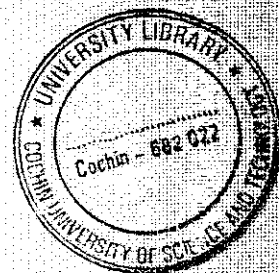# EFFECTS OF THERMAL MODIFICATIONS ON THE RHEOLOGICAL CHARACTERISTICS AND PROTEIN QUALITY OF THREE SPECIES OF FISH OF VARYING COLLAGEN CONTENT

Thesis submited to

Cochin University of Science and Technology

In partial fulfilment of the requirements for the degree of

Doctor of Philosophy

By

MAYA RAMAN

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

October 2005

Om

Sri Sai Ram. . . .

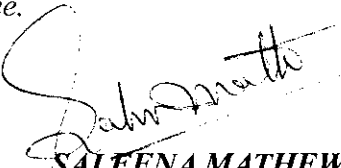*Dedicated to*

My Teacher & My Parents

Prof. (Dr.) Saleena Mathew
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Certificate

This is to certify that this thesis entitled *"Effects of Thermal Modifications on the Rheological Characteristics and Protein Quality of Three Species of Fish of Varying Collagen Content"* is an authentic record of research work carried out by Ms. Maya Raman under my supervision and guidance in the School of Industrial Fisheries, Cochin University of Science and Technology, in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** of the Cochin University of Science and Technology and no part thereof has been submitted before for any other degree.

Cochin – 16.
18.10.2005.

SALEENA MATHEW
(Supervising Teacher)

Declaration

This is to certify that this thesis entitled _"Effects of Thermal Modifications on the Rheological Characteristics and Protein Quality of Three Species of Fish of Varying Collagen Content"_ is a bonafide record of research carried out by me under the supervision and guidance of Prof. (Dr.) Saleena Mathew, School of Industrial Fisheries, Cochin University of Science and Technology, in partial fulfillment of the requirements for the Ph. D degree of Cochin University of Science and Technology and that no part of it has previously formed the basis for award of any degree, diploma, associateship, fellowship or other similar recognition in any University or Institution.

Cochin – 16.
October, 2005

Maya Raman

Acknowledgement

Maya Raman

Contents   Page no.

CHAPTER 1
INTRODUCTION

Chapter 1

INTRODUCTION

1.1. General

1.2. Morphology

1.2.1. Rohu (*Labeo rohita*, Hamilton)

1.2.2. Squid (*Loligo duvaucelli*, Orbigny)

1.2.3. Shark (*Scoliodon sorrokawah*)

1.3. Background of work

1.4. Objectives of the study

1.1. GENERAL

Proteins occupy a unique status among the constituents of biological matter by virtue of their relatively large size, complex, multifaceted and multi reactive structure. These properties give them a superb characteristic for functioning in biological systems in many different ways. But these characteristics also make proteins susceptible to environmental stress. Proteins are also easily attacked by variety of chemicals and are subjected to many deteriorative reactions during processing. Apart from contributing to the nutritional quality, proteins along with other biological constituents also contribute greatly to the textural properties.

Fish muscle is an important food item for man in view of its high protein content, vitamins and polyunsaturated fatty acids. World fish productions from capture fisheries are more or less stagnant at around 100 million tones. For feeding the population at the present level of consumption it is estimated that by 2020 A. D. another 20 – 30 million tones of fish has to be added to the present availability. Another problem of serious threat is that on an average around 20% of the landed fish is wasted due to various reasons. The fishery wealth of the vast expanse of the Exclusive Economic Zone of India remains practically untapped and under – utilized. The augmentation of the present level of catch by adopting need-based technological and infrastructure inputs being the need on one side, the optimum utilization of the landed catch by developing appropriate technologies for their preservation and processing is another priority of equal importance.

Fish muscle proteins depending on their anatomical location and activity, exhibit structural differences that lead to different functional properties and processing abilities. Both intrinsic and extrinsic factors of muscle tissue affect the rheological characteristics. Myofibrillar protein and collagen that comprise 70 – 80% of the total protein content control the structure and the specific rheological properties of the muscle tissue. Post mortem textural changes are caused directly and indirectly by physiochemical changes in the myofibrillar proteins and the collagen of the extra cellular spaces between the fibers. Several scientists have already studied the role of myofibrillar protein in texture. But information regarding the distribution and functional properties of collagen in effecting the textural

properties of fish and fishery products are scarce. Texture is also affected by the structural components of muscle tissue, their pattern of arrangement and the processing techniques employed. Time – temperature profile also plays a significant role in affecting the rheological characteristics of the muscle tissue.

Rohu (*Labeo rohia*), squid (*Loligo duvaucelli*) and shark (*Scoliodon sorrokawah*) belong to three diverse classes. Rohu is a fresh water carp, Squid belongs to cephalopods (molluscs) and Shark is an elasmobranch. The research work carried out earlier suggests that these three species differ in their collagen content and on that basis these three species are selected for the present study.

1.2. MORPHOLOGY

1.2.1. Rohu *(Labeo rohita*, Hamilton) – Plate 1.1

Rohu (*Labeo rohita*) is a freshwater carp and is highly commercial. It is a hot favorite and is commonly preferred in Southeast Asia. And in India it is commonly found in the, Maharastra, Kerala, Meenachil, Manimala, Chilka Lake and Pampa rivers. The fish is commonly distributed in Pakistan, India, Bangladesh, Myanmar and Nepal.

It grows to a maximum size of 200 cm in length and 45 kg in weight with a life span of 10 yrs. The upper body has dark scales. The lower body and the belly is golden brown. Dorsal fin and tail are dark brown in color. The pelvic, pectoral and anal fins have a red tint. The dorsal fin has 12 to 13 branched rays. The body is more linear than Catla. Rohu is considered as tastiest of the Indian carps. Its relatively small pointed head, almost terminal mouth with fringed lower lip and dull reddish scales on the sides easily distinguish it. It grows quickly. The bigger the size of the fish the tastier it is. It is procured, processed and exported to the global market as both whole gutted and also as block frozen steaks.

All landings of this Indian major carp are from aquaculture. Almost all of the rohu production comes from India but in recent years a growing, albeit small proportion is cultured in Myanmar, Thailand and Laos. Currently rohu is the most widely cultured species outside India and Myanmar is the largest non – Indian producer.

Plate 1.1 - *Labeo rohita* (Rohu)

1.2.2. Squid (*Loligo duvaucelli*, Orbigny) – Plate 1.2

Squid is a commercially important cephalopod. They are exclusively marine molluscs and there are about 660 species in the world ocean, of which less than 100 species are commercially important. They are diverse in form, size and nature. In India, cephalopod fishery are found in Bombay, Cochin, Mangalore, Rameshwaram, Mandapam, Kilkarai, Madras, Kakinada, Waltair, Portnova and Vizhinam. It is mostly preferred as raw and in processed forms in Japan and other European Countries. Recently their contribution to the export market has shown an increase from the past. Export of frozen squid in 2004 – 05 has registered a substantial growth of 27.21% in quantity, 27.98% in rupee value and 31.64% in US$ terms. There was also a marginal increase in unit value realization.

Squid has practically a cone shaped sack consisting of several layers of tissue that envelop the organs. The head with 8 – 10 tentacles sprouting around the mouth is loosely attached to the body. There are no bones to support the muscle. Squid refer to those cephalopods having ten circum oral arms, eight of which are short and two slender and tentacular. The suckers of the arms and tentacles are stalked and equipped with armature. The tentacles are slender and long with expanded clubs. Club suckers are arranged in four rows. The suckers on the manus of the club are the largest and the median ones being more enlarged than the marginal ones. The large manus sucker bear about 14 – 17 pointed teeth on the rings. Squid has a chitinous internal shell. The mantle is cylindrically elongate and tubular with almost parallel sides up to the point where fins originate, then tapers to a blunt posterior point. The mid dorsal projection of the anterior margin of mantle is rounded. The fins are either terminal or marginal in position and unite at the apex of the mantle. The fins are small and short and occupy about 50 – 55% of mantle length being ad rhombic in outline. They are broadest near the middle, the anterior margin is nearly straight or slightly convex and the posterior margin is concave. In fresh condition immediately upon capture the squid is colorless and mantle is transparent showing the internal visceral organs. There are numerous light brown chromatophores scattered all over the mantle, fins, head and arms. On the ventral side chromatophores are less dense and appear whitish.

Plate 1.2 - *Loligo duvaucelli* (Squid)

1.2.3. Shark (*Scoliodon sorrokawah*) – Plate 1.3

The annual production of elasmobranchs in India is around 70,000 tones, over 4% of total marine fish landings. Sharks account for between 60 and 70% of this. Tamil Nadu, Gujarat, Maharashtra, Kerala, Karnataka and Andra Pradesh supply around 85% of the shark landings in India. 65 species of shark have been sighted in Indian waters and over 20 of these contribute to the fishery. In India the present annual shark production is around 45,500 tones obtained as a by – catch from a variety of gears. Despite the commercial importance, no serious attempts have so far been made at any targeted exploitation of this valuable resource. Information on the composition of the species of shark landings is very scarce apart from the gross catch statistics.

The sharks are generally large, long and very powerful fish. It is viviparous. Foremost part of the shark is head and comprises of skull and face. First dorsal fin is the first locomotive limb on the back of the body. Second dorsal fin is the rear locomotive limb on the back. The caudal fin is the locomotive limb at the end. Locomotive limb beneath the pelvic girdle is the pelvic fin. Belly is located at the lower part of the trunk of the body. Pectoral fin is the locomotive limb on the chest. Caudal keel is a flattened area that increases forward thrust and speed of swimming. There are 5 to 7 gill openings on either side of the body near to pectoral fin. Mouth is located at the anterior end. Sharks do not have swim bladders. They have large oil – rich livers. The intestines of shark are very compact and take the form of a spiral. Snout is very sensitive and has the nostrils that are extremely sensitive. The snout is also dotted with the *ampullae of lorenzi* organs that can detect electrical signals in the water. Eyes located on either side of the head have a large number of rods that gives them good night vision. In addition to this *tapetum lucidum* in the eyes that lies beneath the retina help in reflecting the light back through the retina thus increasing the light received by the receptive cells.

The fins of sharks are highly valued for the shark fin soup industry, and it is estimated that more than 100 million sharks are killed every year with the trade being driven by the demand for shark fins.

Plate 1.3 - *Scoliodon sorrokawah* (Shark)
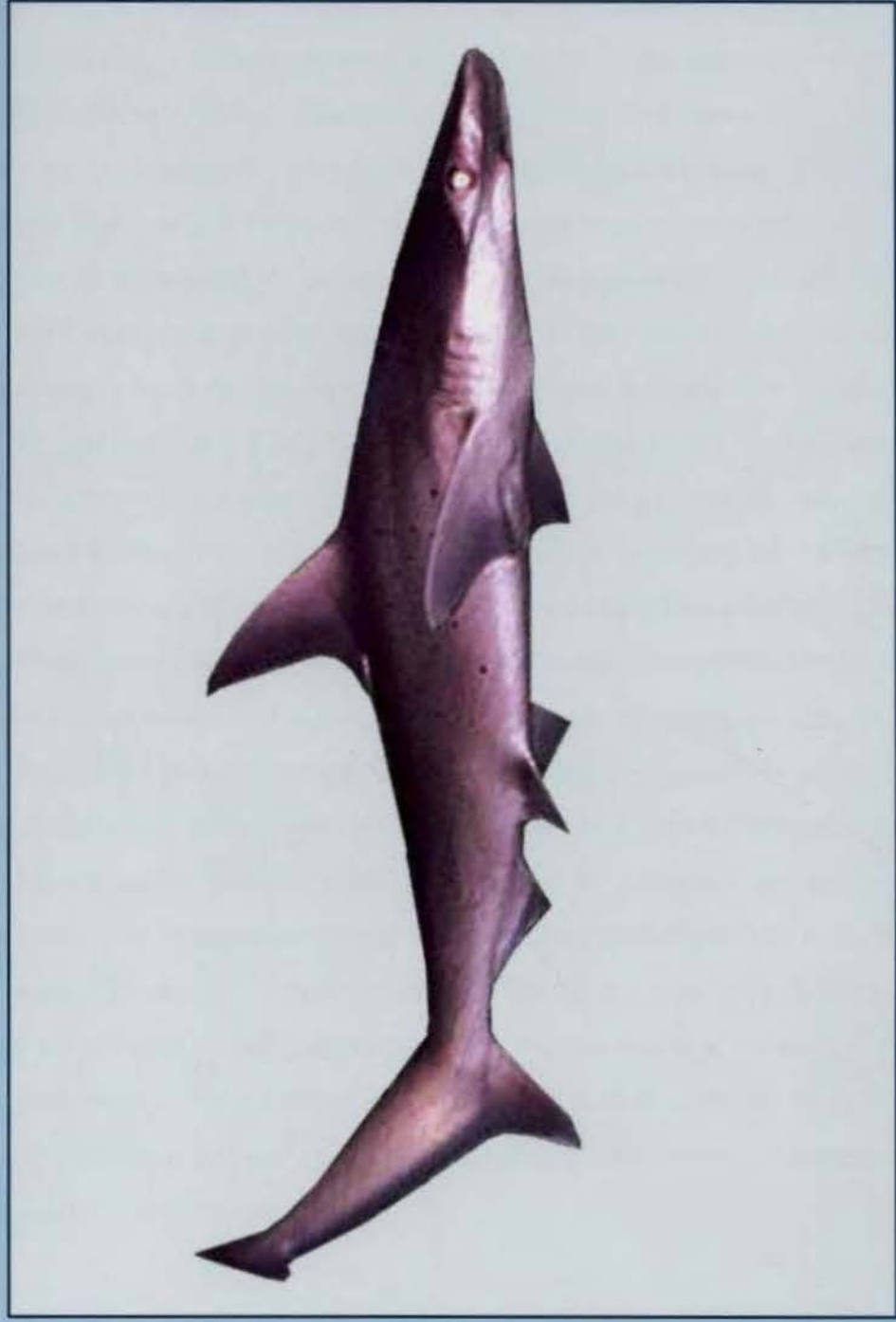
1.3. BACKGROUND OF THE WORK

Collagen is an extracellular matrix protein and is an essential component of muscle connective tissue in multicellular animals. The word collagen has been derived from the Greek words *"kolla"* and *"genos"* meaning *"glue"* and *"formation"* respectively. The basic unit of collagen, tropocollagen occurs in three polypeptide units (α chain) which together form a triple helical structure. On hydrolysis, the collagen is converted to gelatin that finds great commercial importance. In the muscle tissue, the myocommata covering muscle segments and the basement membrane covering muscle fibers are made up of connective tissue and collagen forms its major part. In fish, the largest concentration of collagen is found in the skeleton, fins, skin and air bladder. Mechanical strength, integrity and rheological properties of the muscle tissue, water holding and gel forming capacity of the fish and cooked fishery products and the flow properties of the fishery by – products are some of the attributes of fish protein where collagen has a major functional role. Collagen in fish contributes about one – tenth of total proteins or less. Fish collagen has high biological value, high essential amino acid content and lower content of hydroxyproline. Collagen has great significance in the fish muscle. Belly bursting and gaping are two characteristics found in fish and fishery products that have direct impact of collagen. Collagen is of great technological importance in that it holds fillet together. Recently collagen has received increased attention as an important protein component contributing greatly to the texture of raw material. Tenderization and thermal stability of the muscle products and the toughening are two dual purposes, dependent upon the conversion of collagen to gelatin and heat coagulation of the myofibrillar proteins. Hence, it is necessary to investigate more about the content and properties of collagen so as to optimize the processing conditions for production of quality fish products.

1.4. OBJECTIVES OF THE STUDY

- ✍ To study the physical and biochemical composition of Rohu (*Labeo rohita*), Squid (*Loligo duvaucelli*) and Shark (*Scoliodon sorrokawah*) highlighting their nutritional importance.

- ✍ To study the changes in the protein fractions during frozen storage and heat setting at different temperatures.

- ✍ To characterize the protein fractions using SDS – polyacrylamide gel electrophoresis.

- ✍ To study instrumentally and organoleptically the textural parameters of the meat and correlate these parameters with collagen content.

- ✍ To study the musculature differences in the three species by histochemical analysis during cooking of samples kept under frozen storage.

- ✍ To analyze and characterize the collagen of the squid skin, tentacle and mantle.

- ✍ To analyze and characterize the glycoproteins of the squid skin, tentacle and mantle.

CHAPTER 2

CHEMICAL AND PHYSICAL PARAMETERS

Chapter 2

CHEMICAL AND PHYSICAL PARAMETERS

2.1. Introduction

2.2. Review of literature

> **2.2.1. Proximate composition**

> **2.2.2. Physico – chemical parameters**

> **2.2.3. Freezing and cooking of muscle tissue**

2.3. Materials and Methods

> **2.3.1. Raw material collection**

> **2.3.2. Sample preparation**

> **2.3.3. Analysis of biochemical parameters**

>> **2.3.3.1. Proximate composition**

>>> **2.3.3.1.1. Determination of moisture**

>>> **2.3.3.1.2. Determination of crude protein**

>>> **2.3.3.1.3. Determination of crude lipid**

>>> **2.3.3.1.4. Determination of ash**

> **2.3.4. Analysis of physical parameters**

>> **2.3.4.1. Determination of pH**

>> **2.3.4.2. Determination of Expressible moisture (EM)**

>> **2.3.4.3. Determination of Water Binding Potential (WBP)**

> **2.3.5. Statistical analysis**

2.4. Results

> **2.4.1. Proximate composition**

> **2.4.2. Physico – chemical parameters**

2.5. Discussion

2.6. Conclusion

2.1. INTRODUCTION

Fish is a highly perishable commodity and deteriorates quite fast compared to other muscle meat if not handled properly. The quality of fish depends on their physiological composition, geographical distribution, season, sex, age, maturity and feeding habits. Fish undergoes different stages of post mortem changes from the point of catch to processing. One of the prime factors other than microbiological and biochemical degradation that decisively affect the quality of fish is the proteolytic degradation of the muscle tissue due to the action of enzymes that vary with the type of fish, part of the fish body, temperature and pH. Today there is an ever – increasing awareness about physicochemical aspects of fish as health food because of its nutritional and functional qualities. The thrust area where accurate information on the biochemical composition of fish is a must is in processing and preservation of fish and fishery products. Hence, information on the biochemical composition is helpful in defining the optimum processing and storage conditions in order to preserve the nutritional quality to the maximum and enhance the shelf life of the product. The selection of the species for the study is chiefly based on the variations in their stroma protein (collagen) content.

The present study attempts to investigate the following:

1. To analyze the proximate composition of fresh and frozen samples of rohu (Labeo rohita), squid (Loligo duvaucelli) and shark (Scoliodon sorrokawah) subjected to cooking at different temperatures.

2. To analyze the effect of cooking at various temperatures on pH, expressible moisture (EM) and water binding potential (WBP) of fresh and frozen samples of rohu, squid and shark.

2.2. REVIEW OF LITERATURE

2.2.1. Proximate composition

Analyses of four basic constituents in the edible portion of a fish namely, water, protein, lipid and ash are referred to as proximate analysis (Love, 1988). Biochemical

composition of a species varies widely with environmental conditions, temperature, salinity, pressure and availability of food. Variations in the biochemical compositions are group – specific and species – specific. Within a species, variations could occur for individual fish or with lots of fish taken at different times or under different conditions (Standsby, 1976). Dyer et al. (1977) has reported the proximate composition of frozen fish fillets and steaks. Paul and Southgate (1978) have done work on biochemical composition of raw and cooked fish. The proximate composition of fresh and frozen fish species of fresh water and marine water were reported by numerous scientists (Govindan, 1985; Gopakumar, 1997; Hassan and Mathew, 1999; Chand et al., 2001; Panchavarnam et al., 2003; Smuruthi et al., 2003 and Arannilewa et al., 2005). Lakshmanan and Balachandran (2000) studied and compared the biochemical characteristics of squid and cuttlefish along the Kerala coast. Nair and Mathew (2000) summarized the four major constituents of various species of fish along the Indian Coast. The four components account for 96 – 98% of total tissue components in most cases, of which water contributes 65 – 90%, protein 10 – 22%, fat 1 – 20% and minerals 0.5 – 5% in fish muscle. Carbohydrates, vitamins, nucleotides, other non – nitrogenous compounds account for a minor portion of the chemcial composition. However, these are vital for the growth and development of the organs and maintenance of the system. High content of lipid and vitamin in dark muscle deduced the assumption that the muscle could act as a supplement to liver in transferring metabolites to the adjacent white muscle (Wittenberger, 1972).

Hatagoshi (1938) presented the results of the analysis of mantle (without epidermis) in four types of squid. Takahashi (1965) found that liver, kidney and gonads that constitute about 13 to 20% of body weight of squid is comprised of 50% water, 15% crude protein, 35% crude fat and about 1% ash. Epidermis of the mantle of squid (Todarodes pacificus) was found to contain 82 to 84% water, 1.6 to 2.1% total nitrogen, 2.0 to 3.0% crude fat and 1.5 to 1.8% ashes. Sugiyama et al. (1980) did a similar kind of analysis in squid (Todarodes pacificus) with epidermis and reported that water fraction contributed approximately 75 – 80%, crude protein 16 – 21%, crude fat 1% and ash 1 – 2%. The spawning and seasonal variations were observed to influence the water content and protein content of the tissue. Water content was higher in samples before spawning and was comparatively less after

spawning. Similar was the case with protein that behaved inversely being lower before spawning and higher after spawning.

2.2.2. Physico – chemical parameters

Accumulation of lactic acid in the muscle tissue due to the catabolic degradation of glucose molecules lead to lowering of pH from near neutrality to the acidic range (pH 6.0). Lean meat contained 75% water, a small amount of which is tightly bound to the muscle proteins and the remainder being loosely bound and immobilized to varying extent due to different forces acting upon them (Hamm, 1960). The physical and chemical environment of meat protein influences the loosely bound water and under certain conditions part of water may exude from tissue in the form of drip. About 4 to 5% of tissue water is directly bound to the polar surface of protein molecule viz., carboxyl and amino acid sulphydryl group (Hamm, 1960 and Hamm and Deatherage, 1960). The pH of protein also affects the water holding capacity of the tissue. Fish muscle has lowest water holding capacity at low pH. The amount of water released from the sample was found to be influenced by the inherent water holding capacity and by the extent of micro structural damage that is a function of the textural strength of the sample (Hermansson, 1982). A decrease in pH during post mortem storage is yet another contributing factor to the loss of water from tissue (Hamm, 1966). He reported that $2/3^{rd}$ loss of the water holding capacity of the tissue could be due to the breakdown of ATP molecules and $1/3^{rd}$ could be due to the decrease in pH level. Loss of water holding capacity of tissue is associated with the decrease in the solubility of the structural proteins.

The pH and water holding capacity of the muscle tissue are of great importance in governing the textural quality of the cooked fish. Fish stored in frozen state gradually toughen at a rate that varies with species (Love and Olley, 1964). With the decline of pH the quality of fish tissue is affected in that, flesh becomes firmer and tends to drip loss considerably (Connell, 1990). The pH of flesh exerts a great influence on the tensile strength of the connective tissue. Around pH 5.0 – 5.1 repulsion between proteins was observed to be minimal and consequently the water holding capacity was high (Wismer – Pederson, 1978). Suzuki (1981) has carried out studies on fish protein gelation at near neutral pH

conditions. Gel formation ability of the muscle tissue is also affected by salt and protein concentration (O'Riordan et al., 1989). Hermansson and Lucisano (1982) indicated that gel strength and water holding capacity increased with pH. However, Hermansson (1982) reported that gel strength varied with pH values in different species. The mechanism of gel formation and the nature of the molecular interaction involved in the gelation of plasma proteins were extensively studied in fish (Shimadu and Matsushita, 1981 and Yasuda et al., 1980). Several scientists (Wariss and Brown, 1987; Van Laack et al., 1994 and Warner, 1994) have also studied the relationship between pH and drip loss in various fish species. In the mantle of squids (Ommastrephus sloani pacificus and Loligo pealei) autolytic activity was observed to be higher at lower pH with activity dropping appreciably in the neutral and alkaline range (Sakai and Matsumoto, 1981 and Leblanc and Gill, 1984). Shin – Bin – Lin et al. (2005) reported that the pH and water holding capacity are interrelated.

2.2.3. Freezing and cooking of muscle tissue

Freezing is one of the mostly adopted preservation and processing technique used in increasing the shelf life of fish and to maintain the nutritional quality of fish and fishery products. Freezing and frozen storage affects the textural and biochemical composition of the product. Extensive literature has been published on the effects of freezing and chilling on selected quality parameters of fish (Gormley, 1999; Magnusson and Martinsdottir, 1995 and Refsgaard et al., 1998). Studies have been carried out on the quality characteristics of frozen squid and cuttlefish (Joseph et al., 1985 and Moral et al., 1983). The effects of freezing on foodstuff are also well documented (Bailey, 1986), as are the effects of chilling (Howgate, 1987 and Huss, 1995). Freezing and chilling offers a logistic advantage in preparation and processing of fish and fishery products (O'Leary et al., 2000).

As meat is usually cooked before being eaten it is important to understand the physical changes of meat texture during heating. Davey and Gilbert (1976) defined cooking as the heating of meat to a sufficiently high temperature to denature proteins. Heating of meat is frequently done to destroy some or all of the micro – organisms. Heating could also bring about irreversible changes in several of the components of the foods such as proteins, fat and carbohydrate. Thermal processed foods are known to undergo degradation

in color, flavor, texture and nutritive value (Fenemma, 1985). Hence, temperature and duration of cooking are known to have a profound effect on the physical and nutritional properties of muscle tissue and eating quality.

2.3. MATERIALS AND METHODS

2.3.1. Raw material collection

Rohu (Labeo rohita) was collected live from the freshwater fish culture farms at Thiruvairanikulam, Cochin. They were immediately stored in ice and transported to the laboratory within 2 h of catch for further processing and analyses. Squid (Loligo duvaucelli) and shark (Scoliodon sorrokawah) were captured along the southwest coast of India and the samples from the last haul of catch were selected for the study. After catch they were immediately stored in ice and were brought to the laboratory within 8 h of harvest for further processing. Squid was iced without direct contact with ice by separating the material with a sheet of polyethylene.

2.3.2. Sample preparation

The samples brought to the laboratory under chilled conditions were skinned, eviscerated, cleaned and filleted. In the case of squid, the mantle after evisceration and deskinning was used for the study. All the three species were processed as follows:

1. Fresh samples of the three species – Three samples from each species were used for further study.
2. Frozen – stored samples of the three species – The fillets of rohu and shark and mantle meat of squid were individually quick frozen for at – 40°C using Tunnel freezer. These were packed in polythene bags (high molecular High Density Polyethylene) and stored in 5 ply master cartons in cold store at – 18°C.

Random sampling of these frozen stored lots of rohu, squid and shark were done after 3rd month and 6th month of storage. At each point of sampling three samples were drawn from each species for further study. The frozen samples were thawed under ideal conditions (Jason, 2001).

The fresh and the thawed samples of three species were subjected to cooking as detailed below:

The samples were sliced into small cubical pieces of 2cm^3 and wrapped in aluminum foil (Paul et al., 1966) and cooked at different temperatures ranging from 45°C to 90°C for 1 min after the core temperature reached the required temperature. Cooking time was standardized through repeated experiments and finally, the 1 min cooking time was considered as optimum for the sample size of 2 cm^3. As the samples were of smaller size the time required for the heat to penetrate to the core of the samples was less. Weighed quantities of these samples after cooking at different temperatures were taken for different analysis and calculations were done on these weights taken. Raw sample of the three species (without cooking) were also taken for analyses.

The samples were further analyzed for various physical and biochemical parameters. The analyses were done on raw and cooked weight basis. The studies were performed in triplicates for each sample.

2.3.3. Proximate composition

2.3.3.1. Determination of Moisture

The moisture content was estimated by the method of AOAC (1995). The moisture content was determined by drying 10 g sample at 103°C in a thermostatically controlled hot air oven. The samples were taken in a pre – weighed glass dish with cover and kept in oven till the weight became constant. The weight was checked for constant weight by repeatedly heating and then cooling the sample in desiccators. The percentage solid was determined from the above experiment by using the formula

Percentage solid = Weight of dry sample/ Weight of wet sample x 100

The percentage moisture was calculated by subtracting solid weight % from 100.

2.3.3.2. Determination of Crude Protein

1 g of homogenized sample was used for determining the crude protein content using Micro Kjeldahl method by AOAC (1995). About 1g of the sample was accurately

weighed into a digestion tube. About 2 g of digestion mixture ($CuSO_4$ and K_2SO_4 as catalyst in the ratio of 1: 8) and 10 ml of concentrated H_2SO_4 (AR) were added to the sample taken in the digestion tube. The samples were digested to a clear solution in a KEL PLUS KES 12 digestion unit. 50 ml of distilled water was added to the cooled tube slowly till no heat was generated on adding water. The solution was made up to 100 ml. Pipetted out 5 ml of the prepared sample into the Kjeldahl Micro Distillation Apparatus. The bottom end of the condenser was fitted to a delivery tube immersed in 10 ml of 2% boric acid with added Tachiro's indicator. 40% NaOH was added to the sample in the distillation unit to make it alkaline. The ammonia thus produced on steam distillation was absorbed into the boric acid solution. The distillate collected was back titrated against N/70 H_2SO_4 and using the titer value nitrogen content was estimated. Crude protein content in the sample was calculated by multiplying the nitrogen content by the factor of 6.25.

Percentage of Protein = V x 1 x 100 x 100 x 6.25/ 5 x 5 x Weight of the sample

2.3.3.3. Determination of Crude Lipid

Fat content of the moisture free sample was determined by extracting the fat by using a suitable solvent by soxhlet method (AOAC, 1995). About 2 g of the 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 a water condenser. Petroleum ether (B. P. 40 – 60°C) was used as solvent. The unit was heated in a water bath and temperature was maintained at 40°C – 60°C so that solvent boiled continuously and siphoned at a rate of 5 – 6 times/ h. The extraction was continued till the solvent in the extractor became colorless and fat free. The solvent in the receiving flask was evaporated completely and weighed for fat content.

Percentage of Crude Lipid = Weight of fat/ Weight of sample x 100

2.3.3.4. Determination of Ash

The ash content was measured by the incineration of the sample according to AOAC (1995). 2 g of moisture free sample taken in a pre – weighed clean dry silica crucible was

charred on low heat. Then it was kept at 550°C in the muffle furnace to get a white ash that was cooled in the desiccators and weighed.

Percentage of Ash = Weight of ash/ Weight of the sample x 100

2.3.4. Analyses of physical parameters

2.3.4.1. Determination of pH

The pH of the samples from each treatment was determined at room temperature subsequent to cooling from the specific cook temperature. A digital pH meter Cyber Scan pH – 500 (MERCK) was calibrated with standard buffers of pH 4.0, pH 7.0 and pH 9.0 (SIGMA). 10 g of sample after the treatment was blended for 30 sec with 90 ml of distilled water and the pH of the resultant suspension was measured (AOAC, 1995).

2.3.4.2. Determination of Expressible Moisture (EM)

1 g of meat placed between two pre – weighed clean dry filter papers was pressed under a fixed pressure of 10 kg/cm² for 10 sec (Regenstein and Regenstein, 1984). The weight difference compared to the weight before pressing, in percentage, reflected the expressible moisture (EM).

2.3.4.3. Determination of Water Binding Potential (WBP)

To about 50 ml capacity polycarbonate centrifuge tube 1 g sample was weighed and the weight of the tube plus sample was noted (initial weight). To this, 30 ml of distilled water was added and homogenized and dispersed with a small magnet on magnetic stirrer (2 ML Magnetic Stirrer, Remi). The centrifuge tubes were heated for 30min at 60°C, followed by cooling in tap water for 30 min. Then samples were centrifuged at 3000 rpm for 10 min at 25°C and decanted the supernatant. The weight of the tube with the residue was noted (final weight). From the difference in the final and initial weights of the centrifuge tube the water held per gram sample was calculated (Borresen, 1980).

2.3.5. Statistical analysis

The experimental design was a randomized block design of 10 x 3 with ten temperatures, three months and three species. Analysis of variance (ANOVA) was carried out using the generalized linear model procedure. The difference of means between pairs was resolved by using the least significant difference. The level of significance was set at $p < 0.01$ and $p < 0.05$ (Snedecor and Cochran, 1989).

2.4. RESULTS

2.4.1. Proximate composition

Tables 2.1 – 2.4 show the moisture, crude protein, crude fat and ash content of fresh and frozen samples of rohu, squid and shark subjected to cooking at different temperatures ranging from 45°C to 90°C.

Table 2.1. The percentage retention of moisture in fresh and frozen samples of rohu, squid and shark cooked at different temperatures

| Temperature (°C) | Rohu | | | Squid | | | Shark | | |
| | | | | Months of frozen storage | | | | | |
	0	3	6	0	3	6	0	3	6
raw	72.46	72.46	72.46	74.55	75.53	70.97	72.46	72.46	75.53
45	71.96	71.96	75.69	76.54	75.36	70.73	71.96	75.69	75.36
50	75.94	75.94	79.74	72.73	77.78	75	75.94	79.74	77.78
55	69.03	69.03	74.38	79.49	74.36	75.76	69.03	74.38	74.36
60	79.78	79.78	74.17	73.21	72.96	76.92	79.78	74.17	72.96
65	75.48	75.48	79.19	77.78	73.85	73.33	75.48	79.19	73.85
70	75.46	75.46	73.41	76.6	72.34	71.88	75.46	73.41	72.34
75	79.26	79.26	74.77	76.6	69.57	74.53	79.26	74.77	69.57
80	79.69	79.69	76.62	80	76.32	77.42	79.69	76.62	76.32
90	76.17	76.17	76.51	76.39	71.62	80.48	76.17	76.51	71.62

0 - Fresh, 3 – Three months, 6 – Six months, n - 5.

The moisture content of the three species ranged from 70% to 80% (Table 2.1). Statistical analyses of moisture content using ANOVA showed a high significant difference

with temperature of cooking (p < 0.01). According to the least significant difference, higher significant difference (p < 0.01) were observed between uncooked and 80°C followed by those between 55°C and 80°C, 45°C and 80°C, 70°C and 80°C, uncooked and 50°C and 50°C and 55°C (Appendix 2.1).

Table 2.2. Crude protein content (%) of fresh and frozen samples of rohu, squid and shark cooked at different temperatures

Temperature (°C)	Rohu			Squid Months of frozen storage			Shark		
	0	*3*	*6*	*0*	*3*	*6*	*0*	*3*	*6*
raw	**17.73**	16.22	17.73	**22.17**	17.55	17.5	**22.01**	19.01	18.01
45	17.9	17.13	16.9	19.25	17.33	17	22.11	18.1	18.11
50	16.68	15.83	16.68	19.5	17	17	20.2	19.2	17.2
55	17.45	17.9	15.65	20.42	17.91	17.13	20.64	18.64	17.64
60	18	17.46	16	20.42	17.63	17.2	23.42	18.44	17.42
65	18.9	17.5	17.9	20.42	17.72	17.44	20.7	18.7	17.7
70	17.5	17.81	17.5	20.44	17.67	17.13	20.37	18.37	16.37
75	17.18	17.28	17.18	20.42	17.88	17.67	21.57	17.57	16.57
80	18	17.85	16	22.17	17.5	17.5	22.15	17.15	17.15
90	17.75	16.28	16.75	20.42	17.14	17.13	20.35	18.35	16.35

0 – Fresh, 3 – Three months, 6 – Six months, n = 5.

The highest protein content was recorded for fresh uncooked samples while the least was recorded for fish samples that were frozen stored for six months (Table 2.2). Squid and shark showed high crude protein content (~ 22%). The crude protein content of shark includes non – protein fraction, of which urea contributes mostly. With frozen storage there was a slight variation in the protein content possibly due to the denaturation and leaching of the proteins resulting from cell distortion. Protein content varied significantly with species (p < 0.01) and also with period of frozen storage (p < 0.01). Statistical analysis of means using least significant square difference showed significant variation between the crude proteins content of rohu, squid and shark. Fresh samples showed high significant variation (p < 0.01) compared to frozen samples (Appendix 2.2).

Table 2.3. Crude fat content (%) of fresh and frozen samples of rohu, squid and shark cooked at different temperatures

Temperature (°C)	Rohu			Squid Months of frozen storage			Shark		
	0	*3*	*6*	*0*	*3*	*6*	*0*	*3*	*6*
raw	0.82	0.75	0.7	1.11	1.32	1	0.9	0.84	0.8
45	0.78	0.74	0.69	1.02	1.29	1.12	0.92	0.85	0.8
50	0.77	0.75	0.65	1.13	1.15	1.01	0.85	0.84	0.79
55	0.76	0.77	0.7	1	1.38	1.04	0.88	0.83	0.8
60	0.77	0.73	0.65	1.1	1.32	1.46	0.85	0.82	0.75
65	0.78	0.72	0.66	1.1	0.62	1.47	0.86	0.82	0.74
70	0.8	0.74	0.66	1.1	1	1.46	0.85	0.82	0.77
75	0.78	0.7	0.67	1.1	1.11	1.47	0.85	0.83	0.78
80	0.8	0.69	0.64	1.1	0.64	1.01	0.84	0.84	0.73
90	0.75	0.69	0.63	1.12	0.71	1.12	0.87	0.82	0.7

0 – Fresh, 3 – Three months, 6 – Six months, n = 5.

In squid the lipid content (1.11%) was higher compared to rohu (0.82%) and shark (0.90%) as shown in table 2.3. The highest lipid content was observed in the fresh samples that were not subjected to frozen storage. The changes in lipid content during frozen storage could be associated with the oxidation of lipid. Within the species and frozen storage period, the fat content also showed significant variation ($p < 0.01$). Shark showed significantly greater variation from the other two species. Similarly third month of frozen storage showed significantly greater variation ($p < 0.01$) from zero month and six months of frozen storage (Appendix 2.3).

Table 2.4. Ash content (%) of fresh and frozen samples of rohu, squid and shark cooked at different temperatures

Temperature (°C)	Rohu			Squid Months of frozen storage			Shark		
	0	*3*	*6*	*0*	*3*	*6*	*0*	*3*	*6*
raw	1.48	2.2	2.56	1.2	1.2	2.5	2.36	2.56	2.56
45	1.5	2.1	2.58	1.25	2.3	1.95	2.8	2.58	2.58
50	1.96	2.4	2.45	1.3	1.5	2.2	2.35	2.45	2.45
55	1.87	2.22	2.23	1	2.3	1.56	2.36	2.23	1.96
60	1.45	1.45	2.12	1.45	2.3	1.85	2.69	2.12	2.12
65	1.74	1.98	2	1.25	1.26	1.74	2.45	2	2.23
70	1.89	2.2	1.95	1.2	1.5	1.25	2.74	1.95	1.95
75	1.65	1.56	2.56	1.22	1.26	1.25	2.45	2.56	2.56
80	1.59	1.25	2.45	1.6	1.45	1.98	2.15	2.45	2.45
90	1.89	2.23	2.12	1.2	1.32	1.87	2.78	2.12	2.12

0 – Fresh, 3 – Three months, 6 – Six months, n = 5.

Ash content (Table 2.4 and Appendix 2.4) showed a significant variation between species and months of frozen storage ($p < 0.01$). Squid showed significantly higher ash content. Similarly six month frozen stored samples showed significantly higher variation in ash content. The change in the ash content with respect to frozen storage and cooking could be attributed to drip loss and dehydration that is associated with frozen storage and cooking (Sikorski and Sunpan, 1992).

2.4.2. Physico – chemical parameters

Table 2.5 shows the pH of the fresh and frozen samples (three and six months) of rohu, squid and shark cooked at various temperatures ranging from 45°C to 90°C.

Table 2.5. pH of fresh and frozen samples of rohu, squid and shark cooked at different temperatures

Temperature (°C)	Rohu			Squid Months of frozen storage			Shark		
	0	*3*	*6*	*0*	*3*	*6*	*0*	*3*	*6*
raw	6.3	5.7	5.8	6.3	6.4	6.2	6.4	5.7	5.8
45	6.3	5.9	5.8	6.5	6.2	6.1	6.3	5.9	5.8
50	6.3	6.3	5.8	6.5	6.3	6.1	6.3	6.3	5.8
55	6.3	5.9	5.8	6.3	6.3	5.9	6.5	5.9	5.8
60	6.3	5.7	5.9	6.2	6.4	5.9	6.4	5.7	5.9
65	6.3	5.8	5.8	6.5	6.3	6	6.3	5.8	5.8
70	6.3	5.8	5.8	6.5	6.4	6	6.5	5.8	5.8
75	6.5	5.8	5.7	6.5	6.3	6	6.4	5.8	5.7
80	6.3	5.7	5.7	6.5	6.3	6.1	6.3	5.7	5.7
90	6.4	5.7	5.7	6.5	6.3	6.1	6.3	5.7	5.7

0 – Fresh, 3 – Three months, 6 – Six months, n = 5.

The pH in fresh samples of all the three species ranged from 6.3 to 6.5. The pH was lower than neutral in all the three species when cooked at different temperatures ranging from 45°C to 90°C and reduced further with frozen storage. The pH in squid ranged between 6.0 and 6.5. A significant difference was observed between the species ($p < 0.01$). Statistical analysis showed a significant difference ($p < 0.01$) between the frozen storage months. On cooking pH in fresh sample differed significantly ($p < 0.01$) from frozen samples. On cooking six months of frozen stored samples showed a significantly higher difference in pH than unfrozen and three months frozen stored samples (Appendix 2.5).

Table 2.6. Water binding potential (g) of fresh and frozen samples of rohu, squid and shark cooked at different temperatures

Temperature (°C)	Rohu			Squid Months of frozen storage			Shark		
	0	*3*	*6*	*0*	*3*	*6*	*0*	*3*	*6*
raw	1.84	1.59	1.84	2.23	2.09	2.71	1.68	1.68	1.63
45	1.57	1.46	1.57	2.7	1.57	2.73	1.46	1.46	1.76
50	**2.46**	2.21	2.46	2.39	1.61	3.8	1.82	1.82	2.69
55	1.97	1.24	1.97	2.85	2.02	1.65	1.9	1.9	2.29
60	1.97	2.08	1.97	2.61	2	3.06	1.97	1.97	2.03
65	1.92	1.83	1.92	2.53	2.18	2.53	**2.07**	2.07	2.26
70	1.92	1.44	1.92	2.57	2.32	1.82	1.71	1.71	1.94
75	1.67	1.36	1.67	2.45	2.11	1.84	1.49	1.49	1.86
80	1.39	1.57	1.39	**2.96**	2.05	2.2	1.26	1.26	1.89

0 – Fresh, 3 – Three months, 6 – Six months, n = 5.

Table 2.6 shows the water binding potential of fresh and frozen samples (three months and six months) of rohu, squid and shark cooked at different temperatures. The water binding potential in squid ranged from 2.2 to 3.0. It was high in comparison with rohu and shark. The water binding potential for fresh samples of rohu was highest at 50°C (2.46) while it was highest for shark at 65°C (2.07) and for squid at 55°C (2.85). With further cooking the water binding potential showed a slight decrease. After three months of frozen storage there was a slight variation in water binding potential with species and after six months of frozen storage the water binding property in all the three species were significantly different. Statistical analysis by ANOVA (Appendix 2.6) showed significant variation with species at $p < 0.01$. Freezing was found to play a significant role in affecting the water binding ability of the species. Within the species there was a significant variation with cooking temperature and frozen storage ($p < 0.01$).

Table 2.7. Expressible moisture (%) of fresh and frozen samples rohu, squid and shark cooked at different temperatures

Temperature (°C)	Rohu			Squid Months of frozen storage			Shark		
	0	*3*	*6*	*0*	*3*	*6*	*0*	*3*	*6*
raw	72.42	72.94	82.18	70.09	80.81	81.53	74.19	85.02	87.52
45	70.69	68.1	80.03	66.67	82.76	72.42	71.73	76.12	88.17
50	74.61	74.84	80.73	75.36	77.78	79.98	75.39	88.74	77.81
55	**68.35**	67.15	80.29	**56.79**	65.12	60.56	**67.1**	75.05	73.28
60	79.23	74.42	77.44	58.59	77.98	65.96	77.49	73.54	85.77
65	75.02	72.56	65.4	77.3	75.51	52.06	74.23	80.69	85.76
70	75.04	65.56	80.99	83.54	54.95	63.96	73.9	74.28	86.9
75	78.58	72.77	70.75	71.67	59.46	67.56	76.42	79.67	80.19
80	79.19	61.83	70.59	76.28	64.23	58.41	79.02	74.72	81.19
90	76.33	65.04	63	70.14	65.04	58.76	75.2	80.51	75.64

0 – Fresh, 3 – Three months, 6 – Six months, n = 5.

Table 2.7 shows the expressible moisture of fresh and frozen samples (three months and six months) of rohu, squid and shark cooked at different temperatures. At 55°C the expressible moisture was the lowest with values being 68.35%, 56.79% and 67.1% for rohu, squid and shark respectively. Squid show the minimum expressible moisture (56.79%). Expressible moisture was high in squid upon cooking at 70°C compared to rohu and shark. Statistical analysis showed a significant variation ($p < 0.01$) between the species. A significantly higher difference ($p < 0.01$) was observed between squid and shark, but no significant difference was observed between rohu and shark when compared using least square difference (Appendix 2.7).

2.5. DISCUSSION

The need for the analyses of proximate composition of fish and fishery products is gaining more importance than the past considering the nutritional changes that occur in each component during various processing techniques used. The data regarding the changes in the proximate composition of the species at various cook temperatures are lacking. The proximate compositions of fresh and frozen fresh water and marine water fish were reported

by various scientists (Hassan and Mathew, 1999; Chand et al., 2001; Smuruthi et al., 2003 and Arannilewa et al., 2005). The results obtained for the proximate composition of fresh samples of rohu, squid and shark were in accordance with the results obtained by Gopakumar (1997) and Sankar and Ramachandran (2001). Suyama and Kobayashi (1980) analyzed eight species of squids and observed that water content varied from 75 – 80%, crude protein 16 – 21%, crude fat 1% and ash from 1 – 2%. Comparison of biochemical composition of squid with rohu and shark revealed their considerable similarity with white meat fish. Squid muscle had high nitrogen content compared to that of white meat fish. Squid and shark were found to have about 22% crude protein nitrogen content while rohu contained 18% crude protein. The non – protein nitrogen fraction in shark meat also contribute to the high content of the crude protein. Considering the variations in crude protein content during various cooking regimens of fresh and frozen stored samples (Table 2.2), squid mantle showed lesser decrease compared to rohu and shark possibly due to the unique distribution and arrangement of protein in the mantle tissue. Squid muscle was found to contain lipid content of approximately 1%. The crude fat content in the species studied was also comparable with published data (Sikorski and Kolodziejska, 1986). Lipid content was recorded high for the fresh samples that decreased with the frozen storage (Table 2.3). Ash content showed a slight change with respect to duration of storage possibly due to the drip loss and dehydration of the muscle tissue. Otwell and Hamann (1979) observed that squid meat shrinks to about 30% upon cooking. The cooking loss in weight was observed to be 25 – 42% and took place during the early process of heating. Protein content decreased that could due to leaching out of the protein with drip loss. The percentage of retention of moisture on cooking (Table 2.1) showed almost similar patterns as that of water binding potential. Similar observations were reported in a study of seafood nutritional quality on cooking by Krzynowek, (1988).

The pH affects the water binding properties and subsequent gel forming ability of the proteins in the frozen samples. Between fresh and frozen samples of squid the pH showed a gradual decrease during frozen storage and this variation was seen at each temperature of cooking. But this kind of variation of pH in rohu and shark were more significant compared to squid mantle. Changes in the squid mantle pH during cooking

could be due to the conformational changes in the mantle proteins (Otwell and Hamann, 1979).

Various methods measure various forms of water retention in the tissue indicating that the results may not always be comparable with each other. Hence there is an obvious need for standardization and appropriate selection of methodology for evaluating the water holding capacity in the samples. Water holding capacity is the generic term that describes the ability of the meat system to hold all or part of its own or added water. Methods for analyzing water holding capacity of materials give an indication of how well moisture is held in the matrix by applying some form of stress like pressure and centrifugal force and measuring the amount of fluid released (Jauregui et al., 1981 and Munro and Van Til, 1988). In water binding potential the tissue precipitate retains the maximum amount of water that the sample can hold at a particular set of solution and centrifugation conditions. Expressible moisture measures the actual loss of the moisture from a sample during the application of pressure. Thus, water binding potential and expressible moisture offers different information. The content and the distribution of water influence the sensory properties of the muscle tissue.

The water binding property is a function of protein functionality that is determined by freshness of species of fish and other processing variables (Douglas and Lee, 1988). A certain amount of moisture is necessary for the adequate solubilization of the protein and for the formation of the gel networks. When the moisture level exceeds the water binding capacity of the proteins the product shows a markedly weak texture and is no longer acceptable. Hence in practice, water – binding ability is more useful in optimizing the formulation of fishery products. The formation of a protein network in the gel contributes to textural characteristics and to other functional properties of the product viz., water and fat retention (Sharp and Offer, 1992). The irreversible formation of actomyosin in rigor mortis possibly reduces the water binding capacity of the myofibrils. The changes in the water holding capacity of meat are related to the 95% of the muscle water (Hamm, 1975; 1986). The bulk of the tissue water is immobilized by the weak attraction forces towards the charged groups in proteins, partly by the physical configuration of the myofibrillar proteins and the barriers created by the sarcolemma and connective tissue sheaths. Connective tissue protein holds the myofibrillar bundles and their role in retaining higher

values of water binding potential shown in squid is investigated in the proceeding chapters. The physico – chemical properties of squid mantle were observed to differ significantly from rohu and shark.

2.6. CONCLUSION

Considering the decrease in crude protein content during different cooking regimens of the fresh and frozen – stored samples, squid mantle showed the least change compared to rohu and shark, possibly due to the unique distribution and arrangement of the protein in the mantle tissue. Lipid content was recorded high for the fresh samples which decreased with frozen storage. At a heating temperature of 55°C, the expressible moisture was the lowest with values 68.35%, 56.79% and 67.1% for rohu, squid and shark, respectively. The water binding potential was highest for fresh samples of rohu at 50°C (2.46g), for shark at 65°C (2.07g) and for squid at 55°C (2.85g). Throughout the treatments the water binding potential of squid meat was high (2.2 – 3.0) in comparison with rohu and shark rendering it juicier than rohu and shark. This tissue water is probably located in the three dimensional network of the muscle filaments held by capillary forces. Thus, the amount of water immobilized depends on the space available between the filaments and changes in the myofilament spacing. The expansion or shrinkage of the myofilament lattice cause water movements between intracellular and extra cellular spaces resulting in the swelling or shrinkage of the muscle fiber without much changes in the volume of tissue as whole. Studies to assess and compare the quantitative and qualitative changes in different protein fractions of rohu, squid and shark are presented in the proceeding chapters.

CHAPTER 3

PROTEIN FRACTIONATION AND THEIR CHARACTERIZATION BY SDS – POLYACRYLAMIDE GEL ELECTROPHORESIS

Chapter 3

Protein Fractionation and their Characterisation by SDS– Polyacrylamide Gel Electrophoresis

3.1. Introduction

3.2. Review of literature

    3.2.1. Protein fractions

    3.2.2. Freezing and cooking of muscle tissue

    3.2.3. SDS – Polyacrylamide Gel Electrophoresis

3.3. Materials and Methods

    3.3.1. Raw material collection and sample preparation

    3.3.2. Quantitative fractionation of protein

    3.3.3. SDS – Polyacrylamide Gel Electrophoresis

        3.3.3.1. Preparation of total protein extract

        3.3.3.2. Reagents for SDS – PAGE

        3.3.3.3. Gel preparation

    3.3.4. Statistical analysis

3.4. Results

    3.4.1. Protein fractionation

    3.4.2. SDS – PAGE

3.5. Discussion

3.6. Conclusion

3.1. INTRODUCTION

Based on the differences in the physico – chemical properties, proteins are classified as sarcoplasmic and fibrillar proteins. The sarcoplasmic proteins form approximately 15 – 20% of the total proteins depending upon the fish species. They are generally soluble in water and buffers of low ionic strength. Most of these are low molecular weight proteins in the range of 40 KDa to 70 KDa. The fibrillar proteins are salt soluble and insoluble fractions contributing about 60 – 80% of total proteins that have molecular weight in the range of 400 to 600 KDa. Myofibrillar and connective tissue protein contribute to the fibrillar proteins. Connective tissue protein chiefly comprises of collagen and it contributes to a small fraction of the total protein. They hold together the myotome bundles thus helping in maintaining the texture of meat. Extractability of the protein fractions depends on species and the post mortem stage of the fish. The basic aim of the fish processor and food technologist has to be to control the changes in the functional properties of tissue protein, and thus to preserve and improve the quality of the meat.

Freezing is a commonly employed technique for the preservation and maintenance of the nutritional quality of fish by retarding the biochemical and microbiological reactions in the tissue. The freeze induced physico – chemical changes in the colloidal structure of fish protein pose several technological problems like exudation of drip from thawed fish. The toughness of fish muscle increases with prolonged storage resulting in economic loss and reduced acceptability among the consumers. Thermal processing is yet another means of preservation that also could induce conformational changes in the protein structure. Temperature and duration of cooking have a profound effect on physical properties of meat and eating quality. Cooking causes interaction of muscle protein fraction, enzyme hydrolysis and change in the connective tissue thus affecting the nutritional properties of the muscle tissue. Mechanical properties of meat are known to be affected by the connective tissue protein. Low solubility of collagen plays a positive role in maintaining the texture and morphology of the cooked meat. However, information is still limited about the contribution of collagen to the texture of cooked meat in the species under study.

Several qualitative analytical techniques have been utilized to study the structure of protein molecule with its amino acid sequences as they determine the variations and other properties of the tissue. Electrophoreses is the one of the mostly preferred methodology for the qualitative and quantitative fractionation of the muscle protein. The most common technique used for the separation of protein samples is polyacrylamide gel electrophoreses (PAGE) in the presence of strong ionic detergent such as sodium dodecyl sulphate (SDS) and a reducing agent. This provides a separation of denatured protein subunit partially on the basis of charge but principally on the basis of molecular size. The resolution and sensitivity is balanced by choice of the amount of the gel matrix. This method could be used to analyze microgram quantities of sample. In this regard a number of studies have been carried out to analyze the protein structure and their classification. But studies pertaining to the role of protein in tissue integration are scarce. Hence in the light of this, an attempt is made to study the following aspects of proteins in the three species under study.

1. To quantitatively fractionate muscle proteins from fresh and frozen samples of rohu, squid and shark.

2. To study the effect of heating at various temperatures on the protein fractions viz., sarcoplasmic proteins, myofibrillar proteins, alkaline soluble proteins and collagen.

3. To further fractionate the collagen fraction into pepsin soluble and insoluble collagen.

4. To analyze qualitatively the various protein fractions by SDS – PAGE.

3.2. REVIEW OF LITERATURE

Research work on protein changes during various processing techniques concerning fish muscle has been studied and several excellent reviews have been brought forth by various scientists (Connell, 1990; Dyer and Dingle, 1961; Fennema, 1973 and Shenouda, 1980).

3.2.1. Protein fractions

Protein are major constituents of meat and on the basis of their solubility they are classified into water – soluble, salt – soluble and insoluble fractions containing fibrous protein (actomyosin and collagen) that contribute to the important structural elements of muscle. The structural protein determines the toughness of raw meat and together with the precipitated sarcoplasmic protein they determine the rheology of meat tissue. Hamm (1966) reported that sarcoplasmic protein coagulate amid 40°C to 60°C though in some preparations heat denaturation of sarcoplasmic proteins were not completed below 90°C (Davey and Gilbert, 1974). The myofibrillar protein of muscle tissue could be classed into myosin, actin, tropomyosin and troponin. Shenouda (1980) reported that the properties of fish myofibrillar proteins were comparable with that of mammalian species although, isolated fish actomyosin and myosin preparations aggregated more rapidly. Careche et al. (2002) postulated that some actomyosin aggregate due to the reaction with fatty acids, were depolymerised and separated into actin and myosin in high ionic solutions and form insoluble aggregates. Collagen is an essential component of muscle connective tissue. The membrane myocommata consisting of collagen help in keeping the myotome bundles together. Its importance in the expression of texture of raw and cooked meat has been suggested for several aquatic animals (Ando et al., 1999; Olaechea et al., 1993 and Sato et al., 1997).

3.2.2. Freezing and cooking of muscle tissue

The different factors that cause protein denaturation during frozen storage of fish muscle could be briefly summarized as changes in moisture, changes in the lipid and production of formaldehyde. The changes in moisture cause damage of tissue structure due to the formation of ice crystals due to dehydration and increase in salt concentrations. When muscle tissue was stored at low temperatures actomyosin was readily insolubilised (Powrie, 1977). Myosin is the most sensitive fish myofibrillar protein with respect to freeze denaturation (Shenouda, 1980). Tropomyosin is a stable form of myofibrillar protein. Hamm (1986) reported that sarcoplasmic protein is stable during frozen storage. Haard (1992) suggested that there is an involvement of non – disulfide covalent bonds in the

formation of aggregation in frozen fish muscle. The extractability of actomyosin from cod muscle frozen rapidly and held below − 30°C did not decrease significantly. Minor collagen has received increased attention as an important constituent affecting the post mortem textural changes of fish meat during storage (Ando et al., 1995; Sato et al., 1997).

Effect of cooking on muscle tissue with or without salt was reported to show least changes in the digestibility coefficient, biological value and net protein utility. Careche et al. (1998) reported that moderate heat treatment does not alter the amino acid content. Sarcoplasmic proteins and actin had poor thermal stability and interfered with gel formation (Suzuki, 1981). Hamm (1986) reported that the solubility of myofibrillar proteins decreased greatly between 40°C and 60°C and was accompanied by unfolding of the protein chain and loss of ATP − ase activity. Coagulation resulted due to the random association between unfolded peptide chains. Shrinkage of stroma protein occurred at 60°C and squeezed more water out of the coagulated myofibrillar structure with a concomitant increase in toughness. Balian and Bowes (1977) reported that collagen is denatured at 40°C with the formation of smaller molecules resulting in solubilization and high heat sensitivity of the molecules. Bailey et al. (1974) reported that collagen molecules are polymerized due to the formation of covalent cross linkages that are involved in head to tail longitudinal cross linking. This confers considerable tensile strength to the collagen fibers. An additional transverse, non − reducible, interfibrillar cross links prevent myofibril slippage during mechanical stress. Any changes in any of the cross linkages of collagen will cause change in the textural properties of the muscle tissue.

3.2.3. SDS − Polyacrylamide Gel Electrophoresis

Qualitative fractionation of proteins were carried out by various techniques and among them electrophoresis was the best − suited technique for the qualitative fractionation and analysis of the protein denaturation and their conformational changes during various processing techniques. Tiselius (1937) introduced the electrophoretic technique to distinguish serum protein fractions migrating through a solution under the influence of electric current. Species − specific electrophoretic pattern for muscle myogen and their constancy in several marine and fresh water fishes by electrophoresis were reported by

several scientists (Tsuyuki et al., 1965 and 1968). Electrophoretic technique was also found to be beneficial in describing specific proteins from specific tissues (Davis and Lindsay, 1967). Davis (1964) and Ornstein (1964) reported the finer separation of protein fractions using polyacrylamide disc electrophoretic technique in several species. Electrophoretic studies suggested that cathodic proteins were more thermostable than the anodic protein (Connell, 1990).

Electrophoretic separations of water – soluble proteins were found to be reproducible and distinguishable from one species to another (Mackie and Jones, 1978). Several electrophoretic methods were employed to differentiate species in seafood and seafood products (AOAC, 1995). Other feasible method that has been adopted to identify the species was SDS – PAGE (Scobbie and Mackie, 1988) and urea – isoelectric focusing (Mackie and Ritchie, 1981). The reliability of molecular weight determination by SDS – PAGE was studied by Weber and Osborn (1969). A large volume of literature clearly indicated that electrophoretic techniques has its beneficiary use in areas of science like species classification, fractionation of proteins etc, but were scarce in their use in analyzing the effect of extrinsic factors on the conformational changes and aggregation of muscle protein.

In view of this it could be postulated that SDS – PAGE would be of great significance in showing the finer classification of the tissue proteins and also in presenting the effect of cooking on protein and their denaturation.

3.3. MATERIALS AND METHODS

3.3.1. Raw material collection and sample preparation

The samples of rohu (*Labeo rohita*), squid (*Loligo duvaucelli*) and shark (*Scoliodon sorrokawah*) were collected and tissue samples were prepared as detailed in 2.3.1 and 2.3.2.

3.3.2. Quantitative fractionation of protein

Fractionation of the muscle tissue was carried out in accordance with the modified methodology of Hashimoto et al. (1979). The method of Mizuta et al. (1997) was followed to further extract pepsin soluble collagen from the stroma protein. The flowchart depicting the protocol of protein fractionation is shown in Fig 3.1.

All the steps in the fractionation procedure were performed at a temperature of 4°C. 10 g tissue was thoroughly homogenized with 10 vol. of 0.05 M phosphate buffer, pH 7.5 for 10 min at 5000 rpm using a tissue homogenizer (Yorco Micro Tissue Homogenizer). The homogenate was then centrifuged at 4000 rpm for 10 min in a refrigerated centrifuge (MB – 20 Superspeed Refrigerated Centrifuge) at 4°C. To the supernatant, 5% trichloroacetic acid was added and the residue after centrifugation at 3000 rpm for 10 min was considered as sarcoplasmic protein fraction (SP). The supernatant obtained was the non – protein nitrogenous fraction (NPN). The residue obtained from first extraction was homogenized with 10 vol. of 0.6 M NaCl – phosphate buffer (0.05M), pH 7.5 by continuous stirring using a magnetic stirrer (2 ML Magnetic Stirrer, Remi Equipments) and then centrifuged at 4000 rpm for 10 min at 4°C. The supernatant obtained was taken as myofibrillar protein fraction (MY). The residue obtained hereby was subjected to continuous overnight exhaustive extraction at room temperature with 10 vol of 0.1 N NaOH and centrifuged. This alkali extraction was repeated twice. The combined supernatant was considered as alkali soluble protein fraction (ASF). Finally the precipitate was washed thoroughly with distilled water to remove the traces of alkali. The alkali – free residue (stroma protein) was suspended in 10 vol. (v/ w) 0.5 M acetic acid. The suspension was digested at room temperature for 48 h after adding the enzyme pepsin (SIGMA Co. Ltd., USA) at an enzyme: substrate ratio of 1: 20 (w/w). The collagen extracted in the supernatant on centrifugation was used as pepsin soluble collagen fraction (PSC) and the residue was considered as insoluble fraction (IF).

The nitrogen content of all the above supernatants and residues collected were determined as described in 2.3.3.1.2. The values are presented as percentage of each protein fraction on the total protein content.

Muscle

Homogenized with 10 vol 0.05 M
phosphate buffer, pH 7.5 in glass
homogenizer.

Centrifuged at 4000 rpm for
10 min

Supernatant   Residue

5 % TCA added
and centrifuged.

Homogenized with 0.6 M
phosphate buffer, pH 7.5 in
glass homogenizer.

Centrifuged
at 3000 rpm
for 10 min

Supernatant   Residue
(Myofibrillar protein)

Extracted
overnight
with 10 vol of
1N NaOH.

Supernatant   Residue
(Non – protein nitrogen)   (Sarcoplasmic protein)

Centrifuged
at 3000 rpm
for 10 min

Supernatant   Residue
(Alkali soluble
protein)

Extracted with
10 vol. of
0.5 M acetic
acid and
enzyme: tissue
(1: 20) for 48h.
Centrifuged at
4000 rpm for
20 min

Supernatant   Residue
(Pepsin soluble   (Insoluble
collagen)   fraction)

Fig 3.1 Protocol for protein fractionation.

3.3.3. SDS – Polyacrylamide Gel Electrophoresis

3.3.3.1. Sample preparation for SDS - PAGE

Whole extract of the muscle tissue was prepared using 0.5 M Tris – HCl buffer (pH 6.8) containing EDTA and glycerol (Gordon, 1980). Qualitative fractionation of whole extract, sarcoplasmic protein, myofibrillar protein, alkaline soluble proteins were done using 10% gel strength and pepsin soluble collagen by using 7% gel strength (Lammelli, 1970). The electrophoresis apparatus used was Large Vertical Model Electrophoreses unit.

3.3.3.2. Reagents for SDS – PAGE

☞ **Stock solutions**

a. Acrylamide / bis – acrylamide (30% T, 2.67% C)

   acrylamide 29.2 g / 100 ml

   N′ N′- bis- methylene-acrylamide (0.8 g / 100 ml)

   Made up to 100 ml with deionized water. Filtered and stored at 4°C in the dark.
b. 1.5 M Tris- HCl buffer (pH 8.8)

   18.15 g Tris base / 100 ml

   Dissolved in 80 ml. Adjusted the pH to 8.8 with 6 N HCl and made up the solution to 100 ml with deionized water and stored at 4°C.

c. 0.5 M Tris – HCl buffer (pH 6.8)

   6 g Tris base in 60 ml deionized water

   Adjusted the pH to 6.8 with 6 N HCl. Made up to 100 ml with deionized water and stored at 4°C.

d. 10% SDS

   Dissolved 10 g of SDS in 90 ml of water with gentle stirring and made up to 100 ml with distilled water

e. Sample buffer (SDS reducing buffer). Stored at room temperature.

Deionized water	3.8 ml
0.5 M Tris – HCl buffer (pH 6.8)	1.0 ml
Glycerol	0.8 ml
10% (w/ v) SDS	1.6 ml
1– mercaptoethanol	0.4 ml
1% (w/ v) bromophenol blue	0.4 ml

Diluted the sample at 1: 4 with sample buffer, and heated at 95°C for 4 min.

f. 5 X electrode (running buffer) pH 8.3

Tris base	15.0 g
Glycine	72 g
SDS	5 g

Made up to 1 L and stored at 4°C. Warmed to room temperature before use. Diluted 60 ml of 5 X stock with 240 ml of deionized water for one electrophoresis run.

☞ **Separating gel preparation**

During the study the gel strengths of 7%, 10% and 12% were tried and 7% for pepsin soluble collagen and 10% for sarcoplasmic protein, myofibrillar protein and alkaline soluble protein were standardized and accepted for best results.

	10%	7%
Deionized water	3.3 ml	5.0 ml
1.5 M Tris HCl buffer (pH 8.8)	2.5 ml	2.5 ml
10% (w/ v) SDS	0.1 ml	0.1 ml
Acrylamide/ bis – acrylamide (30% stock)	4.0 ml	2.5 ml
(This mixture was degassed for 15 min at room temperature)		
10% ammonium per sulfate (fresh daily)	0.1 ml	0.1 ml
TEMED (NNN'N' - Tetramethylethylenediamine)	0.1 ml	0.1 ml

☞ **Stain preparation**

Prepared by mixing Coomassie Brilliant Blue R-250 (0.25%) in methanol, glacial acid and distilled water in the ratio (1: 1.5: 17.5).

☞ **Destaining solvent**

Prepared by mixing methanol and glacial acetic acid and distilled water in the ratio (1: 1.5: 17.5)

3.3.3.3. Gel preparation

Clean – dry – wax free plates were fixed to the stand and the acrylamide mixture was polymerized in it. The width and length of the gel was maintained at 1.5 mm (using spacers) and 20 cm respectively. On the top of the separating gel wells were formed using the teflon comb. After polymerization the spacers and comb were removed carefully and were fixed to the electophoresis unit with the gel ends being in contact with the electrode buffer in the upper tank and lower tank.

Sample digestion was performed by incubating the sample with sample buffer containing sodium dodecyl sulphate in 1: 4 ratios at 95°C for 4 min. 30 µl of the sample was applied to each well using eppendorf pipette. Upper tank was closed and electrical connections were made. The power pack initially was adjusted to pass current at a rate of 3 mA/ well and was increased so as to get 10mA/ well till the end. The end of run was denoted by the tracker dye in the sample buffer. The gels from each plate were removed carefully into the dyeing tray.

Before staining, the gels were fixed for half an hour with 10% TCA (Andrews, 1986). Then the protein bands in the gels were stained with Coomassie Brilliant Blue R-250 overnight and destained using the destaining solution. The bands were compared with standard high molecular weight markers and broad molecular weight markers (SIGMA, Co. Ltd., USA). Photographs of the gels were taken. The samples were run in triplicates.

Protein markers used

1. Protein Molecular Weight Marker – Broad Range (PMW – B)

S.no.	Name of protein	Molecular weight (KDa)
1	Myosin	205
2	Phosphorylase b	97.4
3	Bovine Serum Albumin	66
4	Ovalbumin	43
5	Carbonic anhydrase	29
6	Soyabean Trypsin Inhibitor	20.1
7	Lysozyme	14.3
8	Aprotinin	6.5
9	Insulin	3.0

2. Protein Molecular Weight Marker – High Range (PMW – H)

S.no.	Name of protein	Molecular weight (KDa)
1	Myosin	205
2	Phosphorylase b	97.4
3	Bovine Serum Albumin	66
4	Ovalbumin	43
5	Carbonic anhydrase	29

Calculated the relative mobility (R_f) of each fraction and compared with the markers.

3.3.4. Statistical analysis

The experimental design was randomized block design of 10 x 3 temperature and species. Analyses of variance (ANOVA) were carried out using the generalized linear model. The differences of means between pairs were resolved by means of least significance difference. The level of significance was set at $p < 0.01$ and $p < 0.05$ (Snedecor and Cochran, 1989).

3.4. RESULTS

3.4.1. Protein fractionation

Figures 3.1 – 3.5 show the different protein fraction expressed as percentage of the total protein and non – protein nitrogen (mg/ 100g tissue) of fresh and frozen samples of rohu, squid and shark heated at different temperatures. With frozen storage and cooking temperature, the extractions of the protein fractions were significantly altered in the species under study.
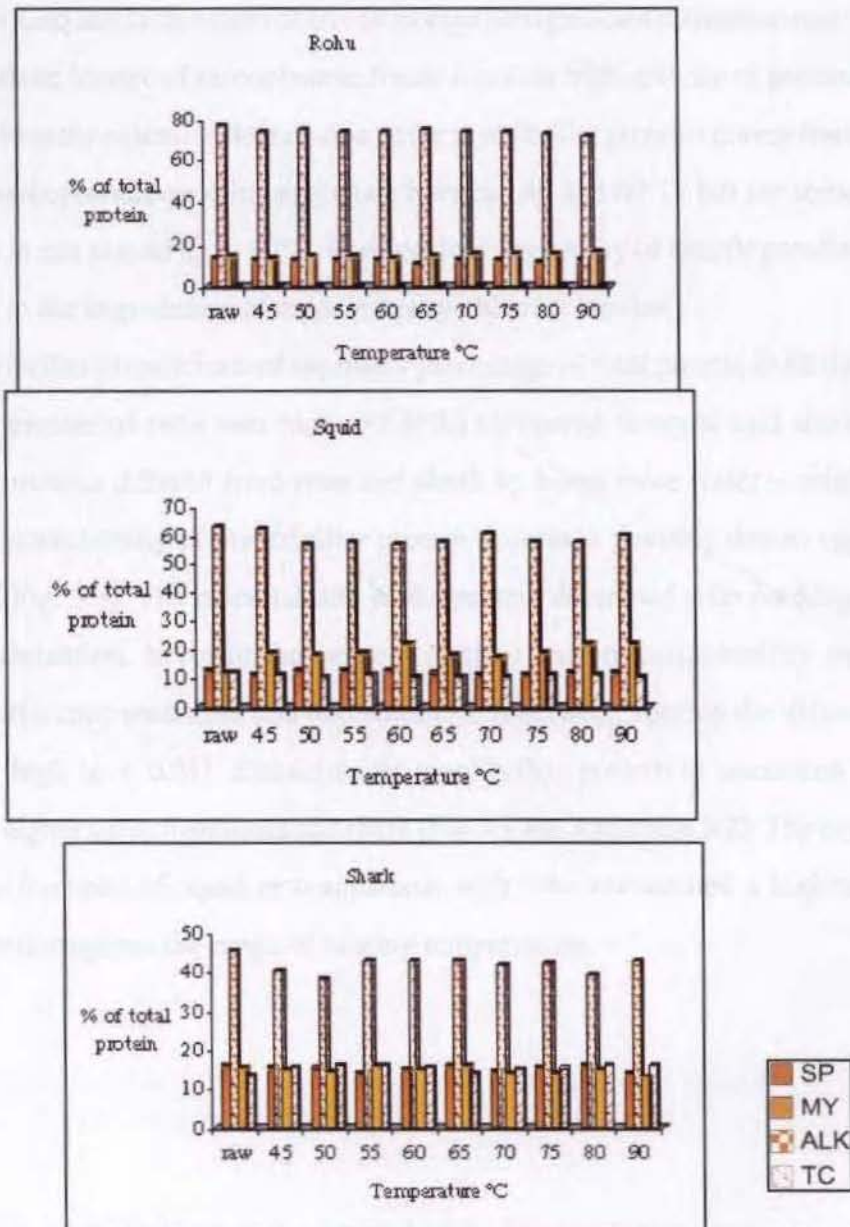
Fig 3.1 Protein fractions of unfrozen rohu, squid and shark cooked at different temperatures.

In fresh uncooked samples the total sarcoplasmic protein content was high in shark (16.07%) compared to rohu and squid. The high non – protein nitrogen content in shark (Fig 3.5) contribute to this high value. With freezing the extractability of sarcoplasmic protein was observed to reduce considerably which may be due to aggregation and accumulation of these in the interfibrillar spaces of the muscle tissue. Statistical analysis of the sarcoplasmic fraction showed significant difference ($p < 0.01$) between frozen months and cooking temperature. Sarcoplasmic protein content showed significantly higher value in the fresh sample compared to the frozen stored samples of three and six months (Appendix 3.1). Between third and sixth month of frozen storage no significant difference was observed. The characteristic feature of sarcoplasmic fraction is their high activity of proteinases that might bring about the extensive degradation of the myofibrillar proteins during fractionation. Most of the sarcoplasmic proteins aggregate between 40 and 60°C, but for some of them the coagulation can extend up to 90°C. Poor gel forming ability of mantle protein could be possibly due to the degradation of myosin heavy chain molecules.

Myofibrillar protein formed the major percentage of total protein in all the species. Myofibrillar protein of rohu was high (77.35%) compared to squid and shark. Squid myofibrillar proteins differed from rohu and shark by being more water – soluble. With freezing the extractability of myofibrillar protein decreased possibly due to aggregation (Fig. 3.2 and Fig. 3.3). The extractability of the protein decreased with cooking possibly due to gel.atinization. Myofibrillar protein content and its extractability showed no significant difference with time and temperature but between species the difference was significantly high ($p < 0.01$). Extraction of myofibrillar protein in uncooked rohu had significantly higher value than squid and shark (Fig 3.1 and Appendix 3.2). The myofibrillar and collagen fractions of squid in comparison with rohu maintained a high and stable extractability throughout the range of heating temperatures.
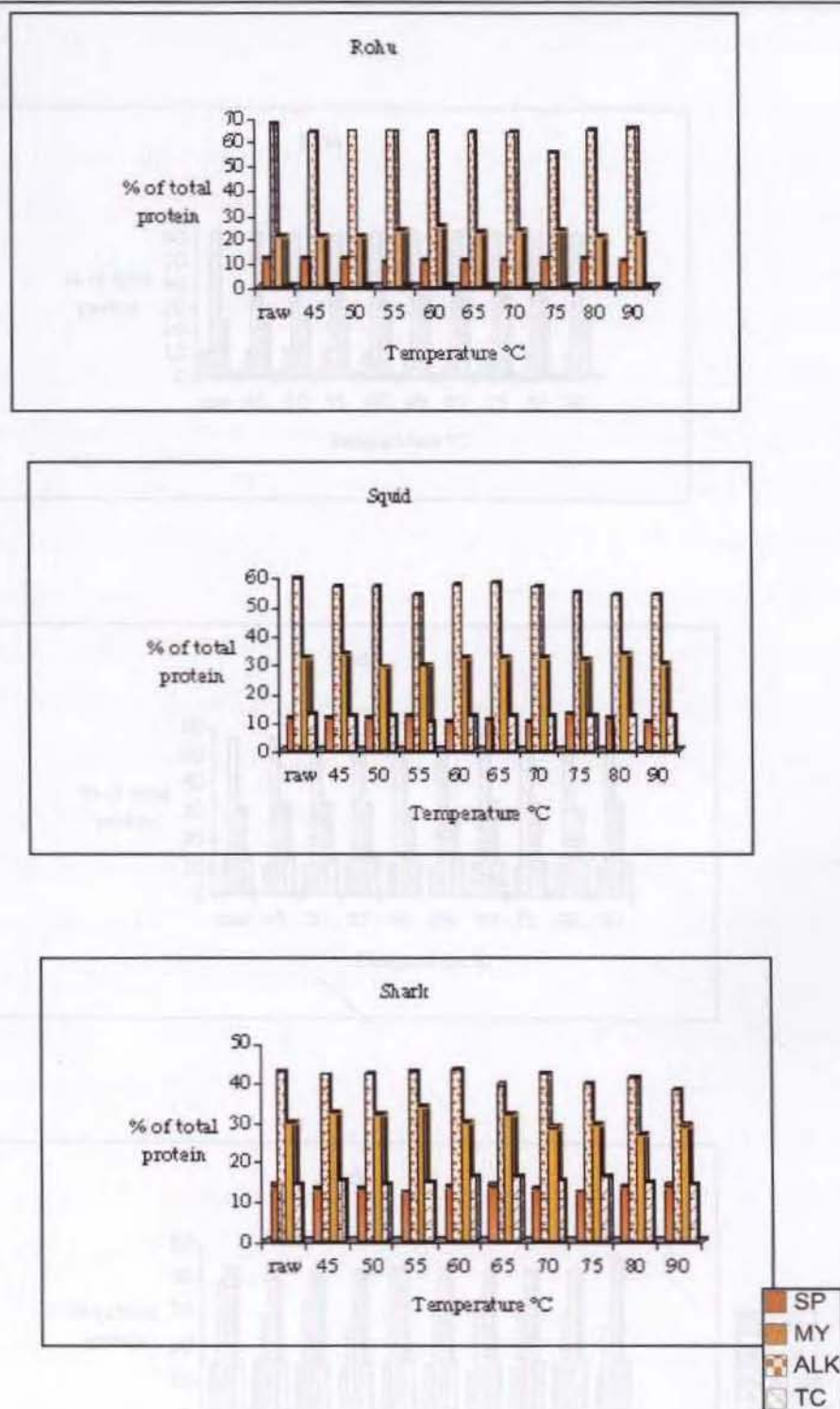
Fig 3.2 Protein fractions of three months frozen samples of rohu, squid and shark cooked at different temperatures.

Rohu

% of total
protein

70
60
50
40
30
20
10
0

raw 45 50 55 60 65 70 75 80 90

Temperature °C

Squid

% of total
protein

60
50
40
30
20
10
0

raw 45 50 55 60 65 70 75 80 90

Temperature °C

Shark

% of total
protein

50
40
30
20
10
0

raw 45 50 55 60 65 70 75 80 90

Temperature °C

SP
MY
ALK
TC
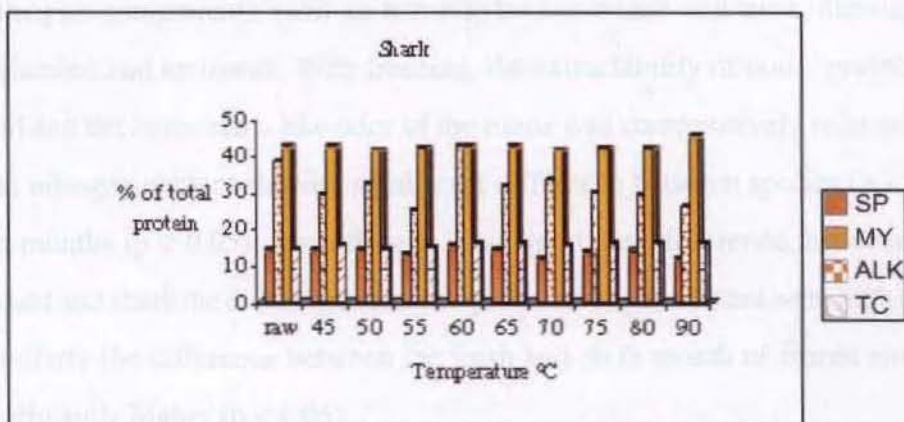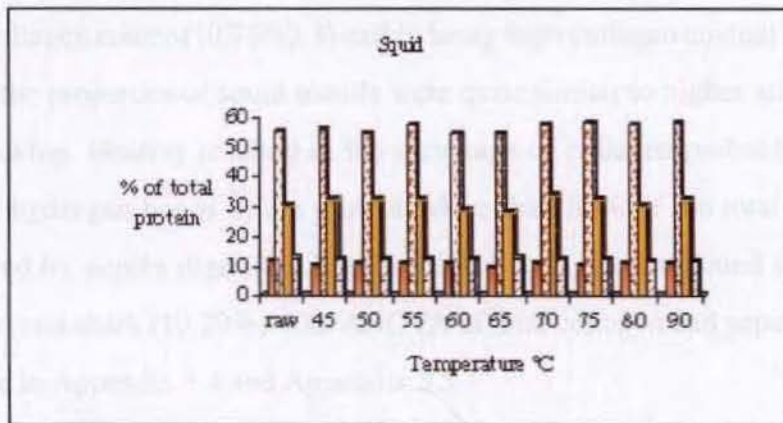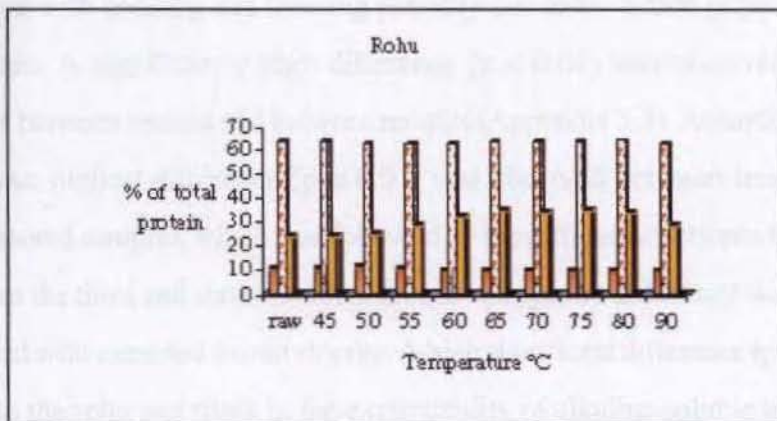
Fig 3.3 Protein fractions of six month frozen samples of rohu, squid and shark cooked at different temperatures.

The alkaline soluble fraction was found to be higher in squid than in rohu. It increased with cooking and freezing possibly due to the action of proteolytic degradation of protein. A significantly high difference ($p < 0.01$) was observed in alkaline soluble proteins between species and between months (Appendix 3.3). According to least significant difference highest difference ($p < 0.01$) was observed between fresh and six months of frozen stored samples, which was followed by the difference between fresh and third month. Between the third and sixth month of frozen storage the difference was least. Denaturation increased with extended frozen storage. A high significant difference ($p < 0.01$) was observed between the rohu and shark in the extractability of alkaline soluble protein. Rohu showed highest denaturation at 65°C possibly due to the activation of proteolytic enzymes.

Squid and shark were observed to have highest collagen content. Rohu had the lowest collagen content (0.76%). Possibly being high collagen content in squid than other species, the properties of squid mantle were quite similar to higher animal muscle tissue upon cooking. Heating resulted in the shrinkage of collagen probably due to the break down of hydrogen bonds in the protein. More than 80% of the total collagen could be solubilised by pepsin digestion. Pepsin soluble collagen was found to be high in squid (10.69%) and shark (10.29%). The ANOVA of total collagen and pepsin soluble collagen are given in Appendix 3.4 and Appendix 3.5.

Highest non – protein nitrogen content was observed in shark possibly due to high urea content in the shark tissue (Fig. 3.5). Shark has ammonia – like odor that is produced from nitrogen components such as trimethylamine oxide and urea, decomposing to trimethylamine and ammonia. With freezing, the extractability of non – protein nitrogen increased and the ammonia – like odor of the tissue was comparatively reduced. The non – protein nitrogen content showed significant difference between species ($p < 0.01$) and between months ($p < 0.05$). According to least significant difference, between rohu and shark; squid and shark the differences in non – protein nitrogen content were high (Appendix 3.6). Similarly the difference between the fresh and sixth month of frozen storage were also significantly higher ($p < 0.05$).
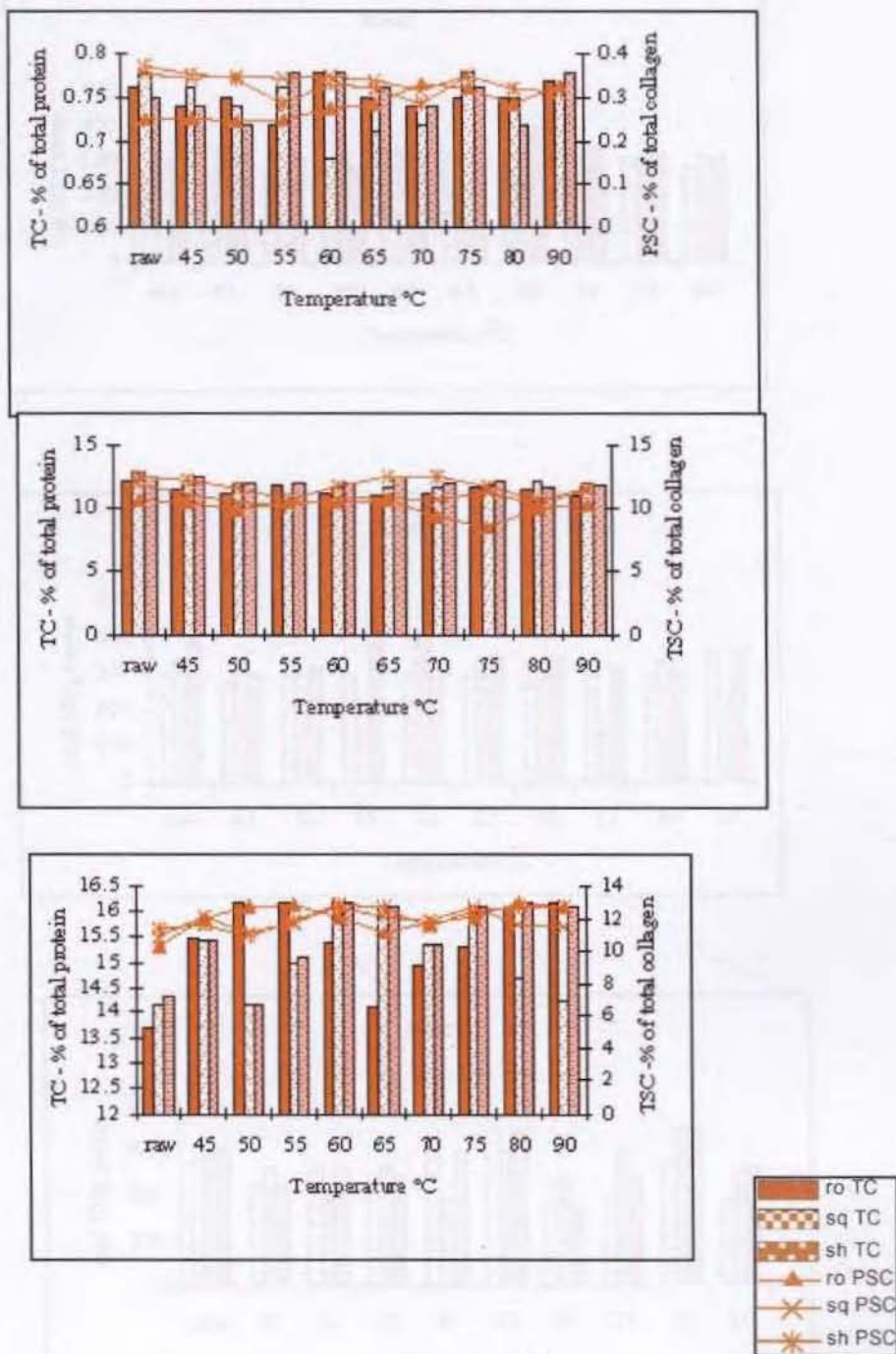
Fig 3.4 Total collagen (TC) % of total protein and pepsin soluble collagen (PSC) % of total collagen of fresh and frozen samples of rohu, squid and shark cooked at different temperatures.
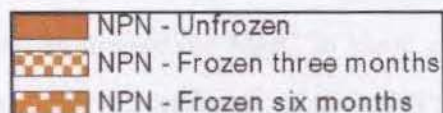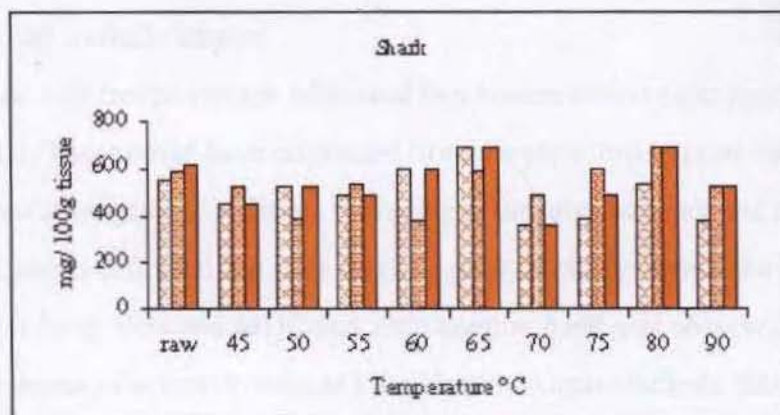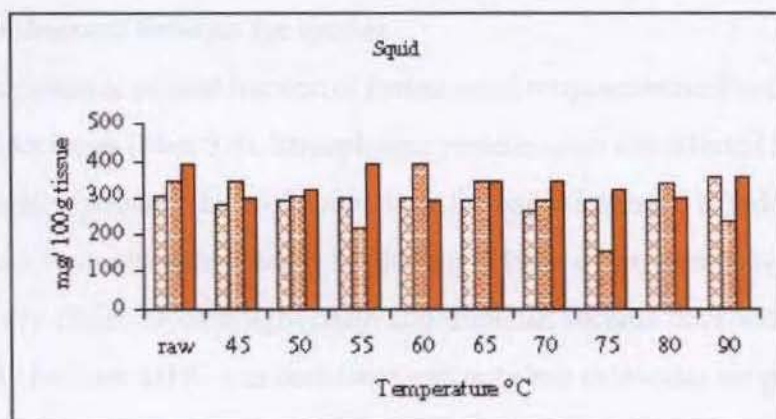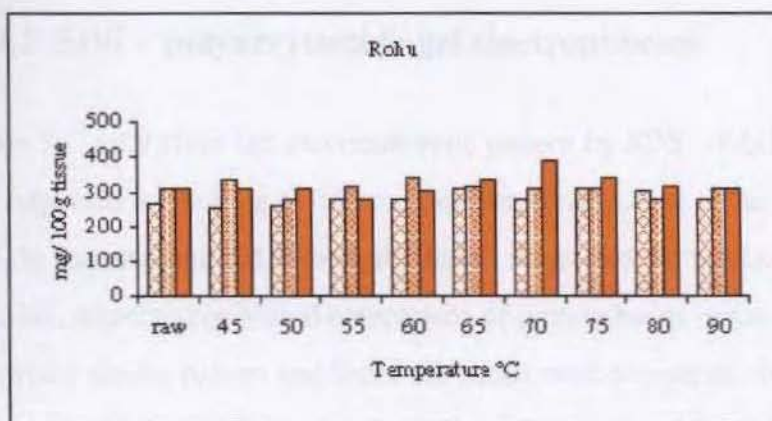
Fig 3.5 Non – Protein Nitrogen content (mg/ 100g) of fresh and frozen samples of rohu, squid and shark cooked at different temperatures.

3.4.2. SDS – polyacrylamide gel electrophoresis

Plate 3.1 – 3.9 show the electrophoretic pattern by SDS – PAGE of rohu, squid and shark subjected to cooking at different temperatures. Lanes at the left extreme (M) end and right extreme end (M') show the broad range protein marker and high range protein marker, respectively. Visual comparison of protein bands in the gels with their R_f values showed a similar pattern and hence the bands were compared visually. The whole protein extract in all the three species showed a large number of protein bands ranging from 205 KDa to 3.5 KDa. Distinct differences in the numbers and the intensity of the bands were observed between the species.

Sarcoplasmic protein fraction of frozen squid on quantitative fractionation showed twenty distinct bands (Plate 3.4). Sarcoplasmic proteins were less affected by frozen storage but with cooking possibly due to denaturation, the bands appeared to fade. Gel patterns of shark protein were characterized by predominant band of myosin heavy chain (MHC). Myosin heavy chain, myosin light chain and troponin became faint with frozen storage and possibly because MHC was denatured and only low molecular weight proteins were observed. SDS – PAGE analysis revealed that the rohu unfrozen composed of MHC, m- and c- proteins, tropomyosin and MLC. An early degradation of MLC in rohu was observed in the frozen and cooked samples.

Further with frozen storage additional bands were observed to appear at 30 to 40, 50 and 80 KDa. These could have originated from the myofibrillar proteins having larger subunit molecular weights. In addition, the fading of the original bands and the appearance of additional bands reflected the post mortem proteolysis occurring during the frozen storage and cooking. Between MHC and actin another band was observed that could be actinin. Appearance of actinin in rohu as a doublet could again indicate the degradation of the myofibrillar protein fraction. A total number of 18 bands were obtained for myofibrillar proteins of frozen rohu (Plate 3.2). With the increased storage the MHC in shark also became narrower and less intense and new band was observed on the top of the gel (Plate 3.7 and Plate 3.8). This could be due to the formation of protein aggregates. Consequently the amount of protein at the origin became greater with the protein having formed aggregates
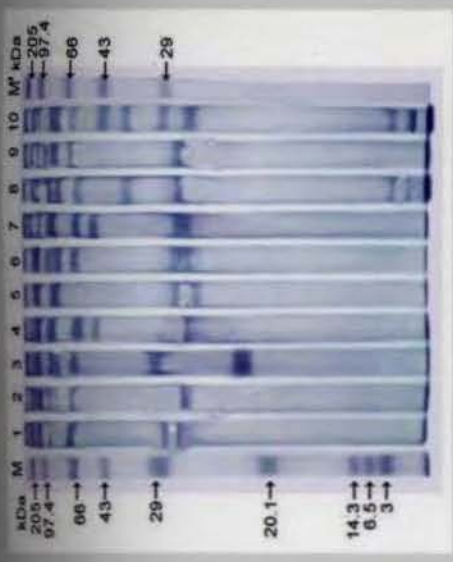
(a) whole extract
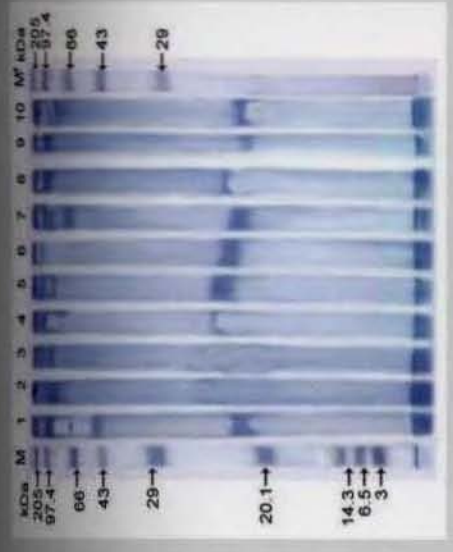
(b) sarcoplasmic protein
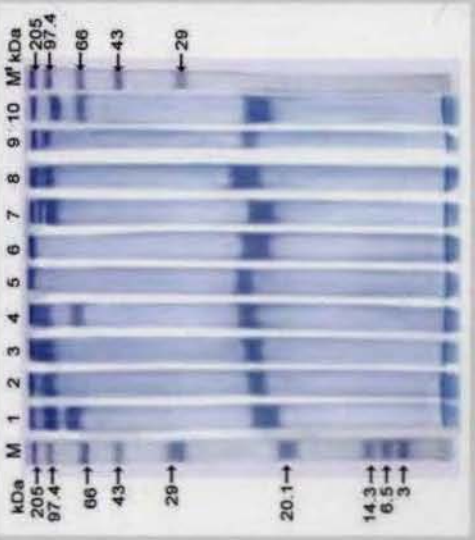
(c) myofibrillar protein

(d) alkaline soluble protein
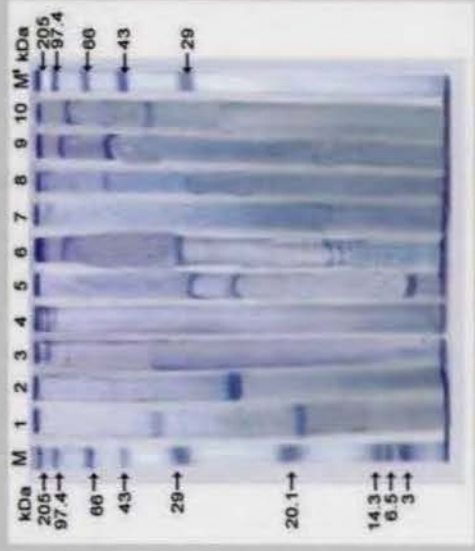
(e) pepsin soluble collagen

Plate 3.1. SDS – PAGE analysis of protein fractions from rohu (unfrozen) cooked at different temperatures.

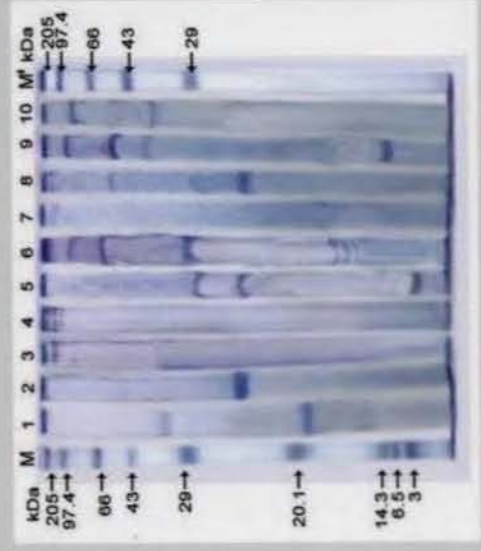M – Broad molecular weight marker, M' – High molecular weight marker.

Lanes 1- uncooked, 2 -45, 3 - 50, 4 - 55, 5 - 60, 6 - 65, 7 - 70, 8 - 75, 9 - 80 and 10 - 90°C.
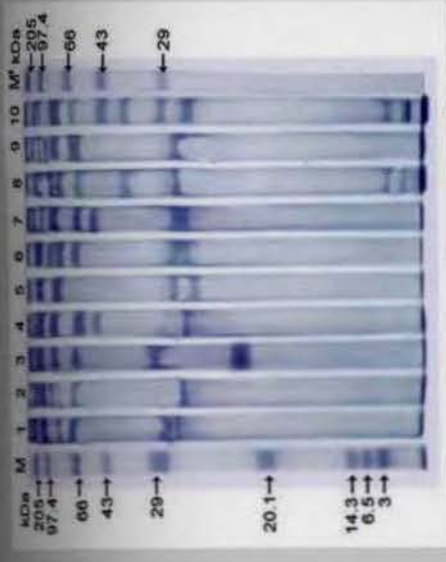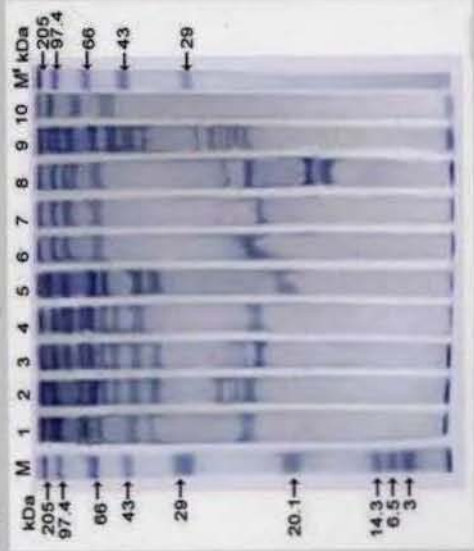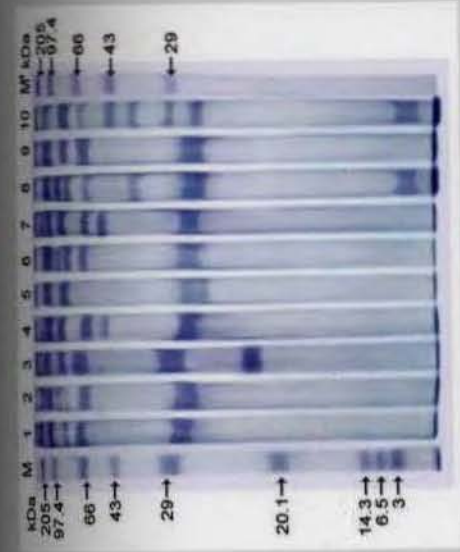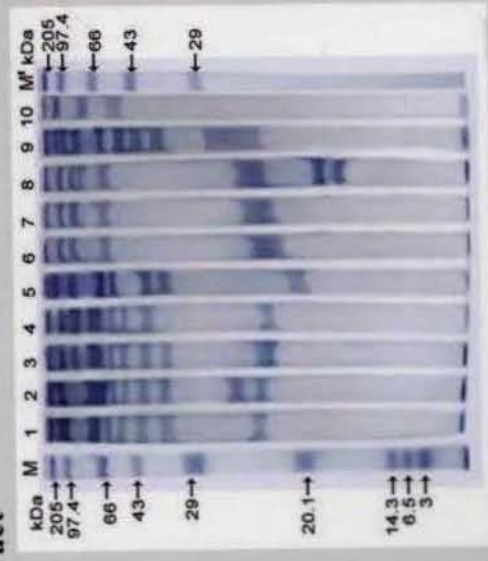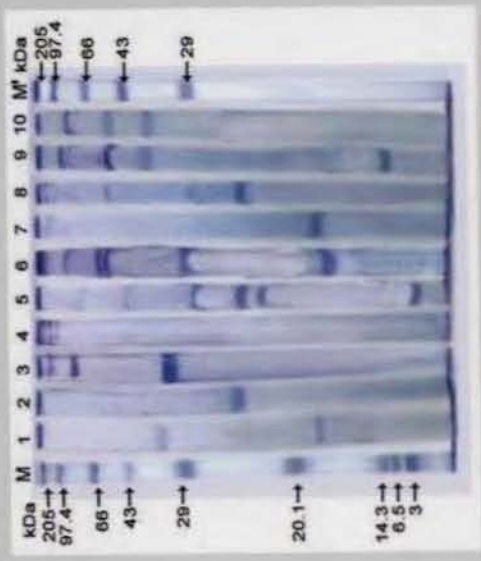
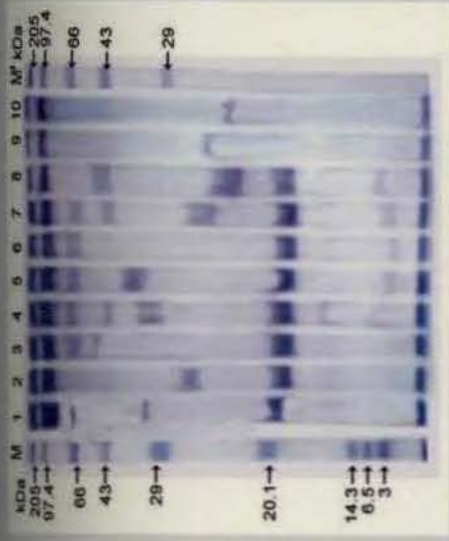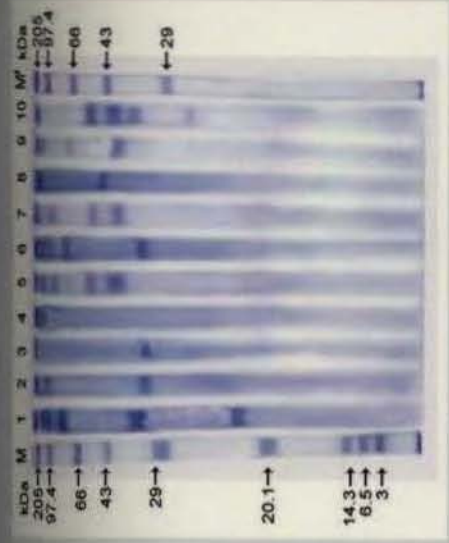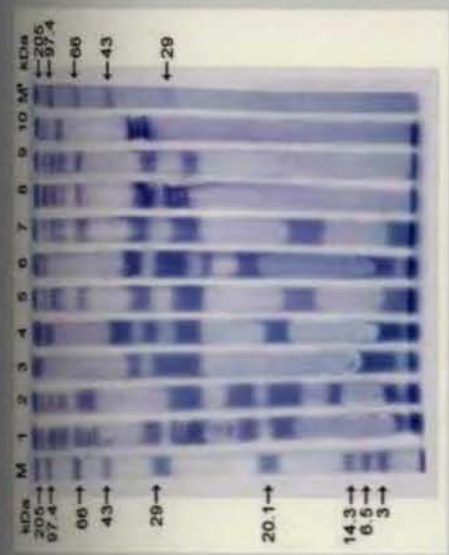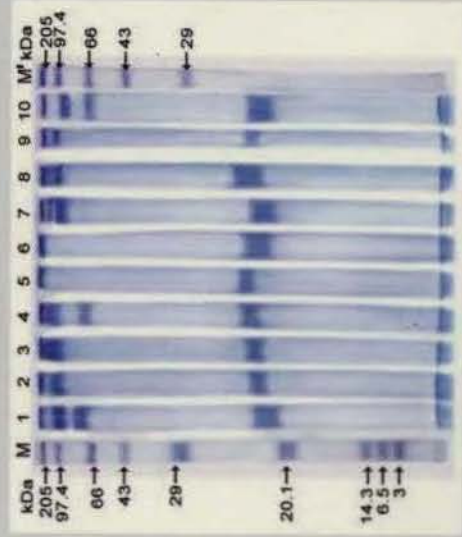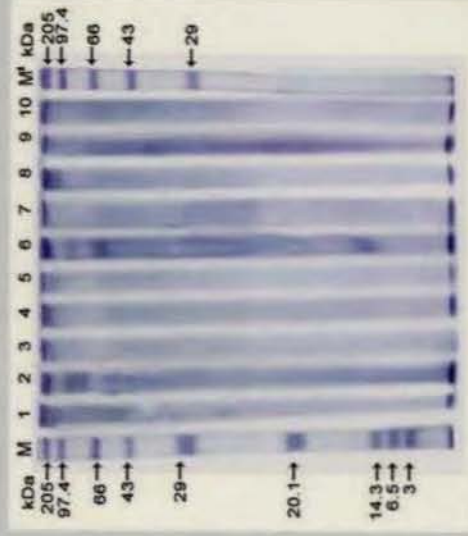(a) whole extract

(b) sarcoplasmic protein

(c) myofibrillar protein
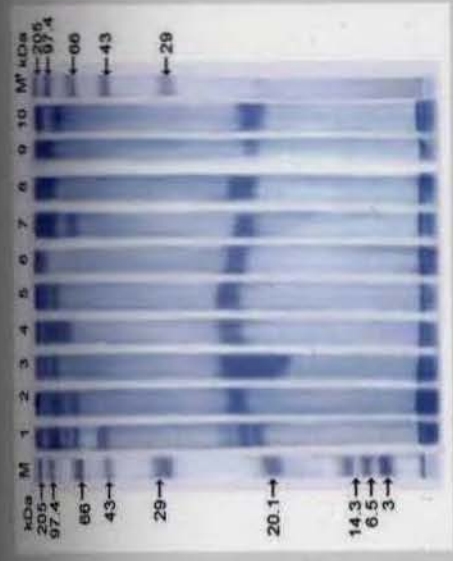
(d) alkaline soluble protein

(e) pepsin soluble collagen

Plate 3.2. SDS - PAGE analysis of protein fractions from rohu (frozen three months) cooked at different temperatures.

M - Broad molecular weight marker, M' - High molecular weight marker.

Lanes 1- uncooked, 2 -45, 3 - 50, 4 - 55, 5 - 60, 6 - 65, 7 - 70, 8 - 75, 9 - 80 and 10 -90°C.
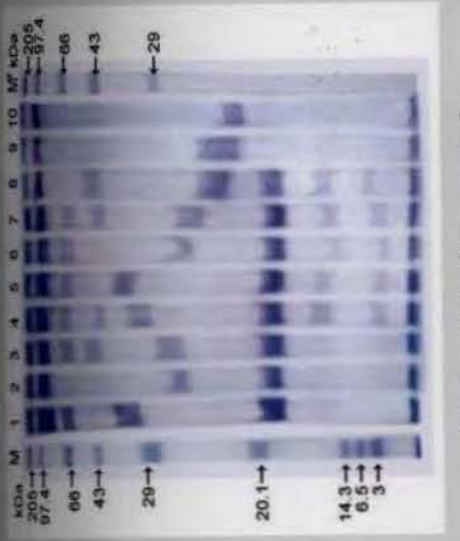
(a) whole extract

(b) sarcoplasmic protein
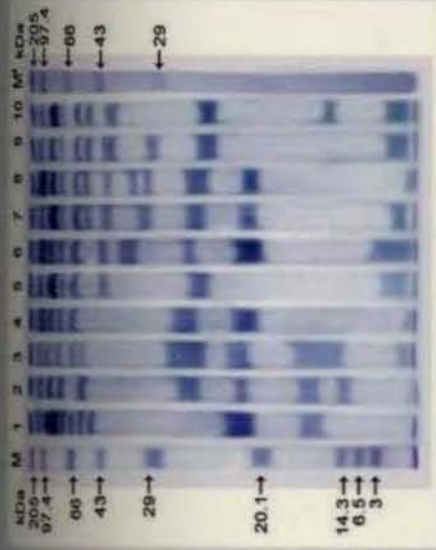
(c) myofibrillar protein

(d) alkaline soluble protein

(e) pepsin soluble collagen

Plate 3.3. SDS - PAGE analysis of protein fractions from rohu (frozen six months) cooked at different temperatures.

M - Broad molecular weight marker, M' - High molecular weight marker.

Lanes 1- uncooked, 2 -45, 3 - 50, 4 - 55, 5 - 60, 6 - 65, 7 - 70, 8 - 75, 9 - 80 and 10 - 90°C.

(a) whole extract

(b) sarcoplasmic protein

(c) myofibrillar protein

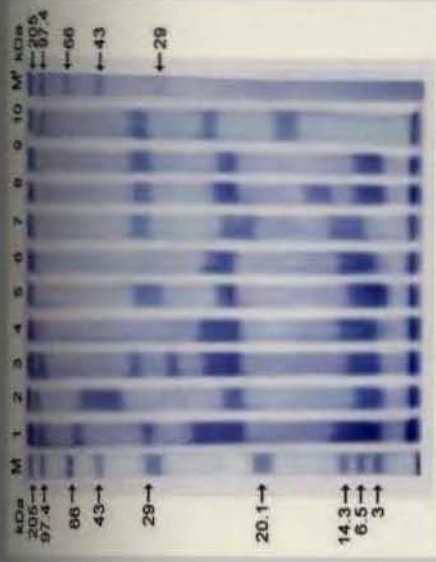(d) alkaline soluble protein

(e) pepsin soluble collagen

Plate 3.4. SDS - PAGE analysis of protein fractions from squid (unfrozen) cooked at different temperatures.

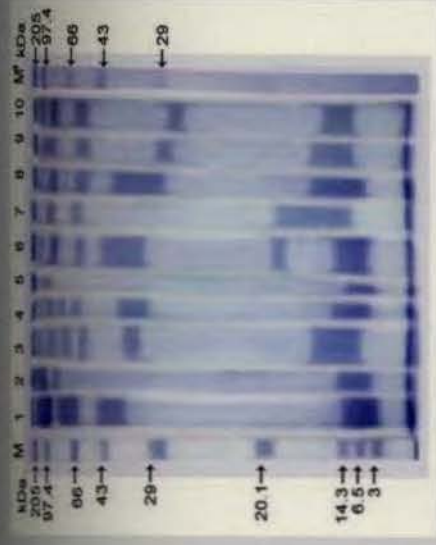M - Broad molecular weight marker, M' - High molecular weight marker.

Lanes 1- uncooked, 2 -45, 3 - 50, 4 - 55, 5 - 60, 6 - 65, 7 - 70, 8 - 75, 9 - 80 and 10 - 90 ^{0}C.
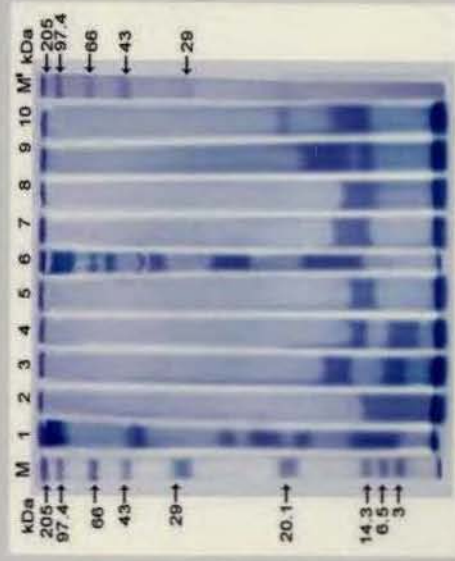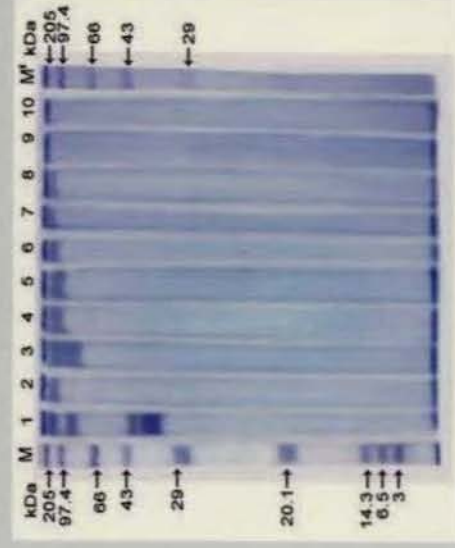
(a) whole extract

(b) sarcoplasmic protein

(c) myofibrillar protein

(d) alkaline soluble protein

(e) pepsin soluble collagen

Plate 3.5. SDS - PAGE analysis of protein fractions from squid (frozen three months) cooked at different temperatures.

M - Broad molecular weight marker, M' - High molecular weight marker.

Lanes 1- uncooked, 2 -45, 3 - 50, 4 - 55, 5 - 60, 6 - 65, 7 - 70, 8 -75, 9 - 80 and 10 - 90 °C.

(a) whole extract

(b) sarcoplasmic protein
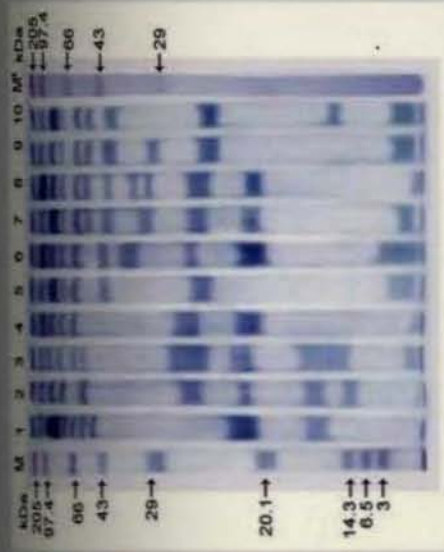
(c) myofibrillar protein
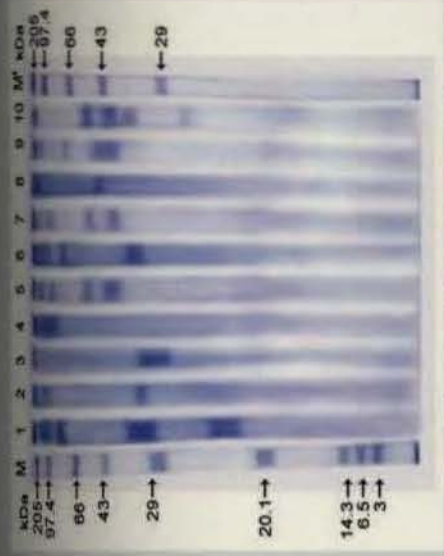
(d) alkaline soluble protein

(e) pepsin soluble collagen

Plate 3.6. SDS - PAGE analysis of protein fractions from squid (frozen six months) cooked at different temperatures.

M - Broad molecular weight marker, M' - High molecular weight marker.
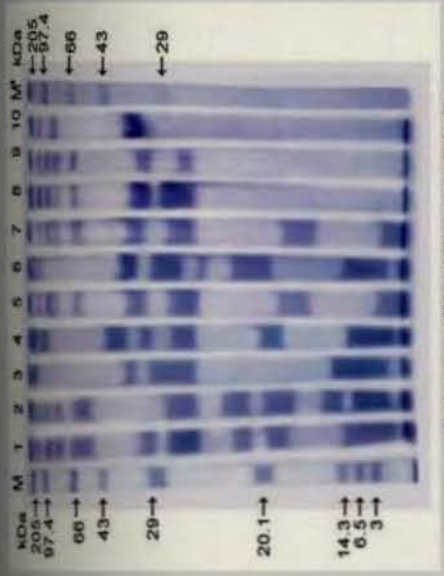
Lanes 1- uncooked, 2 -45, 3 - 50, 4 - 55, 5 - 60, 6 - 65, 7 - 70, 8 - 75, 9 - 80 and 10 - 90 °C.
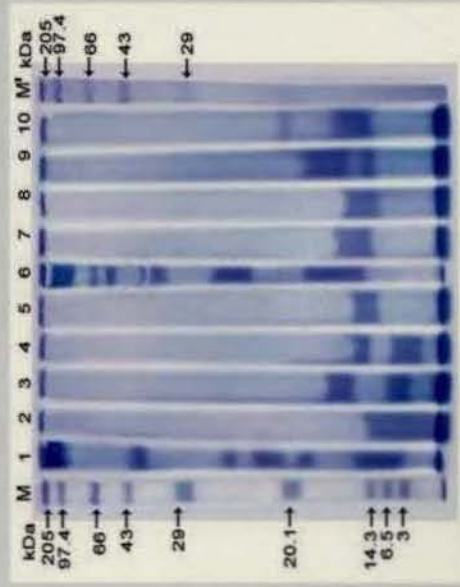
(a) whole extract

(b) sarcoplasmic protein

(c) myofibrillar protein
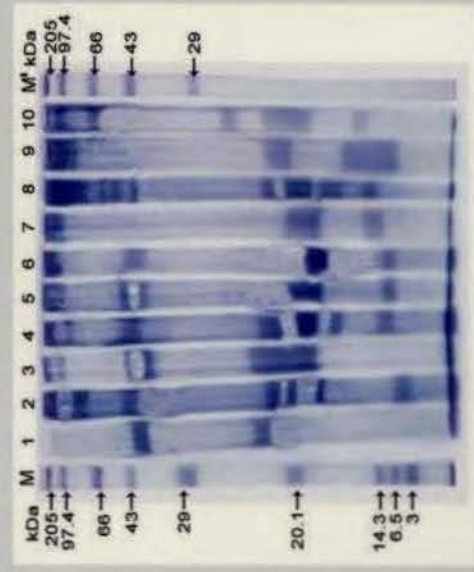
(d) alkaline soluble protein
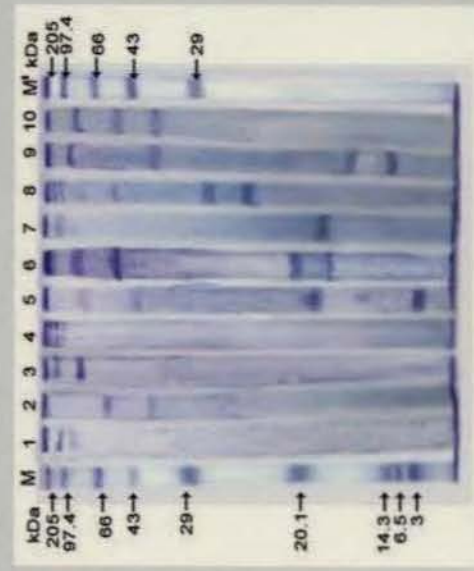
(e) pepsin soluble collagen

Plate 3.7. SDS - PAGE analysis of protein fractions from shark (unfrozen) cooked at different temperatures.

M - Broad molecular weight marker, M' - High molecular weight marker. Lanes 1- uncooked, 2 -45, 3 - 50, 4 - 55, 5 - 60, 6 - 65, 7 - 70, 8 - 75, 9 - 80 and 10 - 90 °C.
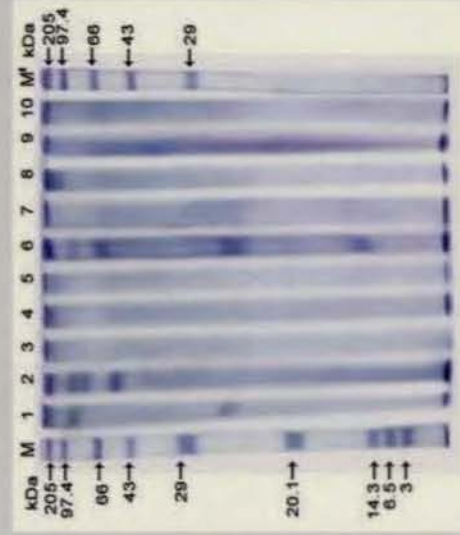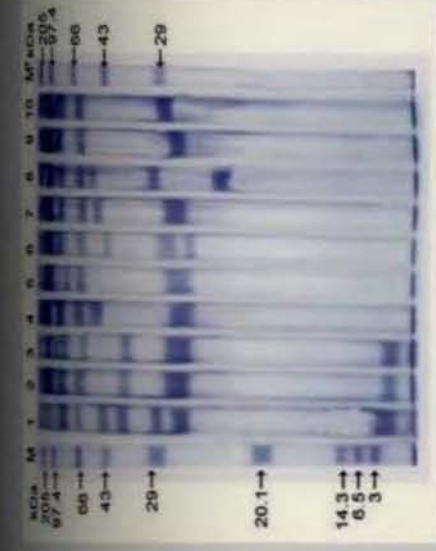
(a) whole extract
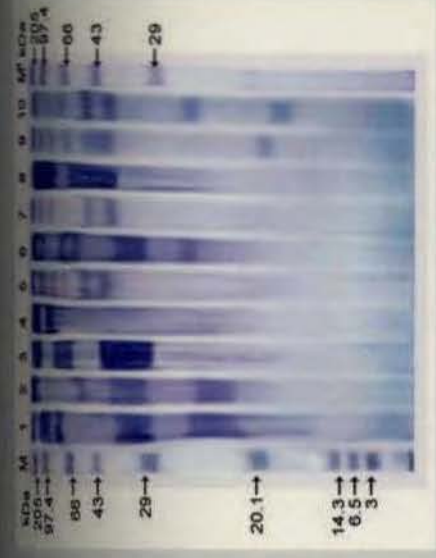
(b) sarcoplasmic protein

(c) myofibrillar protein
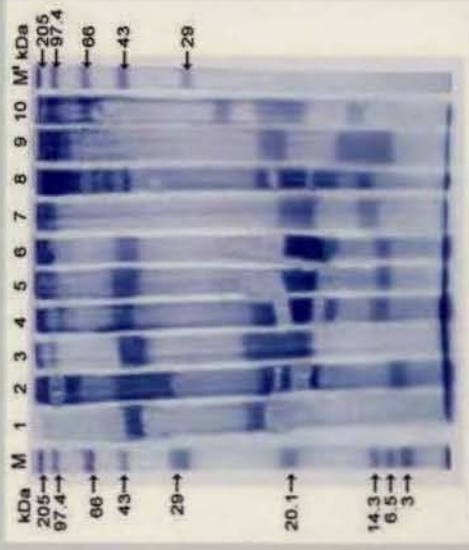
(d) alkaline soluble protein
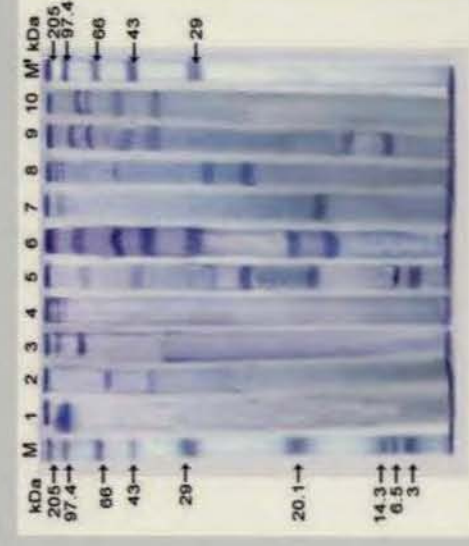
(e) pepsin soluble collagen

Plate 3.8. SDS - PAGE analysis of protein fractions from shark (frozen three months) cooked at different temperatures.

M - Broad molecular weight marker, M' - High molecular weight marker.
Lanes 1- uncooked, 2 -45, 3 - 50, 4 - 55, 5 - 60, 6 - 65, 7 - 70, 8 -75, 9 - 80 and 10 - 90 °C.

(a) whole extract

(b) sarcoplasmic protein
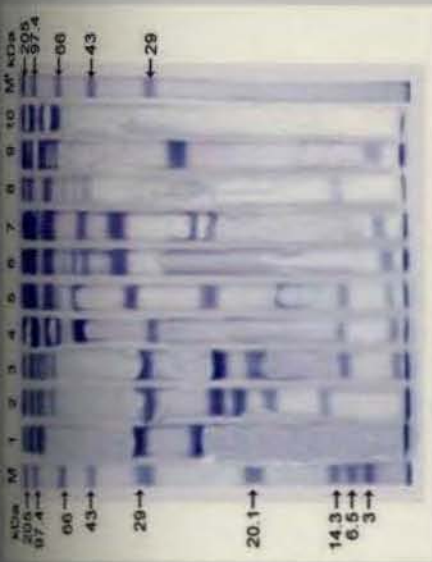
(c) myofibrillar protein

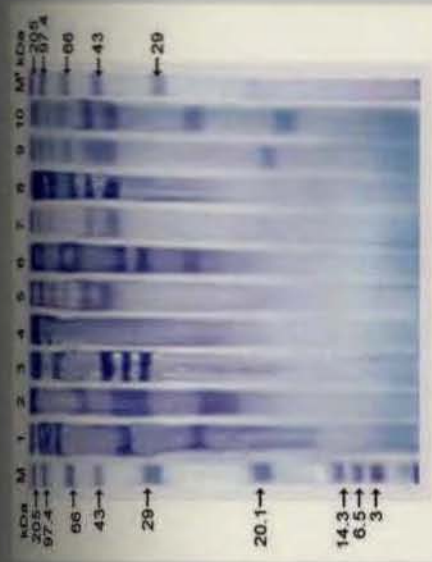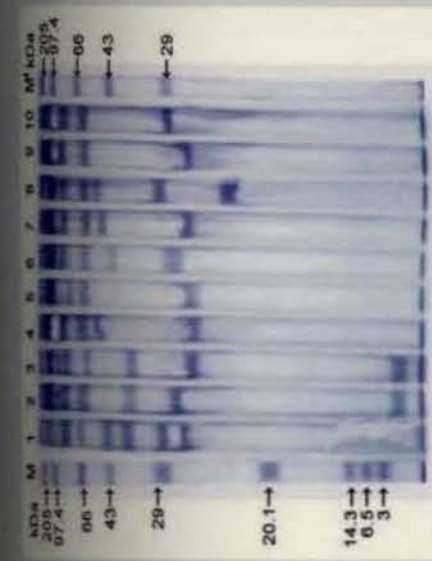(d) alkaline soluble protein
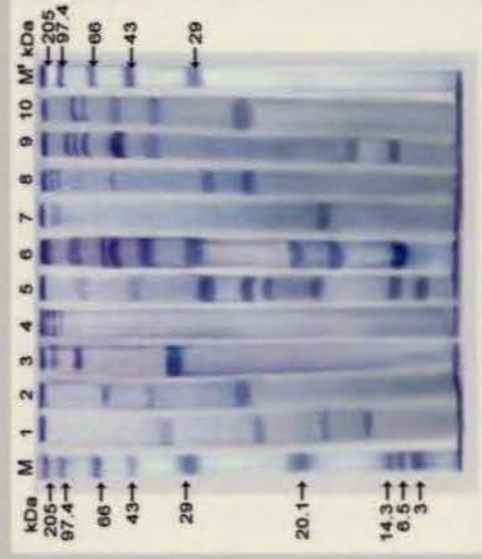
(e) pepsin soluble collagen

Plate 3.9. SDS - PAGE analysis of protein fractions from shark (frozen six months) cooked at different temperatures.

M – Broad molecular weight marker, M' – High molecular weight marker.

Lanes 1- uncooked, 2 -45, 3 - 50, 4 - 55, 5 - 60, 6 - 65, 7 - 70, 8 - 75, 9 - 80 and 10 - 90 $^{\circ}$C.
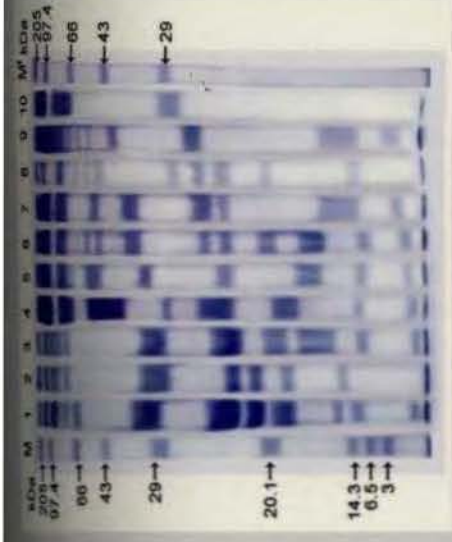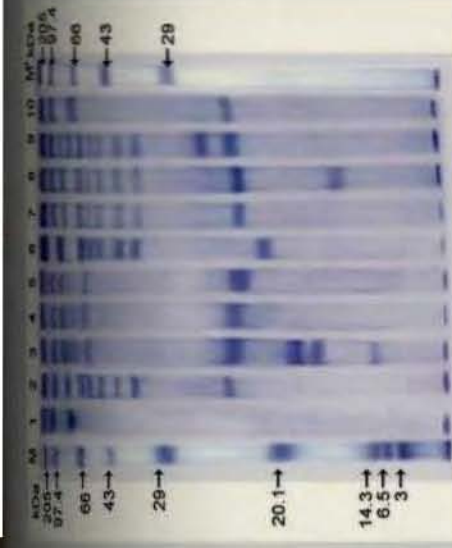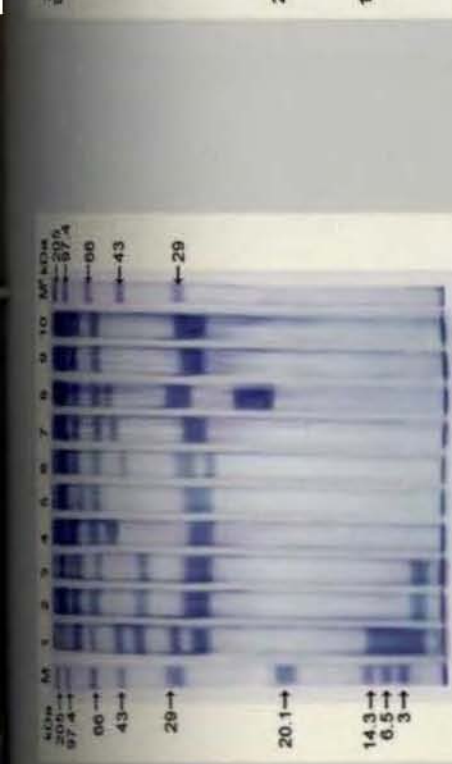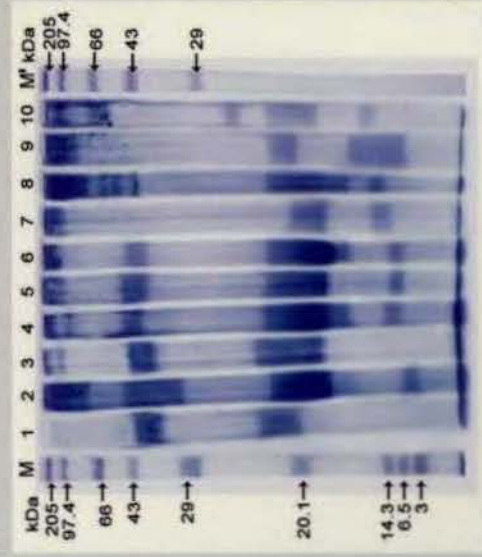
that were too large to enter the gel during electrophoresis and also due to the formation of covalent bonds formed during the cold storage. The electrophoretic profile of squid meat showed three major bands of MHC, paramyosin and globular actin having molecular weights 200, 95 and 42 KDa respectively (Plate 3.4). Myosin is probably cross – linked by disulphide bonds and other non – reducible covalent links. In both fresh and frozen samples of all the three species actin denatured earlier to tropomyosin with increased cooking temperatures.

In frozen samples of rohu the light chain bands noted in alkali soluble proteins were broken down further with cooking resulting in faint bands below 20 KDa probably due to the disintegration of MLC. The electrophorograms of alkali soluble fraction were similar to that of myofibrillar fraction of uncooked samples. The collagen bands in the lane 1 (Plate 3.1) of fresh rohu contained two bands possibly α1 and α2, along with β-chain that faded with cooking and freezing. Fresh squid in lane 1(Plate 3.4) showed two major α-chains designated as α1 and α2, along with β-chain. There was also another band of higher molecular weight. These altogether formed major collagen and a minor collagen band was also visible in fresh squid. With cooking and freezing these bands became faint and only the higher molecular component was visible. The fading of protein bands could be due to the gelatinization of collagen at increased temperature (55 – 90°C). The collagen bands in the lane 1 (Plate 3.7) of shark contained two bands possibly Type I collagen and Type II collagen that are the major collagen types. Another band similar to mammalian collagen (Type XI) also appeared as double of two closely placed bands. With freezing and cooking the bands were found to fade and aggregated at the origin. This could be possibly due to the aggregation and gelatinization of protein molecules that hindered their electrolytic mobility.·

3.5. DISCUSSION

Proteins are important structure builders of food system and would be expected that any alterations in the conformational state would change their functional properties. Applying heat to protein result in altered structure and associated changes in functional properties that have direct consequences on conformation. Denaturation affects the secondary, tertiary and quaternary level and organization. Freezing is an excellent means

of preservation although it is inevitably associated with some irreversible deterioration of muscle protein. Cooking also has a profound effect on the meat texture. With freezing the bands of sarcoplasmic protein in the frozen stored samples faded probably due to their solubilization during frozen storage. On cooking the heat set shark gel had higher expressible water content indicating the degradation of protein molecules and weaker nature of gel network.

The results obtained for the protein fractionation were in accordance with Shimizu and Simidu (1960). Muscle tissue of rohu was found to be rich in myofibrils but poor in muscle stroma when compared with squid and shark (Fig 3.5). Similar observation was also reported by Hassan (1999). Sulfide group of actomyosin and myosin of rohu might be independent of the conformational changes. According to these workers ATP – ase activity during initial stages of frozen storage play a significant role in the formation of myosin heavy chain molecules through their oxidation while squid myosin was susceptible to enzyme digestion. Moreover the squid myosin ATP – ase was inactivated by heating than that of rohu and shark (Tsuchiya et al., 1978 and Kimura et al., 1988). Paramyosin contributed to about 14% of squid myofibrillar protein and could be involved in decreasing the rate of protein denaturation in frozen stored samples.

While the gel patterns of uncooked samples were characterized by predominant band of myosin heavy chain (MHC) it was feeble in cooked and frozen stored samples suggesting degradation of MHC during gel formation. Kamat et al. (1992) reported a similar observation in surimi gel. Loss of meat quality during freezing could be associated with freeze denaturation of muscle protein. Disassociation and denaturation of sarcoplasmic and myofibrillar protein was also believed to result from the higher solute concentration and accompanying changes in ionic strength and pH (Careche et al., 1998).

Collagen in rohu is less stable and could be easily solubilised. In frozen fish fillets connective tissue were weakened and damaged due to the formation of ice crystals. Shark having the highest collagen content was least affected by freezing of fillets (Hassan and Mathew, 1999). Collagen derived their mechanical strength from cross – links. Significant structural, functional and textural changes were produced by relatively few chemical alterations in these (Mizuta et al., 2003). Kolodzeijska (1985) reported a similar observation in squid during cooking. Cephalopod collagen contains eight to ten times more reducible

bonds than mammalian collagen (Shadwick, 1985) and that might account for their high level of acid solubility.

Electrophoretic patterns were influenced by the freshness of squid prior to freezing than by the species (Kolodziejska, 1985). The MHC undergoes intense proteolytic degradation during cooking. Entire bands disappear at high temperatures. The appearance of smaller molecular weight bands could be supported by observations in other animal meats (Jasara et al., 2000). The properties of actin and tropomyosin being stable were in agreement with other reports (Ogata et al., 1998). Tejada et al. (1996) reported that relative to fresh sample there was a decrease in the sarcoplasmic protein bands in the electrophoretic pattern of frozen samples. In this study detectable changes in the electrophoretic pattern of sarcoplasmic protein fraction were observed between the frozen stored samples. The decrease in the intensity and bands with frozen storage were in agreement with Ohnishi and Rodger (1980). The appearance of a new band on the top of the gel probably due to the aggregation of protein was reported at high cooking temperatures (Lim and Haard, 1984). The high peak of actomyosin in the fresh sample could be due to the decreased extractability of the protein. Protein that aggregated during frozen storage could be dissociated with SDS as inter and intra molecular bonding break. Disappearance of sarcoplasmic and myofibrillar protein bands at later stages of cooking could be due to increased concentration of formaldehyde (Amano and Yamada, 1964) due to disintegration of tissue nutrients. The characteristic feature of sarcoplasmic fraction with high activity of proteinases brings about extensive degradation of the myofibrillar proteins in the course of fractionation. Degradation of myosin was noted only in fresh samples.

3.6. CONCLUSION

Detailed investigations on the quantitative fractionation of proteins of fresh and frozen muscle tissue of rohu, squid and shark subjected to heat treatments indicated that with frozen storage and cook temperatures the extraction of protein fractions were significantly altered within the species and between the species. Sarcoplasmic protein was high in shark and its extractability decreased with freezing probably due to their aggregation and accumulation in the interfibrillar spaces of muscle tissue. High non – protein content

in shark was observed that could be due to the presence of urea. Myofibrillar protein was observed to be high in rohu compared to squid and shark. Squid myofibrillar protein was found to be more water-soluble. Cooking disrupted many of the bonds involved in the higher order of structure. As the conformation of the protein is altered under the influence of heat, protein – protein interaction became prevalent. Presence of paramyosin in squid tissue could contribute to the decreased rate of protein denaturation in frozen stored samples. There is a direct correlation between the collagen content of fish and degree of textural changes during frozen storage. The myofibrillar and collagen protein content and their localization contribute to the differences in gelling characteristics of the muscle tissue of the three species. Protein fractionation by SDS – PAGE varied from species to species confirming the species specificity of various protein fractions. With cooking and freezing the protein bands observed in the unfrozen samples became faint and number of protein bands with smaller molecular weights appeared. Due to aggregation and gel formation of collagen and myofibrillar protein the electrophoretic mobility of the proteins were hindered and thick bands appeared at the gel top. The structural protein degraded on cooking and freezing. Hence, the study of effect of structural proteins on texture on cooking and frozen storage is important and is studied in the next chapter. Gel formation is of great interest in food systems as it affords an opportunity to produce under controlled conditions a suitable amenable to rheological measurements and also to food material of commercial interest as it holds in the matrix a large amount of water providing conduct for nutrients, micronutrients, flavor and color. Modifications of the intermolecular interactions of protein by heating were beneficial for alteration of the physical attributes of food.

CHAPTER 4

TEXTURE PROFILE ANALYSIS

Chapter 4

TEXTURE PROFILE ANALYSIS

4.1. INTRODUCTION

Musculature of fish contains a great number of muscles that depending on their anatomical location and activity exhibit structural and compositional differences that lead to different functional properties and processing abilities. Both intrinsic and extrinsic factors of muscle tissue affect the rheological characteristics. Myofibrillar protein and collagen that comprise 70 – 80% of the total protein content control the structure and the specific rheological properties of muscle tissue. Post mortem textural changes are caused by physiochemical changes in the myofibrillar proteins and changes in the extracellular spaces. Texture is also affected by the pattern of arrangement of structural components and the changes occurring during processing techniques employed. Time – temperature profile also play a significant role in affecting the textural characteristics of the muscle tissue.

Texture is a complex sensory experience and is also a multi – faceted concept describing the physical properties of foodstuff related to mouth feel and quality. Mouth feels means feelings associated with the process of mastication, salivation, touching with tongue and swallowing the food. Textural variations are complex and changes with moisture, size, temperature, state of surface and structures of foodstuffs. The rheological properties like elasticity, viscosity, visco – elasticity etc. are ideal for evaluation, if these parameters could be correlated with one's mouth feel. Textural judgments are solicited after visual and non – oral examination of food. Flavor and texture of muscle are delicate factors influencing sensory preferences of consumers. Thus, their evaluation is a critical factor in seafood products, as improper processing might lead to poor quality and rheological characteristics reducing their economic value.

In this study muscle tissues from three species of fish with varying collagen content, subjected to heat treatments are analyzed for texture both instrumentally and sensory methods. The data of instrumental texture profile analysis is correlated to the organoleptic parameters of the three species both fresh and frozen samples subjected to heat treatments at different temperatures.

4.2. REVIEW OF LITERATURE

4.2.1. Texture analysis

Muscle texture could be measured by studying the rheological properties and by observing some of the physical and chemical parameters related to the texture. Texture of fish muscle could be measured by different organoleptic and instrumental procedures. Latter techniques include cell fragility tests, changes in protein solubility and water binding capacity (Dyer, 1951 and Hamm, 1975). Studies of the rheological properties yielded parameters more closely related to the sensory evaluations. Mechanical methods are suitable for quantifying mechanical texture namely hardness, springiness, cohesiveness, toughness (firmness), chewiness, and (stiffness) resistance to mastication (Szezcsniak, 1963 and Peleg, 1996). Protein quality was found to influence strain to failure more than rigidity and that water content influences rigidity more than strain to failure (torsion).

The correlations between rheological properties of food and mouth feel are poor (Meullenet, 1998). The multipoint mensuration texture profile analysis method gave higher correlation with mouth feel than the conventional one point texture profile analysis mensuration method.

4.2.2. Role of muscle proteins

Most textural properties of seafood products are due to the composition and structure of the muscle proteins (Goll et al., 1977). Muscle proteins (sarcoplasmic and myofibrillar) alter human perception of fish quality by enzymatic reaction (by the production of sensory compounds like nucleotides and volatile amine compounds) and by direct changes in protein structure that alter tissue properties like juiciness, toughness, gel, emulsion forming ability and water holding capacity. Chewiness is another function of hardness, cohesiveness and springiness of food (Bourne, 1979). It is another important criterion affected by protein structural changes. Toughness is the most critical quality parameter of tissue. Muscle toughness is a complex property and depend upon the two structural proteins namely connective tissue and myofibrillar protein that give the tissue its mechanical property. Each of the structural components of the connective tissue makes a distinct contribution to

the overall toughness of the meat. The quantity and strength of connective tissue determine the toughness of meat (Hatae et al., 1986). Sato et al. (1986) and Mizuta et al. (1994) reported a close relationship between total collagen content and the raw meat firmness for fish and crustacean muscle. Sato (1988) observed that the total collagen content in the white fish muscle significantly varied with species, and texture of raw meat was affected by the collagen content in the tissue. Hatae et al. (1986) reviewed the possibilities of the collagen as functional material. Culler et al. (1978) observed that tenderness does not vary significantly with soluble form of collagen. Proteolysis also alters the association of the muscle fibers and their interaction between protein and water molecules that might account for tenderness and rheological changes in the muscle (Dunajski, 1979).

4.2.3. Freezing and cooking of muscle tissue

Davey and Gilbert (1974) investigated the effect of cooking temperature on protein – protein interactions, enzyme hydrolysis and textural quality and observed that at temperatures between 55°C and 85°C some tenderizing takes place that could be due to alkaline protease activity. Dunajsky (1979) reported that textural properties of cooked fish meat depend primarily on the state of myofibrillar proteins. He also indicated that the texture of cooked meat was affected by gelatin derived from the muscle collagen. In heat – processed fish meat Sato et al. (1986) observed that texture of cooked meat was affected by gelatinization derived from the muscle collagen on the basis of the fact that cooked meats of species with high collagen content tended to show more elastic texture than those of species with relatively low collagen. This indicates that muscle collagen have an important function in the textural changes of fish muscle tissue during heat processing. Collagen binds the water in blocks of fish mince during frozen storage (Kagawa et al., 2002). They observed that heat denaturation of collagen commenced before 30°C and were substantial at 40°C.

4.3. MATERIALS AND METHODS

4.3.1. Raw material collection and sample preparation

The samples of rohu (*Labeo rohita*), squid (*Loligo duvaucelli*) and shark (*Scoliodon sorrokawah*) were collected and prepared as detailed in 2.3.1 and 2.3.2.

4.3.2. Organoleptic evaluation

A team of 6 panel members performed the sensory evaluation of the samples and a hedonic scale of 7 – point was used for assessment (Borderias et al., 1983). The selected characteristics were tested as defined by Jowitt (1974). The Performa 1 and 2 for the sensory evaluation is given in Appendix 4.1 and Appendix 4.2. The different textural properties evaluated were wateriness (release of water on compression), firmness (force required to compress the material between the molars or between the tongue and palate), elasticity (rubbery mouth – feeling), cohesiveness (extent to which a material could be deformed before it ruptures), juiciness (feeling of liquid in the mouth after chewing 3 to 4 times) and hardness (force required through biting through the sample). The sensory panel also recorded the sensory descriptions of the samples (odor, color flavor, touch and overall acceptability scoring) using 7 – point hedonic scales. Five replicates of each sample were considered. Fig 4.1 illustrates the sampling sites for the cuboids samples   (2 cm^3) used for sensory and instrumental texture analyses. Filleting was done anterior end along the dorsal side and the samples were taken from the middle part of the fillet.

Mechanical and sensory evaluation
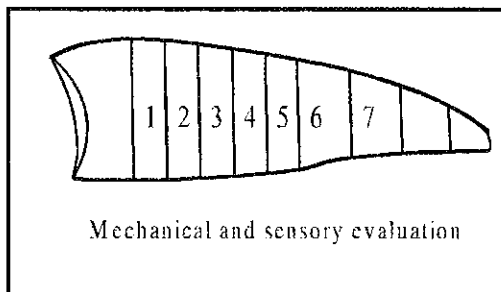
Fig 4.1 Sampling for mechanical and sensory evaluation

4.3.3. Texture profile analysis

Instrumental texture profile analyses of samples were done using Texture Analyzer (Lloyd Instruments, UK, model LRX PLUS) according to Bourne (1978). During measurement a small flat – faced cylindrical probe of 50 mm diameter compressed the bite size of fish 2 cm^3 twice in a reciprocating motion. The test speed and trigger force were standardized to 12 mm/min and 0.5 kgf respectively. It imitated the action of the human jaw resembling the two times reciprocating motion involving the repeated compression of the sample to its original height between two parallel surfaces and recording force versus displacement. From the force – time curve various textural parameters like hardness, cohesiveness, springiness and stiffness were evaluated since they were statistically significant. Five replicates of measurements were taken for each sample. The maximum force required for the first and second compression denoted the hardness 1 and hardness 2, and the ratio of the area under the second cycle of compression curve to the area under first cycle compression curve determined the cohesiveness (Bourne, 1978). A typical force – time curve is shown in Fig 4.2.
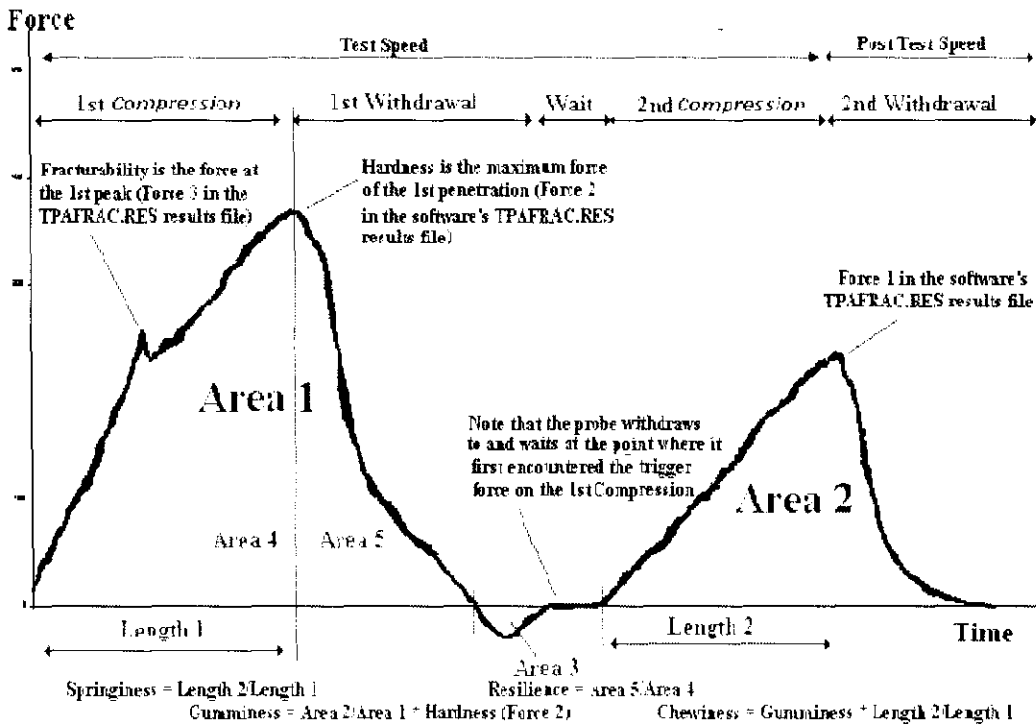
Fig. 4.2 Plot showing a typical force – time curve of texture profile analysis

4.3.4. Statistical analysis

Three–way analysis of variance (ANOVA) as a function of storage time, temperature and species was carried out using the generalized linear model procedure. The differences of means between pairs were resolved by means of confidence intervals using least significance range test. The level of significance was set at $p < 0.01$ and $p < 0.05$ (Snedecor and Cochran, 1989). The SPSS 10.0 for Windows was used to evaluate the correlation between the structural proteins and texture profile parameters.

4.4. RESULTS

4.4.1. Organoleptic evaluation

Hardness and juiciness showed significantly high difference ($p < 0.01$) with temperature, species and frozen storage period. Elasticity and cohesiveness also showed highly significant difference ($p < 0.01$) with temperature and species while firmness showed significant difference ($p < 0.01$) with species and frozen storage period (Appendix 4.2 to 4.7). The overall scoring (table 4.1) showed that the three species showed first phase of hardening at 50°C. At 70°C the samples showed second phase of hardening with maximum juiciness. A slight variation in the second phase of hardening during freezing was observed in the frozen samples that could be possibly due to the differential freeze denaturation of the structural proteins.

Table 4.1. Overall acceptability scoring of fresh and frozen samples of rohu, squid and shark cooked at different temperatures

| Temperature (°C) | Rohu | | | Squid | | | Shark | | |
| | | | | Months of frozen storage | | | | | |
	0	3	6	0	3	6	0	3	6
45	4	4	3	5	5	5	4	4	3
50	5	4	3	5	5	5	5	4	3
55	5	4	4	5	5	5	5	4	4
60	6	4	4	6	6	6	6	4	4
65	6	5	4	6	6	6	6	5	4
70	5	5	5	7	7	6	5	5	5
75	5	5	5	7	7	7	5	5	5
80	5	5	5	7	7	7	5	5	5
90	5	5	5	6	7	7	5	5	5

0 - Fresh, *3* – Three months, *6* – Six months, n - 5.

4.4.2. Texture profile analysis

The results of texture profile analysis with respect to Hardness 1, Hardness 2, Cohesiveness, Springiness and Stiffness are given in tables 4.2 to 4.6 and their ANOVA in Appendix 4.8 to 4.13. Fresh unfrozen samples showed appealing texture at 70°C (Table 4.1 to 4.5). After three months of freezing rohu showed slight variations and optimum temperature was observed at 65°C, 75°C and 80°C for shark and squid, respectively. With further storage this again showed variation at 75°C, 70°C and 75°C for rohu, shark and squid, respectively. In rohu, muscles were softer and softening occurred earlier than squid and shark probably because cultured fishes were fed well and their protein turnover rate was faster than wild indicating a possibility of proteolytic activity higher in cultured fish muscle (Hatae et al., 1986). Statistical analyses of hardness 1, hardness 2, springiness and stiffness showed high significant variation ($p < 0.01$) between temperatures. Fracture force, springiness and adhesiveness showed significant variation ($p < 0.01$) between frozen storage periods and the springiness and adhesiveness showed significant variation ($p < 0.01$) between species. Cohesiveness was high at 50°C probably due to denaturation of collagen. At 70°C myofibrillar proteins that with hold the water molecules with in the tissue also

influenced cohesiveness. An increase in the opacity of fish flesh during cooking was observed that could be probably due to the precipitation of thermally denatured sarcoplasmic proteins beginning at 45°C (Karthikeyan et al., 2004).

Table 4.2. Hardness 1 of fresh and frozen samples of rohu, squid and shark cooked at different temperatures

Temperature (°C)	Rohu			Squid			Shark		
				Months of frozen storage					
	0	*3*	*6*	*0*	*3*	*6*	*0*	*3*	*6*
raw	4.71	5.12	5.22	6.48	4.89	2.3	5.13	4.33	5.63
45	2.51	4.61	7.61	2.73	4.47	1.97	0.89	5.42	4.98
50	**4.99**	5.86	3.26	**11.53**	3.49	1.87	**4.33**	6.41	3.65
55	3.82	3.27	2.99	2.03	3.56	1.34	0.97	2.42	1.83
60	4.33	2.02	2.04	1.9	1.73	2.13	0.87	1.56	1.21
65	1.2	2.14	1.4	2.13	1.84	4.36	0.95	1.51	1.89
70	**2.17**	1.61	1.99	**8.11**	1.51	2.22	**1.93**	1.65	1.76
75	1.26	1.37	2.3	1.9	1.77	6.15	1.1	1.75	1.3
80	1.22	1.26	1.28	1.79	2.04	1.7	1.04	1.37	2
90	2.16	1.45	1.31	5.8	1.97	2.41	2.04	1.53	1.58

0 - Fresh, *3* – Three months, *6* – Six months, n - 5.

Table 4.3. Hardness 2 of fresh and frozen samples of rohu, squid and shark cooked at different temperatures

Temperature (°C)	Rohu			Squid			Shark		
				Months of frozen storage					
	0	*3*	*6*	*0*	*3*	*6*	*0*	*3*	*6*
raw	3.77	3.88	3.6	3.87	3.53	4.56	5.03	3.56	1.5
45	2.04	3.49	5.14	0.72	4.4	3.81	2.12	3.28	1.15
50	4.16	4.54	2.56	3.19	5.08	3.12	9.68	2.56	1.21
55	3.02	2.65	2.42	0.77	1.97	1.49	1.45	2.64	1
60	3.2	1.53	1.6	0.72	1.22	0.99	1.45	1.34	1.64
65	1.02	1.68	1.04	0.8	1.18	1.58	1.63	1.45	3.4
70	1.87	1.27	1.56	1.69	1.31	1.46	7.13	1.19	1.89
75	0.96	1.09	1.82	0.92	1.35	1.07	1.51	1.46	5.17
80	0.99	0.95	0.98	0.89	1.08	1.75	1.43	1.72	1.41
90	1.92	1.18	1.04	1.76	1.24	1.29	5.23	1.62	2.1

0 - Fresh, *3* – Three months, *6* – Six months, n - 5.

Table 4.4. Cohesiveness of fresh and frozen samples of rohu, squid and shark cooked at different temperatures

Temperature (°C)	Rohu			Squid			Shark		
				Months of frozen storage					
	0	*3*	*6*	*0*	*3*	*6*	*0*	*3*	*6*
raw	0.21	0.14	0.1	0.13	0.11	0.05	0.13	0.2	0.18
45	0.19	0.12	0.11	0.15	0.11	0.03	0.1	0.22	0.21
50	**0.26**	0.19	0.17	**0.17**	0.12	0.08	**0.14**	0.23	0.31
55	0.22	0.17	0.25	0.11	0.12	0.13	0.1	0.18	0.17
60	0.16	0.13	0.15	0.17	0.15	0.22	0.11	0.14	0.13
65	0.24	0.12	0.11	0.19	0.14	0.46	0.15	0.15	0.26
70	**0.26**	0.14	0.15	**0.31**	0.14	0.34	**0.28**	0.16	0.22
75	0.13	0.12	0.16	0.21	0.24	0.82	0.18	0.18	0.15
80	0.14	0.12	0.13	0.25	0.29	0.27	0.19	0.14	0.29
90	0.29	0.18	0.16	0.33	0.29	0.4	0.29	0.16	0.21

0 - Fresh, *3* – Three months, *6* – Six months, n = 5.

Table 4.5. Springiness of fresh and frozen samples rohu, squid and shark cooked at different temperatures

Temperature (°C)	Rohu			Squid			Shark		
				Months of frozen storage					
	0	*3*	*6*	*0*	*3*	*6*	*0*	*3*	*6*
raw	0.63	0.53	0.43	0.44	0.32	0.08	0.48	0.7	0.43
45	0.75	0.5	0.45	0.16	0.29	0.08	0.11	0.87	0.64
50	**0.86**	0.71	0.66	**0.58**	0.29	0.11	**0.48**	0.76	0.81
55	0.75	0.67	0.78	0.13	0.31	0.15	0.14	0.58	0.38
60	0.61	0.34	0.57	0.15	0.22	0.24	0.12	0.5	0.31
65	0.45	0.4	0.35	0.21	0.22	0.58	0.17	0.48	0.59
70	**0.62**	0.43	0.55	**0.74**	0.2	0.33	**0.53**	0.58	0.6
75	0.35	0.33	0.65	0.19	0.34	1.04	0.24	0.55	0.4
80	0.3	0.32	0.5	0.21	0.43	0.21	0.24	0.5	0.68
90	0.84	0.41	0.42	0.65	0.41	0.47	0.6	0.53	0.42

0 - Fresh, *3* – Three months, *6* – Six months, n = 5.

Table 4.6. Stiffness of fresh and frozen samples rohu, squid and shark cooked at different temperatures

Temperature (°C)	Rohu			Squid Months of frozen storage.			Shark		
	0	*3*	*6*	*0*	*3*	*6*	*0*	*3*	*6*
raw	6.28	6.34	6.37	16.74	9.15	11.44	6.6	5.45	11.91
45	1.57	4.89	4.65	12.93	10.28	6.49	2.76	5.64	7.31
50	**4.46**	6.05	2.77	**18.23**	6.54	15.43	**5.09**	6.81	3.85
55	3.82	3.04	2.97	7.47	6.16	3.3	3.28	3.55	4.38
60	3.94	3.82	1.68	6.28	3.32	14.48	3.04	1.68	2.57
65	1.83	3.69	1.63	12.62	3.64	19.15	4.18	1.29	2.9
70	2.46	2.37	2.22	**13.44**	2.99	7.29	**8.57**	1.93	3.05
75	5.52	2.74	1.92	6.08	3.14	16.63	3.85	3.95	1.52
80	2.06	5.87	1.04	4.23	3.2	5.75	3.36	1.26	2.83
90	2.47	2.38	2	10.67	4.48	15.79	2.94	1.8	1.89

0 - Fresh, *3* – Three months, *6* – Six months, n = 5.

The collagen and myofibrillar protein fraction on statistical comparison with texture profile parameter indicated that these proteins have a profound role in the textural properties. On statistical analysis using SPSS 10.0 for Windows, correlation between myofibrillar proteins and collagen with selected texture profile parameters were carried out in all the three species. Results indicated that collagen significantly influenced the cohesiveness of the muscle tissue at 50°C ($p < 0.01$). Myofibrillar proteins and collagen collectively influenced the springiness at 50°C. At 90°C myofibrillar protein solely influenced the texture. In frozen samples the influence of collagen was higher at lower temperatures (Appendix 4.14).

4.5. DISCUSSION

Freshness of fish is the most important single criterion for judging the quality of majority of fishery products (Howgate, 1982). Texture varies with species and postmortem storage (Ando et al., 1991). It is a unique quality attribute of meat and meat products and is influenced by many factors (Love, 1979). Tenderness of meat might be defined as a case

of cutting meat fibers with teeth during mastication. The major contributors to meat toughness are muscle fibers. In raw meat the textural parameters were high and at 45°C, it decreased considerably probably due to dissociation of actin – myosin complex (Egelandsdal et al., 1995) and denaturation of myosin tail. At temperatures less than 45°C, dissociation of some myofibrillar components takes place. The conformational changes of these proteins had no significant contribution to rheology initially (Xiong et al., 1999). It is postulated that partial unfolding of the protein structure initiated by the dissociation of myosin light chain subunits from heavy chains may lead to inter – filamental association of myosin and formation of a three dimensional structure.

The third transition at 50°C suggested more rigidity of protein matrix formed probably attributed to the formation of irreversible gel networks. At 70°C (for fresh samples) the hardness was nearly half of the uncooked fresh sample. Shark (70°C) showed high stiffness probably due to higher cross-links between protein aggregates and deposition of denatured protein in the existing protein networks leading to the strengthening of gel matrix. In case of frozen samples this temperature shifted to 75°C and 80°C for shark and rohu, respectively.

Cooking played a significant role in affecting the textural properties of muscle tissue. Certain typical differences were observed between the behavior of the myofibrillar proteins and collagen at elevated temperature. At 60°C collagen fibers become solubilised thus textural changes in flesh at higher temperatures were related to heat denaturation of the myofibrillar protein (Dunajski, 1979). Stanley and Hultin (1982) indicated that frozen storage brought a slight increase in hardness. The sensory quality of cooked squid depends significantly on the characteristics of the raw material. The plot of texture profile analysis showed a considerable depression between raw to 50°C and was typical of gelation of squid muscle. It could be possibly that during gelation myosin molecules undergo partial denaturation followed by irreversible aggregation of myosin heads through formation of disulphide bonds and helix – coil transition of the tail part of the molecules, resulting in a three – dimensional network (Niwa, 1992 and Stone and Stanley, 1992). Modifications of these intermolecular interactions of protein are beneficial for alteration of the physical attributes. Heat induced gelation of myofibrillar proteins is an important functional property. The formation of protein network in the gel contributes to unique textural characteristics

and to the functional properties of the product. Springiness and fracture force are inversely related.

Post mortem storage of squid and shark for different time intervals showed a rapid loss in muscle hardness and a slower resolution of rigor stiffness. High level of proteolytic activity at low temperatures and high collagen content could be the principal factor to trigger elasticity of these muscle tissues. The collagen of the squid muscle was observed to undergo denaturation at 55°C. Comparable thermal denaturation temperature was also reported for collagen in rohu and shark. Maximum rate of tenderization for shark and rohu were observed at 60°C – 65°C that could be due to alkaline proteolytic enzymes cathespin c. This slightly shifted in shark and rohu to 65°C and 80°C with freezing (Makinodan et al., 1987). It was proposed that a significant part of collagen retained the original triple helical configuration and deformation of collagen helical structure (thermal denaturation) completed before the myofibrillar denaturation as this would have interfered with the latter.

The contribution of connective tissue to cooked meat texture is a function of concentration of muscle collagen (amount) and degree of covalent cross linkages in collagen. Though the collagen contributed a minor fraction to the total protein content in the fish meat they are greatly involved in holding together the muscle bundles (myotomes) and therefore, contributed to the overall texture of the meat. Collagen contributes to the toughness of meat and was found to be the major protein to influence the texture of fish meat and other properties like gelling, emulsification, and elasticity. Collagen accordingly has a significant influence on the functional and rheological properties of the meat. Texture that were relatively firm, there existed collagen fibrils of higher density in the pericellular connective tissue. Fish muscles are generally softer with less collagen fibrils that are well arranged. Fish from cooler water have less cross – links than fishes from warmer waters. Upon heating, both endomysial and perimysial collagen shrink and developed tension. Shrinkage and force developed occurred incrementally with every degree of increase in temperature resulting in a biphasic increase in shear force that was observed prior to collagen gelatinization (Davey and Gilbert, 1974).

The functional properties of fish muscle were associated with the ability of the myofibrillar proteins to form a three – dimensional gel patterns upon heating. The thermally induced interaction of fresh fish muscle occurred in three distinct stages, namely setting

(40°C), softening (60°C) and gelation (80°C). It was proposed that setting phenomenon could be due to the hydrophobic interactions; the softening could be related to naturally occurring proteolytic enzymes, and it varied with species and the gelation could be due to the covalent intermolecular cross - linkages between proteins. Aggregation of connective tissue protein occurred during cooking and the proteolytic fragmentation of the connective tissue sheath that might have taken place became a significant influential factor on tenderness of the tissue. Myosin (pre rigor) and actomyosin (post rigor) accounted for most of the gel – forming capacity of the myofibril protein system (Xiong et al., 1999). The rheological properties of collagen and myofibrillar proteins were heavily influenced by the structure, the concentration and extrinsic factors like pH, freezing and cooking.

4.6. CONCLUSION

The changes in fish texture were associated with connective tissue protein and attachments of myofibrils to connective tissue. An increase in temperature above 50°C had an opposite effect on the proteins of connective tissue and myofibrils. Collagen degraded and most of it was being converted to gelatin during first phase of cooking (50°C) and this transition increased the tenderness of the fish muscle tissue. The coagulation and degradation of myofibrillar proteins reduced the toughness. Consequently, different muscle protein reacts differently on cooking. The ultimate textural properties depend largely upon the time – temperature conditions during cooking, freezing and frozen storage. A low correlation was observed between protein solubility in raw tissue and gel texture in cooked fish gels probably resulting from the variations in protein degradation during thermal process. Texture and water holding capacity in cooked fish gel were observed to be interrelated and connective tissue and myofibrillar proteins played the significant role in influencing these properties. Cohesiveness is significantly influenced by collagen. Myofibrillar protein and collagen were found to influence springiness and cohesiveness at 50°C and myofibrillar protein solely influenced the texture at 90°C. Freezing and frozen storage also affected the muscle protein degradation. Collagen probably underwent conformational changes at low

temperatures thereby inducing the effect of cooking on myofibrils at earlier temperature than that observed in fresh samples. Texture profile analysis gives the textural changes in the sample of the species studied during cooking and freezing. The effect of temperature on the structural protein and the musculature pattern could only be confirmed by histochemical analysis of the tissues of the species. Hence, such a study is undertaken in the proceeding chapters.

CHAPTER 5

HISTOCHEMICAL STUDY OF MUSCLE TISSUE WITH EMPHASIS ON COLLAGEN

Chapter 5

HISTOCHEMICAL STUDY OF MUSCLE TISSUE WITH EMPHASIS ON COLLAGEN

5.1. Introduction

5.2. Review of literature

5.3. Materials and Methods

     5.3.1. Raw material collection and sample preparation

     5.3.2. Histochemical studies

5.4. Results

     5.4.1. Freezing and cooking of muscle tissue

     5.4.2. Musculature pattern

5.5. Discussion

5.6. Conclusion

5.1. INTRODUCTION

Histochemistry is an effective tool used for the direct diagnosis of chemical changes occurring in tissues at cellular and sub cellular levels. The normal method of assay in histochemistry involves development of color to flag the biochemical compounds that are used as the biomarkers. In the present study myofibrillar and collagen are used as the biomarkers. The staining techniques developed and employed were sufficient to assess the intensity of reactions during cooking and freezing as evidenced by the histochemical reactions. Essentially the method involves identifying qualitatively and to some extent quantitatively the occurrence of the biochemical markers. Many publications are available that provide information on histochemistry proving the significance of such studies. The method seems to be most reliable in establishing and bringing forth the various changes in the tissue. From the previous chapters it was observed that there were quantitative and qualitative variations in the collagen and myofibrillar protein of the three species that had a significant role on the muscle texture. The distribution and arrangement pattern of these structural proteins could have a key role in determining the texture. Hence histochemical studies of the musculature pattern of the three species under study are essential. In view of this investigations are carried out on the following aspects of the tissue.

1. Histochemical differences in the musculature pattern of rohu, squid and shark with emphasis on collagen.

2. Histochemical changes in the muscle proteins of these species at different heating regimen in both unfrozen and frozen samples.

5.2. REVIEW OF LITERATURE

The histological and histochemical studies were found to be highly beneficial in biology. Histochemical studies were helpful in understanding the content and accumulation of carbohydrates, general lipids, neutral lipids, proteins and RNA in relation to the gonad development and maturation.

The textural and nutritive value of seafood products are duly influenced by the composition and the structure of the muscle proteins (Goll et al., 1977). Firmness of raw

muscle tissue is an important index for freshness detection that were informed to decrease rapidly after death and further storage (Ando et al., 1995) causing quality deterioration. Firmness of raw muscle was found to be directly related to the content of collagen. In addition, it has been established that weakening of pericellular connective tissue collagen cause post – mortem softening of fish muscle (Sato et al., 1997). Extensive degradation of connective tissue in fish and loss of connective tissue structure was reviewed by Ando (1999). Collectively, these results indicated that most of the changes in fish texture were associated with connective tissue changes and attachments of fibers to connective tissue. Similarly in gaping, deterioration of fish muscle and disintegration of collagen fibrils were reported. Collagen is important in fish muscle firmness but there are limited reports on the effects of fish muscle collagen during frozen storage. There have been a few studies on degradation of collagen during freezing of squid (*Loligo* and *Illex* species). Additionally, collagen fibers and cell membranes were not studied earlier in squid muscle tissue. Therefore, effects of collagen are still unclear in softening of the squid (*Loligo duvaucelli*) muscle.

In fish it was observed that the fine collagen fibrils that arise from myocommata progressively deteriorate during frozen storage (Bremner, 1999). Ando (1999) demonstrated that post mortem tenderization of rainbow trout muscle was due to the disintegration of collagen fibers in the pericellular connective tissues. Ando et al. (1999) reported that with frozen storage the solubility of muscle collagen decreased, probably due to the cleavage of the triple helical region by a collagenase – like enzyme.

Histological observations of cooked prawns revealed the different connective tissue layer at relatively high temperatures (70 to 90°C) although endomysium showed partial disintegration (Nip and Moy, 1988). They also observed that prawn meat became firm and solid with further cooking and was unpalatable when the core temperature reached above 100°C. The heat processing with boiling water made prawn meat firmer. Nip and Moy (1979) reported damages to the tissue during freezing probably due to the formation of ice crystals between the fibers. Nip and Moy (1988) examined the various changes in the appearance of prawn meat during heat processing with macroscopic changes. For prawn meat processed at 90°C separation between meats was observed that could be due to the

excessive shrinkage of the muscle protein. Deformation value tended to increase during heat processing showing high values at 70°C and 90°C in contrast to raw meat.

A considerable amount of studies concerning the effects of temperature on tenderization of muscle food has been performed and reported by many scientists (Davey and Gilbert, 1976 and Bouton et al., 1975) of which most of the reports were related to red meat and mammalian muscle and only little work has been done on thermally induced changes on texture of fish and fishery products.

5.3. MATERIALS AND METHODS

5.3.1. Raw material collection and sample preparation

The samples of rohu (*Labeo rohita*), squid (*Loligo duvaucelli*) and shark (*Scoliodon sorrokawah*) were collected and prepared as detailed in 2.3.1 and 2.3.2 for the following analysis.

5.3.2. Histochemical studies

Reagents used:

☞ **Bouin's fixative**

Saturated picric acid solution: 75 ml

Formalin (40%): 25 ml

Glacial acetic acid: 5 ml

☞ **Weigert's iron – haematoxylin stain**

Solution A: A 1% solution of haematoxylin in absolute alcohol.

Solution B: Distilled water – 100 ml

Ferric chloride (30%) – 4 ml

Conc. HCl – 1 ml

These are stock solutions. They were mixed at the time of use. The stain was prepared freshly every day. Before use, one part of Solution A was mixed with one part of Solution B and 2 parts of distilled water was added. It is differentiated very slowly by picric acid and is therefore ideal for using with Van - Geison's staining method.

☞ **Van Geison's stain**

This consists of saturated solution of picric acid in water with 5 ml of a 1% aqueous solution of acid fuschin added to each 100 ml. It stains collagen fibers of connective tissue to bright red; and muscle fibers and epithelium to yellow.

The histochemical analysis was done according to Ando et al. (1999). 1 cm thick samples were fixed in Bouin's fixative for a period of 24 h. The tissues being pliable were larger in size during first fixation and were cut into small pieces after ½ h of soaking. It was then then left in the Bouin's solution for the remaining period. After fixation they were passed through a series of alcoholic dilutions (70%, 96% and absolute alcohol). The tissues were kept in 70% alcohol for a period of 24 h followed by serial transfer to 80% alcohol where the samples were kept for 30 min with two successive changes of the solution. Followed by this the samples were passed to 96% alcohol (30 min with two changes) and absolute alcohol for 20 min (with two changes). The dehydration was completed by a final dip in acetone for 1 min.

After complete dehydration, the samples were passed to xylene till they appeared transparent. The samples were treated with xylene and then passed to xylene – wax mixture (3:1 and 1:1) sparing 5 min at each step. This was for the infiltration of wax into the tissue cavities. After infiltration using xylene: wax mixture, the tissues were infiltrated further using pure wax with two changes for 10 min. The tissues were then embedded in paraffin wax with ceresin (congealing point about 60°C) using L – blocks. The blocks were then sectioned to a thickness of 8 μm using microtome (SIPCON SP 1120 Rotary Microtome, India). The sections were fixed on clean dry slide using glue and dried before further staining.

The slides were dipped in xylene in a decreasing series of alcohol and were stained using Weigert's – Haematoxylin stain for 5 min with an acid: alcohol wash for 30 sec. They were then passed to Van Geison's stain for 5 min and dehydrated through a series of alcohol for 10 min followed by xylene for another 10 min. After staining the permanent slides were made using DPX mountant. The photographs of the sections were taken using camera attached light microscope (Nikon Eclipse E 200 compound light microscopes fitted with Nikon DN 100 Digital Net Camera).

5.4. RESULT

5.4.1. Freezing and cooking of the muscle tissue

Plates 5.1 to 5.18 show the photomicrographs of the samples. Though they are only a very small area of a section, they aid in portraying the post mortem histochemical changes in the tissue. In the muscle sections of fresh samples (plate 5.1, 5.7 and 5.13) the intrinsic conditions are good, the myofibril bundles are regular, closely packed and the cellular architecture is complete with only few extra cellular spaces. In the frozen stored samples (plate 5.3 to 5.6, 5.9 to 5.12 and 5.15 to 5.18) the sections showed large tissue voids probably due to the crystallization of tissue water and ice formation leading to cell structure disruption. This increases with extended period of frozen storage. There exists depletion in protein resulting in low water binding capacity causing the release of water into the surrounding tissue where it accumulates. The myofilaments are bathing in the sarcoplasmic fluid and gathered into myofibrils. In the cross – section these fibrils are of greatly varying sizes and shapes that varied with species. Between fibrils the sarcoplasmic reticulum forms inter – connected vesicles. Sarcolemma, the outer layer often disintegrates and plasmalemma split off from fiber. Fibers are separated by large spaces containing fragmentary and precipitated material. Sarcoplasmic reticulum that was swollen in fresh sample were disintegrated and transformed into smaller ones. Apart from contraction, the predominant change was the gradual increase in permeability of membraneous structures with frozen storage. Upon freezing the ammoniacal odor of the shark tissue was reduced comparatively making the tissue more preferable.

During cooking the collagenous tissue acquired a granular appearance and the fibers fell apart in rohu. Sometimes frayed pieces of collagen were found over the surface of these sections (plate 5.3 to 5.6 and 5.15 to 5.18). Waves and zig – zag pattern were seen in the sections of cooked meat at higher temperatures. These passive contractions could be due to the active contraction of adjoining fibers and the contraction of collagen. The tissue heated at 40° to 60°C (plate 5.1, 5.7 and 5.13) had a wide intercellular gap region with high frequency that might be due to thermal denaturation and shrinkage of intercellular protein and dehydration of muscle fibers. The thin connective tissue of endomysium and

perimysium in muscle tissue heated at 55 to 60°C were observed to be thin although there were separations from the muscle fibers. The tissue heated at 70 to 90°C (plate 5.2, 5.8 and 5.14) showed images of remarkably reduced muscle fibers that shrink and gather in the gap region that were frequent around the muscle fibers. This could be due to the rapid effect of temperature on tissue during cooking at high temperatures. Structure of endomysium in the three species became quite unclear in the tissues cooked at 70 to 90°C although the perimysium was still evident. Structure of epimysium was also maintained. Shark has high collagen content compared to rohu and squid. On cooking collagen was converted to gelatin. At 50°C, since the connective tissue was distorted, the tissue became tender. At 70°C shark muscle tissue showed appealing texture probably due to the gelatin from collagen that held the myotomes together probably retaining the nutritional quality. Unlike rohu where tissue structure was completely distorted at 90°C, shark gelatin was able to hold together the muscle cells.

5.4.2. Musculature pattern

In rohu and shark the connective tissue consisting of collagen formed myocommata sheath that enveloped each myotome bundle and help in holding the myotome bundles together. The musculature pattern of the squid mantle tissue differed from that of rohu and shark as they are composed of several layers of fibers running transversally to each other and are covered with several sheets of connective tissue (plate 5.7). Muscle fiber layer of the mantle made up to about 98% of the mantle thickness. They were observed to consist of 0.1 – 0.2 mm thick bands with fibers running circumferentially sandwiched between thinner sheets of radial fibers. Four sheets of connective tissue cover the muscle fibers: the inner tunic and the visceral lining on the inner side of the body cavity and the outer tunic and outer lining beneath the skin. Just under the skin proper there were pigment cells containing dark red and brown melanin pigment cells, together with the skin.

5.5. DISCUSSION

Connective tissue of the fibrous structures primarily, consisting of collagen, could be categorized into epimysium, perimysium and endomysium as in rohu and shark that

differed from each other in being morphologically discrete collagen depots. Epimysium is the heavy sheath of connective tissue surrounding individual muscle tissue that thickens at both origin and insertion. Endomysium encircles individual muscle fiber and were found to superimpose the basement membrane, perimysium is the thin layer enveloping muscle bundles and muscle fibers (myofibers) and endomysium is the fine layer encasing the muscle fibers. Epimysium is normally removed while de – skinning and only the perimysium and endomysium connective tissues are suggested to be involved in the realistic toughness of meat tissue. Perimysium and epimysium comprise the intracellular connective tissue and were not separable in rohu and shark. The bulk (90%) of intracellular connective tissue consisted of perimysium and was probably the main contributor to meat texture (Light et al., 1985). In squid, perimysium was not clearly distinguishable. The connective tissues of the squid tunics had high collagen content (Otwell and Hamann, 1979). With its complex muscle orientation and high collagen content raw squid was relatively tough and possessed rubbery texture. The muscle patterns of squid (*Loligo duvaucelli*) are comparable with the results of Llorea et al. (2001).

Freezing could alter the quality of frozen samples in comparison to the unfrozen fresh products. Physical and chemical changes in proteins that occur during frozen storage result in texture deterioration that negatively affect the functional, nutritional and sensory properties of the product. Studies showed that fiber separation occurred with increased period of frozen storage. The sample texture changed more extensively resulting from partial denaturation of proteins. Myosin possibly unfolds in frozen samples resulting in the exposure of hydrophobic groups that then cause protein aggregation resulting new cross – linkages during frozen storage. This kind of protein – protein interactions led to decreased water holding capacity and increased expressible moisture that is reported in chapter 2. The variations are in agreement with the findings of Xiong and Mikel (2001). Enzymatic activities can partially unfold the myosin in frozen – thawed samples as observed in rohu and shark (Shenouda, 1980). These interactions during frozen storage cause an increase in extracellular volume with fibers moving apart. On freezing, the ammonia – like components of urea in shark tissue were lost either due to leaching or through evaporation, rendering the shark muscle tissue more preferable. The voids formed in the

shark muscle tissue during freezing were less possibly due to the less spacing of myocommata during ice crystal formation.

Cooking serves dual function of tenderizing the muscle by converting collagen to gelatin, and by toughening due to heat coagulation of the myofibrillar proteins. Hence, the muscle characteristics obviously depend on the relative effects of these two structural components. Diameters of collagen fibrils were smaller in the fishes than in the squid and been focused in as a protein that greatly contribute to meat texture (Olaechea et al., 1993). With frozen storage a partial disintegration of endomysium and perimysium was observed in the muscle, while the structure of the thick connective tissue epimysium was not much changed. On the other hand, a preferential decrease in the relative staining intensity of the components was detected for collagen from the muscle with further cooking which may be due to the conversion of collagen to gelatin. The disintegration of thin connective tissue could be due to some biochemical changes in the components (Ando et al., 1999). It was generally believed that collagen shrinks (toughening effect) at a temperature around 63°C and may be partially transformed to gelatin at higher temperatures that was almost similar to denaturation temperature (60°C) for squid as observed in the present study (Hamm, 1966). The denaturation and the shrinkage temperature for rohu (50°C) and shark (50°C) were further lower. With increasing the temperature from 50°C to 60°C the texture profile parameters decreased in samples than in fresh that was discussed in chapter 4. It could be due to additional factors involved in the cooking effect at these temperatures than just changes in connective tissue alone (Bouton et al., 1975). Shark has high collagen content that was converted to gelatin upon cooking (70°C) and held the muscle cells. It also helped in retaining the nutritional quality of the tissue. Even at 90°C the tissue structure was comparatively maintained than that of rohu. Epimysium was maintained in the tissue, though perimysium and endomysium showed slight denaturation with increased temperature of cooking.

Davey and Gilbert (1976) reported that certain proteolytic enzyme cathepsin C that is relatively heat stable could also support tenderization effect at 60°C. Rohu and shark cooked at 45°C and above 70°C had higher gel strength than at 60°C possibly due to muscle alkaline protease that was found in the fish flesh (Makinodan et al., 1963). The extent of protein – protein interaction in rohu actomyosin probably increased with increasing

temperature. The rate increases sharply with temperature increase from room temperature to 45°C. When heated at various temperatures ranging from 55°C to 80°C, the toughening increased initially and then decreased resulting in softening of the tissue. For meat processed at 90°C separation between meats was observed that could be due to the excessive shrinkage of the muscle protein. Deformation value tended to increase during heat processing showing high values at 70°C and 90°C in contrast to raw meat. At higher temperature, no tenderizing effect was observed in rohu and shark. This might be because of the loss of enzyme activity and only protein denaturation was having significant effect on texture activity (Deng, 1981). Upon cooking the losses of water from the cut ends of the meat were high. This was accompanied with shrinkage of meat in two phases. In squid at 45 to 60°C the shrinkage was primarily transverse to the fiber axis and at higher temperatures, 60 to 90°C the shrinkage were parallel to the fiber axis. This could possibly explain the toughening of the squid meat when compared to rohu and shark muscle tissue.

5.6. CONCLUSION

The histochemical studies show that the musculature pattern of the squid mantle tissue differed from that of rohu and shark as they are composed of several layers of fibers running transversally to each other. These layers were covered with several sheets of connective tissue. The differences in the stain intensity confirm the decomposition of collagen to gelatin with increase in temperature of cooking thus decreasing the integrity between the muscle cells. Even though the intercellular spaces were narrow and collagen fibrils were scarcely visible in raw tissues they played a significant role in affecting the texture during frozen storage and cooking. The muscle pattern became clearer in frozen stored samples in comparison to raw samples of all the species possibly due to formation of intercellular spaces. Cell detachment occurred during cooking as a result of the decrease in the intercellular integrity due to loosening of collagen fibers probably resulting in the decrease of the binding force between cell membrane and collagen. The voids observed in the raw muscle tissue of squid and shark was less compared to cooked samples and those after frozen storage. The histochemical observations were confirmed the results of texture profile analysis discussed in the previous chapter. The firmness of cooked muscle was
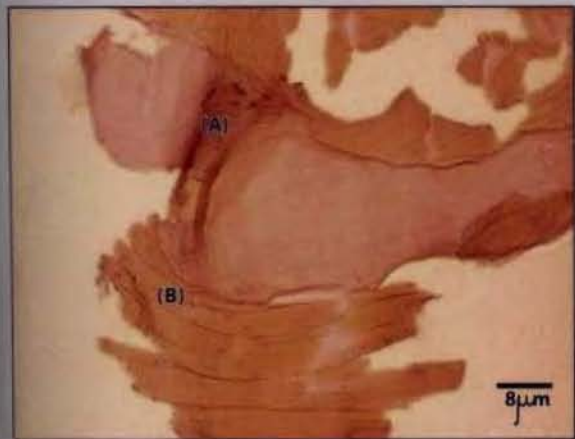
weak but constant in squid and shark and could be explained by considering the occurrence of gelatinization of intercellular collagen. The solubility differences could be due to the differences in the intermolecular cross – links of collagen in the three species studied. The specific influences of connective tissues depend on the thickness, amount of collagen, their density and type of cross – linkages between fibrils. Specific patterns of collagen distribution and its localization in squid mantle have a profound role in textural changes during heat treatments require further studies in this respect.

Plate 5.1 Histographs of fresh samples of rohu, *Labeo rohita* cooked at different temperatures.

(a) *fresh* , (b) 45 °C, (c) 50 °C, (d) 55 °C, (e) 60 °C and (f) 65 °C.

(A)- Collagen, (B)- Myofibrillar protein.

Plate 5.2 Histographs of fresh samples of rohu, *Labeo rohita*
cooked at different temperatures.
(g) 70 °C, (h) 75 °C, (i) 80 °C, and (j) 90 °C.
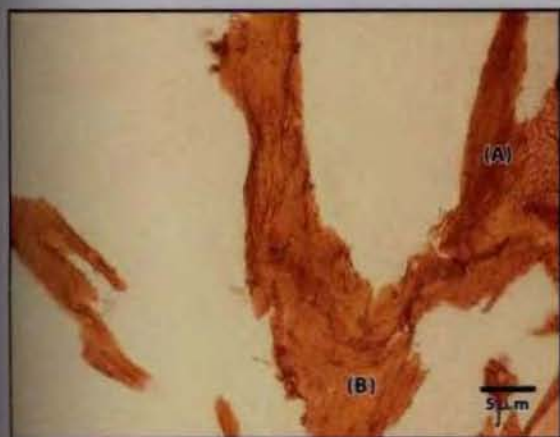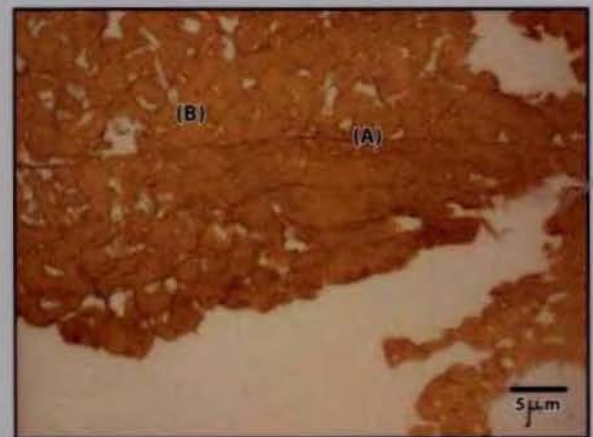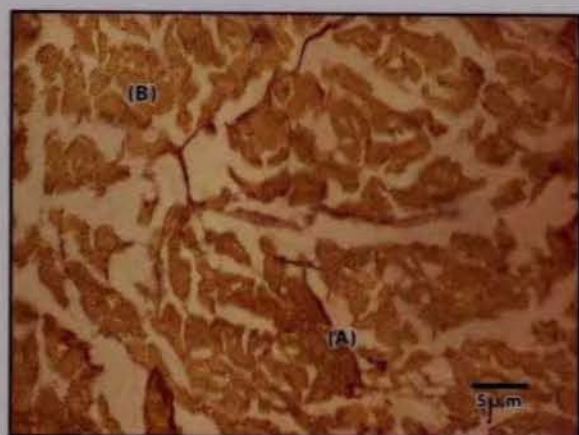(A)- Collagen, (B)- Myofibrillar protein.

Plate 5.3 Histographs of frozen samples (three months) of rohu, *Labeo rohita* cooked at different temperatures.
(a) *frozen(three months)*, (b) 45 °C, (c) 50 °C, (d) 55 °C, (e) 60 °C and (f) 65 °C.

(A)- Collagen, (B)- Myofibrillar protein.
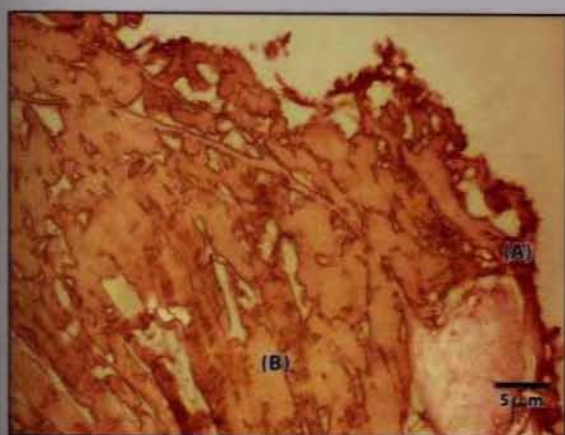
Plate 5.4 Histographs of frozen samples (three months) of rohu, *Labeo rohita* cooked at different temperatures.
(g) 70 °C, (h) 75 °C, (i) 80 °C, and (j) 90 °C.
(A)- Collagen, (B)- Myofibrillar protein.

Plate 5.5 Histographs of frozen samples (six months) of rohu, *Labeo rohita* cooked at different temperatures. (a) *frozen(six months)*, (b) 45 °C, (c) 50 °C, (d) 55 °C, (e) 60 °C and (f) 65 °C.
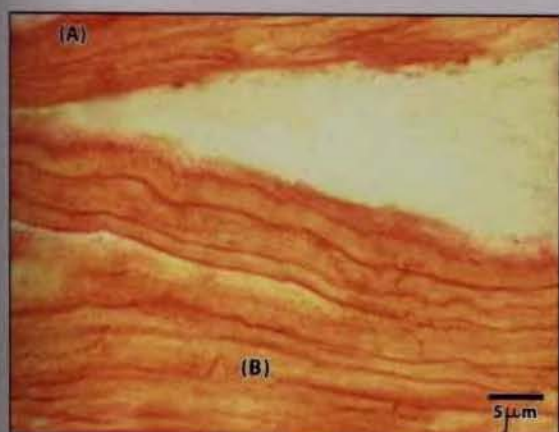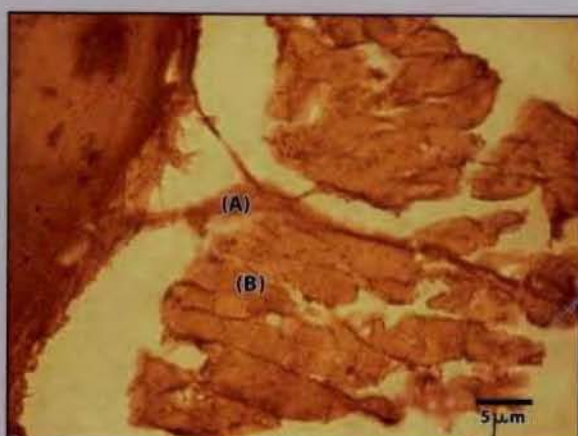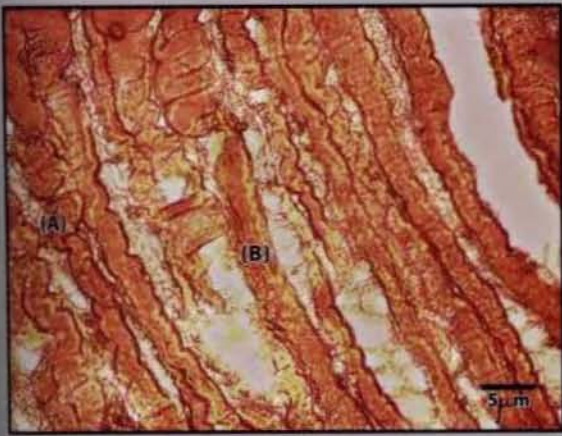
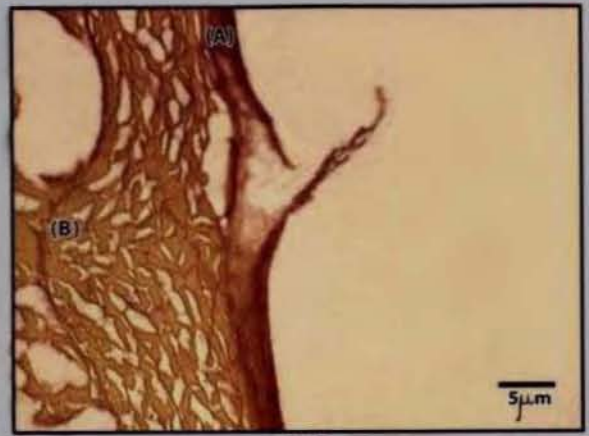(A)- Collagen, (B)- Myofibrillar protein.

(g)

(h)

(i)

(j)

Plate 5.6 Histographs of frozen samples (six months) of rohu, *Labeo rohita* cooked at different temperatures. (g) 70 °C, (h) 75 °C, (i) 80 °C, and (j) 90 °C. (A)- Collagen, (B)- Myofibrillar protein.
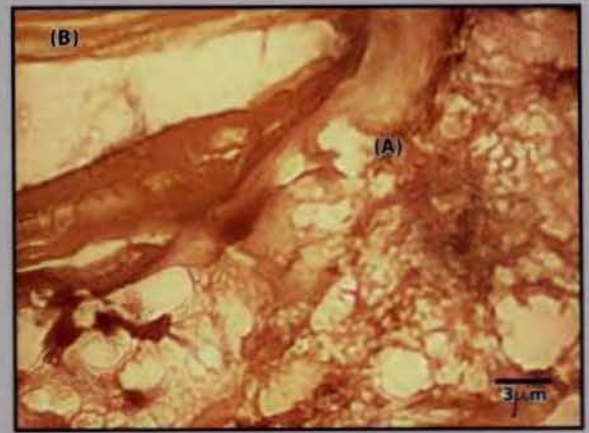
Plate 5.7 Histographs of fresh samples of squid, *Loligo duvaucelli* cooked at different temperatures. (a) *fresh*, (b) 45 °C, (c) 50 °C, (d) 55 °C, (e) 60 °C and (f) 65 °C.

(A)- Collagen, (B)- Myofibrillar protein.
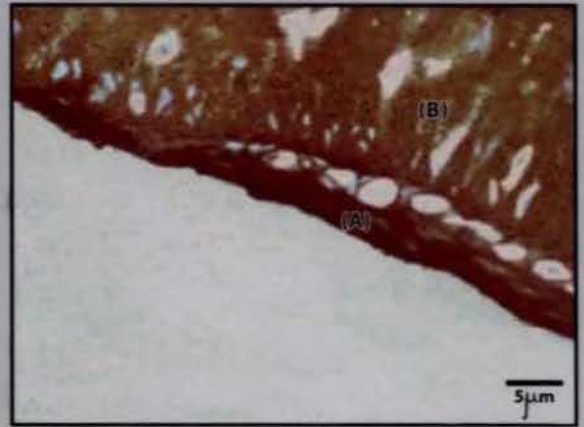
Plate 5.8 Histographs of fresh samples of squid, *Loligo duvaucelli* cooked at different temperatures. (g) 70 °C, (h) 75 °C, (i) 80 °C, and (j) 90 °C. (A)- Collagen, (B)- Myofibrillar protein.

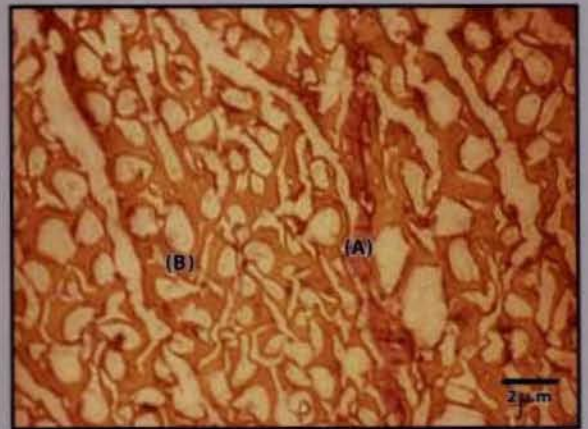Plate 5.9 Histographs of frozen samples (three months) of squid, *Loligo duvaucelli* cooked at different temperatures.
(a) *frozen(three months)*, (b) 45 °C, (c) 50 °C, (d) 55 °C, (e) 60 °C and (f) 65 °C.
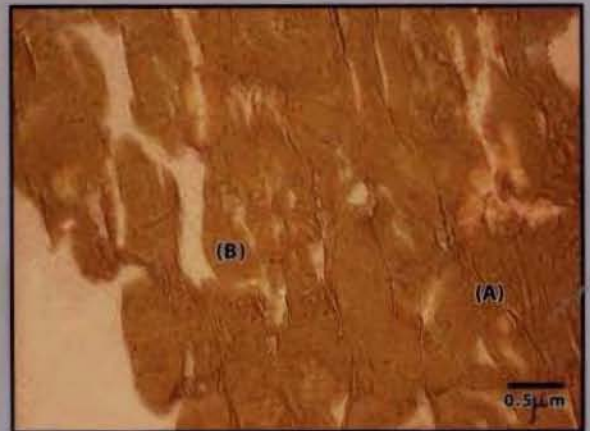(A)- Collagen, (B)- Myofibrillar protein.

Plate 5.10 Histographs of frozen samples (three months) of squid, *Loligo duvaucelli* cooked at different temperatures. (g) 70 °C, (h) 75 °C, (i) 80 °C, and (j) 90 °C.

(A)- Collagen, (B)- Myofibrillar protein.

Plate 5.11 Histographs of frozen samples (six months) of squid, *Loligo duvaucelli* cooked at different temperatures.
(a) *frozen(six months)*, (b) 45 °C, (c) 50 °C, (d) 55 °C, (e) 60 °C and (f) 65 °C.
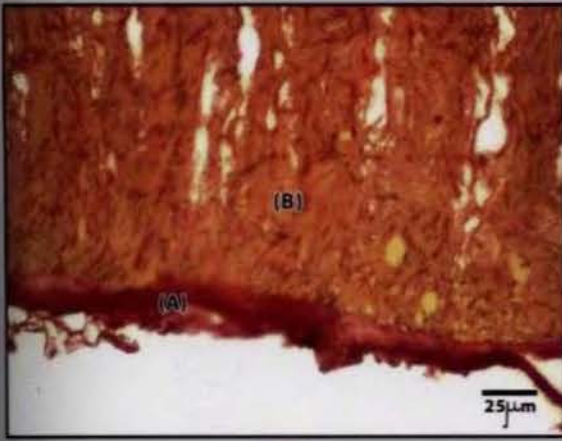
(A)- Collagen, (B)- Myofibrillar protein.

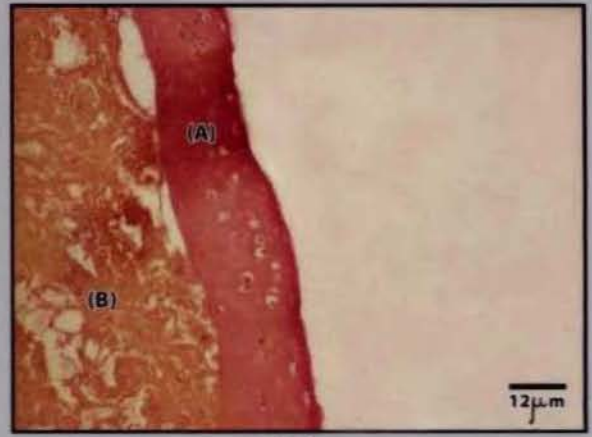**Plate 5.12 Histographs of frozen samples (six months) of squid,** *Loligo duvaucelli* **cooked at different temperatures. (g) 70 °C, (h) 75 °C, (i) 80 °C, and (j) 90 °C. (A)- Collagen, (B)- Myofibrillar protein.**
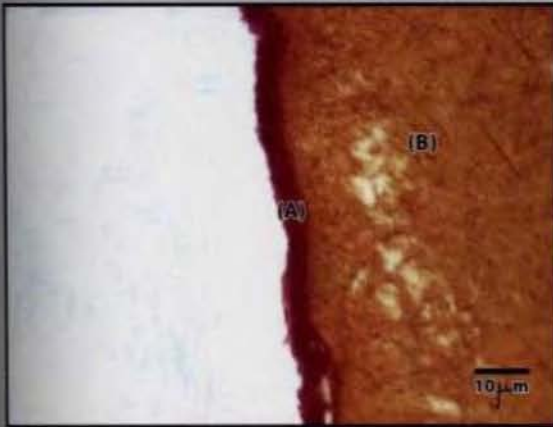
Plate 5.13 Histographs of fresh samples of shark, _Scoliodon sorrokawah_ cooked at different temperatures.
(a) _fresh_, (b) 45 °C, (c) 50 °C, (d) 55 °C, (e) 60 °C and (f) 65 °C.
(A)- Collagen, (B)- Myofibrillar protein.

Plate 5.14 Histographs of fresh samples of shark, *Scoliodon sorrokawah*
cooked at different temperatures.
(g) 70 °C, (h) 75 °C, (i) 80 °C, and (j) 90 °C.

(A)- Collagen, (B)- Myofibrillar protein.
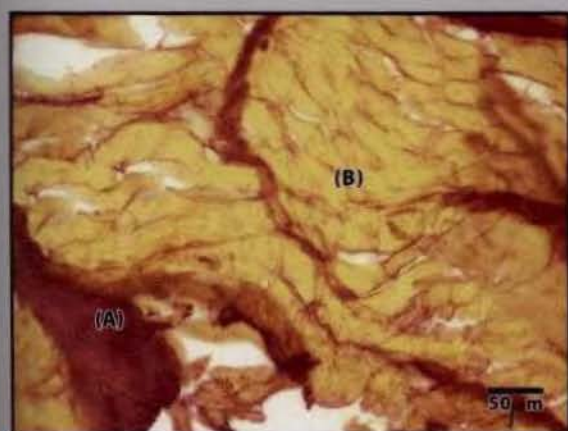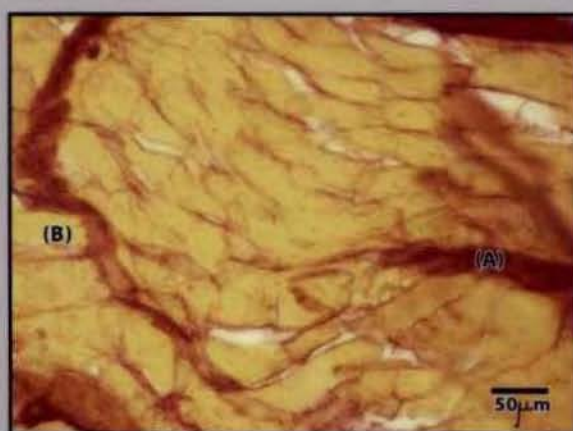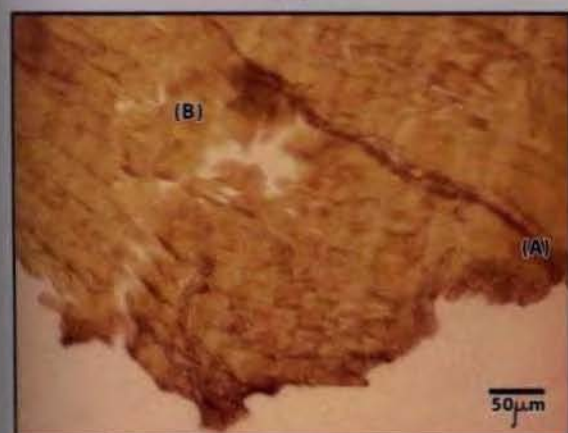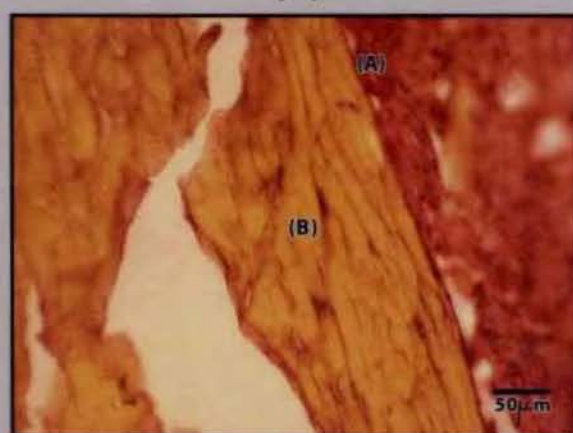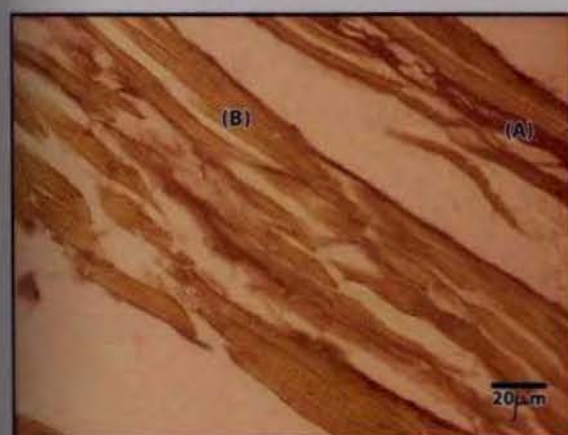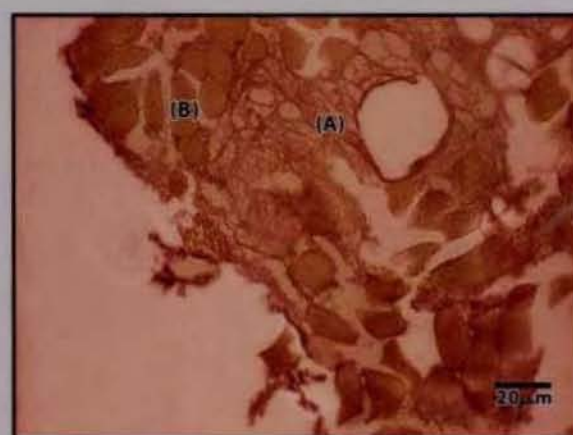
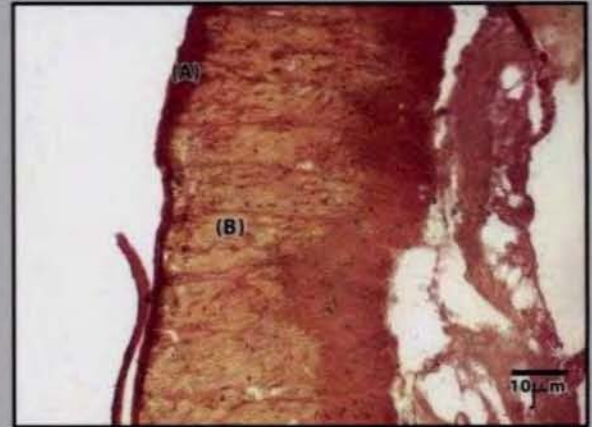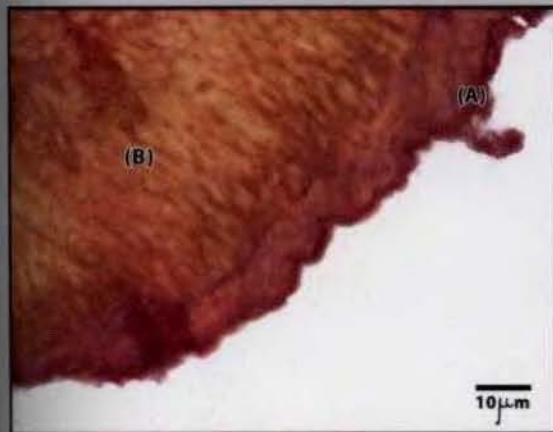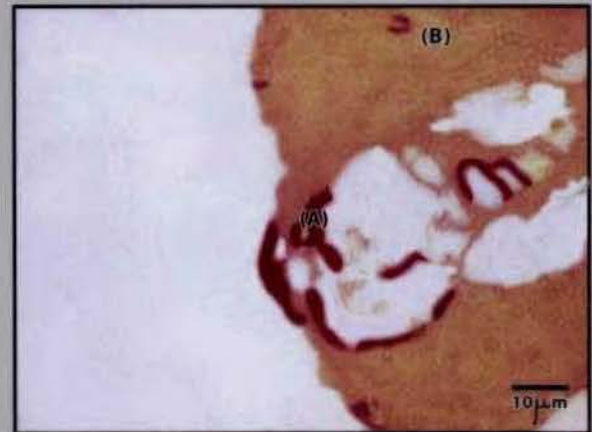**Plate 5.15** Histographs of frozen samples (three months) of shark, *Scoliodon sorrokawah* cooked at different temperatures. (a) *frozen(three months)*, (b) 45 °C, (c) 50 °C, (d) 55 °C, (e) 60 °C and (f) 65 °C.

(A)- Collagen, (B)- Myofibrillar protein.
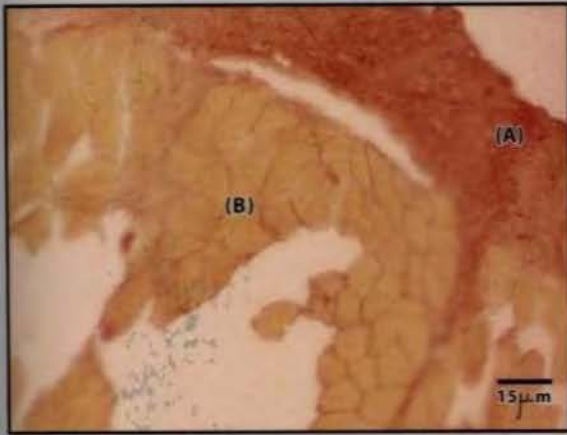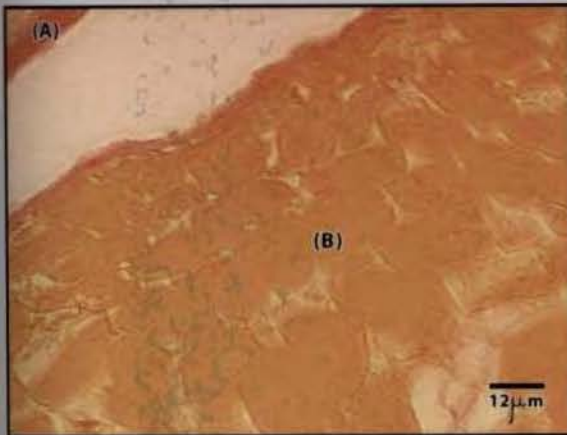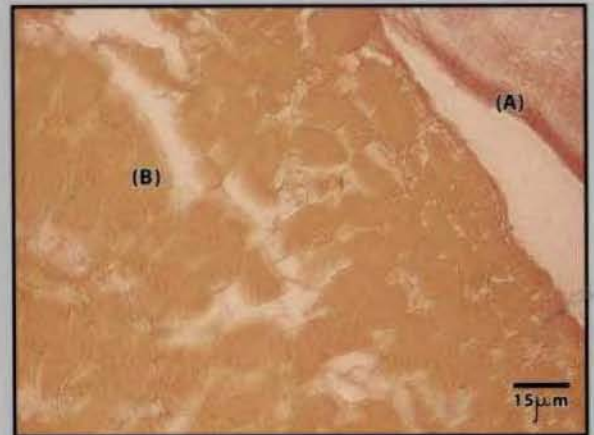
Plate 5.16 Histographs of frozen samples (three months) of
shark, *Scoliodon sorrokawah* cooked at different temperatures.

(g) 70 °C, (h) 75 °C, (i) 80 °C, and (j) 90 °C.
(A)- Collagen, (B)- Myofibrillar protein.
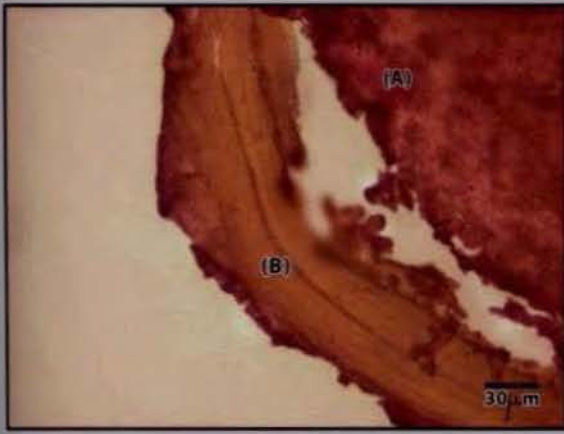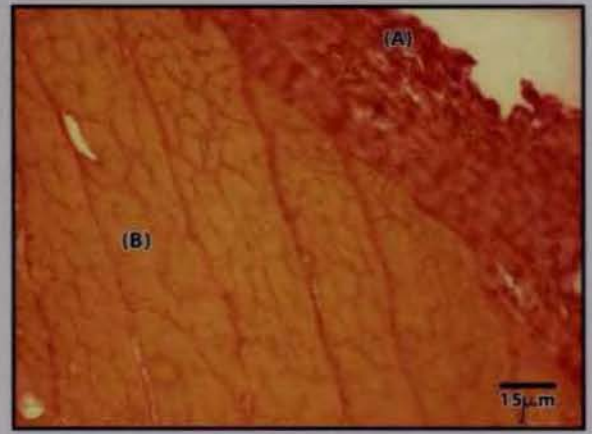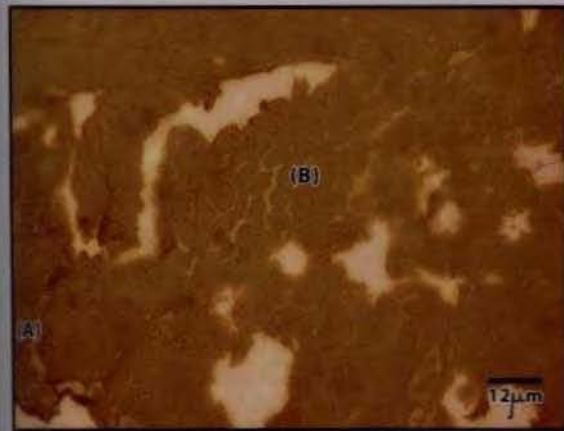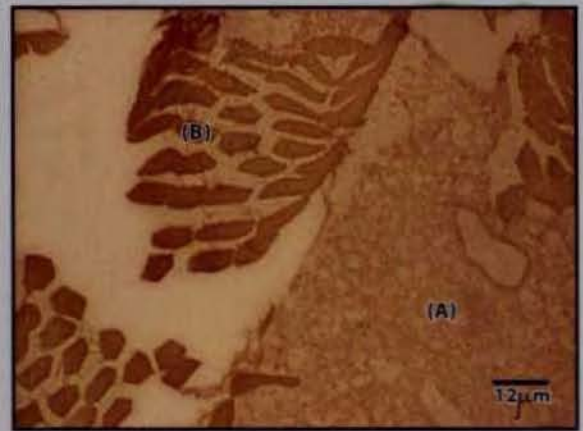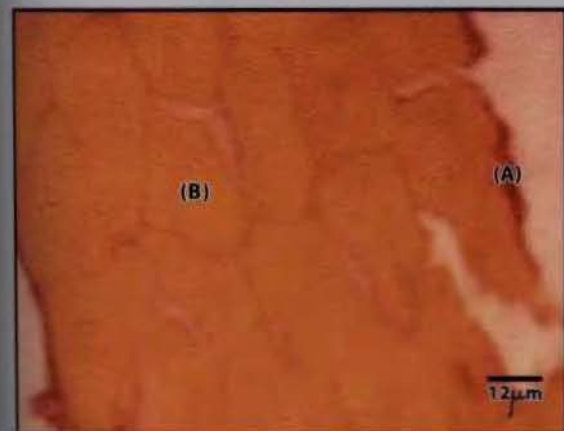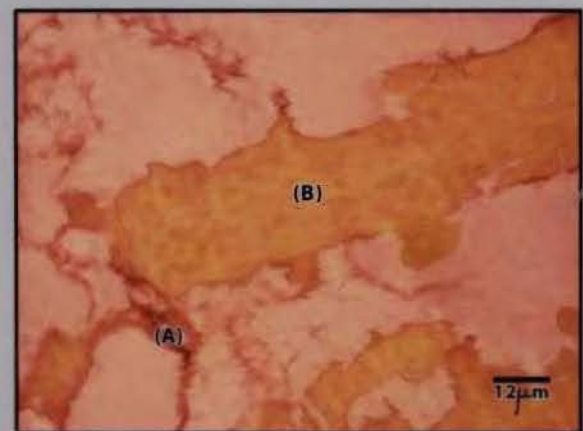
Plate 5.17 Histographs of frozen samples (six months) of shark, *Scoliodon sorrokawah* cooked at different temperatures. (a) *frozen(six months)*, (b) 45 °C, (c) 50 °C, (d) 55 °C, (e) 60 °C and (f) 65 °C.
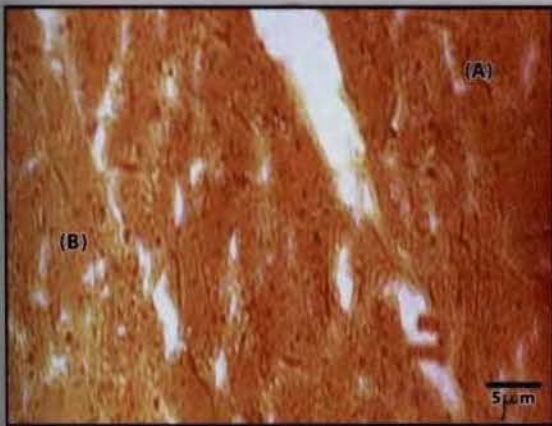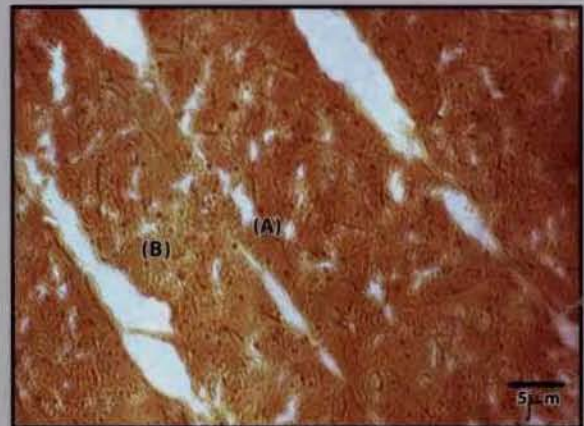
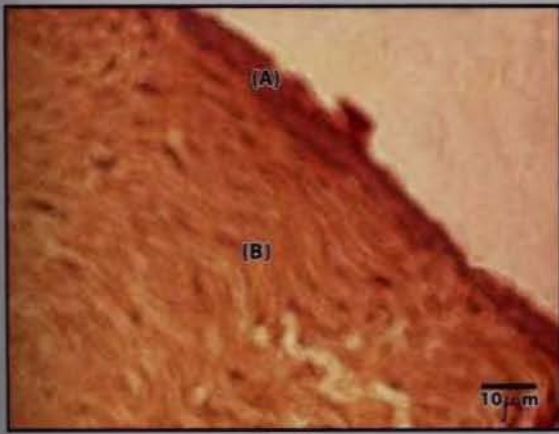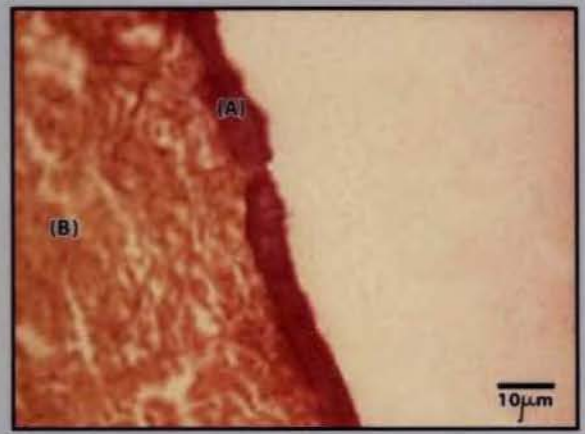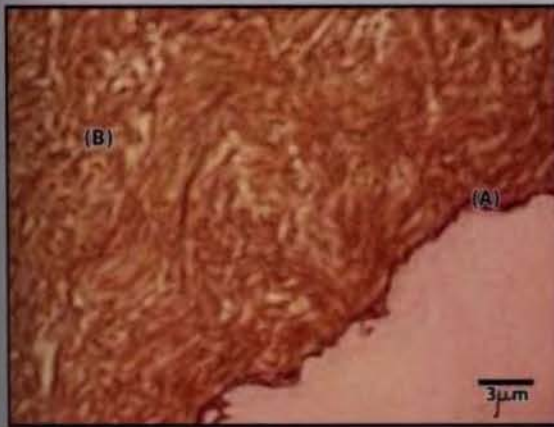(A)- Collagen, (B)- Myofibrillar protein.
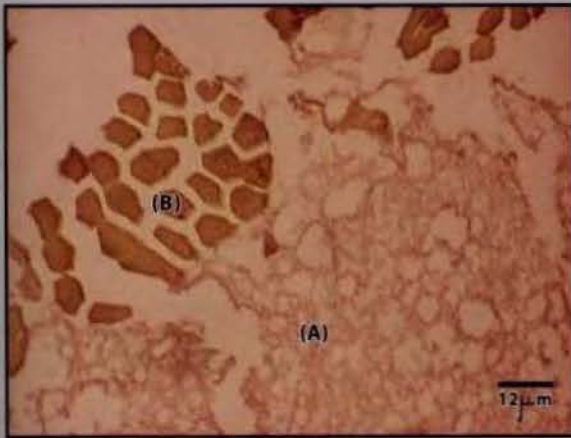
(g)
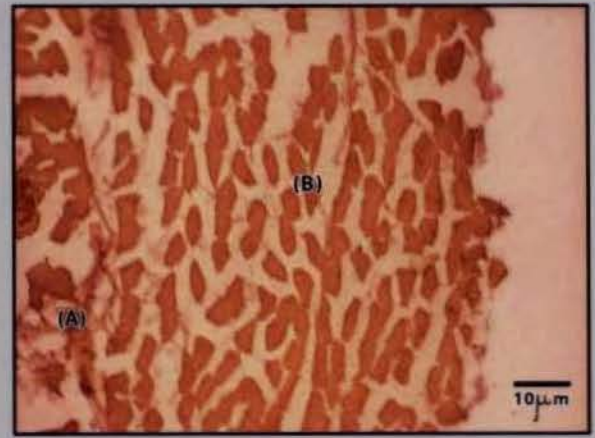
(h)
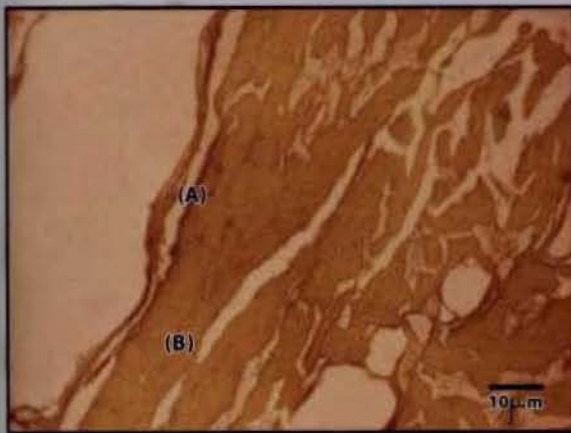
(i)
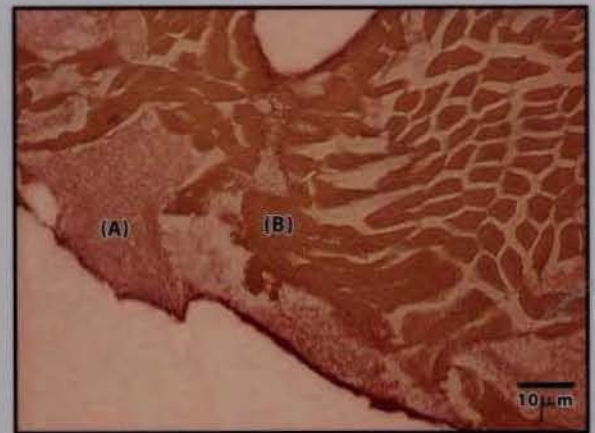
(j)

Plate 5.18 Histographs of frozen samples (six months)
of shark, *Scoliodon sorrokawah* cooked at different temperatures.
(g) 70 °C, (h) 75 °C, (i) 80 °C, and (j) 90 °C.

(A)- Collagen, (B)- Myofibrillar protein.

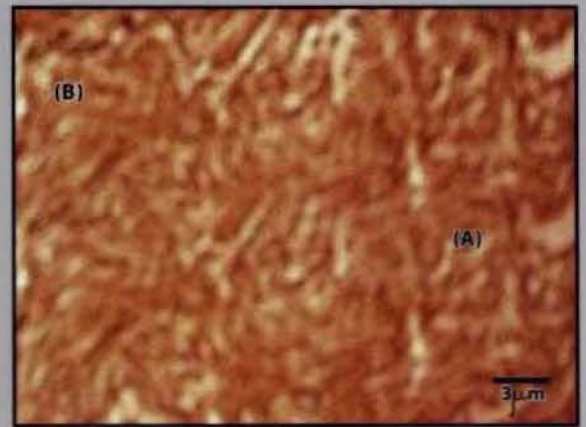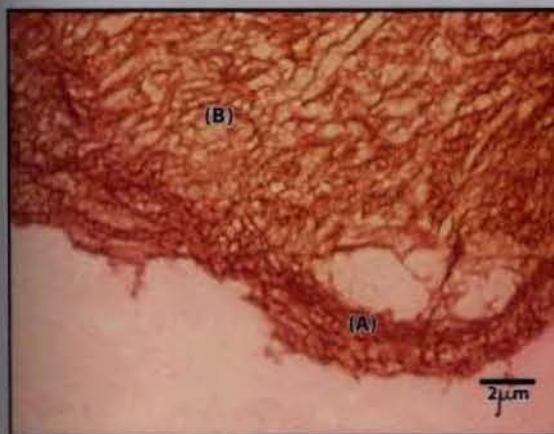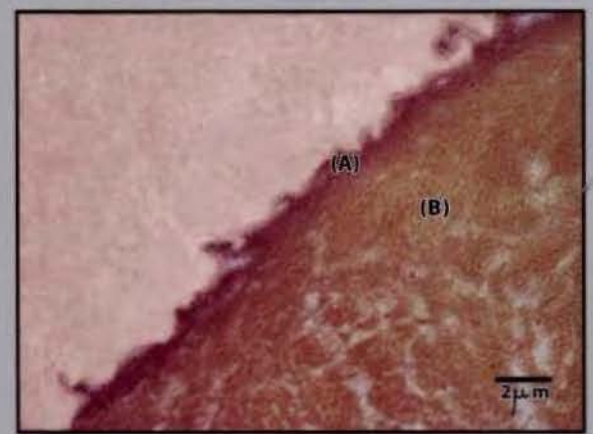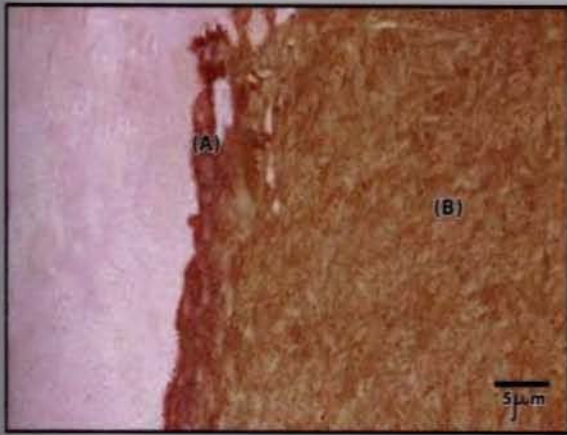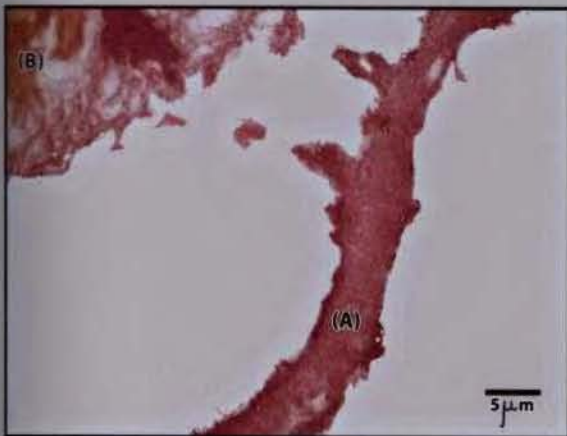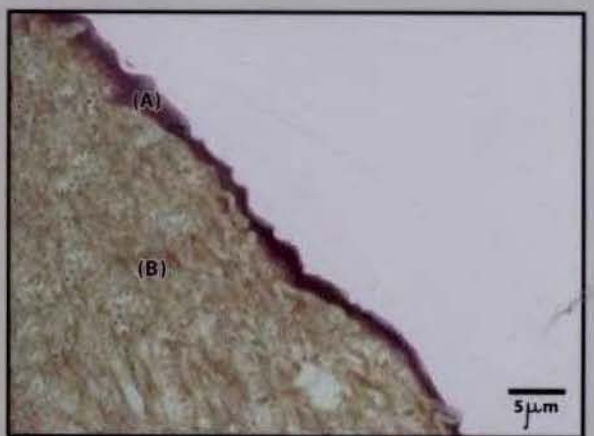perimysium in muscle tissue heated at 55 to 60°C were observed to be thin although there were separations from the muscle fibers. The tissue heated at 70 to 90°C (plate 5.2, 5.8 and 5.14) showed images of remarkably reduced muscle fibers that shrink and gather in the gap region that were frequent around the muscle fibers. This could be due to the rapid effect of temperature on tissue during cooking at high temperatures. Structure of endomysium in the three species became quite unclear in the tissues cooked at 70 to 90°C although the perimysium was still evident. Structure of epimysium was also maintained. Shark has high collagen content compared to rohu and squid. On cooking collagen was converted to gelatin. At 50°C, since the connective tissue was distorted, the tissue became tender. At 70°C shark muscle tissue showed appealing texture probably due to the gelatin from collagen that held the myotomes together probably retaining the nutritional quality. Unlike rohu where tissue structure was completely distorted at 90°C, shark gelatin was able to hold together the muscle cells.

5.4.2. Musculature pattern

In rohu and shark the connective tissue consisting of collagen formed myocommata sheath that enveloped each myotome bundle and help in holding the myotome bundles together. The musculature pattern of the squid mantle tissue differed from that of rohu and shark as they are composed of several layers of fibers running transversally to each other and are covered with several sheets of connective tissue (plate 5.7). Muscle fiber layer of the mantle made up to about 98% of the mantle thickness. They were observed to consist of 0.1 – 0.2 mm thick bands with fibers running circumferentially sandwiched between thinner sheets of radial fibers. Four sheets of connective tissue cover the muscle fibers: the inner tunic and the visceral lining on the inner side of the body cavity and the outer tunic and outer lining beneath the skin. Just under the skin proper there were pigment cells containing dark red and brown melanin pigment cells, together with the skin.

5.5. DISCUSSION

Connective tissue of the fibrous structures primarily, consisting of collagen, could be categorized into epimysium, perimysium and endomysium as in rohu and shark that

differed from each other in being morphologically discrete collagen depots. Epimysium is the heavy sheath of connective tissue surrounding individual muscle tissue that thickens at both origin and insertion. Endomysium encircles individual muscle fiber and were found to superimpose the basement membrane, perimysium is the thin layer enveloping muscle bundles and muscle fibers (myofibers) and endomysium is the fine layer encasing the muscle fibers. Epimysium is normally removed while de – skinning and only the perimysium and endomysium connective tissues are suggested to be involved in the realistic toughness of meat tissue. Perimysium and epimysium comprise the intracellular connective tissue and were not separable in rohu and shark. The bulk (90%) of intracellular connective tissue consisted of perimysium and was probably the main contributor to meat texture (Light et al., 1985). In squid, perimysium was not clearly distinguishable. The connective tissues of the squid tunics had high collagen content (Otwell and Hamann, 1979). With its complex muscle orientation and high collagen content raw squid was relatively tough and possessed rubbery texture. The muscle patterns of squid (*Loligo duvaucelli*) are comparable with the results of Llorea et al. (2001).

Freezing could alter the quality of frozen samples in comparison to the unfrozen fresh products. Physical and chemical changes in proteins that occur during frozen storage result in texture deterioration that negatively affect the functional, nutritional and sensory properties of the product. Studies showed that fiber separation occurred with increased period of frozen storage. The sample texture changed more extensively resulting from partial denaturation of proteins. Myosin possibly unfolds in frozen samples resulting in the exposure of hydrophobic groups that then cause protein aggregation resulting new cross – linkages during frozen storage. This kind of protein – protein interactions led to decreased water holding capacity and increased expressible moisture that is reported in chapter 2. The variations are in agreement with the findings of Xiong and Mikel (2001). Enzymatic activities can partially unfold the myosin in frozen – thawed samples as observed in rohu and shark (Shenouda, 1980). These interactions during frozen storage cause an increase in extracellular volume with fibers moving apart. On freezing, the ammonia – like components of urea in shark tissue were lost either due to leaching or through evaporation, rendering the shark muscle tissue more preferable. The voids formed in the

shark muscle tissue during freezing were less possibly due to the less spacing of myocommata during ice crystal formation.

Cooking serves dual function of tenderizing the muscle by converting collagen to gelatin, and by toughening due to heat coagulation of the myofibrillar proteins. Hence, the muscle characteristics obviously depend on the relative effects of these two structural components. Diameters of collagen fibrils were smaller in the fishes than in the squid and been focused in as a protein that greatly contribute to meat texture (Olaechea et al., 1993). With frozen storage a partial disintegration of endomysium and perimysium was observed in the muscle, while the structure of the thick connective tissue epimysium was not much changed. On the other hand, a preferential decrease in the relative staining intensity of the components was detected for collagen from the muscle with further cooking which may be due to the conversion of collagen to gelatin. The disintegration of thin connective tissue could be due to some biochemical changes in the components (Ando et al., 1999). It was generally believed that collagen shrinks (toughening effect) at a temperature around 63°C and may be partially transformed to gelatin at higher temperatures that was almost similar to denaturation temperature (60°C) for squid as observed in the present study (Hamm, 1966). The denaturation and the shrinkage temperature for rohu (50°C) and shark (50°C) were further lower. With increasing the temperature from 50°C to 60°C the texture profile parameters decreased in samples than in fresh that was discussed in chapter 4. It could be due to additional factors involved in the cooking effect at these temperatures than just changes in connective tissue alone (Bouton et al., 1975). Shark has high collagen content that was converted to gelatin upon cooking (70°C) and held the muscle cells. It also helped in retaining the nutritional quality of the tissue. Even at 90°C the tissue structure was comparatively maintained than that of rohu. Epimysium was maintained in the tissue, though perimysium and endomysium showed slight denaturation with increased temperature of cooking.

Davey and Gilbert (1976) reported that certain proteolytic enzyme cathepsin C that is relatively heat stable could also support tenderization effect at 60°C. Rohu and shark cooked at 45°C and above 70°C had higher gel strength than at 60°C possibly due to muscle alkaline protease that was found in the fish flesh (Makinodan et al., 1963). The extent of protein – protein interaction in rohu actomyosin probably increased with increasing

temperature. The rate increases sharply with temperature increase from room temperature to 45°C. When heated at various temperatures ranging from 55°C to 80°C, the toughening increased initially and then decreased resulting in softening of the tissue. For meat processed at 90°C separation between meats was observed that could be due to the excessive shrinkage of the muscle protein. Deformation value tended to increase during heat processing showing high values at 70°C and 90°C in contrast to raw meat. At higher temperature, no tenderizing effect was observed in rohu and shark. This might be because of the loss of enzyme activity and only protein denaturation was having significant effect on texture activity (Deng, 1981). Upon cooking the losses of water from the cut ends of the meat were high. This was accompanied with shrinkage of meat in two phases. In squid at 45 to 60°C the shrinkage was primarily transverse to the fiber axis and at higher temperatures, 60 to 90°C the shrinkage were parallel to the fiber axis. This could possibly explain the toughening of the squid meat when compared to rohu and shark muscle tissue.

5.6. CONCLUSION

The histochemical studies show that the musculature pattern of the squid mantle tissue differed from that of rohu and shark as they are composed of several layers of fibers running transversally to each other. These layers were covered with several sheets of connective tissue. The differences in the stain intensity confirm the decomposition of collagen to gelatin with increase in temperature of cooking thus decreasing the integrity between the muscle cells. Even though the intercellular spaces were narrow and collagen fibrils were scarcely visible in raw tissues they played a significant role in affecting the texture during frozen storage and cooking. The muscle pattern became clearer in frozen stored samples in comparison to raw samples of all the species possibly due to formation of intercellular spaces. Cell detachment occurred during cooking as a result of the decrease in the intercellular integrity due to loosening of collagen fibers probably resulting in the decrease of the binding force between cell membrane and collagen. The voids observed in the raw muscle tissue of squid and shark was less compared to cooked samples and those after frozen storage. The histochemical observations were confirmed the results of texture profile analysis discussed in the previous chapter. The firmness of cooked muscle was

weak but constant in squid and shark and could be explained by considering the occurrence of gelatinization of intercellular collagen. The solubility differences could be due to the differences in the intermolecular cross – links of collagen in the three species studied. The specific influences of connective tissues depend on the thickness, amount of collagen, their density and type of cross – linkages between fibrils. Specific patterns of collagen distribution and its localization in squid mantle have a profound role in textural changes during heat treatments require further studies in this respect.

CHAPTER 6

PURIFICATION AND EVALUATION OF COLLAGEN IN
Loligo duvaucelli

Chapter 6

PURIFICATION AND EVALUATION OF COLLAGEN IN
Loligo duvaucelli

6.1. Introduction

6.2. Review of literature

      6.2.1. Structure of collagen

6.3. Materials and Methods

      6.3.1 Sample collection

      6.3.2. Extraction of collagen

      6.3.3. Determination of hydroxyproline

      6.3.4. Determination of amino acid profile

      6.3.5. Histochemical studies

6.4. Results

6.5. Discussion

6.6. Conclusion

6.1. INTRODUCTION

Collagen is found to be present in almost all multicellular organisms. It is not a single protein but a family of structurally related proteins. Mechanical strength, integrity and gel forming capacity of the cooked muscle and the flow properties are some of the attributes of fish protein where the collagen was known to have a major functional role. Collagen content was reported to vary from species to species. The largest concentration of collagen is found in fish bone, fins, skin and air bladder. Collagen content in fish muscle is generally one tenth or less than that in the red meat. Air bladder was considered as a source of pure collagen. Collagen content in the squid mantle is high and might level up to 10% of the total protein. Fish collagen differs from mammalian collagen in having high biological value, high essential amino acid content and lower hydroxyproline content. The different types of collagen were characterized by different polypeptide composition. Histochemical analysis confirmed the localized distribution of collagen in the tissues and thence its significant role in contributing to the unique texture to the mantle meat. Based on these observations, an in depth study of the squid collagen was found to be necessary. In this chapter an attempt is made to study the following in squid (*Loligo duvaucelli*):

1. To analyze the amino acid profile of collagen extracted from squid epidermal layer, mantle with dermis layer and tentacle.

2. To study the distribution of collagen in the squid tissue.

6.2. REVIEW OF LITERATURE

Collagen, super family of proteins contains 19 proteins formally defined as collagens and an additional of ten proteins that have been reported to have collagen like domains. The most abundant collagen form extra cellular fibrils and network like structures but the others fulfill a variety of biological functions (Voet and Voet, 1995). Collagen is of great significance in attributing exceptionally a noteworthy role in muscle with regard to their diverse properties. Collagen contributes modestly to the nutritional value of fish.

In the case of squid the collagen content being high, the properties were quite similar to higher animal muscle tissue while cooking. Heating in humid environment resulted in the shrinkage of collagen to 1/4th their original lengths probably due to the break down of hydrogen bonds. The physical strength of the endomysial collagenous fibrils and myocommata were responsible for the integrity of the fish and fish fillets, failure of these structural elements to withstand the strain imposed on the musculature during rough handling after catch and during rigor mortis cause rupture of the fillets (Mathew, 2002). The tensile strength and integrity of the muscles are mostly contributed by collagen. Soluble collagen from the swim bladder of teleost was perhaps the first collagen to be characterized. At temperature of 40°C collagen molecules were reported to rupture and converted to elements of lower molecular weight (Balian and Bowes, 1977).

6.2.1. Structure of collagen

Collagen is about 2 nm in diameter and more than 300 mm long. It has an axial ratio of about 150. The collagen molecule is a triple helical structure with three long polypeptide chains as shown below. Each polypeptide is a left – handed helix but the three helices are wrapped around each other in the right direction. Each polypeptide is made up of roughly 1000 amino acid residues with a repeated Glycine – X – Y sequence. The low molecular weight amino acid glycine is located at the center of the triple helix. X and Y are usually imino acid proline and hydroxyproline, respectively and are arranged towards the outside of the triple helix (Yonath and Traub, 1969).

Collagen forms are broadly classified as Type I, II, III, V and XI. Collagen that form network like structures are classified as Type IV, VIII and X. Type I collagen is found at all body sites and is considered as the most abundant type of collagen. This classification of collagen is based on the differences of the chains in the molecule. The different types of collagen, their structure and location in the body are summarized below. Type I usually contain α and β chains in 2: 1 ratio. In type II collagen, α vary slightly different and is denoted as α(II). In Type III collagen α is denoted by α(III) and in type IV, α(IV) and so on. Type V usually contains α, β and λ chains. Type I is found to occur commonly in skin, tendons and bones of young organisms; Type II is hyaline cartilage; Type III in skin of

relatively young organisms and Type IV is seen in the basement membranes. Type V occurs in the amnion chorion, placenta and cornea of animals (Mathew, 2002). The following diagram describes the triple helical structure of collagen, its cross section and dimension.

Polypeptide chain

N= -3.3 D= 0.29 (nm) P= -0.96 (nm)	N= number of residues per turn of helix (positive numbers indicate a clockwise helix and negative numbers a counterclockwise helix) D = diameter P = pitch of helix. R = radius of backbone helix without side chains.

R= 0.16 (nm)

T= 109 (deg)

Triple helical structure of collagen

Types	Polypeptide composition	Distribution
I	$[\propto 1(I)]_2 \propto 2(I)$	Skin, bone, tendon, cornea, blood vessels
II	$[\propto 1(II)]_3$	Cartilage, in vertebral disk
III	$[\propto 1(III)]_3$	Fetal skin, blood vessels
IV	$[\propto 1(IV)]_2 \propto 2(IV)$	Basement membrane
V	$[\propto 1(V)]_2 \propto 2(V)$	Placenta, skin

Stability of collagen helix is closely correlated with the total imino acid content in the collagen molecule as they limit the rotation of the polypeptide chains. The three polypeptide chains are stabilized by inter chain hydrogen bonds. Collagen also contains rather large amounts of polar amino acids (arginine, lysine and aspartic acid). The other post – translational modification that occurs in collagen is glycosylation. During this process the sugar residues usually glucose, galactose and their disaccharides are attached to the hydroxy group in the newly formed hydroxylysine residues. The amount of attached carbohydrate in collagen varies from 0.4 to 12% by weight depending on the tissue in which they are synthesized (Mathew, 2002).

Several researches were performed in the genetic differentiation of collagen in several species (Mizuta et al., 1996 and Nagai and Suzuki, 2000). Kimura et al. (1981) reported the isolation and characterization of the pepsin – soluble collagen from octopus *Octopus vulgaris* skin. Takema and Kimura (1982) reported two genetically distinct molecular species of collagen with structure $(\alpha 1)_2 \alpha 2$ from *Octopus vulgaris* arm muscle. Pepsin soluble collagen was observed to have the denaturation temperature (T_d) of 28°C. This value for Octopus collagen was similar to those from other marine organisms 16.8 – 31.7°C (Nagai and Suzuki, 2000). It was known that the stability of collagen was correlated with their environmental and body temperature (Rigby, 1968). Mizuta et al. (1997) reported that about 8% of the total collagen in the mantle muscle could be recovered as guanidine hydrochloride – soluble fraction.

The industrial use of collagen has expanded and collagens are now used as materials for foods, cosmetics and biomedicine. Although there are many studies concerned with collagen from marine vertebrates and invertebrates (Nagai et al., 1999) the main source of industrial collagen are limited to those from bovine and pigskin and bones. However, the incidence of cattle's infected with Bovine Spongiform Encephalopathy (BSE) reported in Japan (Yamaguchi, 2002) became a serious threat to the availability of collagen. One alternative is to replace bovine collagen with another source. As part of the study, looking at the effective use of under utilized marine resources, it has been reported that the

preparation and characterizations of collagen from aquatic organisms, mainly marine vertebrates and invertebrates could be a replacement and help in solving the crisis. A few studies about collagen from skin of marine organisms and fish scales have been reported (Nomura et al., 1996).

6.3. MATERIALS AND METHODS

6.3.1 Sample collection

The squid, *Loligo duvaucelli* were collected as mentioned in 2.3.1. In the laboratory, the samples eviscerated. Epidermal connective tissues of skin were carefully removed and the rest was taken as mantle tissue. The tentacles were also removed for analysis.

6.3.2. Extraction of collagen

Collagen was extracted according to the methodology of Kolodziejska et al. (1999). The above samples were minced thoroughly and treated with 5 vol of hydrogen peroxide for 30 min at 20°C, centrifuged and filtered. The residue obtained was then treated with 0.1N sodium hydroxide for overnight at 20°C and filtered. The residue collected was washed with hydrogen peroxide (2.5: 1 ratio) at 20°C and then treated with 1% hydrogen peroxide containing 0.01 M sodium chloride in 5: 1 ratio (residue: solution) for 48 h at 20°C. The residue after treatment was treated with 2.5 vol of hydrogen peroxide, washed and filtered and the residue thus obtained was taken as purified collagen and was used for further analyses. Protein content was measured using microkjeldahl method (AOAC, 1995). The flowchart showing the purification procedure is shown in Fig 6.1.

Sample

H$_2$O$_2$ (5: 1), for 30 minutes at 20 °C ──→ Mixing

Filtration (20 °C) ──→ Extract

0.1 N NaOH, overnight at 20 °C ──→ Mixing

Filtration (20 °C) ──→ Extract

H$_2$O$_2$ (2.5: 1), at 20 °C ──→ Washing

Filtration (20 °C) ──→ Extract

1 % H$_2$O$_2$ in 0.01 M NaCl (5: 1), ──→ Mixing
for 48 hours at 20 °C

Filtration (20 °C) ──→ Extract

H$_2$O$_2$ (2.5: 1), at 20 °C ──→ Washing

Filtration (20 °C) ──→ Extract

Residue
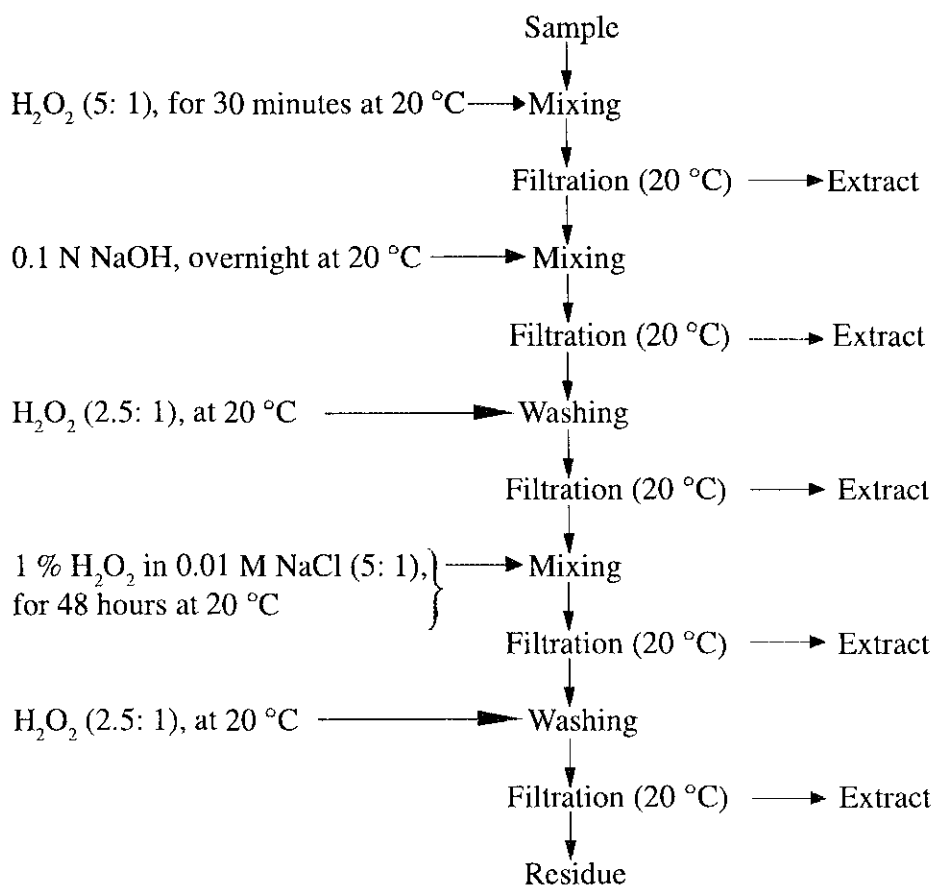
Fig. 6.1 Protocol for the extraction of collagen

6.3.3. Determination of hydroxyproline

Hydroxyproline was quantitatively determined as a measure of collagenous material. Samples of epidermal connective tissue, mantle tissue and tentacle were hydrolyzed in sulphuric acid at 105°C, filtered and diluted. Hydroxyproline was oxidized with choramine – T to pyrrole. Red – purple color that developed after addition of 4 – dimethylaminobenzaldehyde was measured photo metrically at 560 nm (AOAC, 1995). Using hydroxyproline standards containing 0.6, 1.2, 1.8 and 2.4 µg hydroxyproline/ ml, a standard curve was plotted. Here, for *Loligo duvaucelli* the conversion factors for collagen from hydroxyproline were 14.1 and from nitrogen it was 6.25 (Sadowska and Sikorski, 1987).

6.3.4. Determination of amino acid profile

Amino acid profiling of the tissues were done according to AOAC (1995). About 100 mg of the sample in duplicates were weighed accurately into heat stable test tubes. 10 ml of 6 N HCl was added and the tubes were heat – sealed after filling with pure nitrogen gas. Hydrolysis was carried out in hot air oven at 110°C for 24 h. After the hydrolysis was over, the test tube were broke open, contents were removed quantitatively and filtered into a round bottom flask through What man Filter Paper No. 42. The filter paper was washed 2 – 3 times with distilled water. The contents of the flask were flash evaporated to remove final traces of HCl and this process of boiling was repeated for 2 – 3 times with distilled water. The residue was made up to 10 ml with 0.05N HCl. The sample thus prepared was filtered again through a membrane filter of 0.45 µm and 20 µl of this was injected to Shimadzu HPLC LC 10 AS Amino Acid Profiling System. This system of amino acid analysis consists of a column packed with strongly acidic cation exchanger resin (styrene divinyl benzene co – polymerized with sulphinic groups). The column was of sodium type (ISC- 07/ S 1504 Na) with a length of 19 cm and diameter of 5 mm. The mobile phase of the system consists of two buffers – Buffer A (sodium citrate, ethanol of 99.55 %, perchloric acid 60 %, pH 3.2) and Buffer B (sodium citrate, boric acid, 4N NaOH, pH 10.0). The oven temperature was maintained at 60°C and amino acids were eluted from the column by stepwise elution namely acidic amino acids were first eluted followed by neutral and then alkaline amino acids. The analysis was done with non – switching flow method and fluorescence detection after post column derivatization of o – phthaldehyde. In case of proline and hydroxyproline, imino groups were converted to amino groups with sodium hypochlorite. Amino acid standards (Sigma Chemicals Co. Ltd, USA.) were also run to determine the concentration of the sample amino acid based on the standard chromatogram received.

6.3.5. Histochemical studies

Sample fixation and histochemical analyses of the squid mantle with skin were done as shown in 5.3.2.

6.4. RESULTS

The hydroxyproline content in the tissues ranged from 0.14 to 0.2 µg/ ml in mantle, tentacle and epidermal connective tissue as shown in Table 6.1. The collagen content was determined by multiplying the hydroxyproline by the conversion factor 14.1. The collagen content of the mantle tissue obtained was comparable with the values obtained by fractionation (Fig. 3.4).

Table 6.1 Hydroxyproline content (µg/ ml) and collagen (% of total protein) in squid epidermal layer, tentacle and mantle

	Epidermal layer	Tentacle	Mantle
Hydroxyproline	0.20	0.145	0.14
Collagen	17.63	12.78	12.34

n-3

Table 6.2 shows the amino acid profile of skin, tentacle and mantle of the squid (*Loligo duvaucelli*). Alanine and glutamic acid were found to be relatively high. On the contrary, tryptophan was observed to be absent in the collagen of all the three tissues. Amino acids of squid collagen contained smaller proportion of proline and hydroxyproline and larger proportions of serine and threonine. All the twenty amino acids were found to be present in tentacle and mantle lacked cysteine. Mantle and tentacle were found to contain all the ten essential amino acids. The imino group of hydroxypoline was converted to amino part and it was estimated along with aspartic acid. Aspartic acid contributed to only a small portion to the total amino acids.

Table 6.2 Amino acid profiles of squid skin, tentacle and mantle (g/ 100g N)

Amino acids	Epidermal layer	Tentacle	Mantle
Aspartic acid	10.18	23.12	42.08
Threonine	17.78	10.05	16.61
Serine	-	9.61	15.32
Glutamic acid	-	46.72	95.40
Proline	-	3.92	6.52
Glycine	-	19.15	18.15
Alanine	-	17.74	29.99
Cystine	20.08	1.83	-
Valine	-	11.38	19.84
Methionine	18.04	18.72	34.20
Isoleucine	34.85	57.07	173.96
Leucine	10.19	19.30	24.51
Tyrosine	9.52	12.60	20.73
Phenylalanine	-	1.34	0.97
Histidine	5.78	23.22	36.41
Lysine	42.05	41.99	81.34
Arginine	0.42	0.94	272.69

Plate 6.1 shows the distribution of collagen in the dermal tissue of the squid. Squid mantle consists of two types of layers, one running circularly around the longitudinal axis of the body occupying the larger part of the tissue and the second layer running perpendicular to the first layer. The outer side of the skin tissue is divided into epidermal connective tissue, chromatophore layer and dermal connective tissue. The distribution of collagen was observed to be primarily concentrated in outer and inner tunics with sparse fibers traversing the mantle. The outer side of the skin tissue could be divided into three layers, namely epidermal connective tissue, chromatophore layer and dermal connective tissue, a
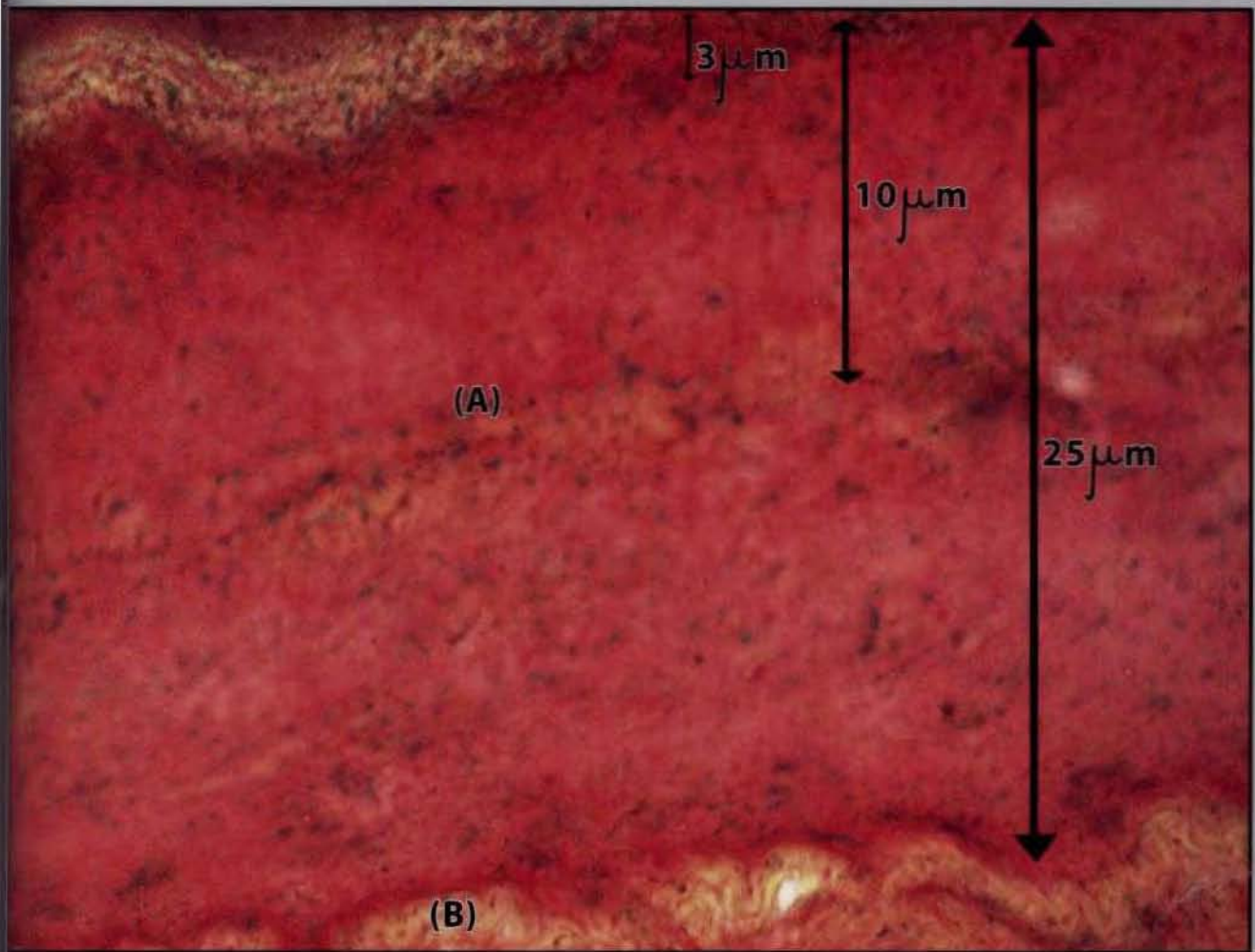
Plate 6.1. Histograph of fresh sample of squid, *Loligo duvaucelli* showing collagen structure.

(A)- Collagen, (B)- Myofibrillar protein.

layer of dermal connective tissue was also observed at the inner skin tissue. Intramuscular connective tissue consists of epimysium and endomysium. Epimysium is the thick connective tissue forming the outer sheath of the muscular tissue, while endomysium is the thin collagen fiber scattered in the inner parts of tissue. These fibers are heterogeneous in distribution, density and diameter. These unique arrangements of collagen fibers play a significant role in demarcating the squid texture from other fishery products.

6.5. DISCUSSION

All the twenty amino acids were found to be present in tentacle and mantle lacked cysteine. Alanine and glutamic acid were relatively high with tryptophan being absent in the collagen of all the three tissues of squid. Only smaller proportion of proline and larger proportions of serine and threonine were also observed. Mantle and tentacle were found to contain all the ten essential amino acids. There is a controversy over the conversion of nitrogen and hydroxyproline to collagenous connective tissue. It was observed that collagenous connective tissue contained 12.5% hydroxyproline when nitrogen to protein factor of 6.25 was used and 14% when nitrogen factor was 5.55%. The conversion factors, for calculating the content of squid collagen from nitrogen and hydroxyproline contents were 6 – 6.5 and 14.1 – 14.3, respectively (Sadowska and Sikorski, 1987).

Takema and Kimura (1982) reported the amino acid composition of collagen from Octopus (*O. vulgaris*) arm muscle. The results were similar to squid (*C. arakawai*). The differences in the proportion of amino acids of the tissues could indicate changes in the molecular structure of proteins leading to the difference in the resolution ability and precipitation of the proteins. Sikorski et al. (1984) observed that the collagen in the muscle and skin of squid contain about twice as many hydroxyproline residues per 1000 residues as the cod skin collagen. Studies show that different samples of collagen prepared by exhaustive extraction of the skin and mantle connective tissue of frozen *Loligo* species and *Ilex* species contained 0.3 – 4.9%, 3.5 – 6.1% and 4.7 – 8.0% hydroxyproline (Sadowsha and Sikorski, 1987). Amino acid profiling of epidermal connective tissue, tentacle and mantle tissues indicated a great difference. Since the dermis contains more collagen as

shown by histochemical results and as it was collected along with mantle, it could be proposed that dermal layer of squid tissue is a rich source of amino acids.

6.6. CONCLUSION

Characterization of fish meat collagen in terms of biochemical and rheological properties are essential for the better understanding of the functional behaviors co these proteins towards specific thermal process. Collagen was found to have localized distribution in squid tissue and was more in the dermal and epidermal region. The outer side of the skin tissue of squid consisted of epidermal connective tissue, chromatophore layer and dermal connective tissue. A layer of dermal connective tissue was also observed at the inner skin tissue. Relatively thick fibers were distributed densely near the outer epimyiusm and relatively thin fibers were scattered sparsely around the central part of the muscle. Collagen fibers that ran almost perpendicularly to the muscle fibers were also observed in smaller density. Similarly amino acid profiling of epidermal connective tissue, tentacle and mantle tissues indicated a difference. It was observed that amino acid content was more in dermal region than mantle and tentacle. Since the dermis contains more collagen as shown by histochemical results and as it was collected along with mantle, it could be proposed that dermal layer of squid tissue is a rich source of amino acids. The collagen is a bioactive protein and hence, a need for detailed study of this bioactive protein is attempted in the next chapter.

CHAPTER 7

IDENTIFICATION AND EVALUATION OF MACROMOLECULAR COMPONENTS IN
Loligo duvaucelli

Chapter 7

IDENTIFICATION AND EVALUATION OF MACROMOLECULAR COMPONENTS OF COLLAGEN IN *Loligo duvaucelli*

7.1. Introduction

7.2. Review of literature

7.3. Materials and Methods

    7.3.1. Sample collection

    7.3.2. Preparation of acetone dry powder

    7.3.3. Estimation of uronic acid

    7.3.4. Collagenase solubilised glycoprotein and glycosaminoglycan

        7.3.4.1. Phenol sulphuric acid method for total hexose

        7.3.4.2. Determination of Fucose

        7.3.4.3. Periodic Acid/ Thiobarbituric Acid Assay

    7.3.5 Analysis of glycosaminoglycans by SDS – Polyacrylamide Gel Electrophoreses.

        7.3.5.1. PAS staining.

7.4. Results

7.5. Discussion

7.6. Conclusion

7.1. INTRODUCTION

Proteoglycan consist of core proteins with glycosaminoglycan chains and are abundant molecules found both in the extracellular matrix and on the cell surfaces. These diverse molecules serve wide range of functions, from affecting the compressive properties of cartilage to growth factor reception. The macromolecular components of connective tissue namely collagen, glycosaminoglycan and glycoprotein, among other factors, are involved in the pathogenesis of several diseases. The glycosaminoglycans are located predominantly on the cell surface and in the extracellular matrix and are of particular interest for their possible role as cellular recognition sites. Some glycosaminoglycans are also well known to have biological functions like anticoagulants and antithrombotic activity.

An understanding of these biological processes is intimately linked to the knowledge of the structures involved. Compared with the progress achieved in the structural analysis of biopolymers like proteins and nucleic acids, progress has been rather slow in the field of glycosaminoglycan. This could be mainly due to the complexity of the glycosaminoglycan structure including their molecular size, sugar component, glycosidic linkages and sulfation. Thus, it is important to verify to what extent these compounds are distributed in squid (*Loligo duvaucelli*) and how it can contribute to the ever raising demands of the same. No systematic investigation seems to have been reported on the macromolecular components (glycosaminoglycans and glycoproteins) in squid skin, tentacle and mantle. In view of the importance of glycoaminoglycans and glycoproteins in the biological systems preliminary study has been done on the following aspects:

1. To study the glycoaminoglycans in the squid skin, tentacle and mantle.
2. To study the glycoprotein contents in the squid skin, tentacle and mantle.

7.2. REVIEW OF LITERATURE

Proteoglycans are far more complex than collagen consisting of amino sugars and organic acids arranged in long chains of repeating disaccharides called glycosaminoglycan (formerly named mucopolysacchrides). The long chain glycosaminoglycans are attached

like a bottlebrush to the core protein. The resulting combination is called proteoglycan. Apart from these, this bottlebrush like proteoglycans are connected to another long chain compound called hyaluronic acid by connecting proteins. These conglomerates are known as an "aggregating proteoglycan". There are many kinds of proteoglycan configurations, from very large complex aggregates to simple forms where there is only a small protein chain with one glycosaminoglycan chain attached to it. Glycoproteins are regarded as proteins that contain carbohydrate (less than 10 %) attached to the peptide portion by covalent linkages. Included in this classification are a large variety of compounds like collagen. Proteoglycans are extremely large molecular complexes (mass > 2000 K Da) made up of carbohydrates (95%) and proteins (5%). The basic units of various glycoaminoglycans are repeating disaccharide unit that contain an uronic acid and an amino sugar (hexosamine) as building blocks. Glycoproteins cannot be distinguished from each other by their amino acid composition. Glycosaminoglycans are complex carbohydrate macromolecules and are important structural components of the connective tissue. They are present in the tissues covalently linked to protein to form proteoglycan forming the basic substance in the extracelluar matrix. The hexosamines (amino sugar) are either D – glucosamine or D – galactosamine, while the uronic acid is either D – glucoronic acid or L – iduronic acid. The amino group of the hexosamine is always acetylated and in some cases sulphated. Sulphate present in several glycosaminoglycans is in the form of O – sulphate. Six major classes of glycoaminoglycans have been recognized based on the acidic polysaccharides as hyaluronic acid (HA), chondroitin sulphate (CS), dermatan sulphate (DS), keratan sulphate (KS), heparin (H) and heparan sulphate (HS). Collagen is a glycoprotein and contains small quantities of galactose and glucose. Five types of collagen have been reported in fishes that differ in their solubility, fiber thickness, amino acid composition, carbohydrate proportion and the extent of hydroxylation. It plays a major role in adhesion of cells, their differentiation and migration with the effect of glycoprotein sites on the collagen fibrils. With mutations in collagens, a variety of human diseases including osteogenesis imperfection, osteoarthritis and the real disease known as Alport syndrome has been reported. The post – translational modification that occur in collagen is glycosylation where the sugar residue, usually glucose, galactose and their disaccharides

are attached to the hydroxy group in the newly formed Hyl residue rather than to Asn or Ser/ Thr residues as they occur in the more wide spread N – & O – linked glycosylation. The amount of attached carbohydrate in collagen varies from 0.4 to 12 % by weight depending on the tissue in which it is synthesized.

Glycosaminoglycans with proteins are used for forming skin, cartilage and connective tissue. In the articular cartilage they might play important function in retaining elasticity of the tissue. Alternately, they are regarded as a basic element for structuring and playing an important role to control the elementary process in living cell body. Pearson and Gibson (1982) discussed the functional implications, different characteristics, probable distribution of proteoglycan and their quantitative variation during tooth eruptions. Molluscs glycosaminoglycan chemical composition susceptibility to specific enzymes; and their physiochemical and pharmacological activities are undistinguishable from those of mammalian heparin. The mucous coat of fish is in constant contact with the external aqueous environment and has been recognized to play a variety of important physical, chemical and physiological functions. The principal constituent of the mucous coat was thought to be skin mucous glycoprotein that differs individually in their carbohydrate moieties depending upon the species. To elucidate the nature and functions of the fish skin mucus glycoproteins, characterization of their carbohydrate chains are important, since the characteristics of glycoproteins depend on their carbohydrate chains.

Connective tissue proteoglycans are distinguishable from mammalian carbohydrate – protein compounds due to the presence of relatively large polysaccharide chains containing repeating disaccharide units. Most of the glycosaminoglycan, with the possible exception of hyaluronate, do not occur as free polymers in the native state, but occur as proteoglycan in which many polysacchride chains are linked to the protein molecules as a the terminal reducing sugar residue. In recent years, as a result of great developments in the field of preparation and fractionation of the proteoglycans, a considerable amount of data has been accumulated regarding the structure of these macromolecules. The versatility of proteoglycan and their capacities for multiple interactions with other molecules give them the ability to function as multipurpose "glue" in the cellular interactions. They can bind together the extracellular matrix components, mediate the binding of cells to matrix, and capture soluble molecules for growth of the matrix and at cell surfaces. Oversulfated

chondroitin sulfate was originally discovered in shark cartilage (Soda et al., 1940) and squid and king crab (Mathew, 1966). Chondroitin sulfate isoform from shark, squid and king crab cartilage had also been characterized by the presence of distinct disulfated disaccharide units in them. An in vitro anticoagulant activity has been reported for squid cartilage chondroitin sulfate – E (Akiyama et al., 1982). Squid cartilage chondroitin sulfate E was known to activate the contact (Hageman factor) system of plasma in vitro, as heparin (Hojima ct al., 1985). These macromolecules have several fundamental biological and pharmacological activities, making them important in clinical and pharmaceutical fields. Heparin is an anticoagulant and antithrombotic drug used as a thrombolytic, fat clearing anti – atherosclerotic and as a promising anti – inflammatory agent. Dermatan sulfate has anticoagulant and antithrombotic activities that essentially depend on binding and activation of heparin co – factor II. Chondroitin sulfate is a chondro – protective drug in patients with osteoartherosis of the knee. One of the most striking features of glycosaminoglycans is their structural diversity, with discrete structural variants found in different tissues. Specific form of glycosaminoglycan (heparan sulfate) was found to be associated with different disease state and age. These findings suggest that different forms of glycosaminoglycan (heparan sulfate) are important in influencing the biological function. Studies pertaining to the isolation of heparin, hepain sulphate and chondroitin sulphate from animal tissues have been performed in order to investigate their biological activities and potential as hypolipidemic agents (Jaques, 1987). Heparin and heparan sulphate have been found to show high potent in shark (Susiki and Strominger, 1960), dog fish (Praus and Goldman, 1970), hagfish, lamprey and Chimera (Seno et al., 1972; Rahematulla and Lovtrup, 1976).The tissues of mrigal and chanos were studied for glycosaminoglycans and were found to differ in the content of the glycosaminoglycans, though both species followed the similar kind of distribution pattern. Tissues of brackish water fish contained higher levels of glycosaminoglycans than the freshwater fish possibly due to the difference in the environmental conditions. Varghese et al. (1986) performed studies in blood vessels of higher animals and reported that total aortic glycoprotein had lower hexose and higher sialic acid content; but the glycoprotein released by collgenase digestion of the venous tissue had lower hexose and higher fucose and glycoprotein released by urea had lower fucose and sialic acid in venous tissue.
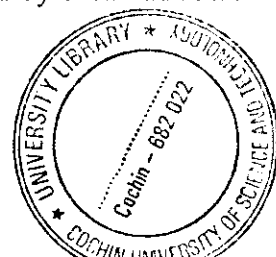
Numerous techniques are available for the fractionation of the glycosaminoglycan but none of these can separate all the glycosaminoglycans from each other. Since many of these separation methods are largely based on the differences on the charge density of individual glycosaminoglycan, heterogeneities present even within a particular polysaccharide species, such as variations in the degree of sulfation, molecular weight polydispersity, or variable amounts of the attached residual peptides, could cause an overlap of chemically distinct glycosaminoglycan types during the separation process. Narita et al. (1995) identified the glycosaminoglycan using High Performance Liquid Chromatography (HPLC). Fractionation and quantification of these in fish have also been reported using enzymes and in other animals, including mammals (Mathew et al., 1982). Glycoproteins and glycosaminoglycans are a large family of heterogeneous polysacchrides widely distributed in animal tissues. Moczar and Moczar (1976) reported the modification of the macromolecular composition of the extracellular matrix of differentiated connective tissues in squid dermis with ontogeny and phylogeny. Very few reports are available on the glycoproteins and glycosaminglycans in fishes and no detailed information is available on the distribution and biological potency of these in squid (*Loligo duvaucelli*). The present paper attempts to study the distribution of these glycoproteins and glycosaminoglycan in skin, tentacle and mantle of squid (*Loligo duvaucelli*).

7.3. MATERIALS AND METHODS

7.3.1. Sample collection

Fresh samples of squid immediately stored in ice were brought to the laboratory under low temperature. They were cleaned and eviscerated and skin, tentacles and mantle were collected separately and acetone dry powder was prepared (Chempakam and Kurup, 1978). Pure chemicals from Qualigens and SIGMA Chemicals Co., St. Louis, USA were used for further analysis.

7.3.2. Preparation of acetone dry powder

Finely divided tissues were kept in acetone at 0°C for 72 h (20 ml/g wet tissue) with intermittent change of acetone every 16 h. It was then filtered off by suction and dried in vacuum. The material was then extracted with ether: acetone (3: 1 vol. /vol.) for 1 h at 37°C followed by ether for 1 h. The residue was finally filtered off under suction and dried in vacuum (Chempakam and Kurup, 1978).

7.3.3. Estimation of uronic acid

Dry defatted tissue were digested with papain (about 100 mg was subjected to digestion with crystalline papain at 65°C for 72 h in 0.2 M acetate buffer, pH 7.0, containing 1 mM cysteine hydrochloride. Fresh papain was added at every 24 hour interval. The uronic was determined as an estimate of isolated glycosaminoglycan by the method of Bitter and Muir (1962).

7.3.4. Collagenase solubilised glycoprotein and glycosaminoglycan

Collagenase solubilised glycoprotein and glycosaminoglycan were extracted from the samples by the method of Varghese et al. (1986). A portion of acetone dry powder was digested twice with collagenase (100 units/ g of original tissue) in tris – buffer (0.05 M, pH 7.4) containing 0.36 M $CaCl_2$ for 24 h at 37°C in the presence of thymol and soyabean trypsin inhibitor (1 mM). Collagenase solubilised protein in the combined supernatants were precipitated with 5% TCA. The precipitated protein was subjected to papain digestion (about 100 mg was subjected to digestion with crystalline papain at 65°C for 72 h in 0.2 M acetate buffer, pH 7.0, containing 1 mM cysteine hydrochloride. Fresh papain was added every 24 h. The residue was used for carbohydrate analysis for the estimation of carbohydrate components.

The uronic was determined as an estimate of isolated glycosaminoglycans by the method of Bitter and Muir (1962). The total hexose of glycoproteins was estimated by phenol – sulphuric acid method (Dubois et al., 1956), fucose by the method of Dische and Sheltes (1948) and sialic acid by thiobarbituric acid method (Warren, 1959). Appropriate

controls with collagenase, but without tissue were also run to correct for the carbohydrate components present in the enzyme.

The residue obtained after collagenase digestion were repeatedly extracted with 6M urea at room temperature till no more protein was extractable. The solubilised macromolecular components in the solutions were determined as described above.

7.3.4.1. Phenol – sulphuric acid method for total hexose

To 2 ml of aqueous solution about 0.05 ml of phenol reagent was added followed by the rapid addition of 5.0 ml of concentrated sulpuhuric acid. After the samples have stood at room temperature for 30 min, the color remained stable for several hours, during this time the optical density was determined at $480 - 490$ mμ. All samples were assayed in triplicate. Total hexose was estimated from the standard plotted using the standards (Dubois et al., 1956).

7.3.4.2. Determination of Fucose

To the pyrex tubes containing 1 ml of sample cooled in an ice bath 4.5 ml of cold sulphuric acid reagent was added with vigorous shaking to ensure thorough mixing followed by sample. The tubes were then brought to the room temperature by placing them in the water bath of $20 - 22°C$ for a few minutes. Subsequently the tubes are capped with glass bulbs, placed in vigorously boiling water bath for exactly 3 min and then cooled to the room temperature in a water bath. To each tube 0.1 ml of cysteine reagent was added with intermediate shaking after each addition. The absorbency was determined at 396 mμ after 1 hour and also at 427 mμ. Separate aliquots of each sample were heated with sulphuric acid, but no cysteine hydrochloride was added after heating (Dische and Sheltles, 1948). The absorption due to the methyl pentoses in a given sample was then determined by subtracting the $O.D._{396} - O.D._{427}$ of the sample analyzed with cysteine from the $O.D._{396} - O.D._{427}$ of the sample analyzed without cysteine.

7.3.4.3. Determination of sialic acid

The reactions were carried out in glass centrifuge tubes with ground – glass stoppers. To 0.5 ml of an aqueous aliquot solution 0.25 ml of periodic acid reagent was added. The oxidation period was 30 min at 37°C. Then 0.2 ml of the arsenite solution was added to destroy the excess of periodate. After about 2 min the yellow color of the liberated iodine disappeared, and 2 ml of the thiobarbituric acid reagent are added. The mixture was heated in a boiling water bath for 8.0 min followed by cooling in an ice bath. The chromophor was extracted by vigorous shaking with 5 ml of the acid/ butanol mixture. After 3 min of centrifugation at 3000 rpm the clear organic phase was transferred to a 10 mm cuvette and the optical density was read at 580 nm against butanol phase of the blank in which the sialic acid and solution was substituted by water (Warren, 1959). The amount of N – acetylneuraminic acid present in the samples taken for hydrolysis was determined in terms of N – acetylneuraminic acid from the following equation.

Micromoles N – acetylneuraminic acid = $V \times A_{580} \times 5/8$

Where,

V = volume of organic solvent phase

A_{580} = Absorbency at 580 nm.

8 ⇒ the molar absorbency index of N – acetylneuraminic acid divided by 1000.

5 ⇒ volume factor

7.3.5. Analysis of glycosaminoglycans by SDS – Polyacrylamide Gel Electrophoreses.

For the qualitative separation of glycoproteins the SDS – PAGE was performed using 7 % gel strength as given in 3.3.3 (Lammelli, 1970).

7.3.5.1. Staining of glycoproeins by Periodic – Schiff's reagent

Reagents used:

☞ **Fixative solution for periodic acid**

Ethanol: 40%

Glacial acetic acid: 5%

Distilled water: 5 %

Stored at room temperature.

☞ **Schiff reagent**

Dissolve 10 g of basic fuschin in 2 L of distilled water with heating. After cooling, added 200 ml of 1 N HCl and 17 g of sodium metabisulfite; mix the solution until it was decolorized. Stir with HCl washed charcoal and centrifuge charcoal to avoid contact with filter paper. Filter the supernatant through glass wool to remove remaining charcoal; the filtrate should be clear and colorless. The solution was stored in a brown bottle at 4°C.

☞ **Periodic acid solution (0.7%)**

Dissolve 1.4 g of periodic acid in 200 ml of 5% acetic acid.

☞ **Sodium metabisulfite (0.2%)**

Dissolve 0.4 mg of sodium metabisulfite in 200 ml of 5% acetic acid.

For PAS staining each gel was fixed overnight in 100 – 200 ml of PAS fixative solution. The gels were then treated with the 0.7% periodic acid solution (covering the gels) for 2 – 3 h followed by treatment with 0.2% sodium metabisulfite for 2 – 3 h with one solution change after 30 min. The gels after clearing were put in tubes and the tubes were filled with Schiff reagent. Only plastic tops should be used to seal the tubes, not corks. Color developed in 12 – 18 h at room temperature.

7.4. RESULTS

Table 7.1 show the uronic acid, neutral hexose and sialic acid content in the skin, tentacle and mantle of the squid (*Loligo duvaucelli*). Skin showed highest sialic acid content, followed by tentacle and mantle. Urea digest showed higher sialic acid (99.50 µmol/ 100g) content than papain and collagenase digest. Neutral hexoses were high in tentacle followed by mantle and skin. Collagenase extract of skin showed high neutral hexoses (8.22%). A significant difference ($p < 0.01$) was noted in the uronic acid and sialic acid content between the tissues with the extraction. It can be postulated that more glycosaminoglycans are bound to collagen of mantle.
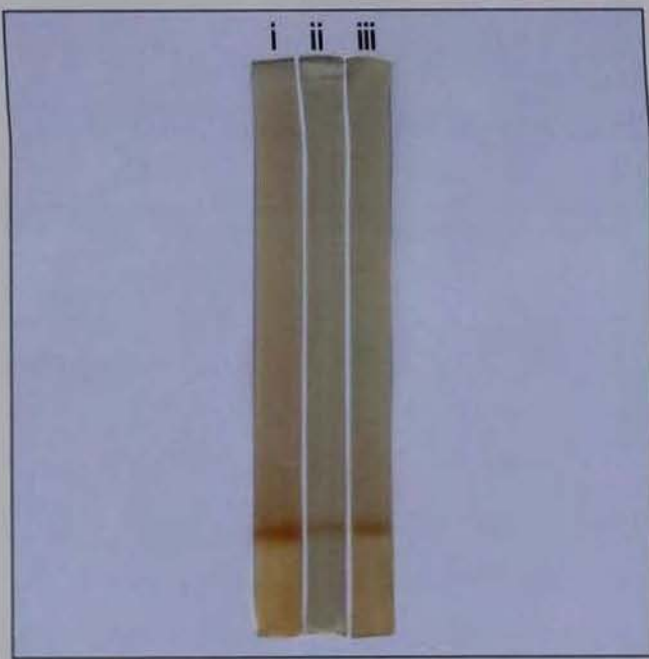
(a)
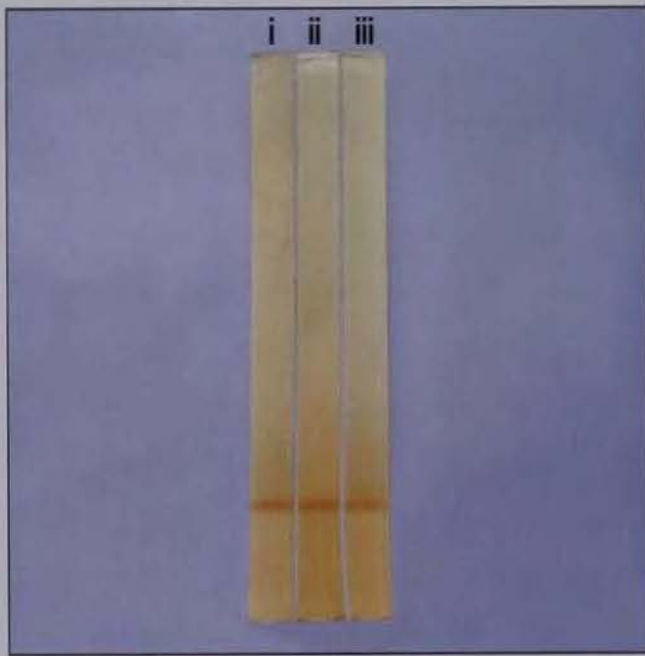
(b)

Plate 7.1 (a) and 7.1 (b) showing glycoproteins in squid skin, tentacle and mantle in the fractions of papain - digest and collagenase - digest, respectively.

Table 7.1 Uronic acid, sialic acid and neutral hexose in the skin, tentacle and mantle of squid

Extracts	Uronic acid (% of dry weight)			Sialic acid (µmol / 100 g)			Total hexose (%)		
	Skin	Tentacle	Mantle	Skin	Tentacle	Mantle	Skin	Tentacle	Mantle
Papain	0.1	0.05	0.09	25.2	14.35	14.08	2.84	15.37	3.4
Collagenase	0.06	0.05	0.04	33.18	8.45	12.29	8.22	1.08	0.55
Urea	0.05	0.04	0.05	99.52	25.4	4.9	0.56	1.21	0.39

n-3

Plate 7.1 and 7.2 show the PAS stained papain and collagenase digested glycoproteins from skin, tentacle and mantle, respectively. It showed that both papain digest and collagenase digest contained glycoproteins. The sugars were attached to low molecular weight proteins.

7.5. DISCUSSION

The results indicate the localized distribution of glycoproteins as part of the collagen structure, probably in the central core of the helical structure of collagen as they were easily extractable using collagenase enzymes. There are a few reports about the distribution of glycoproteins and glycosaminoglycans in different tissues in teleosts but no comparative data is available. The results from the present study show that the skin is rich in total hexose and sialic acid and the collagenase digest of skin has high concentration, it could possibly be concluded that these glycoproteins were tightly bound to collagen and glycosaminoglycans. Presence of sialic acid also indicated the presence of glycoproteins. Hence, both glycoprotein and glycosaminoglycan may be the integral part of squid tissue and could be used as potential sources for isolating these compounds. The amount of total hexoses and uronic acid in squid skin were in accordance with earlier works of Moczar and Moczar (1976) on *Sepia officinalis.*

The glycoprotein fraction recovered from papin – digest and glycoprotein obtained from collagen – glycoprotein precipitate after collagenase hydrolysis have similar electrophoretic pattern. The polyacrylamide gel pattern of the glycoprotein fractions of the three tissues exhibited differences in the band mobility. This is in agreement with the reports on charged heterogeneity for the glycoproteins interacting with collagen (IUPAC, 1985 and Anderson and Jackson, 1972). This charged heterogeneity could be due to

difference in sialic acid (N – acetylneuraminic acid) content of the glycans as reported earlier (Page, 1971).

Uronic acid was primarily in the form of glucuronic acid. Sulfuric acid was virtually absent. The large content of glucose among neutral sugars was noteworthy. In the epidermis of Californian *Doryteuthis* species 70% of mucopolysacchrides were sulfated chondroitin, and chondroitin was only 20 to 25% of the total. The neutral sugars xylose, galactose and glucose were also reported. Kawai et al. (1966) and Suzuki et al. (1968) demonstrated that the squid cartilage is unique in producing chondroitin sulfate containing acetylgalactosamine 4, 6 – disulfate residue. Glucose is an integral part of chondroitin sulfate. As glycosaminoglycans are hypolipidemic agents, they could be used as ready sources for isolating these compounds. Moreover, this will form an excellent means of utilizing unutilized tissues. However, the biological potency of chondroitin sulfate, heparin and heparin sulfate from the tissues are yet to be studied in detail.

7.6. CONCLUSION

Glycoproteins are an integral part of the collagen structure and form central core of the helical structure of collagen. Squid skin was rich in total hexose and sialic acid in the collagenase digested skin. Thus it could possibly be concluded that these glycoproteins are tightly bound to collagen and glycosaminoglycans. Presence of sialic acid also indicates the presence of glycoproteins. Hence, both glycoprotein and glycosaminoglycan may be the integral part of squid tissue and could be used as potential sources for isolating these compounds. From the electrophoretic studies, glycoproteins of the three tissues showed heterogeneity in the charge. This charged heterogeneity could be due to difference in sialic acid (N – acetylneuraminic acid) content of the glycans. Glycosaminoglycans are hypolipidemic agents and squid tissues could be used as ready sources for isolating these compounds. Moreover, this will form an excellent means of utilizing unutilized tissues. However, the biological potency of chondroitin sulfate, heparin and heparin sulfate from the tissues are yet to be studied in detail.

CHAPTER 8
SUMMARY AND CONCLUSION

Connective tissue is of great technological importance in that it holds the fillet together in fish. Recently, collagen has received an increased attention as an important protein component contributing greatly to the texture of raw and cooked muscle tissue. It has been suggested that a slow heating rate may allow more protein – protein interactions to occur to form a more ordered and stronger three dimensional structure. In this respect a coordinated study on biochemical, histochemical and textural changes have been done to analyze the exact changes occurring in three species of fish namely, rohu (*Labeo rohita*), squid (*Loligo duvaucelli*) and shark (*Scoliodon sorrokawah*) of varying collagen content. Detailed studies on the extent of changes in the above parameters, subjected to heat treatments of fresh and frozen stored samples, were also carried out.

Considering the decrease in crude protein content during different cooking regimen of the fresh and frozen – stored samples, squid mantle showed the least change compared to rohu and shark, possibly due to the unique distribution and arrangement of the protein in the mantle tissue (Chapter 2). Lipid content was recorded high for the fresh samples, which decreased with frozen storage. At a heating temperature of 55°C, the expressible moisture was the lowest with values 68.35%, 56.79% and 67.1% for rohu, squid and shark, respectively. The water binding potential was highest for fresh samples of rohu at 50°C (2.46g), for shark at 65°C (2.07g) and for squid at 55°C (2.85g). Throughout the treatments the water binding potential of squid meat was high (2.2 – 3.0) in comparison with rohu and shark rendering it more juicier than rohu and shark. This tissue water is probably located in the three dimensional network of the muscle filaments held by capillary forces. Thus, the amount of water immobilized depends on the space available between the filaments and the changes in the myofilament spacing. The expansion or shrinkage of the myofilament lattice cause water movements between intracellular and extra cellular spaces resulting in the swelling or shrinkage of the muscle fiber without much changes in the volume of tissue as whole.

Detailed investigations on the quantitative fractionation of proteins of fresh and frozen muscle tissue of rohu, squid and shark subjected to heat treatments indicated that with frozen storage and cook temperatures, the extraction of protein fractions were

significantly altered within the species and between the species (Chapter 3). Sarcoplasmic protein was high in shark and its extractability decreased with freezing, probably due to their aggregation and accumulation in the interfibrillar spaces of muscle tissue. High non – protein content in shark was observed that could be due to the presence of urea. Myofibrillar protein was observed to be high in rohu compared to squid and shark. Squid myofibrillar protein was found to be more water-soluble.

Protein fractionation by SDS – PAGE varied from species to species confirming the species specificity of various protein fractions. Presence of paramyosin in squid tissue could contribute to the decreased rate of protein denaturation in frozen stored samples. With cooking and freezing the protein bands observed in the unfrozen samples became faint and number of protein bands with smaller molecular weights appeared. Due to cross – linking, the size of the proteins might become so big that they could not enter the gel or could not be extracted into the media. No such changes were found in unfrozen muscle during heating.

Proteins are important structure builders of food system and any alterations in the conformational state would change their functional properties. Applying heat to proteins result in altered structure and associated changes in functional properties that have direct consequences on conformation. The myofibrillar and collagen protein content and their localization contribute to the differences in gelling characteristics of the muscle tissue of the three species. In this study, there was a direct correlation between the collagen content and degree of textural changes during cooking of fresh and frozen stored samples.

The overall organoleptic scoring showed that the three species showed first phase of hardening at 50°C. At 70°C, the fresh samples showed second phase of hardening with maximum juiciness. A slight variation in the second phase of hardening during freezing was observed in the frozen samples that could be possibly due to the differential freeze denaturation of the structural proteins (Chapter 4). The functional properties of fish muscle were associated with the ability of the myofibrillar proteins to form a three – dimensional gel patterns upon heating. The thermally induced interaction of fresh fish muscle occurred in three distinct stages, namely setting (40°C), softening (60°C) and gelation (80°C). It was proposed that setting phenomenon could be due to the hydrophobic interactions; the softening could be related to naturally occurring proteolytic enzymes, and it varied with

species. The gelation could be due to the covalent intermolecular cross – linkages between proteins. Aggregation of connective tissue proteins occurred during cooking and the proteolytic fragmentation of the connective tissue sheath might have become a significant factor on tenderness of the tissue. The results of texture profile analysis with respect to Hardness 1, Hardness 2, Cohesiveness, Springiness and Stiffness were also studied in detail in chapter 4. Cohesiveness was found to be high at 50°C in fresh samples of squid and shark probably due to their high collagen content and the resultant heat setting. At 70°C myofibrillar proteins that with hold the water molecules within the tissue also influenced cohesiveness. In rohu, muscle softening occurred earlier than squid and shark probably because of low content of collagen. After three months of frozen storage, optimum texture with respect to hardness, cohesiveness and stiffness was observed at 65°C, 75°C and 80°C for rohu, shark and squid, respectively. With further frozen storage, optimum texture again showed variation with the corresponding values of cook temperature being 75°C, 70°C and 75°C for rohu, shark and squid, respectively.

The collagen and myofibrillar protein fractions, on statistical comparison with texture profile parameter, indicated that these proteins have a profound role in the textural properties. Collagen was found to influence at low temperatures of cooking and myofibrillar protein played the key role in textural properties at higher temperatures. In frozen samples the influence of collagen was higher at lower temperatures

The histochemical studies (Chapter 5) revealed that the musculature pattern of the squid mantle tissue differed from that of rohu and shark as they were composed of several layers of fibers running transversally to each other. These layers were covered with several sheaths of connective tissue. Cell detachment occurred during cooking as a result of the decrease in the intercellular integrity due to loosening of collagen fibers, probably resulting in the decrease of the binding force between cell membrane and collagen. The voids observed in the raw muscle tissue of squid and shark were less compared to cooked samples and those after frozen storage. The histochemical observations confirmed the results of texture profile analysis discussed in the previous chapter. The firmness of cooked muscle was weak but constant in squid and shark and could be explained by considering the gelatinization of intercellular collagen. The solubility differences could be due to the differences in the intermolecular cross – links of collagen in the three species studied. The

specific influences of connective tissues depend on the thickness, amount of collagen, their density and type of cross – linkages between fibrils. Specific patterns of collagen distribution and its localization in squid mantle have a profound role in textural changes during heat treatments and further studies are required in this respect.

Based on the above observations, a detailed study of the localization of collagen and its amino acid profile in different squid tissues were carried out in Chapter 6. The outer side of the skin tissue of squid consisted of epidermal connective tissue, chromatophore layer and dermal connective tissue. A layer of dermal connective tissue was also observed at the inner skin tissue. Intramuscular connective tissue consisted of epimysium and endomysium. Relatively thick fibers were distributed densely near the outer epimysium and relatively thin fibers were scattered sparsely around the central part of the muscle. Collagen fibers that ran almost perpendicularly to the muscle fibers were also observed in smaller density. From the studies on amino acid profile in the epidermal layer and the mantle with dermal region it could be concluded that a high content of collagen with an ideal amino acid profile are localized in the dermal region. In Seafood Industry it is a common practice that while preparing squid mantle, along with the skin, the dermal connective tissue is also removed, which is proved to be a rich source of collagen and other bioactive compounds.

Results of a basic study on the macromolecules that form structural components of collagen are reported in Chapter 7. Studies show that the glycoproteins were probably attached to the central core of the helical structure of collagen, as they were easily extractable using collagenase enzymes. The results also showed that the skin is rich in total hexoses, sialic acid and uronic acid in the collagenase-digested skin compared to other tissues. Hence, both glycoprotein and glycosaminoglycans may be the integral part of squid skin and could be used as potential sources for isolating these compounds. From polyacrylamide gel electrophoretic studies, glycoproteins of the three tissues showed heterogeneity in the charge as shown by the difference in their gel pattern. This charged heterogeneity could be due to the difference in sialic acid (N – acetylneuraminic acid) content Glycosaminoglycans are hypolipidemic agents and squid dermal connective tissue could be used as a good source for isolating these compounds. Moreover, this will form an excellent means of utilizing unutilized tissues, otherwise wasted in large amounts in Seafood Industry.

APPENDICES

APPENDIX A

2.1. ANOVA for moisture content of rohu, squid and shark

Source of Variation	SS	df	MS	F	Sig.
Total	743.35	89			
b/n temperature	201.57	9	22.4	3.28	$(p < 0.01)$
b/n species	5.84	2	2.92	0.43	
b/n months	16.82	2	8.41	1.23	
Error	519.12	76	6.83		

2.2. ANOVA for crude protein content of rohu, squid and shark

Source of Variation	SS	df	MS	F	Sig.
Total	250.57	89			
b/n temperature	7.37	9	0.82	0.94	
b/n species	49.19	2	24.6	28.28	$(p < 0.01)$
b/n months	127.66	2	63.83	73.37	$(p < 0.01)$
Error	66.35	76	0.87		

2.3. ANOVA for crude lipid content of rohu, squid and shark

Source of Variation	SS	df	MS	F	Sig.
Total	216.05	89			
b/n temperature	15.32	9	1.7	1.53	
b/n species	97.13	2	49.57	43.76	$(p < 0.01)$
b/n months	19.15	2	9.58	8.63	$(p < 0.01)$
Error	84.45	76	1.11		

2.4. ANOVA for ash content of rohu, squid and shark.

Source of Variation	SS	df	MS	F	Sig.
Total	20.62	89			
b/n temperature	1.01	9	0.11	1	
b/n species	9.54	2	4.77	43.36	(p < 0.01)
b/n months	1.45	2	0.73	6.64	(p < 0.01)
Error	8.62	76	0.11		

2.5. ANOVA for pH of rohu, squid and shark

Source of Variation	SS	df	MS	F	Sig.
Total	7.35	89			
b/n temperature	0.16	9	0.02		
b/n species	1.53	2	0.77	38.5	(p < 0.01)
b/n months	4.23	2	2.12	106	(p < 0.01)
Error	1.43	76	0.02		

2.6. ANOVA for water binding potential of rohu, squid and shark

Source of Variation	SS	df	MS	F	Sig.
Total	19.65	89			
b/n temperature	4.07	9	0.45	4.5	(p < 0.01)
b/n species	5.85	2	2.93	29.3	(p < 0.01)
b/n months	1.83	2	0.92	9.2	(p < 0.01)
Error	7.9	76	0.1		

2.7. ANOVA for expressible moisture of rohu, squid and shark

Source of Variation	SS	df	MS	F	Sig.
Total	9690.82	89			
b/n temperature	697.92	9	77.55	0.78	
b/n species	1349.36	2	674.68	6.74	(p < 0.01)
b/n months	40.08	2	20.04	0.2	
Error	7603.46	76	100.05		

APPENDIX B

3.1. ANOVA for sarcoplasmic protein of rohu, squid and shark

Source of Variation	SS	df	MS	F	Sig.
Total	238.73	89	-	-	
b/n temperature	8.62	9	0.96	2.13	p<0.01
b/n species	145.08	2	72.54	161.8	
b/n months	50.1	2	25.05	55.67	p<0.01
Error	34.93	76	0.45		

3.2. ANOVA for myofibrillar protein of rohu, squid and shark

Source of Variation	SS	df	MS	F	Sig.
Total	14330.44	89	-	-	
b/n temperature	125.82	9	13.98	0.08	
b/n species	12438.27	2	6219.14	35.06	p<0.01
b/n months	919.43	2	459.72	2.59	
Error	13483.52	76	177.41		

3.3. ANOVA for alkaline soluble protein of rohu, squid and shark

Source of Variation	SS	df	MS	F	Sig.
Total	7220.7	89	-	-	
b/n temperature	44.76	9	4.97	0.31	
b/n species	699.24	2	349.62	22.1	p<0.01
b/n months	5275.73	2	2637.87	166.95	p<0.01
Error	1200.97	76	15.8		

3.4. ANOVA for total collagen of rohu, squid and shark

Source of Variation	SS	df	MS	F	Sig.
Total	6850.42	89	-	-	
b/n temperature	13.82	9	1.54	2.2	
b/n species	6779.56	2	3389.78	4842.54	p<0.01
b/n months	3.48	2	1.74	2.49	
Error	53.56	76	0.70		

3.5. ANOVA for pepsin soluble collagen of rohu, squid and shark

Source of Variation	SS	df	MS	F	Sig.
Total	3585.32	89	-	-	
b/n temperature	2.35	9	0.26	1.44	
b/n species	3562.76	2	1781.38	9896.56	p < 0.01
b/n months	6.20	2	3.1	17.22	p < 0.01
Error	14.01	76	0.18		

3.6. ANOVA for non - protein nitrogen of rohu, squid and shark

Source of Variation	SS	df	MS	F	Sig.
Total	1337126.12	89	-	-	
b/n temperature	81761.48	9	9084.61	2.36	
b/n species	938052.51	2	469026.26	122.05	p<0.01
b/n months	25255.43	2	12627.72	3.29	p<0.05
Error	292056.7	76	3842.85		
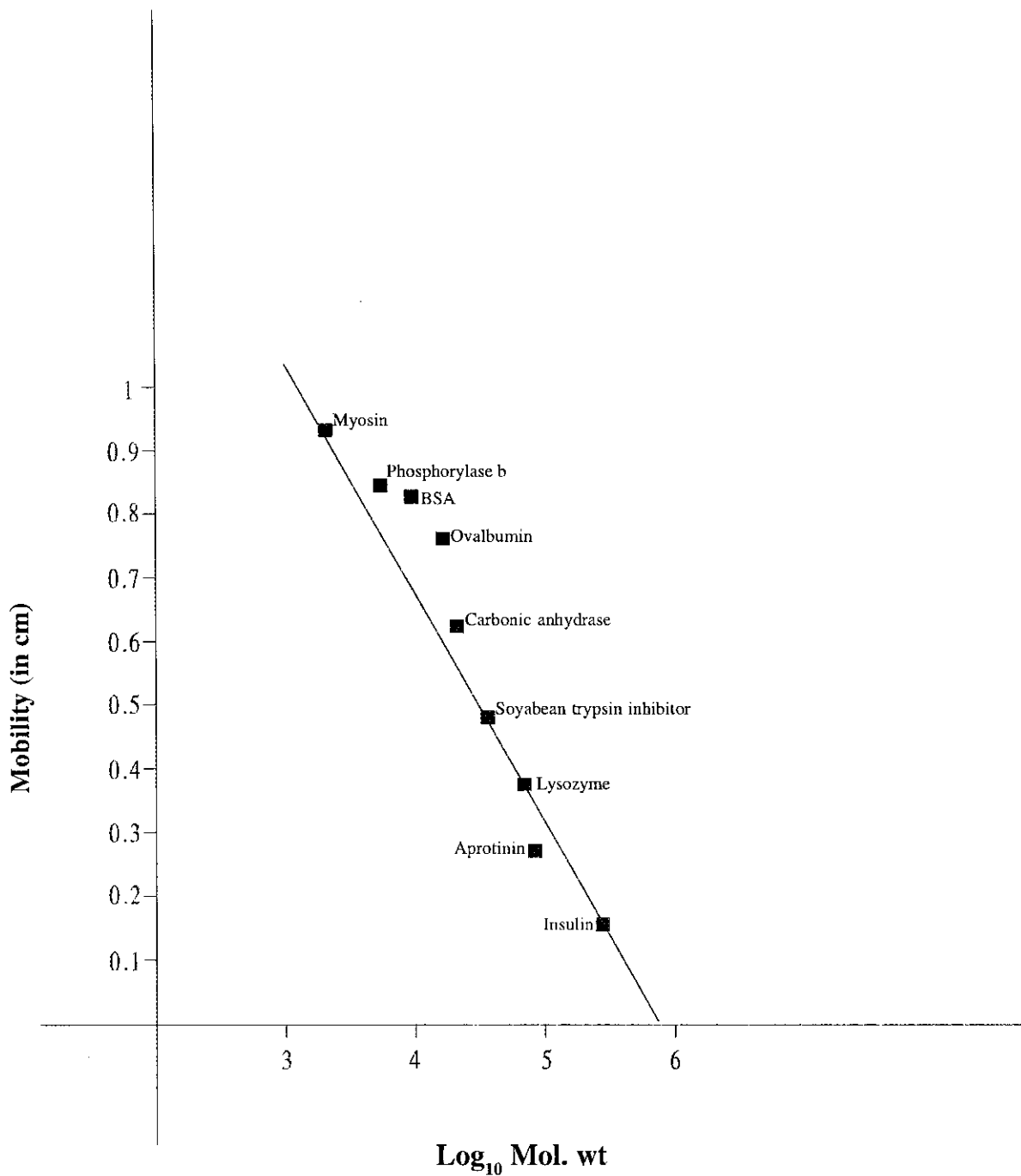
Plot showing standard curve for broad range molecular weight protein markers.

APPENDIX C

4.1. Performa 1 for hedonic scaling (Texture)

The response to the properties of the material on the first bite:

Initial characteristics:

1. Wateriness: The release of water on compression; this is the initial response and is to be distinguished from juiciness - Scale points: 1, much less water released; 4, neither much nor less; 7, much more water released.

2. Firmness: The force required to compress the material between the molars or between the tongue and palate - Scale points: 1, much softer and less consistent; 4, neither soft nor firm; 7, much firmer and more consistent.

3. Elasticity: The ability of the material to return to its original shape after deformation. It is judged by compressing the substance slightly between the molars or between the tongue and palate and noting to what extends the material returns to its original shape - Scale points: 1, much more plastic; 4, neither plastic nor elastic; 7, much more elastic.

4. Cohesiveness: The extent to which a material can be deformed before it ruptures - Scale point: 1, much less cohesive; 4, neither much nor less cohesive; 7, much more cohesive.

The response to the properties of the material after chewing a few times:

Secondary characteristics:

1. Hardness: Resistance to breakdown on chewing to a state, suitable for swallowing - Scale points: 1, much more tender; 4, neither much nor less tender; 7, much tougher.

2. Juiciness: The sensation of a progressive increase of free fluids in the oral cavity during mastication - Scale points: 1, much drier; 4, neither much nor less juicier; 7, much juicier.

Date......... Name.........Product.........

Sample code	Initial characteristics				Secondary characteristics	
	Wateriness	Firmness	Elasticity	Cohesiveness	Hardness	Juiciness

Taste the samples and check how much you like or dislike each one. The ratings are given in numerical values ranging from 1 to 7

Scale: 7 – Like extremely, 5 – Neither likes nor dislike 1 – Dislike extremely.

4.2. Performa 2 for hedonic scaling (Sensory)

Date......... Name.........

Sample code	Odor	Color	Flavor	Touch	Overall acceptability scoring

4.3. ANOVA for wateriness (sensory)

Source of Variation	SS	df	MS	F	Sig.
Total	51.36	80	-	-	
b/n temperature	24.92	8	3.12	13.57	$(p < 0.01)$
b/n species	8.92	2	4.46	19.39	$(p < 0.01)$
b/n months	1.95	2	0.98	4.26	$(p < 0.01)$
Error	15.57	68	0.23		

4.4. ANOVA for firmness (sensory)

Source of Variation	SS	df	MS	F	Sig.
Total	80.54	80	-	-	
b/n temperature	5.21	8	0.65	1.55	
b/n species	30.32	2	15.16	36.10	$(p < 0.01)$
b/n months	16.61	2	8.31	19.79	$(p < 0.01)$
Error	28.4	68	0.42		

4.5. ANOVA for elasticity (sensory)

Source of Variation	SS	df	MS	F	Sig.
Total	121.56	80	-	-	
b/n temperature	40.67	8	5.08	7.7	$(p < 0.01)$
b/n species	34.75	2	17.38	26.33	$(p < 0.01)$
b/n months	1.19	2	0.60	0.91	
Error	44.95	68	0.66		

4.6. ANOVA for cohesiveness (sensory)

Source of Variation	SS	df	MS	F	Sig.
Total	99.51	80	-	-	
b/n temperature	27.07	8	3.38	10.56	$(p < 0.01)$
b/n species	49.36	2	24.68	77.13	$(p < 0.01)$
b/n months	1.66	2	0.33	2.59	
Error	21.42	68	0.32		

4.7. ANOVA for hardness (sensory)

Source of Variation	SS	df	MS	F	Sig.
Total	103.95	80	-	-	
b/n temperature	46.62	8	5.83	34.29	$(p < 0.01)$
b/n species	41.51	2	20.76	122.12	$(p < 0.01)$
b/n months	4.17	2	2.09	12.29	$(p < 0.01)$
Error	11.65	68	0.17		

4.8. ANOVA for juiciness (sensory)

Source of Variation	SS	df	MS	F	Sig.
Total	107.58	80	-	-	
b/n temperature	26.47	8	3.31	19.47	$(p < 0.01)$
b/n species	59.36	2	29.68	174.59	$(p < 0.01)$
b/n months	9.88	2	4.94	29.06	$(p < 0.01)$
Error	11.87	68	0.17		

4.9. ANOVA for Hardness 1

Source of Variation	SS	df	MS	F	Sig.
Total	332.35	89	-	-	
b/n temperature	130.83	9	14.54	5.86	$(p < 0.01)$
b/n species	11.02	2	5.51	2.22	
b/n months	1.96	2	0.98	0.40	
Error	188.54	76	2.48		

4.10. ANOVA for Hardness 2

Source of Variation	SS	df	MS	F	Sig.
Total	216.57	89	-	-	
b/n temperature	73.78	9	8.20	4.66	$(p < 0.01)$
b/n species	6.15	2	3.08	1.75	
b/n months	2.54	2	1.27	0.72	
Error	134.1	76	1.76		

4.11. ANOVA for Cohesiveness

Source of Variation	SS	df	MS	F
Total	0.9	89	-	-
b/n temperature	0.15	9	0.02	2.22
b/n species	0.04	2	0.02	2.22
b/n months	0.04	2	0.02	2.22
Error	0.67	76	0.09	

4.12. ANOVA for Springiness

Source of Variation	SS	df	MS	F	Sig.
Total	4.05	89	-	-	
b/n temperature	0.45	9	0.225	5.62	$(p < 0.01)$
b/n species	0.72	2	0.36	9	$(p < 0.01)$
b/n months	0.03	2	0.015	25	$(p < 0.01)$
Error	2.85	76	0.04		

4.13. ANOVA for stiffness

Source of Variation	SS	df	MS	F	Sig.
Total	1600.76	89	-	-	
b/n temperature	228.93	9	25.44	2.86	(p < 0.01)
b/n species	619.01	2	309.51	34.78	(p < 0.01)
b/n months	76.19	2	38.1	4.28	(p < 0.01)
Error	676.63	76	8.9		

4.14. Correlation between myofibrillar proteins and collagen and texture profile parameters.

Correlations

			TC - 50	MY - 50	H - 50	C - 50	Spr - 50	S - 50
Spearman's rho	TC - 50	Correlation Coefficient	1.000	-1.000**	-.500	-1.000**	-1.000**	.500
		Sig. (2-tailed)	.	.000	.667	.000	.000	.667
		N	3	3	3	3	3	3
	MY - 50	Correlation Coefficient	-1.000**	1.000	.500	1.000**	1.000**	-.500
		Sig. (2-tailed)	.000	.	.667	.	.	.667
		N	3	3	3	3	3	3
	H - 50	Correlation Coefficient	-.500	.500	1.000	.500	.500	.500
		Sig. (2-tailed)	.667	.667	.	.667	.667	.667
		N	3	3	3	3	3	3
	C - 50	Correlation Coefficient	-1.000**	1.000**	.500	1.000	1.000**	-.500
		Sig. (2-tailed)	.000	.	.667	.	.	.667
		N	3	3	3	3	3	3
	Spr - 50	Correlation Coefficient	-1.000**	1.000**	.500	1.000**	1.000	-.500
		Şig. (2-tailed)	.000	.	.667	.	.	.667
		N	3	3	3	3	3	3
	S - 50	Correlation Coefficient	.500	-.500	.500	-.500	-.500	1.000
		Sig. (2-tailed)	.667	.667	.667	.667	.667	.
		N	3	3	3	3	3	3

**. Correlation is significant at the .01 level (2-tailed).

Correlations

			MY	Co	H	C	SPR	S
Spearman's rho	MY	Correlation Coefficient	1.000	.301	.328	.109	.389	.309
		Sig. (2-tailed)	.	.399	.354	.763	.266	.385
		N	10	10	10	10	10	10
	Co	Correlation Coefficient	.301	1.000	-.191	-.671*	-.462	-.055
		Sig. (2-tailed)	.399	.	.596	.034	.179	.880
		N	10	10	10	10	10	10
	H	Correlation Coefficient	.328	-.191	1.000	-.021	.689*	.948**
		Sig. (2-tailed)	.354	.598	.	.953	.028	.000
		N	10	10	10	10	10	10
	C	Correlation Coefficient	.109	-.671*	-.021	1.000	.628	-.255
		Sig. (2-tailed)	.763	.034	.953	.	.052	.476
		N	10	10	10	10	. 10	10
	SPR	Correlation Coefficient	.389	-.462	.689*	.628	1.000	.523
		Sig. (2-tailed)	.266	.179	.028	.052	.	.121
		N	10	10	10	10	10	10
	S	Correlation Coefficient	.309	-.055	.948**	-.255	.523	1.000
		Sig. (2-tailed)	.385	.880	.000	.476	.121	.
		N	10	10	10	10	10	10

*. Correlation is significant at the .05 level (2-tailed).

**. Correlation is significant at the .01 level (2-tailed).

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