STUDIES ON THE ELECTROPHORETIC PATTERN OF FISH/SHELLFISH PROTEINS SUBJECTED TO FROZEN STORAGE

By

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THESIS

Submitted in partial fulfillment of the requirement for the degree

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Faculty of Fisheries
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DEPARTMENT OF PROCESSING TECHNOLOGY

COLLEGE OF FISHERIES

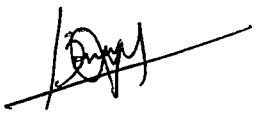
PANANGAD, COCHIN.

2000

To my parents

DECLARATION

I hereby declare that this thesis entitled **"STUDIES ON THE ELECTROPHORETIC PATTERN OF FISH/SHELLFISH PROTEINS SUBJECTED TO FROZEN STORAGE** "is a bonafide record of research work done by me during the course of research and that the thesis has not formed the basis for the award to me of any degree, diploma, associateship, or other similar title, of any other University or Society.



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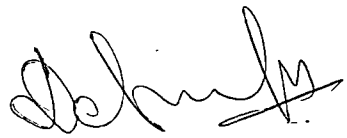

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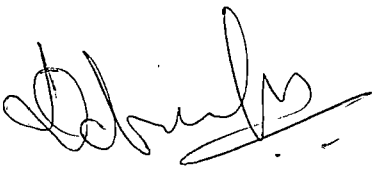

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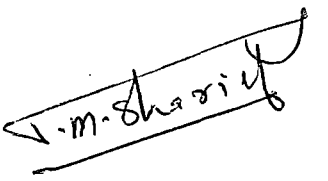

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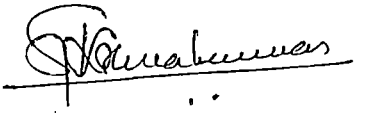

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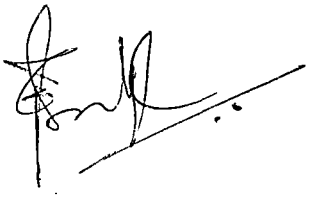

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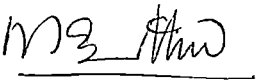

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INTRODUCTION

I. INTRODUCTION

Fish and shellfish are considered to be among the most perishable of food stuffs , as a result of a complex series of chemical, bacteriological and histological changes that occur in the muscle tissue. These inter-related processes are usually accompanied by the gradual loss or development of different compounds that affect fish quality.

Commercially icing or chilling continues to play a major role in slowing down bacterial and enzymatic activities in fish. However, this process is not designed to totally eliminate changes in quality since it offers protection only for 2-3 weeks depending on the species. Freezing is considered as an excellent process for preserving the quality of fish for longer periods of time. Freezing and subsequent cold storage are particularly useful in making seasonal species of fish and shellfish available all year round. In addition, freezing finds its application in a number of products made from different fish or shellfish species.

Good quality fish which has been properly frozen and packaged can normally be held at -20 to $-30\ ^{0}C$ for more than one year without appreciable loss in consumer acceptability (Dyer ,1968; Mackie *et al.*, 1986).

Ideally, there should be no distinguishable differences between fresh fish and frozen fish after thawing. However, deteriorative changes take place in fish and shell fish during freezing, frozen storage and thawing, which influence the quality of the finished product.

These quality changes in turn are accompanied by changes in various fish flesh components such as myofibrillar, sarcoplasmic, connective and stroma proteins, polypeptides, nucleotides, non-protein nitrogenous components etc.

Quality changes are evident from denaturation and solubility loss (Sikorski *et al.*, 1976;Shenouda,1980) as well as deteriorative changes in colour (McGill et.al.,1974;Bunda and Hultin, 1983) and texture (Dingle *et al.*, 1977, Gill *et al.*, 1979). Also the utility of fish as a raw material for fabricated products may be limited by loss of protein functionality (Colomenero and Borderias , 1983).

Many studies have been carried out to detect such changes in the physical and chemical properties of protein, for example measurement of solubility, viscosity, ATP-ase activity and the number of active SH groups (Reviewed by Ohnishi and Rodger, 1980b) . All the studies have shown that these properties are affected by freezing.Studies were also conducted to detect changes in the composition or the structure of muscle proteins. For this techniques such as gel filtration, ultra centrifugation and electron microscopy were used (Reviewed by Ohnishi and Rodger, 1980b).

Sarcoplasmic proteins or waster soluble proteins play a major role in species identification in fish and fishery products, whenever the identification of fish species by sensory evaluation becomes difficult. As long as the sarcoplasmic proteins of flesh remain in the native state, they are readily extractable with water or dilute salt solutions and can be used for fish species identification by Electrophoresis or Isoelectric focussing. Myofibrillar proteins play a major role in the gellying

properties of surimi and surimi based products. So any changes to myofibrillar proteins may affect the quality of the finished products.

Most of the studies on fish muscle proteins have used the techniques such as extractability, electrophoresis or gravity flow gel filtration chromatography. Hence, in the present study, fish and shellfish proteins were examined in an attempt to define any changes in their properties as a result of freezing and to use this information to monitor any changes occurring during frozen storage. With this objective, water soluble proteins and salt soluble proteins were extracted from fresh as well as frozen muscle stored for different periods in order to compare their extractability. The extracted protein was also subjected to SDS - PAGE to identify any changes occurring in the electrophoretic pattern.

1.1. PARAMETERS TESTED INCLUDE

1. Water soluble nitrogen content
2. Salt soluble nitrogen content
3. Non-protein nitrogen content
4. Electrophoretic pattern of water soluble proteins
5. Electrophoretic pattern of salt soluble proteins.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

Among the different constituents of food, it is the properties of carbohydrates, proteins and lipids that affect its quality. Proteins contribute significantly to the quality and behavior of many food systems, including meat and fish. The sensory characteristics of protein -rich foods depend upon particular physical properties and interaction of protein components. For these, the term functional property is more often used by food scientists. The functional properties of proteins are those physical and chemical properties which affect the behavior of proteins in food systems during processing, storage, preparation and consumption (Kinsella, 1982).

The important functional properties of proteins in food systems are solubility, viscosity, gelation, cohesion/adhesion, emulsification, fat and water binding and foaming, which play several roles in the expression of sensory attributes such as appearance, colour, flavour and texture of various foods which determine the food preferences by human beings (Damodaran, 1994).

The protein components of fish muscle that are responsible for various functional properties are mainly the myofibrillar proteins such as actin and myosin (Asghar *et al.*, 1985).

2.1. MUSCLE PROTEINS

As with all contractile muscle, fish flesh contains three main groups of proteins; the sarcoplasmic or water soluble proteins, the myofibrillar proteins and the connective tissue proteins which are readily separated by fractional extraction techniques employing water and strong salt solutions (Mackie, 1993).

2.1.1. SARCOPLASMIC PROTIENS

The soluble proteins of the sarcoplasm, located within the sarcolemma are referred to as "sarcoplasmic proteins". Among them, some albumins and so called myogens, to which belong most of the enzymes of the glycolytic pathway, are the real water soluble proteins (Wahyuni *et al.*, 1998) . The other fractions of sarcoplasmic proteins are soluble in low salt concentrations.

The sarcoplasmic or water soluble proteins make up 20-35% of the total protein content of muscle, depending on the species (Mackie, 1993). They include about 100 different proteins which are globular or rod shaped in conformation with low viscosity (Asghar *et al.*, 1985) and are low molecular weight (40-60 kD) compounds (Mackie, 1997).

The properties of sarcoplasmic fractions have received wide attention as they are known to induce freeze denaturation and some of the sarcoplasmic enzymes are known to degrade gel net work (Wahyuni *et al.*, 1998; Morioka *et al.*,1998). However, Nishioka *et al.*, (1990) and Okazaki *et al.*, (1986) are of the opinion that the sarcoplasmic protein fraction might instead actually supplement the gelling properties of the myofibrillar proteins. Yoon *et al.* (1991) suggested that water soluble proteins retard sarcomere shrinkage resulting from freeze-induced contraction/protein cross - linking.

The genetic difference between species are more pronounced in this than in the other group of proteins as they are responsible for widely divergent enzymatic transformations in the muscle cell. Hence the separation patterns of profiles obtained

on electrophoresis or isoelectric focussing (IEF) can be used for the unequivocal identification of the species (Mackie, 1997).

2.2. OTHER NITROGENOUS COMPOUNDS

Chemically nitrogen containing compounds in fish and shellfish sarcoplasm other than the proteins include ~~pesticides~~ peptides, amino acids, amines, amine oxides, guanidine compounds, quarternary ammonium compounds, purines and urea which contribute about 10-40% of the total nitrogen content. These compounds may directly or indirectly influence the edible qualities of fresh or processed seafood, that is colour, favour, texture, nutrition, and safety (Haard *et al.*, 1994).

2.1.2. MYOFIBRILLAR PROTEINS

The myofibrillar proteins are the proteins present in the myofibrils within the muscle cells. They contribute about 65-75% of the total proteins (Mackie, 1997).

Very concise and informative reviews on the myofibrillar proteins have been published by Maruyama (1985), Squire and Vibert(1987), Nakai and Li-Chan (1988) and Morrissey *et al.*, (1987). Based on the physiological and structural roles in living tissues, myofibrillar proteins can be further divided into three sub groups:

a) the major contractile proteins, including myosin and actin, which are directly responsible for muscle contraction and are the backbone of the myofibril

b) regulatory proteins, including tropomyosin, the troponin complex, and several other minor proteins, which are involved in the initiation and control of contraction, and

c) Cytoskeletal or scaffold proteins, including titin or connectin, nebulin, desmin, and a number of other minor components, which, provide structural support and many functions in keeping the myofibril in alignment or register (Xiong, 1997).

2.1.2.1. CONTRACTILE PROTEINS

The myofibrillar proteins like myosin and actin which are involved in the contraction and relaxation of living muscle are termed contractile proteins. It is the myosin, actin and their conjugated form actomyosin which impart various functionality to the fish meat systems (Mackie, 1997).

2.1.2.1.1. MYOSIN

Myosin contributes 55% of muscle proteins by weight and forms the thick filament in myofibrils (Murray *et al.*,1993). It is the largest myofibrillar protein molecule with a molecular weight of 520 kilodalton (kD) and is highly asymmetrical consisting of two globular heads and a long tail (Rayment *et al.*,1993).

Fish myosin, similar to myosin of other vertebrates, is a hexameric protein consisting of two heavy chains (MW=210 kD each) and four light chains (MW=15-25 kD each) with a total molecular weight of 520 kD (Seki *et al.*,1998).

Rabbit myosin molecules consists of two heavy chains with a molecular weight of 200kD each, and several light chains with a molecular weight of about 20kD each (Suzuki, 1981).Myosin stability of different species have been studied by several workers (Kimura *et al.*,1977; Hasnain *et al.*, 1976). Suzuki (1981) reported that fish myosin is less stable than rabbit myosin.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophonesis (SDS-PAGE) of myosin from any freshwater fish show one heavy chain and three light chains (Takashi *et al.*, 1974; seki,1976). These workers also reported that there are differences between molecular weights of light chains. Among different species, differences in the light chain is clearly visible in the electrophoretic pattern of myosin subunit dissociated by SDS. However, with the heavy chain, this is not the case (seki, 1976).

Martinez *et al.* (1990 a) reported that the myosin found in the same type of muscle (for example, white) of different species is, different. Also myosin found in different types of muscle, such as white and red, in the same fish is different (Martinez *et al.*,1989;Martinez *et al.*, 1990 b; 1990 c).

Myosin is the most important myofibrillar protein as far as functional properties are concerned. It is responsible for many of the functional properties observed in muscle foods and has been extensively studied (Xiong, 1997).

Surimi is the Japanese term for fish meat that has been washed, dewatered, and mixed with cryoprotectants for good frozen shelf-life. It is a concentrate of myofibrillar proteins used in the manufacture of a variety of products ranging from crab meat analogues to ice cream (Vondruska, 1985). One of the most important

properties of surimi is the ability of the myofibrillar proteins, and mainly myosin to form a three dimensional network, or gel upon heating (Niwa, 1985). Hence myosin, which is the main protein component of fish muscle, is also the main determinant of the gel strength of surimi based products (Yasui *et al.*, 1980; 1982).

Myosin extracts of red and white muscles from mackeral (*Scomber scombrus*) were analyzed by Martinez *et al.*, (1989) using native electrophoresis and by two dimensional and SDS gel electrophoresis. They found two isoforms in red muscle comprising one type of heavy chain and two light chains and four isoforms in white muscle made up of one type of heavy chain and three types of light chains.

2.1.2.1.2. ACTIN

Actin is the second most abundant protein, representing 15-30% of the total myofibrillar protein of muscle, and is the major constituent of the thin filaments. Actin can exist either as monomers (G-actin) with a molecular weight of 42 kD or in a fibrous form (F actin) as it exists in muscle (Mackie, 1997).

Purified actin from various fish species showed no difference in molecular weight determined by SDS-polyacrylamide gel electrophoresis (Seki *et al.*, 1973). SDS-PAGE showed that actin is having a molecular weight of 43000 (Sakakibara *et al.*, 1970).

Margulis *et al.* (1982) developed a method combining electrophoresis and isoelectric focussing (IEF) for analyzing actins of different tissues from invertebrates. Jiang *et al.* (1989) reported that actin may increase the thermal stability of myosin in solution, possibly by competition of its SH groups with the myosin SH

groups for oxidation. Fish actin does not contribute in free form to the elasticity of fish gels (Sano *et al.*, 1989). However, in comminuted fish products, the long thin filaments are composed of actin.

2.1.2.1.3. ACTOMYOSIN

Actomyosin is the complex of actin and myosin, which is formed after death when high energy compounds such as ATP are depleted . In actomyosin, myosin and actin are associated via non-covalent bonds (Xiong, 1997).

Oguni *et al.* (1994) is of the opinion that the filamentous structure of actomyosin (in the presence of both F -actin and myosin) is more unstable than crude F-actin in the absence of myosin under the conditions in the higher KCl concentration.

The actomyosin complex is biochemically and physiochemically similar to myosin in many aspects (Xiong, 1997). However, some functional properties of myosin, e.g., emulsifying capacity, are lost when actomyosin is formed (Galluzzo and Regenstein, 1978).

2.1.2.2. REGULATORY PROTEINS

The major regulatory proteins are tropomyosin and the troponins, which are associated with F-actin. They together contribute about 10% of myofibrillar proteins (Mackie, 1997). In addition, myofibrils contain a number of minor regulatory proteins like α-, β- and γ- actinins, C-, M-, H- and X-proteins, creatine kinase, and paratropomyosin (Xiong, 1997).

2.1.2.2.1. TROPOMYOSIN

Tropomyosin which is the most abundant regulatory protein is a dimeric molecule consisting of two dissimilar subunits designated α- and β- tropomyosin with molecular weight of about 34, 000 and 36,000 daltons respectively(Xiong, 1997). Cummins and Perry (1973) reported that on the basis of electrophoresis, rabbit tropomyosin consists of 2 bands, namely the small molecule α-subunit and the large molecules β-subunit. Suzuki (1981) also reported that the molecular weight of tropomyosin is 68,000 and is having two subunit chains.

Seki (1977) compared SDS-electrophoretic patterns of tropomyosin from over 20 different species of fishes. He observed a single band in mackerel , yellow fin tuna and big eye tuna, and one band in skipjack.

Shimomura and Seki (1980) compared the regulatory proteins from fish ordinary and dark muscle using SDS-PAGE and found out that the native tropomyosin were similar in composition.

Tropomyosin is a heat stable protein which can be extracted from heat treated fish products, making it possible to identify the material of fish of the product by SDS-electrophoretic patterns of tropomyosin (Suzuki,1981).

2.1.2.2.2. TROPONIN

Troponin is the second most abundant regulatory protein in the myofibril, comprising of three subunits designated as Tn-C, Tn-I, Tn-T (Xiong, 1997).

Studies on the components of the carp troponin showed that troponin – T is having a molecular weight of 30,000 , Tn-I with a molecular weight of 21,000 and troponin-C with a molecular weight of 19,000 (Shimomura *et al.*,1976).

Shimomura and Seki (1980) reported that Tn-T, Tn-I and Tn –C from ordinary and dark muscles have molecular weights of 30000 , 21000 and 19000 respectively.

2.1.2.2.3. α-ACTININ

α-actinin is the major protein found in the Z-disk accounting for only 21% of total myofibrillar protein , with a molecular weight of about 95,000 daltons (Xiong, 1997).

The total protein pattern of Z-disks from red and white muscle were studied by Slinde and Kryvi (1980) using SDS electrophoresis and they found that a molecular weight of about 55,000 may represent Z-disk protein.

Koohmaraie (1992) and Taylor *et al.*, (1995) reported that the meat tenderness is improved by postmortem aging of meat as a result of the disruption of the Z-disk and the consequent release of α-actinin.

Watson *et al.* (1992) reported the release of a specific doublet of proteins with molecular weights , 42 kD and 46 kD from Z –disk resulting in the proteolysis of myofibrillar proteins of white muscle of yellowfin tuna.

2.1.2.2.4. C-PROTEIN

The C-protein is associated with myosin in the thick filaments and is an impurity in crude myosin preparations. The size of the protein varies with species and muscle type and has a molecular weight of 135,000 daltons for white muscle (Xiong, 1997). C- protein is largely extracted in salt solution at concentrations above 0.6M NaCl (Offer and Trinick, 1983).

2.1.2.2.5. M-LINE PROTEIN

Three proteins – Myomesin, M-protein and Creatine kinase are found in the M-line region. Both Myomesin and M – protein are integral parts of the M-line with a molecular weight of 185,000 and 165,000 daltons respectively. They bind to myosin in the thick filament. Creatine kinase is having a molecular weight of 42,000 daltons (Xiong, 1997).

2.1.2.2.6. H -AND X- PROTEINS

These proteins are associated with myosin at discrete sites on the surface of the thick filaments, and they account for only a small percentage (0.80 and 0.20 % respectively) of total myofibrillar protein. H – Protein is a relatively small protein with a molecular weight of about 69,000 daltons, compared to X-protein which has a molecular weight of 152,000 daltons (Starr and Offer, 1983).

2.1.2.3. CYTOSKELETAL PROTEINS

These proteins provide support and stabilization of the contractile and regulatory proteins and are called as " Scaffold proteins" (Mackie , 1997;

Xiong,1997). They include connectin or titin and other related proteins, desmin and nebulin,, which together comprise about 10% of the total contractile protein of the cell (Robson *et al* ., 1984; Asghar *et al.*, 1985).

Seki and Watanabe (1984) reported high molecular weight connectins from carp and rabbit, showing a doublet band, consisting of band 1 and 2 on SDS -PAGE using a large pore gel.

'Scaffold proteins' can be extracted only in SDS solution in the presence of high concentration of urea while other myofibrillar proteins can be extracted using strong salt solutions (Mackie, 1997).

2.1.3. COLLAGEN

Fish proteins are unique in nature because of their low collagen content and excellent functional properties as compared to mammalian muscle proteins. In fish muscle, 3-10% of the protein is collagen whereas it is 10-15% in mammalian muscle (Mackie, 1997). Sato *et al.* (1986) reported that the ordinary muscles of dark-fleshed fishes contained more collagen than the corresponding muscles from white fishes.

2.2. TECHNIQUES USED FOR SPECIES IDENTIFICATION OF FISH AND FISHERY PRODUCTS

When only a portion of fish flesh such as a fillet is available for examination, identification of fish species by sensory evaluation is often difficult, if not, impossible (Mackie, 1997) . Surimi, the raw material used for the manufacture of

shellfish analogues or substitutes, for example 'crab sticks' (Lainer and Lee, 1992; Mackie, 1992, 1994) has the potential for adulteration and substitution.

Therefore, there is a need for a reliable, objective method of identification which could be used to establish the species when substitution or adulteration is suspected and which could generally be used as a check on the labeling of fish and fishery products. Some of the non-sensory objective methods of identifying the species of fish or shellfish are reviewed below.

2.2.1. ELECTROPHORETIC TECHNIQUES

2.2.1.1. ELECTROPHORESIS

Electrophoresis as an analytical procedure was first introduced by the Swedish scientist, Tiselius, in 1930 (Kleparnik and Bocek, 1991). When first developed, electrophoresis was carried out in free solution in a moving boundary system (Andrews, 1986; Kleparnik and Bocek, 1991).

Earlier workers have employed different media like filter paper (Nikkila and Linko, 1955), starch gel (Tsuyuki *et al.*, 1965), cellulose acetate (Lane *et al.*, 1966) and agar gel (Hill *et al* ., 1966) to obtain electropherograms. These methods have their own advantages and disadvantages.Disc electrophoresis in polyacrylamide gel has since been developed by Ornstein (1964), Davis (1964) and Thompson (1967).

Cowie (1968) on the other hand, employed the slab polyacrylamide gel electrophoresis technique of Akroyd (1967) for the separation of muscle myogen in relation to fish taxonomy. Later sodium dodecyl sulphate (SDS) has been

incorporated into the gel to improve resolution of electrophoretic pattern (Lundstrom 1980 ; Melvin 1987; An *et al.*, 1988).

2.2.1.1.1. ELECTROPHORETIC WORKS ON SPECIES IDENTIFICATION

Since each individual species is chemically composed of different proteins at varying levels, techniques that separate proteins may help to identify different species. Electrophoretic methods such as starch gel-zone electrophoresis, acrylamide disc electrophoresis, thin layer polyacrylamide gel isoelectric focusing and cellulose acetate strip have been accepted as official methods by the Association of Official Analytical Chemists (AOAC, 1990) to differentiate seafood species or seafood products.

Several workers used electrophoresis as an aid in the species identification of fish and fishery products, and is reviewed by LeBlanc and LeBlanc (1989). Although the separation profiles of individual fish, even of the same species, show some degree of variation, they nonetheless have a significant number of major zones in common to enable a profile to be recognized unequivocally (Mackie, 1997).

As long as the sarcoplasmic proteins of flesh remain in the native state, they are readily extractable with water or dilute salt solutions and is then in a suitable form for separation by IEF or electrophoresis, but once the fish is cooked the sarcoplasmic proteins together with the structural proteins are denatured and precipitated thereby becoming inextractable in water(Mackie, 1997).

Mackie(1969) has described a modified polyacrylamide disc electrophoresis method for identifying raw and cooked fish and an extension of the method to

smoked fish was also demonstrated. Polyacrylamide gel electrophoresis method was further extended by Mackie and Taylor (1972) for the identification of heat sterilized canned fish species.

A sensitive SDS-PAGE has been devised by Kokuryo and Seki (1978) for differentiating between species of dried fish sticks. They reported that myosin light chains and tropomyosin are good indices for this purpose. Seki *et al.* (1980) have described a preparative SDS-PAGE method of myosin light chains from both raw and cooked fish products such as kamaboko, fish sausage and canned flesh. Keenan and Shaklee (1985) have developed an electrophoretic methodology for identification of both raw and cooked fish fillets. Lee (1986) has described a method for the identification of fish origin in finished surimi-based products.

Mackie (1997) reported that the connective tissue proteins in their denatured form together with the myofibrillar proteins, are likely to contribute to the differences in the electrophoretic profiles of total muscle extracts.

Seki (1976) reported 3 myosin light chains of molecular weight ranging between 27000 and 14000 which could be used for species identification using SDS-PAGE . However, he observed that the patterns produced by the myosin heavy chain, actin and tropomyosin were too similar to be used for identification purpose.

2.2.1.2. ISOELECTRIC FOCUSING (IEF)

IEF resolves soluble proteins on the basis of their isoelectric points. The extracted water soluble proteins are applied to a polyacrylamide gel or agarose gel

and separated into distinctive protein band pattern (Hamilton , 1982; Lundstrom, 1980, 1981, 1983).

IEF has been used by several workers to identify sea food species such as shrimp (An *et al.*, 1988; Wei *et al.*, 1990) crab (Gangar, 1992), snapper (Huang *et al.*, 1995), ocean perch , monkfish, pollock etc. (Lundstrom, 1980). IEF provides reproducible protein patterns for differentiating related species with >=93% reliability (Lundstrom, 1980). An *et al* .,(1989) incorporated urea to enhance protein separation and resolution.

2.2.2. IMMUNO ASSAY PROCEDURES

Immunological assays with high sensitivity, simplicity and specificity could provide useful methods for rapid identification of meat species. Two immuno assays commonly used for meat identification are agar gel immunodiffusion (AGID), and enzyme-linked immunosorbent assay(ELISA) . ELISA requires less antibody, and is more rapid objective and sensitive than AGID (Hsieh *et al.*, 1996).

Methods for identification of raw meat based on ELISA have been well established and is reviewed by Chen *et al.*, (1998).

Several workers used Monoclonal Antibodies (Mbs) to ELISA's for raw meat identification (Chen *et al.*, 1998), heat processed seafood (An *et al.*, 1990; Chen *et al.*, 1998), while others used polyclonal antibodies (Pbs) to thermally-stable antigens of different preparations for identification of cooked meat (Chen *et al.*, 1998).

2.2.3. CHROMATOGRAPHY

Protein separation methods based on chromatography (Sotelo *et al.*, 1993) are also of potential use in providing species-specific profiles. Pineiro *et al.*, (1997) developed a simple and rapid method to identify raw or frozen gadoid fish species based on HPLC separation of water soluble proteins.

Size exclusion High Performance Liquid chromatography (HPLC) has been used by several workers to separate a wide variety of water soluble organic polymers, proteins, Polypeptides and nucleotides(-reviewed by LeBlanc and LeBlanc, 1989).

Osman *et al.* (1987) reported that fish species identification by liquid chromatographic method is rapid and reliable and can be applicable to fresh and frozen , whole, sliced, filleted and minced fish.

2.2.4. CAPILLARY ZONE ELECTROPHORESIS (CZE)

CZE is another instrumental technique with potential for separation, characterization and quantification of proteins (reviewed by LeBlanc *et al.*,1994). They also studied the applicability of CZE of sarcoplasmic proteins for species identification and for changes during frozen storage.

2.2.5. DNA TECHNIQUES.

There is interest in the application of DNA techniques to identify species of fish which can not be identified by existing methods (Unseld *et al.*, 1995). Basic procedures involved is the polymerase chain reaction (PCR) which enables a

species-distinguishing sequence of DNA, which is targeted and amplified (Mackie, 1997).

McKay *et al.* (1997) developed DNA- based species identification method using polymerase chain reaction (PCR) for the identification of fish species in fresh, frozen and smoked fish samples.

2.3. CHANGES IN PROTEINS DURING FREEZING AND FROZEN STORAGE

The changes that take place during frozen storage of fish are of great commercial importance for, they determine the shelf life of the frozen seafood (Shenouda, 1980).

Sikorski and Kolakowiska (1994) reported that frozen fish stored several months at about -20 ^{0}C may, after cooking , become tough, chewy , rubbery, stringy or fibrous. This is accompanied by a loss in functional characteristics of muscle proteins mainly solubility , water retention, gelling ability and lipid emulsifying properties. These changes are caused by processes known as freeze-denaturation of proteins, involving usually denaturation of proteins with various reactive components of the fish tissue . The palatability of frozen fish stored for extended times is normally limited by losses in either flavour and /texture. Also, the utility of the fish as a raw material for fabricated products may be limited by loss of protein functionality (Colomenero and Borderias , 1983).

The mechanism of freeze denaturation of protein in fish muscle is not completely understood; however, the problem has been extensively studied and

various theories have been proposed (Sikorski *et al.*, 1976; Matsumoto 1979; Shenouda, 1980).

According to Heen (1982) the main phenomena occurring during frozen storage are product drying, oxidative rancidity and muscular protein changes, particularly with myosin.

The quality of frozen fish is dependet on the freshness of fish before freezing and frozen storage. Fukuda *et al.* (1984) observed that there was much more marked denaturation in the muscle when frozen during post-rigor than frozen during pre-rigor. Rao (1983) was also of the opinion that pre-rigor actomyosin was preserved to a greater extent than post rigor actomyosin. However, the influence of post-harvest time on subsequent changes during frozen storage appears to vary with species and other factors. Some studies have shown that quality deterioration in milkfish increased if fish have been held on ice prior to freezing (Joseph and Perigreen ,1980). Jiang *et al.* (1987) reported that adenosine nucleotides (ADP, AMP, IMP) known to occur in fresh fish retard the denaturation of actomyosin during storage at - 20 ^{0}C, whereas nucleotides which accumulate in aged fish (hypoxanthine, inosine) accelerate this reaction.

On the other hand, Ragnarsson and Regenstein (1989) working with cod and perch , observed a slower rate of deterioration in texture when they are aged on ice for several days prior to frozen storage. Their results were attributed to the depletion of trimethylamine oxide (TMAO) and co-factors for the TMAO demethylase reaction and formation of trimethylamine (TMA).

Fish muscle proteins, mainly myosin, are more susceptible to abuse conditions of freezing and frozen storage than those from land animals. The freeze denaturation of myofibrillar proteins depend largely on storage temperature than on freezing rate(Fukuda, 1996) and is less for lower temperature of storage, (Jiang *et al.*, 1988 a ; 1989) regardless of initial freezing temperature (Fukuda *et al.*, 1982) . However, according to Haard (1992) the rate of freezing may influence the quality deterioration of the product when subsequently stored in less than ideal frozen storage conditions.

The most popular tests used to study the changes that occur in fish protein during storage are related to the loss in solubility or the loss in extractability of total fish proteins, or a particular group or proteins, myofibrillar , sarcoplasmic, or the actomyosin, or even of particular species such as myosin, tropomyosin or actin (Shenouda, 1980).

Accoding to Owusu -Ansah and Hultin (1992) the total solubility of protein in neutral 5% NaCl solution may decreased to about 30%, where-by the main loss regards the contractile proteins, mainly myosin heavy chain, M-proteins, tropomyosin, and troponins I and C in descending order. It was also shown by Jarenback and Liljemark (1975), that the myosin microfibrils in fresh cod muscle could be almost totally extracted, whereas myosin in frozen muscle after prolonged storage was resistant to extraction. Joseph and Perigreen (1983) studied the frozen storage changes of minced fish from threadfin bream and observed a good correlation between the organoleptic quality, extractability of protein, cook drip loss and weight loss on thawing.

Protein solubility is affected by several factors such as pH, ionic strength , temperature and organic solvents(Thakker and Grady, 1984). Otani *et al.* (1983) reported that when myofibrils were prepared from various muscles of shellfish and fish by repeated washing with low ionic strength medium, more actin was released from the shell-fish myofibrils than from fish myofibrils.

Washing is the critical step in the production of surimi as it removes water soluble materials such as sarcoplasmic proteins digestive enzymes, inorganic salts and low molecular weight organic substances such as TMAO and enhances the gel strength of surimi (Toyoda *et al.*, 1992). However, Lin *et al.* (1995) reported that a considerable amount of myofibrillar protein was lost in surimi waste streams.

Muscle of salt-water fish contains a variety of salts, about equivalent to a solution with ionic strength 0.145, which corresponds to 0.85% (0.145 M) NaCl, might inhibit solubilization of the proteins of fish muscle (Wu *et al.*, 1991). However, extensive washing of muscle might cause decreased salt concentration in the tissue and allow myofibrillar proteins to become highly associated with water (Lin and Park, 1996). They also reported a high loss of proteins when the mince was washed with distilled water at a high water/meat ratio(20:1). This data suggested that most of the sarcoplasmic proteins were readily solubilized and removed in the first washing step while the amount of MHC, actin, β-tropomyosin /troponin-T and α-tropomyosin bands increased greatly in the subsequent washing steps.

Changes in the moisture phase during freezing or during frozen storage of fish create an environment that is conducive to protein denaturation. Crystallization of ice which depends on the rate of freezing and temperature of storage (Sikorski

and Kolakowiska,1994) may disrupt the water structures contributing to the hydrophobic adherences which participate in buttressing the native protein confirmation (Lewin, 1974).

Freezing also concentrates solids, including mineral salts and small organic molecules, within the remaining unfrozen aqueous phase in the cell (Heldman, 1982) which results in changes in ionic strength and possibly pH, leading to the denaturation of protein molecule (Ota and Tanaka, 1978) . Kolodzieijska and Sikorski (1980) reported that many inorganic salts may contribute to changes in proteins by catalyzing lipid hydrolysis and autoxidation, participating in the formation of lipid-protein complexes.

Another chemical reaction generally associated with quality changes during freezing, frozen storage and thawing is lipid oxidation. The role of lipids in freezing changes in proteins have been investigated and reviewed by several workers (Sikorski and Kolakowska, 1994).

The role of lipids in freeze denaturation of protein depends on the quality and distribution of lipids in the tissues, their hydrolysis and oxidation. Fatty species are more prone to oxidation than lean species, and species with more highly unsaturated fatty acids are less stable than other species (Santos Yap 1995). However, Sikorski and Kolakowska (1994) are of the opinion that the effect of lipids on protein changes in frozen fish is more significant in lean species, where the lipids are limited to the physiologically necessary membrane lipids, the hydrolysis and oxidation of which may damage and increase membrane permeability. This in

consequence may lead to increased activity of enzymes directly or indirectly involved in protein changes.

The unstable free radical intermediates formed during autoxidation result in the formation of protein free radicals (Karel *et al.*, 1975) which may cross-link with other proteins to form protein-protein aggregates (Castells *et al.*, 1973). According to Shenouda (1980) free fatty acids (FFA)are believed to bind myofibrillar proteins, specifically actomyosin, rendering it unextractable . Oshima *et al.* (1984) also reported a negative interrelation between the FFA content and the salt soluble protein extractability.

Oxidation of the thiol groups, has also been regarded as possibly being involved in the denaturation of proteins in frozen fish. Chen *et al.*, (1989) reported a significant decrease in the SH content of milkfish myosin at -20 ^{0}C and Jiang *et al.*, (1988 a, 1988 b)showed that denaturation of myosin in milkfish actomyosin during freezing and storage at -20 ^{0}C was accompanied by the formation of disulfide bonds. It is also evident from the findings of Lim and Haard (1984) that disulfide bonds contribute to protein aggregation.

Trymethylamine Oxide (TMAO) is commonly found in large quantities in marine species of fish, compared to fresh water species Santos Yap(1995).The difference in the rate of freeze denaturation of proteins in fish belonging to various species may be caused by the action of formaldehyde(FA), generated together with dimethylamine(DMA) as the result of enzymatic decomposition of TMAO (Sikorski and Kostuch, 1982; Lundstrom *et al.*, 1982). A large accumulation of FA in the muscle of frozen fish is generally accompanied by a decrease in extractability

of myofibrillar proteins (Ang and Hutin, 1989; Ohnishi and Rodger, 1980 a). Products of demethylation, dimethylamine (DMA) and formaldehyde (FA), have been identified in more than 30 species of fish belonging to 10 families and eight species of invertebrates by Sikorski and Kostuch (1982).

Formaldehyde so produced has been suggested to cause cross-linking of the muscle protein and toughening of the tissue (Castell *et al.*, 1973). According to Ang and Hultin (1989) FA could increase the rate of protein denaturation during frozen storage by interacting with the side chain groups on proteins. However, textural changes may also occur in fish species which do not or cannot produce such FA during frozen storage (Gill *et al.*, 1979). Ang and Hultin (1989) are of the opinion that these changes may be caused by processes which modify protein side chains, probably due to secondary bonds, such as hydrogen or electrostatic bonds(Gill *et al.*, 1979). Del Mazo *et al.* (1994) studied the role of formaldehyde in the formation of natural actomyosin aggregates in hake during frozen storage.

When protein molecules form aggregates , there must be some form of cross-binding which keeps them together. According to Tsuchiya *et al.*, (1980) when myosin and actomyosin are aggregated and insolubilized during frozen storage, the number or cross-bridges between the protein molecules increases. They reported that in this cross-bridge formation, ionic (electrostatic) bonds and disulfide (S-S) bonds all take part.

Loss of protein extractability during frozen storage of fish can be mostly accounted for by non-covalent , hydrophobic interactions and is reviewed by Haard, (1992).Much of the protein denatured during frozen storage is soluble in sodium

dodecyl sulphate (SDS) and most (>95%) is soluble when a disulfide bond reducing agent like β-mercaptoethanol is combined with SDS in the solvent (Haard, 1992). Further evidence that disulfide bonds contribute to protein aggregation is provided by the finding that addition of disulfide reducing agents to mince reduces protein insolubilization during freezing (Lim and Haard, 1984).

Electrophoresis often has been used as an aid in the species identification of fish and fishery products (Hume and Mackie, 1980; Mackie, 1980) . However, it can also be used to monitor changes in specific proteins subjected to a variety of treatments (McBridge and Parrish, 1977; Koohmaraie *et al* ., 1984; Rodger *et al.*, 1984). Several workers used SDS-PAGE to monitor the changes in isolated myofibrillar proteins (Wagner *et al.*, 1979) and sarcoplasmic proteins (Award *et al.*, 1969; Yowell and Flurkey , 1986) of muscle subjected to various frozen storage conditions. These studies have indicated that SDS-PAGE is a time consuming but sensitive method of determining the molecular changes in frozen stored fish muscle tissue.

During iced storage, changes in the composition of the sarcoplasmic protein may occur as a consequence of proteolysis by endogenous and bacterial enzymes along with the leaching out of proteins by melting ice . Lim and Haard (1984) observed a gradual loss in solubility of sarcoplasmic proteins. However, Mackie (1997), reported that the electrophoretic profile remains remarkably constant even to the stage of spoilage when the fish has become unfit for human consumption. Ahmed *et.al.* (1989) reported that the changes in protein electrophoretic patterns after 7 days of icing was more obvious than 8 months frozen storage. Papadopoulos

et al. (1989) studied the ultrastructural changes in fresh water prawn during ice storage and observed a family of proteins ranging in molecular weight form 65 kD to 180 kD due to proteolysis.

Devadasan *et al.* (1978) reported that, the electrophoretic pattern remained unchanged during frozen storage, however, on prolonged storage some of the bands became feeble.

Boerreson *et al.* (1985) used IEF to study changes in the Sarcoplasmic Protein (SPP) fraction of fish fillets. They observed new bands during frozen storage, indicating a change in the iso electric point (IEP) for some components. Significant changes in the SDS-PAGE and HPLC profiles to cod sarcoplasmic proteins due to frozen storage have been shown by LeBlanc and LeBlanc (1992). LeBlanc *et al.* (1994) observed a reduction in the number of sarcoplasmic proteins of fish stored at -12 ^{0}C using capillary electrophoresis.

Al-Dagal (1996) reported that the gel electrophoretic band intensities of SPP fainted in samples with higher bacterial count and comparatively intense in the ones with lower counts, indicating a clear relationship between product degradation and microbial flora.

Umemoto (1970) used sepharose 2B and separated a salt extract of fish muscle into 3 components (myosin , actomyosin and sarcoplasmic proteins)using gel filtration chromatography. He showed that the actomyosin peak which was larger for fresh muscle, decreased after freezing , indicating that the amount of extractable actomyosin decreased as a result of freezing.

Seki and Arai (1974) separated MFP by gel filtration into Myosin Heavy Chain (MHC), Actin(A), Tropomyosin (TM) , Troponin (Tn) and Myosin Light Chain (MLC) in an SDS-buffer system although only MHC. A-Tm mixture, Tn and MLC could be separated using the normal NaCl buffer . So, Ohnishi and Rodger(1980 b) used NaCl and SDS -buffer system to compare their relative dissociative effects on the fresh and frozen muscle and stated that in an NaCl-buffer system A, Tm, Tn, and MLC are all in the form of aggregates stabilized by inter and intra- molecular cross-linkages. They also reported that the elution sequence of A and TM reversed between NaCl and SDS buffer, suggesting that in the NaCl-buffer system, TM retains its native dimeric structure which is broken down by SDS resulting in TM monomeric subunits, being eluted later than actin.

Yoshikawa *et al.* (1995) studied the salt-soluble and insoluble fractions of carp myofibrils during frozen storage. They found out that the main component of myofibrils in the salt soluble fraction decreased and were detectable in the salt-insoluble fraction. They also observed aggregates of proteins which could not migrate into 7.5% polyacrylamide gel was accompanied by the MHC component.

Del Mazo *et al.* (1994) compared the electrophoretic profiles of the proteins from fresh and frozen samples and observed that when FA reacts with natural actomyosin the first protein to be insolubilized is myosin, followed by actin, then the troponins and myosin light chains and lastly tropmyosin; they also found aggregates of high molecular mass at early stages, probably as a result of covalent binding of myosin molecules, which were not found in the soluble fraction.

The effect of triglyceride oxidation products(TGOP) of sardine oil on the subunit composition of carp myofibrils was studied by Kawasaki *et al.*,(1992) using SDS-PAGE and observed that an incubation of myofibrils with TGOP induced a disappearance of MHC bands and an appearance of high molecular weight bands.

Several workers used SDS-PAGE to study the nature of cross bridges formed due to aggregation of protein molecules and also for finding out the molecular weight of these aggregates (Reviewed by Haard1992) .

SDS-PAGE has also gained wide application in monitoring proteolytic degradation of myofibrils (Xiong and Anglemier, 1989; Koohmaraie *et al.*, 1984) as well as sarcoplasmic proteins (Harrington and Henahan, 1982) in skeletal muscle during post mortem aging.

Martinez (1992) reported that the myosin from capelin was affected by proteolytic degradation after 26 days of storage at -20 ^{0}C , showing the presence of an extra band migrating faster than myosin heavy chain. He is of the opinion that myosin susceptibility to degradation is species and tissue specific and that the degradation affects primarily the myosin heavy chains, the myosin light chain being apparently less affected. Martinez (1992) used extracted myosin for storage study. However, according to Sikorski and Kolakowska, (1994) the protein changes are more pronounced in extracted proteins than in the intact muscle.

MATERIALS AND METHODS

III. MATERIALS AND METHODS

Two species of fish and two species of prawn were used for the present study. The species used were,

Megalspis cordyla (Horse mackerel)

Labeo rohita (Rohu)

Penaeus indicus (White prawn)

Parapenaeopsis stylifera (Karikkadi)

The above species were selected on the basis of their commercial importance and availability. Horse mackerel and karikkadi were obtained from the harbour. Rohu and white prawn were collected from the market. The locations were selected based on the availability of materials. The materials were brought to the laboratory in iced condition. Care was always taken to keep the temperature of the material below 5^0C by icing during transportation and the samples were kept in a deep freezer immediately on procurement.

Dressed and filleted fish and prawn were washed with potable water and frozen at -40^0C in a blast freezer for 6 h. and glazed by dipping in cold water. It was then wrapped in polyethyleen bags and kept in wax coated duplex cartons, which were then kept in a master carton and stored at -18^0C. Samples were taken on 0 day (Fresh), 15, 30, 45, 60, 75 and 90 day. Samples were transported from the cold store to the laboratory in thermocole boxes. Samples were allowed to thaw at 5^0C and analyses were done.

3.1. BIOCHEMICAL ANALYSES

3.1.1. TOTAL NITROGEN CONTENT.

Crude protein was estimated by Microkjeldahl's method (AOAC, 1983).

3.1.2. NON- PROTEIN NITROGEN CONTENT (NPN)

NPN content was determined by homogenizing samples for 1 minute with 10 volumes of 10% trichloroacetic acid (TCA) and measuring the Total Kjedahl Nitrogen (TKN) of the filtrate by the AOAC (1984) method.

3.1.3. EXTRACTION AND ESTIMATION OF WATER SOLUBLE NITROGEN.

Samples were thawed and meat collected (white meat was collected from fish). About 15g meat was taken and minced thoroughly. Care was taken to keep the temperature low. From this 5g meat was accurately weighed and homogenized with 10ml cold distilled water for 2 minutes. The suspension was centrifuged at 10,000 rpm for 10 minutes at 4^0C and the supernatant transferred to a 50ml standard flask. The residue was extracted twice with cold distilled water, using 15ml portions and the supernatant decanted into the standard flask. The volume was made up with the same cold distilled water and the water soluble nitrogen content of the aliquot was estimated by Kjeldahl's method.

3.1.4. EXTRACTION AND ESTIMATION OF SALT SOLUBLE NITROGEN.

The residue of the previous extraction was used for the extraction of salt soluble protein using Dyer's buffer (5% NaCl in 0.02 M $NaHCO_3$, pH 7.0). The residue was mixed with about 40 ml Dyer's buffer and stirred at low speed using a

magnetic stirrer for 1 h. at low temperature. The suspension was then centrifuged at 10,000 rpm for 20 min. and the supernatant transferred to a 50ml standard flask. The volume was made up with the same buffer and the salt soluble nitrogen content determined in the aliquot by Kjeldahl's method.

3.2. EXTRACTION OF PROTEIN FRACTIONS FOR ELECTROPHORESIS

3.2.1. EXTRACTION OF SAMPLE FOR ELECTROPHORESIS OF WATER SOLUBLE PROTEINS

About 5g of the minced meat was mixed with 10ml cold distilled water and homogenized using a mortar and pestle for 2 minutes under ice cold conditions. The suspension was then centrifuged at 2000 rpm for 10 min at 4^0C. The supernatant was used for the electrophoresis of sarcoplasmic proteins.

3.2.2. EXTRACTION OF SAMPLE FOR ELECTROPHORESIS OF SALT SOLUBLE PROTEINS.

Protein extracts prepared by the above mentioned (3.1.4) method was used for the electrophoresis of salt soluble proteins.

3.3. ELECTROPHORESIS OF PROTEINS.

Proteins were separated by sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE).

3.3.1. REAGENTS FOR SDS-PAGE

3.3.1.1. ACRYLAMIDE : BIS ACRYLAMIDE SOLUTION

30 g of acrylamide and 0.8g of bis acrylamide were dissolved in distilled water and made up to 100 ml, filtered and stored in dark bottles at 4^0C.

3.3.1.2. SODIUM DODECYL SULPHATE (SDS)

10% (w/v) of SDS was prepared and stored at room temperature. Fresh reagent was prepared every 2-3 weeks time.

3.3.1.3. ELECTRODE BUFFER (TRIS – GLYCINE BUFFER PH 8.3, 0.1% (W/V) SDS)

0.6 gm of Tris, 2.88g of glycine and 0.75 g SDS were dissolved in 750ml of distilled water.

3.3.1.4. SPACER BUFFER (Tris buffer, pH 6.7)

5.98 g Tris was dissolved in 50.3 ml 1 N HCl and made up to 100ml using distilled water and filtered.

3.3.1.5. CONTINUOUS GEL BUFFER (Tris buffer, pH 8.9)

36.3 g of Tris was dissolved in 64ml of 1N HCl and warmed to dissolve. Volume was made up to 100 ml using distilled water and filtered.

3.3.1.6. AMMONIUM PERSULPHATE SOLUTION 10% (W/V)

Prepared just before use.

3.3.1.7. COOMASSIE BRILLIANT BLUE R-250 (0.2%)

The staining solution was prepared by dissolving 0.4g of Coomassie blue in a solvent mixture consisting of 93 ml methanol, 14 ml of glacial acetic acid and 93 ml of distilled water.

3.3.1.8. DESTAINING SOLVENT

Destaining solvent was prepared by mixing methanol, glacial acetic acid and distilled water in the ratio 30 : 10 : 60

3.3.1.9. COMPOSITION OF RESOLVING GEL (7.5%)

Acrylamide : Bis acrylamide	:	5 ml
Tris buffer, pH 8.5	:	5 ml
Water	:	9.5 ml
10% SDS	:	0.2 ml
TEMED	:	0.03 ml
Ammonium persulphate	:	0.3ml

3.3.1.10. COMPOSITION OF SPACER GEL (3.6 %)

Acrylamide : Bis acrylamide	:	1.22 ml
Tris buffer . pH 6.7	:	1.26 ml
Distilled water	:	7.28 ml
10% SDS	:	0.1 ml
TEMED	:	0.02 ml
Ammonium persulphate	:	0.16 ml

3.3.1.11. SAMPLE BUFFER

Sample buffer was prepared by mixing

Tris – buffer pH 6.7	:	2.5 ml
10% SDS	:	4 ml
Glycerol	:	2 ml
Distilled water	:	10.5 ml
β - mercaptoethanol	:	1 ml

3.3.1.12. TEMED (N, N, N, N – Tetra Methyl Ethylene diamine)

3.3.1.13. 0.025% aqueous Bromophenol blue.

3.3.2. CASTING OF THE GEL

Gels were moulded in the form of gel rods. Glass tubes both ends open and having a uniform diameter 0.5 cm and length 7.5 cm were selected. They were placed in a suitable stand in vertical position and the lower end of each tube was sealed with cellophane paper.

Resolving gel solution was prepared and 1.1 ml of this was poured into each gel tube along the side of the tube using a pipette. Care was taken to avoid bubble formation while pouring the solutions. Over this one drop of butanol : water mixture (1:1 V/V) was placed on top of the gel solution to avoid meniscus formation. The gel was allowed to polymerize for 1 h. and the upper layer of butanol water mixture was removed carefully with blotting paper. Spacer gel solution was prepared as mentioned earlier and 0.2ml of this was poured. A drop of butanol-

water mixture (1:1) was then added to avoid meniscus formation and the gel was allowed to polymerize for 1 h. The butanol-water layer was removed after polymerization and these gel tubes were taken for electrophoresis.

3.3.3. SAMPLE APPLICATION AND ELECTROPHORESIS.

The gel tubes were inserted into the groves of the upper buffer tank. This was then placed over the lower buffer tank containing the electrode buffer. Electrode buffer was also added to the upper buffer tank. The protein sample was mixed with an equal volume of sample buffer and 5ml of 0.25% aqueous bromophenol blue solution was added and kept in a boiling water bath for 1 minute. Ten microlitre of this mixture was applied to the top of the gel tube using a hamilton syringe. The upper buffer tank was closed with the lid and electrical connections were given. The power pack was adjusted in such a way as to pass a current of 4 mA per gel tube. When the bromophenol blue reached the lower end of the gel tubes, the supply of current was terminated. Buffer was poured out and gel tubes were removed by injecting electrode buffer in between the gel and tube with the help of a syringe.

3.3.4. STAINING

Coomassie brilliant blue 0.2% (w/v) was used as the stain. The gels were stained for 20 minutes in a test tube.

3.3.5. DESTAINING

Excess dye was removed by repeated washing with the destaining solvent, till the bands appeared as dark blue discs against a clear background.

3.3.6. PRESERVATION

The developed gels were kept in 7% acetic acid and photographed.

3.3.7. MOLECULAR WEIGHT DETERMINATION

Molecular weights of the protein bands were determined according to the methods of Weber and Osborn (1969) and Davies and Stark (1970) using a high molecular weight (MW) protein kit, SDS – 6H (Sigma Chemical Co., St. Louis, MO)

Molecular weight of protein was determined by comparing its electrophoretic mobility with known protein markers. The logarithms of the molecular weights of the standard polypeptide chains were plotted against their respective electrophoretic mobilities (R_f) to obtain the calibration curve , from which the molecular weight of unknown protein was estimated.

$$R_{f.} = \frac{\text{Distance of protein migration}}{\text{Distance of tracking dye migration}}$$

3.4. STASTISTICAL METHODS

Data from the biochemical analysis were subjected to **ANOVA** and **t.** test to determine significant difference between experimental periods of storage.

RESULTS

IV. RESULTS

The results of the experiment can be considered systematically under the following heads.

(1) Salt Soluble Nitrogen (SSN)

(2) Water Soluble Nitrogen (WSN)

(3) Non-Protein Nitrogen (NPN)

(4) Total Nitrogen (TN)

(5) Electrophoretic pattern of Sarcoplasmic proteins

(6) Electrophoretic pattern of Myofibrillar proteins

4.1 SALT SOLUBLE NITROGEN

Fig. 1, shows the changes in the salt soluble nitrogen content in *Labeo rohita, Megalaspis cordyla, Penaeus indicus* and *Parapenaeopsis stylifera* respectively. Table 1, shows the changes in the salt soluble nitrogen content of the species used for the experiment.

From the figure it can be seen that SSN registered a significant decrease during the 12 weeks' frozen storage study. The results of the comparison of the SSN among the treatments using ANOVA technique are given in Tables 2, 3, 4 and 5.

Table 1 : Salt soluble nitrogen content

Species	SSN as % of TN						
	DAYS OF FROZEN STORAGE						
	Fresh	15	30	45	60	75	95
Labeo rohita	48.85^{a} ± 2.5	44.86^{b} ± 1.28	44.15^{b} ± 0.75	42.82^{bc} ± 1.08	40^{c} ± 2.01	38.04^{cd} ± 0.74	36.76^{d} ± 0.74
Megalaspis cordyla	48.73^{a} ± 1.41	46.80^{b} ± 0.79	42.72^{c} ± 0.67	41.56^{cd} ± 0.79	41.1^{de} ± 0	39.06^{f} ± 0.79	37.11^{g} ± 0.50
Penaeus indicus	53.45^{a} ± 0.67	53.38^{a} ± 1.2	51.65^{a} ± 2.4	45.5^{b} ± 1.23	42.96^{c} ± 1.86	42.25^{cd} ± 1.2	39.72^{de} ± 0.7
Parapena-eopsis stylifera	43.52^{a} ± 0.72	42.45^{b} ± 0.6	38.27^{c} ± 1.24	37.44^{cd} ± 0.71	35.98^{de} ± 1.23	34.38^{e} ± 0	34.56^{e} ± 1.23

Superscripts a,b,c,... indicate the results of pair wise comparison, different superscripts showing significantly different means ($P<0.05$).

Table 2 : ANOVA table for SSN of *Megalaspis cordyla*

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F
Treatment	6	303.010	50.502	77.668*
Error	14	9.103	0.650	
Total	20	312.113		

* Significant at 5% level

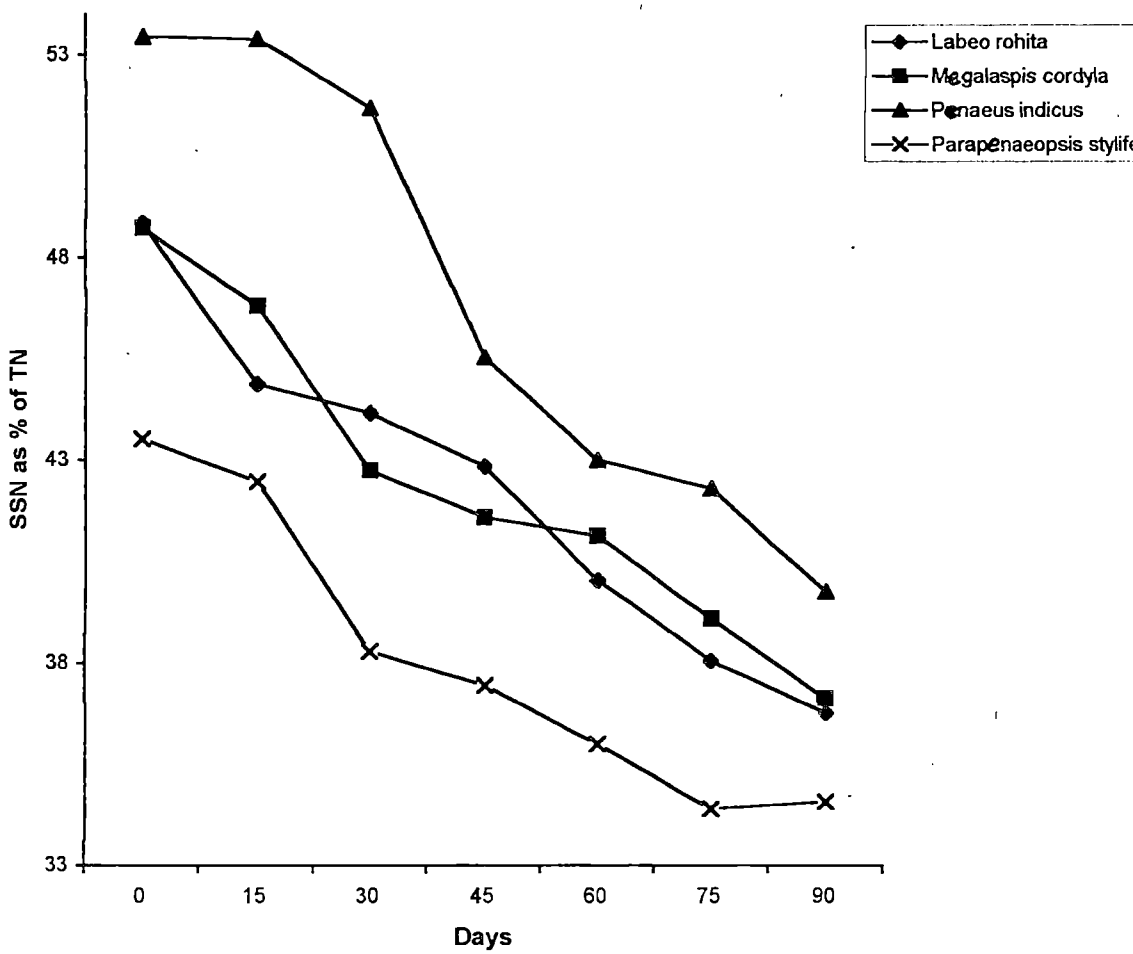

Fig.1 Salt Soluble Nitrogen content

Table 3: ANOVA table for SSN of *Labeo rohita*

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F
Treatment	6	6.241	1.040	7.992*
Error	14	1.822	0.130	
Total	20	8.063		

* Significant at 5% level

Table 4: ANOVA table for SSS of *Penaeus indicus*

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F
Treatment	6	594.338	99.056	47.536*
Error	14	29.173	2.084	
Total	20	623.511		

* Significant at 5% level

Table 5 : ANOVA table for SSN of *Parapenaeopsis stylifera*

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F
Treatment	6	215.323	35.887	35.032*
Error	14	14.342	1.024	
Total	20	229.665		

* Significant at 5% level

4.2 WATER SOLUBLE NITROGEN

Fig. 2 shows the changes in the water soluble nitrogen content of *L. rohita*, M. *cordyla*, *P. indicus* and *P. stylifera* respectively. Table 6 shows the mean WSN content with standard deviation of 4 species.

Table 6 : Water Soluble Nitrogen Content

Species	WSN AS % OF TN						
	DAYS OF FROZEN STORAGE						
	Fresh	15	30	45	60	75	95
Labeo rohita	24.84^{ab} ± 0.39	24.94^{b} ± 0.68	24.22^{ac} ± 0.31	23.75^{cde} ± 0.4	24.09^{cd} ± 0.15	23.55^{de} ± 0.15	23.43^{e} ± 0.13
Megalaspis cordyla	21.1^{a} ± 0.22	20.72^{a} ± 0.82	19.42^{b} ± 0.44	18.91^{bc} ± 0.55	19.09^{bc} ± 0.67	18.44^{cd} ± 0.16	18.53^{cd} ± 0.16
Penaeus indicus	27.78^{a} ± 0.13	27.18^{b} ± 0.25	27.22^{ab} ± 0.12	27.79^{c} ± 0.61	25.54^{cd} ± 0.48	24.14^{d} ± 0.24	24.4^{d} ± 0.14
Parapena-eopsis stylifera	23.8 ± 0.14	24.09 ± 0.14	24.52 ± 1.11	23.29 ± 0.13	22.67 ± 0.28	22.84 ± 0.24	22.22 ± 0.49

Superscripts a,b,c,... . indicate the results of pair wise comparison, different superscripts showing significantly different means ($P<0.05$).

From Fig.2. it can be seen that there is a gradual decrease in the WSN content. The results of the comparison of the WSN among the treatments using ANOVA technique are given in Tables 7,8,9 and 10.

Table 7 : ANOVA table for WSN of *Megalaspis cordyla*

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F
Treatment	6	20.889	3.482	13.967*
Error	14	3.490	0.249	
Total	20			

* Significant at 5% level

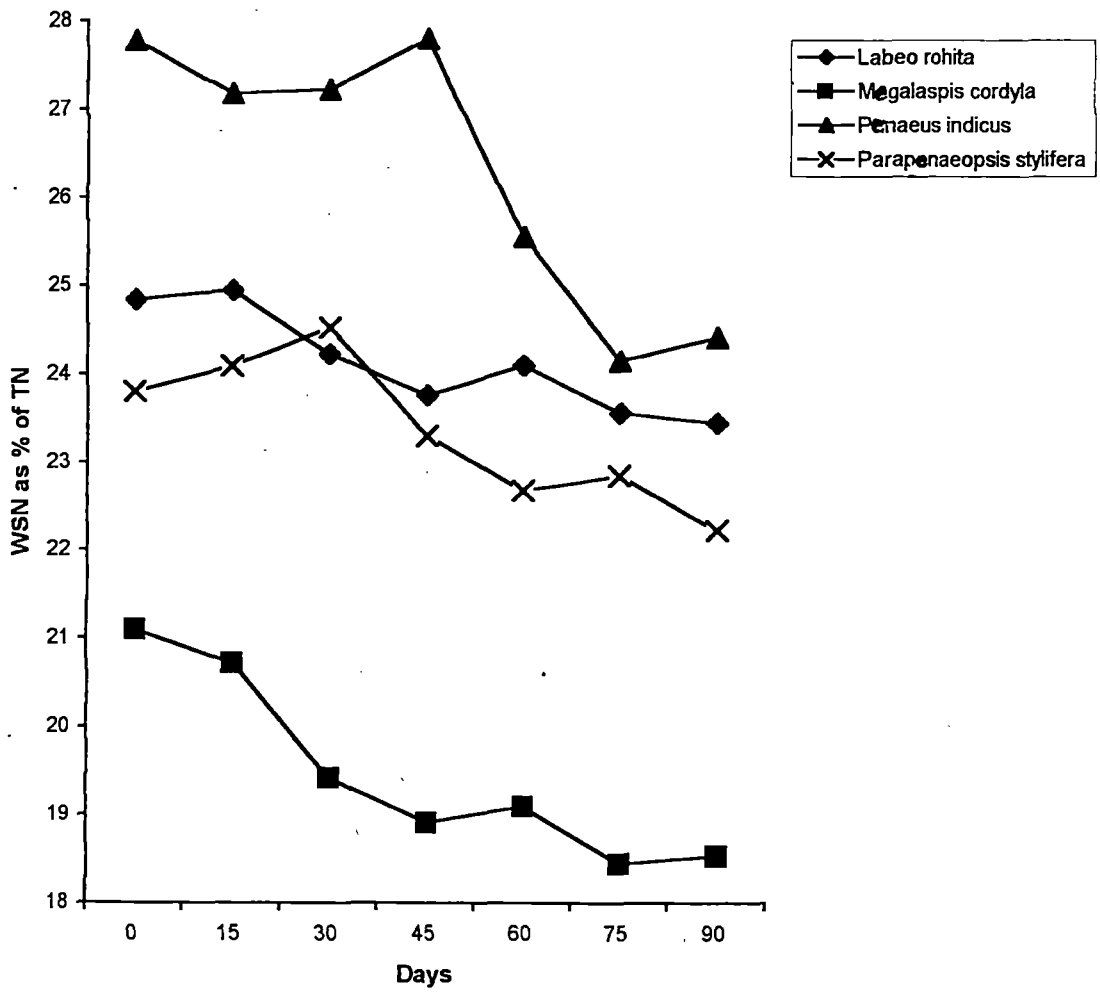

Fig.2 Water Soluble Nitrogen content

Table 8: ANOVA table for WSN of *Labeo rohita*

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F
Treatment	6	6.241	1.040	7.992*
Error	14	1.822	0.130	
Total	20	8.063		

* Significant at 5% level

Table 9: ANOVA table for WSN of *Penaeus indicus*

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F
Treatment	6	37.031	6.172	55.118*
Error	14	1.568	0.112	
Total	20	38.599		

* Significant at 5% level

Table 10 : ANOVA table for WSN of *Parapenaeopsis stylifera*

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F
Treatment	6	10.085	1.681	3.809
Error	14	6.179	0.441	
Total	20	16.264		

* Significant at 5% level

4.3 NON - PROTEIN NITROGEN

Fig. 3 shows the changes in the NPN content in *L. rohita, M. cordyla, P. indicus* and *P. stylifera* respectively. Table 11 shows mean NPN content with standard deviation in the species during 12 weeks' frozen storage study.

Table 11 : Non – Protein Nitrogen Content

Species	NPN AS % TN						
	DAYS OF FROZEN STORAGE						
	Fresh	15	30	45	60	75	95
Labeo Rohto	16.09^{a} ± 0.28	15.45^{b} ± 0.27	15.5^{bc} ± 0.21	14.17^{d} ± 0.07	13.73^{de} ± 0.54	12.24^{f} ± 0.15	11.71^{g} ± 0.07
Megalaspis cordyla	10.57^{a} ± 0.15	10.05^{b} ± 0.34	10.09^{b} ± 0.08	9.27^{c} ± 0.29	8.59^{d} ± 0.21	8.31^{d} ± 0.29	8.01^{d} ± 0.09
Penaeus indicus	18.95^{a} ± 0.18	17.95^{b} ± 0.35	18.81^{ac} ± 0.07	16.65^{d} ± 0.37	16.58^{d} ± 0.18	16.33^{d} ± 0.5	15.12^{e} ± 0.25
Parapen-aeopsis stylifera	18.14^{a} ± 0.3	17.94^{a} ± 0.25	17.69^{a} ± 0.19	17.42^{b} ± 0.14	16.36^{c} ± 0.43	16.24^{c} ± 0.25	16.49^{c} ± 0.07

Superscripts a,b,c,.... indicate the results of pair wise comparison, different superscripts showing significantly different means ($P<0.05$).

NPN content showed a decreasing trend during the 12 weeks frozen storage study which can be seen from the figures. The result of the comparison of the NPN between the treatments by ANOVA technique is shown in Table 12, 13, 14 and 15.

Table 12 : ANOVA table of NPN for *Megalaspis cordyla*

Source of variation	Degrees of freedom	Sum of squares	Means of squares	F
Treatment	6	17.868	2.978	58.044*
Error	14	0.718	0.051	
Total	20	18.586		

* Significant difference at 5% level

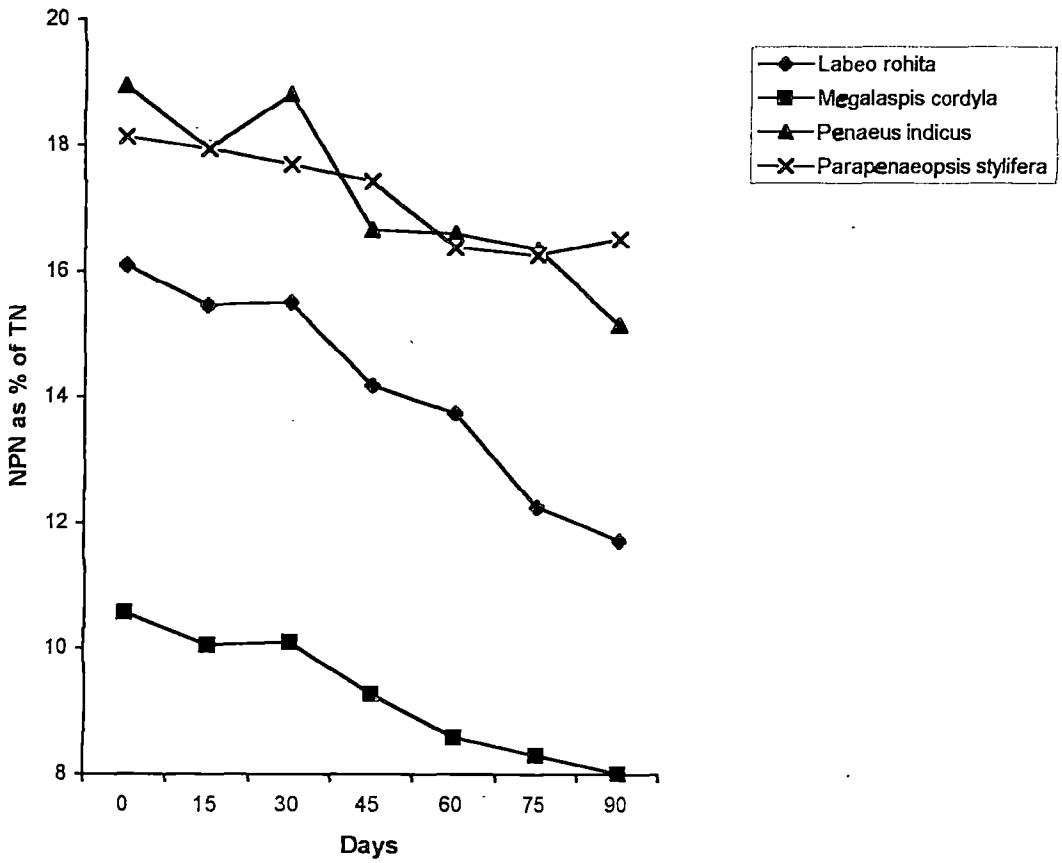

Fig.3 Non Protein Nitrogen content

Table 13: ANOVA table for NPN of *Labeo rohita*

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F
Treatment	6	50.931	8.488	111.830*
Error	14	1.063	0.076	
Total	20	51.993		

* Significant at 5% level

Table 14: ANOVA table for NPN of *Penaeus indicus*

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F
Treatment	6	35.941	5.990	64.380*
Error	14	1.303	0.093	
Total	20			

* Significant at 5% level

Table 15 : ANOVA table for NPN of *Parapenaeopsis stylifera*

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F
Treatment	6	11.334	1.889	18.235*
Error	14	1.450	0.104	
Total	20	12.784		

* Significant at 5% level

4.4 TOTAL NITROGEN (TN)

When the total nitrogen content of the 4 species were compared with the respective TN content on 90^{th} day by the paired t-test there was no significant difference at 5% level.

4.5 ELECTROPHORETIC PATTERN OF WATER SOLUBLE PROTEINS

4.5.1. *Labeo rohita*

Plate 1 shows the electrophoretic pattern of water soluble proteins subjected to various periods of frozen storage. 15 bands were obtained in the electropherogram of WSP in a 7.5% gel.

Table 16. Molecular weights of the protein bands extracted with distilled water

Band No.	1	2	3	4	5	6	7	8	9	10
Mol. Wt. In kD.	226	178	168	133	126	112	89	84	75	71
Band No.	11	12	13	14	15					
Mol. Wt. In kD.	67	56	47	42	40					

Out of the 15 bands, protein bands with molecular weights 112, 75, 71, 67, 56, 47 and 42 (all in kilo Daltons) gave strong bands. Protein bands with molecular weights 266, 178, 168, 133, 126, 89, 84, and 40 gave (all in kD) only a narrow and less intensive bands. Electrophoretic pattern remained almost same throughout the 12 weeks frozen storage study. Protein band with molecular weight 266 kD could not be visualized in the 90 days stored sample. Protein bands with molecular weights 178 and 168 kD could not be visualized from 60th day sample onwards.

4.5.2. *Megalaspis cordyla*

Plate 2 shows the electrophoretic pattern of WSP extracted with distilled water subjected to various periods of frozen storage. 10 bands were obtained in a 7.5% gel.

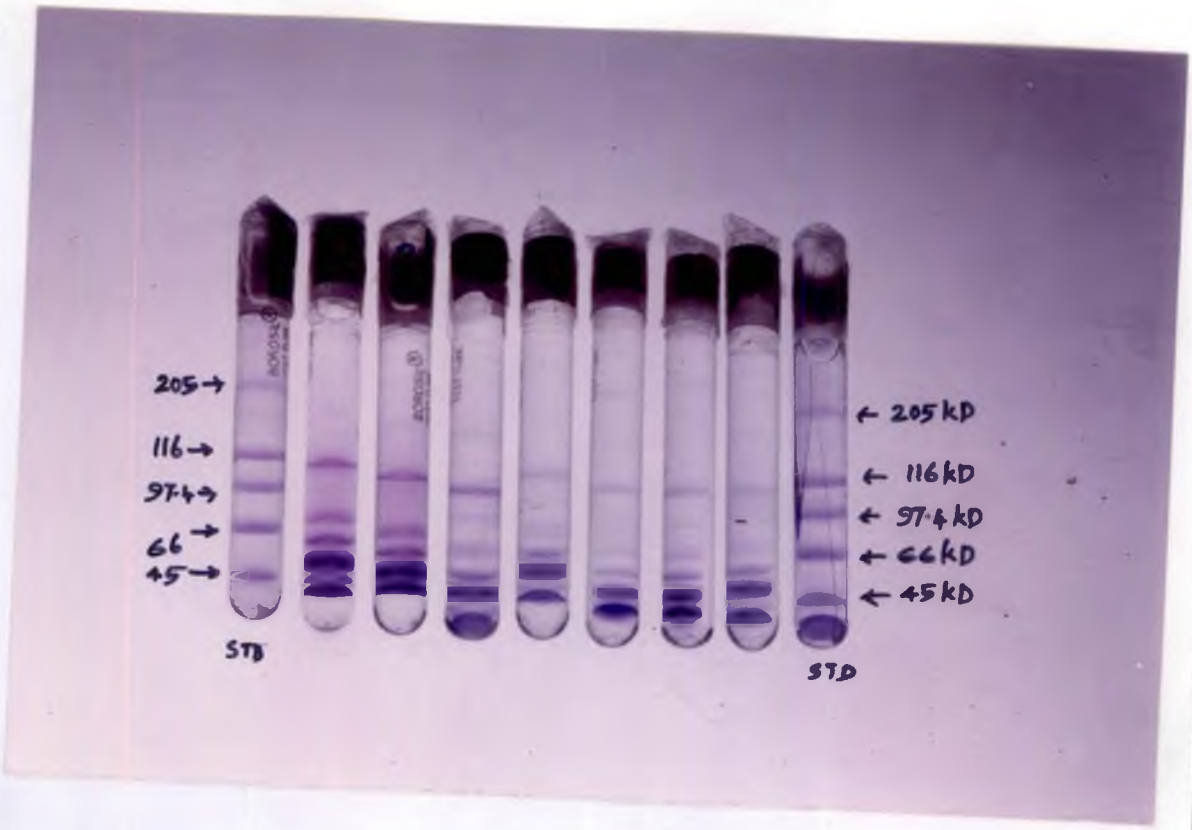

Plate 1 : Electrophoretic pattern of Water Soluble Protein (*L. Rohita*)

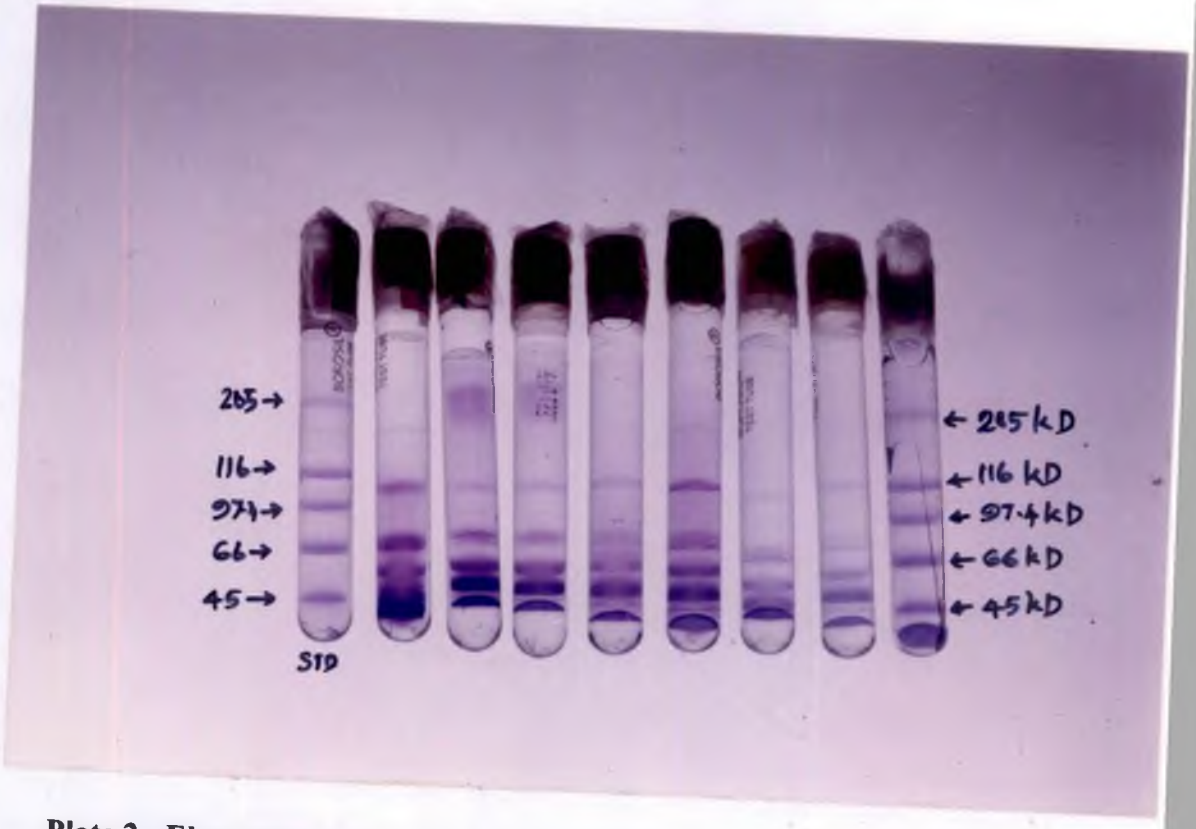

Plate 2 : Electrophoretic pattern of Water Soluble Protein (*M. Cordyla*)

Table 17. Molecular weights of the protein bands extracted with distilled water

Band No.	1	2	3	4	5	6	7	8	9	10
Mol. Wt. In kD.	112	89	75	71	63	56	53	47	42	40

Out of the 10 bands protein bands with molecular weights 112, 75, 71, 56, 47, 42 and 40 (all in kD) gave strong bands. Protein bands with molecular weights 89, 63, and 53 kD gave only a narrow and less intensive bands. Electrophoretic pattern remained almost same through out the 12 weeks frozen storage study. Protein band with molecular weight 63 kD could not be visualized from the 45th day sample onwards.

4.5.3. *Penaeus indicus*

Plate 3 shows the electrophoretic pattern of WSP extracted with distilled water, subjected the various periods of frozen storage. 15 bands were obtained in a 7.5 gel.

Table 18. Molecular weights of protein bands extracted with distilled water

Band No.	1	2	3	4	5	6	7	8	9	10
Mol. Wt. In kD.	200	178	158	133	100	89	84	79	71	67
No of Band	11	12	13	14	15					
Mol. Wt. In kD.	60	56	50	42	40					

Out of these 15 bands, proteins bands with molecular weights 100, 89, 79, 71, 60, 56, 50, 42 and 40 (all in kD) gave strong bands. Protein bands with molecular weights 200, 178, 158, 133, 84 and 67 gave only a narrow and less intense bands.

Electrophoretic pattern remained almost same through out the 12 weeks frozen storage study. Protein bands with molecular weights 200 kD could not be visualized from 30th day sample onwards and protein bands with molecular weights 158 and 133 kD could not be visualized from 15th day sample onwards.

4.5.4 *Parapenaeopsis stylifera*

Plate 4 shows the electrophoretic pattern of WSP extracted with distilled water, subjected to various periods of frozen storage. 12 bands were obtained in a 7.5% gel.

TABLE 19. MOLECULAR WEIGHTS OF PROTEIN BANDS EXTRACTED WITH DISTILLED WATER

Band No.	1	2	3	4	5	6	7	8	9	10
Mol. Wt. In kD.	224	178	150	126	100	84	79	63	60	50
Band No.	11	12								
Mol. Wt. In kD.	45	40								

Out of these 12 bands protein bands with molecular weights 100, 84, 60 and 40 (all in kilo Daltons) gave strong bands while protein bands with molecular weights 224, 178, 150, 126, 79, 63, 50 and 45 (all in kD) gave a narrow and less intense bands. Electrophoretic pattern remained almost the same except those with molecular weights 79 and 63 kilo daltons, which could not be visualised from the 30th day sample.

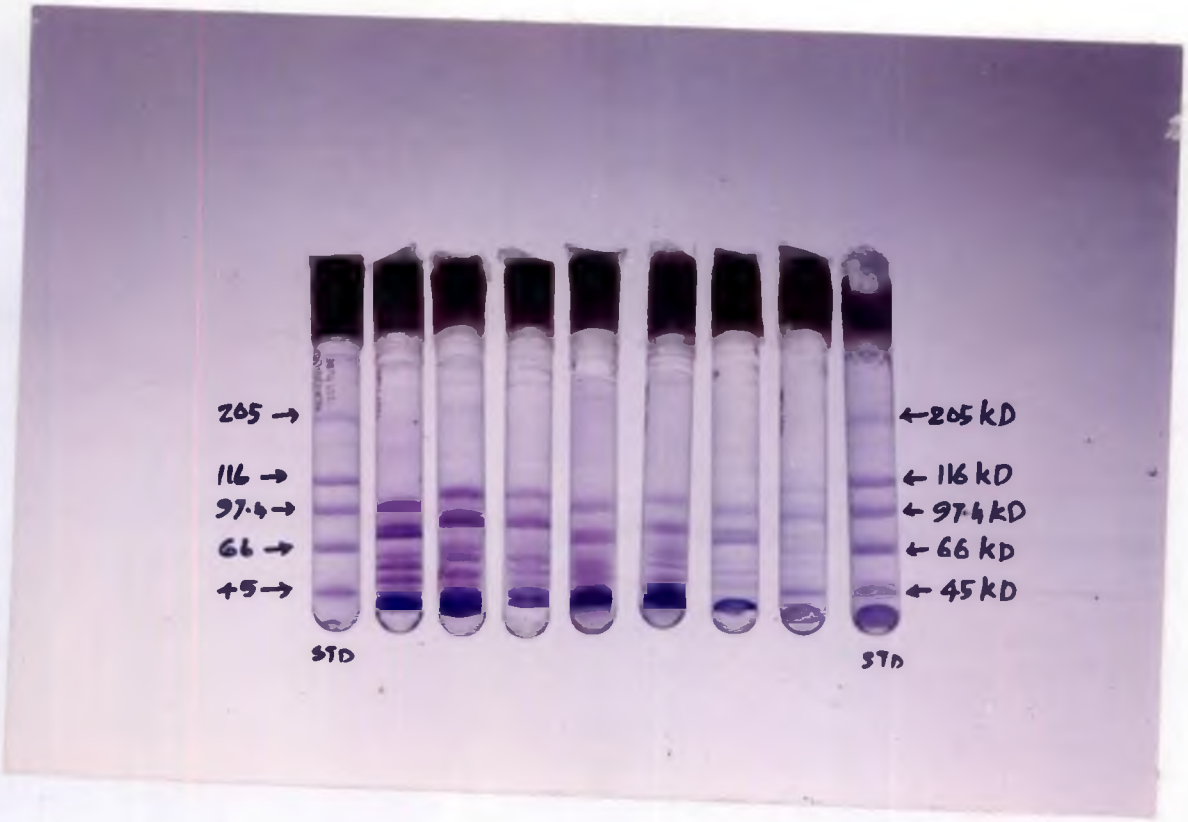

Plate 3 : Electrophoretic pattern of Water Soluble Protein (*P. indicus*)

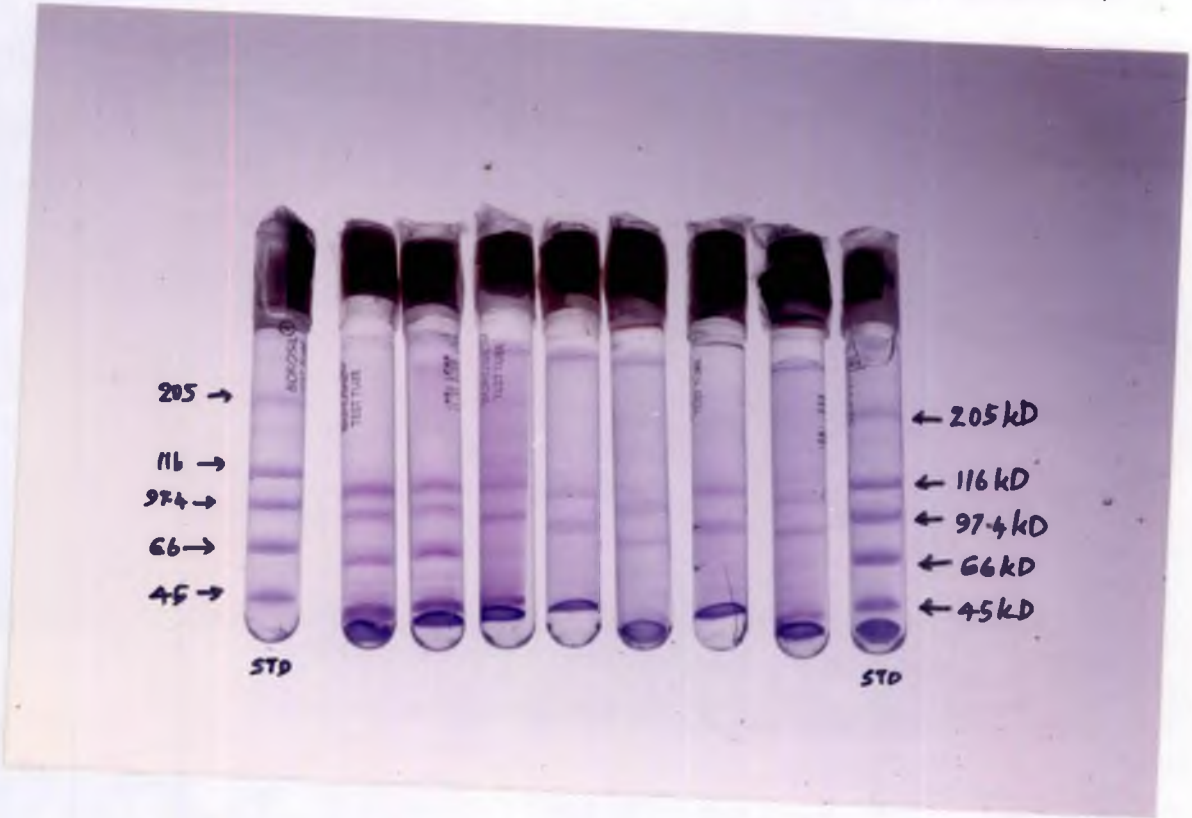

Plate 4 : Electrophoretic pattern of Water Soluble Protein (*P. stylifera*)

4.6. ELECTROPHORETIC PATTERN OF MYOFIBRILLAR PROTEINS

4.6.1. *Labeo rohita*

Plate 5 shows the electrophoretic pattern of myofibrillar proteins extracted from fish meat subjected to various periods of frozen storage. Eighteen bands were obtained in a 7.5%gel.

Table 20. Molecular weights of protein bands extracted with Dyer's buffer (in kilo Daltons)

Band No.	1	2	3	4	5	6	7	8	9	10
Mol. Wt. In kD.	335	316	200	178	168	158	150	133	100	94
Band No.	11	12	13	14	15	16	17	18		
Mol. Wt. In kD.	79	70	63	60	56	50	45	40		

Out of these 18 bands, protein bands with molecular weights 200, 45 and 40 kilo Daltons gave strong bands and the other gave only a narrow and less intense bands. Major change was observed in the myosin heavy chain (200 kD.), which became narrow and less intense with the storage period. Aggregates which could not enter the gel were observed from 45th day sample onwards. Band with molecular weight 178 (kD) was not observed in the 60th day sample onwards, 63 and 56 kD bands from 45th day sample onwards. Band with molecular weight 79 kD was not observed in the 90th day sample.

4.6.2. *Megalaspis cordyla*

Plate 6 shows the changes in the electrophoretic pattern of myofibrillar proteins extracted from fish meat subjected to various periods of frozen storage. Fifteen bands were obtained in a 7.5% gel.

Table 21. Molecular weights of protein bands extracted with Dyer's buffer

Band No.	1	2	3	4	5	6	7	8	9	10
Mol. Wt. In kD.	200	158	141	133	112	100	94	84	79	63
Band No.	11	12	13	14	15					
Mol. Wt. In kD.	60	50	47	42	35					

On 15th day sample onwards one additional band with apparent molecular weight 290 kD was observed, which disappeared in the 60th and 90th day sample. Aggregates could be seen on 30th day sample onwards which became more intense as the storage period progressed. Protein bands with molecular weight 94 kD became less intense in 60th and 90th day samples and the protein band with molecular weight 84 kD became less intense from 30th day sample onwards. Major changes can be seen in the protein band with molecular wt. 200 kD, which could be the myosin heavy chain in width decreased with increasing storage period. The intensity of bands also decreased with the storage period.

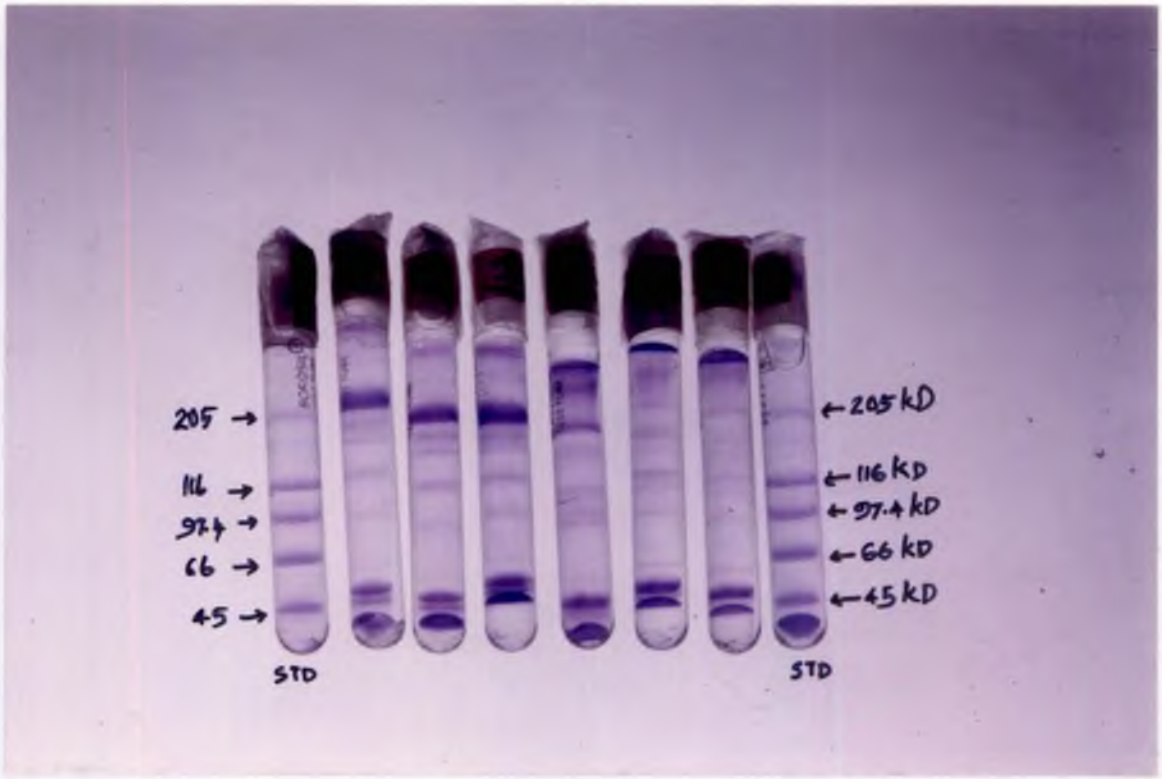

Plate 5 : Electrophoretic pattern of Salt Soluble Protein (*L. rohita*)

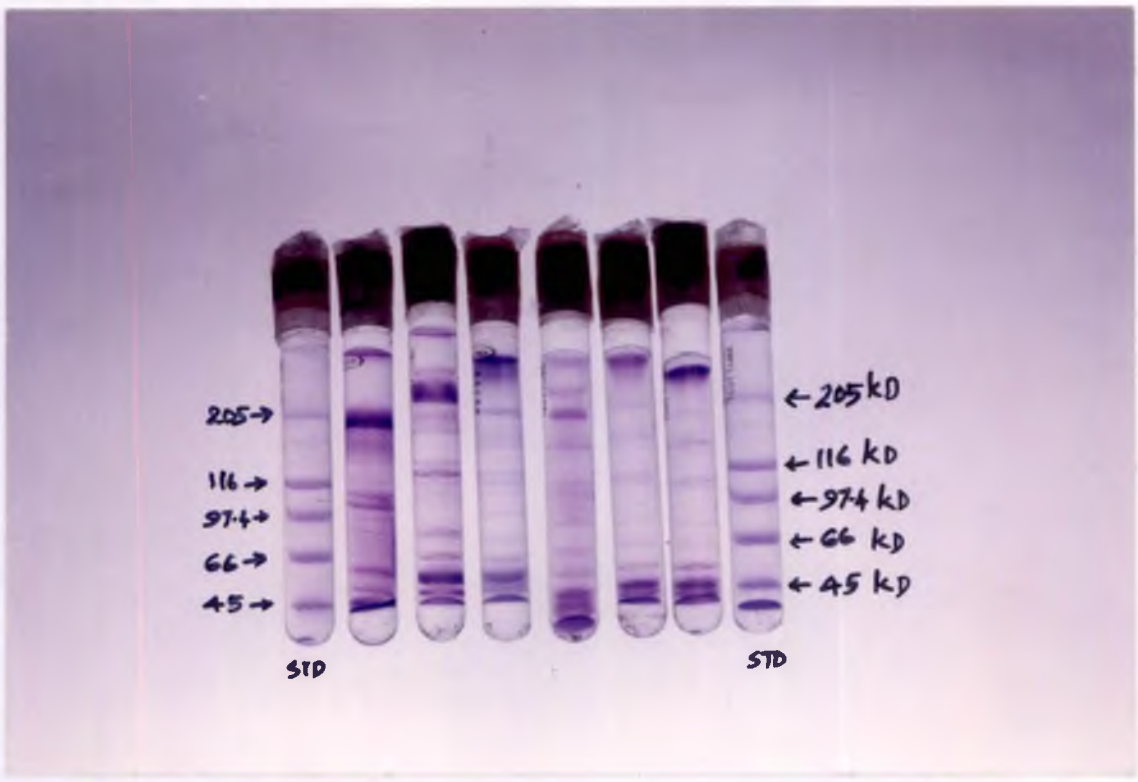

Plate 6 : Electrophoretic pattern of Salt Soluble Protein (*M.cordyla*)

4.6.3. *Penaeus indicus*

Plate 7 shows the changes in the electrophoretic pattern of myofibrillar proteins extracted from fish meat subjected to various periods of frozen storage. Twelve bands were obtained in a 7.5% gel.

Table 22. Molecular weights of protein bands extracted with Dyer's buffer

Band No.	1	2	3	4	5	6	7	8	9	10
Mol. Wt. In kD.	282	200	158	133	126	100	84	79	71	53
Band No.	11	12	13							
Mol. Wt. In kD.	50	42	35							

Protein band with molecular weight 282kD was absent from 75th day sample onwards. At the same time aggregates could be seen at the top of the gel from 45th day sample onwards. Protein bands with molecular weight 100 kD and 71 kD became less intense from 45th day sample onwards. While the protein band with molecular weight 84 kD became less intense from 15th day sample onwards. Intensity of the bands decreased with the storage period.

4.6.4. *Parapenaeopsis stylifera*

Plate 8 shows the changes in the electrophoretic pattern of myofibrillar proteins extracted from fish meat subjected to various periods of frozen storage. Nine bands were obtained in a 7.5% gel.

Table 23. Molecular weights of protein bands extracted with Dyer's buffer

Band No.	1	2	3	4	5	6	7	8	9
Mol. Wt. In kD.	200	188	158	141	126	112	79	42	35

Out of these 9 bands, bands with molecular weights 126, 112 and 79 kD gave less intense bands. Aggregates could be seen at the top of the gel from 15^{th} day sample onwards. On 45^{th} day sample a new band with mol. wt. 282 kD was seen, which was not seen in other samples. Aggregates became stronger in the 60^{th} day sample onwards. Intensity of the bands reduced with the storage period.

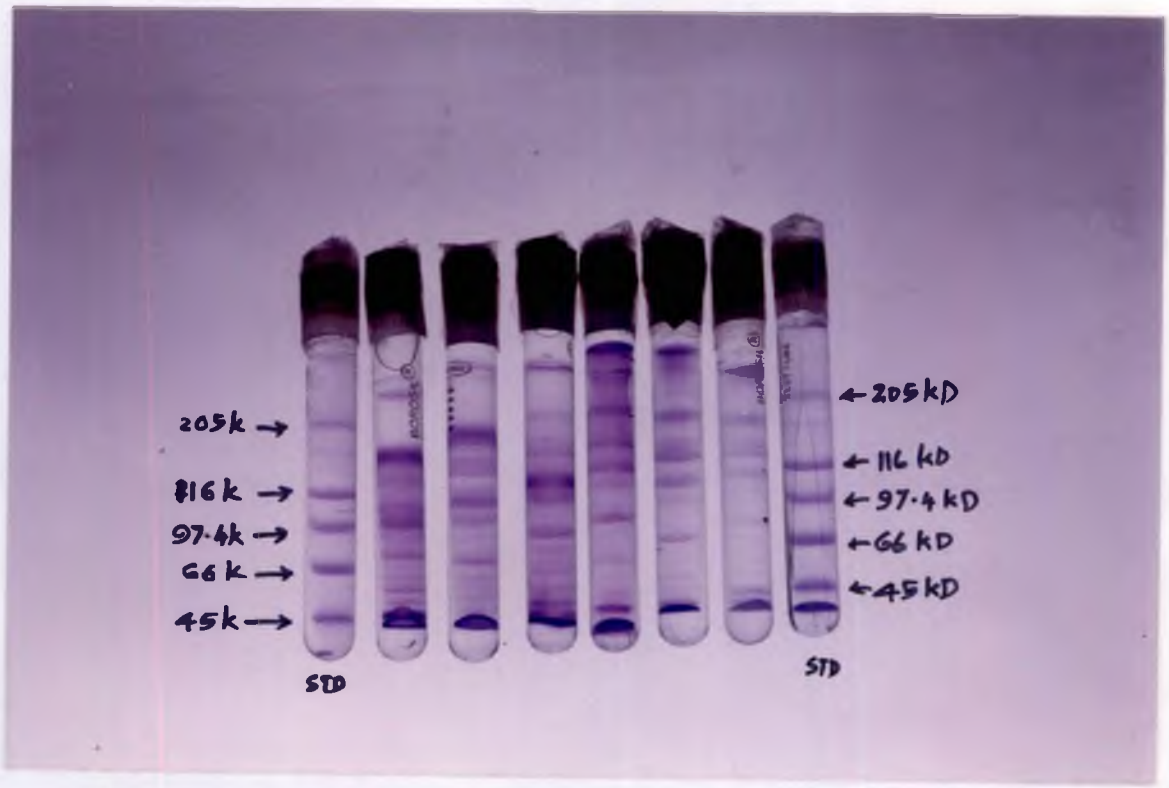

Plate 7 : Electrophoretic pattern of Salt Soluble Protein (*P. indicus*)

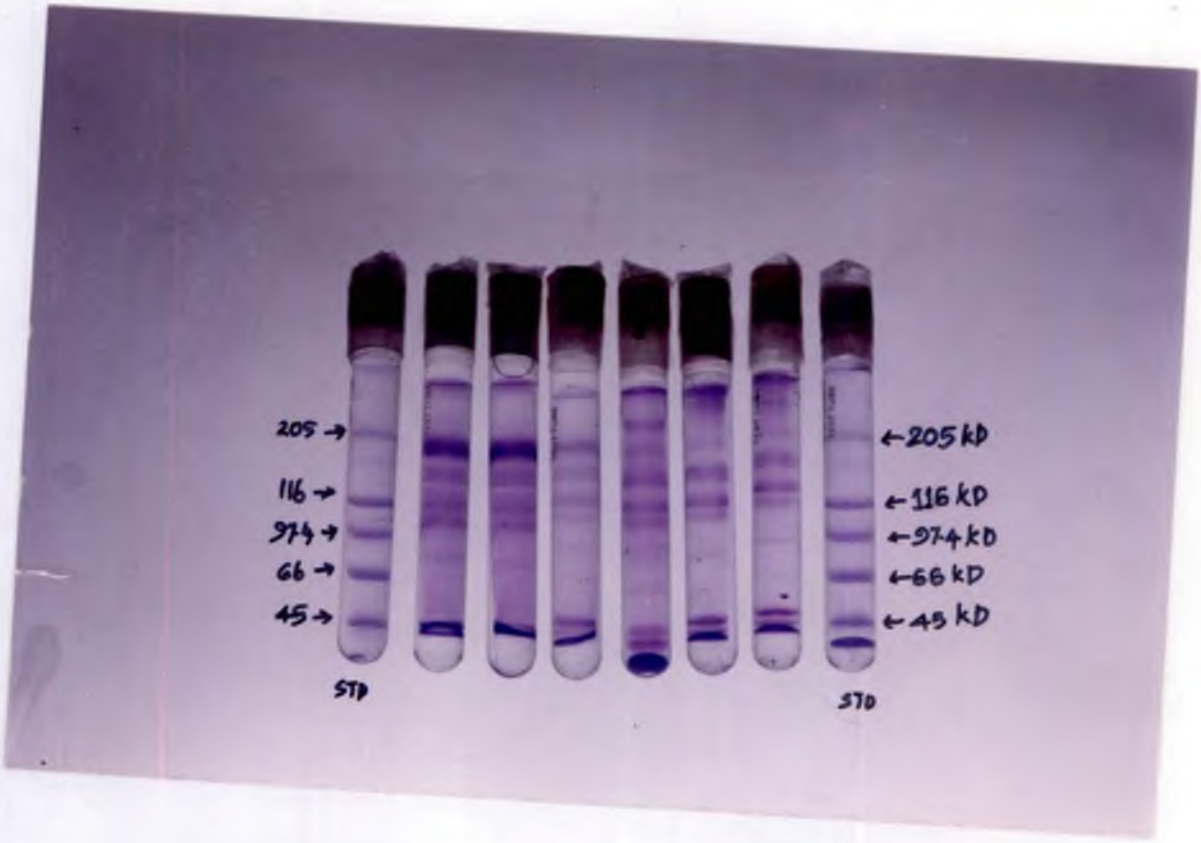

Plate 8 : Electrophoretic pattern of Salt Soluble Protein (*P. Stylifera*)

DISCUSSION

V. DISCUSSION

5.1. SALT SOLUBLE NITROGEN

Salt soluble nitrogen decreased significantly ($P<0.05$) by 23%, 24%, 25% and 20% in *M. cordyla*, *L. rohita*, *P. indicus* and *P. stylifera*, respectively in 3 months at -18^0C. A decrease in the salt soluble protein during frozen storage was observed by several workers (Joseph and Perigreen, 1980; Sarma *et al.*, 1998). This is mainly attributed to the denaturation of proteins.

5.2. WATER SOLUBLE NITROGEN

Water soluble nitrogen showed a gradual decreasing trend in *L. rohita*. This is against the findings of Devadasan *et al.*, (1978), where no significant difference in WSN observed during frozen storage. The loss in WSN may be due to drip loss . A decreasing trend for WSN was also observed in *M. cordyla* and *P. indicus*. Similar results were observed by Badonia and Devadasan (1980) in Ribbon fish and Devadasan *et al.*, (1978) in *M. seenghala* and *W. attu*. However, no significant difference in the WSN was observed in *P. stylifera*. Mathen (1970) reported similar results.

Frozen storage of minced Greenland halibut at -10^0C resulted in a rapid loss in salt solubility of "myofibrillar proteins" (approximately 50% in 15 days), and a gradual loss in water solubility of "Sarcoplasmic proteins" (approximately 40% in 120 days) (Lim and Harad, 1984). Srikar (1979) reported a gradual decrease in the SPP and MFP upto 20 weeks of storage at -20^0C. Suzuki and Kanna (1977) also reported a decreasing trend of MFP and SPP in shrimp during frozen storage.

5.3. NON- PROTEIN NITROGEN

Non – protein nitrogen (NPN) showed an overall significant decline ($P<0.05$) by 24%, 27%, 20%, 9% in *M.cordyla*, *L.rohita*, *P. indicus* and *P, stylifera* respectively. This is the agreement with the findings of Devadasan *et al.*, (1978) and Sarma *et al.*, (1998). Decrease in NPN may be due to drip loss, through which considerable amount of NPN is lost, resulting in a decrease in NPN values in fish muscle.

5.4. ELECTROPHORETIC PATTERN OF WATER SOLUBLE PROTEINS

Since 7.5% polyacrylamide gel was used, only those proteins with molecular weight higher than 40 kD could be separated. In all the 4 species major bands in the electrophoretic pattern of water soluble proteins remained almost the same. This is in agreement with the findings of Devadasan *et al.*, (1978). However in *L.rohita* protein bands with molecular weight 178 and 169 kD from 60^{th} day sample onwards and 266 kD in the 90^{th} day sample could not be visualized. In *M.cordyla*, protein band with molecular weight 63 kD could not be visualized from the 45^{th} day sample onwards. In *P. Indicus*, protein band with molecular weights 200 kD could not be visualized from the 30^{th} day sample onwards and 158 and 138 kD from 15^{th} day sample onwards. In *P. stylifera* protein bands with molecular weight 79 and 63 kD could not be visualized from the 30^{th} day sample. These bands which could not be visualized during frozen storage gave only a narrow and faint band in the fresh sample also. There was a significant difference in the water soluble nitrogen content during frozen storage. Owusu-Anash and Hultin (1992) reported that, relative to the control samples there were decreases in all water soluble protein bands in the

electrophoretic pattern of frozen samples. Dyer and Dingle (1961) found detectable change in the electrophoretic patten of the water soluble protein fraction from frozen cod stored for 7 weeks at -12^0C. Sarcoplasmic protein insolubilization has been reported upon frozen storage of a white fish (Award *et al.*, 1969) and cod fillets (Yowell and Flurkey, 1986). Tiecco (1981) reported a gradual disappearance of the slowest migrating band in frozen beef held at -12^0C which started fading in samples frozen stored for 15 days and had disappeared completely in samples stored for 70 days. LeBlanc and LeBlanc (1989) observed a difference in the SPP Electropherograms of cod fillets frozen at -12^0C for 10 months. LeBlanc *et al.* (1994) found storage changes in SPP from cod and haddock fillets using capillary electrophoresis, indicating both species difference and low and high molecular weight frozen storage changes. However, in the present study no additional bands could be visualized during frozen storage which is in conformation with the findings of Devadasan *et al.*, (1978).

Although the separation profiles of individual fish, even of the same species, show some degree of variation, they none the less have a sufficient number of major zones in common to enable a profile to be recognized unequivocally (Mackei, 1997). So it is not necessary to consider all the bands of the SPP fraction for species identification. Many fish species yield 1- 5 strong bands of acidic low molecular weight proteins, called parvalbumins (Girija and Rehbein, 1988), which are sufficient for species identification. However, changes in these group of proteins could not be detected since 7.5% gel was used for the study.

5.5. ELECTROPHORETIC PATTERN OF MYOFIBRILLAR PROTEINS

5.5.1. *L. rohita.*

Eighteen bands were obtained in a 7.5% gel. A general decrease in the intensity of bands were observed with increase in frozen storage period. This is in agreement with the results obtained by Ohnishi and Rodger (1980 b). With the increase in the storage period extractability also decreased. Major changes were found in the myosin heavy chain, which became narrower and less intense with the increase in storage period. This is in agreement with the previous studies (Ohnishi and Rodger, 1980 b; Ragnarsson and Regenstien, 1989). In the case of *L.rohita* reduction in extractability decreased at a consistent rate through-out the frozen storage period. In the electrophoretic pattern also for the 45^{th} day sample MHC became narrow and became fainter from 60^{th} day sample onwards. Ohnishi and Rodger (1980 b) reported that there was no MHC band on SDS gel electrophoresis for 12 weeks frozen stored sample. From 45^{th} day sample onwards a new protein band at the top of the gel could be seen, which became more intense in the following day sample. This may be due to the formation of protein aggregates, which could not enter the gel. Mathews *et al.* (1980) in studying frozen stored minced cod observed an initial decrease in the amount of MHC and the concomitant appearance of a band of material which did not enter 8.75% polyacrylamide gel. Similar high molecular weight proteins were also observed by Lim and Haard, (1984) in frozen minced Greenland halibut.

5.5.2. *M. cordyla*

Fifteen bands were obtained in a 7.5 % gel. A general decrease in the intensity of bands could be observed with the increase in storage period. Major changes were noticed in MHC, which became narrower from the 35th day sample onwards and its intensity also decreased from the 60th day sample onwards. Here the rate of decrease in the extractability of salt soluble protein was more during the initial period. From the 15th day sample onwards a new band with apparent molecular weight of 290 kD could be seen, which disappeared in the 60th day sample. Concurrently a new protein band at the top of the gel had also been noticed from the 30th day onwards, the intensity of which increased in the following day samples. Similar results were obtained by Ragnarsson and Regenstein, (1989) in cod and whiting. The intensity of protein band with molecular weight 94 kD decreased heavily from the 60th day sample onwards and those with 84 kD from the 30th day sample onwards. These may be due to the reduction in the extractability with frozen storage period. Owusu-Ansah and Hultin (1992) reported that the bands with molecular weights corresponding to those of MHC (200 kD) M- proteins (180-195 k D), probably C-proteins (140 kD) and others (151 kD and 92 kD) were completely absent from the electropherogram of test samples after 6 weeks storage.

5.5.3. *P. indicus*

Thirteen bands were observed in a 7.5 % gel. A general decrease in the intensity of bands could be seen with the increase in storage period. Major changes observed was in MHC, the intensity and width decreased on storage with the appearance of a new band at the top of the gel. This is more pronounced from the

30th day sample onwards. Here also the rate of decrease in the extractability of proteins was more at the initial storage period. The band with molecular weight 282 kD could not be visualized from the 60th day onwards. This may be due to the incomplete transfer of MHC as a result of which the top portion of the gel also took the stain. Protein bands with molecular weight 100 kD and 71 kD became less intense from the 45th day sample onwards and 84kD from the 15th day sample onwards. This may be due to the reduction in the extractability of salt soluble proteins.

5.5.4. *P. stylifera*

Nine bands were obtained in a 7.5% gel. A general decrease in the intensity of the bands could be observed with the increase in the storage period. Major changes were observed in MHC, the intensity and width of which decreased with storage period. This was more pronounced from the 30th day sample onwards. Rate of decrease in the extractability of proteins was also more pronounced at the initial storage period. A concomitant appearance of a new band which could perhaps be the protein aggregates formed could be visualized along with this. Another protein band with apparent molecular weight 280 kD could be seen in the 45th day sample, which could not be clearly seen in the following day samples. This may be due to the incomplete transfer of MHC which might have taken the stain, as a result of which the new band could not be seem.

Owusu-Ansah and Hultin (1992) reported that proteins of molecular weight 102 kD, 82 kD and 73 kD in sarcoplasmic fraction in red hake muscle were most susceptible to frozen storage. They also reported that the bands of proteins with

molecular weights corresponding to those of MHC (200kD) M-proteins (180-195kD), probably C-proteins (140 kD) and others (151kD and 92 kD) were completely absent from the electropherograms of test samples after 6 weeks storage. They also reported that Troponin-I and Troponin-C as well as Tropomyosin are also susceptible to frozen storage. Changes to these proteins could not be observed in the study, since these proteins cannot be separated in a 7.5%gel.

Thus there was a significant decrease in the SSN of all the 4 species during frozen storage. WSN also registered a gradual decrease during frozen storage in all species except for *P. stylifera*, where the decrease in WSN content was not so significant. NPN also showed a decreasing trend during frozen storage. Electrophoretic pattern of water soluble protein remained unchanged in all the 4 species except for a few less intense bands which could not be visualized properly in the subsequent frozen storage period. In the electrophoretic pattern of the salt soluble protein, MHC showed a major change in all the 4 species with the concomitant appearance of a protein band at the top of the gel, which may be the aggregate formed.

SUMMARY

VI. SUMMARY

1. Freezing is considered as excellent process for preserving the quality of fish for longer periods of time.

2. Sarcoplasmic proteins play a major role in species identification in fish and fishery products

3. As long as the Sarcoplasmic proteins of flesh remain in the native state. They are readily extractable with water or dilute salt solutions and can be used for fish species identification by electrophoresis or isoelectric focusing.

4. Myofibrillar proteins play a major role in the jellying properties of surimi and surimi based products, changes to which may affect the quality of the finished products.

5. Four species *Megalspis cordyala Labeo rohita Penaeus indicus, Parapenaeopsis stylifera* were used for the frozen storage study.

6. Frozen fish and prawn were stored for 90 days, during which storage studies were conducted based on various biochemical parameters and the electrophoretic pattern of water soluble proteins and salt soluble proteins.

7. Salt Soluble Nitrogen, Water Soluble Nitrogen and Non Protein Nitrogen showed a significant decrease during 90 days frozen storage

study. However, changes in the WSN content of *P.stylifera* showed no significant difference.

8. Electrophoretic pattern of water soluble proteins remained almost the same through out the frozen storage period with some minor bands becoming less intense with storage period.

9. In the Electrophoretic pattern of salt soluble proteins major change was observed in myosin heavy chain, which became narrower and less intense with the concomitant appearance of high molecular weight protein aggregates at the top of the gel.

VII. REFERENCES

*Ahmed, N.S., Hamza, M.A. and Abdul - Aal, M.H. (1989) Effect of icing and frozen storage on the stability and some quality characteristics of Egyptian eels and catfish. *Bull Nat. Inst. Oceanogr. Fish. Egypt.*, **15(1)** : 107 - 118.

*Akroyd, P. (1967) Acrylamide Gel slab electrophonesis in a simple glass cell for improved resolution and comparison of serum proteins. *Amal. Biochem.*, **19(3)** : 399 - 410.

Al-Dagal, M.M.(1996) Microbial safety and quality and protein integrity of shrimp sold in shops in Riyadh, Saudi Arabia. *J.Food Prot.*, **59(9)** : 988-991.

An, H., Marshall, M.R., Otwell, W.S., Wei, C.I. (1988) Electrophoretic identification of raw and cooked shrimp using various protein extraction systems. *J.Food Sci.*, **53(2)** : 313-318.

An, H., Marshall, M.R., Otwell, W.S. and Wei, C.I. (1989) Species identification of raw and boiled shrimp by urea gel isoelectric focusing technique. *J. Food Sci.*, **54:** 233-236.

An, H., Klein, P.A., Kao,K. J., Marshall, R.R., Otwell, W.S. and Wei, C.I. (1990) Development of monoclonal antibody for rock shrimp identification using enzyme – linked immunosorbent assay. *J. Agric. Food Chem.*, **38** : 2094 - 2100

Ang, J.F. and Hultin H.O.(1989) Denaturation of cod myosin during freezing after modification with formaldehyde *J.Food Sci.*, **54** :814.

*Andrews, A.T. (1986) Monographs on physical biochemistry. **In** : A. R. Peacock. and W. F. Harrington (eds.), *Electrophoresis: Theory, Techniques and Biochemical and clinical Applications.*, 2nd Edn. Clarendon Press, oxford.

AOAC. (1983) Official methods of analysis of the Association of Official Analytical Chemists. W. Horwitz, W.A. Senzel and H. Reynolds. (eds.), *Association of Official Analytical Chemists*, Washington, DC . pp. 1094.

AOAC. (1984) Official methods of analysis of the Association of Official Analytical Chemists, S. Williame, (ed.), Association of Official Analytical Chemists, Arlington Virgima. pp. 1141.

*AOAC. (1990) Official Methods of analysis, 15th ed.,p.885-886. *Assoc.of Official Analytical chemists.*, Arlington, VA.

Asghar, A., Saejima, K. and Yasni, T. (1985) Functionality of muscle proteins in gelation mechanism of structured meat products. C.R.C.*Crit. Rev.Food Sci.Nutr.*, 22(1) : 27-105.

Award, A., Powrie, W.D., and Fennema, O.R. (1969) Deterioration of fresh water white fish muscle during frozen storage at –10 °C. *J.Food Sci.*,**34**: 1-8.

Badonia, R. and Devadasan, K. (1980) Frozen storage characteristics of Ribbon fish *Fish. Tehnol.*, **17** : 125 – 126.

*Boerresen, T., Knudsen, L.B., Nielsen, J.(1985) Denaturation of sarcoplasmic proteins during frozen storage of fish. *Storage lives of chilled and Frozen Fish products-Proceedings of meeting of commissions-C2 and -D3- oct. 1-3, 1985.International Institute of Refrigeration.*, Paris, France.**60(4)**: 71-77.

*Bunda, M.C.M. and Hultin, H.O. (1983) Role of cofactors in breakdown of TMAO in frozen red lake muscle. *J. Food Proc. Preserv.* **7** : 221

Castells, C., Neal, W. and Date.J.(1973) Comparison of changes in TMA, DMA, and extractable protein in iced and frozen stored gadoid fillets. *J. Fish. Res. Bd. Can.*, **30** : 1246-1250.

Chen, C.S., Hwang, D.C., and Jiang, S.T.(1989) Effect of storage Temperatures on the formation of disulfides and denaturation of milkfish myosin *(Chanos chanos) J.Agric.Food Chem.*, **37**: 1228-1231.

Chen, F.C., Hsieh, Y.H.P., Bridgman, R.C. (1998) Monoclonal antibodies to Porcine thermat stable muscle protein for detection of park in raw and cooked meats. *J. Food Sci.*, **63(2)** : 201 – 205.

Colomenero, F.J., Borderias,A.J. (1983) A study of the effect of frozen storage on certain functional propertis of meat and fish protein..*J.Food. Technol.*,**18** : 31.

Cummins, P. and Perry, S.V. (1973) Biochem. J.*, **133** : 765 – 777.

Cowie, W.P.(1968) Identification of fish species by thin slab polyarylamide gel lectrophoresis. *J. Sci.Food Agri.*, **19**: 126-229.

Damodaran ,S.(1994) Structure-function relationship of food protein. **In :** N.S.Hettiarachchy. and G.R.Ziegler (eds.), *Protein Functionality in Food Systems.* IFT basic symposium series, 9. Marcel Dekker Inc. New york. pp. 1-38.

Davies, G., Stark, G. (1970) Proc. Nat. Acad. Sci. USA,* **66** : 651.

*Davis, B.J.(1964) Disc Electrophoresis II. Methods and application to human serum proteins. *Ann .N. Y. Acad. Sci.*, **121** : 404-428.

Del Mazo, M.L., Huidobro, A., Torrejon,P., Tejada, M., Careche, M.(1994) Role of formaldehyde in formation of natural actomyosin aggregates in hake during frozen storage. *Zeitschriftfir Lebensmittel -Untersuchung und-Forschung.* Berlin, Heidelberg; vol. **198(b)** : 59-464.

Devadasan, K., Varma, P.R.G., Venkataraman, R.(1978) Studies on frozen storage characteristics of fillets from six species of fresh water fishes. *Fish. Tech.*, **15** :1-6.

*Dingle, J.R., Keith, R.A., and Lall, B. 1977. Protein instability in frozen storage induced minced muscle of flatfishes by mixture with muscle of red Lake. *Can. Inst. Food Sci. Tech.* Vol. I. **10** : 143

*Dyer, W. J. (1968) Deterioration and storage life of frozen fish. **In:** J. Hawthorn. and E. J. Rolfe (eds.)., *Low Temperature Biology of Foodstuffs.*, pp. 429. Pergamon Press, Oxford.

Dyer, W.J. and Dingle, J.R. (1961) Fish proteins with reference to freezing. **In** :G.Borgstrom. (ed.) vol.1., *Fish As Food. Production, Biochemistry And Microbiology.* P.275. Academic Press, New York, NY.

Fukuda,Y.,Tarakita, Z.,Kawamura, M., Kakehata, K., Arai, K. (1982) Denaturation of myofibrillar proteins in chub mackerel during freezing and storage *Bull. Jap. Soc. Sci. Fish,.* **48(11)** : 1627-1632.

Fukuda,Y., Tarakita, Z ., Arai,K. (1984) Effect of freshness of chub mackerel on the freeze-denaturation of myofibrillar protein. *Bull.Jap.Soc.Sci.Fish.,* **50(5)** : 845-852.

Fukuda, Y. (1996). Denaturation by freezing of fish muscle proteins. *Bull. Nati. Res. Inst. Fish Sci.*, Japan No: 8, pp. 77 – 92.

Galluzzo, S.J. and Regenstein, J.M.(1978) Role of chicken breast muscle protein in meat emulsion formation: myosin, actin and synthetic actomyosin. *J.Food Sci.,* **43**: 1761.

*Gangar, V.(1992) Electrophoretic identification of *(Callinecfes sapidus)* meat. M.S. Thesis, University of Florida.

Gill, T.A., Keith, R.A. and Smith Lall,B. (1979) Textural deterioration of red hake and haddock muscle in frozen storage as related to chemical parameters and changes in the myofibrillar proteins. *J. Food Sci.,* **44**: 66.

Girija, N. and Rehbein, H. (1988) Comparison of parvalbumin patterns from different fish species by isoelectric focussing of muscle extracts. *Comp. Biochem. Physiol.*, **91 B** : 723.

Haard, N.F. (1992) Biochemical reactions in fish muscle during frozen storage. **In**:G.Bligh (ed.), *Seafood Science and Technology.*, Oxford: Fishing News Books.

Haard, N.F., Simpson, B.K., Pan, B.S. (1994) Sarcoplasmic protein and other nitrogenous compounds. **In**: N.E. Sikorski., B.S. Pan and F. Shahidi. (eds.), *Sea Food Proteins.*, Chapman and Hall, London, pp.13-14.

Hamilton, W,D. (1982) Fish species identifiction by thin layer agarose isolelectric focusing and densitometric scanning. *J.Assoc. off.Anal.Chem.*, **65**: 119-122.

*Harrington, M.G. and Henahan,M.M.(1982) Studies of sarcoplasmic proteins by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Meet. *Proc. 28th Europ.Meat Res. Workers.*, **1**: 63.

Hasnain, A., Tamura, H. and Arai, K. (1976) *Bull.Jap.Soc. Sci.Fish.*, **42** : 783-791.

Heen, E. (1982) Development in chilling and freezing of fish. *Int. J. Refrig*, **5(1)** : 5-49.

Heldman, D.(1982) Food properties during freezing. *Food Tecnol.*, **36(2)** : 2-98.

Hill, W.S., Learson, R.J. and Lane, J.P.(1966) Identification of fish species by agar gel electrophoresis. *J.Assoc. Off. Anal. Chem.*, **49** : 1245 - 1247.

Huang, T., Marshall, M.R., and Wei, C.I. (1995) Identification of red snapper (*Lutjanus campechanus*) using electrophoretic techniques. *J.Food Sci.*, **60**: 279- 283.

Hume, A. and Mackie, I.M.(1980) The use of electrophoresis of the water soluble muscle proteins in the quantitative analysis of the species components of fish mixture. **In** :J.J.Connell (ed.), *Advances in Fish science and Technology.*, p.451. Fishing News(Books) Ltd.,Surrrey,UK.

*Hsieh, Y.H.P., Johnson, M.A., Wetzstein, C.J. and Green, N.R. (1996) Detection of species adulteration in park products using agar gel immunodiffusion and enzyme – linked immunosorbent assay. *J. Food Qual.* **19** : 1 – 13.

Jarenback, L. and Liljemark, A (1975) Ultra structural changes during frozen storage of cod *(Gadus morhua.)* II. Structure of extracted myofibrillar proteins and myofibril residues . *J.Food Technol.*,**10**: 309-325.

Jiang, S.T., Hwang, B.S., Tsao, C.Y.(1987) Effect of adenosine nucleotides and their derivatives on the denaturation of myofibrillar proteins *in vito.* during frozen storage at -20 ^{0}C .*J. Food. Sci.*, **52(1)**: 117.

Jiang, S.T.,Hwang ,D.C.and Chen, C. S. (1988 a) The effect of storage temperature on the formation of disulfides and denaturation of milk fish actomyosin. *J. Food Sci.*, **53(5)** :1333-1335.

Jiang, S.T., Hwang, D. C. and Chen, C. S. (1988 b) Denaturation and change in SH group of actomyosin from milk fish *(Chanos Chanos)* during storage at -20^{0} C. *J.Agric. Food Chem.*, **36**: 433-437.

Jiang, S. T., Wang , F. J. and Chen , C. S. (1989) "Properties of actin and stability of actomyosin reconstituted from milk fish *(Chanos chanos)* actin and myosin". *J. Agric. Food Chem.* **37** :1232-1235.

Joseph,J. and Perigreen, P. A. (1980) Iced and frozen storage characteristics of cultured *chanos chanos. Fish. Technol.*, **17(1)**: 21.

Joseph, J., Perigreen, P. A. (1983) Sudies on frozen storage of minced fish from threadfiun bream. *Fish.Technol.*, **20(1)**: 13-16.

Karel, M., Schaich, K. and Roy, R. (1975) Interaction of peroxidizing methyl linoleate with some proteins and amino acids. *J.Agric. Chem.*, **23(2)**: 159-163.

Kawasaki, K.I., Ooizumi, T., Konno, K. (1992) Cross-linking reaction of myosin heavy chain in carp myofibrils induced by peroxidised sardine oil. *Bull. Jap. Soc. Sci. Fish.*, **58(1)** :127-133.

*Keenan, C. P.and Shaklee, B. (1985) Electrophoretic identification of raw and cooked fish fillets and other marine products. *Food Technol.*,(Australia) **37**: 117-128.

*Kimura, I., Murozuka, T. and Arai, K .(1977) *Bull. Jap soc. Sci. Fish.*, **43**: 315-321.

Kinsella, J. E. (1982) Relation between structural and functional properties of food proteins. **In**: R.F. Fox and J.J. Condon. (eds.), *Food Proteins.*, Applied Science publishers, New York. pp.51-103.

Kleparnik, K. and Bocek, P. (1991) Theoretical background for clinical and biochemical applications of electromigration techniques. *J. Chromatog.*, **569:** 3-42.

Kokuryo, H., Seki, N. (1978) Identification of fish species of dried fish sticks (fushi) by SDS-polyacrylamide gel electrophoresis. *Bull. Jap. Soc. Sci. Fish.*, **44(1)**: 67-70.

Kolodziejska, I. and Sikorski, Z. E. (1980) Inorganic salts and extractability of fresh and frozen fish proteins. *Int. J. Refrig.* **3**: 151-155.

*Koohmaraie, M. (1992) The role of Ca^{2+} dependent proteases (calpains) in post-mortem proteolysis and meat tenderness. *Biochemie.*, **74**:239.

Koohmaraie , M., Kennick, W.H., Anglemier, A.F., Elgarim, E.A. and Jones T. K. (1984.) Effect of post-mortem storage on cold -shortened muscle. Analysis by SDS-Polyacrylamide gel electrophoresis. *J. Food Sci.*, **9**: 290.

Lane, J.P., Hill, W.S. and Learson, R.J. (1966)Identification of species in raw processed fishery products by means of cellulose polyacetate strip electrophoresis.*Comm. Fish. Rev.*, **28**: 10-13.

*Lanier, T.C, and Lee, C. M. (1992) **In**:*Surimi Technology.* Marcel Dekker, New York.

LeBlanc, E. L. and LeBlanc, R.J. (1989) "Separation of cod *(Gadus Morhua)* fillet proteins by Electrophoresis and HPLC after various frozen storage treatments." *J.Food Sci.*, **54**: 827-834.

LeBlanc, E. L., Singh, S. and LeBlanc, R. J. (1994) Capillary Zone Electrophoresis of fish muscle sarcoplasmic proteins. *J. Food. Sci.*, **59(6)**: 1267-1270.

Lee, C.M. (1986) Surimi manufacturing and fabrication of surimi-based products. *Food Technol.*, **40(3)**: 115.

Lewin, S. (1974) *Displacement of Water and its Control of Biochemical Reactions.* London : Academic Press.

*Lim, H.K. and Haard., N. F.(1984) Protein insolubilization in frozen Greenland halibut *(Reinhardtius hippoglossoides). J. Food Biochem.*, **8(3)**: 163.

Lin, T.M. and Park, J. W. (1996) Extraction of proteins from Pacific Whiting mince at various washing conditions. *J. Food Sci.*, **61(2)**: 432-438.

Lin, T.M., Park, J.W. and Morrissey, M.T. (1995) Recovered protein and neoconditioned water from Surimi processing waste. *J. Food Sci.*, **60**: 4-9.

Lundstorm, R. C. (1980) Fish species identification by thin-layer polyacrylamide gel isoelectric focusing. Collabarative study. *J. Assoc. Off. Anal. Chem* ., **63**: 69-73.

Lundstrom, R.C. (1981) Rapid fish species identification by agarose gel isoelectric focusing of sarcoplasmic proteins. *J. Assoc. Off. Anal. chem.*, **64**: 38-43.

Lundstrom.(1983) Identification of Pacific rock fish (sebastes) species by isoelectric focusing. *J. Assoc. Off. Anal. Chem.*, **66**: 974-980.

Lundstrom, R.C., Correia, F.F. and Wilhelm, K.A. (1982) Enzymatic dimethylamine and formaldehyde production in minced American Place and Blackback Flounder mixed with a red Hake TMAO-ase active fraction. *J. Food Sci.*, 47 : 1305-1310.

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

Mackie, I.M. (1980). A review of some recent application of electrophoresis and iso-electric focusing in the identification of species of fish in fish and fish products. **In:** J.J. Connell.(ed.), *Advances in Fish Science and Technology.* p. 444. Fishing News (Books) Ltd., Surrey, UK

*Mackie, I.M.(1992) Surimi from fish . **In**: D.E. Johnston., M.K. Knight., D.A.Leeward. (eds.), *The Chemistry of Muscle-based Foods.*, Royal Society of Chemistry, Cambridge, pp.200-207.

Mackie, I. M. (1993), The effects of freezing on flesh on flesh protein. *Food Reviews International.*, **9(4)** : 575-610.

*Mackie, I. M. (1994). Fish protein. **In**: B.J.F. Hudson. (ed.), *New and Developing Sources of Food Proteins.*, Chapman & Hall, London, pp. 95-143.

Mackie, I. M. (1997) Methods of identifying species of raw and processed fish. **In**:G.M. Hall.(ed.), *Fish processing technology.*, II edn., Blackie Academic and Professional, Londopn, pp.292.

*Mackie, I. M., Howgate, P., Craig, A., Liard, W. M., Ritchie, A. H. (1986) Acceptability of frozen stored consumer fish products. *Rev. Int. Froid.*, **9** : 169.

Mackie, I. M. and Taylor, T. (1972) Identification of species of heat-sterilized canned fish by polyacrylamide disc electrophoresis. *Analyst.*, **97**: 609.

*Margulis, B. A., Podgornaya, O. I., Galaktionov, K. I. (1982) Isoelectric focusing analysis of invertebrates actins. *Biofizika.*, **27(1)** : 87 – 90.

Martinez, I. (1992) Fish Myosin degradation upon storage. **In**: H.H. Huss. (ed.), *Quality Assurance in the Fish Industry.*,p.389-397. Elsevier Science Publishers. B.V.

*Martinez, I., Olsen, R.L., Ofstad, R., Janmot, C., d'-Albis, A.(1989) Myosin isoforms in mackerel (*Scomber scombrus*) red and white muscles. *FEBS-LETT.*, **252(1-2)**: 69-72.

Martinez, I., Ofstad, R. and Olsen, R.L. (1990 a) Electrophoretic study of myosin isoforms in white muscle of some teleost fishes. *Comp.Biochem. physiol.*, **96 B** : 221-227.

*Martinez, I., Ofstad, R. and Oslen, R.L. (1990 b). Intraspecific myosin light chain polymorphism in the white muscle of herring *(Clupea harengus harengus,L.) FEBS- LETT.*, **265**: 23-26.

*Martinez, I., Ofstad, R. and Oslen, R.L. (1990 c). Myosin isoforms in red and white muscle of some teleost fishes. *J. Muscle Res.Cell. Motility.*, **11**: 489-495.

*Maruyama, K. (1985) Myofibrillar Cytoskeletal Proteins of Vertebrates striated Muscle". **In**: R.Lawrie. (ed.), *Development in Meat Science.*, Vol. 3, pp.25-50. London/New York Elsevier Applied Sciences.

Mathen, C. (1970) I. Phosphate treatment of frozen prawns. II. Frozen storage characteristic of prawn meat treated with polyphosphates. *Fish. Technol.* **7(1)** : 52 – 57.

Mathews, A. D., Park, G. R. and Anderson, E. M. (1980) Evidence for the formation of cross-linked myosin in frozen stored cod minces. **In:** J. J. Connell.(ed.), *Advances in Fish Science and Technology.* Fishing News Books, Oxford.

Matsumoto, J. J. (1979) Denaturation of Fish Muscle Protein During Frozen Storage . **In**: O.Femmema. (ed.), *Proteins at Low Temperatures.* pp.205-224. Advances in chemistry Series 180. Washington, DC: American Chemical Society.

McBridge, M. A. and Parrish, F.C. (1977) The 30,000 daltons component of *longissimus* muscle. *J. Food Sci.*, **37**: 337.

McGill, A.S., Hardy, R., J.R., and Gunstone, F.D. (1974). Hept – cis – 4 – enal and its contribution to the off-flavour in cold stored cod. *J. Sci. Food Agric.*, **25** : 1477.

*McKay, S. J., Smith, M.J., Devlin, R. H. (1997) Polymerase chain reaction-based species identification of Salmon and coastal trout in British Colubia. *Mol. Mar.Biol.Biotecnol.*, **6(2)**: 131-140.

*Melvin, M. (1987) **In**: *Electrophoresis.* ,p. 40. John Wiley and sons, New York.

Morioka, K, Nishimura, T. and Obatake, A.(1998) Changes in the strengths of heat induced gels from myofibrils in combination with sarcoplasmic proteins from lizard fish and Pacific mackerel. *Fisheries science.*, **64(3)** : 503-504.

Morrissey, P.A., Mulvihill, D. M., and O'Neil, E.M. (1987) Functional properties of Muscle Proteins. **In**: B.J.F. Hudson. (ed.), *Developments in Food Proteins.*, pp.195-256. Vol.7. London/New York : Elselvier Applied Science.

Murray , R. K., Granner, D. K., Mayer, P. A. and Rodwell, V.W. (1993) **In:** *Harper's Biochemistry.*, pp.806. Prantice-Hall International Inc., New Jercey.

*Nakai, S. and Li-chan,S. (1988) **In**: *Hydrophobic Interactions in Food Systems . Boca Raton.*, FL: C.R.C press.

*Nikkila, O. E. and Linko , R. R. (1955) *Biochem .J.*, **60**: 242.

*Nishioka, F., Tokunaga, T., Fujiwara, T., and Yoshioka, S. (1990) Development of new leaching technology and a system to manufacture high quality frozen Surimi. **In**: *Chilling and Freezing of New Fish Products., pp.123-132. Intl. Inst. Refrig. Paris.*

*Niwa, E. (1985) Functional aspects of surimi. **In**: R.E. Martini. and R.L. Colletta. (eds.), *Proceedings of the International Symposium or Engineered Seafood Including Surimi.* National Fisheries Institute, Washington DC, USA, pp. 141-147.

*Offer, G. and Trinick, J. (1983) On the mechanism of water holding in meat the swelling and shrinking of myofibrils. *Meat Sci.*, **8** : 245.

Oguni, M., Inove, N., Shinano, H. (1994) Morphological changes in Carp crude F-actin and reconstituted actomyosin during frozen storage. *Bull. Fac. Fish. Hokkaido-univ.*, **45(4)**: 127-131.

Ohnishi, M., Rodger, G. M. (1980 a) Effect of formaldehyde addition at different ionic strengths on the salt soluble proteins of fish muscle. **In**: J.J.Connell.(ed.), *Advances Fish science and Technology* ., pp.459. Fishing News (Books) Ltd., surrey. UK.

Ohnishi, M. and Rodger, G. W. (1980 b). Analysis of salt-soluble protein fraction of cod muscle by gel filtration. **In**: J.J.Connell. (ed.), *Advances in Fish Sciences and Technology.*, pp.422. Fishing News (Books) Ltd., Surrey, UK.

Okazaki, E., Kanna, K., and Suzuki, T. (1986) Effect of sarcoplasmic protein on rheological properties of fish meat gel formed by retort heating. *Nippon Suisan Gakkaishi.*, **52** : 1821 - 1827.

Oshima, T., Wada S., Koizumi, C. (1984) Effect of accumulated free fatty acid on reduction of salt soluble protein of cod flesh during frozen storage. *Bull. Jap. Soc. Sci. Fish.*, **50(9)**: 1567-1572.

Osman, M. A., Ashoor, S. H., Marsh, P.C. (1987). Liquid Chromatographic identification of common fish species. *J. Assoc. Off. Anal. Chem.* **70(4)** : 618 – 625.

Ota, F, and Tanaka, T, (1978) Some properties of the liquid portion in the frozen fish muscle fluid. *Bull. Jap. Soc. Sci. Fish.*, **44** : 59-62.

Otani, O., Hikichi, S., Nishita, K., Sekii, K., Arai,K. (1983) The loss of actin from shell-fish myofibrills and fish myosin B during wash treatment. *Bull. Jap. Soc. Sci. Fish.*, **49 (3)**: 415-424.

*Ornstein, L. (1964) Disc. Electrophoresis I. Methods and Application to human serum protein. *Ann. N. Y. Acad. Sci.*, **122** : 321 - 349.

Owusu – Ansah, Y. J., and Hultin, H. O. (1992). Differential insolubilization of red hake muscle proteins during frozen storage . *J. Food Sci.* **57** : 265 – 266.

Papadopoulos, L. S., Smith, S. B., Wheeles, T. L., Finne, G. (1989) Muscle ultrastructural changes in freshwater prawns, *Macrobrachium rosenbergii* during iced storage. *J. Food Sci.*, **54(5)** : 1125 - 1128.

Pineiro, C., Sotelo, C. G., Medina, I., Gallardo, J. M., Perez - Martin, R. I. (1997) *Z. Lebensm - Unts – Forsch* (A Food Res. Technol)., **204 (6)** : 411 - 416.

Ragnarsson, K. and Regenstein, J. M. (1989). Changes in electrophoretic pattern of gadoid and non-gadoid fish muscle during frozen storage. *J. Food Sci.*, **54** : 819.

Rao, S. B. (1983) Denaturation of *Labeo rohita* actomyosin on frozen storage-preventive effect of carbohydrates. *Fish. Technol.*, **20(1)** : 29 - 33.

*Rayment, I., Rypniewski, W. R., Schmidt - Base, K., Smith, R., Tomchick, D. R., Benning, M. M., Winkelmann, D. A., Wesenberg, G. and Holden, H. M. (1993) Three dimensional structure of myosin sub fragment - 1 : *A molecular motor Science.*, **261** : 50-65.

*Robson, R. M., O' Shea, J. M. Hartzer, M. K., Rathbun, W. E., La Salle, F., Schreiner, P. J., Kasang, L. E., Stromer, M. H., Lusby, M. L., Ridpath, J. F., Pang. Y. Y., Evans, R. R., Zeece, M. G., Parrish, F. C. and Huiatt, T. W. (1984) Role of new cytoskeletal elements in maintenance of muscle integrity. *J. Food Chem.*, **8** : 1-24.

Rodger, G., Hastings, R., Cryne, C. and Bailey, J. (1984 a) Diffusion properties of salt and acetic acid into herring and their subsequent effect on the muscle tissue. *J. Food Sci.*, **49**: 714.

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

*Sakakibara, I., and Yagi, K. (1970) *Biochem - Biophys, Acta.*, **207** : 178 - 183.

Sano, T., Noguchi, S. F., Matsumoto, J.J., and Tsuchiya, T. (1989) Dynamic viscoelastic behaviour of F-actin on heating. *J. Food Sci.*, **54** : 231-232.

Santos-Yap, E.E.M. (1995) Fish and Seafood. **In** : L.E. Jeremiah (ed.)., *Freezing Effects on Food Quality.*, P. 109-133. Marcel Dekker, Inc. New York.

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

Sato, K., Yoshinaka, R., Sato, M. and Shimizu, Y.(1986) "Collagen content in the muscle of fishes in association with their swimming movement and meat texture. *Bull. Jap. Sco. Sci. Fish.*, **52** : 1595 - 1600.

Seki, N. (1976) Identification of fish species by SDS - polyacrylamide gel electrophoresis of the myofibrillar proteins. *Bull. Jap. Soc. Sci. Fish.*, **48 (2)** : 1169 - 1176.

Seki, N. (1977) **In**: K.K.Koseisha –Koseikaku. (ed.), *Proteins of Fish. Jap. Soc. Sci. Fish.*, Tokyo, pp 7-23.

Seki, N. and Arai, K. (1974) Gel. filtration and electroporesis of fish myofibrillar proteins in the presence of sodium dodecyl sulphate. *Bull. Jap. Soc. Sci. Fish.*, **40** : 1187 - 1194.

*Seki, N., Kitao, M., Konno, K. and Arai, K. (1973) *Bull. Jap. Soc. Sci. Fish.*, **39** : 1211 - 1219.

ɑi, N., Nakahara, C., Takeda, A., Maruyama, N. and Nozawa, H. (1998) Dimerization site of carp myosin heavy chains by the endogenous transglutaminase. *Fisheires Sci.*, **64(I)** : 314 - 319.

ɑi, N., Takayasu, M., Kokuryo, H. (1980) Preparation of myosin light chain as an index for identification of fish species from raw muscle and cooked products. *Bull. Jap. Soc. Sci. Fish.*, **46 (8)** : 1001 - 1006.

ki, N., Watanabe, T. (1984) Connectin content and its post mortem changes in fish muscle. *J. Biochem.*, **95 (4)** : 1161 - 1167.

ıenouda, S. Y. (1980) Theories of protein denaturation during frozen storage of fish flesh. **In**: C.O. Chichester. E.M. Mrak. and G.F. Stewart. (eds.), *Advances in Food Research.*, 26 : 275, Academic press, New York, NY.

ıimomura, M., Seki, N. (1980) A comparative study on the regulatory proteins from fish ordinary and dark muscles. *Bull. Jap. Soc. Sci. Fish.*, **46(9)** : 1159 - 1163.

Shimomura, M., Seki, N. and Arai, K. (1976) *Proc. Annual Meeting of Jap. Soc. Sci. Fish.*, Oct, pp. 86.

Sikorski, Z., Olley, J. and Kostuch, S. (1976) Protein change in frozen fish. *Crit. Rev. Food Sci. Nutri.*, 8 : 97.

Sıkorski, Z. E., and Kostuch, S., (1982) Trimethylamine N- oxide demethylase, its occurrence, properties, and role in technological changes in frozen fish. *J. Food Chem.*, **9** : 213 - 222.

ikorski, Z. E., Kolakowska, A. (1994) Changes in proteins in frozen stored fish. **In**: S. E. Sikorski., B. S. Pan., F. Shahidi. (eds.), *Sea food proteins.*, Chapman and Hall, New York. pp. 99 - 112.

Slinde, E., Kryvi, H. (1980). Studies on the nature of the Z-discs in skeletal muscle fibres sharks, *Etmopterus spimax. J. Fish. Biol.* **16 (3):** 299 – 308.

*Sotelo, C. G., Pineiro, C., Gallardo, J. M. and Perez - Martin, R. I. (1993) Fish species identification is seafood products. *Trends Food Sci. Tech.*, **4**: 395 - 401.

*Squire, J. M. and Vibert, P. J. (1987) **In**: J.M. Squire. and P.J. Vibert. (eds.), *Fibrous protein structure.*, London : Academic Press.

Srikar, L. N. (1979) Protein denaturation in ice stored and frozen marine fish. Changes in muscle proteins of croaker fish during the ice and frozen storage. *Mysore. J. Agric. Sci.*, **13(1)** : 78 – 82.

*Starr, R. and Offer, G. (1983) H-protein and X-protein. Two new component of the thick filaments of vertebrate skeletal muscle. *J. Mol. Biol.* **170** : 675.

Suzuki,T. (1981) **In:** *Fish and Krill Protein : Processing Technology.*, London : Applied Science Publisher.

Suzuki, T. and Kanna, K. (1977) Denaturation of muscle proteins in krill and shrimp during frozen storage. *Bull. Tokai Reg. Fish Res. Lab.*, **91** : 67-72.

Takashi, R., Murozuka, T., and Arai, K. (1974) Light chains of Myosin from Fish Dorsal and Rabbit Skeleted Muscles . *Bull., Jap. Soc. Sci. Fish.*, **40** : 1063 – 1069.

*Taylor, R. G., Geesink, G. H., Thompson, V. F., Koohmaraie, M. and Goll, D. E. (1995) Is Z-disk degradation responsible for postmorten tenderization? *J. Anim. Sci.*, **73** : 1351.

*Tiecco, G. (1980) Electrophoretic patterns of fresh and frozen meat. *Archivio Veterinario Italiano* ., **31(3/4)** : 108 – 110.

*Toyoda, K., Kimura, I., Fujita, T., Noguchi, S. F. and Lee, C. M. (1992) The surimi manufacturing process . **In**: T. C. Larier and C. M. Lee. (eds.), *Surimi Technology.*, ch.4. pp. 79 – 112. Marcel Dekker, Inc. New York.

Thakker, K.G. and Grady,L.T. (1984) Solubility. **In:** G.W. Gruenwedel. and J.R. Whitaker (eds.), *Food Analysis.*,ch.2. Marcel Dekker, Inc. New York.

Thompson, R. R. (1967) Disc Electrophoresis method for the identification of fish species *J. Assoc. Off. Anal. Chem.*, **50** : 282 - 285.

Tsuchiya, Y., Tsuchiya, T. and Matsumoto, J. J. (1980) The nature of cross - bridges constituting aggregates of frozen stored carp myosin and actomyosin. **In**: J. J. Connell.(ed.), *Advances in Fish science and Technology.*, pp. 434. Fishing News Books, Oxford.

Tsuyuki, H., Roberts, E. and Wanstone, W. E. (1965) Comparative zone electrophoresis of muscle myogen and blood hemoglobins of marine and fresh water vertebrates and their application to biochemical systematics. *J. Fish Res. Bd .Can.*, **22(1)**: 203-216.

Umemoto, S.and Knna , K.(1970) Studies on gel filtration of fish muscle protein **IV**. Changes in elution patterns by gel filtration of extractable proteins of fish and rabbit muscle during cold storage at -20 ^{0}C. *Bull .Jap. Soc. Sci. Fish .*, **36**: 798-805.

Unseld, M., Beyermann,B., Brandt. P. and Heisel, R. (1995) Identification of the species origin of highly processed meat products by mitochondrial DNA sequences. **PCR** *Meth. Aapplic.*, **4**: 241-243.

*Vondruska. (1985) Market trends and outlook for surimi based products.**In**: R.E. Martin. R.L. Collette. (eds.), *Proceeding of the International Symposium on Engineered Seafood Including Surimi.*, National Fisheries Institute. Washington DC, USA. pp:329-362.

Wahyuni, M., Ishizaki, S. and Tanaka, M., (1998) Improvement of thermal stability of fish water soluble proteins with glucose-6- phosphate through the maillard reaction .*Fisheries Science.*, **64(6)**: 973-978.

Wanger,P.D. Slater, C.S., Pope, B. and Weedes, A.G. (1979) Studies on the actin activation of myosin subfragment -1 isoenzymes and the role of myosin light chains. *Eur . J. Biochem.*, **99**: 385.

Watson ,C.L., Morrow, H.A., Brill, R.W. (1992) Proteolysis of skeletal muscle in yellow fish tunna (*Thunnus albacares*): Evidence of Calpain activation. *Comp. Biochem. Physiol.*, **103 B(4)**: 881-887.

*Weber, K., Osborn M., *J. Biol. Chem.* **244** : 4406 (1969)

*Wei, C. I., An, H., Chen , J. and Marshall, M.R. (1990) Use of a modified urea gel isoelectric focusing method for species identification of raw or broiled white, pink and rock shrimp. *J. Food Biochem.*, **14**: 91 -102.

*Wu, Y.J., Ataliah, M. T., and Hultin, H.O. (1991) The proteins of washed, minced fish muscle have significant solubility in water. *J. Food . Biochem.*, **15** : 209-218.

Xiong, L.Y. (1997) Structure function relationships of muscle proteins. **In**: S. Damodaran. and A. Parat. (eds.), *Food Proteins and Their Applications.*, Marcel Dekker Inc. New York, pp. 341-386.

*Xiong, Y. L. and Anglemier, A.F. (1989) Gel electrophoretic analysis of the protein changes in ground beef stored at 2^0C. *J. Food Sci.* **54(2)** : 287 – 290.

*Yasui, T., Ishioroshi, M. and Samejima, K. (1980). Heat induced gelation of myosin in the presence of actin *J. Food. Biochem.*, **4** : 61 - 78

*Yasui, T., Ishioroshi, M. and Sanejina, K. (1982) Effect of actomyosin on heat induced gelation of myosin. *Agric. Biol. Chem.*, **46**: 1049-1059.

Yoon, K.S., Lee, C.M., Hufnagel, L.A. (1991) Effect of washing on the texture and microstructure of frozen fish mince. *J. Food Sci.*, **56(2)** : 294 – 298.

Yoshikawa, K., Inone, N., Kawai, Y., Shinano, H. (1995) Subunit components in Salt -soluble and insoluble factions of Carp myofibrils during frozen storage. *Fish. Sci.*, **61(5)**: 813-816.

Yowell, K. and Flurkey, W. H. (1986) Effect of freezing and microwave heating on proteins from cod fish fillets: Analysis by SDS polyacrylamide gel electrophoresis. *J.Food Sci.*, **51**: 508.

* Not consulted in original

ABSTRACTS

STUDIES ON THE ELECTROPHORETIC PATTERN OF FISH/SHELLFISH PROTEINS SUBJECTED TO FROZEN STORAGE

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ABSTRACT OF THE THESIS

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VIII. ABSTRACT

Freezing is considered as an excellent process for preserving the quality of fish for longer periods. But freezing may affect various flesh components, especially the proteins. Sarcoplasmic proteins play a major role in species identification of fish and fishery products. While myofibrillar proteins play a major role in the jellying properties of surimi and surimi based products. The changes to these groups of proteins due to freezing and frozen storage have received much attention.

Four species *Megalaspis cordyla, Labeo rohita, Penaeus indicus* and *Parapenaeopsis stylifera* were used for the frozen storage study. Samples were stored for 90 days at -18^0C and samples were collected at 15 days intervals and Salt Soluble Nitrogen (SSN), Water Soluble Nitrogen (WSN) and Non-Protein Nitrogen (NPN) content were determined. Electrophoretic pattern of both Salt Soluble Proteins (SSP) and Water Soluble Proteins (WSP) of frozen stored sample were compared with that of the fresh sample.

SSN, WSN and NPN content showed significant decrease during 90 days frozen storage period. However changes in the WSN content of *P.stylifera* showed no significant difference. Electrophoretic pattern of WSP remained almost the same throughout the frozen storage period with some minor bands becoming less intense with storage period. Electrophoretic pattern of SSP showed a major change in the myosin. Heavy chain which became narrower and less intense with the concomitant appearance of high molecular weight protein aggregate at the top of the gel.