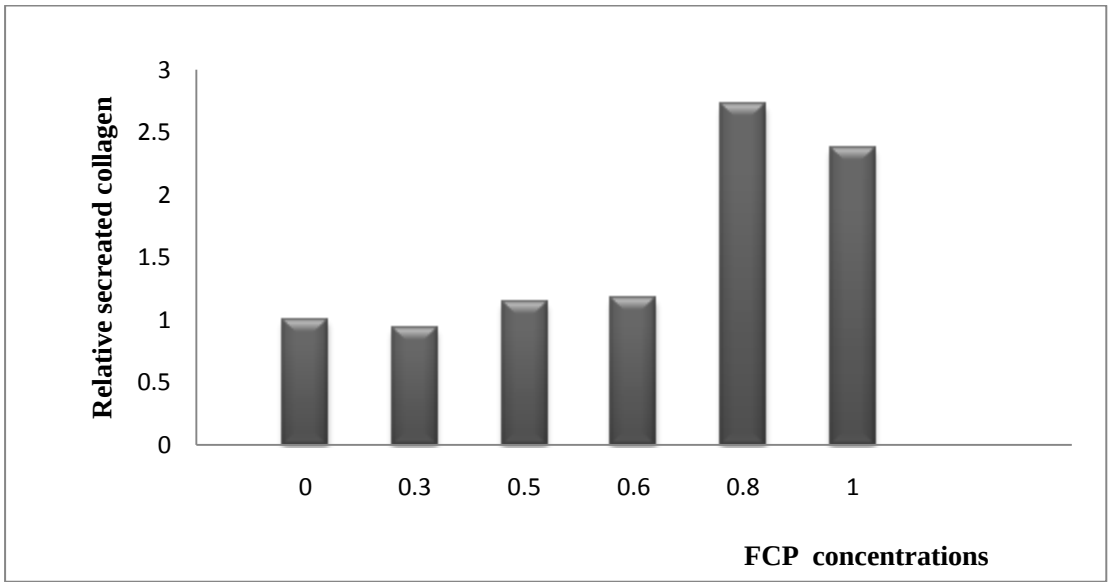


Figure 6.5 Western blotting using antibodies against type 1 collagen.*The expression levelsare normalized to the densitometric value of β actin using image lab software.*
(A)*Lane 2, 3, 4, 5and 6 are respectively of the 0.3, 0.5, 0.6, 0.8 and 1 mg/ml FCP treated cell's protein extract. Lane 1 corresponds to cell lysate of control cells.*



(B)Densitometric analysis



7.3.3.3 Immunocytochemistry

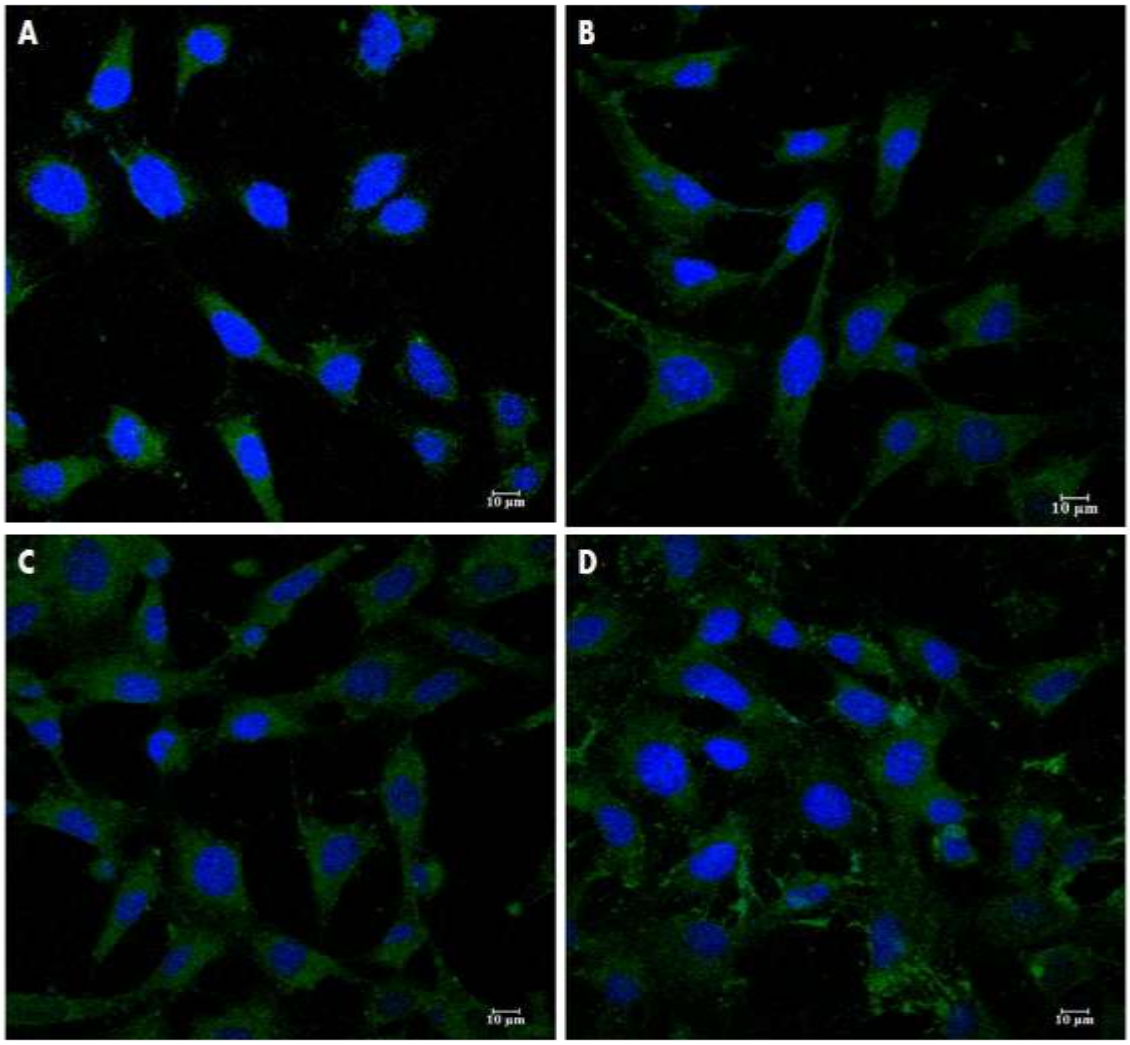
The stimulation of type 1 collagen was fluorescently visualized by means of immunocytochemistry (Fig 6.6). After 24 hours of incubation, osteoblast cultures treated with FCP 0.5, 0.6 and 0.8 mg/mL concentrations significantly deposited tight nets of collagen fibers pericellularly (green fluorescence), where as in normal cultures the measurable amount of cell associated type 1 collagen was considerably reduced.

The specificity of the effect of FCP on collagen biosynthesis in osteoblasts was investigated using native collagen and non collagenous protein hydrolysate. But supplementation of the cell medium with 0.6 mg/ml native type 1 collagen or with a collagen free hydrolysate 0.6 mg/ml, induced no stimulation in collagen biosynthesis in the osteoblast cells. Hasegawa *et al*. (2010) and Ishida *et al*. (1981) used the immuno cytochemical techniques for showing the increased expression of type 1 collagen by cells upon induction with synoviolin. Oesser *et al*. (1999) in his investigations examined the stimulatory effect of bovine collagen hydrolysate in the metabolism of chondrocytes using immunocytochemical techniques.

Col1α1 is the most abundant protein synthesized by active osteoblasts and is essential for mineral deposition, and its expression therefore represents the start of osteoblast differentiation (Franceschi and Iyer, 1992). As bone health supplements, bovine CP compounds are thought to stimulate increased synthesis of collagen (Yamada *et al.*, 2013).

MMPs and extracellular signal-regulated kinases (ERKs) play important roles in osteoblast proliferation and differentiation (Zhang *et al.*, 2002). Recently, it was reported that treatment with collagen hydrolysate from porcine skin gelatin significantly increased the collagen content and expression of the Col1α1 gene through phosphorylation of ERK (Kim *et al.*, 2013).

Figure 6.6 Immunocytochemical visualization of type 1 collagen (green fluorescence pericellularly) secreted by HOS cells. *Staining was performed using type 1 collagen specific antibody (AB765P). The nuclei were counterstained with DAPI (blue fluorescence). A, is control cultures grown in media without FCP; B, C and D are cultured in media with 0.5, 0.6, 0.8 mg/ml FCP respectively.The results were derived from 3independent experiments. Scale bar = 10 µm.*



7.4 Conclusion

In the present study, a primary cell culture model was used to investigate the influence of fish collagen hydrolysate on the type 1 collagen synthesis by osteoblast cells. To elucidate this function regarding collagen synthesis, we have treated the cells with FCP in

both a time and dose dependent manner. After 6, 12 and 24 hours of culture, the number of FCP-treated cells increased significantly compared with untreated cells. Quantification of collagen by chromogenic precipitation with Sirius red showed increased production of collagen from treated cells. The stimulatory effect was confirmed by western blot and immunocytochemistry analysis. The results clearly indicated that the presence of fish collagen hydrolysate led to a dose dependent increase in collagen synthesis by osteoblast cells. However native collagens or collagen free protein hydrolysate failed to stimulate the production of collagen in osteoblast cells.

Present study was carried out in order to understand the molecular mechanisms of the possible structure modifying effect of collagen hydrolysate. The *in vitro* study provides the demonstration of the stimulatory effect of fish collagen hydrolysate on the biosynthesis of collagen by human osteocytes cultures.

Saskia Schadow *et al.* (2010) demonstrated that collagen hydrolysates from various sources differ significantly with respect to both their chemical composition of oligopeptides representing collagen fragments as well as their effect on human articular cartilage. Nomura *et al.*, 2005 showed that administering shark skin gelatin to ovariectomized rats increased not only the bone mineral density of femurs but also the content of type I collagen and glycosaminoglycan in the epiphysis.

Since marked effects on human osteocytes were observed in our *in vitro* study it is suggested that FCP from grouper skin can be used as nutriceutical effective in the treatment of arthritic joints. Hence it is concluded that enzymatically hydrolyzed collagen preparations might contain therapeutically useful peptides. The biomedical properties of FCP have to be studied thoroughly in clinical trials before being applied as a safe and effective nutraceutical in human beings.

Chapter 7

SUMMARY AND CONCLUSIONS

Marine bioresources produce a great variety of specific and potent bioactive molecules including natural organic compounds such as polysaccharides, peptides, proteins, enzymes and fatty acids etc. The protein collagen is widely used for biomedical and pharmaceutical applications owing to its cell attachment capabilities, excellent biocompatibility, biodegradability and weak antigenicity. The conventional source of collagen is from bovine and pig. However, the outbreak of prion diseases, such as bovine spongiform encephalopathy, has resulted in anxiety among users of collagen derived from these land animals. Thus, there is need to find an alternative source of collagen. Fish is one of the best candidates as alternative source because fish is unlikely to be associated with any side effects.

The overall objective of the work is to test if and how to use fish skin as an alternative source of collagen. In the present study, a considerable quantity of ASC and PDC were successfully extracted and characterized from skin of five species of fishes. Pepsin aided extraction proved to be a tool for obtaining the greater yield without a marked effect on the triple-helical structure. Further characterization studies with SDS PAGE, HPLC, FTIR and UV spectral analysis suggest that the extraction process yields collagen in pure form. The extracted collagen could be used for wide applications in food, pharmaceuticals, cosmetics and biomaterials. The following are some of the salient findings of this study.

Fish Collagen Hydrogel

Wound healing efficacy of the fish collagen hydrogel was evaluated in subcutaneous circular incision wound model on albino rats. The study demonstrated that within 2 weeks, the wound covered

with gel were completely filled with new epithelium without any significant adverse reactions.

- ✓ The results clearly suggest that the hydrogel enhances re-epithelialization rather than a repair which was clear from the histopathological and biochemical analyses.
- ✓ There is significant increase in angiogenesis, collagen deposition, hexosamine content, epithelialization and wound contraction in hydrogel treated rats without inflammatory cells compared to the control group, indicating the tissue regeneration potential of fish collagen.
- ✓ The wound healing effects of fish collagen may be attributed to its capability on tissue remodeling process associated with collagen synthesis at the wound site, which is evident from increased tensile strength of skin.
- ✓ Furthermore, increased collagen deposition within the wound bed is effectively potentiated the vascularization process in wound healing. Also the angiogenesis in granulation tissues possibly improved blood supplementation to the wound site, thus providing nutrients and oxygen essential for the healing process.
- ✓ The topical administration of collagen hydrogel renders moist environment to facilitate the smoothness essentially required for fast healing process.

In conclusion, the overall research findings of the present study indicated that the topical application of fish collagen hydrogel increased collagen deposition and enhanced angiogenesis by reducing the duration of the inflammatory phase, which is an important factor not only in the re-epithelialization process, but also in wound contraction. It is concluded that the hydrogel can be effectively used as an active ingredient in the formulation of wound healing ointments.

Fish Collagen Peptide Supplement

Generally, the bioactive properties of collagen-derived peptides especially their resistance to proteolytic process make them potential ingredients in the formulations of health care food supplements. Recently enzymatic hydrolysis has become a valuable tool for modifying the functionality of proteins. In this study, an attempt has been made to prepare enzymatically liberated physiologically active peptides from fish collagen extract.

In order to obtain bioactive peptides, the grouper fish skin collagen was hydrolysed enzymatically using three different enzymes; pepsin, papain and protease consecutively. Since degree of hydrolysis depends on the bioactivity of peptides, the hydrolysis parameters were optimised for each enzyme using RSM in order to get maximum degree of hydrolysis.

- ✓ The optimum hydrolysis conditions were: 2.1 of pH; 36.62°C of temperature; 3.6% of E/S ratio and 5.47 h of time for pepsin.
- ✓ The optimum hydrolysis conditions for papain were 6.38 of pH; 26.22°C of temperature; 4.5% of E/S ratio and 4.25 h of time.
- ✓ In case of protease, the optimum hydrolysis conditions were 6.3 of pH; 39.86°C of temperature; 1.8% of E/S ratio and 4.25 h of time.
- ✓ Under these conditions, the enzymes have exerted maximum activity for the preparation of hydrolysate and the method is also found to be very effective in the optimisation of hydrolysis.
- ✓ The lyophilized hydrolysate is 100% soluble in water.
- ✓ The molecular weight distribution of the hydrolysate carried out by SDS-PAGE combined with MALDI TOF also confirmed the formation of potentially active peptides from fish collagen.

In conclusion, the generation of collagen peptides through enzymatic hydrolysis is highly related to their molecular structure and weight, which are greatly affected by processing conditions such as pH, time, enzyme substrate concentrations and temperature. Depending on the specificity of the enzymes, conditions applied in hydrolysis process, and the extent of hydrolysis, it is possible to generate a wide variety of collagen peptides with varying functional properties. The resultant protein hydrolysate may contain particular desirable characteristics of the new peptides formed.

Anti-arthritic actions of fish collagen peptides

The present research work also aimed at the scientific validation of the protective effect of Fish Collagen Peptide (FCP) against Complete Freund's Adjuvant-induced arthritis in rats. Complete Freund's adjuvant induced arthritis in rat model is the best and most widely used experimental model for testing the efficacy of anti-arthritic drugs as the clinical and laboratory features closely resembles the human rheumatoid disease.

In the present study, an attempt has been made to examine beneficial anti-arthritic potential of FCP on the pathophysiological and pharmacological control of inflammation process in rats. The following are some of the salient findings related to anti-arthritic actions of collagen peptide in attenuating the inflammatory process involved in arthritic condition.

- ✓ The morphological aberrations associated with chronic symptoms of arthritis in the level of paw oedema, changes in body weight, arthritic index values were brought back to near normalcy upon treatment with FCP.
- ✓ No significant changes were observed in biochemical parameters of ALP, ACP, and AST, ALT, blood urea, creatinine and total protein in the liver and kidney of experimental rats after 42 days of per se administration of FCP, showing its non-toxic nature.

- ✓ The significant reduction was noted in level of anti CCP content, most reliable marker for detecting the prognosis of arthritic inflammation, in FCP treated group as compared to arthritic control group, indicating the extent of effectiveness of FCP in ameliorating arthritic disorders.
- ✓ Supplementation of FCP was found to attenuate the liberation of acid phosphatase, a potent lysosomal hydrolase involved in the degeneration process, clearly substantiating the anti-arthritic property of FCP in level comparison with standard anti-arthritic drugs.
- ✓ Interestingly, the supplementation of FCP was found to modulate the inflammatory responses of arthritis without modulating the expression of COX I, which is in generally adversely affected during NASIDs regimen.
- ✓ The evidences from X-ray radiographs, bone histopathology of synovial joints of the study further confirmed the anti-arthritic activity of FCP.

The results of the present research findings indicated that the FCP can be effectively utilized not only in attenuating symptoms of arthritis but also in the treatment of the diseases due to their tissue regeneration capability.

However, further research is necessary to elucidate the exact mechanism of action involved in the anti-arthritic activity of fish collagen hydrolysates. In this scenario, it is hypothesized that the antiarthritic effect of collagen peptide is related to the stimulating effect on collagen biosynthesis in arthritic joints.

In vitro collagen synthesis in cell lines supplemented with fish collagen peptide

An attempt has been made to study the effects of FCP on human osteoblast cell lines (HOS) in both time and dose dependent manner to confirm its role in the collagen biosynthesis.

- ✓ Significant increase in the number of cells was observed upon FCP-treated culture after 6, 12 and 24 hours as compared to that of untreated cell culture.
- ✓ FCP was found to exert a dose dependent effect on modulating collagen synthesis in osteoblast cells.
- ✓ Quantification of collagen by chromogenic precipitation with Sirius red confirmed increased production of collagen in FCP-treated cells.
- ✓ Western blot and immunocytochemistry analyses further confirmed the FCP-mediated stimulatory action on collagen biosynthesis in cell culture.
- ✓ It is interesting to observe that collagen free protein hydrolysate treatments were unable to enhance the collagen production in osteoblast cells.

Conclusions

Collagen purified from marine sources proved as an effective bio molecule of pharmaceutical importance.

Wound healing studies confirms that topical application of collagen hydrogel increased collagen deposition and angiogenesis over the wound area leads to re-epithelialization rather than a repair of wounded tissue. So it is concluded that the hydrogel can be effectively used as an active ingredient in the formulation of wound healing ointments.

Second part of the study confirms that fish collagen hydrolysate is capable of generating bioactive peptides of therapeutic importance required for the treatment of joint disorders. The study optimised the process parameters for enzymatic hydrolysis of collagen for getting maximum degree of hydrolysis.

The study confirms that the developed collagen product stimulates the joint matrix to synthesise collagen and helping to maintain the structure of the joint and potentially aiding joint

comfort. So it is concluded that the fish collagen peptide developed can make a formulation of pharmaceutical drug for joint regeneration. Thorough clinical studies are essential and mandatory to confirm the safety aspects of FCP before recommending it as a pharmaceutical supplement for human applications.

In the present society, aging is widely associated with joint inflammation and disability (conditions of osteoarthritis, osteoporosis and osteopenia). In all cases it is common that there is progressive degeneration of glycoproteins and associated collagens in bone and joint tissues. Current options to promote joint comfort are limited to medicines that can only reduce the symptoms like pain or inflammation and also have adverse health effects. The developed FCP is biocompatible, less immunogenic and can be used effectively for joint regenerative therapy.

REFERENCES

Ahmad, M. and Benjakul, S. (2010) Extraction and Characterization of Pepsin-Solubilized Collagen from the Skin of Unicorn Leatherjacket (*Aluterus monocerous*). *Food Chem*, 120: 817-824.

Alberts, B., Johnson, A., Lewis, J., Raff, M., and Roberts, K. (2002) *Molecular Biology of The Cell*; Garland Science: New York, NY, USA.

Alder-Nissen, J. (1979). Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzene sulphonic acid. *J. Agric. Food Chem.*, 27: 1256-1262.

AOAC, (2012) Official Methods of Analysis. Association of Official Analytical Chemist. (19th edition)

Arend, W.P, and Dayer, J.M. (1990) Cytokines and cytokine inhibitors orantagonists in rheumatoid arthritis. *Arthritis Rheum.*, 33: 305–315.

Arnesen, J.A., and Gildberg, A. (2007) Extraction and characterisation of gelatine from Atlantic salmon (*Salmo salar*) skin. *Bioresource Technology.*, 98(1), 53-57.

Astusi, O., Kawahito, Y., Prudovsky, I., Tubouchi, Y., and Kimura, M. (2005) Copper chelation with tetrathiomolybdate suppresses adjuvants induced arthritis and inflammation associated cachexia in rats. *Arthr. Res. Thera.*, 7: 1174-82.

Badii, F., and Howell, N.K. (2006). Fish gelatin: structure, gelling properties and interaction with egg albumen proteins. *Food Hydrocoll.*, 20: 630-640

Bae, I., Osatomi, K., Yoshida, A., Osako K., Yamaguchi A., and Hara K., (2008) Biochemical properties of acid-soluble collagens extracted from the skins of underutilised fishes. *Food Chem.*, 108: 49–54.

Bailey, A.J, and Light, N.D., (1989). Connective tissue in meat and meat products. London: Elsevier Applied Science.

Bailey, A.J., Paul, R.G., and Knott, L. (1998). Mechanisms of maturation and aging of collagen. *Mech Aging and Develop* 106: 1-56.

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. *Biomat.*, 26: 6335–42.

Balian, G., and Bowes, J.H., (1977).The structure and properties of collagen. In: The science and technology of gelatin, Ward AG, Courts A (Ed), London, Academic Press, 1–31.

Barnett, S.E., and Irving, S.J., (1991) Studies of wound healing and the effect of dressings. In: Szycher M, editor. High performance biomaterials.Lancaster: Technonic. 583-620.

Barrientos, S., Stojadinovic, O., Golinko, M.S., Brem, H., and Tomic-Canic, M. (2008) Growth factors and cytokines in wound healing. *Wound Repair Regen.*, 16: 585-601.

Bazin, S., and Delaumay, A. (1976), Preparation of acid and citrate soluble collagen. In: The methodology of connective tissue research, Hall DA (Ed), Joynson–Bruvvers, Oxford, 13–18.

Bello, A.E., Oesser S. (2006) Collagen hydrolysate for the treatment of osteoarthritis and other joint disorders: a review of the literature. *Cur.Med. Res. Opin.*, 22(11): 2221-2232.

Berg, R.A., and Prockop, D.J. (1973). Thermal transition of a non-hydroxylated form of collagen: Evidence for a role for hydroxyproline in stabilizing the triple-helix of collagen. *Biochem Biophys Res Commun.,* 52: 115–120.

Birk, D.E., and Bruckner, P. (2005). Collagen suprastructures. *Topics in Current Chemistry* 247: 185-205.

Blagojevic, M., Jinks, C., Jeffery, A., and Jordan, K.P. (2010) Risk factors for onset of osteoarthritis of the knee in older adults: a systematic review and meta-analysis. *Osteoarthritis Cartilagen.,*18: 24-33

Bongartz, T., Cantaert, T., and Atkins, S.R., (2007) Citrullination in extra-articular manifestations of rheumatoid arthritis. *Rheumatology (Oxford).,* 46: 70.

Box, G.E.P., and Wilson, K.B. (1951). On the experimental attainment of optimum conditions. *J. R. Stat. Soc. B Met.,* 13: 1-35.

Boyce, S.T., Christianson, D.J., and Hansbrough, J.F. (1988a) Structure of a collagen-GAG dermal skin substitute optimized for cultured human epidermal keratinocytes. *Biomed Mater Res.,* 22: 939–957.

Boyce, S.T., Glafkides, M.C., Foreman, T.J., and Hansbrough, J.F. (1988b) Reduced wound contraction after grafting of full-thickness burns with a collagen and chondroitin-6-sulfate (GAG) dermal skin substitute and coverage with biobrane. *J Burn Care Rehabil.,* 9(4): 364-70.

Brand, D.D., Latham, K.A., and Rosloniec, E.F. (2007) Collagen-induced arthritis. *Nature Protocols* 2: 1269-1275.

Brief, A.A., Maurer, S.G., and Di Cesare, P.E. (2001) Use of glucosamine and chondroitin sulfate in the management of osteoarthritis. *J Am Acad Orthop Surg.,* 9: 71-78.

Brodsky, B., and Persikov, A.V., (2005). Molecular structure of the collagen triple helix. *Adv Protein Chem.,* 70: 301-339.

Broughton, G., Janis, J.E., and Attinger, C.E. (2006). The basic science of wound healing (retraction of Witte M., Barbul A. In: Surg Clin North Am 1997; 77:509-528). *Plast Reconstr Surg* 117(7 Suppl):12S-34S.

Bryant, S.J. and Anseth K.S. (2001) The effects of scaffold thickness on tissue engineered cartilage in photocrosslinked poly(ethylene oxide) hydrogels. *Biomat.,* 22: 619–626.

Burghagen (1999). Collagen. In H. Belitz and W. Grosch (Eds.), Food chem. Berlin, Germany: Springer-Verlag Berlin Heidelberg 2:540-547.

Cai, X., Zhou, H., Wong, Y., Xie, Y., Liu, Z., Jiang, Z., Bian, Z., Xu, H. and Liu, L. (2007). Suppression of the onset and progression of collagen-induced arthritis in rats by QFGJS, a preparation from an anti-arthritic Chinese herbal formula. *J. Ethnopharmacol.,* 110(1): 39-48.

Caissie, R., Gingras, M., Champigny, M.F., and Berthod, F. (2006) *In vivo* enhancement of sensoryperception recovery in a tissue-engineered skin enriched with laminin. *Biomat.,* 27: 2988-2993.

Campos, A.C., Groth, A.K., and Branco, A.B., (2008). Assessment and nutritional aspects of wound healing. *Curr Opin Clin Nutr Metab Care* 11: 281-288.

Chauhan, B. and Gupta, R. (2004). Application of statistical experimental design for optimization of alkaline protease production from *Bacillus sp.* RGR-14. *Process. Biochem.,* 39:2115-2122.

Chen, L.Y., Remondetto, G.E., and Subirade, M. (2006) Food protein-based materials as nutraceutical delivery systems. *Tr Food Sci Technol.,* 17(5): 272-283.

Chen, P., Marsilio, E., Goldstein, R.H., Yannas, and Spector, M. (2005) Formation of lung alveolar like structures in collagen-glycosaminoglycan scaffolds *in vitro. Tissue Eng.,* 11:1436-1448.

Chen, X.G., Wang, Z., Liu, W.S., and Park, H.J. (2002) The effect of carboxymethylchitosan on proliferation and collagen secretion of normal and keloid skin fibroblasts. *Biomat.,* 23: 4609-4614.

Chitra, P., Sajith lal, G.B., and Chandrakasan, G. (1998) Influence of Aloe vera on the glucoaminoglycans in the matrix of healing dermal wounds in rats. *J. Ethnopharmcol*. 59:179-186.

Cho, S.M., Gu, Y.S., and Kim, S.B. (2005) Extraction optimization and physical properties of yellowfin tuna (*Thunnus albacares*) skin gelatine compared to mammalian gelatins. *Food Hydrocoll.*, 19: 221-229.

Choi, Y.S., Hong, S.R., Lee, Y.M., Song, K.W., Park, M.H., and Nam, Y.S. (1999) Study on gelatin-containing artificial skin: I. Preparation and characteristics of novel gelatin-alginate sponge. *Biomat.*, 20: 409-17.

Choi, Y.S., Lee, S.B., Hong, S.R., Lee, Y.M., Song, K.W., and Park, M.H. (2001) Studies on gelatin-based sponges. Part III: a comparative study of cross-linked gelatin/alginate, gelatin/hyaluronate and chitosan/ hyaluronate sponges and their application as a wound dressing in full-thickness skin defect of rat. *J Mater Sci Mater Med.,* 12(1):67-73.

Chvapil, M. (1979). Industrial uses of collagen. In: Fibrous proteins: Scientific, industrial and medical aspects, Parry DAD, Creamer LK (Eds), Academic Press, London, 247– 269.

Ciarlo, A.S., Paredi, M.E., and Alicia,N. (1997) Isolation of soluble collagen from Hake skin (*Merluccius hubbssi*). *J Aquat Food Prod Tech.,* 6 (1): 65-77.

Cioca, G. (1981) Process for preparing macromolecular biologically active collagen. US Patent 4279812.

Clark, R.A.F. (1996) Wound repair: overviewand general considerations. In: Clark RAF, editor. The molecular and cellular biology of wound repair. New York: Plenum Press, 3–50.

Clemente, A. (2000) Enzymatic protein hydrolysates in human nutrition. *Food Sci.Techn*. 11: 254-262.

Conti, B., Giunchedi, P., Genta, I., and Conte, U. (2000) The preparation and in vivo evaluation of the wound-healing properties of chitosan microspheres. *Pharm Sci.,* 10: 101-104

Cooper, G.M., (2000) *The Cell:* A molecular approach. (2nd edition).

Courtenay, J.S., Dallman, M.J., Dayan, A.D., Martin, A., and Mosedale, B. (1980) Immunisation against heterologous type II collagen induces arthritis in mice. *Nature*., 283:666-668.

Dai, L., Ye, Z.Q., and Tang, M.A. (2000) Expression of transforming growthactor β1, in rheumatoid synovia and its relationship with synovial pathological change, *Chinese J. Rheumatol*.,4: 357-60.

Darby, N.J. and Creighton T.E. (1993) Protein structure Oxford University Press.

Davis, S.C., Badiavas, E., Rendon-Pellerano, M.I., and Pardo, R.J. (1999) Histological comparison of postoperative wound care regimens for laser resurfacing in a porcine model. *Dermatol Surg*., 25:387-393.

De Almeida Jackix, E., Cúneo, F., Amaya-Farfan, J., de Assunção, J.V., and Quintaes, K.D. (2010) A food supplement of hydrolyzed collagen improves compositional and biodynamic characteristics of vertebrae in ovariectomized rats. *J. Med. Food,* 13:1385-1390.

De Lustro, F., Dasch, J., Keefe, L., and Ellingsworth, J. (1990) Immune responses to allogenic and xenogenic implants of collagen and collagen derivates, Clin. *Orthop. Relat. Res*. 260: 263-279.

De Rycke, L., Peene, I., and Hoffman, I.E. (2004) Rheumatoid factor and anticitrullinated protein antibodies in rheumatoid arthritis: diagnostic value, associations with radiological progression rate, and extra-articular manifestations. *Ann Rheum Dis*., 63:1587.

Dressler, D.P., Barbee. W.K., and Sprenger, R. (1980) The effect of Hydron burn wound dressing on burned rat and rabbit ear wound healing. *J Trauma.,* 20(12):1024-8.

Drury, J.L., and Mooney, D.J. (2003) Hydrogels for tissue engineering: Scaffold design variables and applications. *Biomat.,* 24: 4337-4351.

Duan, R., Zhang, J., Du, X., and Konno, K., (2009) Properties of collagen from skin, scale and bone of carp (*Cyprinus carpio*) *Food chem.,* 112: 702-706.

Edwards, R., and Harding, K.G. (2004) Bacteria and wound healing. *Curr Opin Infect Dis.,* 17: 91-96.

Emna Soufi Kechaoua, C., Jean-Pascal Bergéb, Pascal Jaouena, and Raja Ben Amarc. (2013) Optimization of common cuttlefish (*Sepia officinalis)* protein hydrolysate using Pepsin by Response Surface Methodology. J *aquat food prod t.,* 24: 270-282.

Exposito, J.Y., Le Guellec, D., Lu, Q., and Garrone, R. (1991) Short chain collagens in sponges are encodedby a family of closely related genes. *J. Biol. Chem*., 266: 21923-21928.

Fahmi, A., Morimura, S., Guo, H.C., Shigematsu, T., Kida, K., and Uemurac Y., (2004). Production of angiotensin I converting enzyme inhibitory peptides from sea bream scales. Process *Biochem.*, 39(10): 1195-1200.

Falguni P., Shilpa V., Mann B. (2010) Production of proteinaceous antifungal substances from *Lactobacillus brevis* NCDC. *Int J Dairy Technol.* 63: 70-76.

Feldmann, M., Brennan, F.M., Maini, R.N. (1996) Role of cytokines in rhematoid arthritis. *Annu. Rev. Immunol.,* 14:397-440.

Felson, D.T., Lawrence, R.C., Dieppe, P.A., Hirsch, R., Helmick, C.G., and Jordan, J.M. (2000) Osteoarthritis: new insights. Part 1: the disease and its risk factors. *Ann Intern Med*., 133: 635-46.

Ferreira, I., Pinho, O., Tavares, P., Pereira, A., and Roque, A.C. (2007) Development and valldation of an HPLC/UV method for quantification of bioactive peptides in fermented milk. *J Liq Chromatogr Relat Technol*., 30 (14): 2139-47.

Foegeding, E.A., Lanier, T.C., and Hultin, H.O. (1996). Collagen. In: Food chemistry, Fennema OR (Ed), 3: 902-906, New York: Marcel Dekker, Inc.

Foulquier, C. (2007) Peptidyl arginine deiminase type 2 (PAD-2) and PAD-4 but not PAD-1, PAD-, and PAD-6 are expressed in rheumatoid arthritis synovium in close association with tissue inflammation. *Arthritis Rheum*. 56: 3541-3553.

Franceschi, R.T., Iyer, B.S. (1992) Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-El cells. *J Bone Miner Res*., 7:235-246.

Francis Suh, J.K., and Matthew, H.W.T. (2000). Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: a review. *Biomat.,* 24: 2589-2598.

Friess, W., and Lee, G. (1966) Basic thermo analytical studies of insoluble collagen matrices, *Biomat*., 17: 2289- 2294.

Froget, S., Barthelemey, E., Guillot, F., and Soler, C. Courdet, M.C Benbaunan, M., Dosquet, C. (2003) Wound healing mediator production by human dermal fibroblasts grown within a collagen– GAG matrix for skin repair in humans. *Eur. Cytokine Netw*., 14: 60-64.

Fujita, H. and M. Yoshikawa, (1999) LKPNM: A prodrug-type ACE inhibitory peptide derived from fish protein. *Immunopharmacology*, 44: 123-127.

Funk, J.L., Oyarzo, J.N., Frye, J.B., Chen, G., Lantz, R.C., Jolad, S.D., Solyom, A.M., and Timmermann, B.N. (2006) Turmeric extracts containing curcuminoids prevent experimental rheumatoid arthritis. *J Nat Prod*., 69(3): 351-355.

Gbogouri, G.A., Linder, M., Fanni, J., and Parmentier, M. (2004) Influence of hydrolysis degree on the functional properties of salmon byproducts hydrolysates. *J Food Sci.,* 69(8): C615-C622.

Gelse, K., Poschl, E., and Aigner, T. (2003). Collagens-structure, function, and biosynthesis. *Adv Drug Del Rev.,* 55: 1531-1546.

Gilsenan, P.M., and Ross-Murphy, S.B. (2000). Rheological characterisation of gelatins from mammalian and marine sources. *Food Hydrocoll*., 14:191-195.

Gomathi, K.D., Gopinath, M.A., Rafiuddin, R., and Jayakumar, (2003) Quercetin incorporated collagen matrices for dermal wound healing process in rat. Biomat., 24: 2767-2772.

Gomez-Guillen, M.C., and Montero, P. (2001) Method for the production of gelatin of marine origin and product thus obtained. International Patent PCT/S01/00275.

Gomez-Guillen, M.C., Turnay, J., Fernandez-Dıaz, M.D., Olmo, N., Lizarbe, M.A., and Montero, P. (2002) Structural and physical properties of gelatin extracted from different marine especies: A comparative study. *Food Hydrocoll.,* 16: 25-34.

Gosain, A., and DiPietro, L.A. (2004) Aging and wound healing. *World J Surg*., 28:321-326.

Greenwald, R.A., (1991) Animal models for evaluation of arthritis drugs. *Experimental and Clinical Pharmacoloy*., 13: 75-83.

Grossman, S., and Bergman, M. (1992) Process for the production of gelatin from fish skins.U.S.Patent 5093474.

Gudmundsson, M., and Hafsteinsson, H. (1997) Gelatin from cod skins as affected by chemical treatments. *J Food Sci*., 62, 37-39.

Guerard, F., Guimas, L., and Binet, A. (2002) Production of tuna waste hydrolysates by a commercial neutral protease preparation. *J Mol Catalysis B.,* 19–20: 489-498.

Guillermin, F., Beaupied, H., Fabien-Soulé, V., Tome, D., Benhamou, C.L., Roux, C., and Blais, A. (2010) Hydrolyzed collagen improves bone metabolism and biomechanical parameters in ovariectomized mice: An in vitro and in vivo study. *Bone*., 46(3): 827-834.

Guo, S. and DiPietro, L.A. (2010) Factors Affecting Wound Healing. *J Dent Res*., 89(3): 219-229.

Hanai, K., Takeshita, F., and Honma, K. (2006) Atelocollagen-mediatedsystemic DDS for nucleic acid medicines. *Ann. N. Y. Acad. Sci.,* 1082: 9-17.

Hansen, S.L., Voigt, D.W., Wiebelhaus, P., and Paul, C.N. (2001) Using skin replacement products to treat burns and wounds, *Adv. Skin Wound Care*., 14: 37-44.

Harirforoosh, S., and Jamali, F. (2009) Renal adverse effects of nonsteroidal anti-inflammatory drugs. *Expert Opin Drug Saf.*, 8(6): 669-681.

Harris, E.D. (1990) Rheumatoid arthritis. Pathophysiology and implications for therapy. *N Eng J Med*., 322:1277-1289.

Hasegawa, D., Fujii, R., Yagishita, N., Matsumoto, N., and Aratani, S. (2010) E3 Ubiquitin Ligase Synoviolin Is Involved in Liver Fibrogenesis. PLoS ONE 5(10): 13590. doi:10.1371/journal.pone.0013590

Hassan, F., and Sherief, P. M. (1994). Role and application of fish collagen. Seafood Export Journal, 25: 19-24.

Hayashi, T. and Nagai, Y. (1980) The anomalous behavior of collagen peptides on sodium dodecyl sulfate-polyacrylamide gel electrophoresis is due to low content of hydrophobic aminoacid residues. *J Biochem*. 87(3): 803-808

Henrotin, Y., Mobasheri, A., and Marty, M. (2012) Is there any scientific evidence for the use of glucosamine in the management of human osteoarthritis? *Arthritis Res Ther.*, 14:201-7.

Hinman, C. and Maibach, H. (1963) Effect of air exposure and occlusion on experimental human skin wounds. *Nature* 26(200): 377-8.

Hinman, C.D. and Maibach, H., (2000) *Nature.*, 377-378.

Hinrich, W,L., Lommen, E.J., Wildevuur, C.R., and Feijen, J. (1963) Fabricationand characterization of an asymmetric polyurethane membrane for use as a wound dressing. *J Appl Biomater*., 3(4):287-303.

Ho, H.O., Lin, C.W., and Sheu, M.T. (2001) Diffusion characteristics of collagen film. *J. Control. Release 77: 97-105*.

Hoffman, S. (2001) Hydrogels for biomedical applications. *Annals of the New York Academy of Sciences.*, 944:62–73.

Holzer, D. (1996) Gelatin production, US Patent 5484888.

Hong, Y. Chen, W.W., Jia, Z.B., and Huang, G.R. (2013) Application of Response Surface Methodology to Optimize Hydrolysis of Collagen from Croaker Scale *Adv. J. Food Sci. Technol.*, 5: 1646-1651.

Hopkins, S.J. (1990) Cytokines and eicasonoids in rheumatic diseases, *Ann Rheum Dis.*, 49:207-210.

Horch, R.E. and Stark, G.B. (1998) Comparison of the effect of a collagen dressing and a polyurethane dressing on the healing of split thickness in graft (STSG) donor sites, Scand. J. Plast. *Reconstr. Surg. Hand Surg*. 32: 407-413.

Huo, H., Li, B.F., Zhao, X., Zhuang, Y.L., Ren, G.Y., Yan, Y.P., Cai, M.Y., Zhang, X.K., and Chen, L. (2009) The effect of pacific cod (Gadus macrocephalus) skin gelatin polypeptides on UV radiation-induced skin photoaging in ICR mice. *Food Chem.*, 115(3): 945-950.

Huo, J.X. and Zhao, Z. (2009) Study on enzymatic hydrolysis of Gadusmorrhua skin collagen and molecular weight distribution of hydrolysates. *Agric. Sci. China.*, 8(6*)*: 723-729.

Hye Kyung Kim, Myung-Gyou Kim, and Kang-Hyun Leem. (2013) Osteogenic Activity of Collagen Peptide via ERK/MAPKPathway Mediated Boosting of Collagen Synthesis and Its Therapeutic Efficacy in Osteoporotic Bone by Back-Scattered Electron Imaging and Microarchitecture Analysis, *Molecules.*, 18: 15474-15489.

Ignateva, N.Y., Danilov, N.A., Averkiev, S.V., Obrezkova, M.V., Lunin, V.V., and Sobol, E.N. (2007) Determination of hydroxyproline in tissues and the evaluation of the collagen content of the tissues, *J. Analytical Chem.* 62: 51–57.

Ishida, Y., Fugita, T., and Asai, K. (1981) New detection and separation methods for amino acids by high performance liquid chromatography. *J Chromatogra*., 204: 143-148.

Iwai, K., Hasegawa, T., and Taguchi, Y. (2005) Identification of food derived collagen peptides in human blood after oral ingestion of gelatin hydrolysates. *J Agric Food Chem.*, 53:6531-6536.

Jamilah, B., and Harvinder, K.G. (2002) Properties of gelatins from skins of fish-black tilapia (*Oreochromis mossambicus*) and red tilapia (*Oreochromis nilotica*). Food Chem., 77: 81–84.

Je, J.Y., Kim, S.Y., and Kim, S.K. (2005) Preparation and antioxidative activity of hoki frameprotein hydrolysate using ultrafiltration membranes. *Eur Food Res Technol.,* 221:157-62.

Jeon, O., Song, S.J., Lee, K.J., Park, M.H., Lee, S.H., and Hahn, S.K. (2007) Mechanical properties and degradation behaviors of hyaluronic acid hydrogels cross-linked at various cross-linking densities. *Carbohydrate Polymers*, 70(3):251-257.

Jerosch, J. (2011) Effects of glucosamine and chondroitin sulfate on cartilage metabolism in OA: Outlook on other nutrient partners especially omega-3 fatty acids. *Int J Rheumatol.*, 20: 969-972.

Jhon, M.S. and Andrade, J.D. (1973) Water and hydrogels. *Journal of Biomedical Materials Research.,* 7:509-522.

Jongjareonrak, A., Rawdkuen, S., Chaijan, M., Benjakul, S., Osako, K., and Tanaka, M. (2010) Chemical compositions and characterisation of skin gelatin from farmed giant catfish (*Pangasianodon gigas*). *Food Sci Technol.,* 43: 161-165.

Jung, W.K., Karawita, R., Heo, S.J., Lee, B.J., Kim, S.K., and Jeon, Y.J. (2006) Recovery of a novel Ca-binding peptide from Alaska Pollack (*Theragra chalcogramma*) backbone by pepsinolytic hydrolysis. *Process Biochem.*, 41(9): 2097-2100.

Karim, A.A., and Bhat, R. (2009) Fish gelatin: properties, challenges, and prospects as an alternative to mammalian gelatins. *Food Hydrocoll*. 23: 563-576.

Katarzna, Dybka, and Piotr walczak. (2009), Collagen Hydrolysate as a new diet supplement. *Food Chemistry and Biotechnology*, 73: 83-84.

Keira, S.M., Ferreira, L.M., Gragnani, A., Duarte, I.S., and Barbosa, J. (2004) Experimental model for collagen estimation in cell culture. *Acta Cir Bras*., 19:17-22.

Keiser, H.W., Stark, G.B., Koop, J., Balcerkiewicz, A., Spilker, G., and Kreysel, H.W. (1994) Cultured autologous keratinocytes in fibrin glue suspension, exclusively and combined with STS allograft, *Burns*., 20: 23-29.

Kim, H.J., Choi, E.Y., Oh, J.S., Lee, H.C., Park, S.S., and Cho, C.S. (2000) Possibility of wound dressing using poly(l-leucine)/poly(ethylene glycol)/ poly(l-leucine) triblock copolymer. *Biomat*., 21(2): 131-41.

Kim, J.S., and Park, J.W. (2004) Characterization of acid-soluble collagen from pacific whiting surimi processing byproducts. *J. Food Sci* 69(8): 637-642.

Kim, S.K., and Mendis, E. (2006) Bioactive compounds from marine processing byproducts –a review. *Food Res Int.*, 39: 383–93.

Kim, S.K., and Wijesekara, I. (2010) Development and biological activities of -derived bioactive peptides: A review. *J Funct Foods*., 2:1–9

Kim, S.K., Kim, Y.T., Byun, H.G., Nam, K.S., Joo, D.S., Shahidi, F. (2001) Isolation and characterization of antioxidative peptides from gelatin hydrolysate of *Alaska pollack* skin. *J. Agric. Food Chem*. 49(4):1984-1989

Kim, U.J., Park, J.C., Li, J., Jin, H. J., Valluzzi, R., and Kaplan, D. L. (2004) Structure and properties of silk hydrogels. *Biomacromolecules.*, 5:786-792.

Kimura, S., Miyauchi, Y., and Uchida, (1991) Scale and bone type I collagens of carp (*Cyprinus carpio*) *Physiol.*, 99B (2): 473-476.

King, S.R., Hickerson, W.L., and Proctor, K.G. (1991) Beneficial actions of exogenous hyaluronic acid on wound healing. *Surgery*., 109: 76-84.

Kinloch, A., Lundberg, K., and Wait, R. (2008) Synovial fluid is a site of citrullination of autoantigens in inflammatory arthritis. *Arthritis Rheum*., 58: 2287.

Kinne, R.W., Brauer, R., and Stuhlmuller, B. (2000) Macrophages in rheumatoid arthritis, *Arthritis Res*., 2: 189-202.

Kisiday, J., Jin, M., Kurz, B., Hung, H., Semino, C., and Zhang, S. (2002) Self-assemblingpeptide hydrogel fosters chondrocyte extracellular matrix productionand cell division: implications for cartilage tissue repair. *Proc Natl Acad Sci U S A.*, 99(15): 9996-10001.

Kittiphattanabawon, P., Benjakul, S., Visessanguan, W., Kishimura, H., and Shahidi, F. (2010). Isolation and characterisation of collagen from the skin of brownbanded bamboo shark (*Chiloscyllium punctatum*). *Food Chem.*, 119: 1519-1526.

Kittiphattanabawon, P., Benjakul, S., Visessanguan, W., Nagai, T., and Tanaka, M. (2005) Characterisation of acid-soluble collagen from skin and bone of bigeye snapper (*Priacanthus tayenus*). *Food Chem*., 89(3): 363-372.

Knapp, T.R., Luck, E., and Daniels, J.R. (1977) Behaviour of solubilised collagen as a bioimplant. *J Surg Res* 23: 96-105.

Korhonen, H., Pihlanto-Leppala, A., and Tupasela, T. (1998). Impact of processing on bioactive proteins and peptides. *Trends in Food Science and Technology*, 9: 307-319.

Kristinsson, H.G., and Rasco, B.A. (2000) Fish protein hydrolysates: production, biochemical, and functional properties. *Crit Rev Food SciNutr*, 40(1):43-81.

Kuberka, M., von Heimburg, D., Schoof, H., Heschel, I., and Rau, G. (2002) Magnification of pore sizes in biodegenerable matrices, *Int. J. Artif. Organs.,* 25: 67-73.

Kubo, K. and Takagi, T. (1984) The alpha 1(I) and alpha 2(I) chains of collagen separate in sodium dodecyl sulphate-polyacrylamide gel electrophoresis due to differences in sodium dodecyl sulphate binding capacities. *Coll. Relat. Res.* 4:201-208.

Kuhn, K. (1987) Structure and Function of Collagen Types (Mayne, R., and Burgeson, R. E., eds) pp. 1-42, Academic Press, Orlando, FL

Kuptniratsaikul, V., Tosayanonda, O., Nilganuwong, S., and Thamalikitkul, V. (2002) The Efficacy of a Muscle Exercise Program to Improve Functional Performance of the Knee in Patients with Osteoarthritis. *J Med Assoc Thailand.*, 85(1):33-39.

Kuriyama, N., Shoji, N., Taniguchi, K., and Omote, M. (2005) Efficient peptide purification by HPLC- effect of pore size, particle size and chemistry. Kyoto, Japan: YMC Co., Ltd.

Laemmli U. K. (1970) Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature*. 227: 680-685.

Lee, C.H., Singla, A., and Lee, Y. (2001) Biomedical applications of collagen. *Int. J. Pharm*., 221: 1-22.

Lee, S.L. (1983) Optimal conditions for long-term storage of native collagens. *Coll Relat Res* 3: 305-315.

Levy, A.S.A., Simon, O., Shelly, J., and Gardener, M. (2006) 6-Shagaol Reduced Chronic Inflammatory Response in the Knees of Rats Treated with Complete Freund's Adjuvant, *BMC Pharmacology*., 6(12): 1-8.

Leyland, K.M., Hart, D.J., and Javaid, M.K. (2012) The natural history of radiographic knee osteoarthritis: A fourteen-year population-based cohort study. *Arthritis Rheum.*, 64: 2243-2251.

Li, C.M., Zhong, Z.H., Wan, Q.H., Zhao, H., Gu, H.F., and Xiong, S.B. (2008) Preparation and thermal stability of collagen from scales of grass carp (*Ctenopharyngodon idellus*). *Euro Food Res Technol.*, 227: 1467-1473.

Li, S.T. (1995) Tissue-derived biomaterials (collagen). In: The biomedical engineering handbook, Bronzino JD (Ed), CRC Press, Boca Raton, FL, pp 627–647.

Lillie, L.E., Temple, N.J., Florence, L.Z. (1996) Reference values for young normal Sprague-Dawley rats: weight gain, hematology and clinical chemistry. *Hum Exp Toxicol.* 15:612–616.

Lin, Y.K., and Liu, D.C. (2006) Comparison of physical-chemical properties of type I collagen from different species. *Food Chem.*, 99: 244-251.

Liu, H. Y., Li, D., & Guo, S. D. (2007). Studies on collagen from the skin of channel catfish (*Ictalurus punctaus*). *Food Chem.,* 101: 621-625.

Liu, W.T., Li, G.Y., Miao, Y.Q., and Wu, X.H. (2009) Preparation and characterization of pepsin-solubilized type I collagen from the scales of snakehead (*Ophiocephalus argus*). *J Food Biochem.*, 33: 20–37.

Loeser, R.F., Goldring, S.R., and Scanzello, C.R. (2012) Osteoarthritis: A disease of the joint as an organ. *Arthritis Rheum*. , 64:1697-1707.

Mathieu, D., Linke, J.C., and Wattel, F. (2006) Non-healing wounds. In: Handbook on hyperbaric medicine, Mathieu DE, editor. Netherlands: Springer, pp. 401-427.

Matsui, R., Ishida, M., and Kimura, S. (1991) Characterization of an alpha 3 chain from the skin type I collagen of chum salmon (*Oncorhynchus keta*), *Comp.Biochem.Physiol.B.*, 99:171-174.

McInnes, I.B., and O Dell J.R. (2010) State-of-the-art: rheumatoid arthritis. *Ann Rheum Dis.*, 69:1898-1906.

McInnes, I.B., and Schett, G. (2011) The pathogenesis of rheumatoid arthritis. *N Engl J Med*, 365:2205-2219.

Mendis, E., Rajapakse, N., and Kim, S.K. (2005) Antioxidant properties of a radical- scavenging peptide purified from enzymatically prepared fish skin gelatin hydrolysate. *J. Agric. Food Chem*., 53(3): 581-587.

Menke, N.B., Ward, K.R., Witten, T.M., Bonchev, D.G., and Diegelmann, R.F. (2007) Impaired wound healing. *Clin Dermatol*., 25:19-25.

Meszaros, A.J., Reichner, J.S., and Albina, J.E. (2000) Macrophage-induced neutrophil apoptosis. *J Immunol*., 165:435-441.

Miller, E.J., Piez, K.A, Reddi, A.H. (1984) Chemistry of collagens and their distribution. In: Extracellular matrix biochemistry, (Ed), Elsevier, New York, 41-82.

Miyata, T., Taira, T., and Noishiki, Y. (1992) Collagen engineering for biomaterial use. *Clinical Materials*., 9: 139–148.

Mizuno, M. and Kuboki, Y. (2001) Osteoblast-related gene expression of bone marrow cells during the osteoblastic differentiation induced by type I collagen. *J Biochem*., 129(1):133-8.

Montero, P. and Borderias, J. (1989) Changes in hake muscle collagen during frozen storage due to seasonal effcts. *International Journal of Refrigeration.*, 12: 220-223.

Montero, P., Jimenez-Colmenero, F., and Borderias, J. (1991). Effect of pH and the presence of NaCl on some hydration properties of collagenous material from trout muscle and skin. *J. Sci. Food Agr.*, 54:137-146.

Moore, R.A., Derry, S., and McQuay, H.J. (2007) Cyclo-oxygenase-2 selective inhibitors and nonsteroidal anti-inflammatory drugs: balancing gastrointestinal and cardiovascular risk. *BMC Musculoskelet Disord*., 8:73.

Morrissey M.T. and Park J.W. (2000) Manufacturing of surimi from light muscle fish. In: Park JW, editor. Surimi and surimi seafood. New York: Marcel Dekker Inc. 23- 58.

Morrissey, M.T., Lin, J., and Ismond, A. (2005) Waste management and by-product utilization. In: Surimi and surimi seafood, (2nd edn), Florida, CRC Press, Taylor and Francis Group, 279-323.

Moskowitz, R.W. (2000) Role of collagen hydrolysate in bone and joint disease. *Semin. Arthritis Rheum*., 30: 87-99.

Mosser, D.M., and Edwards, J.P. (2008) Exploring the full spectrum of macrophage activation. *Nat Rev Immunol.,* 8:958-969.

Mullally, M.M., O'Callaghan, D.M., FitzGerald, R.J., Donnelly, W.J., and Dalton, J.P. (1995) Zymogen activation in pancreatic endoproteolytic preparations and influence on some whey protein characteristics. *J. Food Sci*., 60: 227-233.

Murashita, Y.Y., Nakayama., T. Hirano, S., and Ohashi. (1996) Acceleration of granulation tissue growth by hyaluronic acid in artificial skin. *Br. J. Plast. Surg.,* 49: 58- 63.

Muyonga, J H., Colec, C. G. B. and Duodu, K. G. (2004a) Extraction and physicochemical characterisation of Nile perch (*Lates niloticus*) skin and bone gelatine. *Food Hydrocoll.*, 8: 581-592.

Muyonga, J.H., Cole, C.G.B., and Duodu, K.G. (2004b) Characterization of acid soluble collagen from skins of young and adult Nile perch (*Lates niloticus*). *Food Chem.*, 85: 81-89.

Mewar, D.and Wilson, A.G. (2006) Autoantibodies in rheumatoid arthritis: a review. *Biomed.Pharmacother.*, 60: 648-655.

Myers, R.H., Montgomery D.C., and Anderson-Cook, C.M. (2008) Response Surface Methodology: Process and Product Optimization Using Designed Experiments. 3nd Edn., Wiley, New York

Nagai N., Nakayama, Y., Zhou, Y.M., Takamizawa, K., Mori, K., and Munekata, M. (2008) Development of salmon collagen vascular graft: mechanical and biological properties and preliminary implantation study. *J. Biomed. Mater. Res. Part B.,* 87:432-439.

Nagai, T. and Suzuki, N. (2000a) Isolation of collagen from fish waste material skin, bones and fins. *Food Chem.*, 68(3):277-81.

Nagai, T. and Suzuki, N. (2002) preparation & partial characterization of collagen from paper nautilus (*Argonauta argo*) outer skin. *Food chem*. 76:149-153.

Nagai, T., and Suzuki, N. (2000b) Isolation of collagen from fish waste material - skin, bone and fins. *Food Chem.,* 60: 277-281.

Nagai, T., Araki Y., and Suzuki, N. (2002) Collagen of the skin of ocellate puffer fish (*Takifugu rubripes*). *Food Chem.*, 78: 173-177.

Nagai, T., Yamashita, E., Taniguchi, K., Kanamori, N., and Suzuki N. (2001) Isolation and characterisation ofcollagen from the outer skin waste material of cuttleffish (*Sepia lycidas). Food Chem*. 72: 425-429.

Najafian, L. and Babji, A.S. (2012) A review of fish-derived antioxidant and antimicrobial peptides: Their production, assessment, and applications. *Peptides*, 33(1): 178-185.

Nakatani, S., Mano, H., Sampei, C., Shimizu, J., and Wada, M. (2009) Chondroprotective effect of the bioactive peptide prolyl-hydroxyproline in mouse articular cartilage *in v*itro and *in vivo*. *Osteoarthritis and Cartilage*., 17(12):1620-1627.

Nam, K.A., You, S.G., and Kim, S.M. (2008) Molecular and Physical Characteristics of Squid (*Todarodes pacificus*) Skin Collagens and Biological Properties of Their Enzymatic Hydrolysates. *J. Food Sci*., 73:.249-251.

Ngo, D.H., Qian, Z.J., Ryu, B.M., Park, J.W., and Kim, S.K. (2010) *In vitro* antioxidant activity of a peptide isolated from Nile tilapia (*Oreochromis niloticus*) scale gelatin in free radical-mediated oxidative systems. *J. Funct. Foods*., 2(2): 107-117.

Nissler, K., Pohlers, D., and Huckel, M. (2004) Anti- CD4 monoclonal antibody treatment in acute and early chronic antigen induced arthritis: influence on macrophage activation. *Ann. Rheum.Dis*., 63: 1470-7.

Nomura, Y., Oohashi, K., Watanabe, M., and Kasugai, S. (2005) Increase in bone mineral density through oral administration of shark gelatine to ovariectomized rats. *Nutrition*., 21(11-12): 1120-1126.

Nomura, Y., Sakai, H., Ishii, Y., and Shirai, K. (1996) Preparation and some properties of type I collagen from fish scales. *Biosci Biotechnol Biochem*., 60: 2092-2094.

Noormohamed, S.E. and Ray, T. (1998) Effect of 'Compound R' on thermal burn and full-depth wound contracture in fuzzy rats. *J Burn Care Rehabil.*, 19:213–5.

Oesser, S. and Seifert, J. (2003) Stimulation of type II collagen biosynthesis and secretion in bovine chondrocytes cultured with degraded collagen. *Cell Tissue Research*. , 311(3): 393-399.

Oesser, S., Adam, M., Babel, W., and Seifert, J. (1999) Oral administration of ^{14}C labelled gelatine hydrolysate leads to an accumulation of radioactivity in cartilage of mice. *J Nutr.*, 129(10):1891-1895.

Ogawa, M., Moody, M.W., Portier, R.J., Bell, J., Schexnayder, M.A., and Losso, J.N. (2003). Biochemical properties of black drum and sheepshead seabream skin collagen. *J. Agric. Food Chem*., 51: 8088-8092.

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 (4): 495-501.

Ohara, H., Matsumoto, H., Ito, K., Iwai, K., and Sato, K. (2007) Comparison of quantity and structures of hydroxyproline-containing peptides in human blood after oral ingestion of gelatin hydrolysates from different sources. *J Agric Food Chem., 55(4)*:1532-5.

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-8S.

Palosuo, T, Lukka, M, and Alenius, H. (1998) Purification of filaggrin from human epidermis and measurement of antifilaggrin autoantibodies in sera from patients with

rheumatoid arthritis by an enzyme-linked immunosorbent assay. *Int Arch Allergy Immunol*., 115:294.

Panduranga and Rao, K. (1995). Recent developments of collagen-based materials for medical applications and drug delivery. *J Biomater Sci* Polym., 7(7):623- 645.

Pati, F., Datta, P., Adhikari, B., Dhara, S., Ghosh, K., and Mohapatra, P.K.D. (2012) Collagen scaffolds derived from fresh water fish origin and their biocompatibility. *J Biomed Mater Res* A., 1001068-1079.

Pearson, C.M. and Wood, F.D. (1959) Studies of polyarthritis and other lesions induced in rats by injection of mycobacterial adjuvant. I. General clinical and pathologic characteristics and some modifying factors. *Arthritis Rheumatoid.*, 2:440-459.

Peppas, N.A. and Khare A.R. (1993) Preparation, structure and diffusional behavior of hydrogels in controlled release. *Advanced Drug Delivery Reviews.*, 11:1–35.

Perkins, D.N., Pappin, D.J., Creasy, D.M., and Cottrell, J.S. (1999) Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis*., 20:3551-67.

Persico, F.J., Pritchard, J.F., Fischer, M.C., Yorgey, K., Wong, S., and Carson, J. (1988) Effect of Tolmetin Glycine Amide, a Prodrug of Tolmetin Sodium, on Adjuvant Arthritis inthe rat, *The Journal of Pharmacology and Experimental Therapeutics.*, 247(3): 889-896.

Petibois, C., Gionnet, K., Goncalves, M., Perromat, A., Moenner, M. and Deleris, G. (2006). Analytical performances of FT-IR spectrometry and imaging for concentration measurement within biological fluids, cells, and tissues: *Analysis.* 131: 640-647.

Piez, K.A. (1984) Molecular and aggregate structures of the collagens. In: Extracellular matrix biochemistry, Piez KA, Reddi AH (Ed), Elsevier, New York, pp 1–40.

Piez, K.A. (1985) Collagen. In: Encyclopedia of polymer science and engineering, Kroschwitz JI (Ed), Wiley, New York, pp 699–727.

Pihlanto, A. and Korhonen H. (2003) Bioactive peptides and proteins. *Adv Food Nutr Res*., 47:175-276.

Pihlanto-Leppala, A. (2000) Bioactive peptides derived from bovine wheyproteins: opioid and ace-inhibitory peptides. *Trends Food Sci Tech*, 11:347-356.

Poppe, J. (1997) Gelatin.In: Thickening and gelling agents for food, Imeson A (Ed), Blackie Academic and Professional, London, pp 144-168.

Prabjeet Singh, Benjakul S, Maqsood S, Kishimura H. (2011). Isolation and characterisation of collagen extracted from the skin of striped catfish (*Pangasianodon hypophthalmus*). *Food Chem*. 124(1):97-105.

Prockop, D.J. and Kivirikko, K.I. (1995) Collagens: Molecular biology, diseases, and potentials for therapy. *Annu* Rev *Biochem*., 64: 403-434.

Purna, S.K. and Babu, M. (2000) Collagen based dressings–a review. *Burns*., 26:54-62.

Quinn, K.J., Courtney, J.M., Evans, J.H., Gaylor, J.D.S., and Reid, W.H. (1985) Principles of burn dressings. *Biomat*., 6:369-77.

Raabe, O., Reich, C., Wenisch, S., Hild, A., Burg-Roderfeld, M., Siebert, H.C., and Arnhold, S. (2010) Hydrolyzed fish collagen induced chondrogenic differentiation of equine adipose tissue-derived stromalcells. *Histochemistry and Cell Biology*., 134(6): 545-554.

Rajapakse, N., Mendis, E., Jung, W.K., Je J.Y. and Kim, S.K. (2005) Purification of a radical scavenging peptide from fermented mussel sauce and its antioxidant properties. *Food Res. Int.*, 38: 175-182.

Ramshaw, J.A.M., Werkmeister, J.A., and Glattauer, V. (1995) Collagen based biomaterials. *Biotechnol Rev.*, 13: 336-382.

Rebeca, B.D., Pena-Vera, M.T., and Diaz-Castaneda, M. (1991) Production of fish protein hydrolysates with bacterial proteases; Yield and nutritional value. *J. Food Sci*. 56: 309-314.

Reddy, G.K., and Enwemeka, C.S. (1996) A simplified method for the analysis of hydroxyproline in biological tissues. *Clin. Biochem*. 29: 225-229.

Regenstein, J.M. and Zhou, P. (2007) Collagen and gelatin from marine by-product. In: Maximising the value of marine by-products, Shahidi F (Ed), CRC Press, Boca Raton, Florida, pp 279-303.

Rehn, M., Veikkola, T., Kukk-Valdre, E. Interaction of endostatin with integrins implicated in angiogenesis. (2001) *Proc. Natl. Acad. Sci.*98:1024-1029.

Rennekampff, H.O., Kiessig, V., Griffey, S., Greenleaf, G., and Hansbrough, J.F. (1997) Acellular human dermis promotes cultured keratinocyte engraftment. *J Burn Care Rehabil.*, 18:535-544.

Rigby, B.J. (1968) Amino-acid composition and thermal stability of the skin collagen of the Antarctic ice-fish. *Nature.*, 219:166-167

Roberts, P.R., Burney, J.D., Black, K.W., and Zaloga, G.P. (1999) Effect of chain length onabsorption of biologically active peptides from the gastro intestinal tract. *Digestion*., 60(4):332-337.

Roreger, M. (1995) Collagen preparation for the controlled release of active substances. PCT WO 95/28964

Roth, S.H. (2005) Nonsteroidal antiinflammatory drug gastropathy: we started it, why don't we stop it? *J Rheum*., 32(7):1189-1191.

Ruszczak, Z.B. (2000) Modern aspects of wound healing: an update, *Dermatol. Surg*. 26: 219-229.

Ruszczak, Z.B. and Schwartz, R.A. (1999) Collagen uses in dermatology: an update. *Dermatology (Basel)* 199: 285- 289.

Sachs, D.H. (1998) The immunology of xenotransplantation, in: J. Fishman, D. Sachs, R. Shaikh (Eds.), Xenotransplantation: Scientific Frontiers and Public Policy, *Ann. N.Y.Acad.Sci*., 862:1- 4.

Sadowska, M., Kolodziejska, I., and Niecikowska, C. (2003) Isolation of Collagen from the Skins of Baltic cod (*Gadus morhua*) *Food Chem*. 81: 257-262.

Sano, A., Maeda, M., and Nagahara S. (2003) Atelocollagen for proteinand gene delivery. *Advanced Drug Delivery Reviews*., 55:1651-1677.

Saskia Schadow, Hans-Christian, Siebert, Gunter Lochnit, Jens Kordelle, Markus Rickert, Sawitzke, A.D., Shi, H., *et.al.*, (2010) Clinical efficacy and safety of glucosamine, chondroitin sulphate, their combination, celecoxib or placebo taken to treat osteoarthritis of the knee: 2-year results from GAIT. *Ann Rheum Dis*, 69:1459-1464

Sawitzke, A.D., She, H., Finco, M.F., Dunlop, D.D., Harris, C.L., Singer, N.G., Bradley, J.D., Silver, D., Jackson, C.G., Lane, N.E., Oddis, C.V., Wolfe, F., Lisse, J., Furst, D.E., Bingham, C.O., Reda, D.J., Moskowitz, R.W., Willians, H.J. and Clegg, D.O. (2010) Clinical efficacy and safety of glucosamine, chondroitin sulfate, their combination, celecoxib or placebo

taken to treat osteoarthritis of the knee: 2-year results from GAIT. *Ann. Rheum. Dis.* 69:1459-1464.

Schagger, H., and Von Jagow, G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamidegel electrophoresis for the separation of proteins in the range from 1 to100 kDa *Analytical Biochem.*, 166(2):368-379.

Schellekens, G.A., de Jong, B.A., and Van den Hoogen, F.H. (1998) Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies. *J Clin Invest.*, 101:273.

Schellekens, G.A., Visser, H., de Jong, B.A., van den Hoogen, F.H., Hazes, J.M., and Breedveld, F.C., (2000) The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide. *Arthritis Rheum.*, 43:155-163.

Schoof, H.J., Apel, I., Heschel, G., and Rau, (2001) Control of pore structure and sizes in freeze-dried collagen sponges, *J. Biomed.Mater. Res*. 58: 352-357.

Scott, D.L., Shipley, M., and Dawson, A. (1998) The clinical management of rheumatoid arthritis and osteoarthritis: Strategies forimproving clinical effectiveness. *Br. J. Rheumatol*., 37:546-54.

Senaratne, L.S., Park, P., Kim, S. (2006) Isolation and characterization of collagen from brown backed toadfish (*Lagocephalus gloveri*) skin. *Bioresour Technol*. 97:191-197.

Shahidi, F. and Zhong, Y. (2008) Bioactive peptides. *J AOAC* Int; 9(4):914–31.

Shanmugasundaram, N., Ravichandran, P., Reddy, P.N., Ramamurty, N., Pal, S., and Rao, K.P. (2001) Collagen–chitosan polymeric scaffolds for the in vitro culture of human epidermoid carcinoma cells. *Biomat.*, 22(14):1943-1951.

Shen X.R., Kurihara H., and Takahashi K. (2007) Characterization of molecular species of collagen in scallop mantle. *Food Chem*. 102(4): 1187-1191.

Siedler, S. and Schuller-Petrovic, S. (2000) Allogenic keratinocytes suspended in human fibrin glue used for wound healing support in chronic leg ulcers. *Arch. Dermatol*., 136: 676-678.

Simoes, S.I., Delgadoa, T.C., Lopes, R.M., Jesus, S., Ferreira, A.A., Morais, J.A., Cruz, M.E.M., Corvo, M.L., and Martins, M.B.F. (2005) Developments in the rat adjuvant arthritis model and its use in therapeutic evaluation of novel non-invasive treatment by SOD in transfersomes. *Journal of Controlled Release*., 103: 419-434.

Simon, G., (1965) Alterations in joint space and associated home change, in: Principles of bone X-ray diagnosis, Butlerworth and Co, Great Britain., pp. 157–163.

Singer, A.J., Berrutti, L., Henry, C., Thode, J., and McClain, S.A., (1999) Octylcyanoacrylate for the treatment of partial-thickness burns in swine: a randomized, controlled experiment. *Acad Emer Med*., 6:688-92.

Singh, M.P., Stefko, J., Lumpkin, J.A., and Rosenblatt, J. (1995) The effect of electrostatic charge interactions on release rates of gentamicin from collagen matrices. *Pharm Res*., 12: 1205-1210.

Sionkowska, A., Skopinska-Wisniewska, J., and Wisniewski, M. (2009) Collagen synthetic polymer interactions in solution and in thin films. *J Molecular Liquids*., 145: 135-138.

Slade, L., and Levine, H. (1987) Polymer-chemical properties of gelatin in foods.In: Advances in meat research, collagen as a food, Pearson AM Dutson TR, Bailey AJ (Ed), 4AVI Publishers, New York, pp 251-266.

Smith, W.L., De Witt, D.L. and Garavito, R.M. (2000) Cyclooxygenases: structural, cellular, and molecular biology. *Annu Rev Biochem*., 69: 145-82.

Song, E., Yeon Kim, S., Chun, T., Byun, H.J., and Lee, Y.M. (2006) Collagen scaffolds derived from amarine source and their biocompatibility. *Biomat*, 27:2951-2961.

Soo C.,G. Rahbar, R.L., and Moy. (1993) The immunology of bovine collagen implants, *J. Dermatol. Surg. Oncol.* 19: 431-434.

Spreafico, A. (2006) Osteogenic growth peptide effects on primary human osteoblast cultures: potential relevance for the treatment of glucocorticoid induced osteoporosis. *J Cell Biochem*., 98(4): 1007-20.

Srivastava, S., Gorham, S.D., French, D.A., Shivas, A.A., Courtney, J.M. (1990) *In vivo* evaluation and comparison of collagen, acetylated collagen and collagen/glycosaminoglycan composite films and sponges as candidate biomaterials. *Biomat.*, 11: 155-161.

Stainsby, G. (1987) Gelatin gels. In: Advances in meat research: Collagen on a food. Pearson AM, Dutson TR, Bailey AJ (Ed), New York, Van Nostrand Reinhold Company Inc. 4, 209-222.

Steffen, C., Timpl, R., and Wolff, I. (1968) Immunogenicity and specificity of collagen. V. Demonstration of three different antigenic determinants on calf collagen. *Immunology*., 15: 135-144.

Stemberger, A., Grimm, H., Bader, F., Rahn, H.D., and Ascherl, R. (1997) Local treatment of bone and soft tissue infections with the collagen–gentamicin sponge. *Eur J Surg*., 163: 17-26.

Su CH, Sun C-S, Juan S-W, Hu C-H, Ke W-T, and Sheu M.T. (1997) Fungal mycelia as the source of chitin and polysaccharides and their applications as skin substitutes. *Biomat*.,18(17):1169-74.

Sudaroli, M., and Chatterjee, T.K. (2007) Evaluation of red and white seed extracts of Abrusprecatorius Linn. Against freund's complete adjuvant induced arthritis in rats. *J. Med. Plants. Res*., 1: 86-94.

Suetsuna, K., Ukeda, H., and Ochi, H. (2000) Isolation and characterization of freeradical scavenging activities peptides derived from casein. *J. Nutr. Biochem.,* 11(3): 128-131.

Sugiura H., S. Yunoki, E. Kondo, T. Ikoma, J. Tanaka, and Yasuda, K. (2009) In vivo biological responses and bioresorption of tilapia scale collagen as a potential biomaterial. *J Biomater Sci Polym.,* 20: 1353-1368.

Suzuki, Y., Tanihara, M., Nishimura, Y., Suzuki, K., Kakimaru, Y., and Shimizu, Y. (1997) A novel wound dressing with an antibiotic delivery system stimulated by microbial infection. *ASAIO J*., 43(5): M854.

Swatschek D, Shatton W, Kellermann J, Muller, Keuter J. (2002) Marine sponge collagen: isolation, characterization and effects on the skin parameters surface-pH, moisture and sebum. *Eur J Pharm*., 53(1): 107-113

Takeshi Nagai and Nobutaka Suzuki. (2000) Isolation of collagen from fish waste material - skin, bon and fins, *Food Chem*., 60: 277-281.

Takeshi, K., Shoichi, N., Yasunori, K., Yasuhiko, I. (2002) Report of the committee on the classification and diagnostic criteria of diabetes mellitus (The Committee of the Japan Diabetes Society on the diagnostic criteria of diabetes mellitus) *Diabetes Res Clinical Pract*., 55:65-85.

Timpl, R. (1984) Immunology of the collagen, in: K.A. Piez, AH. Reddi (Eds.), Extracellular Matrix Biochemistry, Elsevier, Amsterdam, 159-188.

Trelstad, R.L. and Birk, D.E (1984) Collagen fibril assembly at the surface of polarized cells, in: Exracellular matrix in Development (R.L.Trelstad, ed) pp.513-543, Liss, New York

Trevitt, C.R. and Singh, P.N. (2003) Variant Creutzfeldt-Jakob disease: pathology, epidemiology, and public health implications. *Am J Clin Nutr.,* 78: 651S-6S.

Vacanti, J.P. and Langer, R.S. (1998) Preparation of three-dimensional fibrous scaffold for attaching cells to produce vascularised tissue in vivo. US Patent 5,770,193 (MIT).

Van der Rest, M. and Garrone, R. (1991) Collagen family of proteins. *FASEB J.,* 5: 2814-2823.

Vijayalakshmi, M.A., Lemieux, L., and Amiot, J. (1986) High performance size exclusion liquid chromatography of small molecular weight peptides from protein hydrolysates using methanol as a mobile phase additive. *J Liq Chromatogr.,* 9:3559-3576.

Vivian Zague, Vanessa de Freitas, Marina da Costa Rosa, Geo´rgia, A., lvares de Castro, Ruy, G., Jaeger, and Gla´ucia, M. Machado-Santelli, (2011) Collagen Hydrolysate Intake Increases Skin Collagen Expression and Suppresses Matrix Metalloproteinase II Activity. *J Med Food.*, 14 (6): 618-624.

Vojdani, F. (1996) Solubility: In Methods of Testing Protein Functionality. London: Blackie Academic and Professional, 1: 11-60.

Wagner, W.D. (1979) A more sensitive assay discriminating galactosamine and glucosamine in mixtures. *Anal. Biochem.*, 94: 394-398.

Wallace, D.G. and Rosenblatt, J. (2003) Collagen gel systems for sustained delivery and tissue engineering. *Advanced Drug Delivery Reviews.,* 55: 1631–1649,

Walrand, S., Chiotelli, E., Noirt, F., Mwewa, S., and Lassel, T. (2008) Consumption of a functional fermentedmilk containing collagen hydrolysate improves the concentration of collagen-specific amino acids inplasma. *J. Agric. Food Chem.,* 56(17): 7790-7795.

Wang, C.L., Miyata, T., Weksler, B., Rubin, A.L., and Stenzel, K.H. (1978) Collagen-induced platelet aggregation and release. I. Effects of side-chain modifications and role of arginyl residues. *Biochem Biophys Acta.,* 544: 555-567.

Wang, L., An, X., Yang, F., Xin, Z., Zhao, L., and Hu, Q. (2008). Isolation and characterization of collagens from the skin, scale and bone of deep-sea red fish (*Sebastes mentella*). *Food Chem*., 108(2): 616-623.

Weadock, K.S., Miller, E.J., Keuffel, E.L., and Dunn, M.G. (1996) Effect of physical crosslinking methodson collagen-fiber durability in proteolytic solutions. *J Biomed Mater Res*., 32: 221-226.

Whitford, D. (2005) Proteins: Structure and Function. John Wiley and Sons Ltd., Chichester England, pp 528.

Williams, R.O. (1998) Rodent models of arthritis: Relevance for human disease. *Clin Exp Immunol*., 114:330-2.

Winter, G.D. (1962) Formation of the scab and the rate of epithelialization of superficial wounds in the skin of the young domestic pig. *Nature*., 193: 293-294.

Winter, G.D. (1972) In: Maibach HI, Rovee DT, editors. Epidermal wound healing. Chicago: Year Book Medical Publishers. p.71.

Winter, G.D., and Scales, J.T. (1963) The effect of air drying and dressings on the surface of a wound. *Nature*., 197: 91.

Wong, D.W.S. (1989) Mechanism and theory in food chemistry. New York: Van Nostrand Reinhold Company Inc.

Wood, A., Ogawa, M., Portier, R.J., Schexnayder, M., Shirley, M., and Losso, J.N. (2008) Biochemical properties of alligator (Alligator mississippiensis) bone collagen. *Comp. Biochem. Physiol. Biochem. Mol. Biol.*, 151:246-249.

Wright, K.A., Nadire, K.B., Busto, P., Tubo, R., Mc Pherson, J.M., and Wentworth, B,M. (1998) Alternative delivery of keratinocytes using a polyurethane membrane and the implications for its use in the treatment of full-thickness burn injury. *Burns*; 24(1):7-17.

Wu, J. (2004) Assessment of effectiveness of oral administration of collagen peptide on bone metabolism in growing and mature rats. *J bone min met*., 22(6): 547-553.

Yamada, S., Nagaoka, H., Terajima, M., Tsuda, N., Hayashi, Y., et al. (2013) Effects of fish collagen peptides on collagen post-translational modifications and mineralization in an osteoblastic cell culture system. *Dent Mater J.*, 32: 88-95

Yannas, and Burke, J.F. (1980) Design of an artificial skin. I. Basic design principles. *J Biomed Mater Res*., 14(1):65-8.

Yannas, Burke, J.F., Orgill, D.P., and Skrabut, E.M. (1982) Wound tissue can utilize a polymeric template to synthesize a functional extension of skin. *Science*., 215:174-6.

Yong-liang Zhuang, Xue Zhao, and Ba-fang Li. (2009) Optimization of antioxidant activity by response surface methodology in hydrolysates of jellyfish (*Rhopilema esculentum*) umbrella collagen. *J. Zhejiang Univ.-SCI. B* 10(8):572-579

Yoshihito Ishida, Hiroshi Kubota, Akisugu Yamamoto, Akira Kitamura, Hans Peter Bachinger, and Kazuhiro Nagata. (2006) Type 1 collage in Hsp47-null cells is aggregated in endoplasmic reticulum and deficient in N-propeptide processing and fibrillogenesis. *Molecular biology of the cell*. 17: 2346-2355.

Yoshikawa, T., Tanaka, H., and Kondo, M. (2004) The increase in lipid peroxidation in rat adjuvant arthritis and its inhibition by Superoxide dismutase. *Biochem. Med,* 33:320-326.

Zague, V *et a.l,* (2011) Collagen Hydrolysate Intake Increases Skin Collagen Expression and Suppresses Matrix Metalloproteinase 2 Activity. *J. Med. Food.,* 14: 618-624

Zague, V. A (2008) New view concerning the effects of collagen hydrolysate intake on skin properties. *Arch. Derm. Res,* 300: 479-483.

Zhang, L., and Webster, T.J. (2009) Nanotechnology and nanomaterials: Promises for improved tissue regeneration. *Nano Today*., 4: 66-80.

Zhang, L.N., Guo, J., Zhou, J.P., Yang, G., and Du, Y.M. (2000) Blend membranes from carboxymethylated chitosan/alginate in aqueous solution. *J. Appl. Polym. Sci.,* 77(3): 610-616.

Zhang, W., Liu, H.T. (2002) MAPK signal pathways in the regulation of cell proliferation in mammaliancells. *Cell Res*., 12: 9–18.

Zhang, Y., Kouguchi, T., Shimizu, K., Sato, M., Takahata, Y., & Morimatsu, F. (2010) Chicken collagen hydrolysate reduces proinflammatory cytokine production in C57BL/6.KOR-ApoEshl Mice. *J Nutr Sci Vitaminol*., 56: 208-210.

Zhang, Y., Liu, W. T., Li, G. Y., Shi, B., Miao, Y. Q., & 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.

Zhang, Z.K., Li, G.Y., and Shi, B., (2006) Physicochemical Properties of Collagen, Gelatin and Collagen Hydrolysate derived from Bovine Limed Split Wastes, J. Soc. *Leather Technol. Chem*., 90: 23-28.

Zheng, Y.Q., and Wei, W. (2005) Total glucosides of paeony suppresses adjuvant arthritis inrats and intervenes cytokine-signaling between different types of synoviocytes. *International Immunopharmacology*., 5: 1560-1573.

Zhong, S., and Yung, L.Y. (2009) Enhanced biological stability of collagen with incorporation of PAMAM dendrimer. *J. Biomed. Mater. Res. A*., 91: 114-122.

Zhongkai Zhang, Guoying Li and Bi Shi. (2005) Physiochemical properties of collagen, gelatin and collagen hydrolysate derived from bovine limited split wastes. *J Soc Leath Tech Ch*., 90:23.

Zhu, A.P., and Fang, N. (2005) Adhesion dynamics, morphology, and organization of 3T3 fibroblast on chitosan and its derivative: The effect of O-carboxymethylation. *Biomacromolecules*., 6(5): 2607-2614.

PUBLICATIONS

1. Hema, G.S., Shyni, K., Suseela Mathew, Anandan, R., George Ninan, Lakshmanan P.T. (2013) A simple method for isolation of fish skin collagen- biochemical characterization of skin collagen extracted from Albacore Tuna (*Thunnus alalunga*), Dog Shark (*Scoliodon sorrakowah*), and Rohu (*Labeo rohita*) *Annals of Biological Research*, 4 (1):271-278.

2. Hema, G.S., Shyni, K., Suseela Mathew, George Ninan, Joshy,C.G. (2014) Comparison of Collagen Extracted from Skin of Double spotted Queen fish and Malabar Grouper. *Fishery Technology* 51: 93 – 97.

Available online at www.scholarsresearchlibrary.com

Scholars Research Library

Annals of Biological Research, 2013, 4 (1):271-278
(http://scholarsresearchlibrary.com/archive.html)

Annals of Biological Research
Scholars Research Library
ISSN 0976-1233
CODEN (USA): ABRNBW

A simple method for isolation of fish skin collagen- biochemical characterization of skin collagen extracted from Albacore Tuna (*Thunnus alalunga*), Dog Shark (*Scoliodon sorrakowah*), and Rohu (*Labeo rohita)*

G.S. Hema[a], K. Shyni[a], Suseela Mathew[a], R. Anandan[a,*], George Ninan[b], P.T. Lakshmanan[a]

[a]*Biochemistry and Nutrition Division, Central Institute of Fisheries Technology, Matsyapuri (PO), Cochin-682029, Kerala, India*
[b]*Fish Processing Division, Central Institute of Fisheries Technology, Matsyapuri (PO), Cochin-682029, Kerala, India*

ABSTRACT

A simple method has been developed for the isolation of Acid Soluble Collagen (ASC) and Pepsin Digestible Collagen (PDC) from the skin of Albacore tuna (Thunnus alalunga), Dog shark (Scoliodon sorrakowah), and one among Indian Major Carps ie, Rohu (Labeo rohita). Biochemical characterization of the collagen extracted was carried out. On wet weight basis the yields of ASC and PDC from shark skin were 8.96% & 7.68% respectively and that from Rohu was ASC 4.13% & PDC 3.68% respectively. The yield of collagen from Tuna skin was ASC 13.97%. No PDC was obtained for tuna skin. Proximate analysis showed all collagens had protein as a major constituent with trace amount of ash and fat. Amino acid analysis revealed that they contained glycine as a major amino acid with high contents of alanine, proline and hydroxyproline. Based on sodium dodecyl sulfate – polyacrylamide gel electrophoretic patterns and subunit compositions, all were identified to be type 1 collagens. A comparison of these collagens with calf skin type 1 collagen indicated the same and α_1,α_2 and β chains were the major components of these collagens. γ components were also found in lesser amounts in these collagens. The results of the present study indicated that comparing the three species Dog shark skin had good yield of collagen and it could be served as an alternative source of collagen for different biomedical applications.

Keywords Fish Skin Collagen• Extraction• Characterization • Amino acid composition

---

INTRODUCTION

Collagen forms the major fraction of connective tissues such as skin, bone, tendon, the vascular system of animals and the connective tissue sheaths surrounding muscle [1]. Its contents vary, depending on fish species [2, 3]. Type 1 collagen has been found as the major collagen in the skin, bone and fins of various fish species [4]. The physical and chemical properties of collagen differ depending on the tissues such as skin, swim bladder & the myocommata in muscle. Fish collagen is heat sensitive due to labile cross links as compared to mammals; the hydroxyproline content is lower, varying from 4-10% [5]. However, different fish species containing varying amounts of collagen in the body tissue that reflect the swimming behavior and it influences the textural characteristics of fish muscle [6]. Most fish collagens have been found to consist two α - chain variants, which are normally designated as α-1 and α-2 [7, 8]. These chain variants, though having approximately the same molecular weight (95,000 Da) can be separated by

SDS PAGE due to their different affinity for SDS. α - 2 have a higher affinity for SDS and consequently exhibit a higher mobility than α 1 [9]. In addition to differences in molecular species, fish collagens have been shown to vary widely in their amino acid composition. In particular, the levels of imino acids (proline and hydroxyproline) vary significantly among fish species [10-12]. The amount of imino acids, especially hydroxyproline, depends on the environmental temperature in which the fish lives and it affects the thermal stability of the collagens and gelatins [10, 13]. Collagens derived from fish species living in cold environments have lower contents of hydroxyproline and they exhibit lower thermal stability than those from fish living in warm environments. This is because of the involvement of hydroxyproline in inter-chain hydrogen bonding, which stabilizes the triple helical structure of collagen. Collagen film proved as a promising carrier for anticancer drug delivery system and ophthalmic drug delivery system because of its inertness, structural stability and good biocompatibility [14, 15]. The objective of the study was to develop a method to isolate collagen from the skin of Albacore tuna (*Thunnus alalunga*), Dog shark (*Scoliodon sorrakowah*), and one among Indian Major Carps ie, Rohu (*Labeo rohita*) which are generally discarded as waste in fish processing industry.

MATERIALS AND METHODS

2.1 Chemicals

All chemicals were of analytical grade. Type 1 collagen from calf skin, pepsin from stomach mucosa, high molecular weight markers, collagen hydrolysate were from Sigma chemical Co. Sodium dodecyl sulphate (SDS), Coomassie brilliant blue R-250 & N,N,N',N'-tetramethylethylenediamine (TEMED) were procured from Bio-Rad laboratories.

2.2 Raw material

The species used for the study were **Albacore tuna** (*Thunnus alalunga*), **Dog shark** (*Scoliodon sorrakowah*), and one among Indian Major Carps ie, **Rohu** (*Labeo rohita*).The skin in the iced condition was procured from Polakkandom market, Cochin, Kerala, India.

2.3 Proximate analysis

The raw skin of the three species and their collagens (both acid soluble and pepsin digestible collagens) were subjected to proximate analysis including moisture, ash, fat and protein contents, according to the method of AOAC (1995)[6].

2.4 Pretreatment of the skin

Acid Soluble Collagen (ASC) & Pepsin Digestible Collagen (PDC) were extracted from Shark Skin, Tuna Skin & Rohu skin. All the extraction procedures were carried out at 4^0C. The source material was minced and mixed with 30 volumes of 0.1N sodium hydroxide and kept stirred for 24h over a magnetic stirrer to remove non collagenous protein. The treated mass was strained through a coarse sieve. The process was repeated twice and the residue was washed twice with 30 volumes of chilled distilled water.

2.5 Collagen extraction

The residue was homogenized in a Polytron homogenizer with 30 volumes 0.5M acetic acid for one minute and the same was stirred over a magnetic stirrer for 24 h. The supernatant after centrifugation (3000 rpm, 20 min) was collected. The residue was once again extracted with acid as above and the combined supernatant was taken as acid soluble collagen (ASC).

The residue from the previous step was homogenized with 30 volumes of 0.5M formic acid for 1 min and stirred for 24 h. A solution of pepsin (enzyme / tissue ratio 1:100) was added to this and kept stirring for another 24h. The supernatant after centrifuging was taken as pepsin digestible collagen (PDC).

Crystalline sodium chloride was added to both supernatants to the level of 10% and stirred for 24 h to precipitate the collagen. The precipitate was suspended in Tris-glycine buffer (50 mM containing 0.2M NaCl, pH 7.4) and dialyzed against the same buffer for 24 h and then centrifuged. The collagen obtained was spray dried to get fine power.

2.6 Amino acid analysis

Collagen samples were hydrolyzed in 6N HCl at 120^0C for 24h. After cooling the test tubes the contents were filtered using Whatman No 1 filter paper. The tubes were rinsed with distilled water and filtered. The filtrate was evaporated in a vacuum flash evaporator. Then deionized water was added into the tubes and continued evaporation

until the contents were acid free. The process was repeated for three times and the free amino acids were dissolved in 0.05M HCl and filtered using 0.45 micro syringe, then injected in to Shimadzu HPLC using the method [17].

2.7 Tryptophan estimation

Tryptophan was estimated after alkali hydrolysis by colorimetry [18].

2.8 UV–Vis measurement

Collagen was dissolved in 0.5 M acetic acid to obtain a concentration of 1 mg/ml. The solution was then subjected to UV–Vis measurement. Prior to measurement, the base line was set with 0.5 M acetic acid. The spectrum was obtained by scanning the wavelength in the range of 220–600 nm with a scan speed of 50 nm/min at room temperature.

2.9 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoretic patterns of different species of collagens were analysed according to the method [19]. The samples were dissolved in 50 g/L SDS solution. The mixtures were then heated at 85^0C for 1 h, followed by centrifugation at 8500g for 5 min to remove undissolved debris. Solubilized samples were mixed with the sample buffer (0.5 mol/L Tris–HCl, pH 6.8 containing 40 g/L SDS, 200 mL/L glycerol in the presence or absence of 100 mL/L βmercaptoethanol) with the ratio of 1:1 (volume ratio). The mixtures were loaded onto a polyacrylamide gel made of 75 g/L separating gel and 40 g/L stacking gel and subjected to electrophoresis at a constant current of 20 mA/gel. After electrophoresis, gels were fixed with a mixture of 500 mL/L methanol and 100 mL/L acetic acid for 30 min, followed by staining with 0.5 mL/L Coomassie blue R-250 in 150 mL/L methanol and 50 mL/L acetic acid for 1 h. Finally, they were destained with a mixture of 300 mL/L methanol and 100 mL/L acetic acid for 1 h and destained again with the same solution for 30 min. High molecular weight protein markers were used to estimate the molecular weight of proteins. Type I collagen from calf skin was used as standard collagens.

2.10 Statistical analysis

All experiments were done in triplicates. Mean values with standard deviations (SD) were reported.

RESULTS AND DISCUSSION

3.1 Proximate composition

Table 1 shows the protein, moisture and ash content of the skin of the three selected fish skins and table 2 that of the extracted collagens. Generally skin of cartilaginous fishes which include shark and rays are low in lipid content. This lean species store majority of their fat in liver whereas skin of clupeid and scombroid species (sardines, mackerels and tuna) is rich in lipid. The extracted collagen contained negligible amounts of ash and fat. Extracted collagens from skin had low contents of ash and fat, indicating the efficacy of removal of both inorganic matter and fat. Collagen samples had low moisture contents, with protein content ranging from 88.8% to 91.72%.

Table 1. Proximate composition of skin

	Shark (%)	Rohu (%)	Tuna(%)
Moisture	68.38 ± 0.43	76.54 ± 0.45	56.54 ± 0.09
Protein	27.73 ± 0.36	18.84 ± 0.06	20.54 ± 0.26
Fat	0.16 ± 0.02	2.93 ± 0.05	18.32 ± 0.11
Ash	4.19 ± 0.03	2.03 ± 0.04	4.39 ± 0.03

Values were given as mean ± standard deviation of triplicate.

Table 2. Proximate composition of extracted collagen

	Moisture	Protein	Fat	Ash
Tuna ASC	7.53 + 0.30	91.08 + 0.71	0.64 + 0.01	0.74 + 0.02
Rohu ASC	8.78 ± 0.06	89.94 ± 0.75	0.33 ± 0.03	0.43 ± 0.02
Rohu PDC	6.66 ± 0.03	91.72 ± 0.53	0.45 ± 0.02	0.50 ± 0.02
Shark ASC	9.13 ± 0.14	88.80 ± 0.59	0.37 ± 0.01	0.76 ± 0.02
Shark PDC	8.32 ± 0.17	90.80 ± 0.12	0.42 ± 0.04	0.80 ± 0.01

Values were given as mean ± standard deviation of triplicate

3.2 Collagen yield

Table 3 shows the yield of the collagen. The yield of collagen in shark skin was higher compared to Tuna and Rohu skin. The skin was not completely solubilized with 0.5 M acetic acid even with two repetitions of extraction except for tuna skin. This result suggested a high amount of cross-links at the telopeptide region as well as other inter-molecular cross-links, leading to low solubility of collagen in acid [20].

Table 3. Collagen yield

Collagen type	Yield(%)
Tuna Skin ASC	13.97
Rohu skin ASC	4.13
Rohu skin PDC	3.68
Shark skin ASC	8.96
Shark skin PDC	7.68

3.3 Amino acid compositions of collagens

Table 4 shows the Amino acid compositions of collagens. Amino acid analysis showed higher content of glycine in all forms of collagen extracted which accounted to one third of the total amino acids. Higher contents of alanine, imino acids - hydroxyl proline and proline which are characteristics of collagen could be obtained in the present study also. The collagens were found to contain no tryptophan or cysteine. They were also very low in methionine, tyrosine and histidine, like other collagens [10, 21]. Generally, glycine is about one-third of the total amino acid residues, hydroxyproline about one fifth and alanine about one-ninth in collagen samples.

Table 4 Amino acid compositions of collagens

	Tuna ASC	**Rohu ASC**	**Rohu PDC**	**Shark ASC**	**Shark PDC**
Alanine	118	130	131	109	108
Arginine	46	53	54	52	55
Aspartate	41	43	42	43	40
Cysteine	0	0	0	0	0
Glutamate	74	62	62	76	78
Glycine	332	328	330	315	321
Histidine	9	7	7	8	7
Isoleucine	9	8	7	21	18
Leucine	18	22	21	24	23
Lysine	25	24	24	26	29
Hydroxylysine	8	6	6	8	4
Methionine	11	11	11	12	12
Phenylalanine	14	18	20	15	14
Hydoxyproline	78	66	68	95	91
Proline	99	115	117	98	109
Serine	43	41	41	32	32
Threonine	23	22	22	23	22
Tyrosine	2	1	1	2	1
Valine	28	29	29	25	26

Values were given as mean ±standard deviation of triplicate

3.4 Tryptophan analysis

No tryptophan could be estimated in the collagen samples.

3.5 Ultraviolet Spectra

From UV–Vis spectra of the extracted collagens, an absorbance near 200-240 nm with high intensity was observed with no absorption peak at 280 nm. The results indicated high efficacy of non-collagenous protein removal. Collagen commonly has a low amount of tyrosine, which could absorb UV-light at 280 nm [22]. The absorbance in this region is similar to those of collagens from channel catfish skin [23], walleye Pollock [24], and largefin longbarbel catfish [20]. Peptide bonds found in the protein also absorb at 205-230nm. The absorbance at 280nm is mainly because of tryptophan, tyrosine & phenyl alanine. Tryptophan is completely absent in collagen and have negligible amount of tyrosine. Previous research indicates that collagen commonly have a low amount of tyrosine which can absorb UV-light at 280 nm [22]. For these reasons, the extracted protein is collagen. Figures 1 to 6 depict various UV spectra analysis plots for the samples.

Figure 1. Ultraviolet Spectra analysis of pure collagen from calf skin

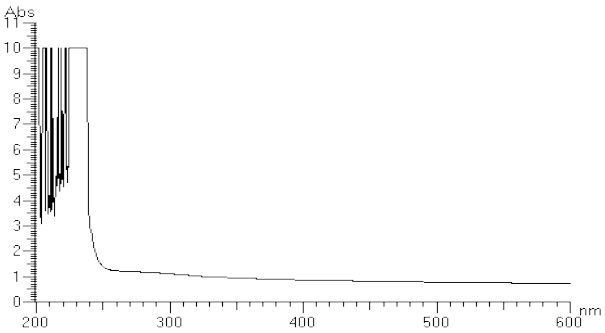


Figure 2. Ultraviolet Spectra analysis of tuna ASC

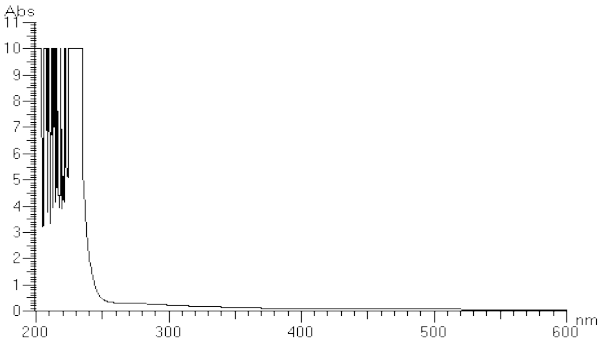


Figure 3. Ultraviolet Spectra analysis of Rohu ASC

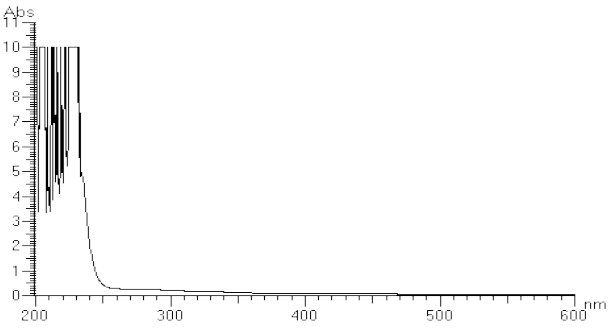


Figure 4. Ultraviolet Spectra analysis of Rohu PDC

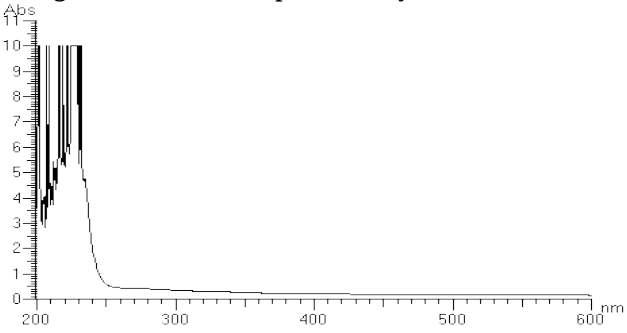


Figure 5. Ultraviolet Spectra analysis of shark ASC

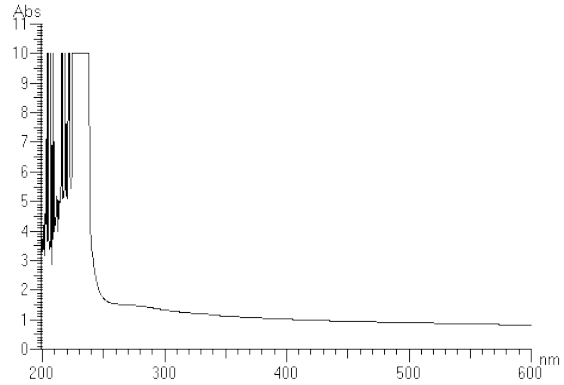
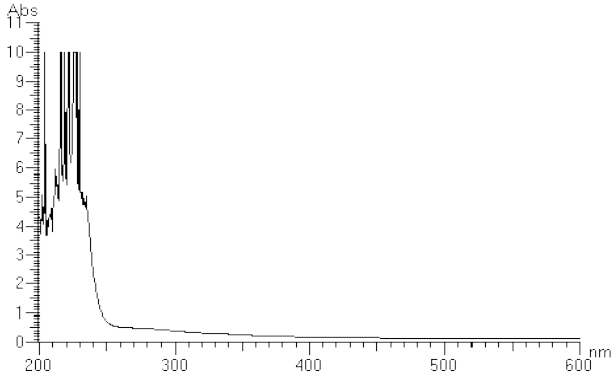


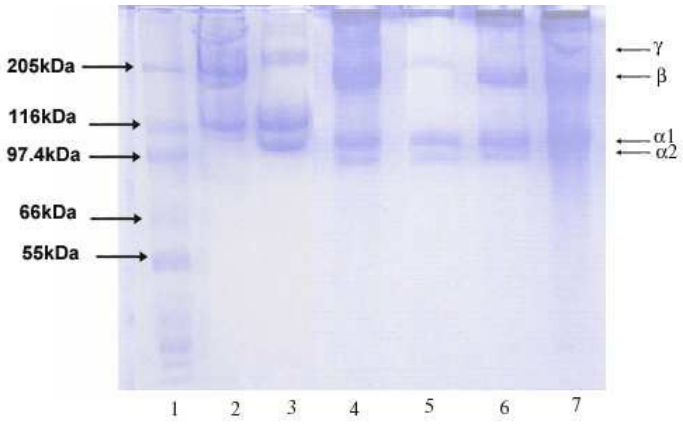
Figure 6. Ultraviolet Spectra analysis of Shark PDC



3.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Plate 1 shows the molecular weight pattern of Collagens against the high molecular weight marker. The protein patterns of ASC & PSC were analyzed by 7.5% resolving gel and it was found that the major constituents of both ASC &PDC consisted of α chains ($\alpha_1\alpha_2$), β, γ chains. These patterns were similar to the type 1 collagen from calf skin (lane 7), and also in accordance with those of collagens from most other fish species previously reported [8, 25]. Type I collagen consists of two identical α_1 chains and one α_2 chain [1, 26]. Fish skin and bone have been reported to contain type I collagen as the major collagen [27-29].The skin collagens of big eye snapper[30], brown banded bamboo shark [31], Nile perch [32], ocellate puffer fish [2], back drum seabream, sheep shead seabream [32], brown backed toadfish [33],Walleye Pollock [24], and large fin long barbel catfish [20] all consisted of two a chains (α_1&α_2), β and γ components.

Plate 1 Molecular weight pattern of Collagens



Lane 1.High molecular weight marker, Lane 2. Shark ASC, Lane 3.Shark PDC, Lane 4.Tuna ASC Lane 5.Rohu ASC, Lane 6.Rohu PDC, Lane 7. Type 1 collagen from calf skin.

CONCLUSION

The acetic acid soluble & pepsin digestible collagens from the skin of three verities of fishes viz Albacore tuna (*Thunnus alalunga*), Dog shark (*Scoliodon sorrakowah*), and Rohu (*Labeo rohita*) were extracted and characterized. The result showed that the pepsin can act as a tool for obtaining a greater yield without having a noticeable effect on the triple helical structure except in the case of tuna skin. All the collagens were of typical amino acid composition of type 1 collagen. All collagens showed maximum absorption at 200-235nm with no absorption at 280. No differentiation could be observed in the collagens from the three species regarding ($\alpha_1\alpha_2$), β, γ chains indicating their type 1 nature. The amino acid pattern, SDS-PAGE and the absorbance at 200-240 nm of collagens extracted in the present study indicates that the process of extraction yielded pure collagen with a purity of greater than 99%.

Acknowledgements
The financial assistance for the study from Department of Biotechnology is acknowledged. Acknowledgements are also due to Director CIFT for providing facilities to carry out the research.

REFERENCES

[1] EA Foegeding, TC Lanier, HO Hultin, Collagen. In O. R. Fennema (Ed.), Food chemistry (3rd ed.,) New York, Marcel Dekker, Inc. **1996**, pp. 902–906
[2] AR Salarzadeh, M Afkhami, KD Bastami, M Ehsanpour, A Khazaali and A Mokhleci, *Annals of Biological Research*, **2012**, 3, 1305-1311
[3] P Montero, F Jimenez-Colmenero, J Borderias, *Journal the Science of Food and Agriculture*, **1991**, 54, 137–146
[4] S Kimura, XP Zhu, R Matsui, H Shijoh, S Takamizawa, *Journal of Food Science*, **1983,** 53, 1315–1318.
[5] K Sato, R Yoshinaka, I Yoshiaki, M Sato, *Comparative Biochemistry and Physiology*, **1989,** 92B(1), 87–91.
[6] P Montero, J Borderias, J Turnay, MA Leyzarbe, *Journal of Agricultural and Food Chemistry*, **1990**, 38, 604–609.
[7] MC Gomez-Guillen, J Turnay, MD Fernandez-Diaz, N Ulmo, MA Lizarbe, P Montero, *Food Hydrocolloids*, **2002**, 16, 25–34.
[8] T Nagai, E Yamashita, K Taniguchi, N Kanamori, N Suzuki, *Food Chemistry*, **2001**, 72(4), 425–429.
[9] K Kubo, T Takagi, *Collagen and Related Research*, **1984**, 4, 201–208.
[10] G Balian, JH Bowes, In A. G. Ward, & A. Courts (Eds.), The science and technology of gelatin, London: Academic Press, **1977,** pp. 1–30.
[11] M Gudmundsson, H Hafsteinsson, **1997**, 62, 37–39.
[12] J Poppe, Gelatin. In A. Imeson (Ed.), Thickening and gelling agents for food Glasgow, UK: Blackie Academic & Professional, **1992**, pp. 98–123.
[13] JK Jakhar, AD Reddy, S Maharia, HM Devi, GVS Reddy and G Venkateshwarlu, *Archives of Applied Science Research*, **2012**, 4, 1353-1358
[14] E Mahdi, K Fariba, *Annals of Biological research*, **2012**, 3, 622-627
[15] HA Patel, JK Patel, KN Patel and RR Patel, *Der Pharmacia Lettre*, **2010**, 2, 100-115.
[16] AOAC, In Official methods of analysis (16th ed.) Association of Official Analytical Chemists, Washington, DC **1995**.
[17] Y Ishida, T Fugita, K Asai,. *J. Chromatogra*. **1981,** 204, 143-148.
[18] CPS Sastry, MK Tummuru, *Journal of Food Science and Technolology* **1985**, 22, 46-47.
[19] UK Laemmli, *Nature*, **1970,** 227, 680-685.
[20] M Zhang, W Liu, G Li, *Food Chemistry*, **2009**, 115, 826–831.
[21] S Grossman, M Bergman, US Patent **1992**, 5,093,474.
[22] R Duan, J Zhang, X Du, X Yao, K Konno, *Food Chemistry*, **2009**,112(3), 702–706.
[23] HY Liu, D Li, SD Guo, *Food Chemistry*, **2007**, 101, 621-625.
[24] M Yan, B Li, X Zhao, G Ren, Y Zhuang, H Hou, *Food Chemistry*, **2008**, 107(4), 1581-1586.
[25] JH Muyonga, CGB Cole, KGDuodu, *Food Chemistry*, **2004**, 85, 81-89.
[26] DWS Wong, In Mechanism and theory in food chemistry. New York: Van Nostrand Reinhold Company Inc. **1989**
[27] AS Ciarlo, ME Paredi, AN Fraga, *Journal of Aquatic Food Product Technology*, **1997**, 6, 65–77.
[28] S Kimura, Y Ohno,. *Comparative Biochemistry and Physiology* Part B, **1987**, 88, 409-413.
[29] T Nagai, N Suzuki, *Journal of Food Biochemistry*, **2000**, 24, 427– 436.
[30] P Kittiphattanabawon, S Benjakul, W Visessanguan, T Nagai, M Tanaka, *Food Chemistry*, **2005**, 89, 363–372.
[31] P Kittiphattanabawon, S Benjakul, W Visessanguan, H Kishimura, Shahidi, *Food Chemistry*, **2010**, 119, 1519-1526.
[32] M Ogawa, MW Moody, RJ Portier, J Bell, M Schexnayder, JN Losso, *Journal of Agricultural and Food Chemistry*, **2003**, 51, 8088–8092.

[33] LS Senaratne, PJ Park, SK Kim, *Bioresource Technology*, **2006**, 97, 191-197.

Fishery Technology 51 (2014) : 93 - 97

Comparison of Collagen Extracted from Skin of Double-spotted Queenfish and Malabar Grouper

G. S. Hema*, K. Shyni, Suseela Mathew, George Ninan and C. G. Joshy
Central Institute of Fisheries Technology, P.O. Matsyapuri, Cochin - 682 029, India

Abstract

Acid soluble collagen (ASC) and pepsin digestible collagen (PDC) from the skin of double-spotted queenfish (*Scomberoides lysan*) and Malabar grouper (*Epinephelus malabaricus*), were isolated and characterized. On wet weight basis, the yields of ASC and PDC from queen fish and grouper were 7.82, 3.92, 12.5 and 6.49% respectively. Amino acid analysis revealed that they contained glycine as a major amino acid with high contents of alanine, proline and hydroxyproline. Based on sodium dodecyl sulfate polyacrylamide gel electrophoretic patterns and subunit compositions, all were identified to be type 1 collagens when compared with calf skin type 1 collagen. α_1, $\alpha 2$ and β chains were the major components of the presently isolated collagens. While comparing these two species, queen fish skin had good yield of collagen which could be served as an alternative source of collagen for different applications.

Keywords: Acid soluble collagen, pepsin digestible collagen, yield, type I collagen

Received 16 August 2013; Revised 25 February 2014 : Accepted 03 March 2014

* *E-mail: hema.sukumaran@yahoo.com*

Introduction

Collagen is the most abundant animal protein polymer, accounting for almost 25 to 30% of total protein in the animal body (Liu et al., 2007; Bama et al., 2010). Being one of the extracellular matrix constituents of multi-cellular animals (Mizuta et al., 2005), it can be found in connective tissues and serves as a major component of bones, cartilage, skin, tendons, ligaments, blood vessels, muscles, teeth and other organs of vertebrates (Senaratne et al., 2006; Quereshi et al., 2010). Collagen contents in fishes vary, depending on fish species (Nagai et al., 2002). Studies on extraction of fish collagens have been extensively carried out recently due to its broad application in cosmetic, biomedical and pharmaceutical industries (Cliché et al., 2003).

The outbreak of bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy and foot and mouth disease (FMD) have created anxiety among consumers of collagen and collagen derived products of land animal origin. Collagen from porcine sources cannot be used as component of some foods due to aesthetic and religious objections. Therefore, alternative sources, such as fish processing waste have received increasing attention for collagen extraction (Jongjareonrak et al., 2005). Several studies have described extraction of collagen from aquatic sources such as skin of ocellate puffer fish (Nagai et al., 2002), black drum and sheepshead sea bream (Ogawa et al., 2003), brown backed toad fish (Senaratne et al., 2006), Baltic cod (Sadowska et al., 2003), Nile perch (Muyonga et al., 2004), big eye snapper (Jongjareonrak et al., 2005), skate (Hwang et al., 2007), grass carp (Zang et al., 2007) deep sea red fish (Wang et al., 2007) dusky spine foot, sea chub, eagle ray and sting ray (Bea et al., 2008) large fin long barbel catfish (Zang et al., 2009) and brown banded bamboo shark (Kittiphattanabawon et al., 2010). These studies described collagens from different species, tissues and living environments which may have different biochemical properties.

Characterization of collagen from warm water species of fish needs further elaboration. The objective of the present study was to isolate and characterize collagen from the skin of two commercially important warm water species of fish, double-spotted queenfish (*Scomberoides lysan*) and Malabar

grouper (*Epinephelus malabaricus*) for better utilization of waste from fish processing industry.

Materials and Methods

All chemicals used were of analytical grade. Type 1 collagen from calf skin, pepsin from bovine gastric mucosa, high molecular weight markers and collagen hydrolysate were from Sigma Chemical Co. Sodium dodecyl sulphate (SDS), Coomassie brilliant blue R-250 & N,N,N',N'-tetra methyl ethylene diamine (TEMED) were procured from Bio-Rad laboratories.

Fresh skin of fishes, grouper and queenfish weighing 1.8 ± 0.87 kg and of total length 46 ± 3.5 cm and 4.6 ± 1.1 kg and of total length 76 ± 5.6 cm respectively were procured from local market near Cochin, Kerala. Skin were stored in ice with a skin/ice ratio of 1:2 (w/w) and transported within 1 h to the laboratory. The skin was washed with cold water (5-8°C) and cut into small pieces (2 ± 0.5 cm^2). The prepared skin samples were packed in polyethylene bags, added glaze water and kept at -20°C prior to collagen extraction.

The raw skin of these two species and their collagens (both acid soluble and pepsin digestible collagens) were subjected to proximate analysis, according to AOAC (2000).

Acid Soluble Collagen (ASC) and Pepsin Digestible Collagen (PDC) were extracted from queenfish skin and grouper skin. All procedures were performed as per Hema et al. (2013). All the extraction processes were carried out at 4°C. To remove non-collagenous proteins, the skin portions were mixed with ten volumes (v/w) of 0.1 M NaOH and stirred for 5 to 6 h. The sample was then washed thoroughly with excess distilled water until the pH was neutral or slightly basic. The residue was filtered using cheese cloth and actively stirred in five volumes (v/w) of 0.5 M acetic acid for 20 h to extract acid soluble collagen. The supernatant after centrifugation (3000 rpm, 20 min) was collected. The residue was once again extracted with acid as above and the combined supernatants were taken ASC. Residue from the previous step was homogenized with 30 volumes of 0.5M formic acid for 1 min and stirred for 24 h. A solution of pepsin having activity >250 units mg^{-1} (enzyme / tissue ratio 1:100) was added to this and stirred for another 24 h. The supernatant after centrifugation was taken as PDC. Crystalline sodium chloride was added to both supernatants to the level of 10% and stirred for 24 h to precipitate collagen. The precipitate was suspended in Tris-glycine buffer (50 mM containing 0.2 M NaCl, pH 7.4) and dialyzed against the same buffer for 24 h and then centrifuged. The collagen obtained was spray dried to get fine powder.

For amino acid analysis 100 mg dry collagen sample was weighed and hydrolyzed with 10 ml 6 N HCl at 110°C for 24 h. The filtered sample was injected to the amino acid analyzer (HPLC- LC 10 AS). The amino acid composition was determined as per the method of Ishida et al. (1981) using Model Hitachi L-2130 Elite La Chrome (Tokyo, Japan) amino acid analyser connected with cation exchange column (Shodex, CX Pak, 4.6×15mm). Electrophoretic patterns of the collagens were analyzed according to Laemmli (1970) by SDS PAGE.

All experiments were done in triplicates. Mean values with standard deviations (SD) were reported. Means were compared using t-test. The significant difference between means was computed at 5% level of significance using SAS 9.3

Results and Discussion

Table 1 shows proximate composition of skin of queenfish and grouper. The protein, fat and ash contents are higher for queenfish compared to grouper. Table 2 shows the proximate values of the collagen extracted from the skin of the two species. From the table it is clear that there is negligible

Table 1. Proximate composition of skin of grouper and queenfish

Sample	Moisture (%)	Protein (%)	Fat (%)	Ash (%)
Grouper skin	70.62 ± 1.05^a	20.78 ± 0.68^b	03.58 ± 0.36^b	02.05 ± 0.11^b
Queenfish skin	64.67 ± 0.40^b	22.43 ± 0.29^a	07.76 ± 0.71^a	04.04 ± 0.09^a

Values are given as mean ± SD. Values with the same superscript letters within a column are not significantly different ($p>0.05$)

Table 2. Proximate composition of collagen extracted from skin of grouper and queenfish

Sample	Moisture (%)	Protein (%)	Fat (%)	Ash (%)
Grouper skin collagen	06.46 ± 0.24[b]	91.07 ± 0.94[a]	00.45 ± 0.12[a]	00.54 ± 0.07[b]
Queenfish skin collagen	07.39 ± 0.14[a]	90.66 ± 0.92[a]	00.61 ± 0.06[a]	00.80 ± 0.06[a]

Values are given as mean ± SD. Values with the same superscript letters within a column are not significantly different ($p>0.05$)

amount of fat and ash contents in the extracted collagen. Table 3 compares the yield of collagen from the two species and the yield is high for grouper skin ($p>0.05$).

Amino acid composition of ASC and PDC extracted from grouper and queenfish skins is given in Table 4. The analysis detected the presence of 18 amino acids and it contained high percentage of glycine, followed by alanine, proline, hydroxyproline and

Table 3. Collagen yield from grouper skin and queenfish skin

Collagen type	ASC (%)	PDC (%)
Grouper skin collagen	12.5 ± 0.63[a]	6.49 ± 0.51[a]
Queenfish skin collagen	7.82 ± 0.70[b]	3.92 ± 0.11[b]

ASC: Acid soluble collagen; PDC: Pepsin digestible collagen
Values are given as mean ± SD. Values with the same super script letters within a column are not significantly different ($p>0.05$)

Table 4. Amino acid composition of acid soluble collagen and pepsin digestible collagen from grouper and queenfish skin (expressed as residues per 1000 total amino acid residues)

Amino Acids	Queenfish skin ASC	Queenfish skin PDC	Grouper skin ASC	Grouper skin PDC
Alanine	118 ± 0.11	130 ± 0.15	131 ± 0.45	109 ± 0.71
Arginine	46 ± 0.02	53 ± 0.53	54 ± 0.67	52 ± 0.66
Aspartic acid	41 ± 0.15	43 ± 0.14	42 ± 0.21	43 ± 0.14
Cysteine	-	-	-	-
Glutamic acid	74 ± 0.21	62 ± 0.10	62 ± 0.54	76 ± 0.44
Glycine	332 ± 0.20	328 ± 0.14	330 ± 0.71	315 ± 0.84
Histidine	9 ± 0.11	7 ± 0.5	7 ± 0.45	8 ± 0.42
Isoleucine	9 ± 0.00	8 ± 0.18	7 ± 0.59	21 ± 0.84
Leucine	18 ± 0.05	22 ± 0.22	21 ± 0.55	24 ± 0.65
Lysine	25 ± 0.09	24 ± 0.09	24 ± 0.23	26 ± 0.43
Hydroxy lysine	8 ± 0.07	6 ± 0.16	6 ± 0.42	8 ± 0.28
Methionine	11 ± 0.05	11 ± 0.20	11 ± 0.12	12 ± 0.54
Phenyl alanine	14 ± 0.15	18 ± 0.14	20 ± 0.67	15 ± 0.23
Hydroxy proline	78 ± 0.14	66 ± 0.17	68 ± 0.47	95 ± 0.75
Proline	99 ± 0.10	115 ± 0.22	117 ± 0.55	98 ± 0.43
Serine	43 ± 0.12	41 ± 0.10	41 ± 0.43	32 ± 0.91
Threonine	23 ± 0.05	22 ± 0.00	22 ± 0.14	23 ± 0.21
Tyrosine	2 ± 0.11	1 ± 0.15	1 ± 0.56	2 ± 0.45
Valine	28 ± 0.17	29 ± 0.08	29 ± 0.87	25 ± 0.19

ASC: Acid soluble collagen; PDC: Pepsin digestible collagen. Values are given as mean ± SD

glutamic acid. On the other hand, histidine and tyrosine were found to be least and cysteine completely absent in the collagens. The imino acid content (proline + hydroxyproline) of queenfish and grouper skin ASC and PDC was 177, 181, 185 and 193 per 1000 residues respectively. The values are comparable to most fish collagens such as grass carp skin collagen (186 residues/1000 residues) and big eye snapper skin collagen (193 residues/1000 residues) (Kittiphattanabawon et al., 2005; Zhang et al., 2007). The variation in imino acid content amongst different species is mostly due to changes in the habitat, particularly temperature.

SDS-PAGE patterns of collagens from the skin of queenfish and grouper are shown in Fig. 1. Collagen extracted from both the species shows similar protein patterns and it was found that the major constituents of both ASC and PDC consisted of α chains (α_1 α_2) and β chains. These patterns were similar to the type 1 collagen from calf skin (lane 6), and also in accordance with those of collagens from most other fish species previously reported (Muyonga et al., 2004; Nagai et al., 2001). Type I collagen consists of two identical α chains (Pearson & Young, 1989; Wong, 1989). Fish skin and bone have been reported to contain type I as the major collagen (Ciarlo et al., 1997; Kimura & Ohno, 1987; Montero et al., 1990; Nagai & Suzuki, 2000b). The skin collagens of bigeye snapper (Kittiphattanabawon et al., 2005), brown-banded bamboo shark (Kittiphattanabawon et al., 2010), Nile perch (Muyonga et al., 2004), ocellate puffer fish (Nagai et al., 2002), back drum seabream, sheepshead seabream (Ogawa et al., 2003), brown backed toadfish (Senaratne et al., 2006), Walleye Pollock (Yan et al., 2008), and large fin long barbel catfish (Zhang et al., 2009) consisted of two α chains (α_1 & α_2) and β components. No difference could be observed in the pattern of α_1 α_2 and β chains of the ASC and PDC of skin of the two fishes in the present study.

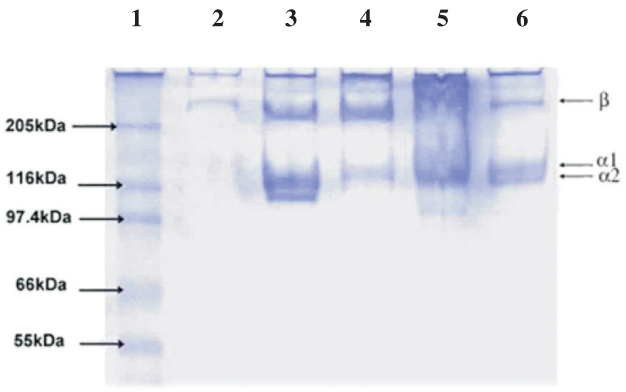


Fig. 1. SDS-PAGE pattern of extracted collagen

Lane 1. High molecular weight marker, lane 2. Queenfish skin ASC, lane 3. Queenfish skin PDC, lane 4.Grouper skin ASC lane 5. Grouper skin PDC, lane 6. Type 1 collagen from calf skin

Collagen yield from grouper skin was found to be high when compared to queenfish skin. All the extracted collagens showed composition typical of collagens. No differentiation could be observed in the collagens from the two species against standard bovine collagen indicating their type 1 nature. The amino acid pattern and SDS-PAGE of collagens extracted in the present study indicate that the process of extraction yielded pure collagen and extraction of collagen by digesting with pepsin increases the yield of total collagen from fish skin.

Acknowledgements

The financial assistance for the study from Department of Biotechnology is thankfully acknowledged. Acknowledgements are also due to Director, CIFT for providing facilities to carry out this study.

References

AOAC (2000) Official Methods of Analysis. 17th edn., Association of Official Analytical Chemists, Washington DC, USA

Bama, P., Vijayalakshimi, M., Jayasimman, R., Kalaichelvan, P.T., Deccaraman, M. and Sankaranarayanan, S. (2010) Extraction of collagen from cat fish (*Tachysurus maculatus*) by pepsin digestion and preparation and characterization of collagen chitosan sheet. Int J Pharm Pharm Sci. 2: 133-137

Bea, k., Osatomi, A., Yoshida, K., Osako, K., Yamaguchi, A. and Hara, K. (2008) Biochemical properties of acid solubilized collagens extracted from the skins of under-utilized fishes. Food Chem. 108: 49-54

Ciarlo, A.S., Paredi, M.E., and Fraga, A. N. (1997) Isolation of soluble collagen from hake skin (*Merluccius hubbsi*). J. Aquat. Food Prod.Technol. 6: 65–77

Cliche, S., Amiot, J., Avezard, C. and Gariepy, C. (2003) Extraction and characterization of collagen with or without telopeptides from chicken skin. Poultry Science. 82: 503-509

Hema, G.S., Shyni, K., Suseela Mathew, Anandan, R., George Ninan and Lakshmanan P.T. (2013) A simple method for isolation of fish skin collagen-biochemical characterization of skin collagen extracted from

Albacore Tuna (*Thunnus Alalunga*), dog shark (*Scoliodon sorrakowah*), and Rohu (*Labeo rohita*). Annals of Biol. Res. 4 (1): 271-278

Hwang, J.H., Misuta S., Yokoyama, Y. and oshinaka. R. (2007) Purification and characterization of molecular species of collagen from the skin of skate (*raja kenojei*) Food Chem. 100: 921-925

Ishida, Y., Fugita, T. and Asai, K. (1981) New detection and separatioin method for amino acid by high performance liquid chromatography. J Chromatogr. 204: 143-148

Jonjareonrak, A., Benjakul, S., Visessanguan, W., Nagai,T. and Tanaka, M. (2005) Isolation and characterization of acid and pepsin solubilized collagens from the skin of brown stripe red snapper (*lutjanus vitta*). Food Chem. 93: 475-484

Kimura, S. and Ohno, Y. (1987) Fish type I collagen: tissue-specific existence of two molecular forms in Alaska pollack. Comp. Biochem. Physiol., B: Comp. Biochem. 88: 409-413

Kittiphattanabawon, P., Benjakul, S., Visessanguan, W., Kishimura, H. and Shahidi, F. (2010) Isolation and characterisation of collagen from the skin of brownbanded bamboo shark (*Chiloscyllium punctatum*). Food Chem. 119: 1519-1526

Kittiphattanabawon, P., Benjakul, S., Visessanguan, W., Nagai, T. and Tanaka, M. (2005) Characterisation of acid-soluble collagen from skin and bone of bigeye snapper (*Priacanthus tayenus*). Food Chem. 89(3): 363–372

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

Liu, H., Li, D. and Guo, S. (2007) Studies on collagen from the skin of channel catfish (*Ictalurus punctatus*). Food Chem. 101: 621-625

Mizuta, S., Fujisawa, S., Nishimoto, M. and Yoshinaka, R. (2005) Biochemical and immunochemical detection of types I and V collagens in tiger puffer (*Takifugu rubripes*). Food Chem. 89: 373-377

Montero, P., Borderias, J., Turnay, J., and Leyzarbe, M. A. (1990) Characterization of hake and trout collagen. J. Agric. Food Chem. 38: 604–609

Muyonga, J. H., Cole, C.G.B. and Duodu, K.G. (2004) Characterization of acid soluble collagen from skins of young and adult Nile perch (*Lates niloticus*). Food Chem. 85: 81-89

Nagai, T. and Suzuki, N. (2000b) Preparation and characterization of several fish bone collagens. J. Food Biochem. 24: 427– 436

Nagai, T., Araki, Y. and Suzuki, N. (2002) Collagen of skin of ocellate puffer fish (*Takifugu rubripes*). Food. Chem. 78: 173–177

Ogawa, M., Moody, M.W., Portier, R.J., Bell, J., Schexnayder, M. and Losso, J.N. (2003) Biochemical properties of black drum and sheepshead seabream skin collagen. J. Agric. Food Chem. 51: 8088–8092

Pearson, A. M. and Young, R. B. (1989) Muscle and Meat Biochemistry, 457 p, San Diego: Academic Press Inc

Sadowska, M., Kololdziejska, I. and Niecikowska, C. (2003) Isolation of collagen from the skin of Baltic cod (*Gadus morhua*). Food Chem 81: 257-262

Quereshi, S., Mhaske, A., Raut, D., Singh, R., Mani, A. and Patel, J. (2010) Extraction and partial characterization of collagen from different animal skins. Recent Res. Sci. Technol. 2: 28-31

Senaratne, L.S., Park, P.J. and Kim, S.K. (2006) Isolation and characterization of collagen from brown backed toadfish (*Lagocephalus gloveri*) skin. Bioresour. Technol. 97: 191-197

Wong, D.W.S. (1989). Mechanism and Theory in Food Chemistry, 327 p, New York: Van Nostrand Reinhold Company Inc

Wang, L., An, X., Xin, Z., Zhao, L. and Hou, Q. (2007) Isolation and characterization of collagen from the skin of deep sea red fish (*Sebastes mentella*). J.Food Sci. 72: 450-455

Yan, M., Li, B., Zhao, X., Ren, G., Zhuang, Y. and Hou, H., (2008) Characterization of acid-soluble collagen from the skin of walleye pollock (*Theragra chalcogramma*). Food Chem. 107(4): 1581-1586

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 (*Ctenopharyn godonidella*) Food Chem. 103: 906-912

Zhang, M., Liu, W., and Li, G. (2009) Isolation and characterization of collagens from the skin of large fin long barbel catfish (*Mystus macropterus*). Food Chem. 115: 826–831