Influence of Processing Variables on Protein Quality and Frozen Storage Stability of Two Commercially Important Species of Squid (*Loligo duvaucelii and Doryteuthis sibogae*)

A Thesis submitted to

Cochin University of Science and Technology

In partial fulfilment of the requirements for the degree of

Doctor Of Philosophy

By

MOHANAN.P.

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

November 2004

Certificate

This is to certify that this thesis entitled "Influence of processing variables on protein quality and frozen storage stability of two commercially important species of squid (Loligo duvaucelii and Doryteuthis sibogae)" is a bonafide record of the research work carried out by Sri Mohanan P under my supervision and guidance in the School of Industrial Fisheries, Cochin University of Science and Technology, in partial fulfilment of the requirements for the degree of Philosophiae Doctor of the Cochin University of Science and Technology and no part thereof has been presented before for any other degree, diploma, associateship, fellowship or any other similar title or recognition.

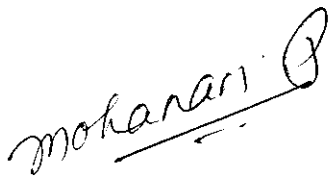
23/11/04

Kochi-16
November, 2004

Dr. Saleena Mathew
Supervising Guide

Declaration

This is to certify that this thesis entitled "Influence of processing variables on protein quality and frozen storage stability of two commercially important species of squid (*Loligo duvaucelii* and *Doryteuthis sibogae*)" is an authentic record of the research carried out by me under the supervision of Prof. (Dr.) Saleena Mathew, School of Industrial Fisheries, Cochin University of Science and Technology, in partial fulfilment 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 for any degree, diploma, associateship, fellowship or any other similar title or recognition in any University.



Kochi
Cochin-16

Mohanan P.
November, 2004

ACKNOWLEDGEMENT

I wish to express with great happiness my deep sense of gratitude to Dr. Saleena Mathew, Professor, School of Industrial Fisheries for suggesting the topic and for her inspiring guidance and ceaseless encouragement rendered all through the progress of this work. Her continuous interest in my work has inspired me throughout.

I am highly grateful to Dr. Ramakrishnan Korakandy, Head of the School of Industrial Fisheries for the valuable help rendered for the successful completion of the Ph.D programme

I take the opportunity to thank all other faculty members in the School of Industrial Fisheries for the valuable suggestions rendered all through the Ph.D programme.

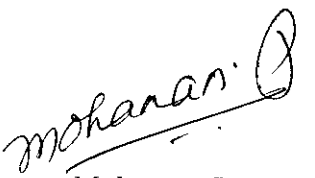
I wish to express my thanks to Shri.H.Krishna Iyer for his assistance in the statistical analysis of the data.

I owe my hearty thanks to my colleagues Miss Maya Raman and Mr. Prasanth for their help, assistance and advice at every stage of my work. My thanks are also to Dr. A.V. Shibu for carefully reading through the manuscript. Without their help, this work would not have been possible. Help rendered by other colleagues by way of fruitful discussions is gratefully acknowledged. I am extending my thanks to all my friends especially to Late Mr. Abdul Gafar and all other Research Scholars of the School of Industrial fisheries for their kind, timely and constant help and co-ordination during the course of my work. With much gratitude, I acknowledge the sincere co-operation and help of non-teaching staff of School of Industrial Fisheries.

I thank the management and staff of M/s. Cherukkat Industries, Chandiroor and M/s Anand Exports, Aroor for their enthusiasm and assistance during sampling and processing during my work. I am grateful to M/s. Bhattsons, Aroor, for providing the necessary facilities to store my samples in their cold storage.

I express my gratitude to Cochin University of Science and Technology for the facilities provided and the financial support in the form of fellowship, which enabled me to carry out this work.

Finally I am indebted to my family especially my wife Smt. Parvathy for their care, enduring support and inspiration given during my research period.



Kochi-16

Mohanan. P.

CONTENTS

Chapter 1

GENERAL INTRODUCTION

1.1. Importance of Cephalopods

Indian fish landings mainly consist of teleost fish, elasmobranch, cephalopods & crustacea. Among the above species, cephalopods play a vital role and their contribution spreads about 3.0-3.5% of the whole fish landings of India (Anon, 1998). Cephalopods namely octopus, cuttlefish and squid – comprise one of the most significant components of marine life. All are large fast growing and active predators with highly evolved and specialized qualities of great inherent interest. Southern coast of the country contributes most of the cephalopod catch, especially the southeast coast. Cephalopods are exclusively marine molluscs and there are about 660 species in the world ocean. (Silas et al., 1985). There are about 80 species of cephalopods of commercial and scientific interest distributed in the Indian seas, the major cephalopods being cuttlefish, squid, and octopus (Silas, 1968; Oommen, 1977). They are an integral part of ocean ecosystem, which are heavily exploited as a major human food source. They are important animals used in biological research in areas such as neuro science.

Large populations of cephalopods are found in the world's entire ocean. They are major food resources for many of the top predators such as whales, dolphins etc. The major species that dominate our squid catch are *Loligo duvaucelii* (Orbigny, 1848) and *Doryteuthis sibogae* (Adam, 1954). In Kerala, the above species contribute about 95-98% of the total squid catch.

1.2.Contribution of Cephalopods

Over the years, the frozen seafood markets for Indian seafood have witnessed rapid changes. USA was the principle buyer for our frozen shrimps for a long time but after 1971, Japan emerged as a principle buyer followed by Western European countries. While Japan accounts for 50% in the volume and 31% in the value, the member countries in European Union emerged as a leading market accounting 20% in volume and 19% in the value during the year 2002-2003, while USA counted for 12% in volume and 24% in value. China emerged as the largest market in terms of volume accounting for 32% of the total volume of marine products exported from the country. There was a decrease during 2001-2002 in quantity and value when compared to 2000-2001. The export of frozen squid recorded an increase of 2162 metric tons (value of Rs.5.24 crore) during 2001-2002. During the year 2001-2002 India exported a total quantity of 424,470 tones of seafood worth US $ 1,253 million (Rs.5957 crores). Frozen squid contributed 9%(39822.3 tonnes) of the total catch. In terms of value, frozen squid contributed about 5.5%(Rs.327.6 crore) of the total earnings (Bojan, 2003).

1.3.Resources

There are 30 families and 460 species of squid known to exist in oceans around the world, which excludes 190 species of octopods. There are about 18 species of decapods and 10 species of octopods along the Indian coast. In the major revision Adam (1939) has redescribed 53 species under 23 genera. Taki (1981) has given a catalogue of the cephalopods, which includes many species that are also distributed in Indian Ocean.

1.4.Morphology

Squid is practically a cone shaped sack consisting of several layers of tissue, which envelops the organs. The head with 8 or 10 tentacles, which sprout around the mouth, is loosely attached to the body. There are no bones to support the muscles. Squid and cuttlefish refer to all 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.

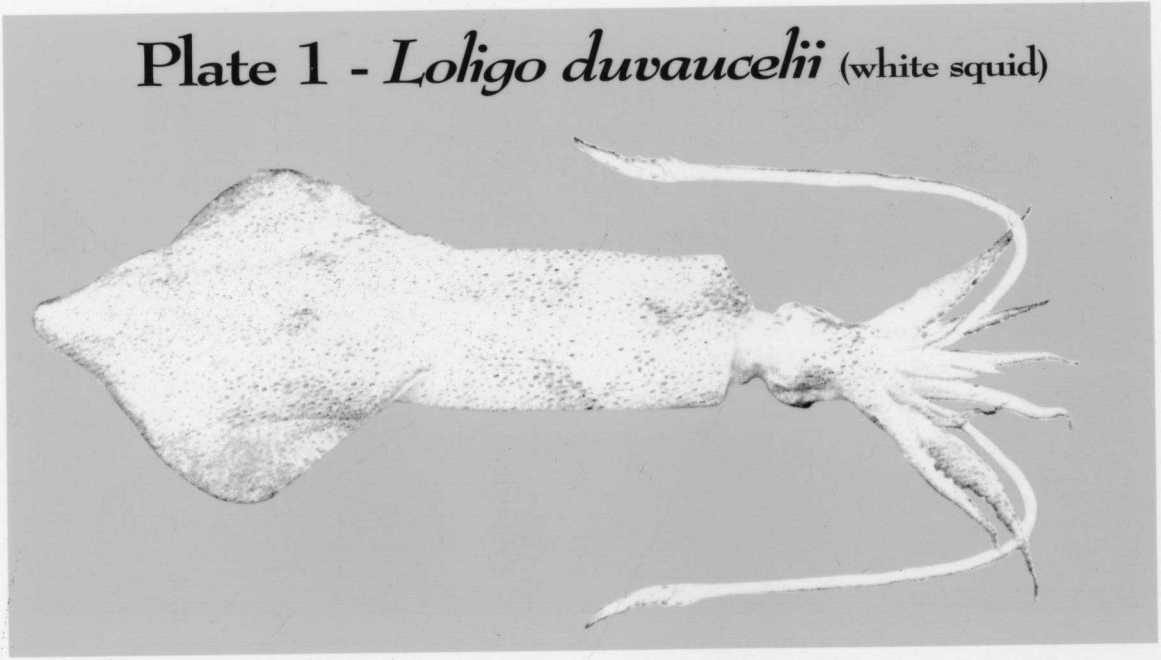
Squids have a chitinous internal shell and a cylindrically elongate body. A large calcified internal shell, the cuttlebone, characterizes Cuttlefish and an ovoid body somewhat flattened dorsoventrally. The fins are either terminal or marginal in position and uniting at the apex of the mantle.

1.4.1. *Loligo duvaucelii* (Orbigny, 1848) -Plate 1

The mantle is cylindrically elongate and tubular with almost parallel sides upto the point where the fins originate, then tapers to a blunt posterior point. The mid-dorsal projection of the anterior margin of mantle is rounded. The fins are small and short, 50-55 per cent of mantle length and rhombic in outline. They are broadest near the middle, the anterior margin is nearly straight or slightly convex and the posterior margin is concave. 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, the median ones being more enlarged than the marginal ones. The large manus suckers bear about 14-17 pointed teeth on the rings.

In fresh condition immediately upon capture, the squid is colourless and mantle transparent showing the internal visceral organs. There are

Plate 1 - *Loligo duvaucelii* (white squid)



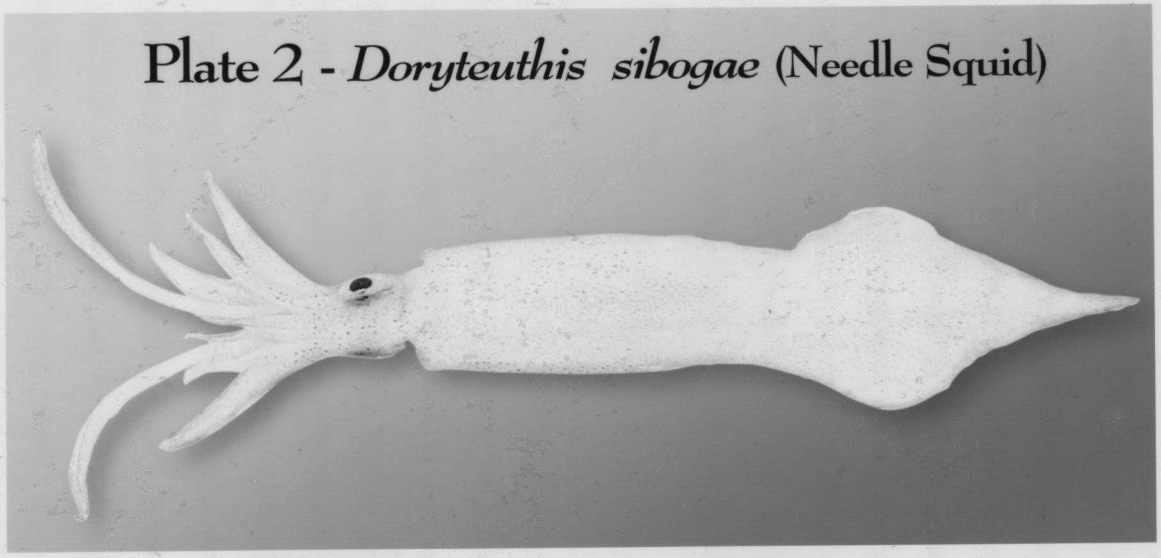


Plate 2 - *Doryteuthis sibogae* (Needle Squid)

numerous light brown chromatophores scattered all over the mantle, fins, head and arms. On the ventral side chromatophores are less dense and appear whitish.

1.4.2.*Doryteuthis sibogae* (Adam, 1954) -Plate 2

The mantle is long and slender, widest at the middle and from the insertion of the fins it becomes narrow and tapers to a sharp end posteriorly. The mid-dorsal projection of the mantle is pointed anteriorly. A distinct longitudinal concentration of chromatophores is present midventrally on the mantle. Such concentration is distinct in males and feeble in females. In fresh condition, the mantle is whitish with dark brownish chromatophores on the dorsal side. On the ventral side the chromatophores are concentrated medially in the form of a line.

1.5. Quality of Squid

Quality is defined as a totality of features and characteristics of a product or service that, bear on its ability to satisfy a given need, degree or grade of excellence, grade of goodness. Quality is often related to the price at which the commodity is purchased or the purpose for which it is to be used. In relation to seafood, the quality is the sum total of its composition, nutritive value, degree of freshness, physical damage, deterioration while handling, processing and storage, distribution and marketing, hazards to health etc. The yardstick for measuring the quality is consumer acceptance. Consumer preferences, however, vary from country to country, region to region and even period to period. In order to assure quality, it is essential to know what the consumer is looking for. But large-scale commercialization of cephalopods has been difficult because of intense autolysis occurring in

the muscle associated with high level of proteolytic activities. These high level activities have been physiologically associated with the growth, which induce rapid post-mortem degradation with accompanying release of high level of nitrogen from the muscle. This will consequently result in enhanced microbial growth leading to tissue spoilage. Acid phosphatase (EC 3.1.3.2) is the marker enzyme of lysosome and exists in a latent form. Enhanced activity of acid phosphatase seems to be characteristic of tissue damage and have become a useful diagnostic and experimental tool. The presence of cathepsin D like activity proteinase of lysosomal origin can play a vital role in the degradation of squid mantle muscle. It is logical to experiment methods/treatments, which can arrest or minimize the release of lysosomal enzymes so that post-mortem degradation of squid tissue can be reduced.

From the consumer's point of view, some of the important factors that determine the quality of the product are: species, odour, appearance of the fish, flavour, texture, presence of parasites, presence of food poisoning bacteria, presence of foreign matter, composition of fish, and finally the packing. Longer the time taken to reach the final consumer higher the degree of spoilage unless, the fish is kept under controlled conditions. Throughout the world, the food business operations are quickly changing from a production-driven into a consumer-driven business. More and more stringent demands are to be met in order to avoid loss of consumer confidence. Also the food industry is facing tighter constraints because of increasing manufacturer's product liability, hence stricter environment and animal-friendly production methods are becoming more important.

To maintain our prime position in the international arena, it is essential that the industry is able to compete in quality, wholesomeness

and price with similar products in the global market. Our fishery products must also meet the organoleptic, bacteriological and chemical standards stipulated by the importing countries. Thus, it becomes imperative for the fish processing industry in India, to establish the most competitive quality systems and take adequate steps to establish Good Manufacturing Practices (GMP). Investigations carried out on the frozen storage characteristics of Squid and cuttlefish show that these products can be stored for a maximum period of 15 weeks at -18°C (Joseph et al., 1977; Joseph and Perigreen 1988).

1.6. Background of the Work

Cephalopods are utilized as an important food item in various countries because of its delicacy as raw consumed food. Mainly sepia and loligo are consumed raw by Japanese and Russians. The freshness of the products is very important when the product is consumed raw. The major species that dominate our squid catch are *Loligo duvaucelii and Doryteuthis sibogae*. There is a noticeable difference in the quality of both the species. The needle squid (*Doryteuthis sibogae*) contributes about 35% of the total squid landing. Due to the fast deterioration, a major portion of the needle squid, which is caught during the first few hauls, is thrown back to sea. The catch in the last hauls only are taken to the landing centers. At present the needle squid is processed as blanched rings and the desired quality is not obtained if it is processed as whole, whole cleaned or as tubes. In this study an attempt is made to investigate the biochemical characteristics in both the species of squid in relation to their quality and, the process control measures to be adopted. The effect of various treatments on their quality and the changes in proteolytic and lysosomal enzymes under various processing

conditions are also studied in detail. Thus this study can provide the seafood industry with relevant suggestions and solutions for effective utilization of both the species of squid with emphasis on needle squid.

1.7. Objectives of the study

- Preliminary composition studies to highlight the nutritional significance of squid
- To study the quality changes during various storage conditions.
- To study the changes in structural protein and other quality parameters under various treatments.
- To characterize the protein fractions using SDS Polyacrylamide gel electrophoresis.
- To study the kinetics of tissue proteolytic enzymes and to compare the changes in the enzyme activity under various processing conditions.
- To study the lysosomal stability as an index of the autolytic spoilage in the two species.
- To recommend an effective treatment to minimize the deteriorative changes by post mortem autolytic changes and thereby to maintain the quality of the products.
- To recommend process control measures to increase the utilization of both the species.

1.7. References

Adam, W., (1939) The Cephalopoda in the Indian Museum, Calcutta. Rec. Indian Mus., **41**, 61-110.

Anon., (1998) MPEDA publication, MPEDA statistical data on total fish landing in India.

Bojan, (2003) Current status and prospects of seafood export. India International Seafood Show 2003 Souvenir.

Joseph, J., Varma, P.R.G. and Venkataraman, R., (1977) Iced and frozen storage of squid (Loligo species) Fish. Tech. **14**, 13-20.

Joseph, J. and Perigreen, P.A., (1988) Studies on frozen storage of cuttlefish fillets. Fish. Tech. **25**, 32- 35.

Oommen, V.P., (1977) Two octopods new to Arabian Sea. Indian J. Fish. **24** (1&2), 25-32.

Silas, E.G., et al., (1985) Bull. CMFRI on cephalopod bionomics, fisheries and resources of exclusive economic zone of India, 37.

Silas. E. G., (1968) Cephalopoda of the west coast of India collected during the cruise of the research vessel 'VARUNA' with catalogue of the species known from the Indian Ocean. Proc. Symp. Mollusca. Mar. Biol. Ass. India. 277-359.

*Taki, J., (1981) A catalogue of the cephalopoda of Wakayama Prefecture in A catalogue of the cephalopoda of Wakayama Prefecture, the province of Kii I. Bivalvia, Scaphopoda and cephalopoda Sato mar. Biol. Lab. Japan, 301.

* Not referred in original

Chapter 2

COMPOSITION OF SQUID MUSCLE WITH SPECIAL EMPHASIS ON PROTEINS

2.1. Introduction

Cephalopods are mainly commercial in the Mediterranean countries and the Far East, but although consumption is much lower in the rest of the world, there has been a considerable increase in recent years. These species are normally processed both fresh and frozen. The percentage of the edible portion of the cephalopods is exceptionally high, between 60 to 80% of the total weight depending on species, size of specimen and sexual maturity, whereas in fish the percentages is only 40-70% (Sikorski & Kolodziejska, 1996)

The main edible portion of the squid is the mantle. A variety of processed food is made from squid as the raw material and each is appreciated for its special flavour and taste. The fresh squid has a sticky feeling, and is not easy to chew. It contracts following cooking and a round shape is assumed due to the shrinkage, especially the skin. The squid meat differs from fish muscle, structurally and biochemically.

The squid muscle is composed of several layers of fibres running transversally to each other and covered with several sheets of connective tissue. The muscle fibres are not only strong which differ from fish meat, but their arrangement is also very different. The protein present can be broadly categorized into myofibrillar protein composed of similar myofibrils as the muscle protein of other animals, sarcoplasmic proteins which fills the gaps of myofibrils and muscle based protein composed of connective tissue. Myofibrillar protein is the major constituent followed by sarcoplasmic protein and muscle based proteins.

The mantles of cephalopods are quite close in chemical composition to low fat fishes and white meat fish. The four major constituents in the edible portion of cephalopods are water, proteins, lipids and ash (minerals). The analysis of these four basic constituents is referred to as proximate analysis (Love, 1970) Different factors like environmental conditions, which include temperature, salinity, pressure and availability of food etc. have profound influence in the biochemical composition. There may be group specific or species-specific in the biochemical composition (Stansby, 1982). By comparing the composition of squid muscle with some selected species of cephalopods, an attempt is made to emphasize the nutritional significance of squid as a food item. Information of the biochemical constituents will also be helpful in defining optimum processing and storage conditions in order to preserve the quality to the maximum.

Micro-organisms play a vital role in the quality and safety of squid muscle, which is being handled by the workers from the point of catch till it reaches the processing plant. Sanitary significant bacteria are the major cause of post mortem changes in the squid muscle. The study of microbiology helps us to define the precautionary measures to reduce the bacterial load to get a premium quality product.

Every fish will have a native flora of micro-organism. The nature of this native microflora will largely depend upon the habitat of fish. Since the washing of seafood items in fresh water is inevitable, the quality of water used should be taken care of.

The microbes of fresh water are mostly a mixture of gram +ve and gram –ve organisms. They are less tolerant to salt; rather, they are killed or inhibited by salt concentrations above 0.5%. The fresh water microbes are

found to be a mixture of spoilage organisms and human pathogens. The presence of human pathogens can be traced to the intervention of human activities and fresh water availability.

2.2.Review of literature

2.2.1. Proximate composition

In order to emphasize the importance of squid and the quality deterioration during various processing steps, elaborate studies in food chemistry have been carried out. In the study done by Tze-kuei-chiou et al., (2000), changes in chemical constituents and quality of Argentina squid before and after drying during various seasons, were investigated. Lakshmanan and Balachandran (2000) in their study on various bio-chemical properties of squid and cuttlefish compared the proximate composition of fresh cephalopods available in Kerala coast. Lakshman et al., (1993) has done a detailed study on the quality of commercially frozen cephalopod product from India, where the selected species were loligo species of squid and sepia species of cuttlefish. The proximate composition and the sweet flavouring compounds like water extractable nitrogen (WEN), non-protein nitrogen (NPN) and alpha amino nitrogen content were studied in fresh and frozen stored cephalopods. Selvaraj (1991) has studied the bio-chemical changes of *Loligo duvaucelii* on frozen storage before and after treatment with ascorbic acid. Sikorski and Kolodziejska (1986) have studied the chemical composition, structure and properties of *Loligo pealei* squid meat. Sugiyama et al., (1980) has studied the structural details and the usage of squid. The yield of edible fleshy parts in the body of squid is exceptionally high, being 60% to 80% of the total weight, depending on the

species, the size of the specimen and the sexual maturity. Pandit and Magar (1972) have found that the squid meat is rich in calcium, phosphorus, and iron and contains moderate amounts of B group vitamins. According to Varela et al., (1962), the biological value of squid proteins is 82 compared to 83.7 for octopus and 74.8 for shrimps.

The muscles of squid mantle differ in structure from the muscles of fish and mammals (Sikorski and Kolodziejska, 1986). Several studies on the proximate composition of various marine species have also been done by several authors (Panchavarnam et al., 2003; Smuruthi et al., 2003; Chand et al., 2001; Pacheco-Aguilar et al., 2000; Kher-un-Nisa et al., 1995).

2.2.2. Fractionation of protein

There are many publications concerning muscle protein solubility in moderate ionic-strength saline solutions in the various myosystems. These studies are of interest in that many of the functional properties of fish muscle are related to the solubility of the constituent Proteins (Jimenez and Borderias, 1983; Hultin et al., 1995).

The myofibrillar proteins of cephalopods are highly water soluble, unlike fish and mammalian proteins. Nearly 85% of the total protein in squid muscle could be solubilized with distilled water by exhaustive extraction with water (Matsumoto, 1958). Squid myosin is also reported to be more susceptible to trypsin and myosin ATPase and is reported to be more easily inactivated by heating than that of fish and mammals (Tsuchiya et al., 1978). Tsuchiya et al, (1977) has isolated and purified the squid actin of *Todarodes pacificus*. A detailed study on the solubility of rabbit muscle protein after various time temperature treatments was carried out by Paul et

al., (1966). Kitabayashi et al., (1954) and Matsumoto et al., (1959) have compared the fraction of squid with other cephalopods in (*Todarodes pacificus)* proteins.

Lakshmanan and Balachandran (2000) have made a comparative study of the protein fractions of frozen squid and cuttlefish. Reghunath (1984) has also carried out experiments on *Loligo duvaucelii* tubes to determine the rate and quantum of the leaching of WEN and NPN when stored in slush ice which showed a high content of water soluble nitrogen fraction The fractions of protein in various species of squid were studied by Sikorski and Kolodziejska (1986). Tsuchiya et al., (1978) has extracted and purified the squid myosin of *Todarodes pacificus*. Purity of each preparation obtained was checked by SDS polyacrylmide gel electrophoresis. The same author has worked on the physico-chemical properties of squid myosin. A characteristic feature of sarcoplasmic fraction is the high activity of proteases, reported in fresh unfrozen muscle, which brings about extensive degradation of the myofibrillar proteins during the course of fractionation. Horie et al., (1975) and Iguchi et al., (1981) has studied the fractionation of squid protein in various species.

2.2.3. Bacteriology

Seafood products pass through physical, chemical and bacteriological hazards at various areas of landing, transportation and processing. Most important of all these is the handling of the material by the workers, which directly influence the quality of the seafood by the contamination due to bacteria. Antony et al., (2003) has studied the presence of sanitary significant bacteria like *Escherichia coli, coagulase positive staphylococcus,*

faecal streptococcus, and salmonella on Tuna-mas and mas based diversified convenience products. The presence of *E- coli*, which is considered as a faecal indicator organism, was studied in the deep-sea water fishes by Madhusudana Rao & Surendran (2003). Nazeem Beena et al., (2002) has studied the growth of cross contaminating bacterial pathogens in fish muscle at different temperatures of storage of 5°C, 20°C and 35°C, to mimic the prevailing conditions in the retail fish markets. The samples at 35°C showed maximum growth of all the microorganisms. Sudha (2002) in her work, explained prevalence of *Vibrio species* in fish from pelagic and demersal habitats. Iyer (2000) has detected presence of various serotypes of salmonella from various seafoods in Cochin, Bombay and Calicut. Lilabati and Viswanath (1999) have studied the changes in the bacterial and fungal quality during storage of smoked *Esomus danricus*. Occurrence of *Vibrio cholerae non-01* and their dispersion phenomena in the coastal waters of Mangalore was investigated by Sreeja and Raveendran (1999). The near-shore stations recorded comparatively higher incidence of these organisms, while in deep waters the occurrence showed a gradual decrease. Selvaraj (1991) has studied the microbiological quality of ascorbic acid treated *Loligo duvaucelii* before and after treatment. Shetty and Shetty (1990) studied the bacteriological changes of *Sardinella longiceps* during the chilled seawater storage.

Fishing from polluted waters or washing in polluted coastal water are also responsible for bacterial contamination. Studies conducted in this respect showed that salmonella was present only in 1.86% of the samples from frozen cuttlefish (Varma et al., 1985). Lakshmanan et al., (1984) reported that 94% of the samples were free from salmonella. Lakshmanan et al., (1993) detected

only 5.5% of salmonella in whole squid, 6% in process cuttlefish and nil in processed squid and whole cuttlefish.

Studies conducted by Varma et al., (1985) and reported that all the samples of cuttlefish were free from *Vibrio cholerae*. Lakshmanan et al., (1993) observed in his study 11.1% of *Vibrio cholerae non-01* in frozen whole squid, 23.8% in processed squid, 6.4% in whole cuttlefish and 13.6% in processed cuttlefish. All the samples were free from *Vibrio cholerae –01*. Since water is used in large quantities during processing, it can be a major source of contamination of *Vibrio cholerae*. (ICMSF 1978).

The objectives of the present study are

- to compare the protein content of various other cephalopods with selected species of squid.
- to evaluate the nutritional significance of squid as a food item with reference to the other species of cephalopods.
- to compare the extractability of various fractions of proteins of squid with other cephalopods.

2.3.Materials and Methods

Fresh samples of *Loligo duvaucelli, Dorytheuthis sibogae, Sepia pharaonis and Octopus globosus,* were caught from the offshore waters of Munambam for the study. The samples immediately after catch were transferred to clean insulated boxes and iced indirectly by separating the samples from ice by a thin layer of polythene sheet. The samples were transported to the processing laboratory where the treatment and processing of the samples were done. The chilled samples were skinned, cleaned and

taken in triplicate for the study. Samples were drawn for various analyses like moisture content, crude fat, ash content, crude protein and various fractions of protein.

2.3.1. Determination of Moisture

The moisture content was estimated by the method of AOAC (1990). The moisture content was determined by drying 10gms of the sample was dried at 103°C in thermostatically controlled hot air oven. The samples were taken in pre-weighed glass dish with cover and kept in oven till the weight become constant. The weight was checked for constant weight by repeatedly heating and then cooling the sample in a desicator. The percentage solid was determined from the above experiment by using the formula

$$\text{Percentage solid} = \frac{\text{Weight of dry sample}}{\text{Weight of wet sample}} \times 100$$

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

2.3.2. Determination of Crude Fat

Fat content of the moisture free sample was determined by extracting the fat by using a suitable solvent by soxhlet extraction method (AOAC 1990). About 2 grams 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 the solvent. The unit was heated over a water bath and the temperature was controlled at 40°C-60° C so that the solvent boiled continuously and siphoned 5 to 6 times per hour.

Extraction was continued till the solvent in the extractor became colourless and fat free. The solvent in the receiving flask was evaporated completely and weighed for fat content.

$$\text{Percentage of fat} = \frac{\text{Weight of fat}}{\text{Weight of sample}} \times 100$$

2.3.3. Determination of Ash Content

Ash content was determined by the incineration of the sample (AOAC 1990). 2 grams of sample was taken in a pre-weighed silica crucible and the sample was charred on low heat. Then it was then kept at 550°C in a muffle furnace to get a white ash, which was cooled in a decicator and weighed.

$$\text{Percentage of ash} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100$$

2.3.4. Determination of Crude Protein

1gm of homogenized sample was used for determining the crude protein content using Micro Kjeldahl method (AOAC 1990). About 1 gram of sample was accurately weighed into a digestion tube. About 2gms of digestion mixture ($CUSO_4$ and K_2SO_4 as a catalyst in the ratio 1:8) and 10 ml of concentrated H_2SO_4 (AR) were added to the sample taken in a digestion tube. The samples were digested to a clear solution in a Kel Plus 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 Kjeldahl micro distillation apparatus. The bottom end of the condenser was fitted to a delivery tube, which was immersed in 10 ml of 2% boric acid solution with added

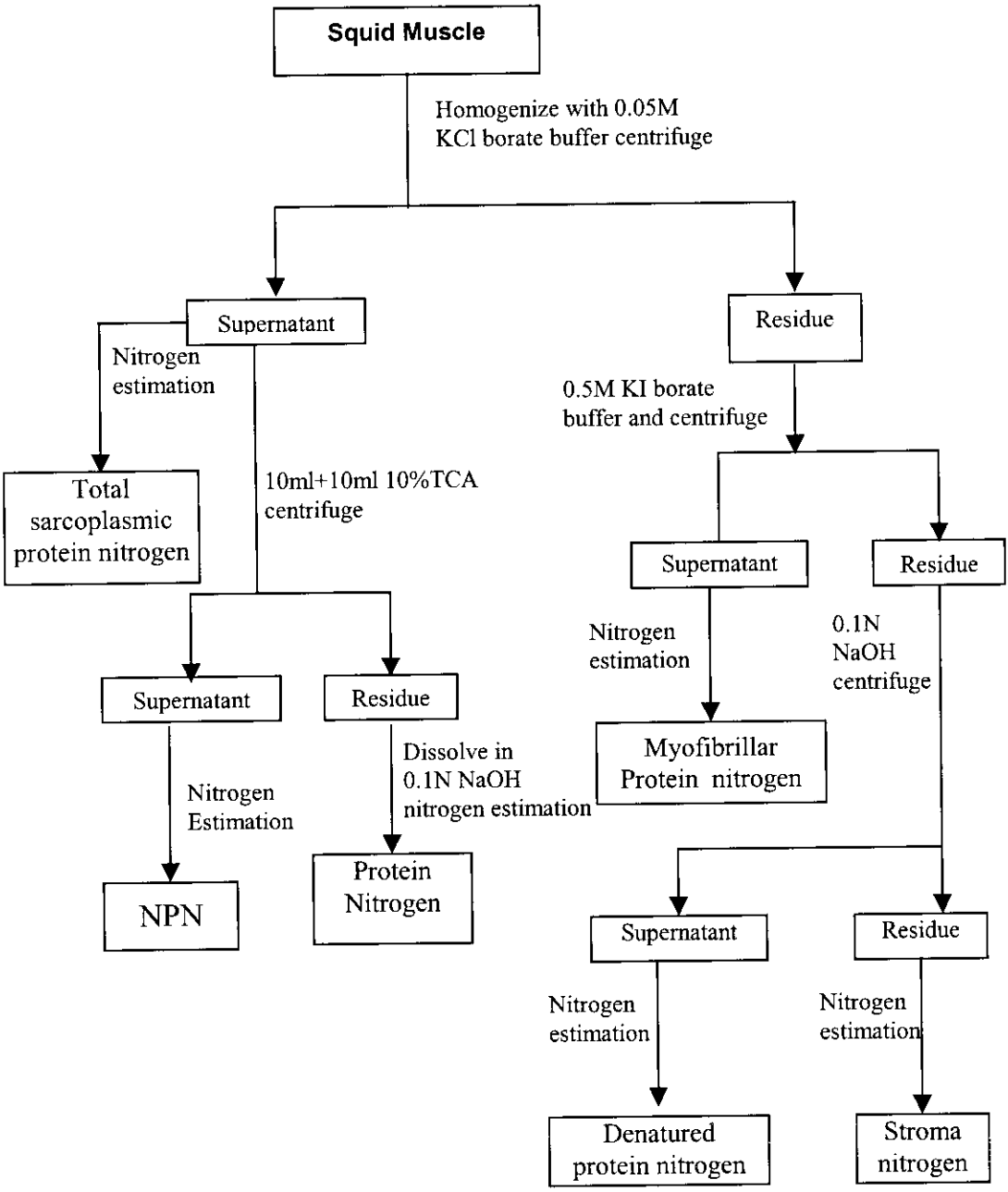
Tachiro's indicator. 40% NaOH was added to the sample in the distillation unit to make it alkaline. The ammonia thus produced on steam distillation was absorbed into the boric acid solution. The distillate collected was back titrated against standard N/70 H2SO4 using Tachiro's indicator and determined the nitrogen content. The nitrogen content thus obtained was multiplied by a factor 6.25 to obtain the crude protein content of the sample.

$$\% \text{ Protein} = \frac{V \times 1 \times 100 \times 100 \times 6.25}{5 \times 5 \times \text{weight of the sample}}$$

2.3.5. Fractionation of protein

The protein of the muscle was fractionated by method of Paul et al., (1966). A detailed scheme of the procedure is shown in Figure 2.1. The extraction scheme was based on separation into sarcoplasmic, myofibrillar and stroma fractions. The samples were disintegrated with a Virtis high-speed homogenizer in 50 ml of KCl- Borate buffer prepared according to Gomori (1955), with an ionic strength of 0.05 and pH 7.5, then centrifuged at 1200 X G for 10 minutes. The supernatant was decanted, and the residue was extracted again with 35 ml of KCl – Borate buffer. The supernatants from the two extractions were combined and made to volume; Aliquots were taken for determination of total sarcoplasmic nitrogen, and for the separation into nonprotein and protein nitrogen of fractions and by precipitation with 10 % trichloroacetic acid. The total nitrogen of this extract is designated as sarcoplasmic protein. The residue from the sarcoplasmic protein extraction was treated with KI- Borate buffer of ionic strength 0.6 and pH 7.5, to obtain the myofibrillar proteins.

Figure 2.1.
SCHEME OF FRACTIONATION OF PROTEINS



Two extractions were made using 50ml and 35 ml of buffer solution followed by centrifugation at 1200 X G for 10 minutes. KI rather than KCl was used to minimize the gelation. Disintegration of the original sample, extraction and centrifugation in KCl and in KI solutions were carried out at 0°C throughout the process, in order to minimize the denaturation of proteins. The residue after extraction of myofibrillar proteins in KI-borate buffer extraction was further extracted with 50ml and 20ml of 0.1N NaOH at room temperature with gentle stirring to remove the denatured protein. The mixture with 50ml NaOH was kept at room temperature for 45 minutes before centrifugation and the second for thirty minutes. The residue from these extractions was designated as stroma fraction. All the above said extracts and TCA precipitated sarcoplasmic and stroma residues were analyzed for nitrogen content by micro Kjeldahl method.

2.3.6. Bacteriological Analyses

2.3.6.1. Total Plate Count

Both Nutrient agar (NA) and TGBE agar (Tryptone glucose beef extract agar) were used to determine the total plate count (TPC). The samples for bacteriological analysis were taken in a sterile stainless steel dish containing about 10gms of the sample aseptically transferred Blended the sample with 90ml of sterile phosphate buffer. Acid washed sterile sand was used to make the slurry homogeneous. The resulting dilution was considered as 10^{-1}. Prepared decimal dilutions by transferring 1ml of inoculum into 9ml of buffer solution taken in test tubes. Poured 1ml of inoculum from 10^{-4} and 10^{-5} to sterile petridishes. Pour about 20ml of TGBE agar and the plates were rotated slowly on a flat surface to ensure uniform

blending and the agar was allowed to solidify. Incubated the petridish at 37±1°C for 48 hours in the inverted position to prevent the condensation of moisture on the surface of the agar medium during incubation. The dilutions having 30 to 300 numbers of colonies were selected. TPC per gram as calculated using the formula

$$\text{TPC per gram} = \frac{\text{Number of colonies} \times \text{reciprocal of the dilution}}{\text{Weight of the sample}}$$

The sampling and the analysis of TPC were done by the method (APHA, 1998). The medium used was of Hi-media. The remaining portion of the inoculum at 10^{-1} dilution was used for isolation and identification of *E.coli* and *staphylococcus aureus*.

2.3.6.2. Escherichia coli

Escherichia coli was isolated from the homogenate using Turgitol 7 agar (APHA, 1998). 0.5 ml was taken and streaked to the medium using a bend glass rod. The plates were incubated for 18–24 hours at 37 °C and were observed for yellow, flat, smooth, circular, non-mucoid colonies with pin pointed pink center. The suspected colonies were taken for further confirmation tests. The suspected colonies from T_7 agar was taken and inoculated to Mac-Conkey broth and incubated at 44 ± 1°C. Indole test, Voges proskeur, Methyl red test, and citrate utilization tests were done. Number of *E.coli* per gram of the sample were calculated by the formula

$$\frac{\text{Number of colonies on } T_7 \times \text{reciprocal of the dilution}}{\text{Weight of the sample} \times 0.5}$$

2.3.6.3. Coagulase positive staphylococcus

Coagulase positive staphylococcus culture was done in Baired Parker Agar (B.P) (APHA, 1998). The staph, a proteolytic bacteria utilizes the egg yolk protein and a clear zone was formed surrounding the colony. Due to the presence of potassium tellurite, black colonies appeared on the medium. Coagulase positive staph can coagulate rabbit blood plasma. The organism was confirmed only after the coagulase test. Number of bacteria per gram of the sample was calculated using the formula

$$\frac{\text{Number of colonies on BP} \times \text{reciprocal of the dilution}}{\text{Weight of the sample} \times 0.5}$$

2.3.6.4. Salmonella

Salmonella was pre-enriched using lactose broth following US FDA (1995) method. Taken 375g sample in 3750ml of lactose broth. It was kept for 24 hours at 37°C and the selective enrichment was done in two separate broths, selenite cystine broth, and tetra thionate broth. In SCB the selenite was reduced to selonium by salmonella and colon bacilli decreased in number during 8 to 12hrs and thereafter increased rapidly. But salmonella multiplied from zero hour onwards. The samples were taken between 6 and 8 hours of incubation. In tetra thionate broth, salmonella reduced thionate to thionite and flourish while other faecal bacteria were inhibited. Further confirmatory test, like bio-chemical and serological test were carried out as per US FDA method (1995).

2.3.6.5. Vibrio cholerae

25g of sample was pre-enriched in 225ml of alkaline peptone water (APW) for 24 hours at 37°C. After incubation 1ml sample was taken from

just below the surface, since *vibrio cholerae* is micro aerophilic, and secondary enrichment was done in 9ml APW. This was incubated for 6-8 hours at 37^0C and was streaked on Thiosulphate citrate bile salt sucrose agar (TCBS). Further confirmatory tests were carried out as per US FDA method (1995).

2.4.Results

2.4.1. Proximate composition

The proximate composition of the selected two species of squid and other cephalopods are given in the table 2.1.

Table 2.1.Proximate composition of different species of cephalopods

Species	Moisture%	Fat %	Ash %	Protein%
Loligo	79.26	1.99	0.96	21.72
Needle	81.27	2.01	0.99	20.32
Octopus	77.25	1.2	0.9	17.49
Sepia	78.21	2	0.7	17.3

The needle squid showed the highest value for moisture content when compared to other species (81.27%). Loligo squid also had higher moisture content when compared with others (79.26%). The moisture content is higher in the case of needle when compared with loligo, which indirectly helps for the faster disintegration. The higher moisture content provides a strong substratum for the bacteria also.

Ash content of all the samples taken, have almost same value throughout the study. The fat content and ash content showed no significant changes in the selected species, but the moisture content and the protein content varied considerably. Fat and ash content in all the species is comparatively less and less susceptible to oxidation changes.

Table 2.2.Total nitrogen content of different species of cephalopods during various months

	Loligo	Needle	Octopus	Sepia
October	3.48	3.25	2.80	2.77
November	3.32	3.06	2.64	2.62
December	3.15	3.00	2.54	2.56
March	3.23	3.09	2.51	2.54
April	3.40	3.23	2.55	2.64
May	3.48	3.29	2.67	2.80
August	3.23	3.11	2.64	2.61
September	3.46	3.17	2.56	2.59

In table 2.2 the total protein content of various cephalopod species during various months was given. Throughout the study, there were no much changes in protein content in all the cephalopod species. In the case of sepia and octopus protein content ranges from 17-18%, but in the case of squid it ranges from 18-22%, which clearly shows that the squid is rich in protein in all seasons.

2.4.2. Fractions of Protein

Table 2.3.Changes in the extractability of proteins expressed as % nitrogen

	Loligo % N	% of protein fraction on total N	Needle % N	% of protein fraction on total N	Sepia % N	% of protein fraction on total N	Octopus % N	% of protein fraction on total N
Total nitrogen	3.48	100.00	3.30	100.00	2.87	100.00	2.84	100.00
Sarcoplasmic	1.84	52.97	2.21	66.97	1.15	40.00	1.10	38.70
Myofibrillar	1.56	44.97	0.99	29.98	1.44	50.32	1.59	56.02
Denatured	0.01	0.40	0.02	0.50	0.25	8.72	0.14	4.81
Stroma	0.07	2.00	0.07	2.00	0.03	1.03	0.02	0.83

Table 2.3.1.Changes in the extractability of fractions of sarcoplasmic proteins expressed as % nitrogen

	Loligo %	% of protein fractions on total sarcoplasmic N	Needle%	% of protein fractions on total sarcoplasmic N	Sepia %	% of protein fractions on total sarcoplasmic N	Octopus%	% of protein fractions on total sarcoplasmic N
Total	1.84	100.00	2.21	100.00	1.15	100.00	1.10	100.00
NPN	1.71	92.97	2.15	96.96	0.95	82.57	0.92	83.78
PN	0.11	5.99	0.07	2.96	0.15	13.29	0.15	13.23

NPN = Non protein nitrogen

PN = Protein nitrogen

Table 2.3 and Table 2.3.1 gives the detailed picture of various fractions of proteins in various species. The sarcoplasmic protein, which was extracted by 0.05 M KCl borate buffer, contributed about 53% of the total proteins in loligo, 67% in needle squid, 40% in sepia and 38% in octopus. Myofibrillar proteins, which were extracted by 0.6 molar KI borate buffer, contributed only 30%(needle) to 45%(loligo) of the total protein content. Denatured protein contributed 0.4(loligo) to 0.5%(needle) and stroma 2% to 3% in loligo and needle squid respectively. Protein nitrogen (PN) in loligo as per the fractionation method showed a very low content of about 6 to 7% only and non-protein nitrogen (NPN) contributed the rest (93-94%) of the total sarcoplasmic fraction. But in the case of needle squid, PN contributed about 3% and the rest by NPN. NPN was found to be very high in the cephalopods, especially in squids.

2.4.3. Bacteriology

Table 2.4. Microbiology of various species of Cephalopods

	TPC	*Ecoli*	*staph*	*Vibrio cholerae*	*salmonella*
Squid loligo	$1.87X10^5$	185	300	nil	nil
Squid needle	$2.03X10^5$	225	325	nil	nil
Sepia	$1.78X10^5$	175	310	nil	nil
Octopus	$1.82X10^5$	200	305	nil	nil

The different types of bacteria enumerated were *E-coli, staphylococcus, vibrio cholerae and salmonella* along with the Total Plate Count. The results are given in table 2.4. Irrespective of the species, TPC in the raw material seemed to be in higher range of $1.875X10^5$. *E-coli* also showed count ranging from 175-225 in all the species of cephalopods studied. *Coagulase positive staphylococci* were present in both the samples of squid, sepia and octopus. *Salmonella and vibrio cholerae* were absent in all the samples studied.

2.5.Discussion

Over the years, the efforts to establish proximate composition of fish have been partially successful, but many gaps in our knowledge still exist. Need for proximate composition of fish and other seafoods is currently greater than in the past because of its nutritional significance and the changes occurring in each chemical components during various stages of processing. The data regarding chemical composition of cephalopods is also vast, but information regarding a species of squid, *Doryteuthis sibogae*, which is scarcely utilized for consumption and in this study a comparative analysis is made with other species of cephalopods. The moisture content of

various species of cephalopods studied was in the range 75-84% (Suyama and Kobayashi, 1980, Tse-Kuei-Chiou, 2000, Lakshmanan & Balachandran, 2000, Selvaraj, 1991, Jose Joseph et al., 1977 and Lakshmanan et al., 1993). The crude protein content varied from 17-22% in the cephalopod species studied with squids showing highest level. The work of Suyama and Kobayashi (1980) showed that the protein content of various species of cephalopods varied from 15-23% and moisture content had an inverse relationship with proteins. The work done on the mantle tissue of fresh *Loligo duvaucelii* in tropical waters also showed some variations of 17.5-22% (Selvaraj, 1995, Jose Joseph et al., 1988 and Raghunath, 1984). Studies of also confirmed that the squid muscle has higher total nitrogen than white lean fish (Sujiyama, 1980). The variations in the value of protein in the same species observed by various workers might be due to the difference in handling, affecting the leaching rate of proteins. In this study no significant variations were observed in total nitrogen in the different cephalopods species during various months of the year. Kawada and Takaschi (1955) had a comparable result showing no significant changes in protein and moisture content of squid muscle (*Todarodes pacificus)* throughout the year. The fat content showed values of 2% and less than 2% among the cephalopods studied. The ash content showed the mineral content in the muscle, which varied from 0.7 to 0.99%.

Although we correlate proximate composition to factors like geographical area or season of the year at which the fish is caught, the ultimate cause of the variation is due to the feed intake and other related factors such as metabolic efficiency of individual fish and the energy expenditure due to more or less active movement of the fish. The

cephalopods under study can be categorized to a group, with low oil content (under 5%) and high protein content (15-20%). Most of the food fishes are found in this category.

2.5.1. Protein Solubility

Proteins of fish muscle are divided into three classes based on their solubilities, water soluble, salt soluble and insoluble proteins. 77-85% of the total protein of squid is water-soluble and can extract more than 80% of the total protein by repeated washing (Matsumoto, 1958). According to Lakshmanan and Balachandran (2000) the myofibrillar protein content in cephalopod was 77-85% and the sarcoplasmic protein showed a value of 12-20%. Sikorski and Kolodziejska (1985) and Kolodziejska et al., (1987) have studied the composition of squid meat and characteristic features of squid protein with high protease activity and high solubility of myofibrillar proteins in water. There are also reports about the changes in the solubility of myofibrillar protein and collagen in squid, *Loligo pealei* (Otwell and Hamann, 1979). The rates of extractable protein nitrogen decrease were usually faster following pre-process holding (Connel and Howgate, 1971). The skinned products showed a greater loss of NPN owing to the leaching effect of these components during washing (Lakshmanan et al., 1993). The work of Joseph et al., (1977) arrived at a similar result showing decrease of non-protein nitrogen during washing. They also noticed that there was a high content of non-protein nitrogen in squid meat. The results also agree with the earlier findings of Japanese scientists (Borgstrom, 1965). Proper presentation of the squid mantle tissue can obtain good quality products with high nutritive value.

2.5.2. Bacteriology

The quality of a seafood product mainly depends upon the number and type of microorganisms present. The toxic metals and pathogens in food items have great public health significance. The bacterial quality of cephalopods is shown in table 2.4. Enumeration of TPC is designed to provide an estimate of the total number of aerobic organisms in a particular food. It reflects the microbiological quality of the food and is useful for indicating the potential spoilage of the perishable food products. It is also an indicator of the sanitary conditions under which the food was produced and /or processed and also of the level of Good Manufacturing Practices (GMP) adopted during the processing. But in raw frozen food, uncontrolled destruction of the organism might have taken place during freezing which makes the above assumption baseless to a certain extent. Inspite of the above limitations, TPC can be taken as a valuable indicator of the effectiveness of any type of processing or chemical disinfections such as cooking, freezing and chlorination. Offshore waters do not contain *E-coli*, but natural water gets contaminated with *E-coli* either by direct contact or by mixing up with terrestrial sewage. The predominant aerobic bacterial flora of the large intestine of the human being and animals is composed of non-spore forming non-acid fast, Gram- negative bacteria. They exhibit general morphological and biochemical similarities and are grouped together in large and complex family of *Enterobacteriaceae*. Members of the coliform, including faecal coliforms are referred to as indicator organisms, since their presence in certain numbers may indicate the potential presence of pathogens in foods.

Lakshmanan et al., (1984) has studied the quality of fish landed at Cochin Fisheries Harbour. Of the total samples 8.5% were considered unacceptable based on TPC and 26.4% of the samples contained E-coli. The crushed ice and the platform had high bacterial load. In the study of Madhusoodana Rao and Surendran (2003) showed that the deep-sea fishes contained high count of E-coli and coliphages in the landing center samples, which was not due to the deep-sea fish or deep-sea waters but could be only from terrestrial sources. According to Nazeem Beena (2002) the temperature abused contaminated fish could act as a potent vehicle of food borne infections in the country. From the above results and the references it was clear that the handling of any perishable item should be done very carefully to avoid health hazards.

The studies conducted by Iyer et al., (1986) on fresh water fish samples showed that 74.2% of the samples had E-coli less than the acceptable limit i.e. 20/g. The studies by Varma et al., (1985) have reported that 0.93% of frozen cuttle fish fillet had *E-coli* above the prescribed limit. The incidence of *E-coli* is mainly due to the external contamination during handling and processing (Liston, 1965 and Cann, 1977).

The role of *Staphylococcus aureus* as a food poisoning organism is a useful indicator of personal hygiene in the process involving human handling (Cann, 1977; ICMSF, 1978; Liston, 1980; Hobbs, 1982). The organism can multiply vigorously and produce toxin at temperatures near and above room temperature (Iyer, 1986). According to Hobbs (1983) the presence of *Staphylococcus aureus*, even in small numbers is an excellent indicator of personal hygiene.

Salmonella finds its entry into the product during handling in preprocessing and processing. The best method to avoid contamination with salmonella is to implement strict sanitary measures during preprocessing and processing.

2.6.Conclusion

Squid was found to be a good source of protein when compared with other seafood items. The repeated washing resulted in the loss of proteins along with the other soluble substances, thus reducing the quality. The time in between the landing and the processing factory is found to be very crucial, since it directly reduces the quality of squid. Utmost care has to be taken to reduce the hazards. Thus practice of storing squid in slush ice, at any point from landing to processing area, does not lead to a decrease in the physical yield of the product, but rather an increase. But as the loss of proteins as well as NPN fraction could affect the organoleptic quality of squid in general, and squid's sweet taste in particular is linked to the NPN fraction. From the findings it is clear that the nutritive value of the squid is more susceptible to loss by leaching. In some particular circumstances even up to 80% of the squid protein can be extracted by repeated washing with water and seems to be more in skinned and processed squid. The leaching rate of needle squid was found to be much higher than that of loligo squid. This leads to a considerable loss of nutrients and higher rate of degradation in needle squid compared to loligo squid. Hence further studies are needed to reduce this nutritional loss and quality degradation.

2.7. References

Antony, K.P., Muraleedharan, V and Mukundan, M.K., (2003) Preparation of quality upgraded Tuna mas and mas-based diversified convenience products. Fish. Technol. **40** (2), 133-138.

AOAC, (1990) Official method of analysis, 15th edn. Association of official analytical chemists, Arlington, Virginia.

APHA, 1998 Standard methods for the examination of water and wastewater, 20th edn. American Public Health Association, Washington DC, USA.

Cann, D.C., (1977) Bacteriology of shellfish with special reference to International trade. Prod. Conf. Handl. Proc. Mark. Trop, Fish. 5-9, Tropical products institute, London.

Chand, B.K., Dora, K.C., Sarkar, S. and Maragal, M.M., (2001) Changes during frying of mackerel at different temperatures. Fish. Tech. **38** (2), 87-91.

Connell, J.J. and Howgate, P.F., (1971) A critical appraisal of tests for evaluating quality loss of frozen fish. In Fish inspection and quality control (Edn. R. Kreuzer) Fishing news (Books) Ltd. Famham, 202-206.

Gomori, G., (1955) Preparations of Buffers for use in Enzyme Studies. Methods of Enzymology Vol **1.** (Ed. Sidney, P. Colowick and Nathan, O. Kapalan). McCollum- Pratt Institute, The Johns Hopkins University, Baltimore, Maryland.

Hobbs, G., (1982) Indicator organism in fresh fish in relation to spoilage and public health. Antonie Van Leeuwenhock **48**, 619.

Hobbs, G., (1983) Marker organism in fresh fish in relation to spoilage. A public health T.D. 1755, Torry Research Station, Aberdeen, Scotland.

*Horie, N., Tsuchiya, T., Matsumoto, J.J., (1975) Bull. Jap. Soc. Sci. Fish. **41**, 1039-1045.

Hultin, H.O., Feng, Y and Stanley, D.W., (1995) A re-examination of muscle protein solubility. J. Muscle Foods. **6**, 91-107.

ICMSF (International commission on Microbiological specifications for food), (1978) Microorganisms in food-1 2nd Edn. University of Toronto Press, Toronto, London.

Iguchi, S.M.M. Tsuchiya, T., Matsumoto, J.J., (1981) Studies on denaturation of squid actomyosin. Bull. Jap. Soc. Sci. Fish. **47** (11), 1499-1506.

Iyer, T.S.G., (2000) Salmonella in seafoods. Quality assurance in seafood processing. (Eds. Gopalakrishnan Iyer, T.S., Kandoran, M.K., Mary Thomas, Mathew, P.T). Pub. Central Institute of Fisheries Technology.

*Iyer, T.S.G., Damle, S.P., Garg, T.K., Nambiar, V.N. and Vasu, N.M., (1986) Fish. Technol. **23**, 78.

Jimenez-Colmenero, F and Borderias, A.J., (1983) A study of effects of frozen storage of certain functional properties of meat and fish protein, J. Fd. Technol. **18**, 731-737.

Joseph, J., Varma, P.R.G. and Venkataraman, R., (1977) Iced and frozen storage of squid (Loligo species) Fish. Tech. **14**, 13-20.

*Kawada, M. and Takaschi, T., (1955) Nissuishi **20**, 888.

Kher-un-Nisa, Riaz Fatima, Qadri, R.B., Ahmed Khan, F. and Neem Mohammed, S., (1995) Proximate composition and macro and micronutrients in finfish from Pakistan coastal area. Tropical science **35** (2), 156-160.

*Kitabayashi Kunitsugu, Kuninori Nakamura, Katsuo Sudo, (1954) Hokusui kenpo, No.**11**, 136.

Kolodziejska, I., Zozislaw, E and Maria Sadowska, (1987) Texture of cooked mantle of squid *Illex argentinus* as influenced by specimen characteristics and treatments. J. Fd. Sci. **52** (4), 932-935.

Lakshmanan, P.T., Cyriac Mathen, Varma, P.R.G. Varma and Iyer, T.S.G., (1984) Assessment of quality of fish landed at the Cochin Fisheries Harbour. Fish. Technol. **21**, 98-105.

Lakshmanan, P.T., Varma, P.R.G. and Iyer, T.S.G., (1993) Quality of Commercially frozen cephalopods products from India, J. Food control, **4** (3), 159-164.

Lakshmanan. P.T.and Balachandran, K.K., (2000) Booklet on commercial important squid and cuttlefish. Advisory series from CIFT, Cochin.

Lilabati, H., and Viswanath, W., (1999) Biochemical and Microbiological quality of *Labeo gonius* stored in ice. Fish. Technol. **39** (1), 24-27.

Liston, J., (1965) Sanitation in seafood production and distribution. J. Milk. Fd. Technol. **28**, 152.

Liston, J., (1980) Microbiology in Fisheries science. Advances in fish science and technology (edn. Connell, J.) Fishing news books ltd, England, 151.

Love, R.M., (1970) The chemical biology of fishes. Academic Press London and New York. 547.

Madhusudana Rao, B. and Surendran, P.K., (2003) Microbial populations in deep-sea water and Fish of southeast Arabian Sea with special emphasis on *Escherichia coli* and coliphages. Fish. Technol. **40** (2), 105-114.

Matsumoto, J.J., (1958) On the protein composition of squid muscle. Bull. Jap. Soc. Scient. Fish. **24**, 133-139.

Matsumoto, J.J., Migita, M. and Aoe, A., (1959) A comparative study on the extractability of muscle proteins of some animals. Bull. Jap. Soc. Sci. Fish. **24** (9), 751-759.

Nazeem Beena, K.P., Jeena, K.G. and Mohamed Hatha, A.A., (2002) Growth of cross contaminating bacterial pathogens in Fish muscle at different temperatures of storage. Fish. Technol. **39** (2), 146-149.

Otwell, S.W. and Hamann, D.D., (1979b) Textural characterization of squid (Loligo pealei, Leseur) instrumental and panel evaluation. J. Fd. Sci. **44** (6), 1636-1643.

Pacheco Aguilar, Lugo sanchez, M. E. and Robles Burgueno, (2000) Post-mortem biochemical and functional characteristics of Monterey sardine muscle stored at 0°C. J. Fd. Sci. **65** (1), 40-47.

Panchavarnam, S., Manisha, K., Basu, S., Warrier, S, B. and Venugopal, V., (2003) Shelflife extension of whole and steaks of Rohu (Labeo rohita) by Gamma Irradiation. Fish. Technol. **40** (1), 36-41.

*Pandit, A.R. and Magar, N.G., (1972) Fish.Technol. **9** (2), 122.

Paul C. Pauline, Lis Buchter and Annemarie Wierenga, (1966) Solubility of rabbit muscle proteins after various Time-temperature Treatments. J. Agr. Fd. Chem. **14** (5), 490-492.

Reghunath, M.R., (1984) Soluble nitrogen loses in squid *(Loligo duaucelii)* during storage in slush ice. J. Food science and Technology. **21,** 50-52.

Selvaraj, P., Jasmine, J.I. and Jeyachandran, P., (1991) Effect of ascorbic acid dip treatment on frozen storage of squid (*Loligo duvaucelii, Oribigny*) Fish. Technol. **2**, 117-121.

Shetty, T.S. and Shetty, T.M.R., (1990) Bacteriology of Indian oil sardine *(Sardinella longiceps valenciennes)* stored in chilled seawater. Fish. Technol. **27**, 141-144.

Sikorski Zdzislaw, E. and Kolodziejska, I., (1986) The composition and properties of squid meat. J. Fd. Chem. **20**, 213-224.

Smruti, K., Rai, S., Yardi, V., Warrier, S.B. and Venugopal, V., (2003) Tenderised, Dehydrated Steaks of fresh water fish Rohu: Preparation and storage characteristics. Fish. Technol. **40** (1), 43-49.

Sreeja, S. and Raveendran, O., (1999) Occurrence of Vibrio cholerae Non 01 and their dispersion phenomena in the coastal waters at Mangalore. Fish. Technol. **36** (2), 96-99.

Stansby, M.E., (1982) Chemistry and biochemistry of marine food products (Eds. Martin, R. Flick, G., Hebard, C. and Ward, C.) A VI, New York.

Sudha, K., Nirmala Thampuran and Surendran, P.K., (2002) Prevalence of *vibrio* species on fish farm Pelagic and Demersal habitats. Fish. Technol. **39** (2), 150-154.

Sugiyama, M., Kousu, S., Hanabe, M., Okuda, Y., (1980) Utilization of squid. Oxonian Press Pvt. Ltd. Calcutta.

Suyama, M. and Kobayashi, H., (1980) Free amino acids and quaternary ammonium bases in mantle muscle of squid. Bull. Jap. Soc. Sci. Fish. **46** (10), 1261-1264.

Tsuchiya Takahida, Noboru Yamada and Matsumoto, J.J., (1978) Extraction and purification of squid myosin. Bull. Jap. Soc. Sci. Fish. **44** (2), 175-179.

Tsuchiya Takahida, Suzuki, H. and Matsumoto, J.J., (1977) Isolation and purification of squid actin. Bull. Jap. Soc. Sci. Fish. **43** (7), 877-884.

Tze-kuei-chiou, Hwong Kwang Chang, Leah Lo, Chyuan Yuan Shiau, (2000) Changes in the chemical constituents and quality of dry Argentina squid during processing. J. Chin. Agri. Chem. Soc. **37** (93), 338-347.

US FDA, (1995) Bacteriological analytical Mammal. 8th edn. AOAC International, Gaithersburg, USA.

Varela, G., Pujol, A. and Morieras, O., (1962) Valor Biologico de Pescados en Espana. Fish in Nutrition. Edited by Heen and Kreuzer R. 259-260.

Varma, P.R.G., Mathan, C. and Mathew, A., (1985) Bacteriological quality of frozen seafood foe export with special reference to Salmonella. Harvest and Post harvest Technology of fish (Ed. K.Ravindran) Society of Fisheries Technologists, India, 665-666.

* Not referred in original

Chapter 3

QUALITY CHANGES DURING ICE – STORAGE

3.1. Introduction

3.2. Review of literature

3.3. Materials and Methods

3.3.1. Experimental Design

3.3.2. Proximate composition

3.3.3. pH

3.3.4. Total Volatile base nitrogen (TVBN)

3.3.5. Trimethylamine Nitrogen (TMA-N)

3.3.6. Alpha Amino Nitrogen

3.3.7. Fractions of Proteins

3.3.8. Sensory Evaluation

3.3.9. Bacteriology

3.3.10. Statistical tools

3.4. Results

3.4.1. Proximate composition

3.4.2. Changes in total protein content

3.4.3. Total volatile bases

3.4.3.1. Total volatile base nitrogen

3.4.3.2. Trimethylamine Nitrogen (TMA N)

3.1. Introduction

Being a highly perishable item, squid must be preserved immediately after the harvest till it is ready for consumption. The main factors responsible for the spoilage at ambient temperatures are autolysis and bacterial spoilage. The rate and extend of the autolytic spoilage is considerably less than bacterial spoilage but, it plays a vital role in flavour development and the onset of bacterial spoilage. Two species of squid, *Loligo duvaucelii* and *Doryteuthis sibogae*, which dominate the squid landings in Kerala, are selected for the study. Due to the unique composition of squid muscle, it is prone to deterioration very easily. Careless handling, faulty processing or improper storage results in the quality deteriorations. The efficiency of the preservation methods depends on its ability to reduce the spoilage rate and thus increasing the shelf life and quality. The selection of a method for preservation depends on the nature and type of the product, economical viability of the method, intended use, adaptability and local tradition. In the case of squid, the handling immediately after harvest is of high priority because it determines, to a large extent, the quality at different stages of processing and the final product.

Generally, the rates of autolytic and microbial spoilage are dependent upon the temperatures at which the squid is stored. Deteriorative processes are retarded at reduced temperatures and when the temperature is low enough, spoilage can almost be stopped. Normally, to keep the squid cool, packing in ice is used, since ice storage is the first and easy method of preservation to maintain the native quality of the squid. Keeping the squid cool, thus extends the high quality life (HQL) of the squid. Even though icing is the easiest and the most economical method of preservation, various

factors like quality of ice, method of icing, and the storage time can affect the results. In this study the quality differences in both the selected species of squid under different conditions of storage in ice, were compared to suggest a better storage technique to maintain the intrinsic quality of the raw material to the maximum.

3.2. Review of literature

Many authors have carried out several investigations on various aspects of storage of *Loligo duavucelii*, but no systematic study has been carried out till date on *Dorytuethis sibogae*.

The chemical, physical and the bacteriological parameters of the industrial samples of squid (Loligo species) and cuttlefish (Sepia species) were studied by Lakshmanan (1993). In the study of Joseph et al., (1977), the quality of the squid tube (*Loligo duavucelii*) stored in ice has been studied in detail. Mathew et al., (1999a) has investigated the distribution of non-protein nitrogenous extractives in the muscles of 41 species of marine fish of India including squid *Loligo duavucelii*. Sophia and Sherief (2003) have investigated the effect of treatments on the iced storage shelf life of cuttlefish (*Sepia aculeate*) fillets. In this, the changes in NPN and its fractions during iced storage and the effect of iced storage duration on frozen storage characteristics of cuttlefish fillets were studied. Paarup (2002) studied the sensory, chemical and bacteriological changes during ice storage of squid (*Todaropsis eblanae).* The effect of storage conditions on sensory properties, colour parameters and psychrotrophic bacterial counts of squid *(Loligo plei)* stored either in contact ice or in non-contact ice were studied by Lapa (2002). Civera (2000) has studied the chemical and

microbial characteristics of cephalopods. Sagedhal et al., (1998) has investigated the post-mortem changes in adenosine triphosphate (ATP) and related compounds in the mantle of squid *Illex argentinus.* The factors involved in the evaluation of the freshness of the squid were also studied by Melaj et al., (1998). The biological evaluation of sea squid found in Pakistan waters as a good source of protein was studied by Begum et al., (1994). Yamasaki et al., (1993) and Nishimura and Shinano (1992) have studied the effect of squid liver and Trimethylamine oxide on the squid product (Ika-shiokara) with respect to the micro flora and the chemical properties and the growth of inoculated *Staphylococcus aureus.*

Various handling and processing methods for Atlantic short finned squid *(Illex illecebrosus)* were studied by Ke et al., (1991) including the effect of contact icing and non-contact icing on the quality of squid. Baldrati (1990) studied the handling, marketing and processing of cephalopods in Italy and the importance of cephalopods (cuttlefish, squid and octopus) in the Italian seafood market. Longer storage of squid tubes and cuttlefish fillet in ice resulted in a noticeable decrease in NPN value (Reghunath, 1984; Joseph et al., 1977; and Joseph and Perigreen, 1988). Matsumotto, (1958) studied quality changes of squid when kept in contact with water and the decrease in PN content during washing. Tanikava et al., (1954) observed mustiness in raw squid indicating the spoilage when TVN level exceeded 30mg/100g tissue.

There are extensive works carried out on iced storage characteristics of many tropical fishes of India (Nair et al., 1971; Nair and Danny, 1975; Guptha and Govindan, 1975; Bandyopadhyay et al., 1986; Joseph et al., 1988; and Sankar and Ramachandran, 2002). Nadia, (2002) investigated the

spoilage of mackerel during storage at ambient temperature and in ice. Jose and Reghunath (2002) studied the tissue proteinase activity of mackerel during iced storage. The biochemical and microbiological qualities of *Labeo gonius* stored in ice were studied by Lilabati and Viswanath (1999). The changes in K value and biogenic amines in Atlantic mackerel during iced storage were studied by Mathew (1999).

The bacteriology of loligo species was studied by Joseph et al., (1997) and the presence of bacteria before and after the treatment with ascorbic acid on *loligo duvaucelii* was studied by Selvaraj (1991). Various authors have also studied bacteriology of other species like Indian oil sardine stored in a mixture of seawater and ice (Shetty et al., 1992) and storage characteristics of catfish by Bhattacharyya and Chaudhuri (1990).

3.3. Materials and Methods

3.3.1. Experimental Design

The samples of the two species of squid (*Loligo duvaucelii* and *Doryteuthis sibogae)* were collected from Munambam landing center as explained in Chapter 2. Collection was by random sampling of catch from the boat. The aseptically brought samples were segregated into three lots and separately iced.

Method 1-with GMP (Good Manufacturing Practices): In this flake ice was used in the ratio 2:1 with material and the ice and melt water was changed daily. Utmost care was taken to maintain the temperature at 0°C-2°C. The insulated boxes used for the study were with smooth interior and not to harbour any contaminants. The plastic boxes were kept clean throughout the study and were covered firmly with their plastic lids. For one

sample two boxes were used during the study. On the second day of storage, the box was emptied and washed thoroughly using permissible detergents and disinfectants. The washed box was kept for drying for the whole day. During this period, the second box was used for icing.

Method 2 -Without GMP In this, samples were kept in a clean box, having holes to drain the melt water off. In this sample block ice was used. Block ice was crushed in an ice crusher up to 2 to 3 inch size and this was used for icing the sample. Till the end of the study, the ice was not removed or replaced. Care was taken to maintain the temperature below 5°C and to avoid stagnant melt water.

Method 3 -With GMP, without direct contact with ice and water: In this, the direct contact of the squid tubes and the ice was prevented using a polythene cover. The method of icing was as in the Method 1. Immense care was taken to avoid breakage of polythene cover and leaching out of the soluble components of the squid muscle. In all the methods, ice and squid were layered alternatively and the number of layers of samples was limited to three in order to avoid crushing of the lower layer.

The three samples were kept in an area where the temperature was below 10°C. The samples were drawn daily for 8 days and tested for various biochemical parameters. The experiment was done in triplicate. The following parameters were analysed in both the species of squid with different storage methods.

3.3.2. Proximate composition

The analyses of moisture, protein, fat and ash were done as in 2.3.

3.3.3. pH

10 g of the muscle was macerated with 90 ml distilled water and the pH was determined using digital pH meter (pH-500) Cyber Scan. Temperatures of the storage media and the storage place were checked and recorded at every two hours interval.

3.3.4. Total Volatile base nitrogen (TVBN)

10gms of minced sample was weighed and ground well with 10% Trichloroacetic acid (TCA). The extraction was repeated 2-3 times and made up the volume of supernatant to 50ml.

Total volatile base nitrogen (TVBN) was estimated using micro diffusion method of Conway (1962) using TCA extract of the sample. Pipetted out 1ml of N/50 H_2SO_4 into the inner chamber of the Conway diffusion apparatus. Fixed the ground glass cover of the unit in such a way that the inner chamber was completely covered and leaving a small portion of the outer chamber uncovered. Pipetted out 1ml of TCA extract into the outer chamber and added 1ml of saturated potassium carbonate solution. The cover glass was slid into position so that the entire unit was covered fully and ensured thorough mixing by rotating the unit. The entire unit was kept overnight at room temperature. Excess acid in the inner chamber was titrated against 1/50 N NaOH using Tachiro's Indicator. A blank was also run simultaneously with 1ml of TCA instead of muscle extract.

$$\text{TVBN as mg\%} = \frac{0.28 \times (\text{blank sample value}) \times 50 \times 100}{\text{Weight of the Mantle tissue (g)}}$$

3.3.5. Trimethylamine Nitrogen (TMA-N)

The procedure for determination of TMA-N was the same as that of TVBN except that in the outer chamber along with 1ml of TCA extract, 1ml of formaldehyde (neutralized with $CaCO_3$) was also added (Shewan 1971). The result was expressed as mg% of nitrogen

$$\text{TMA-N as mg\% of nitrogen} = \frac{0.28 \times (\text{blank sample value}) \times 50 \times 100}{\text{Weight of the Mantle tissue (g)}}$$

3.3.6. Alpha Amino Nitrogen

Alpha amino nitrogen content of muscle in the TCA extract was determined by iodometric titration of Pope & Stevens, (1939). 10 ml of TCA extract was taken in a 100 ml standard flask, added 1-2 drops of thymolphthalein indicator and then neutralized with alkali till a faint blue colour was obtained. After adding 30 ml of cupric phosphate complex it was made up to 100 ml using distilled water, shaken well and kept for 30 minutes. Filtered the solution and 10 ml of the filtrate was pipetted out into a conical flask followed by 10 ml of glacial acetic acid to make the solution acidic. It was then titrated against N/100 Sodium thiosulphate ($Na_2S_2O_3$) using starch as indicator.

The alpha amino nitrogen content was calculated using the following formula

$$\text{Alpha amino nitrogen as (mg\%)} = \frac{TV \times 0.28 \times 100 \times 50 \times 100}{10 \times 10 \times \text{weight of the sample (g)}}$$

3.3.7. Fractions of Proteins

Various fractions of proteins – sarcoplasmic, myofibrillar, denatured and stroma proteins were analysed using the method as explained in Chapter 2 (Sub chapter 2.3).

3.3.8. Sensory Evaluation

Organoleptic parameters viz. colour, texture, odour and flavour of the samples were recorded in all the icing methods of both the species of squid. Trained panelists assessed these characteristics. Sensory tests and the degree of excellence were given by the hedonic scale (Herbert and Joel, 1985). The sensory score is the mean of all sensory characteristics. The sensory quality of the raw and cooked samples of squid and the criteria used in each case is given in Appendix C.0

3.3.9. Bacteriology

The microbiological parameters like TPC, *E-coli, staphylococcus, salmonella and vibrio* were enumerated as per the procedure in 2.3.6.

3.3.10. Statistical tools

For the comparison of parameters between species, between storage days and between methods (with GMP, without GMP and without direct contact.), three way ANOVA was employed. The mathematical model used for the purpose was as follows:

$X_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + E_{ijk}$ where X_{ijk} is the value obtained on ith species using jth method of storage and k^{th} day of storage. μ is the overall

effect and α_i is the effect of ith species, β_j is the effect of j^{th} method of storage and γ_k is the effect of k^{th} day of storage.

3.4. Results

To compare the effect of various methods of icing, between species and the days of storage, the results were subjected to statistical analysis using "3 way anova"(Snedecor & Cochran 1980). Wherever days were found to be significant, the LSD was worked out and the mean was separated.

3.4.1. Proximate composition

Appendix C.1 gives ANOVA table for moisture content of both the species of squid during ice storage. There was significant difference in moisture content, between species, between methods of icing and between days of storage. Between species, there was a significant difference and needle showed a significantly lower value than loligo ($p< 0.001$). There was significant difference between the species, and the squid kept under all the methods showed a gradual increase in moisture content. The rate of increase in the moisture content of loligo squid kept without direct contact with ice and water, under ideal condition with GMP and under conventional method without GMP, has shown an increase of 1.08%, 3.18% and 4.59% respectively and for needle the values were 0.68%, 1.68% and 3.3% respectively. In the loligo squid the rate of increase in moisture content was higher than needle squid. The samples without direct contact could maintain its native quality standards till the end of 8^{th} day of storage. The moisture percentage in needle squid kept under ideal conditions showed 80.43 ± 1.07,

in samples without GMP 81.28 ± 1.73 and without direct contact with ice and water 78.96 ± 0.38, but in loligo with GMP 79.55 ± 1.39, 77.66 ± 0.23 without direct contact with ice and without GMP 79.52 ± 1.24.

Appendix C.2. shows the ANOVA table for changes in fat content of both the species of squid during ice storage. The fat content of loligo sample, with GMP showed a decrease from 2.17% to 1.82%, without GMP showed significantly higher loss of fat during the storage period (2.2% to 2.0%) and in samples with non-contact ice showed a significantly lower value than other methods (2.2%-2.17%). All the values were significant at 5% level.

Appendix C.3. shows ANOVA of the changes in ash content of both the species of ice-stored squid. Ash content showed a significant difference between species, methods of icing and days of storage ($p<0.001$). Ash also showed a decrease of 1.5% to 1.12% in loligo and 1.50% to 0.5% in needle, for samples without GMP. The samples with GMP showed a decrease of 1.6%-0.9% (needle) and 1.56%-1.34% (loligo). The samples with non-contact ice showed a significantly lower loss of minerals during storage (1.55%-1.43% in loligo) and (1.37%-1.12% in needle). Between species, the needle squid showed a significantly lower value in the loss of fat and minerals during ice storage than loligo. Since fat and ash play a very insignificant role in determining the quality of squid, the study of fat and ash has been avoided in the rest of the work.

Table 3.1. Changes of total protein during ice storage (%)

	Loligo			Needle		
Days	with GMP	w/oGMP	w/o DC	with GMP	w/oGMP	w/o DC
1	20.9	20.84	20.87	21.2	20.95	21
2	18.5	16.42	20.54	17.58	16.54	20.45
3	17.2	14.98	20.2	16.45	14.22	19.84
4	16.42	14.02	19.8	15.84	12.8	19.46
5	15.7	13.24	19.5	15.02	11.4	18.96
6	15.1	12.55	19.1	14.52	10.9	18.42
7	14.8	11.85	18.5	13.54	10.01	17.24
8	14.2	11.2	17.5	11.2	8.5	16.54

w/o GMP- without GMP, w/o DC-without direct contact with ice and water

3.4.2. Changes in total protein content

The results showed that there was a great loss of protein in processed squid of both the species during ice storage (Table 3.1.). The protein content showed a significant difference between species, methods of icing, and the days of storage in ice ($p< 0.001$). The F value for protein loss during ice storage in species, methods, and the days were 6.9, 87.95 and 26.3 respectively, having degrees of freedom (1,37), (2,37) and (7,37). Appendix C.4. shows ANOVA for changes in protein during ice storage. Among the species, needle squid showed a significantly higher protein loss than in loligo. Between methods, without direct contact with ice showed a very low protein loss and without GMP showed significantly higher value of protein leaching. Among days, day 1 showed a significantly higher protein content than the rest of the day and day 8 showed the least protein content.

The protein loss was significant in 8th day when compared with 1st, 2nd, and 3rd day of storage. There was no significant difference in 4th to 8th day of storage.

The samples of loligo had an average loss of 32.05%, 46.25% and 17.45% of total protein in samples with GMP, without GMP and without direct contact respectively. Repeated extraction of squid mantle muscle with water resulted in an increase in the amount of protein dissolution, in contrast to fish muscle, with more than 50% of total protein extracted in many cases. The needle squid kept under icing with GMP, without GMP and without direct contact had an average loss of 47.16%, 59.42% and 21.9% of protein respectively.

3.4.3. Total volatile bases

3.4.3.1. Total volatile base nitrogen

Figure 3.1. and 3.2. show the changes of TVBN of both the species during ice storage by the three methods of loligo and needle squid respectively.

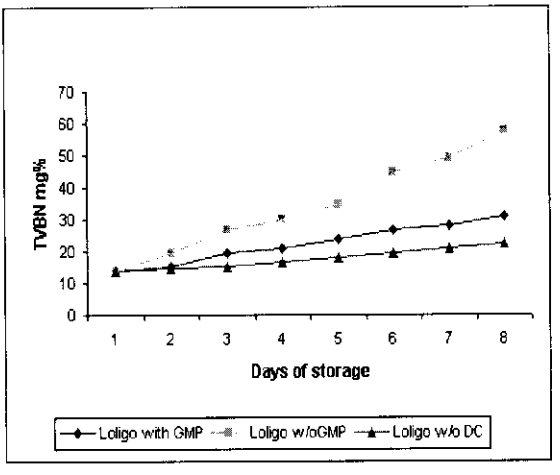


Figure 3.1.Changes in TVBN in loligo squid during ice storage

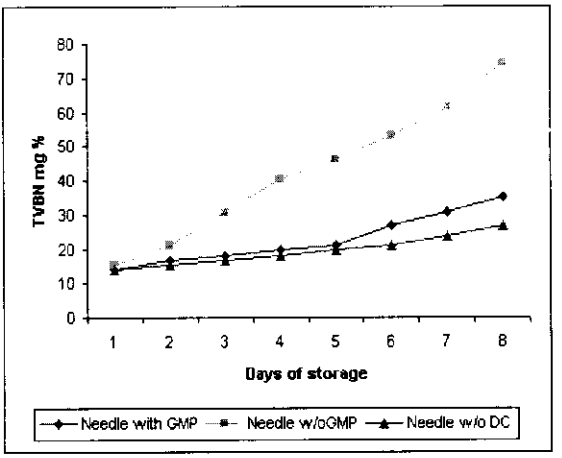


Figure 3.2. Changes in TVBN of *Doryteuthis sibogae* during ice storage

Appendix C.5. gives ANOVA of changes of TVN values during iced storage. There was no significant difference between species. There was significant difference between methods ($p < 0.001$), and days of storage ($p < 0.001$). Between methods, icing without GMP showed significantly higher value than the other two methods. The samples without direct contact showed a significantly lower value. Between days, significantly higher values were observed on 8th day of storage. There was no significant difference between 7^{th} and 8^{th} day. Both the species of squid showed significantly lower value on 1^{st} and 2^{nd} day of storage. The F values were 2.9, 37.82, and 10.64 between species, between methods, and between days of storage respectively.

According to Woyewoda and Ke (1980) the samples exceeding TVN value of 30mg per 100g was not acceptable. In loligo without GMP, TVN crossed 30 mg % on 4^{th} day of storage. But with GMP, it exceeded the value only on the 8^{th} day of storage. The samples without direct contact with ice did not exceed the limit till 8^{th} day of storage. In the case of needle squid,

TVN reached 30mg on 3rd day in the sample without GMP. It attained 30mg only on 6th day when GMP was followed. Samples without direct contact with ice remained within the limit of TVBN till 8th day of storage.

3.4.3.2. Trimethylamine Nitrogen (TMA N)

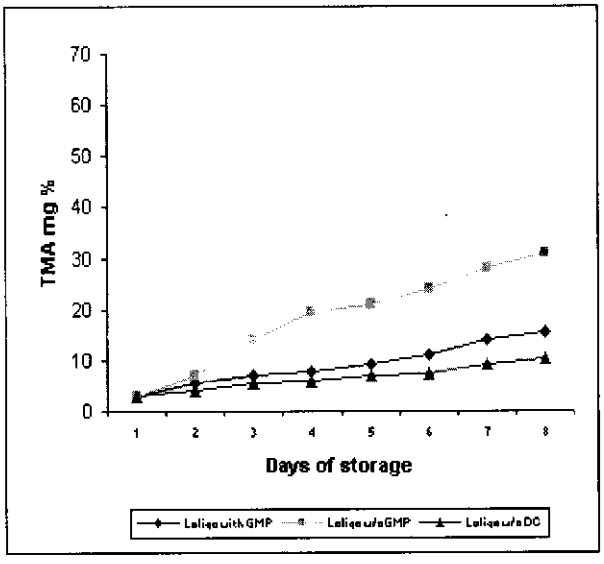


Figure 3.3 Changes of TMA of loligo during ice-storage

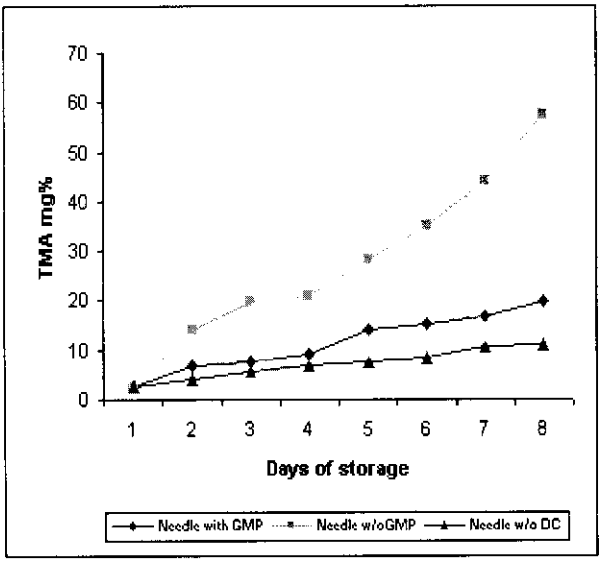


Figure 3.4 Changes of TMA of needle Squid during ice-storage

The changes in TMA content during ice storage by three methods in loligo and needle squid are given in Figure 3.3. and 3.4. respectively.

Appendix C.6. gives the ANOVA of TMA in all the three methods of ice storage. There was significant difference between species ($p < 0.01$), between methods of icing ($p < 0.001$) and between days ($p < 0.001$). Among species needle squid showed significantly higher value than loligo squid. Among methods, without GMP gave a significantly higher value and without direct contact with ice a lower value. 8^{th} day of storage showed significantly higher value when compared to 1 to 6 days. Highly significant lower value was observed in without direct contact with ice. The samples exceeding 3 to 10mg of TMA per 100g of sample is the limit of acceptability (Woyewoda and Ke, 1980). In loligo kept without GMP, TMA crossed the limit of 10mg% and became non acceptable on the 3^{rd} day of storage, with GMP on 6^{th} day of storage and without direct contact on 8th day of storage.

But in needle squid, the rate of increase in TMA was more than that in loligo, the values exceeding the acceptable level on 2^{nd}, 5^{th} and 7^{th} day of ice storage without GMP, with GMP and without direct contact with ice respectively.

3.4.4. Alpha amino nitrogen

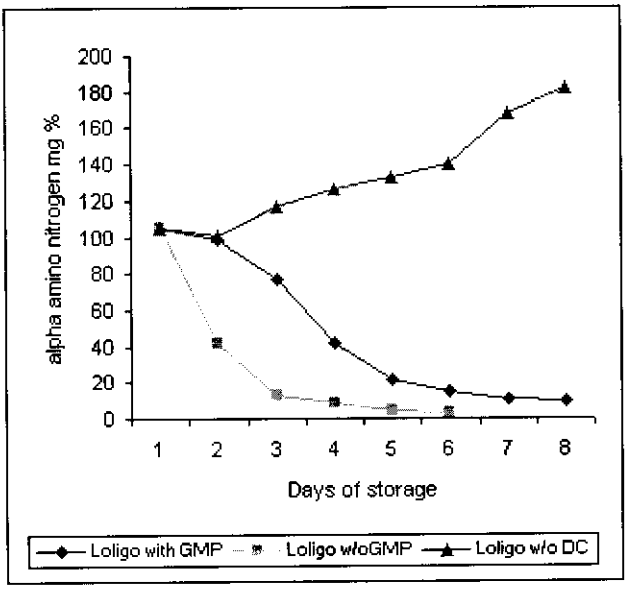


Figure 3.5 Changes of alpha amino nitrogen of loligo during ice-storage

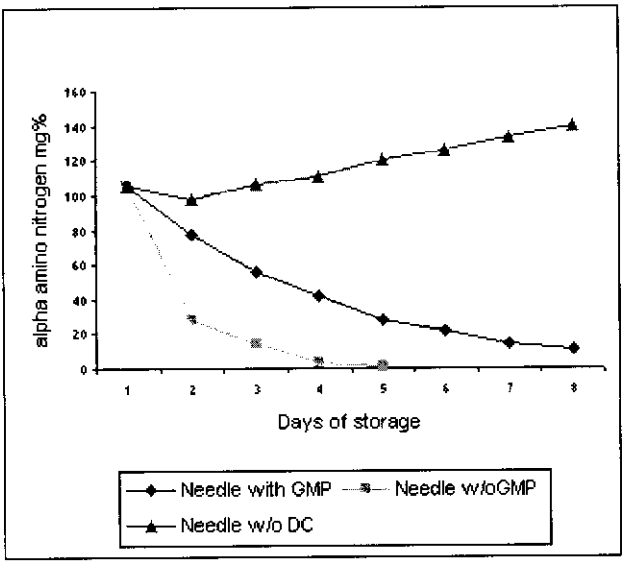


Figure 3.6 Changes of alpha amino nitrogen of needle Squid during ice- storage

Figure 3.5 and 3.6 shows the gradual decrease in alpha amino nitrogen in loligo and needle squid respectively under different methods of ice storage. The leaching of alpha amino nitrogen was not significant at 5% level between days of storage and species (Appendix C.7). In the case of samples without direct contact, a steady increase in alpha amino nitrogen was observed during the storage period, retaining the sweet taste till the end of storage. But in the case of samples with direct contact, the alpha amino nitrogen reduced continuously and attained a very low level. There was a significant difference between days of storage, ($p < 0.001$). The value of retained alpha amino nitrogen in the muscle showed a significant lower value in samples without GMP. The leaching rate of alpha amino nitrogen in needle squid was higher than that in loligo.

3.4.5. Fractions of Proteins

Table 3.2. gives the changes in sarcoplasmic proteins during storage of both the species of squid in ice. According to the study, the percentage of sarcoplasmic protein fractions retained in the muscle tissue were 61%, 54% and 65% in samples with GMP, without GMP and without direct contact respectively.

3.4.5.1. Sarcoplasmic proteins

TABLE 3.2.Changes in sarcoplasmic proteins during ice storage (%)

	Loligo			Needle squid		
Days	with GMP	w/oGMP	w/o DC	with GMP	w/oGMP	w/o DC
1	12.88	11.2	13.6	11.45	10.4	12.8
2	11.5	10.1	13	10.61	8.2	12.4
3	11.1	9.6	12.7	9.82	6.7	11.6
4	10.2	8.4	12.4	8.7	6.1	11.2
5	9.6	7.2	12	8.1	5.8	10.8
6	9.2	6.2	11.7	7.6	5.2	10.3
7	8.7	5.4	11.4	7.01	4.7	9.9
8	7.5	5	11	6.6	4.3	9.4

There was significant difference between species, methods of icing and the days of storage (Appendix C.8.). In species, loligo showed a significantly higher value of protein content in the muscle than in needle tissue. Leaching rate of sarcoplasmic protein showed a higher value in samples without GMP. Among days, 1st day showed a higher protein content in the tissue than all other days. There was significant decrease in protein content from 1 to 6 days but on 7th and 8th day there was no significant difference.

3.4.5.2. Myofibrillar proteins

Table 3.3. gives the leaching rate of myofibrillar proteins during various methods of icing.

TABLE 3.3. Changes in myofibrillar proteins during ice storage (%)

	Loligo			Needle		
Days	with GMP	w/oGMP	w/o DC	with GMP	w/oGMP	w/o DC
1	8.6	8.3	9	9.3	9.04	9.5
2	7.8	7.7	8.6	8.7	8.5	9.2
3	7.2	7	7.8	8.3	8	9
4	6.9	6.7	7.4	7.8	7.5	8.8
5	6.3	6.1	7	7.4	7.2	8.5
6	5.8	5.65	6.8	7.1	6.8	8.3
7	5.2	5	6.6	6.7	6.5	8
8	5	4.8	6.4	6.4	6	7.5

Minimum loss was found in sample without direct contact with ice or water. Maximum loss was reported in samples without GMP. Appendix C.9.gives ANOVA for myofibrillar protein during ice storage. There was significant difference between species, between methods and between days ($p<0.001$ for all). Needle squid showed higher value than loligo and the myofibrillar protein content in samples kept without GMP showed significantly lower value than the other two methods.

3.4.5.3. Denatured proteins

Table 3.4. shows the variation in denatured protein during ice storage. Needle showed a higher degree of denaturation when compared to loligo. Degree of denaturation was much higher in samples without GMP. When compared with other two methods, the samples without direct contact showed lowest degree of denaturation. There was significant difference between all the methods and between days (Appendix C.10).

TABLE 3.4. Changes in denatured protein during ice storage (%)

	Loligo			Needle		
Days	with GMP	w/oGMP	w/o DC	with GMP	w/oGMP	w/o DC
1	0.07	0.07	0.07	0.08	0.07	0.08
2	0.09	0.13	0.08	0.1	0.15	0.1
3	0.1	0.18	0.09	0.13	0.2	0.13
4	0.12	0.2	0.1	0.15	0.25	0.15
5	0.14	0.24	0.12	0.19	0.28	0.17
6	0.15	0.28	0.13	0.2	0.32	0.19
7	0.18	0.3	0.15	0.24	0.35	0.2
8	0.2	0.34	0.17	0.27	0.37	0.23

3.4.5.4. Stroma proteins

TABLE 3.5. Changes in stroma protein during ice storage (%)

	Loligo			Needle		
Days	with GMP	w/oGMP	w/o DC	with GMP	w/oGMP	w/o DC
1	1.1	1	1	0.97	0.95	0.93
2	1.02	0.97	0.96	0.92	0.9	0.9
3	0.97	0.92	0.93	0.9	0.86	0.87
4	0.95	0.9	0.92	0.87	0.82	0.83
5	0.9	0.88	0.9	0.83	0.8	0.8
6	0.87	0.86	0.87	0.8	0.78	0.76
7	0.8	0.82	0.85	0.77	0.75	0.74
8	0.75	0.74	0.8	0.71	0.7	0.7

Table 3.5 gives a detailed picture of stroma protein fraction during ice storage

Loligo showed a lesser leaching rate of stroma while needle exhibited higher leaching rate. Appendix C.11.shows the ANOVA for stroma protein content during ice storage. There was a significant difference between species, between methods and between days ($p<0.001$).

3.4.6. Physical and organoleptic Characters

Samples kept under ideal conditions retained the native pH of the fresh tissue up to 8^{th} day of storage where there was a slight increase (Table 3.6). The samples kept with GMP and without direct contact showed a slight increase in pH of both the species (6.2 to 6.8 and 6.2 to 6.7). The samples without direct contact with ice showed a superior organoleptic quality when compared with other two methods in both the species. Organoleptically the

samples of both the species were acceptable up to 6th day, which were kept under proper GMP, while the samples without GMP were rejected after two days of storage. The samples without direct contact with ice showed an acceptable standards till the end of the storage. Samples without GMP showed a higher rate of increase in pH with the storage days in both the species. They also showed an inferior sensory score, reaching the rejection level by the 3rd day of storage. In all the methods pH did not correlate significantly with sensory score.

Table 3.6. Changes in pH and Organoleptic score during ice-storage

Storage	NEEDLE					
period	with GMP		w/o GMP		w/o DC	
Days	pH	score	pH	score	pH	score
1	6.2	7	6.2	7	6.2	7.5
2	6.3	7	6.5	5	6.2	7.5
3	6.3	7	7	4	6.3	7
4	6.4	6	7.1	3	6.3	7
5	6.5	5	7.3	2	6.5	7
6	6.5	5	7.5	2	6.5	6.5
7	6.6	5	7.8	2	6.7	6.5
8	6.8	4	8	1	6.7	6
	LOLIGO					
	with GMP		w/o GMP		w/o DC	
Days	pH	score	pH	score	pH	score
1	6.2	7	6.2	7	6.2	8
2	6.2	7	6.3	6	6.2	8
3	6.3	7	6.5	5	6.2	7.5
4	6.3	6.5	6.8	4	6.3	7.5
5	6.4	6	6.9	3	6.3	7.5
6	6.5	6	7.1	3	6.3	7
7	6.5	6	7.3	2	6.4	7
8	6.6	5	7.4	2	6.5	7

3.4.7. Bacteriology

Table 3.7. Changes in TPC during ice storage (counts/g)

	LOLIGO			NEEDLE		
Days	**w/o GMP**	**w GMP**	**W/o Dc**	**w/o GMP**	**w GMP**	**W/o Dc**
1	6.67×10^5	4.21×10^4	3.5×10^4	5.3×10^5	2.85×10^4	3.57×10^4
2	1.76×10^5	2.2×10^4	2.28×10^4	1.85×10^5	1.85×10^4	1.97×10^4
3	1.82×10^5	2.25×10^4	2.3×10^4	2.02×10^5	2×10^4	2.05×10^4
4	1.85×10^5	2.38×10^4	2.45×10^4	2.22×10^5	2.15×10^4	2.20×10^4
5	1.90×10^5	2.42×10^4	2.62×10^4	2.28×10^5	2.31×10^4	2.35×10^4
6	2.02×10^5	2.22×10^4	2.85×10^4	2.29×10^5	2.41×10^4	2.46×10^4
7	2.13×10^5	2.58×10^4	2.93×10^4	2.35×10^5	2.48×10^4	2.65×10^4
8	2.22×10^5	2.62×10^4	3×10^4	2.46×10^5	2.75×10^4	2.79×10^4
9	2.25×10^5	2.65×10^4	3.2×10^4	2.50×10^5	2.86×10^4	2.9×10^4
10	2.28×10^5	2.75×10^4	3.25×10^4	2.68×10^5	2.97×10^4	3×10^4
11	2.29×10^5	2.80×10^4	3.37×10^4	2.89×10^5	3.08×10^4	3.2×10^4
12	2.3×10^5	2.95×10^4	3.42×10^4	2.99×10^5	3.28×10^4	3.29×10^4
13	2.55×10^5	3×10^4	3.5×10^4	3.4×10^5	3.34×10^4	3.39×10^4

The analysis of variance of total bacterial count (TPC) after conversion to the logarithmic values showed no significant difference between species. TPC was assessed up to 13 days (Table 3.7). But between days and the methods there were significant differences ($p < 0.001$). Among the methods the icing without GMP showed significantly higher growth rate than the other two methods. The method with GMP showed a lower growth than the other two. There was a reduction of TPC in the first two days and then the number gradually increased with storage days (Appendix C.12). The sanitary significant bacteria like *E.coli* and *Staphylococcus* showed a

higher rate of growth in the samples without GMP irrespective of species. Throughout the study *salmonella and vibrio* were not detected in the samples. There was no significant difference between samples with GMP or without GMP in the growth of *Staphylococcus* in needle ($t = 1.043$, $df = 7$). But in loligo there was a significant difference between the methods in the growth of *Staphylococcus* ($t = 4.686$, $df = 7$, $p < 0.01$). Without direct contact with ice showed no significant growth of *E-coli and Staphylococcus*, since there is no direct contact with ice and water. The number of colonies remains almost same till the end of the storage.

3.5. Discussion

Chemical and physical qualities play vital role in grooming the quality of a product. Since the spoilage of squid starts immediately after the death which largely dependent on the temperature conditions, the sooner the squid can be cooled the better will be the quality and shelf life. In ice storage, it is not only enough that there is sufficient quantity of ice to preserve the squid, but the effectiveness of icing depends on how well the ice is dispersed among the squid. The smaller the size of ice, the greater will be the contact between fish and ice. The rate of heat removal depends on the contact area. Flake ice avoids damaging the squid. The large pieces exerts point forces thereby causing damage which in turn increase the rate of leaching of protein in crushed ice. The major advantage of using ice for chilling fish is that it has a high latent heat of fusion, which can remove large amount of heat as it melts, without changing the temperature at 0° C. During transition from ice to water, 1 kg of ice absorbs 80 kcal of heat and this will be sufficient to cool about 3 kg of fish from 30°C to 0°C. Hence theoretically, ice about 30% of sample weight can bring down the temperature of the

sample from ambient conditions to 0°C. But lot of other factors like surrounding temperature, type of box used for icing, length of time fish need to be kept chilled, thickness of the fish etc., have to be taken into consideration in calculating the amount of ice needed to chill fish. Hence in tropical conditions 1:2 fish to ice ratio is ideal for ice storage.

Dressed squid mantles in seafood industry are stored in crushed ice and its meltwater, in non-perforated containers prior to freezing. It has been observed that the melt water becomes turbid and white mainly due to the leaching of NPN and soluble protein from the squid.

The changes in the moisture content in packed and unpacked samples of *Chanos chanos* stored in ice for 25 days were studied by Subrata and Imam (1985). The moisture content of unpacked sample showed a slight decrease up to three days of ice storage and then a gradual increase till 19th day after which there was a fall. In the case of packed sample, without direct contact, with ice, showed a gradual decrease in the moisture throughout the storage period. According to Joseph et al., (1977) the moisture content showed a gradual increase during the ice storage of squid tubes. Moisture content of the *Loligo duvaucelii* tubes increased from 78.33% to 83.08% at the end of 8th hour of ice storage (Reghunath, 1984). The present study also shows a similar trend. The gradual rise in moisture in samples stored in direct contact with ice is due to the absorption of ice meltwater by the muscle. But studies with prawn stored in ice showed a weight increase in the 1st two days without loss of nutrients, but by prolonging the storage period both solids and water were lost resulting in gradual decrease in weight (Mathen and Thomas, 1988). The moisture content in needle squid reached a difference of 1.68% under ideal conditions. But in the

conventional method, the difference of moisture content reached 3.3% while in samples with non-contact ice showed only an increase of 0.68%. Joseph et al., (1977) have studied the quality of the squid tube stored in ice and found that ice stored tubes were not acceptable after 5 days.

The total nitrogen also reduced considerably on the 5^{th} and 6^{th} day. According to Matsumotto, (1978) 77 to 85% of the total protein of the squid is water-soluble. Similarly, the non-protein nitrogen also showed a sharp decrease when stored in ice. It was noticed that the squid meat contained high percentage of NPN (Joseph et al., 1977) and Brongstrong, 1965). Matsumoto (1958) has also reported high myosin fraction in squid protein. The average value of WEN and NPN were higher in whole squid and cuttlefish (Lakshmanan, 1993). There was a great loss of WEN and NPN in skinned product owing to the leaching effect of this component. Skin acts as a barrier, which prevents the leaching effect to a greater extent. Raghunath (1984) observed that WEN and NPN reduced considerably in squid mantles stored in crushed ice and meltwater after 8 hours. These results are comparable with this study. In this study the percentage of sarcoplasmic protein fractions retained in the muscle tissue were 61%, 54% and 65% in samples with GMP, without GMP and without direct contact respectively.

According to Lapa (2002), the non-contact ice-storage offers no benefits over contact ice-storage in terms of preserving squid quality. But other studies showed that squid kept in contact icing were decolourised in less than 12 hours and could not be considered acceptable for food for more than two days, but non-contact ice stored squid could hold the freshness for a longer time (Ke, 1991). A considerable loss of dry matter in squid due to leaching on contact with water has also been reported by Berg (1974). In

this study, it was noticed that the non-contact ice stored squid showed a better quality when compared with the other methods of icing. Water extractable nitrogen, NPN and alpha amino nitrogen gradually decreased and squid lost their characteristic sweetness and finally became bland in taste (Lakshmanan et al., 1993). Tanikava et al., (1954) observed mustiness in raw squid, which indicated the spoilage when TVN reached 30mg/100g. Lakshmanan et al., (1993) have stated that samples exceeding TMA value of 5mg/100gm lost their culinary characteristics. In loligo without GMP, TVN exceeded 30 mg % on 4^{th} day of storage while storage with GMP and without direct contact TVN remained within the limit even up to 8 days of storage. Almost a similar trend was shown in needle squid also.

Alpha amino nitrogen content of squid tubes stored in ice showed a very low value after two days of ice storage, while total volatile base nitrogen content showed a gradual increase (Joseph et al., 1977). Comparable results were obtained in this study and it is concluded that the squid mantle cannot be stored in ice after six days even with GMP. The changes in TVBN and alpha amino nitrogen showed a gradual increase in iced stored fish species were also reported by Bandyopadhyay (1986) and Sankar and Nair (1988).

From organoleptic evaluation, it is observed that the icing without direct contact was always more acceptable than the one with direct contact with ice even up to 8 days of storage. The flavour was better in the former one, as leaching of flavour compounds was minimum. At any particular stage of storage, the texture and colour were also more acceptable in the case of samples with GMP and without direct contact with ice. The samples kept with GMP and without direct contact showed a slight increase in pH in

both the species (6.2 to 6.8 and 6.2 to 6.7). But the samples without direct contact with ice showed a superior organoleptic quality when compared with other two methods in both the species. It is suggested that the spoilage of iced squid is likely to result from a combination of autolytic and bacterial changes. TPC increased with increasing ice storage time and decreased the quality of cephalopods (Civera, 2000). *Shewanella putrefaciens, Pseudoalteromonas sp and Pseudomonas sp* dominated in spoiled gutted squid (Paarup, 2002). There was an initial reduction in TPC, which later increased and eventually surpassed the original counts. Initial reduction in TPC was due to leaching and cold shock after which psychrophiles dominated (Bandyopadhyay, 1986; Joseph et al., 1977). In a study of icing of both packed and unpacked samples of *Chanos chanos*, initially a definite decrease in TPC was observed due to sudden chilling effect of ice on bacteria. This increased very slowly up to 17^{th} day and then growth became faster up to 21 days of ice storage (Subrata, 1985). From 4^{th} to 13^{th} day, a steady increase in bacterial load was seen. There was a significant leaching effect of ice meltwater on bacterial population.

3.6. Conclusion

In a developing tropical country like India, where distribution of frozen fish in domestic market is not popular due to lack of cold chain facility, short term preservation of fish by icing has gained importance. Average consumers prefer fresh fish to iced fish even if both show the same degree of spoilage. This is probably due to the leaching of components responsible for good organoleptic characteristics, along with some undesirable components of spoilage. The extent of leaching during iced storage of different species of squid and its effect on overall quality has

been studied in detail. Needle squid were more susceptible to spoilage than loligo and leaching rate was also found to be more in the case of needle squid. Hence it is recommended to avoid direct contact with ice when transported from landing centers to the processing units and while storing in ice. Needle squid should be handled more carefully from the point of catching till the final stage of processing, since the post-mortem changes were much faster than in loligo. Ice storage is not advisable after 6^{th} day even where GMP is followed. The samples with GMP and without direct contact with ice were acceptable till 8^{th} day of storage in both the species of squid. The chemical indices as well as organoleptic score showed favourable results in samples without direct contact with ice. Alpha amino nitrogen, which contributes to the sweet flavour of meat, was retained in this method of icing, while in the other two methods, the alpha amino nitrogen as well as WEN were lost and a bland taste was obtained. Finely crushed block ice or flake ice should be used and layering of ice and squid is preferably done in flat boxes. Extended contact with ice and melt water should be avoided. The method recommended to the fishermen is to ice squid without direct contact with ice by using an intervening plastic sheet. Thus under similar conditions of icing (with GMP), storage of squid without direct contact with ice and melt water will have longer shelf life and give more acceptable product than those stored directly in ice.

3.7. References

Balrati, G., (1990) Handling, Marketing and processing of cephalopods in Italy. Industria – Conserve. **64** (4) 353- 355.

Bandyopadhyay, A.K., Chattopadhyay and Bhattacharyya, S.K., (1986) On the Ice-storage characteristics of *Catla catla* and *Labeo fimbriatus* Fish. Technol. **23,** 140-142.

Begum, A., Sadiqa Shakir, Qadri, R.B., (1994) Biological evaluation of Sea Squid in Pakistan water. Pakistan J.of Scientific and Industrial Research **36**(1), 27-30.

Berg, Z., (1974) Processing squid for Food Cambridge, Mass., Massachusetts Institute of Technology, Cambridge, MA

Bhattacharyya, S.K., and Chaudhuri, D.R., (1990) Studies on storage characteristics of *Clariurs batrachus* at different temperatures. Fish. Technol. **27**, 127-129.

Borgstrom George, (1965) Trends in Utilization of fish and shellfish. Fish as food 4, (Ed. Borgstrom, G.) Academic press, New York.

Civera, T., Grassi, M.A. and Pattono, D., (2000) Chemical and Microbial characteristics of Cephalopods during storage in ice. Industrie Alimentari **38**(384), 933-937.

Conway, E.J., (1962) Microdiffusion analysis and volumetric error 5th edn. Parch croskey and Lockwod and sons Ltd, London. 467.

*Guptha, S. and Govindan, T.K., (1975) Fish. Technol. **12**, 151.

Herbert Stone and Joel L. Sidel, (1985) Sensory evaluation practices, Academic Press Inc. 76-77.

Jose, L. & Reghunath, M.R., (2002) Tissue Proteinase activity in Indian Mackerel (*Rastrelliger kanagurta)* during iced storage. Fish. Technol. **39**(1), 39-42.

Joseph, J., Varma, P.R.G. and Venkataraman, R., (1977) Iced and frozen storage of squid (Loligo species) Fish. Tech. **14**, 13-20.

Joseph, J. and Perigreen, P.A., (1988) Studies on frozen storage of cuttlefish fillets. Fish. Tech. **25**, 32- 35.

Ke, P.J., Fierheller, M and Lemon, D.W., (1991) Studies on Processing Technology for Atlantic short-fin squid (Illex illecebrosus). Levensmittel - wissenschaft –und – Technology. **24**(4) 328-333

Lakshmanan, P.T., Varma, P.R.G. and Iyer, T.S.G., (1991) Studies on quality changes of frozen fish on retail cold stores. Fish. Technol. **28**, 42-50.

Lakshmanan, P.T., Varma, P.R.G. and Iyer, T.S.G. (1993) Quality of Commercially frozen cephalopods products from India, J. Food control, **4** (3), 159-164.

Lapa Guimaraes, J., Aparecida Azevedo Da Silva, M., Eduardo De Felicio, P. and Contreras Guzman, E., (2002) Sensory, colour and Psychrotrophic bacterial analyses of squid (*Loligo plei)* during storage in ice. Lebensmittel Wissen Chaft und Technologie **35**(1), 21-29.

Lilabati, H., and Viswanath, W., (1999) Biochemical and Microbiological quality of *Labeo gonius* stored in ice. Fish. Technol. **39** (1), 24-27.

Mathew, P.T., John M. Ryder and Gopakumar, K., (1999) Changes in K value and Biogenic Amines during storage of Atlantic mackerel (*Scombes scomberus*) in ice. Fish. Technol. **36** (2), 78-81.

Mathew, S., Reghunath, M.R. and Devadasan, K., (1999a) Distribution of non-protein Nitrogenous extractives in the muscle of the Indian Fish. Fish.Tech. **36** (1), 1-7.

Matsumoto, J.J., (1958) On the protein composition of squid muscle. Bull. Jap. Soc. Scient. Fish. **24**, 133-139.

*Melaj, M., Sanchez Pasqua, G., Casales, M.R., and Yeannes, M.I., (1998) Factors in evaluation of freshness of squid (*Illex argentinus).* Alimentaria **282**, 93-96.

Nadia Mahmud Omar, (2002) Indole as an index of spoilage of Mackerel (*Rastrelliger kanagurta)* during storage at ambient temperature and in ice. Fish. Technol. **39** (2), 142-145.

Nair, R.B. & Dani, N.P., (1975) Fish Processing industry in India Proc. Symp. Mysore A.F.S.T. (India) and C.F.T.R.I. (Mysore) 20.

*Nair, R.B., Tharamani, P.K and Lahiri, N.L., (1971) J. Fd. Sci. Technol. **8**,53.

Nishimura, M., and Shinano, H., (1992) The Effect of trimethylamine –N-oxide on microbial flora in Ika – shiokara. Bulletin of the Japanese Society of Scientific Fisheries (Nihon – Suisan – Gakki – Shi) **57** (6) 1141-1145.

Paruup, T., Sanchez, J.A., Moral, A., Christensen, H., Bisgaard, M., Gram, L., (2002) Sensory, chemical and Bacterilogical changes during storage of iced squid (*Taropsis eblanae*) J.Applied Microbiology **92** (5), 941-950.

Pope, C.G. and Stevens, M.F., (1939) The determination of amino nitrogen using a copper method. Biochem. J. **33**, 1070.

Reghunath, M.R., (1984) Soluble nitrogen loses in squid *(loligo duaucelii)* during storage in slush ice. J. Food science and Technology. **21,** 50-52.

Sagedal, A., Busalmen, J.P., Roldan, H.A., Paredi, M.E., Curpkin, M., (1998) Post Mortem changes in Adenosine Triphosphate and related compounds in mantle of squid (*Illex argentinus*) at different stages of sexual maturation. J.Aquatic Food Product Technology **6**(4), 43-56.

Selvaraj, P., Jasmine, J.I. and Jeyachandran, P., (1991) Effect of ascorbic acid dip treatment on frozen storage of squid (*Loligo duvaucelii, Oribigny*) Fish. Technol. **2**, 117-121.

Shankar, T.V. and Viswanathan Nair, P.G., (1988) Effect of pre-processing iced storage on deteriorative changes in lipids of Silver pomfret stored at –18°C. Fish. Technol. **25**, 100-102.

Shankar, T.V., and Ramachandran, A., (2002) Behaviour of Mrigal (*Cirrhinus mrigala)* meat components during the washing process in the preparation of Surimi. Fish. Technol. **39** (2), 114-119.

Shetty, T.S., Shetty, T.M.R. and Ravishanakar, C.N., (1992). Temperature growth response of spoilage bacteria isolated form Indian oil sardine *(Sardinella longiceps)* stored in chilled seawater. Fish. Technol. **29**, 158-161.

Shewan, J.M., Gipson, D.M. and Murray, C.K., (1971) Estimation of TMA in fish muscle: Fish Inspection and Quality control. Ed. Krueuzer. 183.

Snedecor, G.W. and Cochran, W.G., (1980) Statistical methods, 7th edn. 505. The Iowa state University press Ames, IA.

Sophia Margaret Joseph and Sherief, P.M., (2003) Effect of Treatments on the Iced storage shelf life of cuttlefish (*Sepia aculeate*) fillets. Fish.Tech. **40**(1), 32-35.

Subrata Basu and Imam Khasim, D., (1985) Studies on the effect of leaching on the quality of ice-stored fish. Fish. Technol. **22**, 105-108.

Tanikawa, E., Akiba, M., and Numakura, T., (1954) Studies on the complete utilization of squid Ommatrophes *soloani pacificus*. Bull.Fac. Fish. Hokkaido Univ. **4** (4), 323-326.

Woyewoda, A.D. and Ke, P.J., (1980) Laboratory quality assessment of Canadian Atlantic squid *(Illex illecibrosus)* Tech. Rep. Fish. Mar.Serv. Can. **902**, 1-26.

Yamasaki, K., Kitamura, F., Inoue, N., and Shinano, H., (1993) Effect of squid liver on microflora of Ika – shiokara. Bulletin of the Japanese Society of Scientific Fisheries (Nihon – Suisan – Gakki – Shi) **58** (10) 1971-1976.

* Not referred in original

Chapter 4

EFFECT OF SUPER COOLING ON THE QUALITY OF PROCESSED SQUID

4.1. Introduction

4.2. Review of Literature

4.3. Materials and methods

4.3.1. Experimental Design

4.3.2. Statistical Analysis

4.4. Results

4.4.1. Total Volatile Bases

4.4.1.1. Total Volatile base Nitrogen

4.4.1.2. TMA

4.4.2. Alpha Amino Nitrogen

4.4.3. Protein loss

4.4.4. Fractions of protein

4.4.4.1. Sarcoplasmic proteins

4.4.4.2. Myofibrillar proteins

4.4.4.3. Denatured proteins

4.4.4.4. Stroma proteins

4.4.5. Bacteriology

4.5. Processing steps

4.5.1. Total Volatile Bases

4.5.1.1. Total Volatile base Nitrogen

4.5.1.2. TMA

4.5.2. Alpha Amino Nitrogen

4.1. Introduction

Two major factors, temperature and sanitation have been identified important in the post harvest extension of shelf life of fresh fish. The objective of chilling is to cool the fish as quickly as possible, to as low a temperature as possible without freezing. Chilling cannot prevent the spoilage fully but in general, the colder the fish, the greater will be the reduction in bacterial and enzymatic activity. Many people have their own names for chilling as, refrigeration process, such as super chilling, super cooling, light freezing, partial freezing etc. For this study, the process is called super cooling and considered as the refrigeration of fish by which the muscle tissue temperature is brought down to -1 to -3°C. (30.2 to 26.6°F). For chilling the fish to this temperature the media temperature was maintained at -6°C throughout the experiment. By cooling the fish to –1 to –3° C approximately half of the unbound water present in the fish gets converted to solid phase. Super cooling is a condition attained by a liquid where its temperature is lowered below the freezing point without crystallization. When the temperature of a biological system is reduced to subzero level, the solution first supercools before solute starts crystallizing. Super cooling, in general, need not impair the quality of fish. As the temperature is lowered to the critical level, nucleation begins and thereafter any further decrease in the temperature will result in abrupt increase in rate of crystallization. Protein denaturation can take place, which is influenced by the temperature; concentration of enzymes and other compounds in the fish is also possible. The objective of the present work is to study the effectiveness of super cooling for retention of acceptable quality during

various stages of processing in two species of squid *Loligo duvaucelii* and *Doryteuthis sibogae.*

4.2.Review of Literature

Many people have worked on changes of muscle tissue occurred during super cooling or chilling. An early reference concerning interest of the Industry on the commercial use of super chill temperatures for fish storage, appeared in the Trade Journal fishing Gazette, (1935). Ranken (1963) mentioned briefly about a system developed in Portugal to hold fish aboard a fishing vessel at temperature –1°C. Scarlette (1965) reported about the patented refrigerative system in which the fish was kept at –1 to –3°C and the quality changes of fish during a storage period of 21 days. Torry Research Station and White Fish Authority have been the leaders in the studies on super chilled fish (Anon., 1964; Merritt, 1965; Merritt et al., 1966; White fish authority, 1964; 1965). They found that regular bulk storage of cod in melting ice gave a storage time of seven days. Studies were also conducted by Federal Research Center for Food Preservation, Karlsruhe, to determine the capability of super chilling process on North Atlantic fish under North Atlantic conditions (Partmann, 1965). He recommended that the super chilled process should be operated at temperatures just above the onset of freezing. The effect of super chilling on gutted cod was studied by the Power and Morton (1965), in which temperature ranged from –1°C to –4°C. Two methods of storage like plain refrigerated air and ice with refrigeration were tried in this study. All the chemical and physical properties of the fish were studied in detail in this study. Quality changes of Pacific salmon indented for canning during partial freezing were studied by Roach and Tomlinson (1969). Balachandran

(2000) has also studied the quality changes of fish during partial freezing. Ninan (2003) studied the properties of washed mince of fresh and chilled stored black Tilapia

4.3. Materials and methods

4.3.1 Experimental Design

Fresh samples of the two species of squid, loligo and needle from Munambam harbour were brought to the processing plant in air-cooled cabs, packed in ice without direct contact with ice. The temperature was maintained at 0 - 1° C.

In the processing plant the samples were cleaned and the mantle tissues were separated. It was divided into three portions.

Sample 1- kept without any Good Manufacturing Practices (GMP). Here the ice was mixed 3% salt and the material was arranged in alternate layers with ice. The ratio of material to ice was 1:2.

Samples 2 & 3 - kept with GMP and the chilling method was same as for given as sample 1. The details of maintaining the boxes with GMP and without GMP were explained in Chapter 3.

3% Salt was mixed with ice to attain sub zero temperature. The samples were kept in chilled storage, where the room temperature was maintained at –5 to -6°C. The chill store temperature was monitored at every 2 hours interval throughout the period of storage.

The samples drawn daily and were analyzed for chemical indices like TMA, TVN, alpha amino nitrogen, and protein fractions and bacteriology. The analyses of the above said parameters were conducted as described in

Chapter 3 (3.3). The third sample (chill stored with GMP) was taken to the processing line where it was subjected to various steps like treatment, sorting, grading, filling and finally freezing. For analyses samples were drawn at different points, like before treatment, after treatment, before filling, after freezing and after 24 hrs frozen storage. The treatment was given by a mixture of 0.3% acetic acid and 0.03% citric acid. The material was kept in the treatment medium for 20 minutes with occasional stirring. After 20 minutes the samples were washed thoroughly and taken to the next processing step. This method of treatment was adopted for this study, as this is a treatment commonly practiced in seafood industry for processing squid.

4.3.2. Statistical Analysis

The analyses of parameters like TMA-N, TVN, αalpha amino nitrogen, proteins and TPC were carried out according to the method already explained in the Chapter 3. For the comparison of parameters between species, between storage days and between methods (with GMP, without GMP and without direct contact.), three way ANOVA was employed. The mathematical model used for the purpose was as follows:

$X_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + \varepsilon_{ijk}$ where X_{ijk} is the value obtained on ith species using jth method of storage and k^{th} day of storage. μ is the overall effect and α_i is the effect of ith species, β_j is the effect of j^{th} method of storage and γ_k is the effect of k^{th} day of storage.

The details of the processing steps and the points of sampling are given in the processing flow chart Figure 4.1.

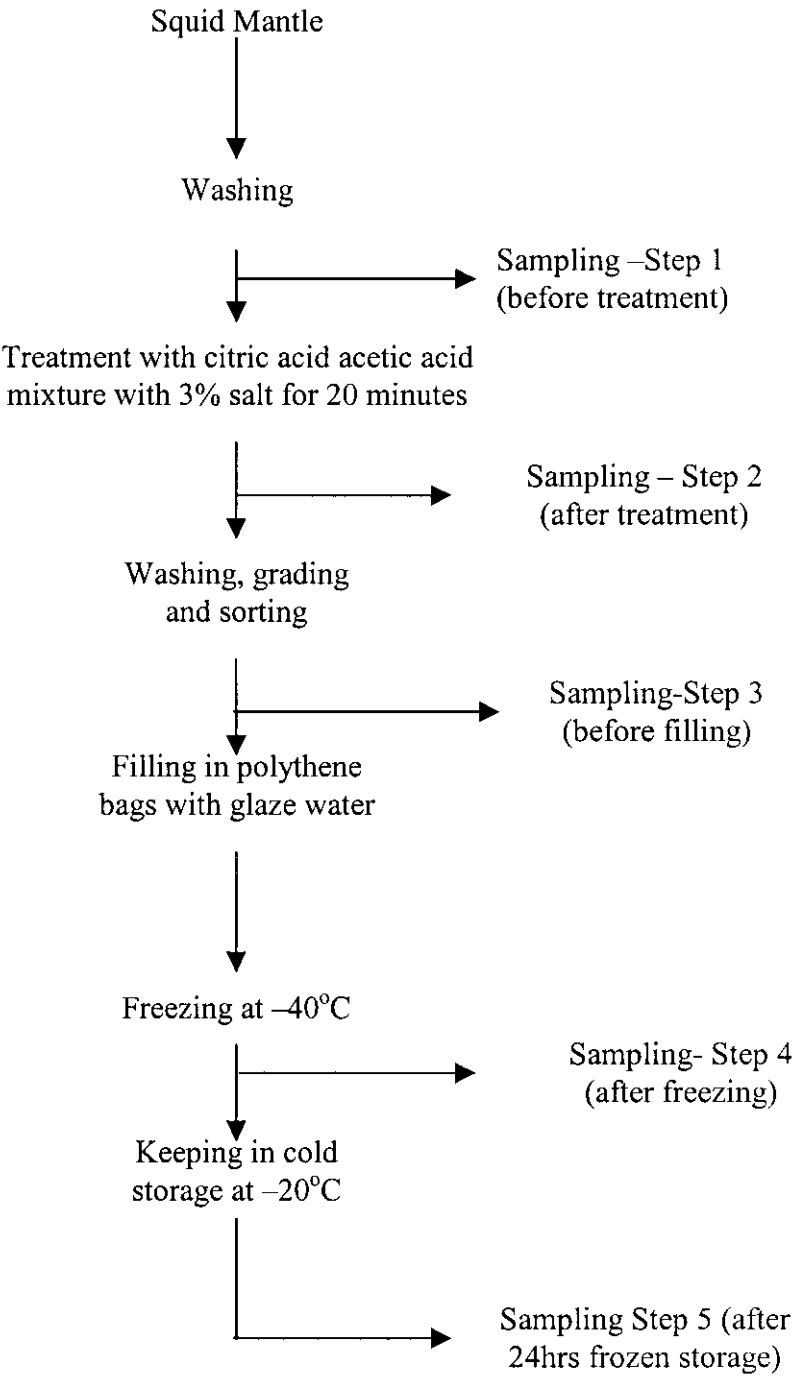


Figure 4.1. **FLOW CHART OF SQUID PROCESSING**

4.4.Results

4.4.1. Total Volatile Bases

4.4.1.1. Total Volatile base Nitrogen

Table 4.1.Changes of TVN mg% during chill storage with/without GMP

	Loligo		Needle	
Days	with GMP	w/oGMP	with GMP	w/oGMP
1	14	15.4	14	15.4
2	15.4	16.8	19.6	21
3	16.8	18.2	25.2	28
4	19.6	20.3	30.8	30.8
5	21	21.7	35	36.4
6	23.8	25.2	44.8	49
7	25.2	25.9	57	60.2
8	26.6	27.3	63	77

w/o GMP - without GMP

Table 4.1 shows the changes in TVBN during chill storage. In both the methods, loligo could keep the quality till the end of 8th day of storage even though it showed a gradual increase in TVBN. But in needle, kept with GMP and without GMP, the TVN content exceeded the acceptable limit on the 4th day of storage. Appendix D.1. gives the ANOVA of TVBN changes during chill storage. The F value between species was 20.612, between methods 0.371 and between days 3.77. There was significant difference between species ($p<0.001$) level. Needle showed a higher value than loligo.

4.4.1.2. TMA

Table 4.2 Changes in TMA mg% during the chill storage with/without GMP

	Loligo		Needle	
Days	**with GMP**	**w/oGMP**	**with GMP**	**w/oGMP**
1	3.5	2.8	2.8	5.6
2	4.2	5.6	4.9	11.2
3	4.9	7	7	14
4	6.3	7.7	11.2	22.4
5	7	11.2	14	28
6	7.7	11.9	28	39.2
7	8.4	12.6	39.2	49
8	8.82	13.3	49	58.8

Regarding the changes in TMA, Table 4.2. gives the changes in TMA-N. The loligo sample with GMP was acceptable till 8^{th} day while the samples without GMP; it lost its quality on the 5^{th} day of chill storage. But in the case of needle, even with GMP, a higher trend of TMA content was observed. On the second day, the needle without GMP crossed the limit of acceptable level while with GMP crossed the limit on the 4^{th} day. Appendix D.2. gives ANOVA for changes in TMA on chill storage. There was no significant difference between species and between days. But between methods there is significant difference. The method with GMP showed a significantly lower value than that of without GMP.

4.4.2.Alpha Amino Nitrogen

Table 4.3 Changes of alpha amino nitrogen during chill storage with /without GMP

	Loligo		Needle	
Days	with GMP	w/oGMP	with GMP	w/oGMP
1	105	98	105	91
2	77	70	28	25.2
3	63	56	14	12.6
4	44.8	53.2	4.2	2.8
5	21	42	2.8	
6	12.6	9.8		
7	7	4.2		
8	2.8	2.1		

Table 4.3. gives the changes in alpha amino nitrogen in chill storage ANOVA-. Appendix D.3.). There was a significant difference between species ($p<0.01$), between methods ($p < 0.05$) and between days ($p<0.001$). The value of alpha amino nitrogen steadily decreased to reach a very low value on 8^{th} day of storage in loligo. But in needle, the leaching rate was very high and almost all the alpha amino nitrogen was leached out by the end of 4^{th} day. Statistically, there was a significant loss of alpha amino nitrogen content between 1^{st} and 2^{nd} day. Then there was a significant difference between 3^{rd} and 6^{th} day.

4.4.3. Protein loss

Table 4.4.Leaching of protein mg% during chill storage with/without GMP

	Loligo		Needle	
Days	with GMP	w/oGMP	with GMP	w/oGMP
1	19.22	21.23	19.47	20.11
2	18.12	16.81	17.25	15.85
3	17.55	15.22	16.92	13.12
4	16.54	14.54	14.8	12.01
5	16	13.82	13.3	11.13
6	15.8	13.07	12.6	10.6
7	14.8	12.13	11.2	9.43
8	14.2	11.88	10.7	8.7

In table 4.4 changes of total protein in both the species of squid on storing under chill storage for eight days are given. In loligo, with GMP, by 8^{th} day of storage almost 25% of the total protein was leached out while in loligo, without GMP, 45% protein was lost.. But in needle with and without GMP, the loss of protein on the 8^{th} day was 46% and 57% respectively. Appendix D.4. gives ANOVA of protein loss during chill storage. The LSD between days was calculated as 1.1048 and there was significant difference between 1^{st}, 2^{nd} and 3^{rd} day, but there was no significant difference between all other days of storage.

4.4.4. Fractions of protein

4.4.4.1. Sarcoplasmic proteins

Table 4.5. Leaching of sarcoplasmic protein during chill storage with/without GMP (%)

	Loligo		Needle	
Days	with GMP	w/oGMP	with GMP	w/oGMP
1	12.88	10.8	11.45	10
2	11.5	9.7	10.61	8
3	11.1	9.3	9.82	6.5
4	10.2	8	8.7	5.7
5	9.6	7	8.1	5.6
6	9.2	5.9	7.6	5
7	8.7	5.1	7.01	4.5
8	7.5	4.7	6.6	4

Table 4.5 gives the loss of sarcoplasmic protein during the chill storage. There was significant difference between species, methods and days at 0.001 levels. Loligo give significantly higher value than the needle. The samples with GMP showed a higher value of protein retention than the samples without GMP. All the days showed significant difference in the loss of protein between adjacent days. About 42% of sarcoplasmic protein was leached out during the storage of the squid, loligo with GMP and a higher rate of leaching was shown 57% in samples without GMP, while in needle squid the loss was 43% and 60% respectively. Appendix D.5 shows the ANOVA table for loss of sarcoplasmic protein during the chill storage.

4.4.4.2. Myofibrillar proteins

TABLE 4.6. Leaching of myofibrillar protein during chill storage with/without GMP

	Loligo		Needle	
Days	with GMP	w/oGMP	with GMP	w/oGMP
1	10.7	9	9.7	8.9
2	10.5	8.9	9.6	8.7
3	10.2	8.5	9.4	8.5
4	9.7	8.4	9	8.4
5	9.6	8.2	8.7	8.3
6	9.2	7.8	8.6	8.2
7	9.1	7.6	8.4	8.1
8	8.9	7.4	8.2	7.8

Table 4.6 shows the loss of myofibrillar protein during chill storage (17-18% in loligo and 16-13% in needle). Here the rate of loss of salt soluble protein in needle kept without GMP was very low because a considerable amount of myosin was already lost in water-soluble fraction. Appendix D.6 shows ANOVA of myofibrillar protein content during chill storage. The F value for species was 8.28, methods 86.43 and days 11.33. There was a significant difference at $p<0.001$ levels.

4.4.4.3. Denatured proteins

Table 4.7. Changes of denatured protein during chill storage with/without GMP (%)

	Loligo		Needle	
Days	with GMP	w/oGMP	with GMP	w/oGMP
1	0.05	0.05	0.08	0.08
2	0.06	0.09	0.1	0.1
3	0.07	0.1	0.12	0.15
4	0.09	0.13	0.14	0.2
5	0.1	0.15	0.18	0.25
6	0.11	0.18	0.2	0.28
7	0.12	0.2	0.23	0.3
8	0.14	0.23	0.25	0.32

Table 4.7 shows the changes in denatured protein. There was a gradual increase in denaturation during the storage. There was a significant increase in the denatured protein between 1st and 8th day. Between species, needle showed significantly higher value than loligo. The samples without GMP showed a higher degree of denaturation (Appendix D.7).

4.4.4.4. Stroma proteins

Table 4.8 Changes of stroma protein during chill storage with/without GMP (%)

	Loligo		Needle	
Days	with GMP	w/oGMP	with GMP	w/oGMP
1	1	1	0.97	0.97
2	1.09	0.97	0.92	0.9
3	1.07	0.92	0.9	0.8
4	1.02	0.9	0.87	0.7
5	0.9	0.8	0.83	0.65
6	0.8	0.7	0.8	0.55
7	0.7	0.6	0.65	0.5
8	0.6	0.5	0.55	0.48

Table 4.8 shows the changes in stroma protein during super cooling. There was a significant loss of stroma protein between species, between methods and between days ($p<0.001$) as shown in Appendix D8. The LSD between days was calculated (0.0708). There was no significant difference between first 4 days, but on 5th day a noticeable loss was found.

4.4.5. Bacteriology

Table 4.9 Changes in TPC during chill storage

	Loligo		Needle	
Days	W/o GMP	With GMP	W/o GMP	With GMP
1	1.75 X 10^4	1.8 X 10^4	1.67 X 10^4	1.76 X 10^4
2	2.0 X 10^4	2.2 X 10^4	1.95 X 10^4	1.85 X 10^4
3	2.5 X 10^4	2.25 X 10^4	2.3 X 10^4	2.0 X 10^4
4	2.9 X 10^4	2.3 X 10^4	2.85 X 10^4	2.15 X 10^4
5	3.4 X 10^4	2.4 X 10^4	3.33 X 10^4	2.3 X 10^4
6	3.8 X 10^4	2.5 X 10^4	3.8 X 10^4	2.4 X 10^4
7	4.4 X 10^4	2.57 X 10^4	4.3 X 10^4	2.48 X 10^4
8	5.3 X 10^4	2.62 X 10^4	5.33 X 10^4	2.7 X 10^4
9	5.7 X 10^4	2.7 X 10^4	5.7 X 10^4	2.9 X 10^4
10	6.0 X 10^4	2.78 X 10^4	6.0 X 10^4	2.96 X 10^4
11	6.4 X 10^4	2.8 X 10^4	6.8 X 10^4	3.07 X 10^4
12	6.85 X 10^4	2.94 X 10^4	7.12 X 10^4	3.27 X 10^4
13	7.5 X 10^4	3.0 X 10^4	7.8 X 10^4	3.34 X 10^4

Table 4.9 shows the Total Plate Count (TPC) during chill storage. Irrespective of species, the count of bacteria increased enormously when kept without any Good Manufacturing Practices. In loligo without GMP, the TPC increased from 1.75 X 10^4 to 7.5 X 10^4, while in samples with GMP,

the TPC showed an increase from 1.8 X 10^4 to 3.0 X 10^4 . The sampling for bacteriology was prolonged 13 days in order to observe the trend of bacterial growth rate. There was a significant difference between days and methods. ($p<0.001$). There was significantly lower value on 1st day and a significantly higher value on 13th day in both the species. The LSD value was 0.1223 (Appendix D.9).

4.5.Processing steps

Here the quality changes during various processing steps in both the species of squid were studied. The samples were drawn at various points in the processing line (Fig.4.1) and analyzed for the various parameters.

4.5.1. Total Volatile Bases

4.5.1.1. TVBN

Table 4.10 changes in TVBN on chill storage during various processing steps

	Loligo					Needle				
Days	step1	step2	step3	step4	step5	step1	step2	step3	step4	step5
1	14	15.4	15.4	15.4	15.4	14	15.4	19.6	21	21
2	15.4	16.8	16.8	16.8	16.8	19.6	21	23.8	26.6	26.6
3	16.8	18.2	18.2	18.2	18.2	25.2	28	30.8	32	32
4	19.6	20.3	20.3	20.3	20.3	30.8	30.8	38	44.8	44.8
5	21	21.7	21.7	21.7	21.7	35	36.4	40.2	49	49
6	23.8	25.2	25.2	25.2	25.2	44.8	49	57.4	60.2	60.2
7	25.2	25.9	25.9	25.9	25.9	57	60.2	65.8	72.4	72.4
8	26.6	27.3	27.3	27.3	27.3	63	77	78.4	84	84

step1-before treatment, step2-after treatment, step3-before filling, step4-after freezing, step5- after 24hrs frozen storage

Table 4.10 shows the changes of TVBN during various processing steps. Here the values were within the limit (below 30mg %) for loligo, but the TVN value exceeded the limit on the 4th day of chill storage in the case of needle squid. During processing steps also, TVN showed a gradual increase. There was no significant difference between species, steps and days, even though the value showed a gradual increase during the storage period (Appendix D.10).

4.5.1.2. TMA-N

Table 4.11 Changes in TMA-N content in chill stored samples during various processing steps

mg%	Loligo					Needle				
Days	step1	step2	step3	step4	step5	step1	step2	Step3	step4	step5
1	3.5	3.5	3.5	3.5	3.92	2.8	5.6	5.6	5.88	6.02
2	4.2	4.2	4.2	4.2	4.48	4.9	7.7	8.68	9.1	11.9
3	4.9	4.9	4.9	4.9	5.32	7	8.82	9.1	14	19.6
4	6.3	6.3	6.3	6.3	6.72	11.2	14	19.6	23.8	25.2
5	7	7	7	7	8.4	14	19.6	22.4	28	33.6
6	7.7	7.7	7.7	7.7	8.68	18.2	22.4	28	33.6	39.2
7	8.4	8.4	8.4	8.4	9.1	23.8	28	33.6	39.2	44.8
8	8.82	8.82	8.82	8.82	9.52	28	33.6	39.2	44.8	56

Table 4.11 shows the changes of TMA in chill stored samples (1-8 days) during various processing steps. Even though the TMA value of loligo squid showed a gradual increase, it was in the limit (below 10mg). But needle, exceeded the limit on the 3rd day after freezing. The TMA content in needle squid reached 56 mg from 2.8mg on 9th day after 24 hrs frozen storage. Irrespective of species, there was no much difference in the TMA content during processing steps. The statistical analysis revealed that there

was no significant difference in changes in TMA content between steps (Appendix D.11). There was significant difference between species and between days. Needle showed a significantly higher value than loligo. There was a gradual increase in the value during the storage time.

4.5.2. Alpha amino nitrogen

Table 4.12 Changes in Alpha amino nitrogen in chill stored samples during various processing steps

	Loligo					Needle				
Days	step1	step2	step3	step4	step5	step1	step2	step3	step4	step5
1	105	98	93.8	91	91	105	91	84	77	63
2	77	70	67.2	64.4	64.4	28	25.2	22.4	21	19.6
3	63	56	53.2	51.8	51.8	14	12.6	11.2	9.8	6.3
4	44.8	42	36.8	35	28	4.2	2.8	2.1	1.82	1.4
5	21	19.6	15.4	14	12	2.8				
6	12.6	9.8	9.1	8.4	8.4					
7	7	4.2	3.92	3.5	3.5					
8	2.8	2.1	1.82	1.54	1.54					

Table 4.12 gives the changes in alpha amino nitrogen during the various processing steps of both the species stored under chill storage (1-8 days) with and without GMP. There was no significant difference between species and steps. But there was a significant difference between days of storage (Appendix D.12)

4.5.3. Protein loss

Table 4.13 Changes in Leaching of proteins in chill stored samples during various processing steps

	Loligo					Needle				
Days	step1	step2	step3	step4	step5	step1	step2	step3	step4	step5
1	19.22	18.78	18.55	17.85	17.55	19.47	17.99	17.43	16.5	16.1
2	18.12	17.35	17	16.9	16.81	17.25	16.75	16.45	16.03	15.85
3	17.55	17.05	16.65	15.8	15.22	16.92	16.22	15.73	15.25	13.12
4	16.54	15.82	15.43	14.77	14.54	14.8	14.13	13.84	13.27	12.01
5	16	14.93	14.44	13.83	13.82	13.3	12.65	12.01	11.64	11.13
6	15.8	14.25	13.72	13	13.07	12.6	12	11.63	11.2	10.6
7	14.8	13.9	13.2	12.7	12.13	11.2	10.7	10.25	9.99	9.43
8	14.2	13.5	13	12	11.88	10.7	10.3	10	9.73	8.7

Almost 39% and 55% of total protein were lost at the end of 24 hrs frozen storage in 8^{th} day of chilled stored samples in loligo and needle respectively. There was significant difference between species, between steps and between days ($p<0.001$) (Appendix D13). There was significant difference between all the days except between 7^{th} and 8^{th} day. Between step 1 (before treatment) and step 5 (after 24hrs frozen storage), there was a significant difference with the protein values showing a gradual decrease in all the processing stages.

4.5.4. Fractions of protein

4.5.4.1. Sarcoplasmic proteins

Table 4.14 Changes in sarcoplasmic proteins in chill stored samples during various processing steps

	Loligo					Needle				
Days	step1	step2	step3	step4	step5	step1	step2	step3	step4	step5
1	12.88	11.2	10.8	10.4	10	11.45	10.4	10	9.6	9.4
2	11.5	10.1	9.7	9.3	9	10.61	8.2	8	7.7	7.5
3	11.1	9.6	9.3	9	8.6	9.82	6.7	6.5	6.1	5.8
4	10.2	8.4	8	7.6	7.2	8.7	6.1	5.7	5.5	5.3
5	9.6	7.2	7	6.6	6.4	8.1	5.8	5.6	5.2	5
6	9.2	6.2	5.9	5.7	5.5	7.6	5.2	5	4.8	4.7
7	8.7	5.4	5.1	4.8	4.6	7.01	4.7	4.5	4.4	4.3
8	7.5	5	4.7	4.5	4.2	6.6	4.3	4	3.9	3.7

The sarcoplasmic protein content showed a gradual decrease about 68% was in both the species of squid during various processing steps (Table 4.14). ANOVA for sarcoplasmic protein is given in Appendix D.14. There was significant difference between species, steps and days. In the processing line, after the treatment step, there was a significantly high loss of sarcoplasmic protein. All the days showed significant difference in the leaching rates and, specifically needle showed a higher value of leaching rate during the processing.

4.5.4.2. Myofibrillar proteins

Table 4.15 Changes in myofibrillar proteins in chill stored samples during various processing steps

	Loligo					Needle				
Days	step1	step2	step3	step4	step5	step1	step2	step3	step4	step5
1	10.7	9.2	9	8.9	8.7	9.7	9	8.9	8.6	8.5
2	10.5	9.1	8.9	8.7	8.5	9.6	8.9	8.7	8.4	8.2
3	10.2	8.7	8.5	8.4	8.2	9.4	8.8	8.5	8.2	8
4	9.7	8.5	8.4	8.2	8	9	8.6	8.4	8.1	7.8
5	9.6	8.3	8.2	8.1	7.7	8.7	8.5	8.3	8	7.6
6	9.2	7.9	7.8	7.6	7.5	8.6	8.4	8.2	7.9	7.5
7	9.1	7.7	7.6	7.5	7.2	8.4	8.2	8.1	7.6	7.4
8	8.9	7.5	7.4	7.2	7	8.2	8	7.8	7.5	7.2

About 35% and 25% of the total myofibrillar protein were leached out from loligo and needle squid respectively during processing of chilled stored samples (Table 4.15). There was a significant difference between species, steps and days ($p < 0.001$). There was a significant difference between chill stored samples of 1st and 8th day. There were no significant changes in the myofibrillar protein between various steps in the case of both the species (Appendix D.15).

4.5.5. Bacteriological

Table 4.16 Changes in TPC in chill stored squid during various processing steps

	Loligo					Needle				
Days	step1	step2	step3	step4	step5	step1	step2	step3	step4	step5
1	1.15×10^5	5.5×10^3	6.5×10^3	5.5×10^3	5.5×10^3	1.25×10^5	1.8×10^4	2.0×10^4	1.8×10^4	1.8×10^4
2	1.65×10^5	7.8×10^3	8.0×10^3	6.0×10^3	6.0×10^3	1.46×10^5	2.2×10^4	2.4×10^4	2.0×10^4	2.0×10^4
3	1.78×10^5	9.4×10^3	9.5×10^3	7.4×10^3	7.5×10^3	1.85×10^5	2.4×10^4	2.5×10^4	2.4×10^4	2.4×10^4
4	1.87×10^5	11.4×10^3	11.6×10^3	10.0×10^3	10.2×10^3	2.02×10^5	2.5×10^4	2.65×10^4	2.52×10^4	2.54×10^4
5	2.15×10^5	16.5×10^3	16.5×10^3	15.0×10^3	15.5×10^3	2.22×10^5	2.8×10^4	2.96×10^4	2.8×10^4	2.8×10^4
6	2.31×10^5	19.2×10^3	19.4×10^3	17.0×10^3	18.0×10^3	2.5×10^5	2.85×10^4	3.05×10^4	2.85×10^4	2.95×10^4
7	2.57×10^5	22×10^3	22.4×10^3	18.0×10^3	18.5×10^3	2.78×10^5	2.98×10^4	3.18×10^4	2.98×10^4	3.0×10^4
8	2.75×10^5	24.5×10^3	24.8×10^3	23.0×10^3	23.5×10^3	2.95×10^5	3.2×10^4	33.6×10^4	3.25×10^4	3.42×10^4
9	2.95×10^5	29.5×10^3	30.0×10^3	29.0×10^3	30.0×10^3	3.1×10^5	3.3×10^4	3.45×10^4	3.4×10^4	3.48×10^4
10	3.15×10^5	31.4×10^3	32.0×10^3	31.5×10^3	32.0×10^3	3.3×10^5	3.5×10^4	3.64×10^4	3.5×10^4	3.56×10^4
11	3.41×10^5	35×10^3	35.6×10^3	34.7×10^3	35.5×10^3	3.5×10^5	3.65×10^4	3.7×10^4	3.65×10^4	3.7×10^4
12	3.61×10^5	38.7×10^3	39.8×10^3	37.4×10^3	37.5×10^3	3.8×10^5	3.9×10^4	4.0×10^4	3.94×10^4	4.0×10^4
13	3.87×10^5	39.7×10^3	40.0×10^3	39.6×10^3	39.8×10^3	4.2×10^5	4.25×10^4	1.8×10^4	4.12×10^4	4.2×10^4

Table 4.16. gives the changes in TPC of samples drawn at various processing steps. The TPC of one day chill stored sample showed a reduction of 5% in loligo and 14%in needle after treatment. At the end of 13^{th} day, the reduction in TPC of loligo sample was decreased from 95% to 90% and for needle from 90% to 86%. There was significant difference between species, steps and days (Appendix D.16.). Among the steps, 1^{st} and 2^{nd} steps showed significant differences than the remaining steps.

4.6.Discussion

Owing to the high ambient temperature, postmortem changes in fish captured in tropical region occur more rapidly than in fish taken from cold or temperate waters. Proper chilling practices are fundamental to obtain high

quality products, When the temperature of biological system is reduced below 0°C, the solution is first super cooled before solute start crystallization. By using ice in salt mixture as chilling medium, the freezing point of the ice can be lowered to sub zero temperature and the fish thus can be super cooled to –1°C. A reduction in storage temperature of 2.8 to -3°C results in doubling the storage life, which completely correlate with the result of this study on squids. On lowering the temperatures, 19% of the water gets frozen at –1°C and about 76% at –4°C, (Mahadevan and Carter, 1948). Love and Elerian (1964) have shown that the rate of protein denaturation in fish frozen at -30°C was maximal in chill stored samples at –1.5°C, possibly due to increased concentration of tissue salts due to the removal of water as ice. Lovern (1961) has indicated that the maximum rate of hydrolysis of lipids to fatty acids took place between –2.4°C and -10°C. Glycolysis also reached a maximum in this region. Sharp (1934) showed that the temperature at which glycolsis takes place at a maximum rate lies between –3 and –3.5°C. According to Power et al., (1969) cod stored at super chilled temperatures showed a superior organoleptic quality and a considerable increase in the storage life.

The findings of Chapter 3 showed that the protein content of ice-stored loligo squid was reduced to 11.2, 14.2, and 17.5% from 20.9% in ice stored samples without GMP, with GMP and without contact ice respectively. In the present study on super cooling, the reduction of protein content in both the species was found to be much more than that of ice storage. The needle squid showed a higher rate of leaching of protein during all the three storage systems. The result of this study on needle squid is comparable with the results of Power et al., (1969) which showed a higher

leaching rate on super cooling than the ice storage and frozen storage. The fish stored at –1°C were reported to be perfectly fresh for 21 days, and when the temperature was reduced to –2°C and –3°C the freshness was extended to 25 days and 35 days respectively; but had a tendency to harden (Scarlatti, 1965).

The needle squid, which is stored in super chilling temperature with GMP and without GMP showed a higher rate of TMA value that exceeded the limit of 10mg% on third day of storage (with GMP) and second day (without GMP) reached 11.2mg%. But in the case of ice stored needle squid (without GMP) crossed the limit of 10mg% on the second day, reached 14mg% and samples stored with GMP on fifth day, and without contact ice on seventh day. The super chilling should not be used for storage period less than 12 days (Peters, 1967). Iced storage at –2°C to –1°C extended the storage life to about 7 days in comparison to regular iced storage (Power and Morton, 1965). The fish stored in –3°C in air gradually dehydrated and reached a value of 78.5% moisture after 35 days of storage (Power & Morton, 1965). The authors have studied the physical and chemical properties of cod during super chilling. The samples stored in ice showed a lesser amount of protein denaturation than in super cooling (Power & Morton, 1965). A similar result was obtained from the present study. In this study, the tissue samples showed a higher rate of extractable nitrogen due to the breakdown of myofibrils during the storage period.

Early it was believed that the storage of fish just below the freezing point gave rise to mediocre quality production due to the cellular damage and accompanying acceleration of bio-chemical reactions (Ronsivalli and Baker, 1981). This lead to leaching of nutrients and loss of texture and

appearance. But recent study have shown that storage of fish at a temperature of –3°C where about 70% of the water is in the ice state can retard the biochemical reactions and extend the quality (Lee and Park, 1985; Simpson and Haard, 1987; Uchiyama, 1988). It is also reported that excellent quality was maintained 1.5 to 8 fold longer for different species held at –3°C compared with ice storage (Ehira and Uchiyama, 1986).

4.7. Conclusion

Good chilling practices on board fishing vessel and onshore, result in better quality of fish and this can demand higher prices at auction and greater acceptance by the consumers. Super chilling is not advisable in the case of squid especially needle squid which showed a higher degree of deterioration during storage. The processed squid are liable to loose the nutrient fractions very much in water and ice. Hence minimum contact with material with ice is recommended throughout the processing. Treatment of the sample with proper additives at lower temperatures could reduce the leaching effect. The repeated washing of the squid tube before freezing should be avoided. The time taken to pass through grading, sorting, filling, before freezing should be minimum as possible. After filling the trays for freezing, the sample should be frozen immediately without delay. Individually quick freezing (IQF) is preferred to block freezing as in IQF, there is no direct contact of the sample with ice and water. Also pressing of samples in block freezing increase the leaching rate of nutrients.

4.8. References

*Anon., (1935) Holding fish without ice. Trade Journal Fishing Gazette.

*Anon., (1964) Super chilling-a report from the White Fish Authority. J. Refrig. **9**,10.

Balachandran, K.K., (2000) Post-Harvest Technology of Fish and Fish products. Pub. Daya Publishing House, Delhi. Chap. **10**. 4. 8.

Ehira, I and Uchiyama, H., (1986) Improvement of preserving freshness of Amberjack striped jack and other fish species by partial freezing. J. Inst. Cold chain. **12** (4), 1.

Lee, Y.W. and Park, Y.H., (1985) Effect of partial freezing as a mean of retaining freshness. Changes in freshness and forming ability of Mullet muscle during storage by Partial freezing. Bull. Korean Fish. Soc. **18** (16), 529.

*Love, R.M. and Elerian, M.K., (1964) J. Sci. Fd. Agric. **15**, 11.

Lovern, J.A., (1961) Lipids of Fish and changes occurring in them during processing and storage. Fish in Nutrition (Ed. Heen, E., Kreuzer, R). Fishing News (Books) Ltd. London 86.

Mahadevan, V. and Carter, N.M., (1948) Prog. Rep. Pacif. Cst. Stns. N0.**76**, 85-87.

Merritt, J.H, Livingston, I., Shewan, J.M., (1966) Super chilling of fish-the position today. Mod. Refrig. **69** (818), 438-443.

Merritt, J.H., (1965) Super chilling onboard trawlers. Annex 1965-5. Bull. Intl. Inst. Refrig. 183.

Ninan, G., (2003) Proceedings of short course on Harvest and Post-harvest technologies of Marine and Inland fishes of India organized by CIFT.

Partmann, W., (1965) Some experiences concerning super chilling of fish Annex 1965-5 Bull. Intl. Inst. Refrig. 191.

*Power, H.E. and Morton, M.L., (1965) New series circular No.23 Halifax Lab. Fish. Res. Board of Canada.

Power, H.E., Margaret L. Morton and Ruth E. Sinclair, (1969) The chemical and physical characteristics of cod stored at Super chill temperatures. Freezing and Irradiation of Fish Ed. Kreuzer, R. 104-113.

Ranken, M.B.F., (1963) Some recent advances in refrigeration machinery installation in fishing vessel. Progress in refrigeration Science and technology, International Institute of refrigeration and Commission. **8**, 1773.

Roach, S.W. and Tomlinson, N., (1969) Partial freezing of Pacific salmon intended for canning. Freezing and Irradiation of Fish Ed. Kreuzer, R. 114-118.

*Ronsivalli, L.J. and Baker, D.W., (1981) Low temperature preservation of seafood- A review, Mar. Fish. Rev., **43** (4), 1.

*Scarlatte, E., (1965) System for preserving fresh fish on board deep-water vessels. In fish handling and Preservation, OECD, Paris. 57.

*Sharp, J.G., (1934) Rep. Fd. Invest. Bd., HMSO, London, 96.

Simpson, M.P., Haard, N.F., (1987) Temperature acclimation of Atlantic cod (*Gadus morhua*) and its influence on freezing point and

biochemical damage of post-mortem muscle storage at 0°C and –3°C. J. Fd. Biochem. **11**, 69.

Uchiyama, H., (1988) Biochemical determination of Fish freshness and Partial freezing as a mean of keeping freshness of fish and its products. In Aquaculture International Congress and exposition, Vancouver, 171.

*White Fish Authority, (1964) Ibid., No.16.

*White Fish Authority, (1964) Rd. Bull. No.2.

* Not referred in original

Chapter 5

CHANGES IN QUALITY ON FROZEN STORAGE

5.1. Introduction

During frozen storage of fish, deterioration in quality due to microorganisms and some biochemical processes is decreased. Properly frozen and packaged good quality lean fish can normally be stored at –20°C to –30°C for more than 1 year without much loss in consumer acceptability. But the quality of fish deteriorates during storage as shown by organoleptic, chemical and physical changes. Frozen fish stored for extended period can have reduced palatability by loss of flavour or texture. The denaturation and aggregation of fish muscle proteins particularly myofibrillar fraction are associated with the texture deterioration of frozen fish. Many factors influence the deterioration during frozen storage, like rate of freezing, temperature and time of frozen storage, post harvest history of fish prior to freezing etc.

In recent years, the production of cephalopods, mainly squid and cuttlefish from India, is on the increase amounting to greater than 56,000 metric tonnes in 2002. The export of frozen squid and cuttlefish in the same year was greater than 28,000 tonnes and formed second major seafood item exported from the country. The factories in Cochin account for roughly 60% of the production and around 24 factories are involved in the processing of cephalopods. Since in foreign market, this is considered as highly favoured seafood delicacy, high quality products are required for exports. Quality standard of frozen squid and cuttlefish were established by Indian Standards Institution in 1976 (IS 8076) and emphasis was laid on physical, sensory and bacteriological quality assessments. However, the quality evaluation based on objective indices has assumed greater importance in International trade in recent years. Many studies pertaining to the iced and frozen storage

characteristics of squid and cuttlefish have been made, of which the major studies in squid have been done only on Loligo (*Loligo duvaucelii)* and no systematic work on needle squid (*Doryteuthis sibogae)* seems to be done. The present study surveyed the quality of two the species of frozen squid based on physical, sensory, chemical and bacteriological methods and compares the acceptability with the chemical parameters and the degree of protein denaturation.

5.2.Review of literature

The history of squid freezing was studied by Hansen and Aagaard (1969). Various authors have studied the importance of freezing cephalopods especially squid and the significance of squid in the international trade (Borgstrom, 1965; Learson and Ampola, 1977; Thrower 1978). Thrower (1978) found that the squid remained for a maximum period of eight months in the cold storage. James and Iyer (1998) have studied the quality of frozen squid and cuttlefish of export trade where the commercial samples of frozen squid and cuttlefish were evaluated by organoleptic, microbiological and biochemical means. Sophia and Sheriff (2003) have investigated the effect of iced storage duration and treatment on frozen storage of cuttlefish fillets. Similar work has been carried out by Selvaraj et al., (1991) on the effect of ascorbic acid dip treatment on frozen storage of squid *(Loligo duavucelii).* Several studies have been reported on the storage characteristics of iced and frozen stored squid and cuttle fish (Joseph et al., 1977; Dhananjaya et al., 1987; Joseph and Perigreen, 1988). Ke et al., (1979) in his study on the frozen storage life of Canadian squid at various temperatures, reached a conclusion that the round squid stored at – 30°C could remain in good condition for more than 18 months and the split

mantle for more than 12 months. Lakshmanan et al., (1993) have studied the quality levels of industrial samples of squid (*Loligo sp*) and Cuttlefish (*Sepia sp*) for export following sensory, biochemical and microbiological characteristics. Some studies pertaining to the iced and frozen storage characteristics of squid and cuttle fish have also been made (Raghunath, 1984, Sastry and Sirkar, 1985, Bykowski et al., 1990). Sanjeevan et al., (1987) Lakshmanan et al., (1993) and Varma et al., (1985) have studied the bacteriology of frozen cuttle fish and squid. Iyer et al., (1990) has investigated the presence of *Vibrio cholerae non–01* in fresh fish and was introduced into the product during handling. Various other workers have also studied the bacteriology of frozen squid (Joseph et al., 1977, Lakshmanan et al., 1993, Selvaraj et al., 1991).

The quality changes during the frozen storage of other marine species have been studied by many workers. Comparative effects of frozen storage on biochemical changes in pink perch and oil sardine were investigated by Sarma et al., (1998). Simeonidou et al., (1997) studied the effect of frozen storage on the quality of whole fish and fillets of horse mackerel. Engvang and Nielsen (2000) evaluated the activity of chymotrypsin from herring intestine during frozen storage. Rodriguez et al., (1998) in their study stated the importance of the reduction of TMAO for the evaluation of the quality of frozen fish. Shenoy and Pillai (1971) have studied the changes of *Sardinella longiceps* on frozen storage and Radhakrishnan et al., (1973) on Bombay duck.

The types of proteins and their functional status are the two factors that most influence the texture of cephalopod muscle. There are many publications concerning muscle protein solubility in moderate-ionic-strength

saline (0.6M NaCl or KCl). In these studies, the functional properties of fish muscle are related to the solubility of the constituent proteins (Jimenz-Colmenro and Borderius, 1983; Hultin et al., 1995). The most affected ones are myofibrillar proteins and stroma proteins, which undergo aggregation during frozen storage (Sikorski et al., 1976; Jimenz-Colmenro et al., 1983).

5.3.Materials and Methods

5.3.1. Sample collection

Freshly collected Samples of Loligo squid (*Loligo duvaucelii*) and needle squid (*Doryteuthis sibogae*) (from 6-8hrs in ice after capture) were gutted and skinned. Tentacles were removed and the mantles were immediately frozen in a fluidized bed freezer at –40°C for 40-50 minutes. The core temperature of the mantle was recorded to be –18°C using a digital thermometer (Casio 168X). The frozen material was in a cold store at –20°C for six months. Samples of 100 to 200g were packed separately in 150gauge polythene bags and stored after packing in 7 ply corrugated master cartons. Samples were drawn on 0, 30., 60, 90, 120, 150 and 180 days and were subjected to physical, chemical and microbiological analyses. All the samples were analyzed in triplicate. The temperatures of the cold store and the frozen material were checked periodically.

5.3.2. Expressible moisture

One cm^2 squid piece was taken and placed between two filter papers and pressed under a fixed pressure ($10kg/cm^2$) for 10 seconds. The weight difference compared to the weight before pressing in % reflects the expressible moisture.

5.3.3. Statistical analysis

The results of various quality parameters were analyzed statistically using two way ANOVA. Two- factor ANOVA was employed to compare the effect of each parameter like TMA-N, TVBN, alpha amino nitrogen, protein fractions and TPC, between species and days of frozen storage. The mathematical model was employed for this purpose was as follows:

$$X_{ij} = \mu + \alpha_i + \beta_j + \varepsilon_{ij}$$

In the case of frozen storage, Xij is the observed value corresponding to the i^{th} species on j^{th} day of storage. μ is the overall effect, α_i is the effect of ith species and β_j is the effect of j^{th} day of storage and epsilon ij (ε_{ij}) is the random error.

5.4. Results

5.4.1. Total volatile bases

5.4.1.1. Total volatile base nitrogen

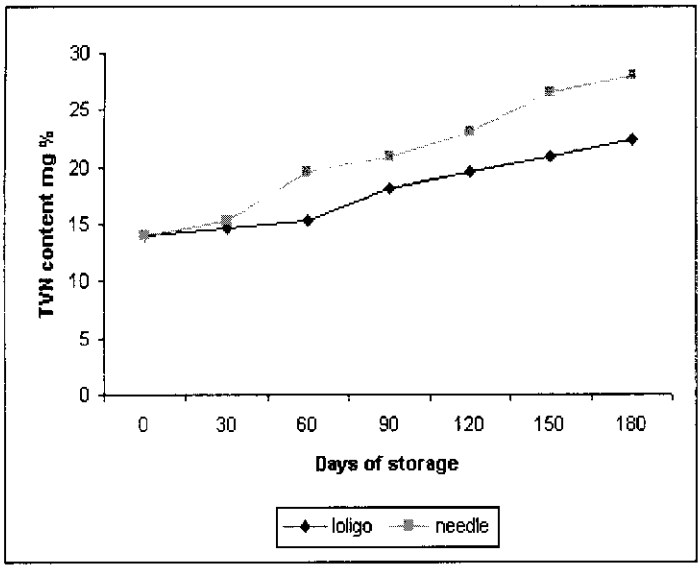


Figure 5.1. Changes in TVN content during frozen storage

Figure 5.1. shows changes in TVN content of both the species during frozen storage. This also showed an increasing trend in both species. The TVN value did not exceed the limit of 30mg N/100g during the period of frozen storage. Appendix E.1. represents the ANOVA table for changes in TVN (which showed a significant difference between species and days) during frozen storage. The F values between species and days were 14.59 and 14.82 respectively. ($p<0.001$).

5.4.1.2. Trimethylamine

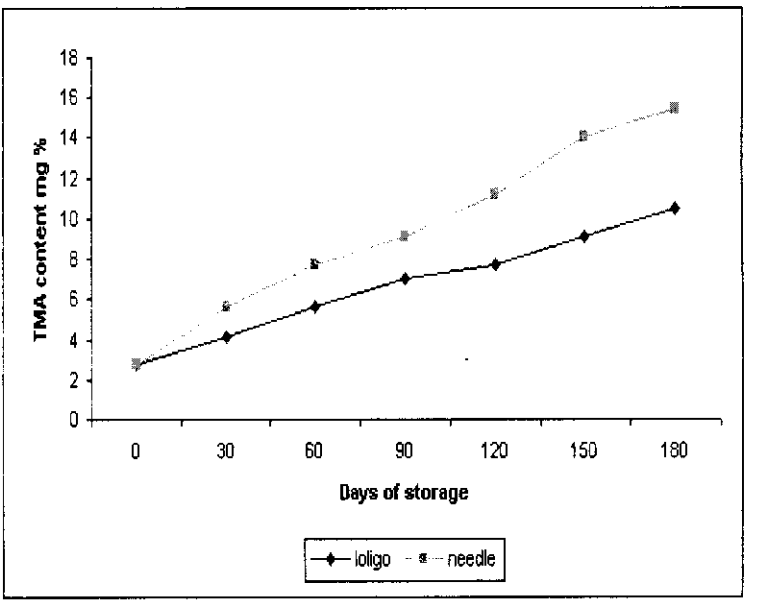


Figure 5.2. Changes in TMA content during frozen storage

Figure 5.2. showed the variations in TMA content during frozen storage of loligo and needle squid. In both the species TMA content showed a gradual increase, but the rate of increase of TMA value was faster in needle squid (exceeded the limit by 90 days) than Loligo (exceeded the limit by 150 days). Appendix E.2. shows that there was a significant difference

between species and days ($p<0.001$). Needle squid showed a significantly higher value, compared to loligo. LSD for the days was calculated as 3.18.

5.4.2. Alpha amino nitrogen

Alpha amino nitrogen also showed a gradual increase in both the species of squid during frozen storage (Figure 5.3.). In the beginning, it showed a decline and then a gradual increase. Appendix E.3. shows that there was no significant difference in alpha amino nitrogen content between the species, but there was a significant difference between days ($p<0.001$). The LSD was calculated as 14.11.

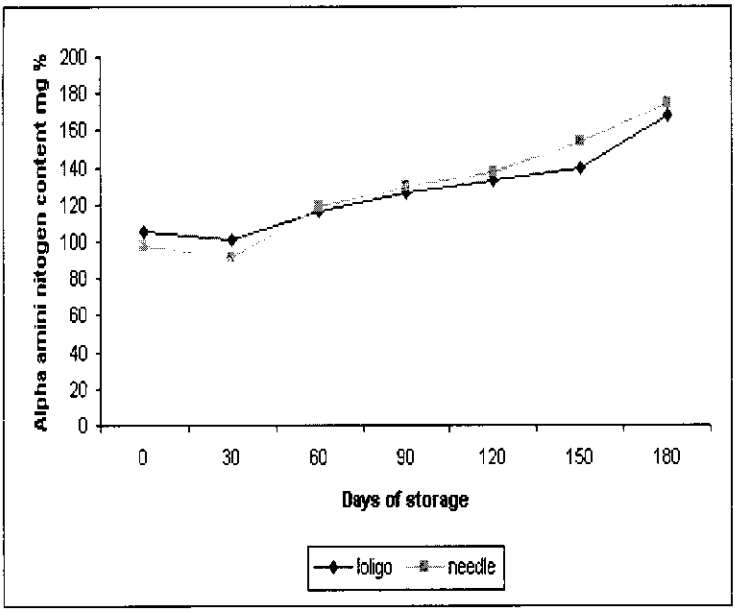


Figure 5.3. Changes in Alpha amino nitrogen content during frozen storage

5.4.3. Total Protein

Table 5.1. gives the variation in total protein content of loligo and needle squid during frozen storage. The decrease in protein content in loligo

and needle squid on frozen storage up to 180 days, was 20% and 34% of the total protein before freezing respectively

Table 5.1. Changes in Total protein content during frozen storage (%)

Days	Loligo	Needle
0	20.71	20.83
30	19.25	18.28
60	18.55	16.31
90	18.2	15.1
120	17.8	14.9
150	17.2	14
180	16.6	13.8

Appendix E.4 shows the ANOVA table for protein changes, which revealed a significant difference between species and days ($p<0.001$).

5.4.4. Fractions of proteins

Table 5.2. Changes in various fractions of protein during frozen storage

	Sarcoplasmic Proteins %		Myofibrillar Proteins %		Denatured Proteins %		Stroma Proteins %	
Days	Loligo	Needle	Loligo	Needle	Loligo	Needle	Loligo	Needle
0	12.62	11.75	9.82	8.68	1.1	0.7	0.8	0.9
30	11.13	10.2	7.24	6.84	1.7	1.4	0.75	0.83
60	9.97	9.17	6.12	5.9	2.3	1.9	0.72	0.8
90	9.24	8.35	5.3	5	2.82	2.5	0.7	0.75
120	8.8	7.83	4.9	4.7	3.4	3.2	0.68	0.72
150	8.3	7.2	4.27	4.01	3.8	3.8	0.64	0.68
180	7.6	6.8	4	3.95	4.2	4.3	0.6	0.65

Table 5.2 presents the changes in various protein fractions during frozen storage. In loligo, about 40% of sarcoplasmic protein was lost, but in the case of needle 43% was lost during storage.

Appendix E.5 represents ANOVA table for changes in sarcoplasmic protein during the frozen storage. There was a significant difference at the level of $p < 0.001$ for both species and days.

During frozen storage, there was an apparent loss of extractability of salt soluble proteins, which was 40% less from the zero day in loligo squid and about 45% less in needle squid. A significant difference between species and days ($p<0.001$) was observed (Appendix E.6). LSD was calculated to 0.618 and the degrees of freedom were as in the case of sarcoplasmic protein. Needle showed significantly lesser solubility of salt soluble proteins, when compared with loligo.

A gradual increase in the denatured protein content was observed during the frozen storage in both the species of squid. There was a significant difference between species ($p < 0.05$) and days ($p < 0.001$) during frozen storage (Appendix E.7).

A gradual decrease in stroma protein was observed during the period of frozen storage. The decrease was about 25% in loligo and 28% in needle from the initial value, showing a significant difference of $p<0.001$ between species and days of storage (Appendix E.8).

5.4.5. Organoleptic quality

Table 5.3.Changes in pH, organoleptic score and Expressible moisture content during frozen storage

	Needle	Grade	EM %	Loligo	Grade	EM %
0	6	7.5	30	6	8.5	25
30	6.3	7	35	6.2	8	28
60	6.4	6	39	6.3	8	34
90	6.5	5	47	6.4	7.5	40
120	6.6	5	54	6.5	7	45
150	7.1	3	58	6.8	7	48
180	7.5	2	60	7.1	6	53

E.M. Expressible moisture % in the muscle

The pH of both the species showed a gradual increase on frozen storage, while the organoleptic quality showed a gradual decrease (Table 5.3.). The needle squid lost its organoleptic acceptance by 3rd month of storage, but loligo retained the quality till the end of the storage. There was no correlation between pH and organoleptic quality, even though the pH showed a gradual increase towards the end of the storage. The expressible moisture showed a gradual increase in both the species and a higher retention of moisture was observed in loligo compared to needle squid.

5.4.6. Bacteriology

Table 5.4. Changes in Total Plate count during frozen storage at - 20°C

Days	Loligo	Needle
0	1.75×10^5	2.12×10^5
30	1.83×10^5	2.15×10^5
60	1.86×10^5	2.18×10^5
90	1.89×10^5	2.19×10^5
120	1.9×10^5	2.22×10^5
150	1.91×10^5	2.23×10^5
180	1.93×10^5	2.25×10^5

The total plate count was calculated after converting the original value into its logarithm (Table 5.4.). In loligo, TPC 1.75 X 10^5 was increased to1.93 X 10^5. But in the case of needle the count of 2.12 X 10^5 increased to 2.25 X 10^5. There was a significant difference between days and species ($p<0.001$). The F values were 752.2 for species and 11.56 for days (Appendix E.9).

5.5.Discussion

The TMA content is often used as an indicator for decomposition in fish. TMA production is believed to be the consequence of microbial action on TMAO present in marine species. The variation in the content of TMA can be related to the availability of TMAO for enzymic degradation or due to some inhibitory mechanism operating for TMAO degrading enzymes. In the present study, a steady increase in the TMA, TVN and alpha amino nitrogen content was observed in both the species on frozen storage, of which needle squid showed a higher rate of increase. According to Thrower (1978) squid could be stored in cold storage for a maximum period of 8 months. Ke et al., (1979) has reported that Canadian squid could be stored in good condition at –30°C for more than 18 months and split mantle for more than 12 months.

The TVN content showed an increase from 14mg N/100g tissue on zero day to 22.4mg N and 28mg N/100g tissue on 180 days, in loligo and needle squid respectively. Thus the TVN content was within the permissible level in both the species till 180 days of frozen storage. Studies on frozen storage characteristics of treated and untreated meat from mussel also showed a steady increase in TVN and TMA-N (Sawanth and Patange,

2002). Similar results were reported by Sophia and Sherief (2003) in their study on the effect of different treatments on frozen storage of cuttlefish fillet. Similar results were reported by Rodriguez (1988), Sarma (1998) and Simeonidous (1997). According to latter, TMA-N and TVN increased during the frozen storage while sensory attributes like odour, texture and taste reduced. There are reports that mention TMA and hypoxanthine concentration did not change much during frozen storage and can therefore be used as indices of pre-freezing quality (Connell, 1969; Rodriguez, 1998). But this opinion that TMA-N does not change during frozen storage is a matter of conflict and does not agree with the present study and many other reported work.

A significant steady increase was observed in alpha amino nitrogen during frozen storage period of loligo and needle, the variations between the species being not significant. The protein content of cephalopod meat varies from 15-20%. Alpha amino constituents of NPN are important from the point of view of bacterial spoilage as they provide a source of nitrogen that can be readily assimilated by the micro flora associated with spoilage of fish. They contribute substantially to the flavour of the fish. In this study, during pre-freezing stage, the squid mantles were kept with GMP and without direct contact with ice and hence leaching of alpha amino nitrogen was kept minimum.

The muscle pH varied between 6.22 to 7.38 in cuttlefish and 6.17 to 7.34 in squids, but did not correlate with sensory scores (Lakshmanan et al., 1993). On frozen storage, the squid lost its organoleptic quality at the end of 19^{th} week, but seems to be acceptable and good when treated with salt and polyphosphate (Joseph et al., 1977)

In general, the results show that the solubilization of sarcoplasmic and myofibrillar proteins in the respective extraction media decreased during frozen storage. In the present study, the concept of solubilization and that of denaturation, is that amount of the protein remaining in the supernatant solution after centrifugation. Similar results were obtained by Joseph et al., (1977) in frozen squid, Shenoy and Pillai, (1971) in sardine and Radhakrishnan, et al., (1973) in Bombay duck. . The solubility in 0.6M KCl decreased with increasing time of frozen storage. The decrease in solubility reflects the increase in the number of cross bridges other than ionic bonds being formed during frozen storage.

Sarcoplasmic proteins, which are extracted by low ionic strength solution, also showed a decrease during frozen storage of both the species of squid. The high extractability of myosin and other structural proteins in squid has been confirmed in the previous chapters. The aggregation of these fractions due to the freeze denaturation can lead to lesser extractability during storage. The sarcoplasmic proteins in fish are not much affected during freezing and frozen storage. In various species of fishes like cod, plaice and halibut, the albumin fraction remained unaffected during frozen storage (Dyer and Dingle, 1961; Connell 1962). But Tomlinson and Geiger (1963) reported results different from this. Studies also showed that extractable myosin decreased gradually where as actin was unaffected for a long time during frozen storage (Connell, 1962). Creche et al., (1998) has observed that in cod stores at –20°C, there was a loss of protein solubility than the fish stored at –30°C. In this study also, there is a significant loss of protein content for which, loss of soluble protein and NPN in drip during thawing is also to be taken into account. Between the two species studied,

needle squid showed lesser extractability of sarcoplasmic and myofibrillar fractions compared to loligo sp; thus explaining the high content of denatured protein fraction during the frozen storage period.

The rate of survival of different types of faecal organisms like *E-coli* along with the normal flora in the frozen squid mantles has been studied. TPC showed a slight increase during storage, where as *Coagulase positive staphylococci* showed a gradual decrease. Towards 60 days of frozen storage their count dropped to nil and *Salmonella* and *Vibrio sp* were not detected in frozen samples. Similar observations were reported by Joseph et al., (1977) in their study of iced and frozen stored squid (*Loligo sp*)

5.6.Conclusion

When compared with ice storage and chill storage, frozen storage could preserve the squid mantles for longer period. For onboard vessel in order to preserve the material before processing, most effective method is the freezing. The quality of individual lots of the different species of squid will vary considerably depending on the initial quality of the raw material. Their proper processing and preparation for freezing is very important. Squid mantles handled with Good Manufacturing Practices and with minimum contact with ice and water could retain its quality parameters during frozen storage. While chemical and physical changes in the proteins are primarily responsible for quality changes, the results of this study confirm the protein aggregation during frozen storage modifying the water-holding capacity and extractability of the proteins. This leads to the texture modification with less tenderness and development of undesirable flavours if the samples are frozen stored beyond the recommended period.

5.7. References

Borgstrom George, (1965) Trends in Utilization of fish and shellfish. Fish as food 4, (Ed. Borgstrom, G.) Academic press, New York

Bykowski, P.J., Zalewski, J. and Gora, A., (1990) Shelf life of frozen squid (*Illex patogonicus*). In: Chilling and Freezing of New Fish products International Institute of refrigeration, Aberdeen, U.K, 317-322.

Connell, J.J., (1969) Changes in the eating quality of frozen stored cod and associated chemical and physical changes. In Freezing and Irradiation of Fish. (Ed. Kreuzer, R.) Fishing News Books, London. 323-338.

Connell, J.J., (1962) Fish muscle proteins. Recent Advances in Food Sciences Vol I, 136-146. Ed. Hawthorne, J. and Leitch, J.M., Butterworths, London.

*Creche, M., Mazo, M.L., del, Torrejon, P., Tetada, M., (1998) J. Agri. Fd. Chem. **46**, 1539-1546.

Dhananjaya, S., Krishnakumar, S. and Hiremath, G.G., (1987) Effect of handling on the pink discolouration in fresh squid during iced storage. Seafood export Journal. **19**, 13-18.

Dyer, W.G. and Dingle, J.R., (1961) In fish as food. (Ed. Borgstrom, G). Academic Press, New York Chapter 9, 275.

Engvang, K. and Nielsen, H.H., (2000) In situ activity of chymotrypsin in sugar-salted herring during cold storage. J. Sci. Fd. Agri. **80** (8), 1277-1283.

Hansen, P. and Aagaard, J., (1969) Freezing of shellfish. Freezing and Irradiation of fish (Ed. Kreuzer, R.) 147-158.

Hultin, H.O., Feng, Y and Stanley, D.W., (1995) A re-examination of muscle protein solubility. J. Muscle Foods. **6**, 91-107.

Iyer, T.S.G., Varma, P.R.G., Zacharia, S.and Joseph, M.A., (1990) Studies on the contamination of fishery products with *V. cholerae non-01* during different stages of handling and processing. Mysore J. Agri. Sci. **24**, 394-398.

James, L. and Iyer, T.S.G., (1998) Quality of frozen squid and cuttlefish of the export trade. Advances and priorities in Fisheries Technology. (Eds. Balachandran, K.K., Iyer, T.S.G., Madhavan, P., Jose Joseph, Perigreen, P.A., Reghunath, M.R. and Varghese, M.D.) 280-283.

Jimenez-Colmenero, F and Borderias, A.J., (1983) A study of effects of frozen storage of certain functional properties of meat and fish protein, J. Fd. Technol. **18,** 731-737.

Joseph, J., Varma, P.R.G. and Venkataraman, R., (1977) Iced and frozen storage of squid (Loligo species) Fish. Tech. **14**, 13-20.

Joseph, J.and Perigreen, P.A., (1988) Studies on frozen storage of cuttlefish fillets. Fish. Tech. **25**, 32- 35.

Ke, P.J., Woyewoda, A.D. and Fierheller, M., (1979) Handling methods and quality evaluation of fresh Canadian Atlantic squid (Illex Illecebrosus). Tech. Rep. Fish. Mar. Serv. Can., 898, 8.

Lakshmanan, P.T., Varma, P.R.G. and Iyer, T.S.G., (1993) Quality of Commercially frozen cephalopods products from India, J. Food control, **4** (3), 159-164.

Learson, R.J. and Ambola, V.C., (1977) Care and maintenance of squid quality. Mar. Fish. Rev. **39** (7), 15-16.

*Radhakrishnan, A.G., Solanki, K.K. and Venkataramanan, R., (1973) Fish. Tech. **10** (2), 124.

Reghunath, M.R., (1984) Soluble nitrogen loses in squid *(loligo duaucelii)* during storage in slush ice. J. Food science and Technology. **21**. 50-52.

Rodrigues, C., Masoud, T. and Huerta, M.D., (1998) Degradation of trimethylamine oxide for evaluation of quality of frozen fish. Alimemtaria, **288**, 125-129.

Sanjeev, S., Iyer, T.S.G., Varma, P.R.G., Rao, C.C.P. and Iyer, K.M., (1987) Carriages of enterotoxigenic staphylococci in workers of fish processing factories. Ind. J. Med. Res. **85**, 262-265.

Sarma, J., Srikar, L.N. and Vidyasagar Reddy, G., (1998) Comparative effects of frozen storage on biochemical changes in pink perch (*Nemipterus japonicus*) and oil sardine (*Sardinella longiceps*). J. Fd. Sci. Technol. Indis, **35** (3), 255-258.

Sastry, H.H.C. and Srikar, L.N., (1985) Protein and related changes in cuttlefish (*Sepia aculeate*) during iced storage. In Harvest and Post harvest Technology of Fish (Ed. K. Ravindran) society of fisheries Technologists, India, 386-388.

Sawant, S.S. and Patange, S.B., (2002) Frozen storage characteristics of treated and untreated meat from male and female mussel (*Perna virdis*). Fish. Technol. **39** (1), 27-33.

Selvaraj, P., Jasmine, J.I. and Jeyachandran, P., (1991) Effect of ascorbic acid dip treatment on frozen storage of squid (*Loligo duvaucelii, Oribigny*) Fish. Technol. **2**, 117-121.

*Shenoy, A.V. and Pillai, V.K., (1971) Fish. Technol. **8**, 37.

Sikorski, Z.E., Olley, J. and Kostuch, S., (1976) Protein changes in frozen fish. Crit. Rev. Fd. Sci. Nutr. **8**, 97.

Simeonidu, S., Govaris, A. and Vareltzis, K., (1997) Effect of frozen storage on the quality of whole fish and fillets of horse mackerel (Trachurus trachurus) and Mediterranean hake (Merluccius mediterraneus). Zietschrift fuer lebensmittel Untersuchung and Forschung A. Fd. Res. Technol; **204** (6), 405-410.

Sophia Margaret Joseph and Sherief, P.M., (2003) Effect of Treatments on the Iced storage shelf life of cuttlefish (*Sepia aculeate*) fillets. Fish.Tech. **40** (1), 32-35.

*Thrower, S.J., (1978) Catching and handling squid. Aust. Fish., **37** (9), 20-25.

*Tomlinson, N and Geiger, S.E., (1963) J.Fish. Res. Bd. Canada. **202**, 187-194.

Varma, P.R.G., Mathen, C and Mathew, A., (1985) Bacteriological quality of frozen seafood to export with special reference to *Salmonella*. In Harvest and Post harvest Technology of Fish (Ed. K. Ravindran) society of fisheries Technologists, India, 665-666.

*Not referred in original

Chapter 6

EFFECT OF VARIOUS TREATMENTS ON QUALITY

6.1. Introduction

Owing to high ambient temperatures, post-mortem changes in fish captured from tropical regions occur more rapidly than in fish from temperate waters. Proper icing practices are fundamental to obtain high quality products. Freezing and frozen storage prolong the shelf life of seafood by retarding enzymatic and microbial activity. An alternative approach to extend the storage life of fish is, application of more than one treatment like addition of anti-microbial compounds, anti-oxidants, polyphosphates etc to improve the muscle quality. In the previous chapters, the effect of icing, super cooling, freezing and frozen storage as short term and long term preservative methods of two species of squids under study, Loligo species and *Doryteuthis* species and the importance of Good Manufacturing Practices, the processor has to adhere to, have been discussed. But it is important to have a better understanding of the effect of possible treatments in prolonging the frozen storage characteristics of the two species of squid. Hence a comparative study has been undertaken here with regard to the use of various treatments, and to recommend the most appropriate one to retain the physico-chemical, organoleptic and microbiological properties of the frozen product.

6.2.Review of Literature

Several work have been carried out on the effect of treatments on quality changes of different species of squid and other marine fishes during ice-storage and frozen storage.

6.2.1. Effect of Sodium Salts

Sodium chloride treatment is widely used in cephalopod processing. The firmness of the meat was regained by the use of sodium salts, especially sodium chloride. Sodium salts play an anti-oxidative/anti-microbial role in the meat processing (Rhee et al., 1997). The sensory quality was found to be increased when treated with sodium ascorbate, lactate and phosphate (Kulashekthra and Rhee, 1996). Hennigar (1988) studied the effect of washing and sodium chloride concentration on mechanical properties of fish muscle gel. NaCl is required to solubilise myofibrillar protein, which then repolymerise to form protein network responsible for the gel (Sakai, 1981). Chang et al., (1995) studied the effect of sodium acetate on catfish fillet *(Ictalurus punctatus).* Matlock et al., (1984) studied the increasing water holding capacity of salt treated sample through extraction of salt soluble protein and the enhanced juiciness of cooked meat. The texture of cooked mantle of squid *(Illex argentinus)* as influenced by the specimen characteristics and treatments was studied by Ilona et al., (1987)

The effect of salt in the range of 0% to 5% on the textural property of the minced squid product was studied by Pan et al., (1979). He found that the maximal breaking force was found at a concentration of 3%-4% salt, and 2.5% salt is the maximum concentration to give maximal elasticity. Scanning Electrons Microscopy (SEM) showed a structure of thread like bundles on mixing with salt for 10 minutes and prolonged mixing resulted in a cross linking network. It was suggested that uniformity and compactness of the protein matrix, played an important role in textural quality of minced fish product.

6.2.2. Treatment with Mild acid

Treatment with 2% salt and 0.2% citric acid was found to improve the overall quality of cuttlefish fillet on frozen storage (Sophia and Sherief, 2003). The NPN fraction and alpha amino nitrogen content were found to be higher in treated sample when compared to control and were organoleptically in good conditions up to 8 weeks of frozen storage.

In addition to the use of acetic acid and acetates in anti-microbial preservation they are used as sequestrants, acidulants and flavouring agents. Spirit vinegar is made from distilled grain alcohol, which contained 4gms of acetic acid per 100ml (Desrosier, 1959). Six types of vinegar are recognised by USFDA and are cider, vine, malt, sugar, glucose and spirit vinegar (Furia, 1968). Acetic acid in the form of vinegar has been used in the food preservation since 5000B.C. (Lueck, 1980). The acid must penetrate the cell wall of microorganism and denature the protein. In order for acetic acid to accomplish this, it must be present in a concentration above 0.5% and acetic acid is 10 to 100 times powerful as preservative at a pH of 3.0 (Lueck, 1980). Gelation of shark myofibrillar proteins by weak organic acids was studied using acetic acid, citric acid, tartaric acid and hydrochloric acid, in which the water formed gel associated with an increase in viscosity was formed, when the pH was lowered to 4.5 using acetic acid (Venugopal, 1994). Venugopal et al., (1995) also reported that a thermo stable gel was prepared from shark myofibrillar protein by reducing the pH to 4.0 using acetic acid. Addition of 1.5 to 2.5 % of NaCl showed significantly higher gel strength as gelation enhancers (Gomez guillen, 1996). Drop wise addition of glacial acetic acid resulted in its slow thickening due to gelation dependent on viscosity increase. In this study the proteins were precipitated

on heating the dispersion either by increasing the pH to 6.0 or addition of salts (Venugopal et al., 1997). Venugopal et al., (2002) and Venugopal (2003) studied the physico chemical and rheological characterization of gel from shark treated with mild acid.

Anti microbial action of acetic acid on cut surface of apple slices was studied by Liao et al., (2003) and reported that washing with a mixture of acetic acid and H_2O_2 was most effective in removing salmonella from apple discs. Acetic acid is generally recognised as safe and has been approved for use as a food additive (FDA, 1982). Dickson (1992) reported that washing beef tissue with 2% acetic acid reduced the number of Salmonella by 0.5 to 0.8 logs. Similar effect of acetic acid on lamb carcass on reduction of bacterial count was also reported by Anderson et al., (1988). Treatment of fruits or seeds with acetic acid or vinegar has also been shown to be effective in reducing fungal decays and food borne pathogens (Shilberg and Gaunce, 1995; Parnell and Harris, 2003). The protection of quality loss by applying a fish protein glaze prepared by gelatin of fin protein by soaking in dilute acetic acid was studied by Kakatkar et al., (2004) and Smruti et al., (2004).

Ascorbic acid treatment inhibited lipid oxidation and preserved the desirable odour in meat products (Boles and Parrish, 1990, Kulshrestha and Rhee, 1996, Rhee et al., 1997). According to Selvaraj (1991), ascorbic acid treated squid (*Loligo sp*) sample was found to have improved quality and shelf life. Ascorbic acid treated sample developed no discolouration even after nine months of storing. Several studies showed that ascorbic acid treatment could improve the quality of seafood.

6.2.3. Treatment with Tripolyphosphates

Pre-dip treatments in sodium chloride (NaCl) or sodium tripolyphosphate (STPP) were found to reduce drip loss and maintain good quality of a number of species of fish during frozen storage (Kumta and Gore, 1970; Tanikava et al., 1963). Sawant and Patange (2002) investigated the usefulness of tripolyphosphates in enhancing the sensory qualities, and retarding progression of rancidity in frozen mussels. Polyphosphates also sequester transition metal ions such as copper and Iron. These metal ions accelerate lipid oxidation, which leads to premature flavour deterioration. Phosphates have anti-microbial effect due to their ability to chelate metal ions, essential for microbial cell division (Davidson and Juneja, 1990). Although STPP had anti-microbial activity in laboratory culture media, its effect on microorganisms in meat products has been less conclusive (Molins et al., 1984 and Venugopal et al., 1984). STPP have no anti-microbial effect in temperature abused frozen raw ground beef (Molins et al., 1987) and refrigerated raw pork containing salt (Choi et al., 1987). Sodium tripolyphosphate in meat products increased water holding capacity and juiciness (Matlock et al., 1984 and Molins et al., 1991), prevented oxidative rancidity development (Choi et al., 1987; and Stoick et al., 1991) and showed anti bacterial effect. When judiciously applied to seafood, phosphate bind the inherent juices and to produce a tender juicy product (Henson and Karen, 1992). According to the author, the thaw – drip was reduced considerably in seafoods when dipped for two minutes in 12% STPP solution.

6.2.4. Effect of Cooking

Since properties and reactions of proteins are involved in determining the textural quality of cooked squid meat, composition and properties of the cooking medium (pH, ion composition, etc.) would also be needed to be determined. The cooking medium should quickly gelatinise the tunics, which consists of connective tissues, and should prevent excessive moisture loss from the muscle fibres (Otwell and Hamann, 1979). According to these authors, squid should be cooked either very quickly, for example, by frying or sautéing for 2-3minutes, or be simmered in a stew or Studies showed that cooking yield was not always sensitive to small differences in WHC (Trout, 1988). Cooking losses can also be influenced by the shape and size of the meat product, temperature profile cooking rate and pH.

6.2.5. Effect of Pigmentation on quality

Yellow, orange and violet – purple chromatophores are present on the body of squid and the chromatophores expand and contract in response to the ambient temperature, there by altering the colour of the body while swimming (Sugiyama et al., 1980). The pigment is produced in the squid from tryptophan as the starting material, can be divided into two molecules ommin and ommatin, in which former is strong in alkali, and latter is weak in alkali (Vuillaume, 1969). The market value of squid is related to the contraction state of its epidermal chromatophores named omnochromes (Hinks, 1985). Improper storage and handling result in chromatophores disruption and red discolouration of the meat.

6.3.Materials and Methods

Fresh samples of the two species of squid *Loligo duvaucelii and Doryteuthis sibogae* (needle squid) were used for the study. The dressed mantles were divided into 8 batches. In this study six methods were compared in order to find out the best treatment method. Additives were mixed in specific ratio to get a best combination. The systems used were

a. Treatment control

b. Citric acid 0.3%

c. Citric acid 0.3% + Acetic acid 3%

d. Acetic acid 3%

e. STPP 3% +Acetic acid 3%

f. Ascorbic acid 0.3%

g. Lime Juice 3%

The above treatment media were mixed with NaCl (3% of the sample weight) and the sample was added along with ice in the ratio 1:2 and was kept at –1°C for 20 minutes with occasional stirring.

Immediately after the treatment, samples were kept in ice without direct contact for three days and analysed for TMA-N, TVN, alpha amino nitrogen, Total protein content, protein fractions and microbiological parameters. The treatment control contained no additives except NaCl. Fresh samples of both species of squids at zero day were also analysed for comparison of results. Samples were then taken for freezing and the biochemical, physico-chemical and organoleptic changes were studied for 120 days of frozen storage at an interval of 30 days.

Samples weighing approximately 2g were packed water tight in aluminium foil, and immersed in a constant temperature water bath and cooked at different temperatures (45°C, 50°C, 60°C, 70°C, 80°C, 90°C and 100°C) as per the method of Paul, et al., (1966). The time required for the samples to reach the temperature of the water bath averaged 3 minutes and afterwards heated for 1 minute. These samples were organoleptically assessed for optimum temperature, which retained the best qualities, by six trained panellists on the basis of colour, appearance, texture and flavour, using a 10-point hedonic scale. The pH of the samples were also checked. Treatment and analyses were done in triplicate.

6.3.1. Statistical analysis

Two-factor ANOVA was employed to compare the effect of each parameter like TMA-N, TVN, alpha amino nitrogen, leaching of protein, various fractions of proteins and TPC, between species and between treatments.

Mathematical model employed for the study was as follows:

$$X_{ij} = \mu + \alpha_i + \beta_j + E_{ij}$$

Where, Xij is the observed value corresponding to the i^{th} species on j^{th} day of storage. μ is the overall effect, α_i is the effect of i^{th} species and β_j is the effect of j^{th} day of storage and epsilon ij (E_{ij}) is the random error.

The comparison of parameters between species and treatment was done for treatment study; X_{ij} is the value obtained corresponding to i^{th} species on j^{th} treatment. μ is the overall effect, α_i is the effect of i^{th} species and β_j effect of jth treatment, E_{ij} random error.

6.4.Results

6.4.1. Total volatile bases

6.4.1.1. Total Volatile Nitrogen

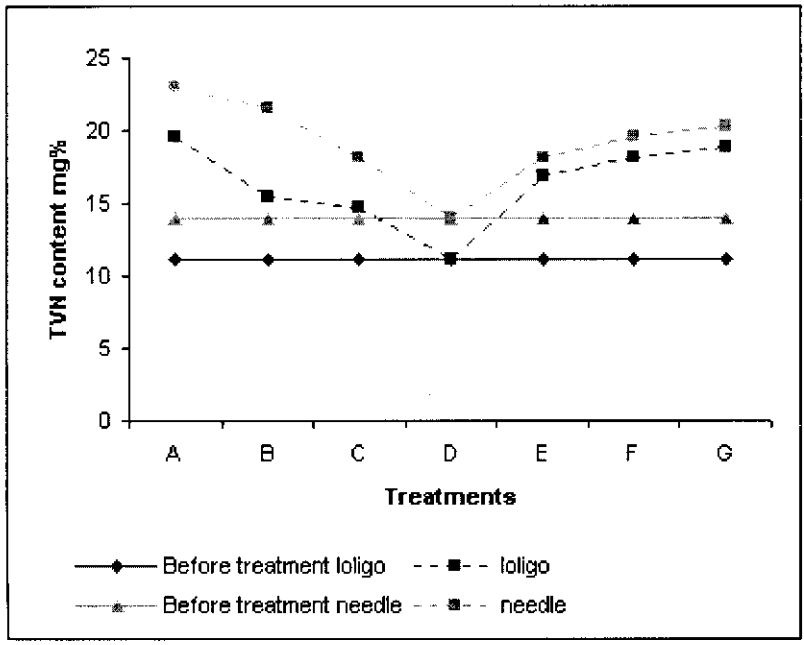


Figure 6.1.Effect of various treatments on TVN content

A-Control B-Citric acid C-Acetic acid citric acid mixture
D-Acetic acid alone E-STPP acetic acid mixture
F-Ascorbic acid G-Lime Juice

Figure 6.1 gives TVN content during various treatments where the trend was similar to TMA-N. Here also sample D showed the least content of TVN, (loligo 11.2mg% and needle 14mg%). The second best was sample C. Appendix F.1. revealed the ANOVA of TVN content. There was a significant difference ($p<0.001$) between both species and treatments.

6.4.1.2. Trimethylamine

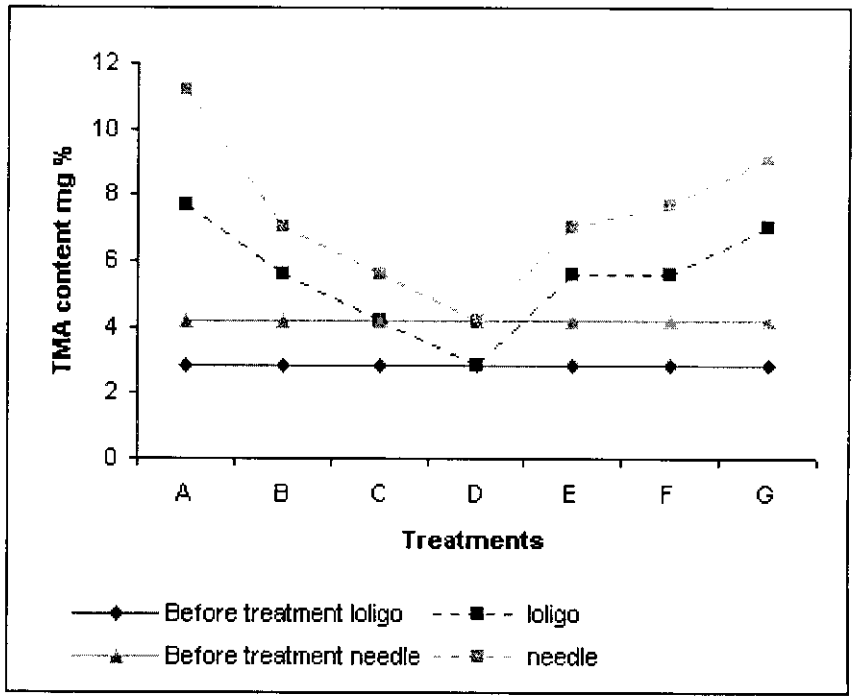


Figure 6.2. Effect of various treatments on TMA-N content

Figure 6.2 gives the TMA content during various treatments and ANOVA table of TMA changes is given in Appendix F.2. There was a significant difference between species and between treatments ($p<0.001$). Similar trend in the changes of TMA-N due to various treatments was observed in both the species, with needle showing slightly higher value than loligo. The TMA-N content for the control group (without treatment) reached a high value of 7.7mg% and11.2mg% in loligo and needle respectively. Minimum value was shown in the sample D, loligo showed 2.8mg% and needle showed 4.2mg%. All the samples were kept chilled with GMP. The next lower value of TMA was shown with sample C.

6.4.2. Alpha amino nitrogen

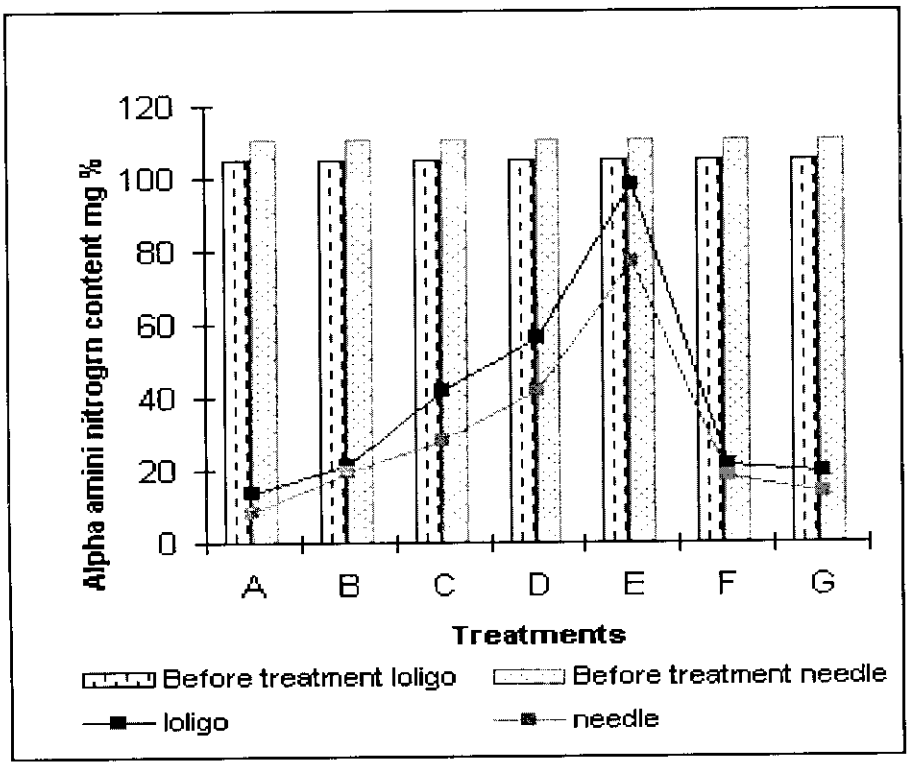


Figure 6.2.Effect of various treatments on alpha amino nitrogen content

Figure 6.3.shows the changes in alpha amino nitrogen content of both the species of squid with various treatments. Here the sample E showed the highest value of alpha amino nitrogen (loligo 98mg% and needle 77mg%). Sample D showed only 56mg% and 42mg% in loligo and needle squid respectively and among the treatments sample G showed the maximum leaching rate with alpha amino nitrogen content reaching 19.6mg% in loligo and14mg% in needle squid. In sample A, where there was no treatment, the value reached 14mg% and 8.4mg% for loligo and needle respectively. There was a significant difference (Appendix F.3.) between species ($p<0.01$) and treatments ($p<0.001$).

6.4.3. Protein loss

Table 6.1. % of protein retained during various treatments

	Needle					Loligo				
	1	2	3	4	5	1	2	3	4	5
B.T.	20.75	11.7	8.22	0.04	0.93	19.75	11.65	7.22	0.08	1.3
A	9.46	6.23	11.26	1.4	0.8	10.43	8.51	9.51	1.13	0.9
%	45.6	30.0	54.3	6.7	3.9	52.8	43.1	48.2	5.7	4.6
B	12.75	10.54	8.42	1.5	1.1	13.42	12.17	6.43	1.23	1.11
%	61.4	50.8	40.6	7.2	5.3	67.9	61.6	32.6	6.2	5.6
C	15.27	12.14	7.21	1.67	1.17	16.63	13.52	6.32	1.27	1.15
%	73.6	58.5	34.7	8.0	5.6	84.2	68.5	32.0	6.4	5.8
D	**15.97**	**13.22**	**5.97**	**1.7**	**1.18**	**17.44**	**15.71**	**3.85**	**1.4**	**1.2**
%	**77.0**	**63.7**	**28.8**	**8.2**	**5.7**	**88.3**	**79.5**	**19.5**	**7.1**	**6.1**
E	**16.83**	**15.83**	**3.43**	**1.8**	**1.14**	**18.91**	**16.67**	**3.27**	**1.45**	**1.13**
%	**81.1**	**76.3**	**16.5**	**8.7**	**5.5**	**95.7**	**84.4**	**16.6**	**7.3**	**5.7**
F	11.1	12.17	7.01	1.65	1.2	13.28	13.44	6.62	1.32	1.1
%	53.5	58.7	33.8	8.0	5.8	67.2	68.1	33.5	6.7	5.6
G	10.11	9.43	9.27	1.45	1.6	12.78	11.68	7.68	1.27	1.16
%	48.7	45.4	44.7	7.0	7.7	64.7	59.1	38.9	6.4	5.9

B.T. Before Treatment, 1- Total Protein, 2-Sarcoplasmic Protein,

3-Myofibrillar Protein, 4-Denatured protein, 5-Stroma Protein

Table 6.1. gives the leaching rate of the protein as a result of various treatments and the % of retained protein was calculated using the formula

$$\frac{\text{Protein content in each fraction}}{\text{Total protein (B.T.)}} \times 100$$

Sample E provided the best result in retaining the protein content (95% and 81% in loligo and needle squid). Sample D could hold 82% protein in loligo and 78% in needle squid. But in sample A, about 51% of the total protein was leached out in loligo and 54% in needle. Appendix F.4. shows that there was a significant difference between species and treatments ($p<0.001$). Between treatments, sample E showed a significantly higher value of protein while untreated sample showed the lowest value.

Maximum retention of sarcoplasmic protein was found in sample E followed by sample D. The LSD was calculated for treatments as 1.06. Appendix F.5. shows a significant difference in the sarcoplasmic protein between species and treatments ($p<0.001$). Among species, needle showed a higher rate of leaching when compared to loligo.

Here the sample E showed the least extractability of myofibrillar protein followed by sample D. Appendix F.7.6. shows there was significant difference myofibrillar proteins extractability between species and treatments ($p<0.001$).

Maximum denatured protein was found in sample E. Denaturation was minimum in control (zero time). Sample D also showed high level of denatured protein. Appendix F.7. shows a significant difference of denatured proteins between species and treatments ($p<0.001$).

With regard to stroma proteins, sample E in loligo showed maximum retention, while in needle squid sample D showed the maximum retention. There was no significant difference between species; while between treatments there was significant difference ($p<0.001$, Appendix F.8.). Minimum value of stroma protein was found in treatment control (A).

6.4.4. Bacteriology

Table 6.2.Changes in total plate count during various treatments.

	Loligo	Needle
A	$1.79X10^5$	$2.2X10^5$
B	$2.8X10^4$	$3.3X10^4$
C	$1.2X10^4$	$1.5X10^4$
D	$1.5X10^4$	$1.7X10^4$
E	$1.8X10^4$	$1.9X10^4$
F	$1.75X10^4$	$2.01X10^4$
G	$2X10^4$	$2.4X10^4$

Table 6.2. shows changes in Total Plate Count (TPC) in samples after various treatments. Here sample C showed maximum reduction in the bacterial count. The second best treatment system was sample D alone, which could reduce the bacterial count to 10%. Appendix F.9. gave significant difference between species and treatments ($p<0.001$).

6.4.5. Organoleptic quality

Table 6.3. Organoleptic Quality changes and pH during various treatment systems

		pH	washed	Flavour	colour	Texture	Odour	grade
Loligo	A	7	7.3	P	Pink	Flabby	P	4
Needle	A'	7.3	7.5	P	Pink	Flabby	P	3
Loligo	B	6	6	G	W	Soft	G	7
Needle	B'	6.3	6.3	G	W	Soft	G	7
Loligo	C	4	4.4	G	W	Soft-Firm	G	6
Needle	C'	4.2	4.5	G	W	Soft-Firm	G	6
Loligo	D	4.6	4.8	E	W	Soft-Firm	E	8
Needle	D'	4.8	5.1	E	W	Soft-Firm	E	8
Loligo	E	4.9	5.3	S	S P	Soft	S	6.5
Needle	E'	6	6.2	S	S P	Soft	S	6.5
Loligo	F	6.4	6.7	P	S P	Soft	P	5
Needle	F'	6.8	7	P	S P	Flabby	P	4
Loligo	G	6.5	6.8	P	S P	Flabby	P	4.5
Needle	G'	7	7.3	P	S P	Flabby	P	3.5

Flavour/Odour: P-Poor, G-Good, E-Excellent, S-Satisfactory
Colour: W-White, SP-Slight Pink

Table 6.3. describes the organoleptic quality and the pH of the mantle after various treatment systems. The pH of sample D was about 4.6 to 4.8 whereas in the treatment media pH was 4.2. In the case of sample C the pH was below 4 and developed a severe sour taste creating an unpleasant flavour when cooked.

In table 6.4. the organoleptic quality of both the species in cooking at various temperatures is shown. The sample E was very tough and rubbery after cooking. The cooking temperature was optimised for 1 minute at 70°C. The temperature more than 70°C gave a yellowish colour to the product and a slightly tougher texture, which in turn gave the panel members a dislike while tasting it.

6.4.5.1. Cooking

Table 6.4. Organoleptic Quality changes and pH during various treatment systems after cooking at different temperatures

		45°C	50°C	60°C	70°C	80°C	90°C	100°C
Loligo	A	5	5	5	6	4	3	3
Needle	A'	5	5	5	5.5	4	3	3
Loligo	B	6	6.5	7	7.5	7	7	6.5
Needle	B'	6	6.5	7	7.5	7	7	6.5
Loligo	C	6	6.5	6.5	7.5	7	7	6
Needle	C'	6	6.5	6.5	7.8	7.5	7	6.5
Loligo	D	6.5	7	7.5	8	7.5	7.5	7
Needle	D'	6.5	7	7.5	8	7.5	7.5	7
Loligo	E	4.5	5.5	5	6	5	4	4
Needle	E'	4.5	5	5	6	4.5	3	3
Loligo	F	5	5.5	5.5	6	5.5	5.5	5
Needle	F'	5	5.5	5.5	6	5.5	5.5	5
Loligo	G	5	5.5	5	6.2	4.5	4	4
Needle	G'	5	5	5	5.8	4.5	3	3

The sample D cooked at 70°C gave a juicy, soft and firm texture and a mixed sweet and sour taste. This was ranked best by all the panel members. The needle squid developed a pink discolouration on sample E and was organolepticaly graded unacceptable.

6.4.6. Frozen Storage

6.4.6.1. Total volatile nitrogen

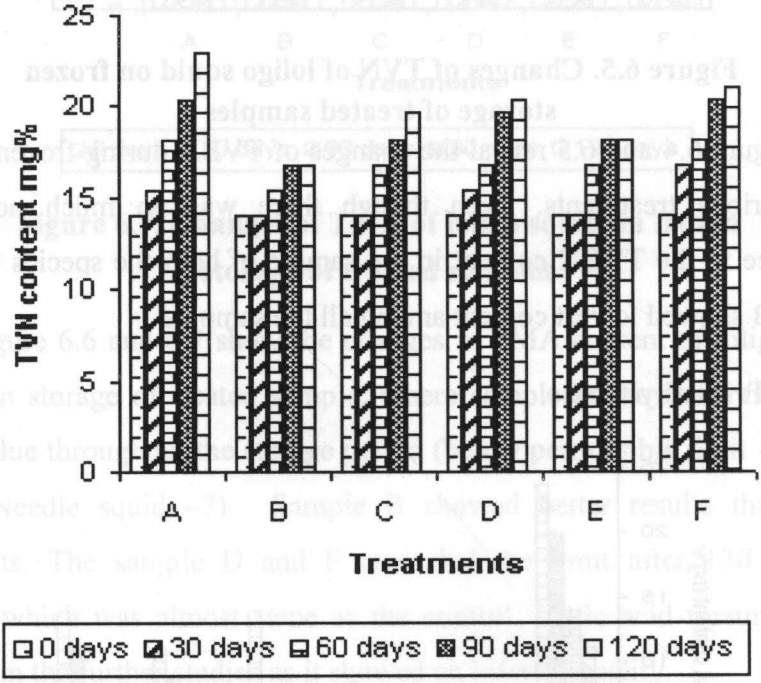


Figure 6.4. Changes of TVN of needle squid on frozen storage of treated samples

A-Control		C-Acetic acid citric acid mixture
B-Acetic acid alone		D-STPP acetic acid mixture
E-Ascorbic acid		F-Lime Juice

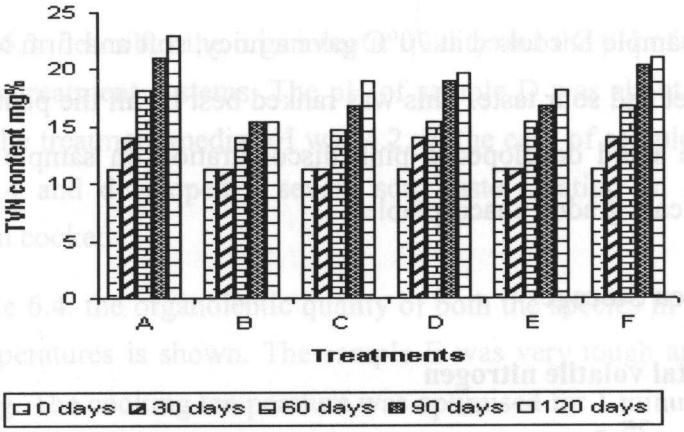


Figure 6.5. Changes of TVN of loligo squid on frozen storage of treated samples

Figure 6.4 and 6.5 reveal the changes of TVBN during frozen storage after various treatments. Even though there was no much noticeable difference in the TVBN content in the sample of both the species of squid sample B showed lowest content among all the samples.

6.4.6.2. Trimethylamine

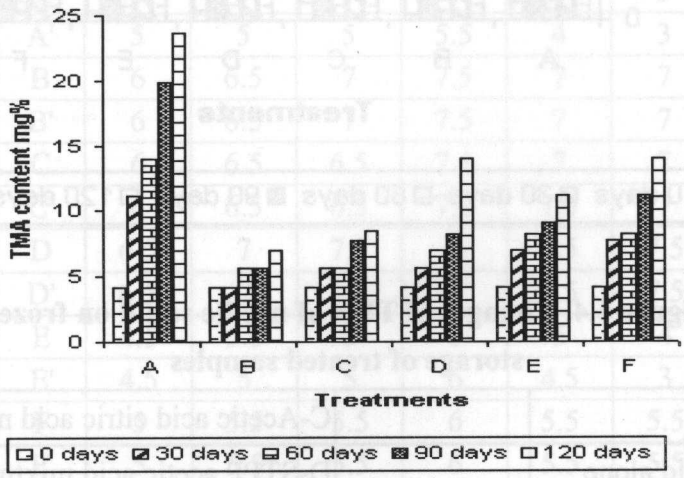


Figure 6.6. Changes of TMA of needle squid on frozen storage of treated samples

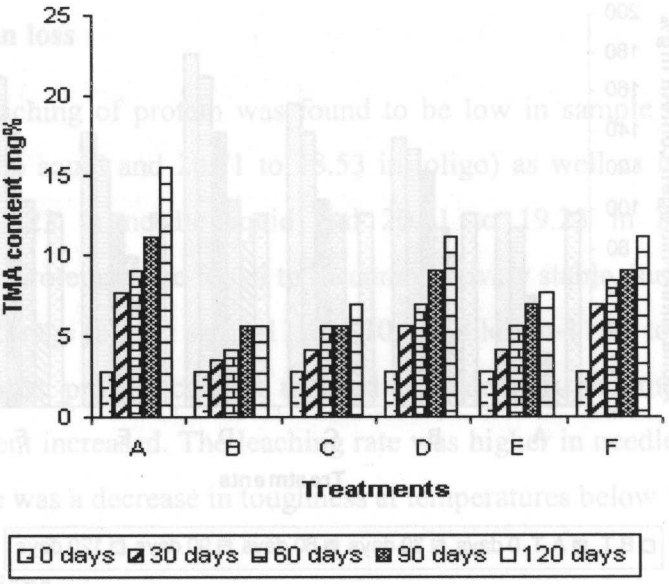


Figure 6.7. Changes of TMA of loligo squid on frozen storage of treated samples

Figure 6.6 and 6.7 show the changes in TMA content of loligo squid on frozen storage of treated sample, where sample B showed a minimum TMA value throughout the storage period (below permissible limit -.Loligo-5.6 & Needle squid –7). Sample B showed better results than other treatments. The sample D and F exceeded the limit after 120 days of storage, which was almost same as the control. Citric acid treatment was avoided in the further studies as it showed an inferior quality.

6.4.7. Alpha amino nitrogen

Alpha amino nitrogen content showed a maximum rate (180 in needle and 140 in loligo) in the sample D, while sample A showed maximum rate of leaching during treatments (Figure 6.8. and 6.9).

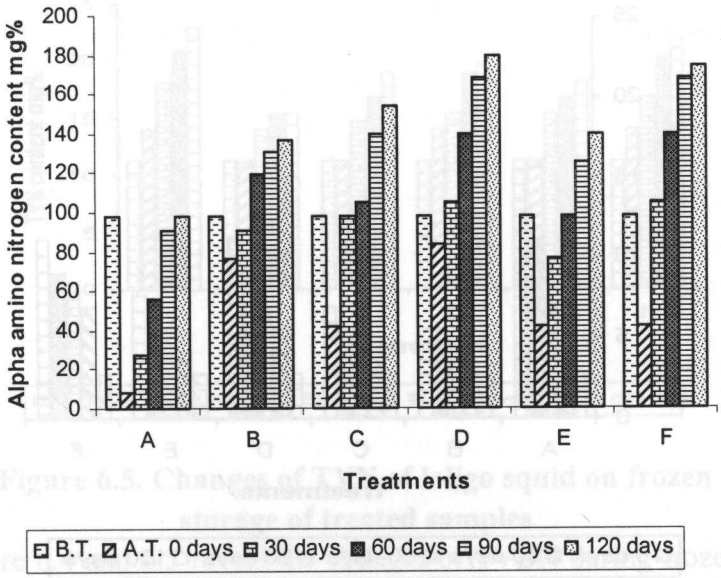


Figure 6.8. Changes of alpha amino nitrogen of needle squid on frozen storage of treated samples

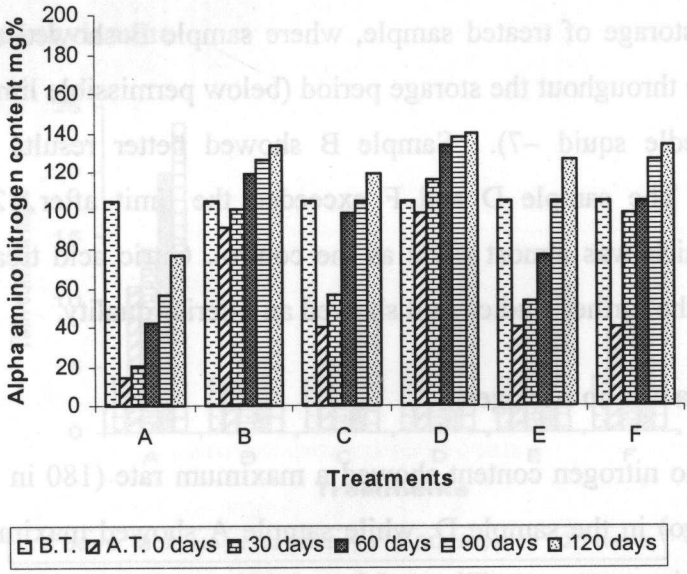


Figure 6.9. Changes of alpha amino nitrogen of loligo squid on frozen storage of treated samples

6.4.8. Protein loss

The leaching of protein was found to be low in sample B (19.71 to 17.2 in needle squid and 20.71 to 18.53 in loligo) as well as in sample D (19.71 to 18.23 in needle squid and 20.71 to 19.23 in loligo). The sarcoplasmic proteins were found to be comparatively stable during freezing and frozen storage (needle squid-11.4 to 10.4 and loligo-11.91 to 10.32), but the myofibrillar protein content reduced considerably and the denatured protein content increased. The leaching rate was higher in needle squid than loligo. There was a decrease in toughness at temperatures below 100°C

6.5.Discussions

(Otwell & Hamann, 1979b) and heating at 60-80°C could result in the softening of the meat and in retaining the juiciness of the product. Such temperatures are widely applied in cooking tender muscles to avoid severe toughness (Bykowski & Kolodziejski, 1985). These findings agree with the results of this study, in which cooking temperature of 70°C produced products with highest organoleptic score. According to Stanley and Hultin (1982) significant changes in texture of the squid mantle took place only during the first 8 minutes of the cooking. The cooking times in excess of five minutes would limit the advantage of heat tenderisation by decreasing the mantle moisture (Otwell and Hamann, 1979a). Otwell and Hamann (1979b) suggested the application of a cooked medium best suited to rapidly gelatinise the tunics of connective tissue in squid to produce tender product. The pH of rabbit muscle tissue increased while cooking. The tenderness of the meat between 65°C -75°C was mainly due to the changes in myofibrillar protein (Paul et al., 1966). The squid (*Loligo duvaucelii*) treated with 0.5%

ascorbic acid for 10 minutes found to improve the quality and shelf life when compared to the control (Selvaraj et al., 1991). The thaw drip increased gradually on cooking, which may be due to the protein denaturation and consequent decrease in water holding capacity (Joseph et al., 1985). Sodium acetate treated sample of cat fish fillet resembled the order and appearance of the fresh fillet up to six days and, could reduce the bacterial load to a great extent (Chang et al., (1995). Effect of NaCl in the range 0.1% to 5% on the textural property of minced squid meat showed that 2.5% gave the maximum elasticity and, prolonged mixing resulted in a cross linking network, Uniformity and compactness of protein matrix played an important role in the textural quality.

The findings of this study confirms that a dip treatment with dilute acetic acid could retain the sarcoplasmic and myofibrillar fractions of proteins in both loligo and needle squid which otherwise get leached out (control). On cooking also, acetic acid treated samples were organoleptically ranked highest.

Venugopal et al., (1997) prepared a stable gel from shark myofibrillar proteins by reducing pH to 4.0 by acetic acid. The stability of the protein in dispersion was dependent on the pH. In the presence of acetic acid, the positively charged protein molecules may repel among themselves and cause solublisation, to form a stable gel without any aggregation and precipitation. In the presence of acetic acid, myosin heavy chain could breakdown with the formation of a fragment having molecular weight 160kDa (Chawla et al., 1995). The repulsion between protein molecules at low pH can be so strong as to hold the proteins in solution even at high

temperatures of cooking. The microbial count in the acetic acid treated sample was considerably lowered due to its antimicrobial action.

Tripolyphosphates act on skeletal muscle proteins and can split actomysoin complex into extract myosin. Extracted myosin binds water and this helps to retain water-soluble proteins, minerals and vitamins and natural juices of seafood. Thus, this imparts a favourable effect in the texture and flavour. Here though STPP acetic acid mixture treatment showed the highest retention of proteins, cooking resulted in the development of a rubbery, gelled texture. Also, STPP acetic acid mixture treatment develops a pink discolouration in needle squid on storage. This may be due to the structural deterioration of the Omnochrome membrane in alkaline pH leading to bleeding of pigments, which downgrades the quality of the product. It is interesting that this type of discolouration was not prominent in loligo species during STPP treatment. Compared to the other four treatments used in this study namely citric acid, acetic acid + citric acid, ascorbic acid and limejuice which are being commonly used in the seafood industry, the Acetic acid treatment is most acceptable for extending the quality of chilled stored mantle tissue before going for freezing.

6.6. Conclusion

Due to highly perishable nature of squid and squid products, various processes have been developed for shelf life extension of the mantle tissue. Chilling alone has limitation in extension of shelf life. But combination of chilling with other treatments with permitted food additives, as single or in mixtures, has been applied to further augment the shelf life. Treatment with acetic acid showed the best chemical and microbial characteristics, like high

retention of total proteins, sarcoplasmic proteins and myofibrillar proteins, in both the species of squid. On cooking at 70°C, the acetic acid treated mantles showed best organoleptical quality while STPP treated samples developed a rubbery gelled texture. Even though STPP treated samples showed maximum protein retention, during storage they developed a pink discolouration in needle squid. Compared to the other four treatments namely citric acid, citric acid + acetic acid, ascorbic acid and lime juice, which are being used in the seafood industry, the acetic acid treatment is most acceptable for extending the quality of chilled stored mantle tissue before going for freezing. Other aspects of the treatment like tissue protease activity, lysosomal activity and pattern of protein by SDS-PAGE will be dealt in detail in the proceeding Chapters.

6.7. References

Anderson, M.E., Huff, H.E., Naumann, H.D. and Marshall, R.T., (1988) Counts of six types of bacteria in limits carcasses dipped or sprayed with acetic acid at 25°C or 55°C and stored vacuum packaged at 0°C. J. Fd. Prot. **51**, 874-877.

Boles, J.A., Parrish, F.C. Jr., (1990) Sensory and chemical characteristics of pre-cooked microwave reheatable pork roasts. J.Fd. Sci. **55**, 618-620.

Bykowski, P and Kolodziejska, W., (1985) Chemical composition and quality of frozen roller-peeled krill meat. Intl. Inst. Refrig. Commissions C_2 and D_3, 257, Aberdeen, U.K.

Chang, R.K., James, O.H., Amy, P.V., Charles, H.W. and Douglas, L.M., (1995) Sodium acetate and bifidobacteria increase shelf life of refrigerated catfish fillet. . J. Fd. Sci. **60** (1), 25-27.

Chawla, S.P., Venugopal, V. and Nair, P.M., (1995) Gelation of proteins from washed muscles of threadfin bream under mild acidic conditions. J. Fd. Sci. **61**, 362-366.

Choi, Y.I. Kastner, C.L and Kropf, D.H., (1987) Effect of hot boning and various levels of salt and phosphates on microbial, TBA and pH value of pre blended pork during cooler storage. J. Fd. Prot. **50**, 1037- 1040.

Davidson, P.M. and Juneja, V., (1990) Antimicrobial agents. Ch.4 in food additives. (Eds.A.L. Branen, P.M. Davidson and S. Saliminan). 83-138. Marcel Dekker Inc. New York.

*Desrosier, N.W., (1959) The technology of food preservation. AVI, Westport, CT.

Dickson, J.S., (1992) Acetic acid action on beef tissue surfaces contaminated with salmonella typhimurium. J. Fd. Sci. **57**, 297-301.

FDA, (1982) Food and Drug administration. GRAS status of acetic acid, ammonium acetate, Sodium acetate and Sodium diacetate. Fed. Reg. **47**, 27813.

*Furia, T.E., (1968) Handbook of food additives. (Ed. Furia) The chemical rubber company, Cleveland, OH.

Gomez-Guillen, M.C., Hurtado, J.L. and Montero, P., (2002) Autolysis and protease inhibition effects on dynamic viscoelastic properties during thermal gelation of squid muscle. J. Fd. Sci. **67**, 2491-2495.

Gomez-Guillen, M.C., Solas T. Borderias, J.and Montero, P., (1996) Ultrastructural rheological changes during the gelation of giant squid (*Dosidicus gigas)* muscle. Z.Lebensm Unters Forsch 202, 215-220.

Hennigar, C.J., Buck, E.M. Hultin, H.O., Peleg, M. and Vareltzis, K., (1988) Effect of washing and sodium chloride on mechanical properties of fish muscle gel. . J. Fd. Sci. **53** (3), 963-964.

Hensen, S.L. and Karen, M.K., (1992) Use of phosphate in seafood. Infofish Intl. 5/92, Sep/Oct, 50-54.

Hinks, M.J. and Stanley, D.W., (1985) Colour measurement of the squid (*Illex illecebrosus*) and its relationship to quality and chromatophore structure. Canadian Inst. Fd. Sci. and Technol. J. **18** (3), 233-241.

Illona Kolodziejska, Zdzislaw, E. Sikorski and Maria Sadowska, (1987) Texture of cooked mantle of squid *Illex argentinus* as influenced by specimen characteristics and treatments. J. Fd. Sci. **52**, 4.

Joseph, J., Perigreen, P.A. and Nair, M.R., (1985) Paper presented at the meeting of International Institute of Refrigeration Commission C2 and D3 on storage lives of chilled and frozen fish and fish products. University of Aberdeen, Scotland.

Kakatkar, A.S., Sreekar, S.V. and Venugopal, V., (2004) Fish protein dispersion as a coating to prevent quality loss in processed fishing products. Fish. Technol. **41**, 29-36.

Kulashrestha, S.A. and Rhee, K.S., (1996) Precooked reduced – fat beef patties chemical and sensory qualities as affected by sodium ascorbate, lactate and phosphate. J. Fd. Sci. **61**, (5) 1053- 1057.

*Kumta, U.S. and Gore, M.S., (1980) J. Fd. Sci. **36**, 456.

Liao, C.H., Shollenberger, L.M. and Phillip, J.G., (2003) Lethal and sub lethal action of acetic acid in salmonella invitro and on cut surfaces of a apple slices. J. Fd. Sci. **68**, 2793-2798.

*Lueck, E., (1980) Antimicrobial food additives: Characteristics, uses, effects. Springer-Verlag, New York, 280.

Matlock, R.G., Terrell, R.L., Sawal, J.W., Rhee, K.S. and Dutson, T.R., (1984) Factors affecting properties raw-frozen pork sausage patties made with various NaCl / Phosphate combination. J.Fd. Sci. **49**, 1363-1366,1371.

Molins, R.A. Kraft, A.A. Olson, D.G. and Hotchkiss, D.K., (1984) Recovery of selected bacteria in media containing 0.5 % food grade poly and pyro phosphates. J. Fd. Sci. **49**, 948-949.

Molins, R.A. Kraft, A.A. Olson, D.G. and Walker, H.W., Rust, R.E. and Mekenich, K., (1987) Effect of Inorganic polyphosphates on

ground beef characteristics. Microbiological effects on frozen beef patties. . J. Fd. Sci. **52**, 46-49.

Otwell, S.W. and Hamann, D.D., (1979a) Textural characterization of squid scanning electron microscopy of cooked mantle. J. Fd. Sci. **44**, 1629

Otwell, S.W. and Hamann, D.D., (1979b) Textural characterization of squid (*Loligo pealei, Leseur*) instrumental and panel evaluation. J. Fd. Sci. **44** (6), 1636-1643.

Pan G.S., Lee, D.J. and Lin, L.P., (1979) Studies in a minced squid product. Effect of raw material and ingredients on the texture of the product. Advances in Fish Science and Technology. Ed. Connell, J.J., Fishing News Book, London. 232-236.

Parnell, T.L. and Harris, L.J., (2002) Reducing salmonella on apples with wash practices. Commonly used by consumers. J. Fd. Prot. **66**, 741-747.

Paul C. Pauline, Lis Buchter and Annemarie Wierenga, (1966) Solubility of rabbit muscle proteins after various Time-temperature Treatments. J. Agri. Fd. Chem. Vol. **14** (5), 490-492.

Rhee, K.S., Krahl, L.M., Lucia, L.M. and Acuff, G.R., (1997) Anti-oxidative / Anti-microbial effects and TBARS in Aerobically Refrigerated Beef As Related to Microbial Growth. J.Fd. Sci. **62** (6) 1205-1210.

Sakai, J.S. and Matsumotto, J.J., (1980) Proteolytic enzymes of squid mantle muscle. J. Comp. Biochem. Physiol. Vol. **68B**, 389-395.

Sawant, S.S. and Patange, S.B., (2002) Frozen storage characteristics of treated and untreated meat from male and female mussel (*Perna virdis*). Fish. Technol. **39** (1), 27-33.

Selvaraj, P., Jasmine, J.I. and Jeyachandran, P., (1991) Effect of ascorbic acid dip treatment on frozen storage of squid (*Loligo duvaucelii, Oribigny*) Fish. Technol. **2**, 117-121.

Sholberg, P.L. and Gaunce, A.P., (1995) Fumigation of fruit with acetic acid to prevent post-harvest decay. Hortscience **30**, 1271-1275.

Smruti, K., Rai, S., Yardi, V., Warrier, S.B. and Venugopal, V., (2004) Shelf life enhancement of hard head catfish patties making use of acetic acid induced gelation of the fish proteins. Fish. Technol. **41**, 121-126.

Sophia Margaret Joseph and Sherief, P.M., (2003) Effect of Treatments on the Iced storage shelf life of cuttlefish (*Sepia aculeate*) fillets. Fish.Tech. **40** (1), 32-35.

Stanley, D.W. and Hultin, H.O., (1982) Quality factors in cooked North Atlantic squid. Can. Inst. Fd. Sci. Technol. J. **15**, 277.

Stoick, S.M. Gray, J.I., Burren, A.M. and Bucklie, D.J., (1991) Oxidative stability of restructured of beef steaks processed with oleoresin rose mary, tertiary butylhydroquinone and sodium tripoly phosphate. J. Fd. Sci. **56**, 597-600.

Sugiyama, M., Kousu, S., Hanabe, M., Okuda, Y., (1980) Utilization of squid. Oxonian Press Pvt. Ltd. Calcutta.

Suzuki, T., (1981) Fish and Krill Processing Technology, London. Applied Science Publishers.

Tanikawa, E., Akiba, M. and Shitamori, A., (1963) Fd. Technol. **17**, 1425.

Trout, G.R., (1988) Techniques for measuring water-binding capacity in muscle foods- A review of methodology. Meat Sci. 235-252.

Venugopal, V., (1997) Functionality and Potential Application of Thermo Stable Water Dispersion of Fish Meat. Trend Fd. Sci. Technol. **8**, 271-276.

Venugopal, V., (2003) Gel formation of fish structural proteins under mild acidic conditions: comparison with conventional surimi gelation and applications. Fish. Technol. **40** (2), 61-70.

Venugopal, V. Doke, S.N., Nair, P.M., (1994). Gelation of shark myofibrillar proteins by weak organic acids. J.Fd. Chem. **50**, 185-190.

Venugopal, V., Kakatkar, A. Bongirwar, D.R., Karthikeyan, M., Mathew, S. and Shamasunder, B.A., (2002) Gelation of Shark Meat Under Mild Acid Condition: Physico-Chemical and Rheological Characterisation of the Gel. J. Fd. Sci. **67**, 2681-2686.

Venugopal, V., Martin, A.M. Patel, T.R., Vasanthan, T., and Omar, S., (1995) Rheological and Solubility Characterisation of Washed Capelin (*Mallotus villosus*) mince in Water. J.Fd. Biochem. **19**, 175-190.

*Vuillaume, M., (1969) Less pigments des invertebres. Masson and cie, Paris, 81-88.

*Not Referred in Original

Chapter 7

CHARACTERIZATION OF SOLUBLE PROTEINS USING SDS - POLYACRYLAMIDE GEL ELECTROPHORESIS

7.1.Introduction

Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) is probably the most widely used technique for analyzing mixtures of proteins with a high resolving power. With SDS, an ionic detergent, proteins loose their individual charges and a net negative charge is formed due to complexing of SDS with protein. The update of detergent is the same per unit mass (1.4g SDS per gm of protein) for all proteins and hence the mobility on electrophoresis is proportional to the molecular mass. Depending upon the size of the proteins to be separated, the concentration of acrylamide is selected to optimize the sieving effect. In addition, a stacking gel of low acrylamide concentration allows rapid movement of the sample to the top of the separating gel in the form of a narrow zone of proteins.

From the previous Chapters, significant differences in the various fractions of proteins in the two species of squid under study, during different methods of preservation were reported. In this study, a detailed investigation of the electrophoretic protein profile (SDS-PAGE) in samples during iced, chilled and frozen storage and after different treatments is attempted. The loss in protein functionality can be attributed to proteolytic degradation, loss of proteins by leaching or protein denaturation, leading to aggregation of proteins.

7.2. Review of literature

The electrophoretic pattern of squid *Ommastrephes sloani pacificus* actomyosin and myosin were carried out by several workers Matsumotto (1959), Tsuchiya et al., (1978). The reliability of molecular weight determination by SDS –PAGE was studied by Weber and Osborn (1969). According to Connell (1965), the cold stored cod fillet cod proteins

dissolved easily in 1% SDS, but the technique of room temperature solubilization overnight was found unsuitable due to bacterial growth. Analysis of protein by Sodium dodecyl sulphate (SDS) Polyacrylamide gel electrophoresis on squid *Ommastrephes sloani pacificus* was done by Sakai and Matsumotto (1981), where the whole extract of the squid mantle was incubated at various pH for the study. The basic experimental details were followed according to Laemmli (1970). The analysis of salt soluble protein fraction of cod muscle by gel electrophoresis was done by Ohnishi and Rodger (1979). Connell et al., (1978) studied the changes in proteins during frozen storage of cod meat as detected by SDS-PAGE.

Mackie (1979) has reviewed some recent application of electrophoresis and iso-electric focusing in the identification of species of fish and fish products. It is reasonable to claim that the various electrophoretic techniques are the most reliable method for identifying the species (Mackie, 1969; Morel, 1977). Hume and Mackie, (1979) investigated the use of electrophoresis of water soluble muscle proteins in the quantitative analysis of the species components of a fish mince mixture. Attempts have been made to apply quantitatively, to the detection and estimation of Soy bean proteins in cooked meat products (Penny and Hofmann, 1971; Llewellyn and Flaherty, 1976). The electrophoretic study to identify cod and saithe from a mixture was carried out by Mackie (1979). Ohnishi and Rodger (1979) have studied the effect of formaldehyde at different ionic strength on the salt soluble proteins of fish muscle using PAGE.

The electrophoretic profile of squid meat showed three major bands of myosin heavy chain (MHC), paramyosin and globular actin having molecular weights 200, 95 and 42 kDa respectively (Collignan and Montet,

1998). The disappearance of myosin heavy chain was observed in mild acid treated samples of surimi (Venugopal, 2003). According to this study, the acid-induced gelation of threadfin bream resulted in disappearance of MHC and appearance of a protein band of about 186 kDa.

Solubility of the gel in various solvents containing SDS, urea and β-mercapto-ethanol suggested the structural changes in the proteins during gelation. Westermeier (1982) has explained the various types of electrophoretic method in practice. Many authors have explained the various problems and the remedies for trouble shooting in SDS PAGE (Smith, 1994; Walker, 1994).

7.3. Materials and Methods

7.3.1. Sampling method

The samples selected include *Loligo duaucelii* and *Doryteuthis sibogae* kept under different conditions.

A. Fresh samples- Extraction carried out with different extraction media
   - Distilled water,
   - Borate Buffer 0.2 M, pH 7.5
   - Phosphate Buffer 0.2M, pH 6.5.

B. Samples under ice stage by three methods (Chapter 3- 3.3).
   - With GMP and with out direct contact with ice and water.
   - With GMP
   - Without GMP

Sampling was done at first day and eighth day of ice storage.

C. Samples under super cooling by two methods (Chapter 4- 4.3.1.)

D. Samples at various processing steps (Chapter 4- 4.3)

- Before treatment.
- After treatment.
- Before freezing.
- After freezing.
- After 24 hours frozen storage.

E. Samples under frozen storage (Chapter 5 –5.3). sampling done at

- 30 days
- 60 days
- 90 days
- 120 days
- 180 days of storage.

F. Samples after various treatments (Chapter 6- 6.3)

- Treatment Control
- Acetic acid
- Acetic acid +citric acid
- Acetic acid + STPP
- Ascorbic acid
- Lime Juice

7.3.2. Preparation of aqueous extract

Sample (1g) was homogenized in a high-speed homogenizer with 20 volumes of cold distilled water at 4°C. After centrifugation of the homogenate at 5000 X G for 20 minutes, the supernatant was diluted in the ratio 1:4 with the sample buffer containing SDS (anionic detergent),

Mercaptoethanol, bromophenol blue dye and glycerol and heated at 100°C for 1 minute.

SDS –PAGE was performed by the method of Laemelii (1970) using 10% separating gel and 4% stacking poly acrylamide gel. Gels were stained with Coommassie brilliant blue R-250. The detailed procedure is as given below.

7.3.3.Reagents and gel preparation of SDS – PAGE

- **Stock solutions**

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

87.6gms acrylamide (29.2g/100ml)

2.4gof N'N'-bis-methylene-acrylamide (0.8g/100ml)

Made up to 300ml of solution with deionised water. Filtered and stored at 4^0C in dark.

b. 1.5M Tris – HCl buffer (pH 8.8)

27.23g of Tris base (18.15g/100ml) in 80ml of deionized water

Adjusted to pH 8.8 with 6N HCl. Made up to 150ml with deionised water and stored at 4^0C.

c. 0.5M Tris HCl pH 6.8

6g Tris base in 60ml-deionised water

Adjusted to pH 6.8 with 6N HCl. Made up to 200ml with deionised water and stored at 4^0C.

d. 10% SDS.

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

e. Sample buffer (SDS reducing buffer). Mix the following and stored at room temperature

Deionised water	3.8 ml
0.5M Tris-HCl, pH 6.8	1.0 ml
Glycerol	0.8 ml
10% (w/v) SDS	1.6 ml
2-mercaptoethanol	0.4 ml
1% (w/v) bromophenol blue	0.4 ml

Diluted the sample with the sample buffer in the ration1:4 and heated at 95^0C for 4 minutes.

f. 5 X electrode (running buffer) pH 8.3

Tris base	9.0 g
Glycine	43.2 g
SDS	3.0 g

Dissolved in 600ml of deionised water.

Stored at 4^0C. Warmed to room temperature before use if precipitation occurs. Diluted 60ml 5 X stock with 240ml deionised water.

➢ **Separating gel preparation**

During this study, the gel strengths 7.5%, 10% and 12% were tried and 10% gel was accepted for the best results.

Deionised water	4.1ml
1.5M Tris HCl buffer (pH8.8)	2.5ml
10% SDS stock	100µl
Acrylamide/ Bis (30% stock)	3.25ml

(This mixture was degassed for 15 minutes at room temperature)

10% ammonium per sulphate (fresh daily)	50µl
TEMED (NNN'N'-Tetramethylethylenediamine)	5µl

- **Stacking gel preparation (4%)**

Deionised water	6.1 ml
1.5M Tris HCl (pH8.8)	2.5 ml
10% SDS stock	100.0 µl
Acrylamide /Bis (30% stock)	1.33 ml
(This mixture was degassed for 15 minutes at room temperature)	
10% ammonium per sulphate (fresh daily)	50µl
TEMED	10µl

- **Destaining solvent**

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

7.3.4.Gel Preparation

The acrylamide mixture was polymerized in glass tubes usually with an internal diameter of 5mm and 7-10cm long. The bottom ends of the tubes were temporarily sealed before introducing the gel solution usually. The tubes were put vertically into a rack and separating gel solution was introduced into each tube with a syringe or pasteur pipette. Within 5 minutes the top layer was sealed with water. This water level reduces the surface tension forces at the top and gives a flat surface. After polymerization of separating gel, a small amount of stacking gel was added to the top and sealed with water as explained above. The gel tube were inserted into the grommets of the upper buffer tank and placed over the lower buffer tank containing the electrode buffer. Electrode buffer was also added to the upper buffer tank.

50µl of the sample was applied to the top of the gel tube using syringe or pasteur pipette. Upper buffer tank was closed with a lid and electrical connections were made. The power pack was adjusted to pass current at 3mA/gel tube for half the run. Then the power was increased so as to get 4mA/tube till the end of the run denoted by the tracker dye. The gel from each tube was removed after switching off the power.

Before staining, gel was prefixed with 10% TCA for half an hour (Andrews, 1986). Then the gel was transferred to the staining solution of Coomassie blue R250 in ethanol and water mixture. The staining process was being carried out overnight and the gels were transferred into destaining solution. Photographs of the electropherograms were taken.

PAGE of whole extract (WE) of fresh raw sample of both the species was also carried out simultaneously for comparison. Two standards (Broad range and Short range) as protein markers were also run simultaneously to compare the molecular weights.

Protein markers used for the study

Sl.No.	Name of the protein	Molecular weight kDa
1.	Myosin	205
2.	Phosporylase b	97.4
3.	BSA Bovine serum albumin	66
4.	Ovalbumin	43
5.	Carbonic anhydrase	29
6.	Soybean trypsin inhibitor	20.1
7.	Lysozyme	14.3
8.	Aprotinin	6.5
9.	Insulin	3.0

7.4. Results

A preliminary experiment was conducted to investigate the protein solubility in three extracting media: water, borate buffer and phosphate buffer. The electrophoretic pattern of the protein extracted in the two species Loligo duaucelii and Doryteuthis sibogae (needle squid) are shown in plate 3.

In both the species, water extraction showed the maximum number of protein bands having molecular weights in the range 200 kDa to 29 kDa. Distinct differences in the number and the intensity of the bands were observed between the two species of squid. In needle squid, the high molecular weight proteins (200 kDa – 50 kDa) pattern were similar in all the three extraction media, while in loligo the number of bands extracted in this range showed difference. Moreover, borate buffer could not extract low molecular weight proteins.

The electrphoretic protein profiles of ice-stored sample of loligo and needle squid kept under three methods of storage are shown in plate 4. Samples of both the species iced under ideal conditions (with GMP and without direct contact with ice), retained the protein bands even after 8 days of ice-storage. But sample with GMP with direct contact with ice (lanes 3 and 4 for loligo and 9 and 10 for needle) showed lesser bands after 8 days of ice storage, with needle showing more leaching. In samples without GMP (Lane 5 and 6 for loligo and 11 and 12 for needle) the protein bands were less and after 8 days storage only few faint bands were visible, with needle showing a faint band of actin. In all the cases MHC (myosin heavy chain) bands gradually became faint, with MLC (myosin light chain) becoming prominent in the first two methods of storage. In needle squid one or two bands in 43

Plate 3 - Protein profile on extraction with different solvents

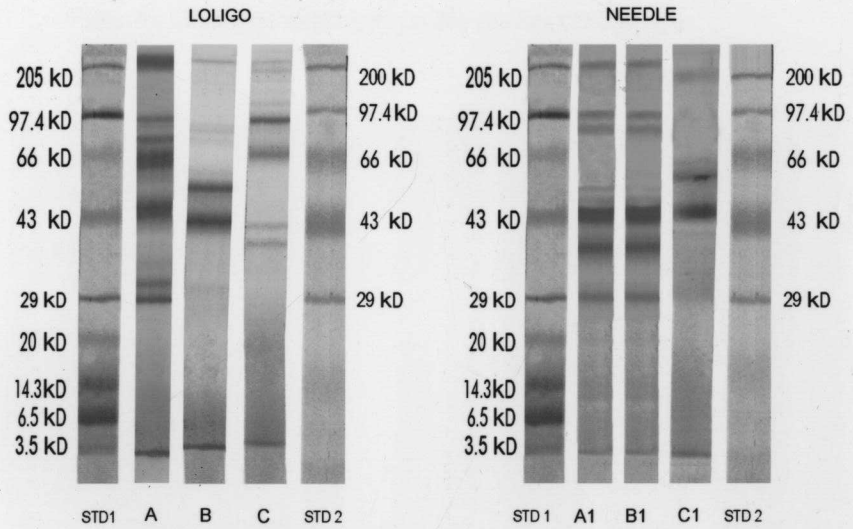
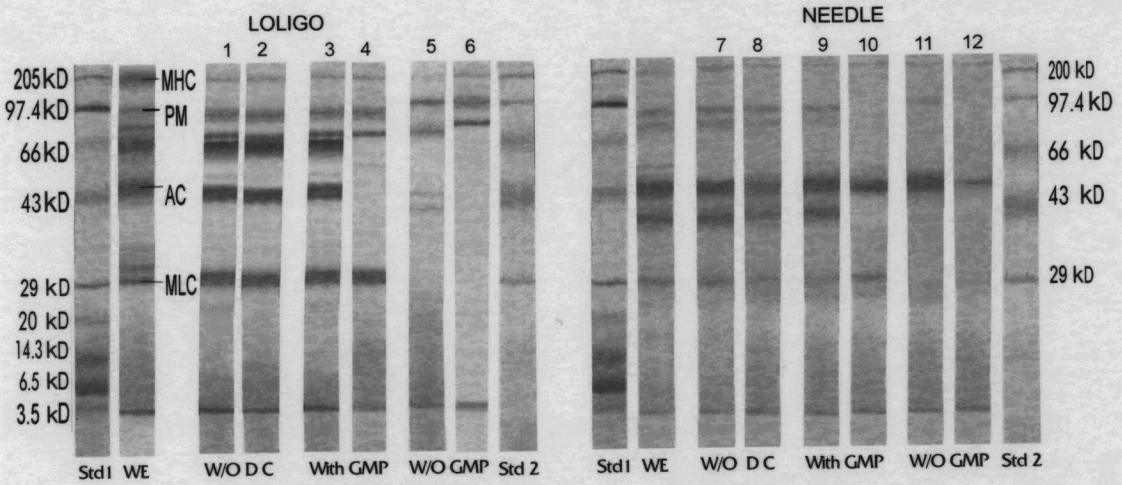


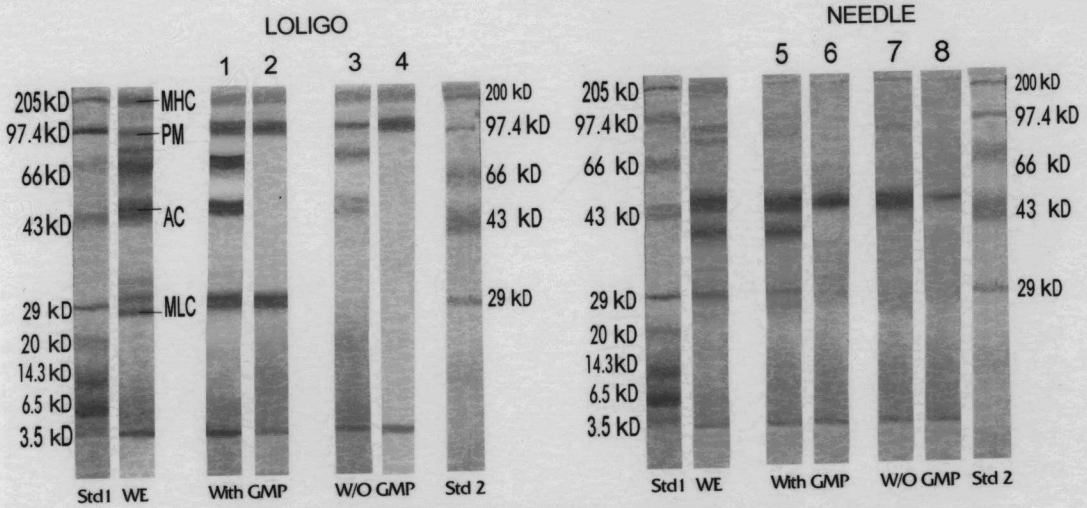
Plate 4 - Changes in protein profile during ice storage



1,3,5,7,9,11 - 1st day of storage 2,4,6,8,10,12 8th day storage

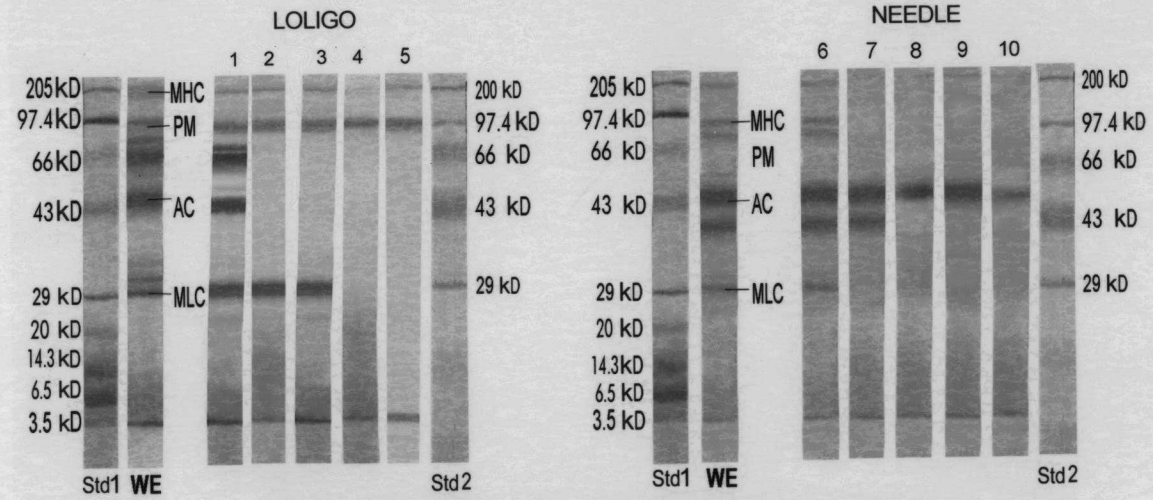
WE-Whole extract

Plate 5 - Changes in protein profile during super cooling



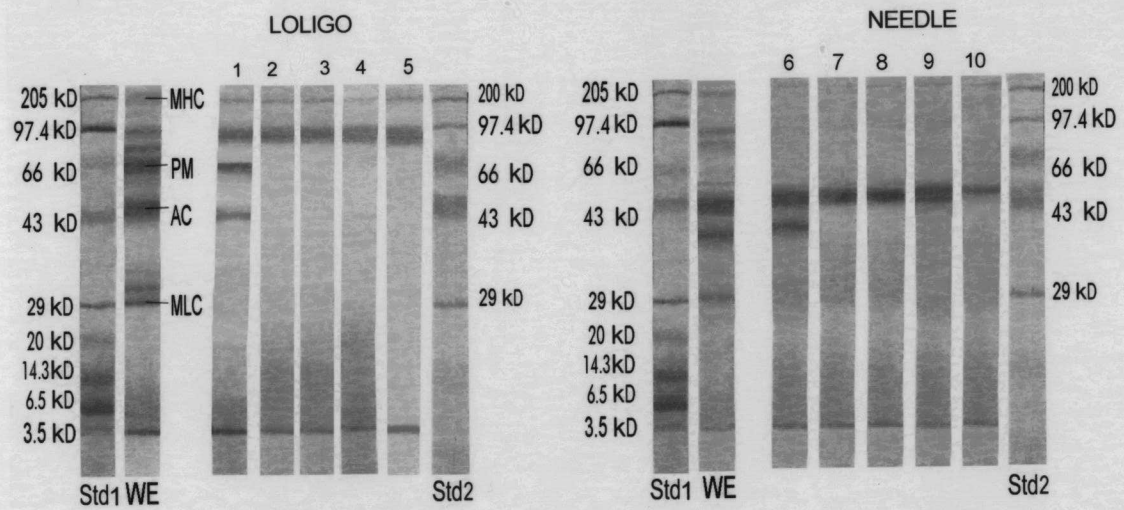
1,3,5,7 - 1st day of storage 2,4,6,8, 8th day storage

Plate 6 - Changes in protein profile during various processing steps



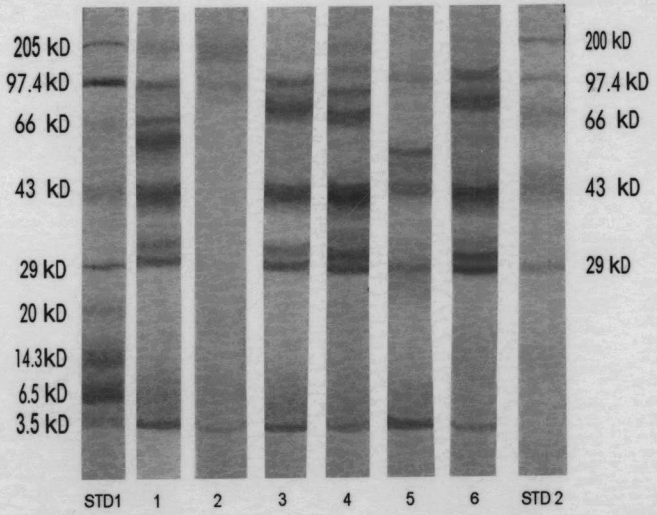
1,6- BEFORE TREATMENT 2,7- AFTER TREATMENT 3,8 -BEFORE FILLING
4,9 - AFTER FREEZING 5,10 - AFTER 24 HOURS FROZEN STORAGE

Plate 7 - Changes in protein profile during frozen storage



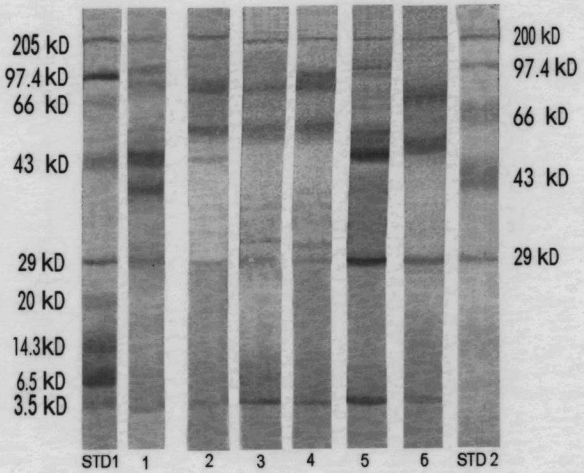
1,6- 30 Days 2,7- 60 days 3,8- 90 days 4,9- 120 days 5,10- 180 days

Plate 8 - Protein profile after various treatments in loligo squid



1 - Treatment control, 2 - Acetic Acid, 3 - Acetic Acid + Citric Acid
4 - Acetic Acid + STPP, 5 - Ascorbic Acid, 6 - Lime Juice

Plate 9 - Protein profile after various treatments in needle squid



kDa ranges were visible which might due to the actin component. Bands corresponding to paramyosin (98 kDa) were visible in all methods of storage in loligo, but only in the ideal method for ones in needle squid.

Changes in electrophoretic protein profile during super cooling of loligo and needle are shown in Plate 5. In this, one set of storage was with GMP and the other without GMP, both in direct contact with ice. In both the methods, most of the protein bands disappeared by 8days of storage in both species of squids. Here also, as in the case of ice storage, needle squid showed bands at 43kDa.

Plate 6 shows the differences in the protein profile of loligo and needle squid sampled at various steps during processing. Here also gradual disappearance of bands was observed. In needle squid, band corresponding to actin was extracted into the water in all the steps, A prominent band at 29kDa, (probably MLC) was seen in loligo till the stage before freezing, while in needle, the corresponding protein was not visible after the treatment.

During frozen storage, number of protein bands in loligo and needle squid (Plate 7) becomes feeble and lesser in number as the frozen storage advanced. On the 30^{th} day of frozen storage, the MHC band was fainter than that of squid muscle super cooled for one day, this could be due to the freeze induced aggregation of MHC and hence lesser extraction into soluble medium. New bands around 90kDa seen in loligo squid may be product of proteolysis. Paramyosin (98kDa) band was visible in frozen stored loligo squid. A distinct species-difference in the pattern of protein profile was visible during frozen storage. In needle squid, MHC and bands around 90-98kDa were totally absent. The component actin was found to be stable in

needle squid during frozen storage days. In all the days of frozen storage, the sarcoplasmic protein (around 3.5kDa) retained the same intensity.

Plate 8 and 9 show the protein profile of soluble extract from loligo and needle respectively after various treatments. Visible differences in the number and intensity of bands were observed between different treatments. In acetic acid treatment sample, very feeble bands were visible, with needle showing more number of protein bands compared to loligo squid. In all other treatments in needle squid, a shift in the position of protein bands from untreated raw sample was observed, may be due to the different degree of proteolytic activity.

7.5. Discussions

As with all contractile muscle, fish flesh contains three main groups of proteins, the sarcoplasmic or water-soluble proteins, the myofibrillar and the connective tissue proteins, which are readily separated by fractional extraction technique with water and salt solutions. Sarcoplasmic proteins are of low molecular weight (40-60kDa) is readily extractable with water. The unique nature of sarcoplasmic proteins of each species is such that the electrophoretic profile can be used for species identification (Mackie, 1969). During iced and chill storage changes, in the composition of sarcoplasmic proteins may occur as a consequence of proteolysis by endogenous or bacterial enzymes. In addition, proteins can be leached out by melting ice. But sarcoplasmic proteins are established to be more resistant to denatuarion during frozen storage, compared with myofibrillar proteins (Mackie, 1979).

Myofibrillar proteins consist of thick and fine filaments running in the longitudinal axial direction in the oblique muscle myofibrils of squid. The

thick filaments contain myosin and fine filaments contain F-actin, G-actin and tropomyosin. Thus, the myofibrils of squid act in the same way as vertebrate skeletal muscles. However, the thick filaments of squid have a core of paramyosin which is a protein unique to invertebrates, the filaments are thicker and longer in vertebrates.

The molecular weight of myosin in *T. pacificus* was approximately 450 kDa and composed of heavy chains of 180 kDa to 200 kDa. It is similar to that of vertebrates such as fish and rabbit. While three types of light chains are seen in vertebrates, only two types are seen in squid, *T.pacificus.* The major constituent of thin filament is G- Actin and in *T pacificus*, G actin had a molecular weight of 43 kDa (Tsuchiya et al., 1977)

Paramyosin is a specific protein of invertebrates. This protein exhibits the highest content after myosin and actin in myofibril protein in *Tadarodes pacificus.* It is composed of two subunits with molecular weight (98 kDa), which was similar to that in shellfish, clams and scallops, and other invertebrates like horseshoe crab, sea urchin and insects. Tropomyosin of *T.pacificus* is composed of two different subunits with a molecular weight of 37 kDa to 35 kDa.

According to Collignan and Montet (1998) the profile obtained for unprocessed broad tail short finned squid showed three major bands, which consists of myosin, paramyosin and actin ranging from 205 kDa to 42 kDa. According to Konno and Fukazawa (1993), squid mantle consists of myosin heavy chain (a -band) b & c band corresponding to the protein with molecular weight around 95 and 42 kDa respectively. Sakai and Matsumotto (1981) had previously noted the instability of myosin, which is the target of enzymes that are active at neutral pH and room temperature.

According to Matsumoto (1958), nearly 85% of the total protein in squid muscle could be solubilized with distilled water by exhaustive extraction with water. The result of this study is fully agreeing with this finding and observed the solubility of proteins is maximum in water when compared with other media. Almost all the proteins were extracted using water in both the species.

During ice storage, composition of sarcoplasmic protein may change as a consequence of proteolysis by endogenous and bacterial enzymes. In addition, protein can be leached out by melting ice (Mackie, 1979). The effect of ice storage in this study on the solubility of proteins has shown a similar trend in the case of samples with direct contact with ice. The non-protein nitrogen also showed a sharp decrease when stored in ice. Raghunath (1984) observed that WEN and NPN reduced considerably in squid mantles stored in crushed ice and melt water after 8 hours. Squid kept in contact icing were decolourised in less than 12 hours and could not be considered acceptable for consumption after two days. But non-contact ice stored squid could hold the freshness for a longer time (Ke et al., 1991). Water extractable nitrogen and NPN gradually decreased and squid lost its characteristic sweetness and finally became bland in taste (Lakshmanan, et al., 1993). The electrophoretic study on ice storage revealed similar results in both the selected species.

During super cooling, the loligo sample without GMP showed a greater loss of bands, which indicated the loss of proteins, especially of molecular weight from 97.4 to 3.5 kDa. But in the case of samples with GMP, MLC retained till the end of the storage. In needle squid, MHC and other proteins in higher molecular weight were less and feeble. This may be

the remit of proteolytic degradation and subsequent loss into melt water due to leaching during storage. These findings are fully supportive with the result of the Power, et al., (1969) that showed a higher rate of leaching of protein in super cooling. The samples stored in ice showed a lesser amount of protein denaturation than in super cooling. The rate of protein denaturation in super cooling as measured by the percentage of extractable protein nitrogen, dropped rapidly after first week in storage (Power & Morton, 1965).

In cephalopods, MHC is the primary myofibrillar protein followed by paramyosin. According to Hurtado et al., (1999), MHC in chill stored octopus was hydrolyzed during autolysis but actin showed no signs of hydrolysis. In frozen storage of squid (*Loligo vulgaris)*, MHC band was found to be fainter than that of super cooled squid muscle (Gomez-guillen et al., 2003). This protein was not present in the soluble fractions because it was retained in the precipitate as a result of freeze-induced aggregation. Their study also confirm that paramyosin and actin remained much more stable and unaffected by the storage. Extensive degradation during frozen storage in North American squid also has been reported by Stanley and Hultin, (1984). They found that actin was the component most resistant to break down. This agrees to our present observations with chilled and frozen stored squid, although slight differences were observed between loligo and needle squid.

Mild acid induced gelation of fish meat is associated with decrease in sulphydryl contents and formation of disulphide bonds. This has been verified with respect to shark and threadfin bream gel (Chawla et al., 1996). Decrease in SH groups in the proteins is comparable with conventional

surimi gelation (Stone and Stanley, 1992). In the study of Sakai and Matsumotto (1981) on proteolytic activity of squid mantle muscle, the SDS-PAGE pattern of whole extract of squid showed a very few bands at pH 4.3 which is similar to our findings in loligo species. But acetic acid treated needle squid showed a more number of bands. The results of the study of Collignan and Montet (1998) showed that the tenderization of squid mantle rings processed at pH 2 and 45°C was the result of proteolysis, which nonspecifically broke down muscle proteins (especially myosin) into many small fragments

Another change was the disappearance of myosin heavy chain (MHC). SDS-PAGE data was characterized by predominant band of MHC in unacidified meat while, it was feeble in the case of acidified gel, suggesting degradation of MHC during gel formation (Venugopal et al., 2002). This study suggests the formation of gel on acidifying myofibrillar protein to pH 4.5 using acetic acid. The gel formation is a result of initial denaturation causing protein unfolding, protein-protein interaction and aggregation (Mulvihill and Kinsella, 1987). The lesser number of protein bands in SDS-PAGE of acetic acid treated sample may be due to the above phenomenon, hence the lesser extractability into aqueous solution.

7.6. Conclusion

The bands obtained were characterized by comparing with protein molecular weight markers. The extractability of the proteins in water, phosphate buffer and borate buffer were also compared. Characterization of the proteins in samples from various storage systems and treatments were also carried out. From the protein profile, it was observed that the rate of

extraction of proteins from needle squid was more compared to loligo. Leaching of protein in the sample kept in direct contact with ice and water was confirmed by the loss of protein bands. Myosin, which is a component of myofibrillar protein, was also solublised in aqueous extract. Mild acid treated sample showed feeble bands of Myosin Heavy Chain (MHC) suggesting possible disappearance due to acid-induced gelation.

7.7. References

Andrews, T.A., (1986) Electrophoresis Theory, Techniques and Biochemical and clinical applications. Clarendon Press, Oxford.

Chawla, S.P., Venugopal, V and Nair, P.M., (1996) Gelation of proteins from washed muscle of threadfin bream (*Nemipterus japonicus*) under mild acidic conditions. J. Fd. Sci. **61**, 362-366.

Collignan, A and Montet, D., (1998) Tenderizing squid mantle by marination at different pH and temperature levels. Lebensm Wiss. U. Technol. **31**, 673-679.

Connell, J.J., (1965) The use of sodium dodecyl sulpate in the study of protein interactions during the storage of cod flesh at –14°C. J. Sci. Fd. Agric. **16**, 522-527.

Connell, J.J., Laird, W.M., Mackie, I.M. and Ritchie, A.H., (1978) Changes in proteins during frozen storage of cod as detected by SDS – electrophoresis. Proc. V Intl. Congress Fd. Sci. Technol.

Gomez-Guillen, M.C., Martinez-Alvarez, O. and Montero, P., (2003) Functional and thermal gelation properties of squid mantle proteins affected by chilled and frozen storage. J. Fd. Sci. **68**, 1962-1967.

Hume, A. and Mackie, I.M., (1979) The use of electrophoresis of the water-soluble muscle proteins in the quantitative analysis of the species components of a fish mince mixture. Advances in Fish Science and Technology (Ed. Connell, J.J.) 451-456.

Hurtado, J.L., Borderias, J., Montero, P., An H., (1999) Characterization of proteolytic activity in Octopus (*Octopus vulgaris*) arm muscle. J.Fd. Biochem. 469-483.

Ke, P.J., Fierheller, M and Lemon, D.W., (1991) Studies on Processing Technology for Atlantic short-fin squid (*Illex illecebrosus*). Levensmittel - wissenschaft –und – Technology. **24**(4) 328-333

Konno, K. and Fukazawa, C., (1993) Autolysis of squid mantle muscle proteins as affected by storage conditions and inhibitors. J. Fd. Sci. **58**, 1198-1202.

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

Lakshmanan, P.T., Varma, P.R.G. and Iyer, T.S.G., (1993) Quality of Commercially frozen cephalopods products from India, J. Food control, **4** (3), 159-164.

Llewellyn, J.W. and Flaherty, B., (1976) The detection and estimation of soya protein in food product by Iso-electric processing. J. Fd. Technol. **11**, 555-563.

Mackie, I. M., (1969) Identification of fish species by a modified polyacrylamide disc electrophoresis technique. J. Assoc. Publ. Anal. 83-87.

Mackie, I. M., (1979) A review of some recent application of electrophoresis and iso-electric focusing in the identification of species of fish in fish and fish products. Advances in Fish Science and Technology (Ed. Connell, J.J.) 444-450.

Matsumoto, J.J., (1958) On the protein composition of squid muscle. Bull. Jap. Soc. Scient. Fish. **24**, 133-139.

Matsumotto, J., (1959) An electrophoretic study of the squid actomyosin. Bull. Jap. Soc. Sci. Fish. **25** (1), 27-29.

*Morel, M., (1977) Identification des especes de poissions par electrofocalisation en gel de polyacrylamide. Sci. Peche. 275, 128.

Mulvihill, D and Kinsella, J.E., (1987) Gelation characterization of whey proteins and β lactoglobulins. Fd. Tech, **41**, 102-111.

Ohnishi, M and Rodger, G.W., (1979) Protein studies: Analysis of the salt-soluble protein fraction of cod muscle by gel filtration. Advances in Fish Science and Technology (Ed. Connell, J.J.) 422-428.

Penny, I.F. and Hofmann, K., (1971) The detection of soyabean protein in meat products. 17th meeting European Meat Research Workers, Bristol. 809-812.

*Power, H.E. and Morton, M.L., (1965) New series circular No.23 Halifax Lab. Fish. Res. Board of Canada.

Power, H.E., Margaret L. Morton and Ruth E. Sinclair, (1969) The chemical and physical characteristics of cod stored at Super chill temperatures. Freezing and Irradiation of Fish Ed. Kreuzer, R. 104-113.

Reghunath, M.R., (1984) Soluble nitrogen loses in squid *(Loligo duaucelii)* during storage in slush ice. J. Food science and Technology. **21**. 50-52.

Sakai, J.S. and Matsumotto, J.J., (1981). Proteolytic enzymes of squid mantle muscle. J. Comp. Biochem. Physiol. **68B**, 389-395.

Smith, B.J., (1994) SDS polyacrylamide gel electrophoresis of Proteins Methods in Molecular Biology. 32 Basic Protein and Peptide protocols. (Ed. Walker, J.M.)

Stanley, D.W., Hultin, H.O., (1984) Proteolytic activity in North Americal squid and its relation to quality. Com. Fd. Sci. Technol. J. **17**, 163-157.

Stone, D.W. and Stanley, A.P., (1992) Gelation of fish muscle proteins. Fd. Res. Intl. **25**, 381-388.

Tsuchiya, T., Suzuki, H. and Matsumotto, J. J., (1977) Isolation and purification of Squid actin. Bull. Jap. Soc. Sci. Fish. **43** (7), 877-884.

Tsuchiya, T., Noboro Yamada and Matsumotto, J. J., (1978) Extraction and purification of squid myosin. Bull. Jap. Soc. Sci. Fish. **44** (2), 175-179.

Venugopal, V., (2003) Gel formation of fish structural proteins under mild acidic conditions: comparison with conventional surimi gelation and applications. Fish. Technol. **40** (2), 61-70.

Venugopal, V., Kakatkar, A. Bongirwar, D.R., Karthikeyan, M., Mathew, S. and Shamasunder, B.A., (2002) Gelation of Shark Meat Under Mild Acid Condition: Physico-Chemical and Rheological Characterization of the Gel. J. Fd. Sci. **67**, 2681-2686.

Walker, J.M., (1994) Nondenaturing polyacrylamide gel electrophoresis of proteins. Methods in Molecular Biology. 32 Basic Protein and Peptide protocols. Ed. Walker, J.M.

Weber, K and Osborne, M., (1969) The reliability of molecular weight determination by dodecyl sulpate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**, 4406-4412.

Westermier, R., (1982) Electrophoresis in practice. (Ed. Westermier).

*Not referred in original

Chapter 8

STUDIES ON TISSUE PROTEOLYTIC ENZYMES

8.1. Introduction

Squids are known to have only one to two years of life span and some species of squid grow very fast from spring till autumn and die after spawning. In order to achieve its rapid growth, the protein turnover must be very fast which usually results from an active proteolytic system. Active protenases were found not only on the visceral part of the squid but also in the mantle muscle as well. On post-mortem, squid enters a state of uncontrollable protein degradation from both natural and bacterial sources. Rough handling and pressure due to icing apparently facilitates subsequent release of proteolytic enzymes. Proteins of squid muscle differ significantly from those of fish muscle with specific catheptic activity, approximately twice as high as that found in other finfishes. Proteolytic degradation is probably the single most important characteristic relating to the eating quality of the squid. Little information is known on the proteolytic activities in muscle of squids and other cephalopods from tropical waters. It is being noticed that the needle squid underwent softening even during thawing, while the loligo squid was rather stable. A comparison of their autolysis seemed to be a logical approach to look for the reasons for the above difference between the two squids.

8.2. Review of Literature

The squid mantle showed extensive protease activity and observed that squid mantle actomyosin was much less stable than those of carp and rabbit (Magita et al., 1958). The intact squid myosin was successfully isolated only when proteinase inhibitors like trasylol and soybean trypsin inhibitor, or ethylene diamine tetra acetic acid (EDTA) were added to the

extraction media (Tsuchiya et al., 1978). Sakai and Matsumotto (1980) studied the assay of proteolytic enzymes of squid *Ommastreohes sloani pacificus* in the presence of inhibitors.

A comparative study of autolysis of Argentina squid (*Illex argentinus)* and Falkland squid (*Martilia hyagesi)* was done by Lee and Bonnie, (1990) in which the optimum temperature for autolysis and the optimum pH have been found out. The visceral proteinase activity was studied by Hameed and Haard (1985). Doke and Ninjoor (1987) have studied the alkaline proteinases and exopeptidases from shrimp muscle. The effect of alkaline protease activity on some properties of comminuted squid (*Loligo forbesi)* was studied by Roger et al., (1984). Luten et al., (1992) studied the proteolytic enzyme activity as a measure of cooking of tropical shrimps and found that cooking time was inversely proportional to the enzyme activity. Schober et al., (1992) studied the isolation, characterization and application of proteolytic enzymes from fish viscera. Mateos et al., (2002) studied the thermal gelation profiles of frozen squid with added proteinase inhibitors. Visessanguan et al., (2000) suggested porcine plasma proteins as a surimi protease inhibitor and found effective on actomyosin gelation.

Characterization of proteolytic activity in octopus arm muscle was studied by Hurtado et al., (2000). Yamashita and Konagaya (1991) analyzed the participation of cathepsin L into extensive softening of muscle of Chum salmon caught during spawning migration. Mireles-DeWitt and Morrissey (2002) suggested the parameters for the recovery of proteases from surimi wash water. The effect of proteinases on meat texture and seafood quality was described by Shann (2000). Ayensa et al., (2000) studied the proteolytic

activity of squid muscle *(Todaropsis eblanae)* in the presence of various chemical inhibitors.

Aoki and Ueno (1999) explained the involvement of cathepsins B and L in the post-mortem autolysis of mackerel muscle. The cathepsin-D like proteinase has a maximum activity at pH 3.1 in the mantle muscle of *Ommastreohes sloani pacificus (*Sakai and Matsumotto, 1985). Cathepsin-D and E were present in the Atlantic short finned squid (*Illex Illecebrosus*) and long finned (*Loligo plealeii leseur*) (Leblanc and Gill, 1982). The effect of temperature on alkaline protease was studied by Deng (1981). Warrier et al., (1972) has studied the effect of cold storage on the proteolytic enzyme of fish muscle. Rodger et al., (1984) also studied the effect of alkaline protease activity on some properties of squid *Loligo forbesi*. The proteolytic activity of Atlantic croaker was studied in minced fish gel by Cheng et al., (1979) using casein as a substrate. Work on proteolytic inactivation using protease inhibitors to improve the quality of fish surimi has been carried out by Jiang et al., (2000). Cao et al., (1999) studied the proteolysis of a myofibril bound serine proteinase from *Cyprinus carpio* on myofibrillar proteins and their gel formation ability.

Simpson et al., (1991) have made a through review of these enzymes in fish. Endogenous proteinases in seafoods in particular have been reviewed exhaustively by Haard (1992).

8.3.Materials and Methods

8.3.1. Sampling method

Squid mantle muscle of both the species of squid, L*oligo duvaucelii* and *Doryteuthis sibogae* were used for the study. For the study of

proteolytic enzymes, sampling was done from fresh stored and treated samples, the methods being given in the previous chapters.

- Fresh sample (2.3.)
- Samples ice-stored with GMP and without GMP (3.3.1.)
- Frozen stored samples (5.3.1.)
- Samples after treatment (6.3.)

8.3.2. Preparation of Enzyme extract

The samples of squid mantle tissue was cut into small pieces and homogenized in four volumes of cold distilled water using a high-speed homogenizer for 3-4minutes, keeping the container in an external pack of ice. The homogenate was centrifuged at 15000g for 30 minutes in a refrigerated centrifuge at –5°C. The supernatant was used immediately for the determination of enzyme activity.

8.3.3. Proteolytic enzyme assay

Enzyme activity was assayed by the method of Leblanc and Gill, (1981) using 2% haemoglobin in appropriate buffers. To cover the test pH range, the following buffers were used: 0.2M glycine HCl buffer for pH 2.2 and 3.0, acetate buffer for pH 4.2 and 5.6, phosphate buffer for pH 6.0 and 7.0, boric acid borate buffer for pH 8.0 and 9.0 and glycine NaOH buffer for pH 9.5, 10.0 and 10.5 (Gomori, 1955).

1ml of aliquot of the extract was added to 2ml of buffer containing 2% hemoglobin and incubated for two hours at 37°C. The activity was terminated by adding 2ml of freshly prepared 10% TCA. Controls were prepared by the addition 10% TCA to the buffered Hb substrate. After

mixing, 1ml extract was added immediately and incubated for 2 hours at 37°C. These terminated mixtures were stored overnight at 0-4°C and then filtered through Whatman No.4 filter paper. The liberated TCA soluble peptides were determined by the method of Lowry et al., (1951). The enzyme activity was expressed in µg of tyrosine per ml per minute. The specific activity was expressed as in µg of Tyrosine per mg protein per minute. Enzyme assays were conducted in triplicate.

8.3.4. Optimization of pH, Temperature and Time

After determining the pH optima, a separate experiment was conducted to study the effect of in temperature of incubation (30°C-75°C) on the activity of proteases. Separate enzyme assay was carried out to optimize the time.

8.3.5. Enzyme assay in the presence of inhibitors

The inhibitors and their concentration in the incubation mixture were as follows:

PMSF (Phenyl Methane Sulphonyl Fluoride), 1mM;

Soybean trypsin inhibitor, 100µg/ml;

Iodoacetic acid, 1mM;

EDTA (Ethylene diamine tetra acetic acid) 2mM

1ml of the inhibitor solution was added to 1ml of extract and 2ml of buffered substrate haemoglobin and incubated for 2 hours at 37°C. The incubation tests were carried out at pH 3.0, 6.0 and 8.0/9.0 with the buffers described above. The activity was arrested by adding 2ml of 10% TCA.

This was filtered and liberated acid soluble peptides were estimated by Lowry's method.

8.4.Results

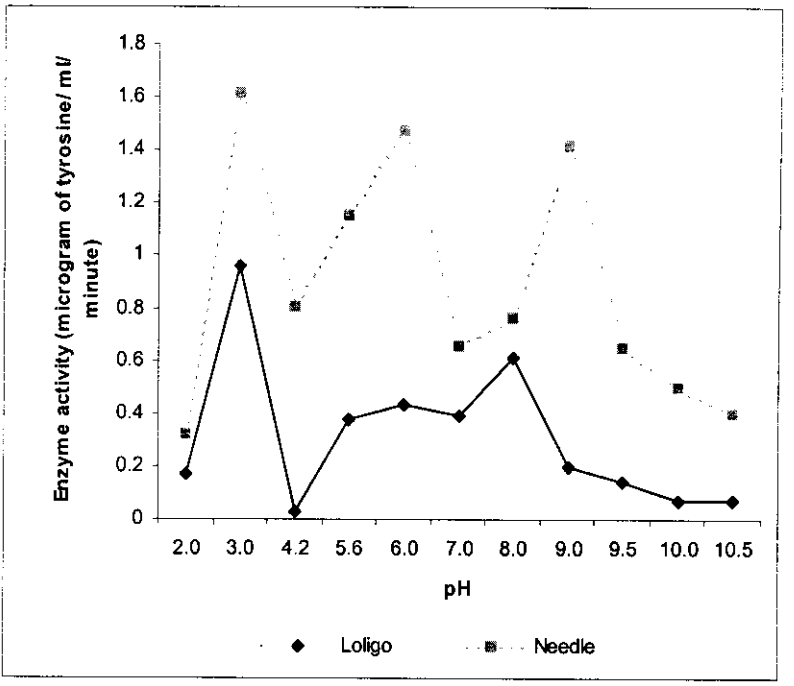


Figure 8.1.Proteolytic activity of both species of squid at various pH

Figure 8.1. shows the proteolytic activity at various pH in both the species of squid. The optimum pH was determined from the above figure. Both the species showed a high degree of proteolytic activity. But needle showed a higher activity than loligo at all the pH range. A high activity was found in the acid pH range with a maximum at pH 3.0 in both the species (loligo 0.95 units and needle 1.61 units). In loligo, there were two peaks for enzyme activity, at pH 3.0 and 8.0. At pH 6.0 also showing a slight increase in the activity was observed. A significant observation in this study was that at pH 4.2, there was a sharp dip in the activity of both the species with

loligo showing least activity of 0.28 units. The pH, 4.2 is same as the effective pH of the tissue in acetic acid treated squid mantle.

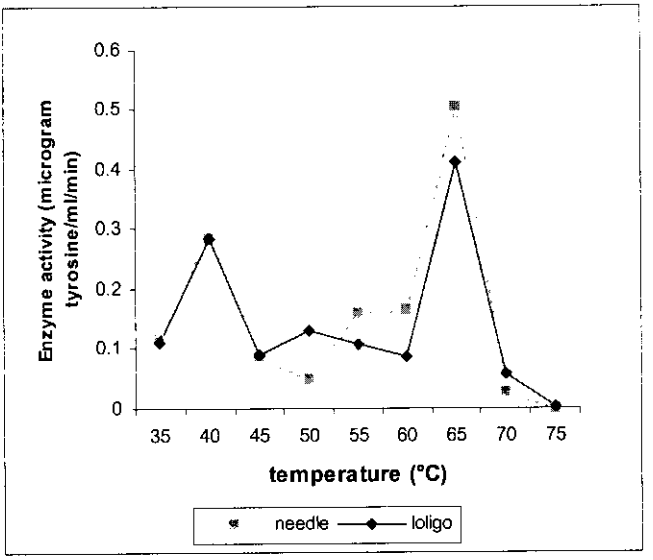


Figure 8.2. Proteolytic activity of both species at pH 3.0 and various temperatures

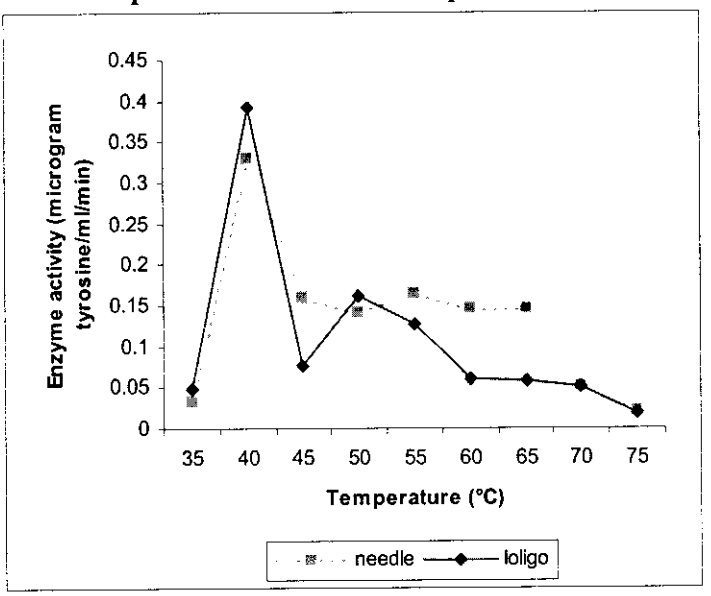


Figure 8.3. Proteolytic activity of both species at pH 6.0 and various temperatures

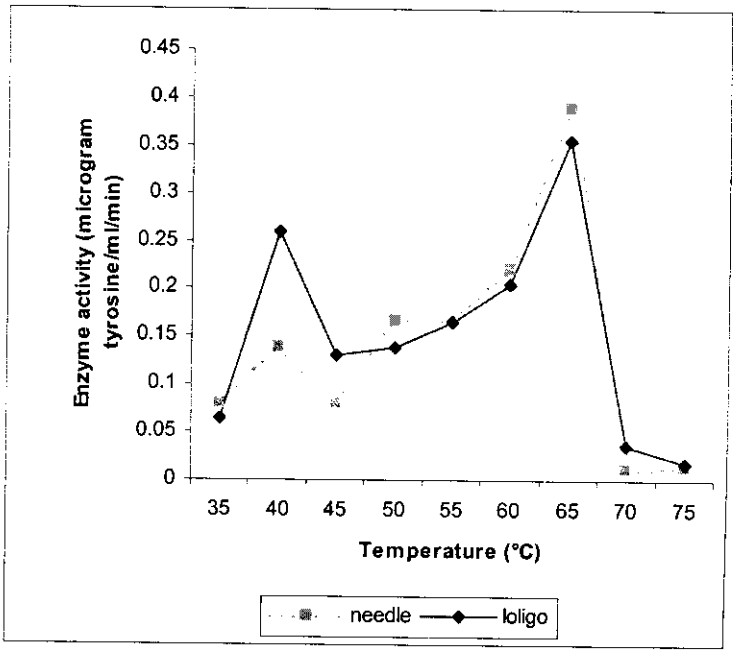


Figure 8.4. Proteolytic activity of both species at pH 8.0/9.0 and various temperatures

The optimum temperature for protease activity was found to be 40°C for both the species at pH 6.0. At pH 3.0 and pH 9.0/8.0 two peaks were observed (40°C and 65°C), where the maximum activity was obtained at 65°C (Figure 8.2, 8.3 and 8.4).

pH optimization was repeated at 40°C and 65°C. The trend in the enzyme activity was found to be same in both temperatures.

To determine the optimum time for incubation, the reaction media was incubated at 40°C for various time intervals (pH 3.0). The minimum time to reach maximum activity was found to be 2hours after which, a gradual decrease in the activity was observed (Figure 8.5).

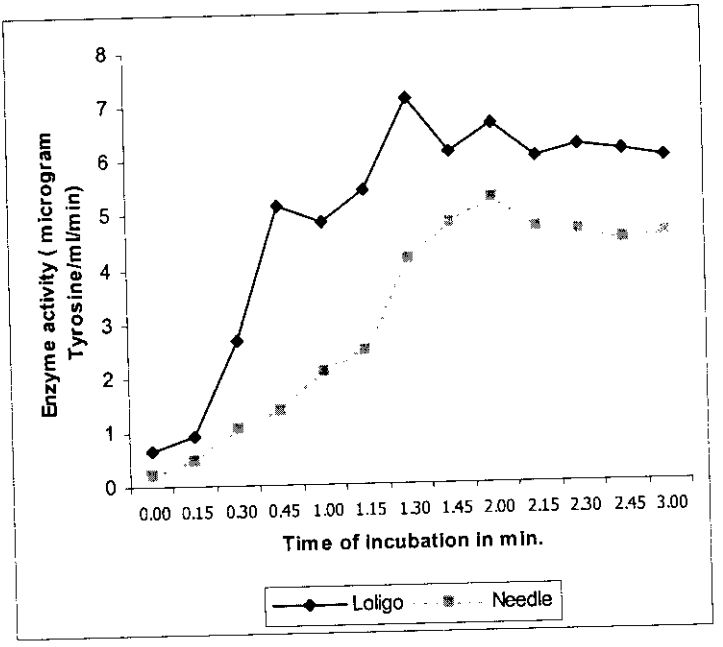


Figure 8.5. Optimization of time of incubation for protease assay in both species of squid at pH 3.0

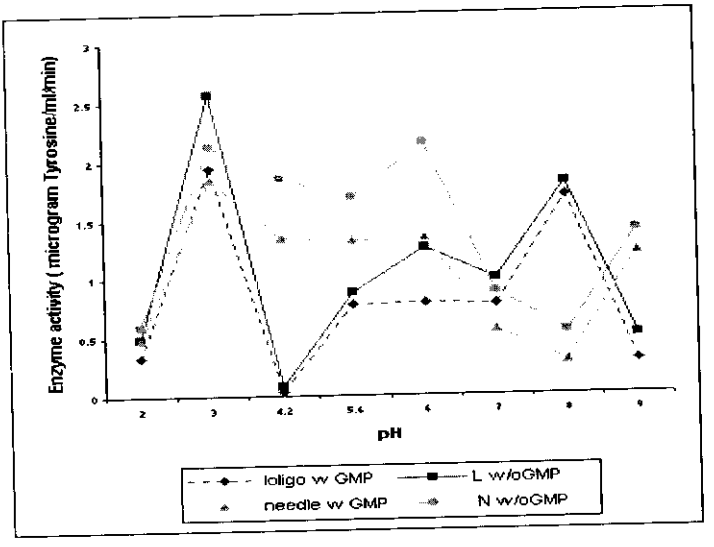


Figure 8.6. Proteolytic activity of both species of squid Ice-stored with GMP/without GMP with varying pH.

The proteolytic activity of both the species ice stored with GMP and without GMP at various pH were studied (Fig 8.6.). In the case of loligo, with

GMP and without GMP, the enzyme activity was found to be minimum at pH 4.2. In all the cases the activity showed a maximum pH 3.0. In both the species, the pattern of proteolytic activity changes in the pH range were specific, with samples without GMP, showing higher activity than with GMP.

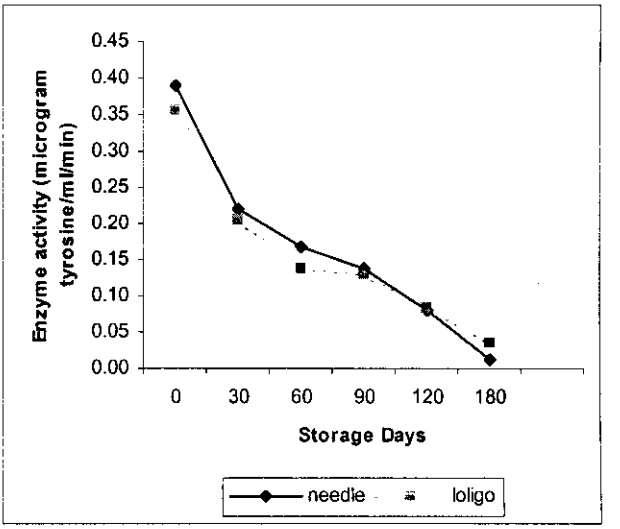


Figure 8.7. Proteolytic activity of both species of squid during frozen storage

Figure 8.7 shows the proteolytic activity of loligo and needle squid mantle tissue during frozen storage. The protease activity showed a gradual decrease during the period of 8 months frozen storage.The change in the activity in both the species were almost equal.

Table 8.1. Proteolytic activity in the drip of frozen stored samples

Storage	Needle	Loligo
Months	E.A.	E.A.
1	34.95	26.09
2	11.10	8.03
3	9.53	6.86
4	7.40	6.40
5	3.63	3.17
6	2.45	0.17

E.A. – Enzyme activity

The drip of frozen stored sample was collected by thawing the frozen samples. In both the species, the enzyme activity showed a decreasing trend and the drip from six months stored sample, showed a very low activity (Table 8.1).

Table 8.2. Effect of treatments on proteolytic enzyme activity

	Needle			Loligo		
Treatments	**E.A.**	**Protein mg/ml**	**S.E.A.**	**E.A.**	**Protein mg/ml**	**S.E.A.**
A	90.76	1.63	55.82	78.27	1.95	40.17
B	9.86	3.01	3.27	4.96	3.80	1.31
C	27.36	2.33	11.76	21.16	2.93	7.21
D	26.42	3.01	8.78	16.96	3.54	4.79
E	18.03	2.38	7.59	16.43	2.79	5.90
F	30.49	1.98	15.39	24.96	2.17	11.51

E.A. = Enzyme activity (µg tyrosine/ml/hr)

S.E.A. = Specific enzyme activity (µg tyrosine/mg protein/hr)

A-Control

B-Acetic acid alone

C-Acetic acid citric acid mixture

D-STPP acetic acid mixture

E-Ascorbic acid

F-Lime Juice

The effect of various treatments (as described in 6.4.6) on the proteolytic activity is given in the table 8.2. The samples treated with acetic acid showed the minimum activity in both the species while the untreated sample showed maximum activity. Among all the treatments, limejuice treated sample showed maximum activity. The specific enzyme activity also showed a minimum value in acetic acid treated sample of loligo and needle squid.

Table 8.3. Effect of inhibitors on the residual protease activity (%) of autolysis of the mantle muscle of both species of squid at 40°C

	Needle					
	pH 3.0		pH 6.0		pH 9.0	
Inhibitors	S.E.A.	R.E.A.	S.E.A.	R.E.A.	S.E.A.	R.E.A.
PMSF	15.73	45.83	12.98	34.09	24.08	48.61
Soy try	15.19	44.26	25.59	67.20	18.22	36.78
Iodo acetate	26.40	76.94	19.90	52.26	20.16	40.69
EDTA	31.61	92.13	10.39	27.29	9.62	19.42
Control	34.31	100	38.08	100	49.54	100
	Loligo					
	pH 3.0		pH 6.0		pH 8.0	
Inhibitors	S.E.A.	R.E.A.	S.E.A.	R.E.A.	S.E.A.	R.E.A.
PMSF	9.20	40.68	8.27	37.06	17.01	57.78
Soy try	9.63	42.56	11.88	53.26	16.04	54.50
Iodo acetate	10.99	48.61	11.76	52.70	17.51	59.49
EDTA	26.54	117.37	3.20	14.34	4.29	14.58
Control	22.61	100	22.31	100	29.43	100

S.E.A. Specific enzyme activity

R.E.A. Residual enzyme activity –Value represent the percentage relative to the activity of control

Table 8.4. Inhibitors and inhibiting enzyme

Inhibitors	**Proteinases**
PMSF and Soy bean trypsin inhibitor	Inhibit Serine Proteinases
Iodo acetic acid, PCMB and Leupeptin	Inhibit Thiol Proteinases
Pepstatin	Inhibit Carboxyl Proteinases
EDTA	Inhibit Metalloproteinases

The proteolysis at pH 3.0, pH 6.0 and pH 8.0/9.0 was carried out in the presence of inhibitors, to investigate what kind of proteinases function at those pH. Based on the essential catalytic group of the enzymes, Hartley (1960) classified proteinases in the four classes namely Serine, Thiol, Carboxyl and Metalloproteinases. Barrett (1977) has suggested that the class assignment can be done on the basis of different sensitivities to different inhibitors. The inhibitors and inhibiting enzymes are given in the Table 8.4 by different inhibitors. At an optimum temperature of 40°C, the inhibition of various inhibitors of the protease enzymes varied with pH (Table 8.3).

The residual activity in loligo was found to be very low when PMSF and soy trypsin inhibitors were added. In other words, at pH 3.0 the cathepsin D like proteinases (with an optimum pH 3.0) was very likely to be present in the aqueous extract of both the species studied. At physiological pH, (6.0-7.0) PMSF inhibited the proteolysis to a residual activity of 37.06 % in loligo and 34.09% in needle. The inhibition due to soy trypsin inhibitor was not much effective at this particular pH for both the species. A chymotrypsin – like serine proteinase was likely to be present in the muscle of both the species. At this pH, EDTA was more effective in suppressing the protein degradation. Iodoacetic acid inhibition was not significant when compared with inhibition due to EDTA. To some extent, iodo acetic acid inhibited the proteolysis in both the species (Needle squid –52.26% and Loligo –52.70%) indicating the presence of a thiol proteinases. EDTA was found to be a very effective inhibitor at neutral and alkaline pH. Suggesting that, there exist highly active metalloproteinases.

In contrary to this, at pH 3.0, EDTA did not inhibit but rather increased the activity (117% of residual activity in loligo and 92.3% in needle squid),

8.5.Discussion

The activity – pH profiles of squid mantle muscle aqueous extract, exhibited a high proteolytic activity in the acid pH range, with a low activity in neutral and alkaline pH ranges. A similar observation was reported by Sakai and Matsumotto (1980) in squid *Ommastrephes sloani pacificus.* In the mantle muscle of Falkland squid, optimum temperature for autolysis was 55°C to 60°C and at 25°C, optimal pH was 6.8 (Lee and Bonnie, 1990). Drabikowski et al., (1977) has observed a similar profile in the skeletal muscle of various vertebrates.

The activity at pH 3.1 was strongly inhibited by pepstatin (Sakai and Matsumotto, 1980, Lee and Bonnie, 1990), which was specific to the carboxyl proteinases such as pepsin and cathepsin D (Aoyagi et al., 1972, Barrett, 1977). Thus the results indicate that a cathepsin D like proteinase contributes mainly to the protein degradation in the squid mantle muscle at this pH. Since cathepsin D is believed to constitute the major proteolytic activity in the lysosomes, (Barrett, 1972), lysosomes may be involved in protein degradation of the squid mantle muscle. The autolytic activity at pH 3.0 was also inhibited by other inhibitors to some extent, a thiol proteinases was inhibited by Iodo acetic acid. cathepsin B is thiol proteinase, which can be inhibited by Iodo acetic acid. The inhibition of activity by iodo acetic acid may be due to a presence of an identical enzyme other than cathepsin B in the squid mantle. Inaba et al., (1978, 1976) established presence of cathepsin B in various species of squid.

In fish skeletal muscle, several of these proteinases such as cathepsin D (Doke et al., 1980), neutral proteinases (Makinodan et al., 1983), cathepsin B (Chen and Zal, l 1986), alkaline proteinase (Makinodan et al.,

1983) and some peptidases (Osnes and Mohr, 1985) have been identified and characterized. Among these, alkaline proteinases seemed to mediate changes in the muscle texture when processed at 50°C to 70°C (Makinodan et al., 1985) apparently due to its heat stability. A comparative result was obtained in the present study, where a significantly active enzyme was observed in both squid at pH 8.0 with optimal temperature of 65°C.

Although alkaline proteinase has been purified from the muscle fish varieties such as Anartic krill (Osnes and Mohr, 1985), Atlantic croaker (Lin and Lanier, 1980), White croaker (Busconi et al., 1984) and carp (Iwata et al., 1973), information on the enzymes from crustacean's species is scarce. While some investigators (Lin and Lanier, 1980, Busconi et al., 1984) suggested that the enzyme is sulphydryl dependent, others showed to be either a metallo or a serine proteinase (Kozlovskoya and Elvakova, 1975; Busconi et al., 1984). Tissue proteinases have been implicated as adversely affecting the quality of stored muscle foods due to the sustained action of endopeptidases and exopeptidases that are involved in the complete break down of tissue proteins (Goll et al., 1983).

In this study also, an attempt was made to assay the tissue proteolytic enzyme of squid mantle at various pH and to characterize these proteases by using specific inhibitors. Between the loligo and needle squid, a distinct variation in the enzyme activity of various proteases was observed which might be due to the species-specific differences. The result obtained for the protease activities in the samples with and without GMP, frozen-stored sample and treated sample are comparable with the pattern of protein bands in SDS-PAGE (Chapter 7). The fluctuation in the endogenous muscle proteinase content and their activity observed at various storage conditions

are affected by post-rigor condition and consequent softening of muscle. The release of lysosomal proteases in the squid at acidic pH and their activity as a result of various treatments, will be discussed in the next chapter

Subjecting to various pre-treatments to extent the quality of frozen stored squid, had significant difference in the proteolytic activity of which, acetic treated sample showed a very low protease activity compared to all other treatments. Venugopal et al., (1994) had reported that acid protease activity of shark protein dispersion at pH values of 3.8, 6.0 were 13.6 and 15.3 units respectively, which decreased to 8.5 units when the pH was lowered to 4.5 by addition of acetic acid. The sharp decrease in the protease activity at pH 4.2 observed in the squid mantle, specifically in loligo, is in agreement with the above observation. The acetic acid treated sample were thus found to be organoleptically and physico-chemically of the best quality (6.4). In addition, the presence of proteases of microbial origin could also be inactivated due to the anti-microbial effect of acetic acid.

8.6.Conclusion

Optimal pH of the autolysis was found to be at three different Points pH 3.0, pH 6.0 and pH 8.0 in the case of loligo and pH 3.0, pH 6.0 and pH 9.0 in the case of needle. In loligo squid, the protease activity was found to be minimum at pH 4.2 (acetic acid alone treated sample pH). Optimum temperature for protenase activity was found to be 40°C at pH 6.0 and 65°C and 40°C at both pH 3.0 and pH 8.0/9.0. The optimum time of incubation for the enzymal assay was found to be 2 hours.

Between the two species studied, needle squid showed higher protease activity than loligo. But samples iced with GMP showed a lesser activity than samples without GMP. During frozen storage a gradual reduction in the activity was observed and it may due to freeze denaturation of the enzymes. The quantity of protein was gradually reduced in the drip, which also indicated the denaturation of proteins during frozen storage. Among treatments, acetic acid treated sample was found to be the best, which offers minimum proteolytic activity and high degree of protein retention. The proteolytic activity was strongly inhibited by specific protease inhibitors, suggesting the presence of serine proteinase, thiol proteinase, cathepsin D and metallo proteinase in the squid mantle tissue.

8.7. References

Aoki, T. and Ueno, R., (1999) Involvement of Cathepsins B and L in the postmortem in the mackerel muscle. Food Research Intl. **30**(8), 585-591.

Aoyagi, T., Morishima, H., Nishizawa, R., Kunmoto, S. Takeuchi, T. and Umezawa, H,. (1972) Biological activity of pepstatins, pepstanone A and partial peptides on pepsin, cathepsin D, and rennin. J. Antibiot. **25**, 689-694.

Ayensa, M. G. An, H. Gomez Guillen M.C. Montero, P. Borderias, A.J., (2000) Partial Protease activity characterization of squid *(Todaropses eblanae)* mantle. Food Sci. and Technol. Intl. **5** (5), 391-396.

Barrett, A. J., (1972) Lysosomal enzymes. Lysosomes A Laboratory handbook (Ed, Dingle, J.T.), North- Holland, Amsterdam. 46-135.

Barrett, A. J., (1977) Cathepsin D and other carboxyl proteinases. Proteinases in mammalian cells and tissues (Ed. Barrett, A.J.) North- Holland, Amsterdam. 209-248.

Busconi, L., Falco, H.J., Marton, C., Trucco, R.E., Sanchez, J.J., (1984) Identification of two alkaline proteases and a trypsin inhibitor from the muscle of white croaker *(Micropogon opercularis)*. FEBS Letts. 176: 211.

Cao, M.J. Ostami K. Pang Key .H Haara. K. Ishihara. T., (1999) Cleavage specificity of a myofibril-bound serine proteinase from carp *(Cyprinus carpio)* muscle. Comp. Biochem. Physiol. Biochem. Mol. Biol. **123** (4), 399-405.

Chen, M.C. and Zall, R.R., (1986) Partial purification and characterization of cathepsin D-like and B-like acid proteases from surf clam viscera. J. Fd. Sci. **51**, 71.

Cheng, C.S. Hamann D.D. and Webb N.B., (1979) Effects of species and storage time on minced fish gel structure. J. Food Sci. **44**, 1087-1092.

Darbikowski, W. Gorecka, A. and Jaribeic-Puka, A., (1977) Endogenous proteases in vertibrate skeletal muscle. Int. J. Biochem. **8**, 61-71.

Deng, J.C., (1981) Effect of Temperatures on fish alkaline protease protein interaction and texture quality. J. Food Sci. **46**, 62-65.

Devadas, K., (2002) Proteins and Lipids of Marine Products and Their Changes during Processing and Preservation. Fish Processing Technology, (Ed.) Gopakumar, K.

Doke, S.N. and Ninjoor, V., (1987) Characteristics of a alkaline protease and a exopeptidase from Shrimp (*Penaeus indicus*) muscle. J. Food Sci. **52**, (5), 1205-1211.

Doke, S.N., Ninjoor, V. and Nadkarni, G.B., (1980) Characterization of cathepsin D from the skeletal muscle of fresh water fish, Tilapia mosambica. Agric. Biol. Chem. **44**, 1521.

Goll, D.E., Olsuka, Y., Nagainis, P.A., Schannon, J.D., Sathe, S.K. and Muguruma, M, (1983) The roll of muscle proteinases in maintenance of muscle integrity and mass. J. Fd. Biochem. **7**,137.

Gomori, G., (1955) Preparations of Buffers for use in Enzyme Studies. Methods of Enzymology Vol 1. (Ed. Sidney, P. Colowick and

Nathan, O. Kapalan). McCollum- Pratt Institute, Baltimore, Maryland.

Haard, N.F., (1992) A review of proteolytic enzymes from marine organisms and their application in food industry. J. Aqua. Food Prdn. Technol., **1**, 17-35.

Hameed, K.S. and Haard, N.F., (1985) Isolation and characterization of cathepsin C from Atlantic short finned squid (*Illex illecebrosus*). Comp. Biochem. Physiol. **82**, 241-246.

Hurtado, J.L. Borderias, J. Montero, P. Haejung, and An., (2000) Characterization of proteolytic activity in octopus (*Octopus vulgaris),* J. Food Biochem. **23**, (4) 469-483.

Inaba, T. Shindo N. and Fujii M., (1976). Purification of cathepsin B from squid liver. Agric. Biol. Chem. **40,** 1159-1165.

Inaba, T. Yamada, K. and Takei, H., (1978) Purification of cathepsin B1 from the liver of Squid *(Ommastrephes Sloani pacificus)(in* Japanese) Agric. Bull. Saga. Univ. **45**, 27-35.

Iwata, K., Kobashi, K., and Hase, J., (1974) Studies on muscle alkaline protease I Isolation and purification and some physico-chemial properties of a alkaline protease from Carp muscle. Bull. Jap. Soc. Sci. Fish. 39:1325.

Jiang S.T. Hsieh J.F, Ho M.L., Chung, Y.C., (2000) Combination effects of Microbial Transglutaminase, reducing agent and protease inhibitor on the quality of hair tailed surimi. J. Fd. Sci. **65** (2) 241-245.

Kozlovskaya, E.P. and Elyakova, L.A., (1975) Certain properties of preparations from star fish proteinases II. Com. Biochem. Physiol. 50V: 255.

Leblanc, E.L. and Gill, T.A., (1982) Comparative study of Proteolysis in short finned (Illex *illecebrosus*) and long finned *(Loligo pealei lesseur)* squid. Comp. Biochem. Physiol. **73** B, 201-210.

Lee May-Lin and Bonnie Sun Tan, (1990) Comparison on the autolyses of Argentina squid (*Illex argentinus*) and Falkland squid (*Martilia hyagesi*). The second Asian Fisheries forum Asian fisheries society, Manila, Philippines. 881-884.

Lin, T.S. and Lanier, T.C., (1980) Properties of an alkaline protease from the skeletal muscle of Atlantic croaker. J.Fd. Biochem. **4**: 17.

*Luten, J.B; Bouquet, W.; Barneveld, E. Van; Bruenner, K.K., (1992) Proteolytic enzyme activity as a measure of cooking of tropical shrimp. Voedingsmiddelen technologie; **25** (2), 10-11.

Makinodan, Y., Toyohara, H and Niwa, E., (1985) Implication of muscle alkaline protease in the textural degradation of fish meat gel. J.Fd. Sci. **50**, 1351.

Mateos, P.M. Montero, P., Gomez Gullien, M.C., (2002) Addition of microbial transglutaminase and protease inhibitors to improve gel properties of frozen squid muscle. European Food Research and Technology. **214** (5), 377-381.

Migita, M., Matsumoto, J.J., Aoe, N., (1959) A Comparative study on the extractability of muscle protein of some animals. Bull. Jap. Soc. Sci. Fish. **24**, 751-759.

Migita, M., Matsumotto, J.J., Aoe, N. and Saishu, T., (1958) On a specific behaviour of "myosins" of squid muscle- I. Precipitability of "myosins'. Bull. Japan. Soc. Sci. Fish. **24**, 51-58.

*Mireles Dewitt C.A. and Morrissey M.T., (2002) Parameters for the recovery of proteases from surimi wash water. Bioresource Technol. **81** (3) 241-247.

Osnes, K.K. and Mohr, V., (1985) Peptide hydrolases of Antarctic krill (*Euphausia superba*). Comp. Biochem. Physiol. 82B: 599.

Rodger, G., Weddle, R.B., Craig, P., Hastings, R., (1984) Effect of alkaline protease activity on some properties of comminuted squid. J. Food Sci. Vol. 49, 117-123.

Sakai, J.S. and Matsumotto, J.J., (1980) Proteolytic enzymes of squid mantle muscle. J. Comp. Biochem. Physiol. Vol. 68B, 389-395.

*Schober, B., Jansen, G., Behnke, U., (1992) Studies on isolation, characterization and applications of proteolytic enzymes from fish viscera. Fiseherei, Forschung, **29**, (2), 89-96.

Shann Tzong Jiang, (2000) Effect of proteinases on the meat texture and seafood quality. Food. Sci. and Agr. Chem **2** (2) 55-74.

*Simpson, B.K., Smith, J.P., and Haard, N.F. (1991) Marine Enzymes, Encyclopedia of Food Science and Technology. New York, Wiley.1645-1653.

Tsuchiya, T., Yamada, N. and Matsumotto, J.J., (1978) Extraction and Purification of squid myosin. Bull. Japan. Soc. Sci. Fish. **44**, 175-179.

Venugopal, V. Doke, S.N., Nair, P.M., (1994) Gelation of shark myofibrillar proteins by weak organic acids. J.Fd. Chem. **50**, 185-191.

Visessanguan, W. Benjakul, S. and An, H., (2000) Porcine plasma proteins as surimi protease inhibitor: effects on actmyosin gelation. J. Food Sci. **65** (4) 607-611.

Warrier, S. B. K. Ninjoor, V. and Ghadi, S.V., (1988) Effect of Cold Storage on the proteolytic enzymes of fish muscle. J. Fd. Sci. Technol **25** (5) 313-314.

Yamashita, M. Konagaya, S., (1991) Participation of Cathepsin L into extensive softening of the muscle of Cham Salmon caught during spawning migration. Bull. Jap. Soc. Sci. Fish. (Nihon. Suisan Gakkai. Shi) **56** (8) 1271-1277.

*Not referred in original

Chapter 9

EFFECT OF TREATMENTS ON LYSOSOMAL STABILITY

9.1. Introduction

The problem that could arise out of stored flesh foods are the tenderisation, change in colour and texture, production of undesirable flavours and spoilage by microorganisms.

The spoilage of flesh foods stored at refrigerated temperatures and freezing temperatures has been attributed to intracellular autolysis. The autolytic enzymes comprise proteases, including lysosomal hydrolases. These enzymes are known to tenderise the meats, and the hydrolytic products released may also influence the acceptability of the stored flesh foods. The release of lysosomal enzymes occurs at a faster rate when the samples are stored at ambient temperature. Storage in freezer and subsequent thawing facilitates the release of hydrolases. Hence it is very important to consider the role of lysosomes in the preservation of fish and fishery products that are stored either in iced storage or in freezer.

In this chapter studies are undertaken regarding the stability of lysosomes of two species of squid, loligo and needle squid under various processing variables, using the enzyme acid phosphatase as an indicator. Assessment of the total lysosomal enzyme activity of the squid muscle was done using mantle homogenate in 0.25M sucrose containing EDTA in the presence of a non-ionic detergent like Triton X-100. Latent and free activity of the enzyme was determined in a homogenate in a manner wherein the integrity of lysosomal particles was maintained as much as possible.

9.2. Review of Literature

Various factors are capable of altering the structure of the lysosomal membrane and releasing the hydrolases. The occurrence, distribution and

properties of lysosomes were reviewed by Ninjoor, et al.,(1969). The storage stability of cattle spleen lysosomal enzymes was investigated using refrigerated storage and frozen storage by Melendo et al., (2001). Shann (2000) recommended that the lysosomal cathepsins and calpains, and their endogenous inhibitors were considered to be involved in meat tenderisation and deterioration of fish protein gel. Characterisation of crude lysosomal extract from bovine spleen for its use in processing muscle foods, was studied by Melendo et al., (1999) and (1998). Lysosomal cathepsins are calcium-activated muscle proteinases, involved in the post-mortem rheological changes in tissues of fish and marine invertebrates (Kolodziejska and Sikorski, 1996). Assessment of lysosomal nature of hydrolytic enzymes of skeletal muscles is rendered difficult for different variety of fish, owing to drastic homogenisation procedures required to obtain a uniform homogenate and need for prolong incubation periods. Such treatments cause disorganization of intact lysosomes and hence the latency of these enzymes cannot be established (Duve, 1959; Weinstok and Iodice, 1969).

The lysosomal enzymes in drip and the muscle of *Harpadon nehereus* were studied during repeated freezing and thawing in the presence of non-ionic detergent before and after irradiation. Irradiation and freezing and thawing treatments bring about increased exudation of soluble proteins of Bombay duck into the drip, possibly by the denaturation of myofibrillar proteins (Warrier et al., 1972). This treatment may also enrich the solubility of enzymes in the drip. Hari Kumar et al., (1974) have developed a method for evaluation of latency of lysosomal enzymes from chicken skeletal muscle tissue. Several mechanisms have been put forward to explain the increase in acid phosphatase activity. A direct correlation was noticed

between the duration of exposure to mercury and activity of acid phosphatase in catfish (Rema and Philip, 1999).

Lesly et al., (1996) investigated the role of myosin in the endosomal traffic and lysosomal system. The biosynthetic pathway for lysosomal hydrolysis has been extensively characterised by Cardelli (1993). The transport of materials to and from lysosome requires the proper movement and fusion of membrane vesicle.

Since cathepsin D is believed to constitute the major proteolytic activity in lysosomes (Barrett, 1972), lysosomes may also be involved in the protein degradation of squid mantle muscle. The activity at pH 3.0 is mainly contributed by carboxyl proteinases such as pepsin and cathepsin D. Migita et al., (1958) has observed that the squid mantle muscle actomyosin was much less stable than that of carp and rabbit. Sakai et al., (1986) have purified and characterised the acid cysteine proteinase from squid mantle. In studies on the proteinases of the squid mantle muscle, authors have found that there is high autoproteolytic activity in squid muscle. The activity was observed in both acidic and alkaline pH ranges with a maximum pH 3.0 (Sakai and Matsumotto, 1981). Sub cellular distribution study revealed that the acid proteinase activity is localised in lysosomes. These studies indicated the presence of a cathepsin D like proteinase and a cysteine preoteinase in squid mantle muscle, the former being the major enzyme in lysosomes. When the extraction was carried out in the presence of Dithiotritol (DTT), the acid proteinase activity increased, indicating that the squid mantle muscle contains a considerable amount of cysteine proteinase, thus refuting the presence of cathepsin B, H and L in the squid mantle muscle. The cathepsin D-like proteinase and cysteine proteinase were

separated by Diethylaminoethane (DEAE)-Sephadex A-50 column chromatography.

9.3. Materials and Methods

The analyses of lysosomal enzymes were done as per the method of Warrier, et al., (1972).

9.3.1. Reagents

- 0.25M sucrose containing 1mM EDTA (8.5575g of sucrose and 40mg of EDTA in 100ml of distilled water).
- 0.25M sucrose containing 1mM EDTA with 0.1% Triton X-100.
- Citrate buffer (100mM, pH 4.8) 4.1g of citric acid and 11.25g of sodium citrate. Dissolved and made up to 500ml with distilled water and stored at 4°C.
- Substrate- *p-nitro phenyl phosphate* (400mg dissolved in 100ml of distilled water).
- 0.1N NaOH
- Standard *p-nitro phenol* -1mM, 0.13911 g/1000ml.

9.3.2. Preparation of tissue homogenate

➢ Total Activity

Fresh and treated samples of both species of squid, loligo and needle, as explained in 8.3 were taken for this study. The sample tissues were finely minced with scissors and 10% (w/v) tissue suspension was prepared in 0.25M sucrose containing 1mM EDTA using a homogeniser set at minimum speed for 20 sec. The homogenates were centrifuged at 4°C and collected

the supernatant. The residue was washed twice in the same media and washings were combined with the supernatant. A similar homogenate was also prepared by thorough homogenisation of the sample (10% w/v) in the above solution with 0.1% Triton X –100 and taken as total activity.

For each assay, samples were taken in triplicate.

- **Free Activity**

The free activity was determined from 10% w/v tissue cut into small pieces using scissors and suspended in cold 0.25 M sucrose solution containing I mM. EDTA. The suspensions were passed through a single layer of surgical gauze and the residue obtained were washed 2 or 3 times with the same media and the washings were added together.

- **Drip**

The frozen samples were thawed and the exudate or drip was collected.

- **Bound Activity**

Bound Activity (Latent activity) was determined by subtracting free activity from total activity.

$$\% \text{ Bound Activity} = \frac{\text{Total activity Free activity}}{\text{Total Activity}} \times 100$$

9.3.3. Assay of Acid phosphatase

0.5ml of Buffer was taken in a test tube, added 0.5ml of substrate and 0.5ml of the enzyme extract. Incubated the test tubes at 37°C for 30 minutes and stopped the reaction by adding 4ml of 0.1N NaOH. To the control extract was added after adding the NaOH. The optical density was read at

405nm along with standards and blank. The protein in the extracts was estimated by Lowry, et al., (1951).

The lysosomal enzyme activity was expressed in terms of acid phosphatase activity as micrograms of p-nitro phenol liberated per minute per ml (EU) and specific activity as micrograms of p – nitro phenol liberated per minute per mg protein.

9.3.4. Protease activity

Protease (cathepsin-D like) activity in all the above extracts- total, latent and free were assayed according to the method described in 8.3. The pH of the medium was 3.0. Here also enzyme assays were conducted in triplicate. The protease activity was expressed as μg of tyrosine per ml per minute.

9.4. Results

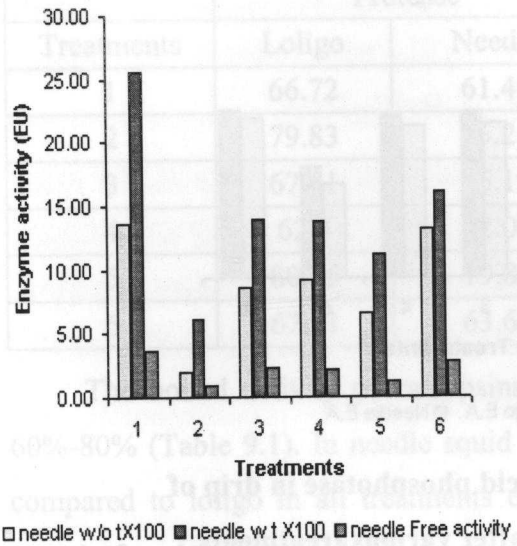


Figure 9.1 Effect of treatments on lysosomal on acid phosphatase in needle squid

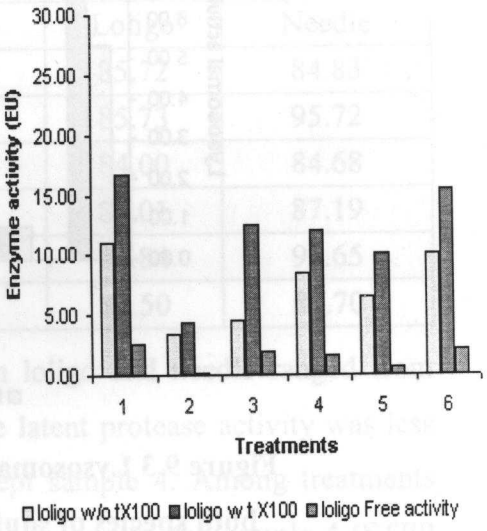


Figure 9.2 Effect of treatments on lysosomal on acid phosphatase in loligo

1-Control, 2-Acetic acid alone, 3-Acetic acid citric acid mixture

4-STPP acetic acid mixture, 5-Ascorbic acid, 6-Lime Juice

Profiles of lysosomal activity of both the species studied under various treatments are given in Fig 9.1 and 9.2. The activity was observed to be higher in needle squid when compared with loligo. As in the case of proteolytic enzymes, lysosomal enzymes also showed a very low activity in samples treated with acetic acid alone. Untreated sample showed maximum activity followed by limejuice. Sample treated with acetic acid citric acid mixture and acetic acid STPP mixture exhibited almost similar activity in both the species. The samples treated with ascorbic acid showed a better result when compared with the above treatments

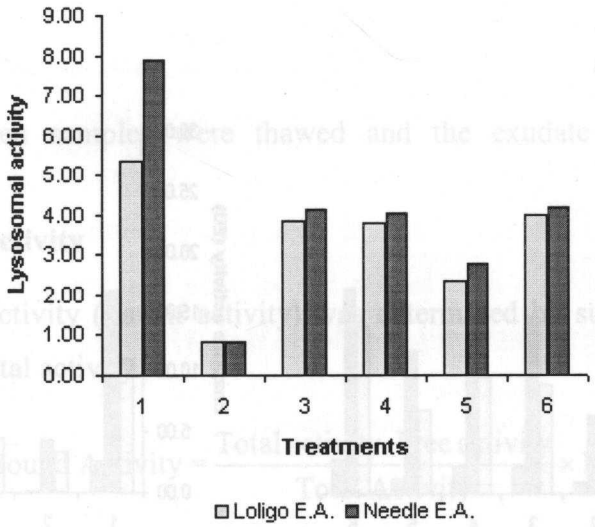


Figure 9.3 Lysosomal acid phosphotase in drip of both species of squid after various treatments.

Figure 9.3. explains the lysosomal acid phosphotase activity in drip of both the species under various treatments. The quantity of drip was very less

in samples treated with acetic acid STPP mixture, but the drip showed a high activity. Acetic acid alone treated sample showed minimum activity in the drip also. The quantity of drip in samples treated with acetic acid alone was less when compared with other treatment systems, but more than the samples treated with acetic acid STPP mixture. The quantity of drip and the enzyme activity was maximum in the untreated samples. Ascorbic acid showed favourable results in the drip also. In all the treated samples, acid phosphatase activity was slightly higher in needle squid than loligo, while the untreated samples showed significant difference between the two species.

Table 9.1. Profile of percentage of bound activity (latent activity) of lysosomal acid phosphatase and protease in the squid mantle tissue

	Protease		Acid phosphatase	
Treatments	Loligo	Needle	Loligo	Needle
1	66.72	61.49	85.72	84.83
2	79.83	75.25	85.73	95.72
3	67.61	65.19	84.00	84.68
4	62.3	72.02	85.01	87.19
5	80.75	79.85	88.84	93.65
6	67.81	63.61	82.50	86.70

The bound activity of cathepsins in loligo and needle ranged from 60%-80% (Table 9.1). In needle squid the latent protease activity was less compared to loligo in all treatments except sample 4. Among treatments sample 5 and 2 showed the highest values. In needle, the sample 4 (STPP treated samples) resulted in higher retention of lysosomal bound enzyme activity compared to loligo.

The latent activity in terms of acid phosphatase showed higher values in needle compared to loligo in all the treated samples, while in untreated samples needle showed a lower value.

Table 9.2 Lysosomal Protease activity in the mantle tissue and the drip of both species of squid after treatments.
(EU microgm of tyrosine / ml / minute)

Treatments	Loligo (EU)				Needle (EU)			
	A	B	C	D	A'	B'	C'	D'
1	63.04	90.76	34.95	7.89	46.38	78.27	26.09	5.35
2	5.76	9.86	2.45	0.84	4.76	4.96	0.17	0.81
3	15.59	27.36	9.53	3.06	15.23	21.16	6.86	3.78
4	15.49	26.42	7.40	3.83	14.73	16.96	6.40	3.86
5	14.63	18.03	3.63	1.78	12.99	16.43	3.17	2.33
6	19.89	30.49	11.10	4.20	16.53	24.96	8.03	2.97

A, A'- without Triton X100, B, B' – with Triton X100,
C, C'-Free activity, D, D'– Drip

Table 9.2 shows the lysosomal protease activity in the mantle tissue and the drip in both species of squid. In this also, a same trend was obtained as in the case of protease activity, which showed a minimum activity in sample 2 and maximum activity in sample 1. Sample 2 and 5 showed significantly lower activity than the other treatments.

9.5. Discussions

Sakai et al., (1983) have separated a cysteine proteinase and cathepsin D like proteinase from squid mantle muscle by using a buffer containing Triton X-100, acid treatment, ammonium sulphate fractionation and column

chromatography. The latter proteinase has been recently purified and confirmed to be cathepsin D. The optimum temperature was at 45°C and pH 3.0 and the activity was almost zero at pH 5.0 (Sakai, 1986). Most of the cathepsin D activity was removed by pepstatin and leupeptin, which strongly inhibited the squid cysteine proteinase activity (Umezawa and Aoyagi, 1977). From the study, it was clear that cathepsin D is present in the squid meat, which was taken for the present study. Thus the result indicated that cathepsin D like proteinase contributed mainly to the protein degradation in squid mantle muscle at this pH. Since cathepsin D is believed to constitute the major proteolytic activity in lysosomes (Barrett, 1972), lysosomes may be involved in the protein degradation of squid mantle muscle.

When 10% homogenate of the mantle tissue prepared in 0.25M sucrose containing 1mM EDTA using a homogeniser at minimum speed for 20 seconds were employed for assessing the free and bound activity of Cathepsins (EC 3.4.4.2) and acid phosphatase (EC 3.1.3.2), it was observed that these enzymes were mostly in free form in muscle homogenate. The tissue was subjected to homogenisation with Triton X100 to solublize the lysosomal membrane lipoproteins and to liberate all enzyme into solutions. Treatment such as blenderisation, repeated freeze -thaw cycles, addition of Triton X100 etc. bring about almost complete release of the enzymes.

Homogenisation technique due to limitations could give high free activities, as evidenced by the high activity in the homogenate prepared without Triton X100, in control as well as test samples. Therefore using homogenisation technique to monitor endogenous free activity will be misleading. A method reported by Hari kumar et al., (1974), using tissue slices was used to study the *invivo* changes occurring in the lysosome due to various

treatments. The difference in the total and free activities is computed as bound or latent activity thus accounting for 60-80% for cathepsins and 82-95% in acid phosphatase. The results on acid phosphotase activity (both total and free) show significant variation in the release or solubilisation of the enzymes under various treatments in both the species of squid. The homogenisation with and without triton X 100 showed almost similar values confirmed the high solubility of squid proteins including enzymes into the extracting medium. Irradiation or freezing of muscle of *Harpodon nehereus* (Bombay duck) enhanced the levels of lysosomal activities (Warrier, et al., 1972).

9.6. Conclusion

The presence of cathepsin D like proteases of lysosomal origin is involved in the protein degradation in squid mantle muscle. Treatment is inevitable in the case of squid in order to maintain the storage quality. The treatment with acetic acid, acetic acid STPP mixture reduced the quantity of drip, while acetic acid STPP mixture treated sample showed a higher lysosomal enzyme activity in the drip than the acetic acid treated sample. In the present study, the level of lysosomal enzyme released into the medium was reduced by various treatments. The reduced amount of lysosomal enzymes in the drip of acetic acid treated sample and their low activity in the drip confirm the ability of acetic acid to maintain the inter-protein network and retain proteins including water soluble enzymes in the gel matrix to the maximum. The difference in the rates of release of enzymes could be due to the difference in the binding of these enzymes to the lipoprotein membrane matrix. This is very well supported by the increased bound activity in the respective samples.

9.7. References

Barrett, A. J., (1972) Lysosomal enzymes. Lysosomes A Laboratory handbook (Ed, Dingle, J.T.) North- Holland, Amsterdam. **46**-135.

Cardelli, J.A., (1993) Regulation of lysosomal trafficking and function during growth and development of *Dictyostelium discoideum*. In advances in cell and molecular biology of membranes, Vol. I (ed. B. Storrie and R. Murphy), 341-390, JAI Press, Inc.

*Duve De, C., (1959) Cited in subzellular particles, edited by Hayashi, T (Ronald Press Newyork) 128.

Harikumar, P., Ninjoor, V., and Kumta, U.S., (1974) A method for evaluation of latency of lysosomal enzymes from chicken skeletal muscle tissue. Manuscript translated and published in "DIB FLEISEHWIRTSCHAFT" Jahrgang-Mai.

Kolodziejska, I. and Sikorski Zdzislaw, E., (1996) Muscle cathepsins of marine fish and invertebrates. Polish J. Fd. Nutr. Sci. **4/45** (3), 3-10.

Lesly A. Temesvari, John M. Bush, Michelle D. Peterson, Kristine D. Novak, Margret A. Titus and James A. Cardelli, (1996) Examination of the endosomal an lysosomal pathways in the *Dictyostelium discoideum* myosin 1 mutants. J. Cell Sci. **109**, 663-673.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**, 265-275.

Melanto, J.A., Beltran, J.A., Roncales, P., (1998) Tenderisation of squid with bromelain and a bovine spleen lysosomal enriched extract. Fd. Research Intl. **30** (5), 33-341.

Melanto, J.A., Beltran, J.A., Roncales, P., (1999) Characterisation of a crude lysosomal extract from bovine spleen in the processing of muscle foods. Fd. Biotech. **12** (3), 239-262.

Melanto, J.A., Beltran, J.A., Roncales, P., (2001) Preservation of the proteolytic activity of a bovine spleen lysosomal enriched using various freezing conditions. Enzyme and microbial technology, **28** (4/5), 453-459.

Migita, M., Matsumotto, J.J., Aoe, N. and Saishu, T., (1958) On a specific behaviour of "myosins" of squid muscle- I. Precipitability of "myosins'. Bull. Japan. Soc. Sci. Fish. **24**, 51-58.

Rema, L.P. and Philip, B., (1999) Effect of heavy metals on some metabolically important enzymes of *Oreochromis mossambicus.* Fish. Technol. **36** (1), 8-12.

Sakai, J.S. and Matsumotto, J.J., (1981) Proteolytic enzymes of squid mantle muscle. J. Comp. Biochem. Physiol. Vol. **68B**, 389-395.

Sakai, J.S., Sakaguchi Hoshino, S. and Matsumotto, J.J., (1983) Separation of cathepsin D like proteinase and acid thiol proteinase of squid mantle muscle. Comp. Bio. Chem. Physiol. **75B**, 409-414.

Sakai, J.S., Tsuchiya, T. and Matsumotto, J.J., (1986) Purification and characterisation of acid cysteine proteinase from squid mantle muscle. J. Comp. Biochem. Physiol. Vol. **85B**, (4), 887-893.

Shann Tzong Jiang., (2000) Effects of proteinases on the meat texture and seafood quality. Fd. Sci. Agri, Chem. **2** (2), 55-74.

Umezawa, H and Aoyagi, T., (1977) Activities of proteinase inhibitors of microbial origin. In Proteinases in Mammalian cells and Tissues

(Edited by Barrett A. J.) 637-662. North-Holland, Amsterdam, The Netherlands.

Warrier, S. B. K. Ninjoor, V. Sawant P.L. and Kumta, U.S., (1972) Lysosomal in drip and Muscle of *Harpodon nehereus*. Indian J. of Biochem. Biophys. Vol **9** (2) 207 – 209.

*Weinstok and Iodice, A.A., (1969) Cited in lysosomes in biology and pathology Vol I Eds. Dingle J.T. and Fell, H.B. (North Holland publishing company, Amsterdam) 451.

* Not referred in original

Chapter 10

SUMMARY AND CONCLUSION

The thesis on the topic "Influence of processing variables on protein quality and frozen storage stability of two commercially important species of squid (*Loligo duvaucelii and Doryteuthis sibogae*)" is presented in 10 chapters.

Chapter 1. This chapter gives a brief account of the present export status of seafood from India and the role played by squid in the export. This chapter also describes the significance of the research work undertaken and the objectives to which the study is focusing.

Chapter 2. The protein content of the squid was compared with other marine cephalopods and thereby to emphasize the nutritive value of squid. Proximate composition of both species of squid was compared with other cephalopods. The variations in protein during various months were also studied in different species. The fractions of proteins namely sarcoplasmic, myofibrillar, denatured and stroma proteins were extracted and analyzed. From the results, the squid was evaluated to be a good source of protein. The repeated washing resulted in the loss of proteins along with the other soluble substances, thus reducing the quality. The time in between the landing and the processing factory is found to be very crucial, since it directly reduces the quality of squid. Utmost care is to be taken to reduce the hazards. Thus practice of storing squid directly in slush ice at any point from landing to processing is not advisable due to the high dissolution of

myosin fraction and other proteins in water. The loss of proteins as well as Non-protein nitrogen (NPN) fraction could affect the organoleptic quality of squid in general. The sweet taste of squid is linked to the NPN fraction. The leaching rate of needle squid was found to be much higher than that of loligo squid. This leads to a considerable loss of nutrients and higher rate of degradation in needle squid compared to loligo squid. Hence further investigations were undertaken to reduce this nutritional loss and quality degradation. The microbiological study was done for total plate count, sanitary significant bacteria like *Escherichia coli*, *Coagulase positive staphylococcus* and pathogens like *Salmonella* and *Vibrio cholerae*.

Chapter 3. In this chapter various changes in quality parameters during different methods of ice-storage were studied in detail. The methods adopted for the icing were with GMP, without GMP and without direct contact with ice and water. In method-1- with GMP, (Good Manufacturing Practices) flake ice was used and the ice was changed with fresh ice daily. In method-2- without GMP, block ice was used for icing and samples were kept in slotted boxes, allowing the melt water to flow out. In this case, the ice was not changed daily but was replenished as required. In Method–3- without direct contact with ice and water, the samples were packed in polythene covers and kept in ice (with GMP) to avoid direct contact with ice and water. In all the three methods insulated boxes were used.

Moisture, fat and ash were calculated and statistically compared between methods, species and days of storage using students t test. The chemical indices like trimethyl amine (TMA), total volatile base nitrogen (TVBN) and alpha amino nitrogen were estimated using trichloro acetic acid extract. Organoleptic quality and pH of the tissue were monitored in all the

above methods. Organoleptic assessment was done by sensory test and the degree of excellence was given by hedonic scale. Microbiological quality changes were also studied during ice- storage.

Needle squid showed a faster rate of deterioration than loligo. Leaching rates of protein and alpha amino nitrogen were more in needle squid than in loligo. Non-contact ice-stored material showed a low rate of leaching. As per the results, the ice-storage was not found to be advisable beyond 6 days even with GMP. The storage time could be prolonged using non-contact ice.

Chapter 4. The material was kept at –2° C for 8 days. The temperature was maintained by mixing 3% sodium chloride with ice and the material was layered with the ice in the ratio 1:2 and the samples were kept in a chill store maintained at -10°C. The physical, chemical and microbiological indices were estimated and statistically compared between species, methods and days of storage using 3-way ANOVA. The results showed that the samples kept with GMP had a better quality than the samples without GMP.

A similar lot with GMP was frozen by IQF (individually quick frozen) and the samples were drawn at different points of the processing line for analyses. Results showed that the repeated washing of squid tube during processing should be avoided in order to minimize the nutrient loss during the process. The time of direct contact with ice during processing also should be reduced. Leaching of protein showed significant difference between species, steps and days of storage. Fractions of protein also showed significant difference in leaching. From the study, it is also suggested to adopt a proper treatment to reduce the leaching effect.

Chapter 5. Frozen samples were stored at –20°C to -25°C and samples were drawn at every 30 days for six months of frozen storage to evaluate physical, chemical and microbiological parameters. 2-way ANOVA was used for comparison of different quality parameters between species and days of storage. The variations in water soluble and salt soluble fractions of protein were very significant during the storage. But the difference in denatured protein was not significant between days and species. Among the various storage systems, the storage without direct contact with ice is proved better for short period preservation, while frozen storage is advised for longer periods.

Chapter 6. The effects of six treatments in the two species of squid were assessed by physico-chemical, microbiological and organoleptic studies. The systems used were (a) 0.3% Citric acid (b) 0.3%Citric acid + 3%acetic acid (c) 3% Acetic acid (d) 3% Sodium tripolyphosphate (STPP) + 3 % acetic acid (e) 0.3%Ascorbic acid (f) 0.3%Limejuice. A control was also taken without any treatments. All the samples were mixed with 3% salt and material to ice in the ratio 1:2. These were kept for 20 minutes. After the treatment, the samples were kept in non-contact ice with GMP for 3 days and samples were drawn for various analyses. The results were compared using 2-way ANOVA.

Another set of treated samples were immediately frozen and stored for 6 months at –20°C to -25°C and analyzed for physico-chemical, microbiological and sensory evaluation at thirty days of interval. The results were analyzed using 3way ANOVA. Even though the leaching rate was minimum in STPP acetic acid mixture treated sample, its bacteriological and organoleptic qualities were inferior to that of the sample treated with acetic

acid alone. The effect of cooking was studied at various temperatures from 40°C to 100°C. Even though STPP treated sample reduced the drip loss in the sample, the organoleptic quality was found to be lost while cooking. Among all the treatments, acetic acid treated sample was most acceptable in both the species. The treatments like citric acid treatment, STPP treatment were avoid due to the poor quality standards of the products.

Chapter 7. Aqueous extract of mantle was used for the study and PAGE was done using disc electrophoretic apparatus according to the method of Laemmli. The samples selected for this study included ice stored (with GMP, without GMP and without direct contact with ice and water), chill stored (with and without GMP), from various processing steps, frozen stored and the treated sample. PAGE was carried out in the presence of sodium dodecyl sulphate (SDS). It was found that 10 % gel strength was appropriate for a distinct separation of squid proteins.

The bands obtained were characterized by comparing with protein molecular weight markers. The extractability of the proteins in water, phosphate buffer and borate buffer were also compared. Characterization of the proteins in samples from various storage systems and treatments were also carried out. From the protein profile, it was observed that the rate of extraction of proteins from needle squid was more compared to loligo. Leaching of protein in the sample kept in direct contact with ice and water was confirmed by the loss of protein bands. Myosin, which is a component of myofibrillar protein, was also solublised in aqueous extract. Mild acid treated sample showed feeble bands of Myosin Heavy Chain (MHC) suggesting possible disappearence due to acid-induced gelation.

Chapter 8. Squids are known to have only one to two years of life span. In order to achieve this rapid growth, the protein turnover rate must be very fast which usually results from an active proteolytic enzyme activity in the squid muscle. In this chapter tissue proteolytic enzyme activity of squid muscle was determined using haemoglobin as substrate. The effect of pH, temperature and the period of incubation time on proteolytic enzyme activity was studied and arrived at the optimum conditions with respect to specific proteases present. The influence of GMP and various treatments on the proteolytic enzyme activity was studied in both the species. The protease enzymes in squid mantle were also characterized by studying the effect of various inhibitors like phenylmethylsulphonil fluoride (PMSF), soybean trypsin inhibitor, iodo-acetic acid and ethylenediaminetetraacetic acid (EDTA). The proteolytic activities were considerably high at pH 3.0, 6.0 and 8.0. Maximal proteolytic activity in squid muscle extract at physiological pH was observed at 40°C. Between the two species the needle squid exhibited a higher proteolytic activity compared to loligo.

Chapter 9. A detailed study was done on lysosomal enzyme activity of squid muscle as an index of autolysis. The lysosomal stability of both the species of squid under various processing variables adopted was studied in detail. The enzyme acid phosphatase was used as an indicator for lysosomal stability. The lysosomal activity of the drip from freezing and thawing procedure was done. The results showed that the lysosomal activity was higher in needle squid than in loligo. Among the various treatments acetic acid treatment reduced the lysosomal activity considerably. The presence of cathepsin D like proteases of lysosomal origin is involved in the protein degradation in squid mantle muscle. Treatment is inevitable in the case of

squid in order to maintain the storage quality. In the present study, the level of lysosomal protease enzyme released into the medium was reduced by various treatments. Reduced amount of lysosomal enzymes in the drip of acetic acid treated sample and their low activity in the drip confirm the ability of acetic acid to maintain the inter-protein network and retain proteins including water soluble enzymes in the gel matrix to the maximum. The difference in the rates of release of enzymes could be due to the difference in the binding of these enzymes to the lipoprotein membrane matrix. This is very well supported by the increased bound activity in the respective samples.

If fishermen, processor and retailers understand the factors, which affect seafood quality and make a conscientious effort to control these quality factors, problems associated with autolytic and microbial changes could be significantly minimized. Every stage of handling from harvest to consumption affects quality. In the processing plants and at retail outlets good sanitary conditions and strict temperature conditions must be maintained so as to provide high quality products to the consumer. The time- temperature management throughout the process line should be strictly followed.

APPENDICES

APPENDIX C.0.

Product	Overall quality	Range of score	Criteria
Whole squid (raw)	Excellent/ very good	10-> 7	Squid odour, sheen white colour with red spots firm elastic texture.
	Good to fair	7-5	Slight squid odour, red brown spots firm and elastic texture
	Unacceptable	Below 5	Offensive odour moderate to intense pink spot flabby texture.
Processed squid	Excellent/ very good	10-> 7	Squid odour, sheen off-white colour firm elastic texture.
	Good to fair	7-5	Slight cabbage odour, creamy to yellowish appearance. Rubbery firm texture
	Unacceptable	Below 5	Slight to intensive ammoniacal odour. Slight to intense yellow discolouration. Curdy texture.
Whole squid cooked	Excellent/very good	10-> 7	Fresh Squid odour, Off-white or cream white. Firm and juicy texture. Sweetmeat flavour.
	Good to fair	7-5	Cooked cabbage odour, No discolouration. Firm and slightly tough texture. Slight squid to cabbage flavour.
	Unacceptable	Below 5	Ammoniacal to foul odour. Yellow or brown discolouration. Mushy or Curdy texture. Slight to very bitter flavour.
Processed squid (cooked)	Excellent/very good	10-> 7	Fresh Squid odour, Off-white or cream white. Firm and juicy texture. Sweetmeat flavour.
	Good to fair	7-5	Cooked cabbage odour, No discolouration. Firm and slightly tough texture. Slight squid to cabbage flavour.
	Unacceptable	Below 5	Ammoniacal to foul odour. Yellow or brown discolouration. Mushy or Curdy texture. Slight to very bitter flavour.

Appendix C.1.

ANOVA table for moisture content during ice storage

Source	SS	df	ms	F	significance
Total	109.26	47			
bt sps	20.66	1	20.66	52.24	p< 0.001
bt mthds	39.28	2	19.64	49.66	p< 0.001
bt dys	34.69	7	4.96	12.53	p< 0.001
error	14.63	37	0.40		

Appendix C.2.

ANOVA table for fat content during ice storage

Source	SS	df	ms	F	significance
Total	2.706037	47			
bt sps	0.153567	1	0.153567	5.49259165	p<0.05
bt mthds	0.232489	2	0.116244	4.15767406	p<0.05
bt dys	1.285499	7	0.183643	6.56829647	p<0.05
error	1.034482	37	0.027959		

Appendix C.3.

ANOVA table for ash content during ice storage

Source	SS	Df	ms	F	significance
Total	2.915	47			
bt sps	0.587	1	0.587	38.304	p< 0.001
bt mthds	0.847	2	0.424	27.615	p< 0.001
bt dys	0.914	7	0.131	8.510	p< 0.001
error	0.567	37	0.015		

Appendix C.4.

ANOVA table for protein leaching during ice storage

Source	SS	df	ms	F	significance
Total	552.91	47			
bt sps	9.50	1	9.50	6.94	p< 0.001
bt stps	240.76	2	120.38	87.96	p< 0.001
bt dys	252.02	7	36.00	26.31	p< 0.001
error	50.64	37	1.37		

Appendix C.5.

ANOVA table for TVN with or without direct contact with ice on storage

Source	SS	df	ms	F	significance
Total	9141.12	47			
bt sps	141.80	1	141.80	2.95	NS
bt stps	3637.01	2	1818.51	37.83	p< 0.001
bt dys	3583.53	7	511.93	10.65	p< 0.001
error	1778.78	37	48.08		

LSD for methods = 11.44

LSD for days = 7.006

Appendix C.6.

ANOVA table for TMA during ice storage

Source	SS	df	ms	F	significance
Total	5953.41	47			
bt sps	204.8477	1	204.8477	5.611	p< 0.01
bt stps	2321.923	2	1160.962	31.798	p< 0.001
bt dys	2075.754	7	296.5363	8.122	p< 0.001
error	1350.885	37	36.510		

LSD for methods = 9.97

LSD for days = 6.1058

Appendix C.7.

ANOVA table for alpha amino nitrogen during ice storage

Source	SS	df	ms	F	significance
Total	119513	42			
bt sps	277.4673	1	277.4673	0.29976757	ns
bt stps	76703.35	2	38351.68	41.4340317	p< 0.001
bt dys	12912.72	7	1844.674	1.99293225	ns
error	29619.46	32	925.6081		

LSD for methods = 47.9

Appendix C.8.

ANOVA table for changes in sarcoplasmic protein during ice storage

Source	SS	df	ms	F	significance
Total	304.3354	47			
bt sps	21.18692	1	21.18692	63.4227582	p< 0.001
bt mthds	160.6548	2	80.32741	240.459042	p< 0.001
bt dys	110.1335	7	15.73335	47.0975864	p< 0.001
error	12.36017	37	0.334059		

LSD for methods = 0.9438

LSD for days = 0 .5779

Appendix C.9.

ANOVA table for myofibrillar protein leaching during ice storage

Source	SS	df	ms	F	significance
Total	71.2046	47			
bt sps	14.509	1	14.509	240.109392	p< 0.001
bt mthds	10.77263	2	5.386315	89.1380891	p< 0.001
bt dys	43.68718	7	6.241026	103.282702	p< 0.001
error	2.235785	37	0.060427		

LSD for methods = 0.4014

LSD for days = 0 .2458

Appendix C.10.

ANOVA table for changes in denatured protein during ice storage

Source	SS	df	ms	F	significance
Total	0.3108	47			
bt sps	0.0169	1	0.0169	22.059473	p< 0.001
bt mthds	0.0890	2	0.0445	58.14169	p< 0.001
bt dys	0.1767	7	0.0252	32.9903893	p< 0.001
error	0.0283	37	0.0008		

LSD for methods = 0.0451

LSD for days = 0 .02765

Appendix C.11.

ANOVA table for changes in stroma protein during ice storage

Source	SS	df	ms	F	significance
Total	0.3888	47			
bt sps	0.0690	1	0.0690	188.842527	p< 0.001
bt mthds	0.0079	2	0.0040	10.8149461	p< 0.001
bt dys	0.2984	7	0.0426	116.637552	p< 0.001
error	0.0135	37	0.0004		

LSD for methods = 0.03119
LSD for days = 0 .0191

Appendix C.12.

ANOVA table for TPC during ice on storage

source	Ss	df	ms	F	significance
total	15.504	77			
bt sps	0.000185	1	0.000185	0.283	NS
bt metds	15.008	2	7.504024	11520.694	p < 0.001
bt days	0.456	12	0.03798	58.310	p < 0.001
error	0.040	62	0.000651		

LSD for methods = 0.04166
LSD for days = 0 .02001

Appendix D.1.

ANOVA table for TVN changes during chill storage

Source	ss	df	ms	F	significant
Total	7902.035	31			
between species	2346.125	1	2346.125	20.61	$p< 0.001$
between methods	42.32	1	42.32	0.37	NS
between days	3009.545	7	429.935	3.78	NS
error	2504.045	22	113.82		

Appendix D.2.

ANOVA table for TMA changes during chill storage

Source	ss	df	ms	F	significant
Total	11405.33	31			
bt sps	346.3712	1	346.37	2.12	NS
bt methods	4459.584	1	4459.58	27.33	$p< 0.001$
bt days	3009.545	7	429.94	2.63	NS
Error	3589.828	22	163.17		

Appendix D.3.

ANOVA table for alpha amino nitrogen changes during chill storage

Source	ss	df	ms	F	significant
Total	32040.29	24			
bt sps	1982.005	1	1982.01	7.29	$p< 0.01$
bt methods	1344.289	1	1344.29	4.94	$p< 0.05$
bt days	24633.37	7	3519.05	12.94	$p< 0.001$
Error	4080.63	15	272.04		
LSD	22.59				

Appendix D.4.

ANOVA table for leaching of protein during chill storage

Source	ss	df	ms	F	significant
Total	314.4756	31			
bt sps	35.57	1	35.57	34.84	p< 0.001
bt methods	25.96	1	25.96	25.42	p< 0.001
bt days	230.48	7	32.93	32.24	p< 0.001
Error	22.47	22	1.021		
LSD		1.099			

Appendix D.5.

ANOVA table for leaching of sarcoplasmic protein during chill storage

Source	ss	df	ms	F	significant
Total	171.96	31			
bt sps	15.11	1	15.11	87.37	p< 0.001
bt methods	51.94	1	51.94	300.34	p< 0.001
bt days	101.11	7	14.44	83.51	p< 0.001
Error	3.80	22	0.172947		
LSD for days		0.609			

Appendix D.6.

ANOVA table for leaching of myofibrillar protein during chill storage

Source	ss	df	ms	F	significant
Total	20.00875	31			
bt sps	0.845	1	0.845	8.28	p< 0.001
bt methods	8.82	1	8.82	86.43	p< 0.001
bt days	8.099	7	1.156964	11.34	p< 0.001
Error	2.245	22	0.102045		
LSD	0.4684				

Appendix D.7.

ANOVA table for changes in denatured protein during chill storage

Source	ss	df	ms	F	significant
Total	0.175822	31			
bt sps	0.038503	1	0.038503	59.68	p< 0.001
bt methods	0.018528	1	0.018528	28.72	p< 0.001
bt days	0.104597	7	0.014942	23.16	p< 0.001
Error	0.014194	22	0.000645		
LSD	0.3724				

Appendix D.8.

ANOVA table for leaching of stroma protein during chill storage

Source	ss	df	ms	F	significant
Total	1.009097	31			
bt sps	0.073153	1	0.073153	31.38	p< 0.001
bt methods	0.093528	1	0.093528	40.11	p< 0.001
bt days	0.791122	7	0.113017	48.47	p< 0.001
Error	0.051294	22	0.002332		
			LSD	0.0708	

Appendix D.9.

ANOVA table for TPC on Chill Storage with or without GMP.

Source	ss	df	ms	F	significant
Total	1.8177	51			
bt sps	0.0003	1	0.00029	0.03939	NS
bt methods	0.5508	1	0.55082	75.19154	p <0.001
bt days	0.9955	12	0.08296	11.32481	p <0.001
Error	0.2710	37	0.00733		

LSD between days 0.1223

Appendix D.10.

ANOVA table for TVN changes on chill storage during various processing steps

Source	ss	df	Ms	F	significant
Total	673685.3	79			
bt sps	10026.24	1	10026.24	1.013	NS
bt steps	559.667	4	139.92	0.014	NS
bt days	258.68	7	36.95	0.004	NS
error	662840.8	67	9893.15		

Appendix D.11.

ANOVA table for TMA changes on chill storage during various processing steps

Source	ss	df	ms	F	significant
Total	11544.62	79			
bt sps	4367.195	1	4367.19	103.18	$p< 0.001$
bt steps	654.6513	4	163.66	3.87	NS
bt days	3687.046	7	526.72	12.44	$p< 0.001$
error	2835.728	67	42.32		

LSD between days -12.93

Appendix D.12.

ANOVA table for alpha amino nitrogen changes on chill storage during various processing steps

Source	ss	df	Ms	F	significant
Total	74907.6	60			
bt sps	741.99	1	741.99	1.48	NS
bt steps	546.61	4	136.65	0.27	NS
bt days	49476.17	7	7068.02	14.05	$p <0.001$
error	24142.82	48	502.98		

LSD between days -22.4271

Appendix D.13.
ANOVA table for protein content on chill storage during various processing steps

Source	ss	df	Ms	F	significant
Total	547.46	79			
bt sps	73.73	1	73.73	224.46	p<0.001
bt steps	49.72	4	12.43	37.85	p<0.001
bt days	402.00	7	57.43	174.84	p <0.001
error	22.01	67	0.33		

LSD between steps- 0.8105
between days - 0.5731

Appendix D.14.
ANOVA table for sarcoplasmic protein on chill storage during various processing steps

Source	ss	df	Ms	F	significant
Total	420.60	79			
bt sps	36.71	1	36.71	204.23	p<0.001
bt steps	98.33	4	24.58	136.78	p<0.001
bt days	273.52	7	39.07	217.40	p <0.001
error	12.04	67	0.18		

LSD between steps-0.5362, between days-0.4239

Appendix D.15.
ANOVA table for myofibrillar protein on chill storage during various processing steps

Source	ss	df	ms	F	significant
Total	44.28	79			
bt sps	0.26	1	0.26	4.74	p<0.01
bt steps	21.89	4	5.47	98.12	p<0.001
bt days	18.38	7	2.63	47.08	p <0.001
error	3.74	67	0.06		

LSD between steps- 0.2987, between days-0.2357

Appendix D.16.
ANOVA table fr TPC on chill storage during various processing steps.

source	ss	df	ms	F	significant
total	28.31	129			
bt sps	1.02	1	1.02	103.51	p <0.001
bt steps	21.90	4	5.47	553.77	p <0.001
bt days	4.28	12	0.36	36.09	p <0.001
error	1.11	112	0.01		
lsd between steps		0.07799	lsd between days		0.08892

Appendix E.1

ANOVA Table for changes in TVN during Frozen storage

source	total	df	ms	F	Significant
ss	266.69	13			
bt sps	35.52	1	35.52	14.59	$p < 0.001$
bt days	216.56	6	36.09	14.83	$p < 0.001$
error	14.60	6	2.43		
LSD 3.817					

Appendix E.2.

ANOVA Table for changes in TMA during Frozen storage

source	total	df	ms	F	Significant
ss	190.86	13			
bt sps	25.52	1	25.52	15.29	$p < 0.001$
bt days	155.33	6	25.89	15.52	$p < 0.001$
error	10.01	6	1.67		
LSD 3.18					

Appendix E.3.

ANOVA Table for changes in Alpha amino nitrogen during Frozen storage

source	total	df	ms	F	Significant
ss	8505.46	13			
bt sps	16.94	1	16.94	0.5090144	NS
bt days	8288.84	6	1381.473	41.510617	$p < 0.001$
error	199.68	6	33.28		
LSD	14.11				

Appendix E.4.

ANOVA Table for changes in protein during Frozen storage

source	total	df	ms	F	Significant
ss	66.66	13			
bt sps	16.26	1	16.26	20.45	$p < 0.001$
bt days	45.63	6	7.60	9.56	$p < 0.001$
error	4.77	6	0.80		
LSD 2.1818					

Appendix E.5.

ANOVA Table for changes in sarcoplasmic protein during Frozen storage

source	total	df	ms	F	Significant
ss	39.407	13			
bt sps	2.889	1	2.89	523.06	$p < 0.001$
bt days	36.484	6	6.08	1100.82	$p < 0.001$
error	0.033	6	0.01		
LSD	0.181				

Appendix E.6.

ANOVA Table for changes in Myofibrillar protein during Frozen storage

source	total	df	ms	F	Significant
ss	42.82	13			
bt sps	0.47	1	0.472	7.405	$p < 0.001$
bt days	41.97	6	6.995	109.791	$p < 0.001$
error	0.38	6	0.064		
LSD	0.62				

Appendix E.7.

ANOVA Table for changes in Denatured protein during Frozen storage

source	total	df	ms	F	Significant
ss	18.04	13			
bt sps	0.17	1	0.17	8.52	$p < 0.05$
bt days	17.76	6	2.96	152.88	$p < 0.001$
error	0.12	6	0.02		
lsd	0.34				

Appendix E.8.

ANOVA Table for changes in Stroma protein during Frozen storage

source	total	df	ms	F	Significant
ss	0.0870	13			
bt sps	0.0138	1	0.013829	49.64	$p < 0.001$
bt days	0.0715	6	0.011917	42.78	$p < 0.001$
error	0.0017	6	0.000279		
LSD	0.0408				

Appendix E.9.

ANOVA Table for TPC during Frozen storage

source	total	df	ms	F	Significant
ss	0.019	13			
bt sps	0.017	1	0.02	752.28	$p < 0.001$
bt days	0.002	6	0.00003	11.56	$p < 0.001$
error	0.0001	6	0.00002		
LSD	0.01177				

Appendix F.1.

ANOVA Table for changes in TVN during various treatment systems

source	total	df	ms	F	Significant
ss	130.25	13			
bt sps	28.86	1	28.86	18.97	$p < 0.001$
bt trmts	92.27	6	15.38	10.11	$p < 0.001$
error	9.13	6	1.52		
LSD-3.018					

Appendix F.2.

ANOVA Table for changes in TMA during various treatment systems

source	total	df	ms	F	Significant
ss	59.95	13			
bt sps	12.63	1	12.63	41.65	$p < 0.001$
bt trmts	45.50	6	7.58	25	$p < 0.001$
error	1.82	6	0.30		
LSD-1.34					

Appendix F.3.

ANOVA Table for changes in Alpha amino nitrogen during various treatments

source	total	df	ms	F	Significant
ss	9050.16	13			
bt sps	296.24	1	296.24	11.36	$p < 0.01$
bt trmts	8597.4	6	1432.9	54.93	$p < 0.001$
error	156.52	6	26.09		
LSD-12.49					

Appendix F.4.

ANOVA Table for changes in protein during various treatments

source	total	df	ms	F	Significant
ss	116.98	13			
bt sps	9.28	1	9.28	36.62	$p < 0.001$
bt trmts	106.17	6	17.70	69.80	$p < 0.001$
error	1.52	6	0.25		
LSD-1.23					

Appendix F.5.

ANOVA Table for changes in sarco plasmic protein during various treatments

source	total	df	ms	F	Significant
ss	109.74	13			
bt sps	10.53	1	10.53	55.25	$p < 0.001$
bt trmts	98.07	6	16.35	85.78	$p < 0.001$
error	1.14	6	0.19		
LSD-1.068					

Appendix F.6.

ANOVA Table for changes in Myofibrillar protein during various treatments

source	total	df	ms	F	Significant
ss	70.48	13			
bt sps	5.65	1	5.65	18.19	$p < 0.001$
bt trmts	62.97	6	10.50	33.81	$p < 0.001$
error	1.86	6	0.31		
LSD-1.36					

Appendix F.7.

ANOVA Table for changes in Denatured protein during Frozen storage

source	total	df	ms	F	Significant
ss	0.51	13			
bt sps	0.31	1	0.31	127.70	$p < 0.001$
bt trmts	0.18	6	0.03	12.39	$p < 0.001$
error	0.01	6	0.001		
LSD-0.12					

Appendix F.8.

ANOVA Table for changes in Stroma protein during various treatments

source	total	df	ms	F	Significant
ss	0.15	13			
bt sps	0	1	0	0	NS
bt trmts	0.15	6	0.02	20.71	$p<0.001$
error	0.01	6	0.001		
LSD-0.084					

Appendix F.9.

ANOVA Table for TPC during various treatments

source	total	df	ms	F	Significant
ss	1.93	13			
bt sps	0.02	1	0.02	56.39	$p < 0.001$
bt trmts	1.91	6	0.32	992.61	$p < 0.001$
error	0.002	6	0.0003		
LSD-0.043					