A COMPARATIVE STUDY OF THE MUSCLE PROTEINS OF FISHES AND SHELL FISHES OF TROPICAL WATERS

THESIS

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DECLARATION

I hereby declare that this thesis is a record of bonafide research carried out by me undet the supervision of Dr. K.Gopakumar, Scientist S-3, CIFT, Cochin-29, and that it has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles from this or any other University or Society.

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This is to certify that this thesis entitled "A comparative study of the muscle proteins of fishes and shellfishes of tropical waters" embodies the results of original work conducted by Shri K. Devadasan under my supervision and guidance from 17-6-1979 to 1-12-1982. I further certify that no part of this thesis has previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles of this or any other University or Society. He has also passed the Ph.D. qualifying examination of the University of Cochin held in December 1981.

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CONTENTS

1. INTRODUCTION

Nutritionists all over the world are unanimous in their opinion that man will ultimately have to turn to the vast and renewable fishery resources, for meeting the protein requirements of the growing world population. Fish protein is easily digestible and is rich in all essential amino acids. As an abundant and renewable source of cheap, high quality protein, fish is the ideal answer to the problem of protein malnutrition prevalent in developing nations. But, being a perishable commodity, fish must be processed and preserved properly for ensuring its optimum utilisation. For this, a clear idea about the nature and

composition of the different species of fishes and shell fishes is essential.

Proteins are perhaps the most abundant and important constituent of fish muscle, constituting more than 50% of its dry weight. Changes in the muscle proteins during processing and preservation directly affect the texture of the processed fishery product. Besides, as ensymes, they play a vital role, in deciding the postmortem deteriorative changes in all muscle constituents. Proteins like myoglobin have a bearing on the colour of fish muscle also.

Fish muscle proteins are basically similar to meat proteins in many respects. But, for several reasons fish proteins demand separate attention. In the first place, post rigor changes in fish muscle is basically different from those in warm blooded animals. Secondly the number of different species of edible fishes, vastly different from each other in their composition, is much more than the variety obeserved in land animals exploited for their meat. Moreover, unlike the terrestrial animals, fish, mostly caught from the open sea, cannot be subjected to any pretmatment aimed as its better preservation, postmortem. The infinite variety of species makes generalisation difficult in the case of fish. The different species show wide variations in their composition. Within a single species itself there

are the inevitable variations due to season, size, sex, maturity, nutritional status and various environmental factors. All these necessitate systematic studies on the composition of different species of fishes. As the most important muscle constituent, affecting the quality of processed fishery products, studies on fish muscle proteins demand maximum attention.

Compared to the volume of work reported on meat proteins, very little information is available in the literature on the composition and changes during processing, of fish proteins. This is especially so, in the case of fishes and shell fishes of tropical waters. This becomes all the more significant in view of Connell's (1961) observation that though they are similar to meat proteins in many respects, fish muscle proteins need not behave in the same way as mammalian or avian muscle proteins. Dyer (1967) also has expressed the same opinion, thereby stressing the need for basic research on the composition and processing characteristics of the muscle proteins of different edible fishes and shell fishes. Whatever we know on the nature of fish proteins today, is mostly from the studies reported on the fishes of temperature waters. Fishes and shell fishes of tropical waters cannot be expected to behave in a similar way, since the environmental conditions in the seas are

totally different.

India has vast marine and freshwater fishery resources. But our present day knowledge on the composition and processing characteristics of our fishes is grossly inadequate. In the tropical conditions existing in the country, spoilage is fast also. Since fishing in India is traditionally done by small boats, not equipped with onboard cooling or processing facilities, spoilage becomes a big problem in our country, resulting in the loss of valuable protein.

Southwest coast of India, had traditionally been the main fish landing centre in India, landing substantial quantities of prawns, sardines, mackerels etc. and other fishes. But information on the composition of the muscle proteins of these fishes and their changes during processing and preservation is scanty. There is a dirth of authentic data on these vital aspects. This thesis is an attempt to provide this much needed basic data on the proteins of our major fishes and shell fishes.

2. REVIEW OF LITERATURE

In this chapter, an attempt is made to review the work reported so far on muscle proteins of fishes and shellfishes.

As already stated, compared to meat, very little work has been reported on muscle proteins of fish. However, in the last two decades, we have received several excellent reviews on fish muscle proteins and their changes during processing and preservation (Hamoir, 1955; Dyer & Dingle, 1961; Jebsen, 1962; Connell, 1962, Connell, 1964, Connell, 1970, Suzuki, 1981). Fish muscle proteins can be broadly classified into three groups, namely, sarcoplasmic, myofibrillar and connective tissue proteins.

2.1 Sarcoplasmic proteins

Sarcoplasmic proteins of fish muscle contribute 20-30% of the total muscle proteins (Hamoir, 1955; Dyer & Dingle, 1961; Shimizu & Simidu, 1960; Baliga et al. 1962). They are proteins soluble in salt solutions of low ionic strengths (μ = 0.05). They are mostly enzymes; but Hamoir (1962) has suggested that sizeable proportion of fish sarcoplasmic proteins have no enzymic activity. Being highly soluble proteins, they do not influence the water holding capacity or texture of the fish muscle which are properties attributed to myofibrillar proteins. Fish sarcoplasmic proteins contain some proteins with very low sedimentation coefficients. These proteins are rich in tyrosine and phenylalanine and are suspected to be responsible for the allergic reactions of some people to fish (Connell, 1958; Jebsen & Hamoir, 1958; Connell, 1970). In general, the enzymology of fish muscle is similar to that of other mammalian muscle (Gumbmann et al. 1858; Jones, 1962; Tomlinson & Geiger, 1962). But some enzymes like anserinase and thiamenase are supposed to be peculiar to some fish species. Shimizu et al. (1976) & Suzuki (1976) have reported that pelagic fishes have in general a higher content of sarcoplasmic proteins compared to demersal species.

Fractionation of fish muscle sarcoplasmic proteins has been attempted by several workers employing different

techniques. Dingle et al. (1962) tried fractionation using zinc, whereas Tsuyuki et al. (1962) employed chromatography on DEAE cellulose. Tsuyuki (1962 A) and Tsuyuki & Roberts (1963) tried zone electrophoresis on starch gel for the separation of fish sarcoplasmic proteins. Odense & Shimmers (1962) used a technique employing intact muscle rather than extracts, for similar separations. Connell (1953) and Dingle et al. (1955) studied the electrophoretic behaviour of pre and post rigor sarcoplasmic extracts of cod using moving boundary electrophoresis. Electrophoretic patterns of extracts of fresh and frozen cod or yellow striped rockfish were compared by several workers (Connell, 1952; Seagran, 1958; Dyer & Dingle, 1961). Electrophoretic patterns of the muscle myogens of fish muscle were found to be characteristic of the species. These species specific patterns remained unaltered during processing and preservation. These patterns were thus found suitable for the species identification in cases of doubtful authenticity. Different support media were used by different workers in studies on this interesting aspect. Nikkila & Linko (1955) studied the electrophoretic patterns of proteins from ten species of fish on paper. Connell (1953) compared the electrophoretic patterns of the sacroplasmic proteins of twenty species of fish using free boundary electrophoresis. Thompson (1960) and Tsuyuki et al. (1962, 1965, 1965a) used starch gel as the support medium for similar

studies. Manwell et al. (1963) and Tsuyuki & Gadd (1963) used separation patterns of haemoglobins on starch gel for the same purpose. Thomson tried to refine the technique further by (1968) separating the esterases of fish tissue electrophoretically, for species identification. The perfection of disc electrophoretic technique by Ornstein & Davies (1964) have lead to the replacement of all the other support media for electrophoresis by polyacrylamide. Many workers used acrylamide as the medium for the electrophoretic separation of muscle myogens of fish with a view to using them for species identification (Payne, 1963; Mancusso, 1964, Thomson, 1967; Mackie, 1969). Cowie (1968) on the other hand employed the slab technique of electrophoresis on polyacrylamide gel as described by Akroyd (1967). Mackie (1968) tried to extend the use of electrophoretic patterns for species identification to cooked fish also by a simple modified extraction procedure. The U.S. Food and Drug Administration has now decided to recognise electrophoretic patterns of the muscle myogens of fish as an authentic method for species identifications. Robertson et al. (1967) has shown that nutritional status of the fish does not affect the electrophoretic patterns of its muscle myogens materially. Uthe & Tsuyuki (1967) observed that electrophoretic patterns of muscle myogens do not undergo any significant change when the juvenile turns adult although this transformation may

lead to changes in blood proteins. Lillevik & Schloemer (1961) also used electrophoresis of myogens for species identification in fish.

The coloured proteins myoglobin and cytochrome-c form part of sarcoplasmic proteins. Compared to meat, their content in fish is very small. There is wide variation in the content of these proteins between different species of fish. Fishes having red meat (eg: tuna, black pomfret etc.) have them in appreciable quantities. Oxidation of these proteins often lead to discolouration problems in such fish eg: greening of canned tuna. Several workers have studied fish myoglobins and their oxidation (Brown et al. 1962; Matsuura & Hashimoto, 1962; Matsuura et al. 1972; Brown & Dolev, 1963, 1963a). Sarcoplasmic proteins are involved in other types of discolouration problems also in fish by virtue of their control over concentration of other tissue constituents like sugars, sugar phosphates, amino acids, carbonyl compounds etc. These compounds in turn are involved in reactions of the Maillard type which cause other types of discolourations (Jones, 1962).

Sarcoplasmic proteins include enzymes of glycolytic pathway (Tarr, 1966; Nagayama, 1966; Siebert & Schmitt, 1965; Gumbmann & Tappel, 1962; Gould, 1965; Martin & Tarr, 1961) and also enzymes of autolysis (Siebert, 1958,

Siebert & Bottke, 1963, Bird et al. 1969; Harrier et al. 1972a). They are generally low molecular weight proteins, molecular weight being in the range of 4,00,00 to 7,00,00.

The sarcoplasmic extract of squid muscle and also octopus (Matsumoto, 1957) showed a peculiar feature. In these cases some structural protein with characteristics streaming birefringence, gets extracted in the water extract. Matsumoto has done detailed studies on the characteristics of this fraction which he designates as actomyosin M. (Matsumoto, 1957, 1958, 1958a, 1959, 1959a, Katsumi & Matsumoto, 1969; Migita et al. 1958).

2.2 Myofibrillar proteins

To the fish processing technologist, myofibrillar proteins are perhaps the most important group of proteins, as they are responsible for the textural qualities of fish like fibrousness, water holding capacity, gel forming ability, plasticity etc. Denaturation of these proteins directly result in deterioration of fish texture.

Myofibrillar proteins are proteins soluble in salt solutions of high ionic strength. They are relatively more easily denatured also. In fish, the percentage of myofibrillar proteins (as percentage of total protein) is generally higher than in meat. This group of protein

accounts for 65-75% of the total protein in fish (Hamoir, 1955). All the major myofibrillar proteins isolated from meat namely, actin, myosin and tropomyosin have been found in fish also. In other miner myofibrillar components also fish resembles meat, but in fish, these proteins have been less well characterized compared to meat.

2.2.1 Myosin

Myosin accounts for about 40% of the total protein of cod (Connell, 1962). Preparation of fish myosins in pure state is very difficult, since actin gets extracted easily when myosin is prepared from fish (Guba, 1943). This emphasizes the necessity to exercise caution in extending the methods used in preparation of myosin from meat to fish. Such procedures may yield myosins heavily contaminated with actin and actomyosin. This can be due either to the low connective tissues content of fish muscles or to the labile nature of the so called "relaxing factor", which maintains the ATP level. In fish, contamination with actomyosin can be minimised by using an acidic extraction media (Hamoir, 1955) or extractants containing ATP (Hamoir et al. 1960) or pyrophosphate (Connell, 1954, 1960). Many workers have isolated myosins from different species of fish and have studied their properties. Takashi et al. (1970) prepared pure myosin from carp muscle. Syrovy et al. (1970) isolated

myosin from the red and white meats of carp separately. Properties of tuna myosin were studied by Chung et al. (1967). Buttkus (1966) made a similar study of trout myosin. Buttkus (1971) later compared trout myosin with rabbit myosin correlating their-SH contents and protein stability. Myosin from cod is more exhaustively studied compared to myosins from other species of fish. Mackie and Connell (1964) studied the molecular properties of purified cod myosin. Molecular weight and amino acid composition of cod fibrillar proteins were studied in detail by Connell (1958, 1959) Connell & Mackie (1964) made a comparison of molecular weights of rabbit and cod myosins. Hafmoir et al. (1960) also isolated and studied the properties of fish myosins. Connell (1961a) made a comparative study on the relative stabilities of myosins from the skeletal muscles of different animals. Aggregation of cod myosin during frozen storage was studied in detail by Connell (1959, 1960). The effect of adenosine triphosphate (ATP), inorganic pyrophosphate and inorganic tripolyphosphate on the stability of cod myosin was the subject of a detailed study by Mackie (1966). Dingle & Hines (1960) followed the ATP-ase activity of cod myosin and actomyosin. Connell & Olcott (1961a) treated cod myosin preparation with alkali and low concentrations of urea and studied the nature of components liberated by these treatments. Cod myosin is very similar to

rabbit myosin in molecular weight, and helical content (Connell & Mowgate, 1959; Connell 1958a). But it is more unstable when heated or treated with urea. Trypsin, chymotripsin etc. hydrolyse it faster than mammalian or chicken myosin (Connell, 1961). This difference in the digestibility is an interesting point which has not been explained satisfactorily. Studies have shown that cod and carp myosins are split in the same way as rabbit myosin by trypsin (Connell, 1961a; Stainier Lambrecht, 1962). But the instability of the resulting meromyosins have retarded the progress of work in this field. Probably this difference in the trypsin digestibility of meat and fish myosins explain the greater digestibility of fish proteins.

Myosins of cold blooded vertebrates in general have been considered similar to myosins of warm blooded vertebrates (Hamoir, 1953; 1955; 1955a; 1956). But myosin of fish aggregates very fast and the speed of this aggregation varies from species to species.

The most important biochemical characteristics of myosin is its enzymatic activity with respect to hydrolysis of adenosine triphosphate (ATP). This enzymic activity is associated with the heavy meromyosin fragment of the myosin molecule. This activity discovered by Engelhardt et al. (1939) is related to the ATP-ase activity of reconstituted

actomyosin (Barany, 1967) and to the interaction between actomyosin and ATP-in vivo i.e. muscular contraction: (Mommaerts, 1950; 1966; Davies, 1963). Fish muscle myosin also shows this activity. Its changes in myosins from different fishes and shell fishes are reported by many workers (Nobuo Seki et al. 1980, 1980a; Kimura et. al. 1980). ATP-ase activity of fish muscle myosins decreases during storage of fish in ice. ATP-ase of myosin in frozen and stored muscle is rather stable if temperature of storage is low. At higher temperatures there will be an appreciable decrease in this activity (Connell, 1960; Sawant & Magar, 1961). Nowa et al. (1980) prepared myosin heavy chain from fish muscle and studied its properties in detail: work on meat myosin ATP-ase has shown it to be an adaptive enzyme which becomes more and more active when the animal grows. up. White muscle myosin is known to have a higher ATP-ase activity compared to red muscle myosin. Sakamoto et al. (1979) found that fish myofibrillar system resembled closely that of rabbit in its reaction towards ATP and divalent cations. Uchiyama et al. (1978) studied the thermo stability of Ca^{++} activated myofibrillar ATP-ase of epipelagic and mesopelagic fishes. Mesopelagic fish muscle ATP-ase was more unstable than ATP-ase of epipelagic species, suggesting an adaptation to the environmental temperature in which the fish lives. The Ca^{++} activated ATP-ase of actomyosins also

were similar in all species studied. Myosin ATP-ase in Kcl solution is inhibited by Mg^{++} and is activated by Ca^{++} Takashi (1973) compared myosin ATP-ase activity of rabbit and freshwater fishes.

Sulfhhydryl content is another important property studied for characterisation of myosins. Fish species do not differ significantly in this respect from one another (Takashi, 1973; Buttkus, 1967; Connell, 1961; Woods et al. 1963; Kimura et al. 1979).

In presence of urea or guanidine Hcl, myosin is split into its sub units. Freshwater fish myosin was found to contain one heavy and three light chains when analysed in the presence of sodium dodecyl sulphate (Takashi, 1974; Seki & Arai, 1974; Seki, 1976).

When myosin is digested with trypsin or chymotrypsin, it is divided into two components, one fragment, havy meromyosin, sediments fast. This fragment on further digestion with papain is split into a head and neck part. The head part has the actin binding site. Light meromyosin does not seem to have any specific biological function.

2.2.2 Actin

Actin is one of the most important myofibrillar proteins. It is located in the thin filaments of the muscle.

Compared to actin from meat, information on fish actins is scanty. The monomeric or globular G-actin polymerises in presence of salt to form F-actin filament. Interaction of actin with myosin is the main reaction involved in muscle contraction. The relaxing protein (tropomyosin-troponin complex) also plays a significant role in this by regulating the Ca^{++} and Mg^{++} concentration.

Fish actins are prepared in the same way as meat actins (Connell, 1954; Connell & Howgate, 1959; Dingle, 1959). As reported in the case of meat (Laki & Cairns, 1959; Laki et al. 1962; Drabikowksy & Gergely, 1964; Lewis et al. 1963), these preparations are also often contaminated with tropomyosin. Connell & Howgate (1959) have estimated that actin accounts for 15-20% of the total protein in cod. Tadao Ueda et al. (1967) and Shenoda & Pigott (1975) have described methods for the preparation of pure actin from fish muscle.

Actin is more stable than myosin and can survive processing treatments without appreciable denaturation. In the muscle, it offers protection to myosin against denaturation by covering up some -SH groups. Weber & Portzehl (1952) observed that extractability of F-actin from muscle was largely dependant on the stroma content of the muscle. Stroma somehow prevents removal of the long F-actin microfibrils. Stroma content being very low in fish, fish actins

are more easily extracted compared to meat actins. Actin is a highly charged molecule with acidic amino acids predominating in the molecule. The amino acid composition of cod actin has been reported by Connell & Howgate (1959). G-actin-F-actin transformation has been extensively investigated by different workers (Mommaerts, 1951; Laki et al. 1951; Strohman, 1959). The rate of G-F transformation in the presence of potassium chloride is decreased by ageing, heating and exposure to high pressure. Alkaline pH inhibits this transformation. The role of -SH groups in the G-F polymerisation is not clear. Bridgen (1972) studied the reactivity and function of the -SH groups of trout actin. The effect of the protein concentration and Mg^{++} concentration on the G-F transformation was studied by Gergely et al. (1960). Below a certain concentration of salt (Kcl & $Mgcl_2$) actin exists as a dimer. In 0.6 M. KI also, cod G-actin exists as a dimer (Tseo, 1953). The unit of active actin appears to be a dimer having two polymerising sites (Ooi, 1961). Addition of a little F-actin to G-actin generally accelerates G-F transformation.

2.2.3 Actomyosin

Freshly minced muscle when extracted with salt solutions (μ = 0.5), extracts most of the proteins. Twenty fold dilution of this extract precipitates a globulin which

was described by Von-Furth as myosin. Later studies showed this protein to be a complex of two proteins, the water soluble actin and the salt soluble myosin. The interaction of these two proteins forming actomyosin and the dissociation of the actomyosin in presence of ATP was soon recognised as the fundamental reaction involved in muscle contraction. In earlier literature there is considerable confusion with regard to the term myosin. Thus actomyosin has been described variously as myosin B and myosin $\propto$ also whereas myosin A, myosin T, L-myosin, myosin B etc. were terms used to describe myosin proper.

Fish actomyosin have been prepared from different species of fish by different workers Ikeda & Taguchi (1967, 1968) and Taguchi & Ikeda (1968a, 1968b) studied the properties of yellow tail actomyosin. Reiji Takashi et al. (1970) and Ueda et al. (1964, 1968) also studied the properties of actomyosins from other species. According to Connell & Howgate (1959) ratio of myosin to actin in cod actomyosin varied between 2 and 4. Taguchi & Ikeda (1968a) found that fish muscle actomyosin contains lecithin. According to them actomyosin with a high lecithin content usually had a high myosin content. However, Olcott et al. (1968) contradicted this report. They could not find any lipids in their actomyosin preparations from rabbit. Ueda et al. (1964, 1968) did not observe any difference

in intrinsec viscosity, electrophoretic mobility or salting in and salting out range, between actomyosins from different species of fish. But the temperature of denaturation as well as velocity of denaturation did vary from species to species. Seki et al. (1980a, 1980b) made detailed studies on the changes in the ATP-ase activity and other properties of carp and sardine myofibrillar proteins during ice storage. Hashimoto et al. (1979) did not see any actomyosin fraction in red muscles of sardine and mackerel in their ultra centrifugal analysis. But white muscle showed a clear peak corresponding to actomyosin. Fujimaki et al. (1965) observed that with progressive ageing of fish there is a corresponding decrease in the interaction between actin and myosin in fish muscle. Yoshiaki Itoh et al. (1980, 1980a) studied the -SH groups of carp actomyosin and their role in the gel formation during heating. Kimura et al. (1980) subjected yellowfin and carp actomyosin to tryptic digestion and studied the nature of the heavy meromyosin fragments released. They found these fragments to be similar to heavy meromyosins from rabbit and skipjack tuna.

2.2.4 Tropomyosin

Tropomyosin is another major myofibrillar protein isolated from fish. It was first isolated by Bailey (1948) from rabbit muscle. It is present in all muscles and can

be observed by the flumrescent antibody staining method (Pepe, 1966) as associated with the actin of the thin filaments. Tropomyosin has been isolated from muscles of oyster, crayfish, blowfly and abalone (Woods et al. 1971) clams (Kamins et al. 1957) squid (Bailey, 1956; Tsuchiya et al. 1980; Yoshimura 1955) and prawns (Tsao et al. 1956). All the tropomyosin were basically similar in their molecular properties. Average molecular weight was in the range of 7,00,00. Sub unit molecular weight of all vertebrate tropomyosins were found to be the same by McCubbin et al. (1967) and Woods (1968, 1969). However, ammonium sulphate precipitation range varied between different tropomyosins. Glutamic acid, aspartic acid and histidine are high whereas tryptophan is lacking in this protein. Tropomyosin often gets contaminated with the minor proteins like paramyosin and troponin unless prepared carefully. In addition to muscles of vertebrate and invertebrates tropomyosin has been isolated recently from non muscle cells like platelets (Cohen & Cohen, 1972), Physarum (Kato & Tonomura, 1975) and fibroblast (Masaki, 1975). The biological role of tropomyosin remained unknown until Ebashi (1963) discovered its role in the contraction-relaxation system in muscle.

Fish tropomyosin is basically similar, to rabbit tropomyosin (Hemoir 1951, 1951a, Kubo, 1957, 1957a; Dingle &

Odense, 1959; Hoogland et al. 1961). Truscott et al. (1962) attempted to study the amino acid composition of cod tropomyosin. Odense et al. (1969) described a simple procedure for its isolation from cod muscle. The procedure involved heat treating the muscle and then extracting the heated muscle with appropriate extractants. Hamoir's (1951a) earlier procedure for isolation of tropomyosin gave two fractions, one with a low and another with a high nucleic acid content. The lower nucleic acid content product resembled Bailey's tropomyosin (1948) from rabbit. Tropomyosins lack in tryptophan which is helpful to check the purity of preparations.

Tropomyosins can be crystallised under suitable conditions, in various polymorphic forms (Casper et al. 1969). All tropomyosins have the characteristic tactoidal form. Millward & Woods (1970) showed that the tactoids from cray fish tropomyosin show the same axial periodicity as those formed from vertebrates. Maruyama (1959) found two fractions of tropomyosin in crayfish, one salting out at 35-40% saturation and another between 40 and 60% saturation. Maruyama et al. (1968) found out that the fraction precipitating at 22.5-37.5% saturation of ammonium sulphate contained a good percentage of troponin. Hartshorne and Mueller (1969) have discussed the various variables involved in preparing pure tropomyosin and troponin from vertebrate muscle.

2.2.5. Other minor protein components of fish muscle

2.2.5.1 Paramyosin

Paramyosin was first discovered in Molluscan muscle by Bailey (1956). It has a unique X-ray diffraction pattern. Szent-Gyorgyi et al. (1971) and Olender et al. (1967) isolated paramyosin from different molluscs. Bailey (1960) later isolated the same protein from octopus. Tsuchiya et al. recently (1980) isolated and characterised squid paramyosin. Paramyosin was originally described as water insoluble tropomyosin and later as tropomyosin A. In squid, paramyosin accounts for 14% of the myofibrillar proteins. Squid paramyosin showed some differences in its amino acid composition from other paramyosins. The physiological role and technological significance of paramyosin is not fully understood.

2.2.5.2 Troponin

Troponin with tropomyosin forms the so called "trigger" mechanism in muscle contraction reactions. Troponin and tropomyosin are relatively low molecular weight proteins. They combine with each other in the myofibril. Troponin appears to bind the tropomyosin molecules together. This protein complex in turn attaches itself to the actin in the myofibril. Troponin first isolated by Ebashi & Kodama, acts as the Ca^{++} binder. Troponin in its bound form with tropomyosin (Native tropomyosin) sensitizes the interaction

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of myosin and actin to Ca^{++}. Considerable work on troponin from meat has been reported (Ebashi et al. 1965, 1966, 1967 1968). More recent work has shown troponin (Mole. wt. 40000) itself to be composed of two fragments, troponin A (Mole. wt. 18000) and troponin B (Mole. wt. 22000). But it is not clear whether they exist as two separate fragments within the myofibril. Troponin does not bind to myosin directly. It comes in the contractile system attached to tropomyosin and thereby to the actin moiety. Mild tryptic digestion release it. However, little work has been reported so far on troponins of fish muscle.

2.2.5.3 α-actinin

Ebashi et al. (1964) isolated another minor myofibrillar protein which is thought to be the protein responsible for joining the ends of the actin filaments of adjoining sarcomeres. It combines with F-actin and promotes actin aggregation. Tropomyosin tends to remove aggregation of actin and α-actinin. α actinin with a molecular weight of 17,00,00 binds with actin in the ratio, 1 molecular of α actinin to 10 molecule of G-actin units in the F-actin chain. α-actinins role if any on the contractite system is not yet clear.

2.2.5.4 γ-component is yet another minor myofibrillar peotein isolated by Liwen Lee & Shisuo Watanabe (1970). The

biological function of this protein is also yet to be established. Practically no work has been reported on these two proteins from fish.

2.3 Connective tissue proteins

The third major group of proteins in fish muscle is the connective tissue or stroma proteins. These are insoluble in salt solutions and even in dilute acids and alkalies. Compared to meat, fish muscle contains very little stroma. Teleost muscle contains around 3% stroma. However, elasmobranchs contain around 10%. Fish muscle stroma is constituted mainly by collagens which are rich in hydroxy proline. Fish muscle collagens are noted for their easy gelatinization. On heating, collagens are degraded to water soluble gelatins. This releasesthe myotomes of muscle. On cooking myotommata gets softened and the released myotomes come off as flakes. The low content of stroma in fish muscle and its easy gelatinisation are important properties which confer the characteristic texture to fish muscle. Stroma is the protein on the outer side of the muscle cell. Recently the existance of elastic fibres, containing a protein called connectin has been established in these cells. Hashimoto et al. (1979) and Kimura et al. (1979) have studied the connectin content of different fishes.

Technologically fish muscle stroma is important due

to its role in the so called "gaping". Gaping is the phenomenon observed, especially in frozen cod, by which the connective tissue (myocommata) fails to hold the myotomes (muscle segments) together. This condition causes the formation of slits in the fish fillets. Gaping gives a bad appearance to the frozen fish fillet and reduces the market value of the fish. Love et al. (1968) have described this condition. The effect of rigor mortis (Love et al. 1969) muscle pH, and temperature at which fish enters rigor (Love et al. 1970), conditions of freezing and frozen storage (Love et al. 1970a) and other relevant factors (Love et al. 1972a, 1972b) have all been studied in great detail and in a very systematic manner.

2.4 Changes in fish muscle proteins during preservation and processing

2.4.1 Icing

Icing is the most common method of preservation employed both onboard, as well as by retail marketers. For transportation over reasonably long distances, to the interior parts of the country also, icing is the preferred method. Changes in fish muscle protein when fish is stored in ice have been studied by many workers. Moorjani et al. (1962) and Baliga et al. (1962, 1962a, 1969) reported the changes in the protein fractions of some freshwater fishes

during storage in ice. Govindan (1962, 1969) studied the changes in total nitrogen and water soluble nitrogen in prawns during ice storage. Postmortem changes of horse mackerel muscle proteins stored in ice were reported by Maruyama & Suzuki (1968). Changes in the ATP-ase activities and other properties of sardine and carp myofibrillar proteins during ice storage were studied by Nobuo Seki et al. (1980, 1980a) Cruptin et al. (1979) correlated the log of reduced viscosity of a high ionic strength extract of ice stored Hake, to the number of days of storage of fish in ice. Relation between loss of freshness and the denaturation of myofibrillar proteins in stone flounder, plaice and carp was examined by Ehira et al. (1979). Use of ice treated with various preservatives and antibiotics have also been tried to enhance the ice storage shelf life of various species of fish. Wessels et al. (1972) tried ice treated with benzoates, EDTA, polyphosphates, sodium propionate etc. singly as well as in various combinations to enhance the shelf life of hake. Billinsky & Jonas (1975) could correlate the freshness of iced salmonids to the formation of lactic acid in muscle from fructose 1,6 diphosphate.

Several workers have tried to follow the postmortem changes in the extractability of fish muscle proteins with a view to correlating it to protein denaturation during

storage near 0°C. But the results are found contradictory. Deuticke (1932) observed the phenomenon (named after him) of reduced extractability during rigor followed by a recovery. Dyer & Dingle (1961) Otake and Yamamoto (1954) and Piskarev et al. (1960) supported this theory. However, Love (1962) observed the opposite. Moorjani et al. (1962) and Van Den Broeke (1947) on the other hand found very little change in proteins as a result of rigor. These variations in the results are only to be expected when we take into account the variable factors like different experimental techniques, possible misjudgement of onset and resolution of rigor and species difference in the time of rigor onset, etc. Postmortem ice storage ultimately makes the product soft and unacceptable. The complex changes causing this can be proteolysis by bacterial enzymes or autolysis by muscle cathepsins (Bramstedt, 1962).

In a tropical country like India crushed ice is essential for preservation of catch onboard as well as in the pre-processing stage. Considerable attention has accordingly been paid to the spoilage of fish during ice storage (Susamma Jacob et al. 1962; Nair et al. 1962; Iyer et al. 1966, Chinnamma et al. 1970) Shenoy et al. 1974; Solanki et al. 1977, 1978)

2.4.2 Changes in fish muscle proteins during freezing and frozen storage

From the food technological point of view this is perhaps the most important and interesting problems in the biochemistry of fish proteins. Understandably this is the field where maximum work is reported also. As early as 1929, Huntsman had observed the deteriorative changes in frozen fish muscle proteins (Huntsman, 1929). The most noticeable change in frozen stored fish is the development of a tough texture. This is attributed to protein denaturation. Protein denaturation in frozen fish has three important aspects, namely, (1) the changes brought about by the process of freezing in the protein micelles and its effect on the protein structure (2) the changes in the muscle protein during frozen storage and (3) the influence of other tissue constituents on the protein structure.

Protein denaturation is a complex phenomenon which is perhaps more easily followed experimentally than defined. It can at best be described as a conformational disorganization of the polypeptide chains. Experimentally it is studied best, by the changes in the solubility characteristics of the proteins although Anderson et al. (1963) have cast serious doubts on accepting solubility as a criterion of protein denaturation. The best approach so far to the problem of protein denaturation in frozen fish has been to

study the loss of solubility in a neutral salt solution of high ionic strength. Bate Smith (1934, 1937) and Reay (1933, 1934, 1935) observed that during frozen storage of fish, there is a progressive loss in solubility of its muscle proteins, especially the globulins. Dyer et al. (1950) improved the extraction procedure by the introduction of a blending technique, which has subsequently become a standard method for determining protein solubility in frozen fish. Using this method, it was established that the actomyosin fraction of fish becomes progressively insoluble during frozen storage. This fall in solubility was successfully correlated to the sensory tests on the fish samples also. The loss in solubility is usually associated with a loss in "water holding capacity" of muscle proteins.

Water molecule being dipolar is attracted to polar groups like the carboxyl, amino and sulphhydryl groups present in proteins. These combined water molecules further combine with other molecules of water forming aggregates thereby stabilising the native protein structure.

Hamm (1960) suggested that only 4-5% of the total water of the muscle is tightly bound to the muscle proteins. This water is not affected by changes in the protein structure. The remaining free water is retained within the protein molecules, and is affected by changes in protein structure.

The free water can be forced out from the protein by application of forces like pressing, centrifugation etc. Water holding capacity of fish muscle is its ability to hold water when forces like pressure or centrifugation are applied. Methods have been developed for measuring the "water holding capacity" (Wierbecki & Deatherage, 1958; Wierbicki et al. 1957; Urbin et al. 1962). Muscle pH is a critical factor in deciding its water holding capacity. Around pH 5, i.e. near to the isoelectric point of contractile proteins, fish muscle generally has the lowest water holding capacity (WHC) (Grau et al. 1953). The decrease in pH during rigor can thus cause loss in WHC. Hamm (1960) has suggested that breakdown of ATP in muscle during postmortem storage is another factor contributing to loss in WHC. Breakdown of ATP releases bound Ca^{++} ions which are bound by protein molecules. This leads to a contraction in muscle leading to loss in WHC. He proved this by showing that addition of ATP to muscle postmortem increases its WHC. In beef, he attributed two thirds of the loss in WHC to breakdown of ATP and one third to decrease in pH. ATP and inorganic pyrophosphates can under certain conditions cause relaxation of muscle which is sometimes referred to as plasticizer effect. This is presumably due to the dissociation of actomyosin complex into actin and myosin by ATP and pyrophosphate (Bendall, 1954). Fish muscle contains various

inorganic salts and also organic compounds. These salts, mostly phosphates, play an important role in its biochemistry. The salts in fish muscle can be thought to be equivalent to roughly a 1.0% solution of sodium chloride. When fish is frozen, due to the dissolved salts, there is a lowering of freezing point. When that temperature is reached salts get concentrated in tissues as a result of the freezing of free water. This further lowers the freezing point till about -5°C. In this range (0° to -5°C) cooling is very slow. After this cooling is rapid. During the freezing out of tissue water, tissue salts get more and more concentrated in the remaining unfrozen water. This concentration of salts can cause protein denaturation. Another factor causing protein denaturation is the physical damage to the cell walls when ice crystals are formed. Slow freezing leads to formation of larger ice crystals causing greater damage, whereas quick freezing results in small ice crystals, which minimises the cell damage (Callow, 1952). Love (1955, 1958a,b) attempted to measure the cell damage by estimating the DNA in the drip from frozen and thawed fish. DNA released as a result of cell wall bursting during freezing must serve as a good index of cell damage and thereby indirectly to protein denaturation. Both these factors namely, physical damage of cell walls by ice crystals and concentration of tissue salts, may be contributing to the

protein denaturation in frozen fish. However, salts like sodium chloride and sodium polyphosphates are known to increase the water holding capacity. Chloride ion brings about greater repulsion between polypeptide chains, thereby increasing the WHC. There are different theories with regard to the mode of action of phosphates. Bendall (1954) attributed their effect to the ability of polyphosphate to split actomyosin into actin and myosin. Hamm (1960) suggested that polyphosphates remove alkaline earth metals from muscle by forming complexes or by precipitation. Their removal prevents further loss in WHC. But Sherman (1961) Inklar (1967) and Hollendoorn (1962) do not support this view. Fukazawa et al. (1961) is of the opinion that polyphosphates facilitates better extraction of proteins from the fibrils. Yashi et al. (1964) and Linko et al. (1961) made extensive studies on the extractability of muscle proteins in the presence of different inorganic phosphates in the presence and absence of sodium chloride. High concentration of sodium chloride enhanced the activity of polyphosphates. Tripolyphosphate gets dissociated to pyrophosphate by tripolyphosphatase enzyme and the pyrophosphate formed acts similar to ATP. Hexametaphosphate binds directly to the actomyosin. Various workers have reported the protective effect of polyphosphates on the WHC of muscle proteins (Mac Callum et al. 1964; Boyd & Southcott, 1965). Mahon & Schneider, 1964;

Migita & Otake 1960; Tanikawa et al. (1963), Kunta & Gore (1970). Many workers have attributed an antioxidant action also to polyphosphates which can prevent protein denaturation due to oxidised lipid-protein interactions (Tims & Watts, 1958; Zisper & Watts, 1961; Ramsey & Watts, 1963; Thomson, 1964; Zisper, Taiwan Kwon & Watts, 1964).

2.4.3. Protein denaturation in frozen fish

The sarcoplasmic proteins are not affected to any significant extent during freezing and frozen storage of fish. In cod upto 2 years, the albumin fraction remained unaffected (Dyer, 1953). In other species of fishes like plaice, halibut and rose fish also the albumin fraction remained unaffected during frozen storage (Dyer et al. 1956). Similar results were reported by other workers also (Dyer & Dingle 1961; Nishimoto & Tanaka 1960; Sawant & Magar, 1961; Connell, 1962). But Tomlinson & Geiger (1963) reported results different from this. Seagran (1958) found no change in the electrophoretic pattern for yellow striped rockfish stored for two months at -20°C. Awad et al. (1969) observed no change in solubility of sarcoplasmic protein in frozen freshwater white fish muscle, stored at -10°C. However, they have reported appearance of two additional protein bands in the electrophoretic patterns of the sarcoplasmic protein at the end of sixteen weeks' storage at -10°C. Hashimoto

et. al. (1979) made a comparison between the protein fractions of the red and white meats of sardine and mackerel stored at -80°C. during storage. In red meat, sarcoplasmic protein registered an increase. This phenomenon was not observed in white meat. They attributed this unusual behaviour to the instability of myofibrillar proteins like myosin and troponin which gets degraded into smaller soluble fragments and thus appear in the sarcoplasmic extract. Red meat is known to contain higher proportion of sarcoplasmic protein compared to white meat.

It is the myofibrillar proteins which are affected the most during frozen storage of fish. These changes have been studied by different workers both by actual studies on fish as well as studies using model systems. The solubility technique of Dyer et al. (1950) has been the main method for following protein denaturation in all these studies. In the normal course and if properly done, blending with a neutral high ionic strength salt solution of pH 7.2 should extract 85-95% of the muscle proteins from fresh fish. But this generalisation need not apply in all cases. Considering the wide variety of species of fishes, this can only be a general statement. However, under standardised conditions extractability data for a particular species of fish in the fresh condition is fairly reproducible. During frozen storage with the development

of toughness in texture, there is a parallel decrease in solubility of proteins. This is in most cases well correlated with sensory evaluation also. This loss in solubility is usually taken as a measure of protein denaturation. But caution should be exercised in equating the two phenomena. A variety of other reasons also can cause loss insolubility which need not necessarily mean denaturation. For example, during rigor, solubility of muscle proteins is considerably reduced. But on resolution of rigor solubility shows an increase again. This is caused by the actin-myosin complexing and subsequent dissociation of the complex by autolysis. As such, this cannot be interpreted as denaturation and renaturation.

Anderson et al. (1963) have raised another pertinent point in this regard. In their experiments they found that fatty acids when added externally to the extractant, during extraction can reduce the extractability considerably. The mechanism of this is not yet established. But it clearly shows that solubility is not a fully reliable criterion to follow the course of protein denaturation in frozen fish. Love et al. (1972) accordingly tried a new approach to the problem. They tried to measure the toughness developed in the muscle as a result of frozen storage, by measuring the muscle resistance to disruption by a standardised high speed

homogeniser. The extent of disruption is measured by the turbidity in the 2% trichloro acetic acid in 1.2% formalin medium used for blending the fish muscle. However, the connective tissue content, variations in experimental conditions etc. can cause considerable experimental error leading to mistaken conclusions in this case also. Klerian (1965) tried the refractive index of the muscle juice as a criterion for protein denaturation. This also showed erratic values. In the absence of a fool foolproof criterion, salt solubility thus continues to be the accepted method for measuring protein denaturation in frozen fish.

There is considerable variations in the data presented by different workers on the loss of solubility of fish muscle proteins in salt solutions during frozen storage. Many workers have found the curve relating extractable protein to period of storage as nearly exponential (Dyer & Dingle, 1961; Love, 1962 A; Olley et al. 1962; Sawant & Magar, 1961; Nikkila & Linko, 1956). But sigmoid curves (Dyer & Dingle 1961; Nishimoto, 1962) and curves showing a series of dips (Husaini & Alm, 1955) Simidu & Simidu, 1957; Simidu et al. 1958) were also reported. Since sarcoplasmic protein remains unaffected the changes in the solubility can only be due to changes in the myofibrillar proteins. Considering the difficulties in extracting all the soluble proteins, attempts were made to see if extractability of a single protein like

myosin (Connell, 1962) or actin (Connell, 1960B) alone can give a better correlation with cold storage denaturation. Extractable myosin decreased gradually whereas actin was unaffected for a long time.

Changes in the -SH groups during frozen storage of fish was another factor explored by many workers as a possible criterion for protein denaturation in frozen fish. (Husaini & Alm, 1955; Smidu & Simidu, 1957; Connell, 1960A; Simidu et al. 1958). But the results showed too much variations to make it a reliable method.

ATP-ase activity of myosin and viscosity of myofibrillar protein extracts (Seagran, 1956; Ueda et al. 1962; Crupkin et al. 1979) were also tried as methods for monitoring protein changes in frozen fish muscle. But they have not been fully successful. Hamada et al. (1979) observed a decrease in the viscosity of actomyosin during storage at 4°C.

It is generally agreed that cross linking and other bondings as well as aggregation of the myofibrillar proteins causes the toughening of texture in frozen stored fish. But the nature of these bonds is not clear. Three types of bonds are possible.

i) bonds formed by interaction of denatured protein molecules

ii) bonds between native protein molecules and

iii) cross linking between protein molecules caused by small molecules or atoms.

Apart from these bonds and cross links resulting in loss of solubility, there are two more factors to be considered in protein denaturation in frozen fish.

i) concentration of tissue salts during freezing and

ii) lipid hydrolysis in frozen stored fish.

The salt denaturation theory is an old one (Reay, 1930). This theory assumes that the maximum rate of damage to proteins in freezing, observed in the temperature range of 0°C to -5°C, is due to the concentration of tissue salts during this phase. But Lovern & Olley (1962) have proved that this holds good for the other theory also. Lipid hydrolysis in frozen fish muscle is also found to be maximum in the same phase. Slow freezing resulting in extended time to pass through this temperature range results in the formation of larger ice crystals damaging the cell walls more. Another evidence in favour of the salt denaturation theory was Duerr & Dyer's observation (1952) that when immersed in concentrated brine, myofibrillar proteins of fish, becomes rapidly insoluble. The critical salt concentration was found to be 10%. Simidu & Hibiki (1952) Nikkila & Linko (1954) Linko & Nikkila (1961) and Simidu & Simidu (1957) supported this view in their

studies using different species of fish, though the critical salt concentration varied between different species.

Lipid hydrolysis theory of protein denaturation was suggested by Dyer (1951, 1953). He suggested that lipids protect proteins against denaturation. Fatty fishes were found to be less susceptible to protein denaturation during frozen storage. When lipids were hydrolysed, leading to the production of free fatty acids, proteins were denatured at a correspondingly faster rate (Dyer & Fraser (1959), Dyer et al. 1956; Dyer & Morton, 1956; Dyer & Dingle, 1961). Two possibilities were suggested (1) that the free fatty acids formed by lipid hydrolysis get absorbed on the surface of protein micells giving a hydrophobic surface and (2) lipid hydrolysis results in the loss of protective effect of lipids on proteins. Olley & Lovern (1960) observed that formation of free fatty acids and protein insolubilisation were parallel phenomena in cod stored at different temperatures. However, when some other species were used for the study (Olley & Lovern, 1962) this correlation was not observed. But according to King et al. (1962) this can be due to the difference in the capacity of different fatty acids to make myofibrillar proteins insoluble. Variations in storage characteristics of different species of fish may be due to the differences in free fatty acids produced from their lipids. Anderson

et al. (1963) suggested that the quantity of unhydrolysed lipid is critical. Fish with high lipid content needed large amounts of free fatty acid or its sodium salt to insolubilise proteins.

In addition to fatty acids and sodium soaps of fatty acids, oxidised lipids cause insolubilization of proteins. In fact, in situ, this is a problem demanding more attention. Oxidised lipids-protein interaction have been studied by various workers (Narayan & Kummerow, 1958; Lewis & Wills, 1962; Narayanan & Heyrka, 1962). Pokorny et al. (1975) suggested that four types of products are possible when proteins react with oxidised lipids.

i. Protein polymers formed by intramolecular cross-linking of proteins without any lipid in the final cross linked molecule.

ii) Polymers of protein and oxidised lipids held together by physical forces like multiple hydrogen bonds.

iii) Polymers of oxidised lipids and protein held together by covalent bonds.

iv) Polymers of oxidised lipids and small protein fragments formed by break up of protein molecules.

The relative preponderance of each type of these products depends on the conditions of reaction and also on

the nature of the reacting protein and lipid. High temperature accelerates this reaction. However, Kwon et al. (1965) isolated reaction products between malonaldehyde, a product of lipid oxidation, and protein from tuna stored at -18°C for 8 months. Buttkus (1967b) found that interaction between malonaldehyde and the free ω -amino groups of proteins were twice as fast at 20°C as at 0°C in a reaction mixture of suspended fish muscle. But the reaction rate at -20°C was almost as fast as 20°C. They explained this phenomenon as an effect of the concentration of reactants as a result of freezing. Ice crystals also was supposed to have a catalytic effect.

2.4.4 Changes in fish muscle proteins during freeze drying

Freeze drying results in considerable denaturation of the muscle protein. These products have a fibrous texture. Connell (1962) has reviewed the work reported on freeze drying of fish and its effect on the muscle proteins. Sarcoplasmic proteins are not affected to any significant extent. But total myofibrillar proteins extracted is reduced considerably. Urea and -S-S-reducing substances could solubilise this inextractable, denatured protein. Actin is not affected by denaturation during freeze drying whereas myosin is rendered insoluble to a considerable extent. Free fatty acids do not denature muscle proteins during freeze dying.

Dehydration of the unfrozen phase is thought to be responsible for the protein denaturation and texture damage during freeze drying (Luijet, 1962). Govindan (1969; 1970; 1974; 1975) and Govindan et al. (1968) have reported some detailed studies on the processing and storage characteristics of freeze dried prawns and some other fishery products.

2.4.5 Changes in the muscle protein of fish subjected to Irradiation

Though not commercially applied so far in India, irradiation is another method to enhance shelf life of fishery products. Several workers have reported results of studies on the effect of irradiation on the keeping quality of fish. Venkataraman et al. (1969) reported enhanced shelf life as a result of low dose irradiation of fish. Warrier et al. (1973) studied differences in the sarcoplasmic and myofibrillar proteins of irradiated Bombay duck 0.25 M rad irradiation along with mild heat treatment (60°C for 10 minutes) resulted in 83% precipitability of fibrillar proteins without causing precipitation of sarcoplasmic proteins. Irradiation-denatured sarcoplasmic proteins had a higher tryptic digestibility whereas trypsin digestibility of fibrillar proteins was not affected by irradiation. Madhavan & Kunta (1971) studied the radiation induced alterations in myoglobin from different fish species. Keay (1968)

made a study on the effect of low dose irradiation on the plastic packaging materials of fish on its quality and shelf life.

2.4.6 Effect of dehydration and salt curing of fish on the muscle proteins

Sun drying and salt curing are perhaps the oldest methods of fish preservation. Curing results in the loss of substantial amount of soluble proteins in the self brine. But qualitatively salting or smoking does not have any pronounced adverse effect on the proteins (Munro & Morrison, 1965). Linko et al. (1961) found that sodium chloride at a concentration of 2% could cause denaturation of muscle myosin in baltic herring. This denaturation could be prevented by small quantities of alakli phosphates in solution. The higher the concentration of sodium chloride in solution the greater the pyrophosphate needed to inhibit the denaturation. Linko et al. (1961) found sodium citrate also effective in inhibiting denaturation of muscle proteins by salt. Duipjen (1957) had opined that protein denaturation during different processes like freezing, freeze drying heating, salting etc. need not be of the same type. Hamm & Deatherage (1960) showed that denaturation of muscle proteins during heating and freeze drying are basically different. But surprisingly many workers have found the denaturation due to salt and that due to freezing to be similar (Love, 1958;

Simidu & Simidu, 1957) Simidu & Hibiki, 1951 and Luipjen, 1957). This in fact lead to the suggestion that denaturation during freezing is due to the concentration of tissue salts. Duerr & Dyer (1952) suggested that sodium chloride specifically denatures myosin fraction and that this denaturation is similar to the denaturation of myosin during freezing. Subsequent work done by Nikkila & Linko (1954) also supported this view. Connell (1957) observed that in dried fish the protein gel system of the fresh fish is in a disorganised state resulting in a much lower solubility. He concluded that actomyosin gets denatured during drying. Sawant & Nagar (1961) in their studies on drying of Bombay duck reported that denaturation during drying is not restricted to actomyosin fraction alone. They found evidence to suggest that sarcoplasmic protein are also affected during drying. The behaviour of these proteins in urea and urea containing thioglycollate solutions, suggests the formation of hydrogen bond and disulphide crosslinkages during drying. Kishimoto et al. (1956) have also concluded from their studies on air dried cuttle fish that hydrogen bonds are formed in proteins during drying. Nakamura & Ishikawa (1980) studied the changes in chemical contents in different parts of salted and dried sardine during storage. Nakamura et al. (1980) made a detailed study on the influence of raw material freshness on the quality of cured sardines.

2.4.7 Changes in muscle proteins of fish during heating

Protein denaturation during heat treatment of fish muscle has not received as much attention as the corresponding changes during freezing and frozen storage of fish. Hamm (1977) has recently given an excellent review on the changes in muscle proteins during heating of meat. Fish proteins behave basically in a similar way.

Heat denaturation of muscle proteins can be studied as three stages (1) upto 55°C the protein coagulation reactions dominate. This involves formations of unstable bonds between the free groups in the side chains. Ham & Hofmann (1965) observed that -S-S- bonds are formed in meat only when heated above 70°C. But coagulation of meat starts at around 45°C itself. They, therefore, suggested that coagulation of muscle proteins during heating is due to inter molecular association of other side groups of the protein chains. Samejima (1969) has also supported this view

(2) Above 55°C-60°C, the polypeptide chains undergo an uncoiling. As a result, -SH groups "burried" in the interior of the native protein helix so far, get exposed. This is proved by the initial increase in the number of -SH groups available to react with reagents like N-Ethyl Maleimide (NEM) or $AgNO_3$. Hamm and Hoffman (1965) and Tinbergen (1970) have observed this phenomenon. Above 70°C

there is a reduction in the -SH groups suggesting formation of S-S bonds. Other stable crosslinks are also formed during this stage (Hamm & Hofmann, 1965). Itoh et al. (1980) 1980a) have also presented evidence to suggest the formation of intermolecular -S-S-bond during heating of carp actomyosin.

Another type of intermolecular cross links formed during heating are the so called isopeptide links. These are links formed by the free NH_2 groups of the lysine residues with the amide groups of asparagine and glutamine. Bjarnasen & Carpenter (1970) have done some pioneering studies on this aspect. According to them isopeptide links are formed when proteins are heated at ordinary drying temperatures. Lysine can react with the destruction products of cystine also. The isopeptide bonds are resistant to enzymatic hydrolysis. So formation of such bonds results in decreased digestibility of the protein.

(3) At temperatures above 70°C, oxidation of -SH groups and associated changes occur in the protein molecules. As a result hydrogen sulphide (H_2S) is released. With further increase in temperature the rate of H_2S release increases upto 120°C. The pH of the muscle has an important bearing on the production of H_2S. Alkaline pH (upto pH 10) favours production of more H_2S (Johnson et al. 1964). Muscle of higher

content produced higher amount of H_2S (Kunsman et al. 1975) than lean muscle. H_2S production is commercially significant because it causes corrosion of tin containers. It also means a reduction in nutritive value because it indicates destruction of the essential sulphur amino acids. Above 120°C, the protein chains are likely to get fragmented. Hokuryo Hiro et al. (1980) observed that above 100-120°C carp muscle myosin got degraded into smaller fragments. After heating for one hour at 120°C the bands corresponding to heavy meromyosin chain disappeared from the electrophoretic pattern of carp myosin. But the light meromyosin chain appeared unaffected. Under the same conditions actin band showed only a reduced intensity of dye binding. Some small new bands were also visible. Mihalji-Kengyel et al. (1975) also reported break up of protein molecule on heating at 120°C.

Actin is more resistent to heat denaturation compared to myosin (Kaho, 1968a,b). Moreover actin protects myosin from heat denaturation to a limited extent, at the actin binding sites (Perry, (1967) Yasui et al. (1973)). Tropomyosin is the stablest of myofibrillar proteins.

Heat denaturation of sarcoplasmic proteins result in gel formation. Nishioka & Shimizu (1979) studied the coagulation properties of fish sarcoplasmic proteins at different pHs, and ionic strengths. Among the sarcoplasmic proteins,

the thermal denaturation of myoglobin is of special interest because this determines the change in colour of cooked muscle from red to greyish brown. This reaction takes place at around 65°C.

Mackie (1968) extracted heated fish muscle using 6 M urea and subjected this extract to disc electrophoresis. Urea disrupted the corroslinks formed during thermal denaturation. This pattern obtained was found to be species specific and useful for species identification in cooked fishery products. Kako (1968) also has suggested an alkaline buffer containing high consentration of urea for solubilising heat denatured proteins for further analysis.

Roberts and Lawrie (1974) reported that thermal denaturation caused a loss in electrophoretic mobility of muscle proteins. This was attributed to the destruction of some net charge in the protein molecule. However, SDS electrophoresis overcomes this problem. Hofmann (1977) made a remarkable study on the influence of heat on meat proteins using SDS electrophoresis. He came to some significant and interesting conclusions. 1) proteins of lower molecular weight are more resistant to thermal denaturation 2) Presence of salts increases the reactivity of proteins and thereby leads to increased damage during heating 3) During heating of muscle there is a loss of free $-NH_2$ and -COOH groups in the

protein molecule due to the formation of isopeptide bonds. Because of the loss in basic groups, dye binding capacity of the protein is reduced. This explains the reduced intensity of protein bands in electrophoretic pattern of heated muscle proteins 4) Isopeptide bonds, being resistant to proteolysic enzymes, reduce the digestibility of the protein.

Heating of muscle or isolated myofibrils usually result in an increase of pH (Hamm, 1966; Roberts et al. 1974; Fogg et al. 1975). The rate of this increase is found to be maximum in the temperature range 40-60°C. Simultaneously there is a shift in isolectric point to the higher side. These changes are presumably due to the release of "hidden" basic groups like imidazole group of histidine residues (Hamm, 1966). Thermal denaturation of myofibrillar proteins lead to the release of protein bound Ca^{++} and Mg^{++} ions also. The correlink formation and related changes during heating decreases the water holding capacity of the muscle and as a result muscle loses its tenderness.

Various workers have used different criteria like protein solubility, viscosity, fluorescence measurements, ATP-ase activity of myosin, number of -SH groups available for various -SH reagents etc. for following thermal denaturation of muscle proteins. Uchiyama et al. (1978) compared some epipleagic and mesopelagic fishes in respect of

the thermo stability of their myofibrillar Ca^{++} activated ATP-ase activity. Epipelagic ATP-ase was found to be more resistant to higher temperatures. This may be an adaptation to environmental conditions.

Thermal denaturation affects the connective tissue of muscle proteins also. In fish this is althemore pronounced. Fish muscle collagens gets easily degraded to gelatin on heating. Myocommata on easy gelatinization becomes lose and each myotome separates as a flake. This is a peculiar feature of fish muscle.

Maillard reaction is another important change to reakon with when fish muscle proteins are subjected to heat treatment. Sugars from muscle glycogen and amino acids react at temperatures above 80°C forming Maillard type of products leading to non enzymic browning. This leads to loss in nutritional quality by making unavailable certain essential amino acids.

From the foregoing review it is seen that our present knowledge on the proteins of Indian fishes and shell fishes is inaduquate. This study was therefore taken upto understand the composition of the muscle proteins of our major food fishes (including shell fishes) and to follow the changes in their proteins during preservation and processing.

As a part of this, the distribution of the major protein fractions in the muscle of several species of marine fishes (oil sardine, mackerel, lactarius) and shell fishes (different species of prawns) was worked out. The changes in these protein fractions during storage of fish/prawn, in ice and during quick freezing and subsequent frozen storage at -18°C were also followed. Along with these, the protein composition of six species of freshwater fishes and their changes during frozen storage were also studied for the purpose of comparison.

In these studies, solubility was the main criterion used for the classification of muscle proteins. Results of the comparative study between marine fishes, shell fishes and freshwater fishes revealed some interesting differences. These results threw up some interesting problems which lead to a study on the protein - fatty acid interaction in fish and their effect on the muscle protein solubility. The results of this study in turn lead to a comparison of solubility and cell fragility techniques, as methods for following protein denaturation in fish.

Effects of other factors like ionic strength and pH of the extractant and nature of the ions present therein, on the solubility of muscle proteins of fishes and shell fishes were also studied in this connection. Denaturing

effect of salt on muscle proteins and its possible prevention by alkali phosphates was another aspect investigated in detail using fish as well as prawns. Effect of salts like acetates, citrates etc. and sugars like glucose on protein denaturation in fishes and prawns also were worked out.

3. CHAPTER I

Icing is the cheapest and most convenient method for preservation of fish onboard as well as for transportation of fish over long distances. Since the textural qualities of fish are directly associated with the changes in its muscle proteins, such changes during storage of fish in ice assume special significance.

Compared to the volume of work reported on the changes of meat proteins during storage under different conditions, very little information is available on the changes in fish muscle proteins during storage. Connell

(1961) has observed that proteins of fish do not necessarily behave like avian or mammalian muscle proteins. Studies aimed at understanding the changes of fish muscle proteins during storage under different conditions, thus become very important, to evolve better methods for its preservation.

Seagran (1958a) fractionated the muscle proteins of King crab using a differential solubility method. Maruyama & Suzuki (1968) investigated the changes in the muscle proteins of horse mackerel during storage in ice. Hashimoto et al. (1979) studied the protein composition of the red and white meats of sardine and mackerel from Japanese coast and followed their changes during frozen storage of the fishes. Awad et al. (1969) reported results on the changes in the freshwater white fish muscle proteins during frozen storage.

In India, Govindan (1962) reported results of his studies on the total and water soluble nitrogen in ice stored prawns. Moorjani et al. (1962) and Baliga et al. (1962, 1962a, 1969) have attempted to follow the changes in the muscle proteins of freshwater fishes during storage in ice. But a systematic study on the distribution of major protein nitrogen fractions in our commercially important marine fishes and shell fishes, and their changes during storage in ice have not been attempted so far. Results of such a study are reported in this chapter.

3.1 MATERIALS AND METHODS

Marine prawns (*M. dobsoni* and *P. indicus*), oil sardines (*Sardinella longiceps*), Mackerel (*Rastrelliger kanagurta*) and Lactarius (*Lactarius lactarius*) were procured in the prime condition of preservation, by immediately icing after capture, for this study from the boats of the Central Institute of Fisheries Technology, Cochin. Prawns were beheaded, washed clean of adhering dirt, slime etc. and stored in crushed ice. Oil sardines, mackerels and lactarius were washed well in good potable water, and similarly stored in crushed ice.

All buffers and solutions used for extraction of the protein fractions were prepared using Analar chemicals. Except when otherwise stated all other chemicals used in this study were also of Analar grade.

3.1.1 Fractionation of proteins

Protein fractions from the fishes and shell fishes were extracted by the preferential solubility technique of Seagran (1958a) as modified by Paul (1966). Potassium chloride - Borate buffer, μ = 0.05, pH 7.5, was used for extraction of the sarcoplasmic proteins in all cases. But myofibrillar proteins were extracted using KCl-NaH_2PO_4 - Na_2HPO_4 buffer (μ = 0.6, pH 7.5) at 4°C and not by KI-Borate buffer as done by Paul. All operations were done in a cold room at 4°C. For extraction of the denatured protein, 0.1 N sodium hydroxide at room temperature (25-27°) was used. Residue after these extractions was directly digested to estimate the stroma.

3.1.2 Extraction procedure

Extraction procedure adopted is outlined in figure 1. 10 gm of well minced muscle was accurately weighed into a steel centrifuge tube. About 30-35 ml of the previously cooled potassium chloride-borate buffer was added to the muscle and mixed well using a glass rod. The mixed slurry

was kept at 0-4°C overnight with frequent stirring. Next day the slurry was centrifuged at 5000 r.p.m. at 0°C in a high speed refrigerated centrifuge (International Equipment Co., Needham, USA) for 30 minutes. The supernatant was carefully decanted into a 100 ml standard flask kept at 4°C. The residue in the centrifuge tube was similarly extracted two times more with 25 ml portions of the cooled buffer. Each time the residue was mixed well with the buffer using the glass rod and kept at 4°C for two hours (with occasional stirring) before centrifuging. Supernatant after centrifugation was transferred carefully to the same standard flask each time. The volume finally was made up to the mark with the cooled buffer and protein nitrogen content in the extract was determined by Kjeldahl's method according to A.O.A.C. (1960).

Residue after extraction of the sarcoplasmic protein nitrogen was extracted similarly at 4°C with cold KCl-phosphate buffer (μ=0.6, pH 7.5). The myofibrillar protein nitrogen content was calculated from the nitrogen content of an aliquot of the made up extract.

The residue after this extraction was extracted in a similar way with 0.1 N sodium hydroxide at room temperature to dissolve the denatured albumins and globulins. In this case the extracts were centrifuged at 8000 r.p.m. to ensure

clear settling of the residue. Nitrogen content of an aliquot from this extract was also determined to calculate the denatured protein.

Residue after this extraction consisting mostly of connective tissue was directly digested with sulphuric acid and nitrogen content determined, to calculate the stroma content of the muscle.

Total nitrogen in the muscle was determined on each day by digesting 1 gm portions of the minced muscle with sulphuric acid and determining the nitrogen content. Total non protein nitrogen was determined by precipitating all protein from an aquous extract of the muscle (10 gms muscle in about 200 ml) by blending with 30% trichloroacetic acid and filtering off the precipitate. The filtrate was made upto 250 ml washing the precipitate with distilled water and nitrogen content was determined to calculate the total non protein nitrogen in the muscle. Non protein nitrogen content of the protein extracts were also separately determined each time in a similar way by precipitating the protein with trichloroacetic acid. Content of the different protein nitrogen fractions were then calculated and expressed as percentage of total protein nitrogen.

3.2 Results

The distribution of the major protein nitrogen

fractions in the muscle of different prawns, and fishes and the changes in these fractions during storage of fish/prawn in ice are given in tables 1,2,3,4 and 5.

3.3 Discussion

Table 1 gives the protein nitrogen distribution in prawns P. indicus and its changes during storage in ice. During storage in ice for 12 days, the sarcoplasmic protein nitrogen decreased by about 5% only. But in the case of myofibrillar protein nitrogen there was a reduction of about 20%. Simultaneously proteins extracted with dilute sodium hydroxide showed a steep increase from the initial 37.19% to 62.15%. Stroma protein fraction did not show any appreciable change. After a few days in ice there was a small inevitable decrease in total protein nitrogen which is partly responsible for the slight increase in stroma when expressed as percentage of TPN.

Table 2 gives the corresponding figures for prawns (M. dobsoni) held in ice for 13 days. In this case also the pattern of changes was the same. Sarcoplasmic protein accounted for about 30% of the total protein. After 13 days in ice, extractable sarcoplasmic protein was about 25% of the total protein. From the residue after extraction of sarcoplasmic proteins a further 25% of the total protein nitrogen could be extracted with KCl-phosphate buffer, μ = 0.6, pH 7.5

in the fresh state. After 13 days in ice, this value came down to 10.27%. Alkali soluble fraction during the same period increased from the 35.92% to 57.90%. Stroma registered negligible change in this case also.

As different from prawns, the three species of fish examined showed some peculiar features. In the case of oil sardine (Table 3) and lactarius (Table 5) the sarcoplasmic protein accounted for only about 25% of the total protein. However, in mackerel (Table 4) sarcoplasmic protein came to the same level as in prawns i.e. about 30% of the total protein.

In all the three species of fishes, the amount of protein extracted with KCl-phosphate buffer (μ = 0.6, pH 7.5) from the residue after extraction of sarcoplasmic protein was very low. Whereas in fresh prawns, 25-28% of the total protein could be extracted like this, only 7-8% of the total protein could be extracted from the fishes.

In sardines (Table 3) again sarcoplasmic protein extractability did not show any significant fall during storage in ice (26-28%). In lactarius (Table 5) sarcoplasmic protein registered a slightly higher loss in extractability during ice storage (25.68% to 15.[illegible]%). Mackerel showed a distinctly different trend in this case also. From 30.55%, the percentage of extractable sarcoplasmic protein decreased to 15.4%

during 13 days storage in ice.

The percentage of proteins extracted with KCl-phosphate buffer μ = 0.6, pH 7.5 from the residue after removal of sarcoplasmic protein decreased from the initial value of 8.77% to 5.43% in sardines during storage in ice for 13 days. In lactarius the corresponding change was from 7.64% to 3.08% and in mackerel from 7.62% to 2.92%. Denatured protein extracted with alkali in all cases showed a corresponding increase (55.6% to 66.47% in sardine, 50.38% to 74.19% in lactarius and 57.16% to 75.26% in mackerel). In all the three cases stroma showed little change during ice storage.

For comparing this successive extraction technique with direct extraction, 10 gm portions of muscle from each fish were extracted directly with 100 ml of KCl-phosphate buffer (μ = 0.6, pH 7.5) in a waring blender fitted with a baffle plate to minimise foaming. The protein nitrogen content of this extract was similarly determined after centrifuging and estimation of nitrogen in the supernate. All samples were similarly extracted with Dyer's buffer (5% Nacl in 0.02 M $NaHCO_3$ μ = 0.8747, pH 7.2) also, to see the effect of a higher ionic strength on the solubility of muscle proteins. Results are presented in Table 6.

Direct extraction with 0.6μ buffers in all cases gave

a higher extraction than extraction in two steps (Table 6). It appears, the initial extraction of sarcoplasmic protein modifies the remaining myofibrillar proteins in some way, reducing their subsequent extraction. This effect was more pronounced in the case of fishes than in prawns. Perry (1953) and Bailey (1954) had also found that the residue after extraction of albumins is not a suitable starting material for extracting myofibrillar proteins. Dyer (1961) endorses this view.

It is also seen that an ionic strength of 0.6 does not give complete extraction of muscle proteins in fish as is clear from a comparison between the values of direct extraction with $\mu = 0.6$ buffer and with Dyer's buffer (Table 6). Dyer's buffer gave near complete extraction in prawns. However, with fishes, this buffer also did not give complete extraction.

Results of a separate study on the extraction of muscle proteins from different fishes using buffers of varying ionic strenghts (KCl-phosphate buffers were used in all cases; ionic strengths were adjusted by changing the KCl concentration) are reported in Table 7. Dyer's buffer gives maximum extraction in all cases.

From the results it is clear that it is the myofibrillar proteins which become increasingly insoluble during

iced storage of fish. Sarcoplasmic proteins, except in mackerel were not affected to any significant extent during chill storage. Stroma, remained unaffected throughout in all cases. These observations are in agreement with the findings of Dyer et al. (1950) and Sawant & Magar (1961). Incomplete extraction of myofibrillar proteins from the residue after extraction of sarcoplasmic proteins observed in this study can be at least partly due to the procedure adopted for the extraction. In this study, no mechanical homogenisation was employed for extraction. Dyer (1950) observed that very fine subdivision of the muscle myofibrils is one of the prerequisites for complete extraction of myofibrillar proteins. Unless a waring blender is used, this condition is not satisfied. But for successive extraction with different buffers, waring blender cannot be used, as losses in the blender will render the results unsuitable for quantitative estimations. This was why the present procedure was used in this study which ensured quantitative results. Sarcoplasmic proteins, on account of their high solubility, get extracted even in the absence of mechanical blendering whereas myofibrillar proteins may not get fully extracted in the absence of blending. Cowie & Mackie (1968) have in fact observed that even the type of homogeniser used is critical.

However, even in the present method, there is a clear difference in the amount of myofibrillar proteins extractable

from fishes and prawns as can be seen from Tables 1,2,3,4 and 5.

The low extraction of myofibrillar proteins from all species of fishes examined, even in fresh state, can be due to the action of free fatty acids also. Free fatty acids when added externally to the extraction buffer at the time of extraction are known to cause insolubilisation of muscle proteins (King et al. 1962; Andersen et al. 1963). Fishes, which have a higher fat content than prawns will naturally have a higher free fatty acid content also. This may be the reason for the greater protein insolubilisation in fishes compared to prawns. The fact that formation of free fatty acids in frozen fish is accompanied by a corresponding increase in protein insolubilisation also supports this view. However, the effect of externally added f.f.a and f.f.a produced in situ, need not be the same (Dyer & Fraser, 1959 Olley & Lovern, 1961; Bligh E.G. 1961).

Free fatty acids may be getting absorbed on the surface of the protein micelles making them more hydrophobic and thus less extractable. Free fatty acids can react with sarcoplasmic proteins without resulting in insolubilisation (Putnam, 1948). Bull & Bruse (1967) have shown that about 10 moles of fatty acid per mole of protein is to be bound, before denaturation begins. In prawns, this level of fatty acids is not present in the fresh state. Whereas in the

fatty fishes this level may be present in the initial stage itself. They bind with the sarcoplasmic proteins and during extraction of the sarcoplasmic protein, these protein - FFA complexes may modify the hydration characteristics of the remaining contractile proteins inhibiting their subsequent extraction (Seagran, 1958).

In the case of mackerel, the content of sarcoplasmic protein was higher compared to other fishes. In this respect it was similar to prawns. In these cases, the contribution of ions from the muscle to the total ionic strength of the buffer may be higher. Usually the contribution of the muscle ions to the total ionic strength of the buffer is ignored. But at high fish: buffer ratio (as in the case of the present procedure of repeated extraction of 10 gm muscle with 30 ml portions of buffer), this factor can be significant (Dyer & Dingle, 1961). Such an increased ionic strength may result in the extraction of some myofibrillar fraction also in the sarcoplasmic fraction. In squid, Matsumoto (1957) found that part of the actomyosin got extracted in the water extract itself. He referred to this fraction as M-actomyosin and made detailed studies on its properties. Such a phenomenon cannot be altogether ruled out in mackerel also. The presence of such an easily denatured myofibrillar protein in the sarcoplasmic extract may

also explain the unusual rapid fall in the percentage of sarcoplasmic protein during storage in the case of mackerel, as distinct from other fishes.

This study thus reveals some characteristic differences between the muscle proteins of fishes and shell fishes and also between different species of fishes. The results also suggest a possible role of free fatty acids in rendering the muscle proteins insoluble. This effect was therefore studied in detail.

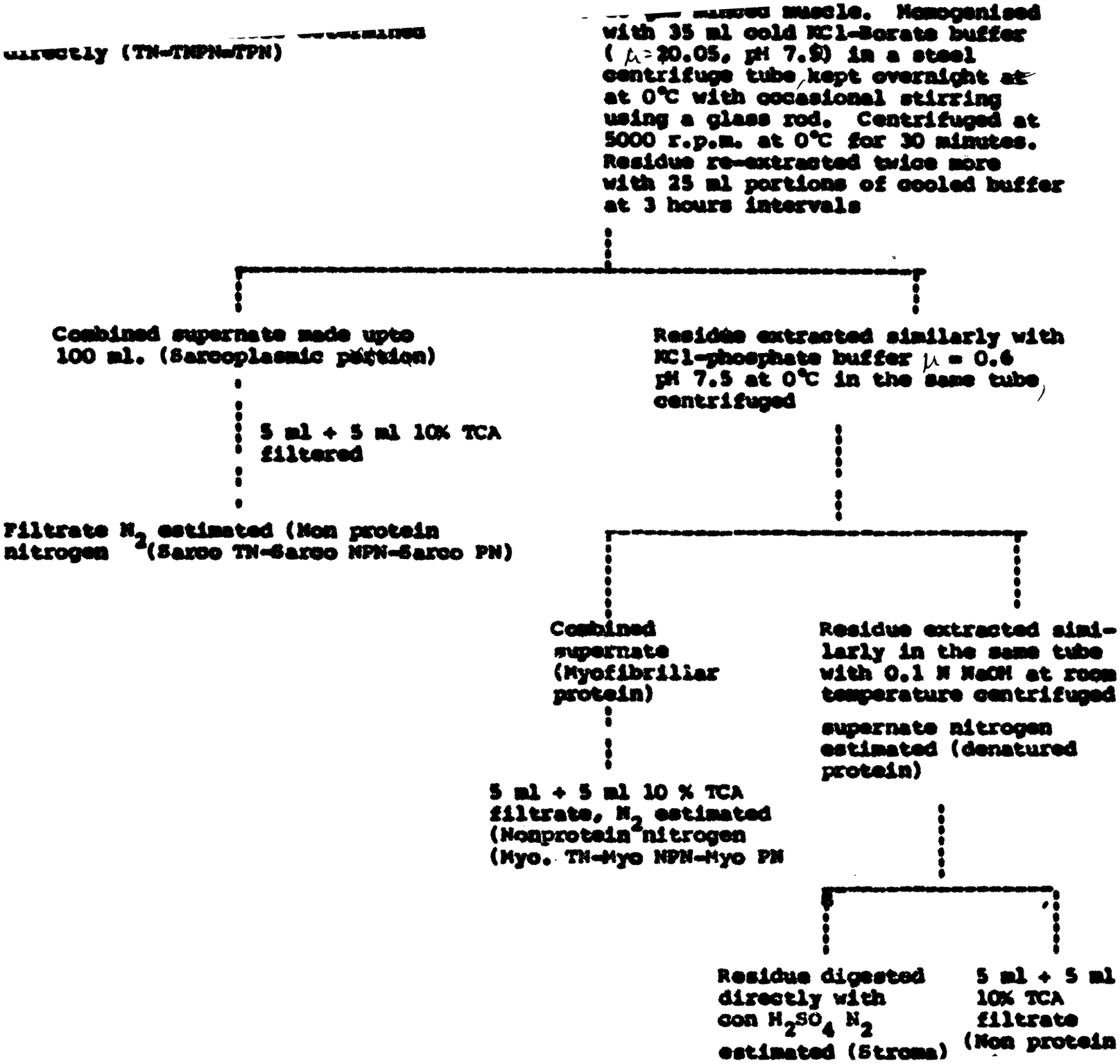
directly (TN-TNPN=TPN)
with 35 ml cold KCl-Borate buffer (μ=0.05, pH 7.9) in a steel centrifuge tube kept overnight at at 0°C with occasional stirring using a glass rod. Centrifuged at 5000 r.p.m. at 0°C for 30 minutes. Residue re-extracted twice more with 25 ml portions of cooled buffer at 3 hours intervals
Combined supernate made upto 100 ml. (Sarcoplasmic pattion)
Residue extracted similarly with KCl-phosphate buffer μ = 0.6 pH 7.5 at 0°C in the same tube centrifuged
5 ml + 5 ml 10% TCA filtered
Filtrate N2 estimated (Non protein nitrogen (Sarco TN-Sarco NPN-Sarco PN)
Combined supernate (Myofibrillar protein)
Residue extracted similarly in the same tube with 0.1 N NaOH at room temperature centrifuged
supernate nitrogen estimated (denatured protein)
5 ml + 5 ml 10 % TCA filtrate, N2 estimated (Nonprotein nitrogen (Myo. TN-Myo NPN-Myo PN
Residue digested directly with con H2SO4 N2 estimated (Stroma)
5 ml + 5 ml 10% TCA filtrate (Non protein

Table 1. CHANGES IN PROTEIN NITROGEN FRACTIONS OF PRAWNS P. INDICUS HELD IN ICE

No. of days in ice	Sarcoplasmic protein nitrogen (extracted with KCl-borate buffer μ = 0.05, pH 7.5) (a)	Myofibrillar protein nitrogen extractable from the residue from (a) with KCl-phosphate buffer μ = 0.6, pH 7.5 (b)	Protein nitrogen extractable with 0.1 N NaOH from the residue from (b) (c)	Stroma protein nitrogen (d)
	(Results expressed as % of total protein nitrogen)			
0	28.95	27.31	37.19	3.77
2	27.38	27.06	37.20	3.77
5	24.86	22.29	45.86	4.70
7	25.46	16.67	49.12	5.57
10	23.48	11.89	59.94	5.57
12	23.35	7.23	62.15	5.82

Table 2. CHANGES IN PROTEIN NITROGEN FRACTIONS OF PRAWNS (M. DOBSONI) HELD IN ICE

No. of days in ice	Sarcoplasmic protein nitrogen (extracted with KCl-borate buffer μ = 0.05, pH 7.5) (a)	Myofibrillar protein nitrogen extractable from the residue from (a) with KCl-phosphate buffer μ = 0.6, pH 7.5 (b)	Protein nitrogen extractable with 0.1 N NaOH from the residue from (b) (c)	Stroma protein nitrogen (d)
	(Results expressed as % of total protein nitrogen)			
0	29.36	25.01	35.92	4.91
2	29.29	19.43	39.91	5.04
4	-	18.01	41.91	5.22
6	27.77	16.98	49.27	5.81
9	27.77	16.90	52.01	5.93
11	25.98	13.63	55.34	5.93
13	25.20	10.27	57.90	6.11

Table 3. CHANGES IN PROTEIN NITROGEN FRACTIONS OF OIL SARDINES S. LONGICEPS HELD IN ICE

No. of days in ice	Sarcoplasmic protein nitrogen (extracted with KCl-borate buffer μ = 0.05, pH 7.5 (a)	Myofibrillar protein nitrogen extractable from the residue from (a) with KCl-phosphate buffer μ = 0.6, pH 7.5 (b)	Protein nitrogen extractable with 0.1 N NaOH from the residue from (b) (c)	Stroma protein nitrogen (d)
	(Results expressed as % of total protein nitrogen)			
0	26.33	8.77	55.60	6.84
2	23.71	7.86	59.15	7.03
4	23.15	6.66	61.24	7.15
6	22.35	6.32	62.86	6.98
8	22.24	5.96	64.91	7.08
10	21.80	5.47	65.47	7.24
13	20.39	5.43	66.48	7.39

Table 4. CHANGES IN THE PROTEIN NITROGEN FRACTIONS OF MACKEREL (*RASTRALLIGER KANAGURTA*) HELD IN ICE

No. of days in ice	Sarcoplasmic protein nitrogen (extracted with KCl-borate buffer μ = 0.05, pH 7.5 (a)	Myofibrillar protein nitrogen extractable from the residue from (a) with KCl-phosphate buffer μ = 0.6, pH 7.5 (b)	Protein nitrogen extractable with 0.1 N NaOH from the residue from (b) (c)	Stroma protein nitrogen (d)
	(Results expressed as % of total protein nitrogen)			
0	30.55	7.62	57.16	5.23
2	27.26	5.23	62.36	5.81
5	15.24	4.82	73.61	6.22
10	15.52	3.92	74.72	6.08
13	15.40	2.92	75.26	6.23

Table 5. CHANGES IN THE PROTEIN NITROGEN FRACTIONS OF LACTARIUS (LACTARIUS LACTARIUS) HELD IN ICE

No. of days in ice	Sarcoplasmic protein nitrogen (extracted with KCl-borate buffer μ = 0.05, pH 7.5 (a)	Myofibrillar protein nitrogen extractable from the residue from (a) with KCl-phosphate buffer μ = 0.6, pH 7.5 (b)	Protein nitrogen extractable with 0.1 N NaOH form the residue from (b) (c)	Stroma protein nitrogen (d)
	(Results expressed as % of total protein nitrogen)			
0	25.68	7.64	59.38	7.13
4	23.32	5.37	64.10	7.26
6	17.50	4.28	70.50	7.62
12	15.76	3.08	74.19	7.82

Table 6. EXTRACTABILITY OF MUSCLE PROTEINS OF PRAWNS AND FISHES BY DIRECT AND SUCCESSIVE EXTRACTION TECHNIQUES: A COMPARISON

All values expressed as % of total protein nitrogen

Species	Soluble protein nitrogen in buffer of μ = 0.05 pH 7.5 (a)	Soluble protein nitrogen extracted from the residue after extraction with buffer μ = 0.05, pH 7.5 using buffer μ = 0.6, pH 7.5 (b)	Total soluble protein nitrogen extracted by the two extractions (a+b)	Soluble protein nitrogen by direct extraction with buffer μ = 0.6, pH 7.5	Soluble protein nitrogen in Dyer's buffer μ = 0.8747
Prawns (*P. indicus*)	28.95	27.31	56.26	80.06%	82.63
Oil sardine	26.33	8.77	35.10	52.86%	57.68
Mackerel	30.55	7.62	38.17	50.28%	62.36
Lactarius	25.68	7.62	33.32	56.84%	60.84

Table 7. SOLUBILITY OF MUSCLE PROTEINS OF PRAWNS AND FISHES AT DIFFERENT IONIC STRENGTHS (pH KEPT AT 7.5 IN ALL CASES)

Ionic strength of the buffer (NCl-phosphate buffer pH 7.5 in all cases from 0.6 to 0.05	Species			
	Prawns (P. indicus)	Oil sardine	Mackerel	Lactarius
	(All values expressed as % of Total protein nitrogen)			
0.8747 (Dyer's Buffer 5% Nacl in 002 $NaNH_2IO_3$	82.63%	56.28%	60.32%	62.28%
0.6	80.06%	52.86%	58.28%	56.84%
0.50	80.06%	50.48%	40.68%	54.46%
0.25	38.03%	34.86%	34.82%	38.86%
0.15	33.26%	35.18%	34.82%	37.16%
0.05	30.02%	27.33%	30.55%	25.68%
Water Extract	26.02%	25.82%	29.86%	23.83%

4. CHAPTER II

4.1 Introduction

In view of the results presented in chapter I, it was found desirable to see whether free fatty acids actually cause insolubilisation of fish muscle proteins. It has been reported by several workers that in frozen stored fish muscle, lipid hydrolysis and accumulation of free acid is accompanied by a corresponding increase in protein inextractability. (Dyer & Fraser, 1959; Olley & Lovern 1960; Olley & Lovern, 1962; Boyd *et al.* 1967). King *et al.* (1962) and Anderson *et al.* (1963, 1964) showed that

free fatty acids or their salts, if added to the extractant during extraction, can cause protein insolubilisation of fish muscle proteins. The effectiveness of free fatty acids as denaturants is known to increase with the chain length of the acid. Anderson & Steinberg (1964) demonstrated the species differences in the reactivity of fish muscle proteins towards fatty acid salts in muscle homogenates. The nature of the reactions between proteins and fatty acids (or their sodium salts) may vary depending upon the proteins as well as the free fatty acid reacting with it. Since fish oils generally contain appreciable amounts of highly reactive unsaturated fatty acids, the effect of two common unsaturated acids (oleic and linoleic) on the solubility of the muscle proteins of typical fishes and shell fishes of tropical waters was studied for this purpose. Two species of prawns, a typical fatty fish (oil sardine) and a lean fish (jew fish) were used in this study.

4.2 Materials and Methods

For solubility determinations, Dyer's buffer (5% NaCl in 0.02 M $NaHCO_3$) was used in all cases. The waring blendor technique using the baffle plate for minimising foaming, was used for extraction. All operations were done in a cold room at 4°C, using precooled solutions and glasswares. All solutions were prepared from Analar grade chemicals. Oleic

acid and Linoleic acid were from Koch - Light Laboratories, England.

10 gm of well minced muscle of the fish/prawn was blended with 200 ml of cooled Dyer's buffer for 2 minutes with a 30 second break after the first minute. To the extractant, measured quantities (measured volumetrically using a pipette) of the acid concerned was added just before blending. A control sample with no acid added to it was also taken each time. The blended samples were centrifuged in all cases at 5000 r.p.m. at 0°C for 30 minutes in an I.E.C. (International Equipment Co., Needham, Mass, U.S.A.) model high speed refrigerated centrifuge. Nitrogen in the supernate was determined by the microkjeldahl method in all samples (AOAC, 1960). Total nitrogen and non protein were determined as described in chapter 1.

In a separate experiment, actomyosin from oil sardine was prepared by the method of King et al. (1962) by washing off the sarcoplasmic protein first with low ionic strength buffer and subsequently extracting the actomyosin from the residue by a high ionic strength buffer. To 40 ml portions of this preparation, calculated amounts of linoleic acid was added using a capillary jet tube. The solutions, after gentle but thorough mixing, was kept in a cold room at 5°C for several days. At regular intervals the soluble protein

nitrogen of the solution was determined by centrifugation and estimation of the protein in the supernate. This was meant to study the effect of prolonged contact of free fatty acid with muscle protein in solution.

4.3 Results

Tables 8 and 9 give the results of the study on the effect of added oleic and linoleic acids on the solubility of protein of prawns (P. indicus and M. dobsoni)

Tables 10 and 11 gives the corresponding results in the case of oil sardine and jewfish (Johnius argentius)

Table 12 summarises the results of the study on the effect of exposing actomyosin from oil sardine to varying amounts of linoleic acid for different periods.

4.4 Discussion

In the case of prawns (Tables 8 and 9) an interesting phenomenon was observed when small amounts of fatty acids are added to the extracting buffer, the extractability is slightly enhanced in the beginning. But after reaching a critical concentration, fatty acids cause regular insolubilization. The effect was the same in the case of oleic as well as linoleic acids. In both cases when more than 0.2 ml of the acid is added to 200 ml of the buffer, solubility begins to register a regular decrease. The initial small but consistant increase in the solubility is in clear contrast

to the regular fall in solubility observed in the case of fishes (oil sardine and jewfish (Sardinella longiceps, Johnius argentia) (Table 10 and 11). In similar experiments using cod, reported by Anderson et al. (1963), also this phenomenon was not observed. This appears to be peculiar to prawn, as different from the fishes.

In the case of oil sardine and jewfish there was a regular fall in solubility when increasing quantities of fatty acids were added to the extractant. In these cases, 0.25 ml of acid in 200 ml Dyer's buffer caused insolubilization of almost all extractable myofibrillar proteins leaving only the sarcoplasmic protein in solution. In prawns at this level of acid a sizeable portion of the myofibrillar proteins still remained in solution. This may be partly due to the increased extraction of myofibrillar protein observed in prawns compared to fishes.

Extracted actomyosin from oil sardines is also found to get insolubilised, when held in solution in contact with added fatty acids. The extent of insolubilisation caused, depends upon the concentration of the acid added and also on the period of storage in contact with it (Table 12). Although the actual mechanism involved is not clear, the main factor involved appears to be the loss in water holding capacity of the proteins and the changes in its hydration

characteristics (Seagran, 1958) brought about by the formation of a more hydrophobic surface on the protein micelles due to adsorption of the fatty acids.

According to Bull & Bruce (1967) the denaturation of muscle proteins by fatty acids follows first order kenetics with respect to proteins. But order with respect to fatty acids is higher. At least 10 moles of fatty acids per mole of protein is to be bound to the protein before denaturation begins. In prawns added fatty acids in low levels, probably interact with the sarcoplasmic proteins, which occur to the extent of about 30% of the total protein. These initial interactions result in better emulsification during blending and consequent improved extraction. In the lean prawn muscle, sufficient free fatty acids are not available to saturate the binding sites of sarcoplasmic proteins. C_{18} acids are not dissociated completely in buffered salt solutions during blending (Ralston, 1948) and their interaction with muscle sarcoplasmic proteins do not result in insolubilization (Putnam, 1948). When binding sites of sarcoplasmic proteins are saturated, further addition of fatty acids will result in contractile protein -f.f.a interaction. These interactions lead to the formation of cross links resistant to fragmentation and solubilisation by the extractant buffer, reducing solubility (Anderson et al. 1968).

In the case of fishes (Oil sardine and jewfish) the fat content is higher and sarcoplasmic protein content is lower compared to prawn. Due to the higher fat, lipolysis in situ, must have produced enough f.f.a. in these cases to saturate the binding sites of sarcoplasmic proteins in the initial stage itself. Addition of fatty acids therefore result in a regular fall in solubility due to contractile protein-f.f.a. interaction. This may thus offer an explanation to the lower amount of salt soluble protein observed in these cases in the fresh state itself, in comparison with prawns.

Table 8. EFFECT OF ADDED OLEIC ACID AND LINOLEIC ACID ON EXTRACTABILITY OF MUSCLE PROTEINS OF PRAWNS (P. INDICUS)

Sl. No.	Volume of acid added to 200 ml extractant (ml)	Salt soluble protein nitrogen as % of total protein nitrogen	
		Series I	Series II
Oleic acid			
1	0.00	81.87	75.77
2	0.10	84.13	77.62
3	0.20	80.88	75.39
4	0.30	51.49	52.85
Linoleic acid			
1	0.00	78.86	75.41
2	0.20	84.01	81.32
3	0.40	63.40	61.75
4	0.60	30.63	40.88

Series II
* Same experiment repeated with another batch of fish.

Table 9. EFFECT OF ADDED OLEIC AND LINOLEIC ACID ON THE EXTRACTABILITY OF MUSCLE PROTEINS OF PRAWNS (M. DOBSONI)

Sl. No.	Volume of acid added to 200 ml extractant (in ml)	Salt soluble protein nitrogen as % of total protein nitrogen
Oleic acid		
1	0.00	77.76%
2	0.10	87.28%
3	0.15	79.48%
4	0.20	70.00%
5	0.25	68.28%
6	0.30	63.96%
Linoleic acid		
1	0.00	77.76%
2	0.10	82.24%
3	0.15	78.46%
4	0.20	75.36%
5	0.25	70.34%
6	0.30	58.64%

Table 10. EFFECT OF ADDED OLEIC AND LINOLEIC ACIDS ON THE EXTRACTABILITY OF MUSCLE PROTEINS OF OIL SARDINES (SARDINELLA LONGICEPS)

Sl. No.	Volume of acid added (ml) to 200 ml of Dyer's buffer	Salt soluble protein nitrogen as % of total protein nitrogen
Oleic acid		
1	0.00	47.10
2	0.05	46.82
3	0.10	43.28
4	0.15	38.86
5	0.20	36.28
6	0.25	28.88
7	0.30	26.84
Linoleic acid		
1	0.00	47.10
2	0.05	45.04
3	0.10	40.12
4	0.15	29.88
5	0.25	25.77
6	0.30	25.31

Table 11. EFFECT OF ADDED LINOLEIC ACID ON THE EXTRACTABILITY OF MUSCLE PROTEINS OF JEW FISH

Sl.No.	Volume of linoleic acid added (in ml) to 200 ml Dyer's buffer	Salt soluble protein nitrogen as % of total protein nitrogen
1	0.00	58.56%
2	0.05	53.23%
3	0.10	45.23%
4	0.15	45.78%
5	0.20	46.85%
6	0.25	44.72%
7	0.30	26.73%

Table 12. EFFECT OF ADDED LINOLEIC ACID ON INSOLUBILIZATION OF FISH ACTOMYOSIN FROM SARDINELLA LONGICEPS HELD AT 4°C

Sl. No.	Amount of linoleic acid added mg/gm of soluble protein nitrogen	Soluble protein nitrogen (mg/ml) in actomyosin preparation held at 4°C	
		24 hrs stored	120 hours stored
1	0.000	0.409	0.349
2	0.088	0.296	0.279
3	1.978	0.245	0.191
4	2.054	-	0.175
5	5.005	0.232	0.141
6	10.380	0.134	0.107

5. CHAPTER III

5.1 Introduction

Solubility in Dyer's buffer has been generally accepted as a criterion for protein denaturation in frozen fish. But the results reported in the previous chapter casts doubts on its reliability as a food proof test of protein denaturation, especially in fatty fishes. Love tried an entirely new approach to the problem. Frozen fish muscle develops toughness with progressive denaturation of the muscle proteins. With increased toughness in texture, the muscle develops increased resistance to fragmentation and

cell disruption during homogenisation using a blendor. He attempted to correlate the degree of toughness to protein denaturation. The procedure evolved was simple. A small portion of the muscle is blended in a specially designed homogeniser with a protein precipitating medium (2% trichloroacetic acid in 1.2% formaldehyde). When about 100 mg of the muscle, freed as far as possible from connective tissue, is blended with this solution in the standardised Torry-Brown homogeniser at a constant speed, the turbidity formed will be directly proportional to the extent of cell disruption during blending. When cell disruption becomes progressively lesser due to increased toughness in frozen stored fish muscle, the turbidity in the solution will correspondingly decrease. The optical density of the homogenate, if measured immediately after blending, can thus give an idea about the denaturation of muscle proteins. Love et al. (1972) reported good correlation between storage time of frozen fish and its cell fragility value and found it useful in routine quality control tests.

As solubility was not found to be a very satisfactory criterion, especially in tropical fatty fishes, cell fragility test was tried as a criterion for protein denaturation in different species of fish and prawns, frozen and stored under conditions adopted by the fish processing industry. This was compared with the standard salt solubility test to

assess the relative merits and demerits of each.

5.2 Materials and Methods

Prawns (*P. indicus*) Mullet (*Mugil cephalus*) Kilimeen (*Nemipterus japonicus*) and Mackerel (*Rastralier kanagurta*) were used for this study. The fish procured in fresh condition were washed and frozen in a plate freezer at -40°C and stored at -20°C. Prawns were peeled and deveined, washed well, and frozen as blocks in a plate freezer as done by the industry and stored at -20°C. Samples were drawn at regular monthly intervals and the extent of protein denaturation during frozen storage was assessed by both methods, namely,

(1) The salt solubility technique by the Dyer's procedure (loc. cit) and

(2) The modified cell fragility (Love *et al*. 1972) test, to make a comparison.

All reagents used were made from Analar grade chemicals. Cell fragility test was conducted using a Torry-Brown homogeniser (A.G. Brown Electronics, U.K.) and optical density of the homogenate was measured at 420 nm. using a photo electric colourimeter. From each fish duplicate samples were taken each time, for both measurements. In the case of cell fragility to avoid connective tissue in the portion of muscle taken and to get comparable values, several determinations were often necessary. Two represen-

tative values are given and their mean value taken in the tables.

5.3 Results and Discussion

Tables 14, 15, 16 and 17 give the results of this comparative study for mackerel, mullet, kilimeen and prawns respectively.

In the case of the species tested, the two methods were comparable. However, when fatty fishes were used, the emulsions formed in the homogenate in cell fragility test, caused considerable variations in optical density measurements. Love et al. (1972) had realised this and had observed that cell fragility can be recommended for measuring protein denaturation in non fatty species of fishes only. Seasonal changes in fat content of many species may also introduce errors in cell fragility values. Presence of connective tissue in the portion of muscle taken for cell fragility test is another factor that can introduce errors in the value. Toughening of the muscle during frozen storage, is a complex process involving aggregation of the myosin molecules and various other associated changes. These changes are affected by many biological and processing factors in the case of each species. Aggregation of protein resulting from crosslinking of protein chains is affected by many factors like amino acid composition, conformation of the poly-

peptide chains etc. Amino acid composition of contractile proteins vary only marginally from species to species but as Connell (1961) has observed, the rates of aggregation of protein may vary, due to other factors. Differences in the rates of aggregation may affect cell fragility value, as cell disruption is inhibited by aggregation.

pH also affects the texture of fish muscle. Fish connective tissue is very sensitive to pH changes. Weakening of connective tissue releases and weakens the myotomes. Since postmortem changes in pH varies due to various factors, this may also influence their behaviour in cell fragility measurements. All these introduce different variable factors in the cell fragility values. As such, except for its ease and quickness, cell fragility is not found to offer any added advantage over the conventional salt solubility test in measuring protein denaturation in frozen fish. Salt solubility is, in fact, found to be more reliable as a standardised technique which can be done without fear of unusual variations due to small changes in experimental conditions. With its limitations, salt solubility is thus found to be the best available method as yet, for following protein denaturation in frozen fish.

Table 13. COMPARISON OF SALT SOLUBILITY AND CELL FRAGILITY METHODS AS INDICES OF PROTEIN DENATURATION IN FROZEN MACKERELS (RASTRALIGER KANAGURTA)

Storage time in weeks at -20°C	Salt solubility protein content expressed as % of Total Protein			Optical Density in cell Fragility test		
	1	2	Mean	1	2	Mean
0	58.24%	60.32%	59.28%	0.958	0.922	0.940
4	44.10%	43.14%	43.62%	0.699	0.721	0.710
8	32.90%	33.80%	33.35%	0.482	0.456	0.444
12	15.20%	14.56%	14.88%	0.312	0.328	0.320
16	19.10%	17.66%	18.38%	0.337	0.340	0.339

Table 14. COMPARISON OF SALT SOLUBILITY AND CELL FRAGILITY METHODS AS INDICES OF PROTEIN DENATURATION IN FROZEN MULLETS

Storage time in weeks at -20°C	Salt soluble protein content expressed as % of total protein			Optical Density in cell fragility test		
	1	2	Mean	1	2	Mean
0	49.20%	47.46%	48.33%	0.831	0.854	0.843
4	41.42%	43.14%	42.28%	0.547	-	0.547
8	31.29%	29.81%	30.55%	0.658	0.678	0.668
12	34.68%	35.20%	34.94%	0.569	0.585	0.577
16	32.55%	32.55%	32.55%	0.315	0.323	0.319

Table 15. COMPARISON OF SALT SOLUBILITY AND CELL FRAGILITY METHODS AS INDICES OF PROTEIN DENATURATION IN FROZEN KILEMEEN (NEMIPTERUS JAPONICUS)

Storage time in weeks at -20°C	Salt soluble protein content expressed as % of total protein			Optical density in cell fragility test		
	1	2	Mean	1	2	Mean
0	44.20%	43.34%	43.77%	0.770	0.733	0.752
4	28.70%	30.20%	29.45%	0.284	0.292	0.288
8	24.20%	22.10%	23.15%	0.272	0.284	0.278
12	28.06%	30.26%	29.16%	0.220	0.229	0.225
16	29.91%	28.82%	29.16%	0.2072	0.2078	0.2075

Table 16. COMPARISON OF SALT SOLUBILITY AND CELL FRAGILITY METHODS AS INDICES OF PROTEIN DENATURATION IN FROZEN PRAWNS (P. INDICUS)

Storage time in weeks at -20°C	Salt soluble protein content expressed as % of total protein			Optical density in cell fragility test		
	1	2	Mean	1	2	Mean
0	71.40%	74.20%	72.80%	0.979	0.969	0.974
4	67.20%	65.60%	66.40%	0.658	0.678	0.668
8	54.32%	54.32%	54.32%	0.695	0.703	0.699
12	52.86%	53.24%	53.05%	0.643	0.643	0.643
16	49.82%	50.34%	50.13%	0.628	0.628	0.634

6. CHAPTER IV

6.1 Introduction

The results presented in the previous chapter indicate, that, with all its limitations, solubility in 5% salt solution remains the most acceptable method for following the course of protein denaturation in frozen fish. Solubility of muscle proteins in salt solutions is affected by various factors like pH and ionic strength of the extractant, nature of the ions etc. At neutral pH, Dyer's buffer extracts most of the soluble proteins from fishes like cod and also prawns. In the case of tropical fatty fishes complete extraction was,

however, not observed even with this buffer. Reasons for this difference is not clear, as already discussed in chapters I and II. Extraction of muscle proteins is affected by presence of varying amounts of connective tissue in the sample taken for determination of solubility, the homogenisation procedure, centrifugal field applied etc. Even after giving allowance to possible variations in all these experimental factors there remains a clear difference between the proteins of fishes and prawns in the ease of extraction. While around 80% of the proteins could be extracted with Dyer's buffer from fresh prawns the corresponding figures was only 50-70% for fishes, depending on the species. As opined by Dyer (1967), many species of fishes and shell fishes differ greatly from one another and from mammalian and avian species in this respect. Connell (1961) also observed that myosins from different animals differ greatly in stability either *in situ* or after extraction. Connell reported two significant findings in this connection (Connell, 1960b, 1962). He found that actin as well as myosin from cod when extracted separately remained extractable for a long period when frozen cod was stored at -14°C. But extractable actomyosin reduced to very low levels during the same period. This suggested that rather than denaturation by any unfolding of protein chains, a crosslinking or aggregation resulting in reduced solubility

is taking place during frozen storage of fish. Disulphide, amide, or inter chain -c-c bonds through oxidised unsaturated lipids could be implicated in such a crosslinking. The formation of such crosslinks by disulphide or amide linkages may vary from species to species depending upon the amino acid composition and sequence.

As already seen, denaturation of muscle proteins in fatty fishes, as followed by solubility, shows a clearly different picture, tending to emphasise the role of oxidised lipids in causing such crosslinks. Dyer (1967) reported that binding of fatty acids to protein was maximum when the ionic strength of the extractant was 0.5. When ionic strength was increased to 0.8747 (Dyer's buffer) this binding is considerably reduced. Solubility as such is not increased significantly when ionic strength is raised from 0.6 to 0.8747 as seen from table 7. But for following the changes during frozen storage, in the proteins of fatty fish, an extractant with an ionic strength 0.8747 was found more ideal, probably due to the above mentioned effect. Dyer (1967) also observed that in the dissociated condition myosin requires more fatty acids for insolubilisation than does the actomyosin complex. In view of these findings, it was thought desirable to see if incorporation of phosphates, capable of dissociating actomyosin, in the extraction buffer could improve the solubility test as a method

for following protein denaturation.

Dyer (1950) in his original paper, has studied the efficiency of various salts at neutral pH to extract fish muscle proteins. He found sodium chloride, the best and accordingly recommended 5% sodium chloride buffered with sodium bicarbonate at 0.02 M level, as the ideal extractant. He however, has not seen the effect of using various phosphates, many of which has the capacity to dissociate actomyosin, in place of sodium bicarbonate. The effect of incorporating these phosphates in Dyer's buffer in place of sodium bicarbonate was therefore studied in detail using several phosphates. Total ionic strength was kept at 0.8747 in all cases. Concentration of the phosphates were varied, but sodium chloride was suitably adjusted to keep the ionic strength constant. Since ionic strength was kept constant, there was the inevitable variations in pH when different phosphates were used at different levels. Sodium dihydrogen orthophosphate, sodium hexameta phosphate and tetra sodium pyrophosphate were tried in these experiments.

6.2 Materials and Methods

In this set of experiments, buffers containing the different phosphates at different levels were prepared. In all cases Dyer's Buffer (5% sodium chloride in 0.02 M sodium bicarbonate) served as the control. Total ionic

strength of all buffers were adjusted to 0.8747 by varying the concentration of sodium chloride suitably. Ionic strength contributed by the phosphate varied from 0.00 to 0.15, balance ions being contributed by sodium chloride. In the phosphate containing buffers no sodium bicarbonate was used.

10 gm portions of the well minced muscle, from oil sardine as well as prawns, were extracted with 200 ml of the test buffer cooled to 0°C. Extractions in all cases were done by blending in a high speed blendor for 2 minutes with a break of 30 seconds after the first minute. A baffle plate was used to minimise foaming as recommended by Dyer (1950). The blended extract was centrifuged at 5000 r.p.m. at 0°C for 30 minutes in an IEC model high speed refrigerated centrifuge (International Equipment Co. Needham, Mass, U.S.A.). Nitrogen content of the supernate was determined by the microkjeldahl method (A.O.A.C. 1960). Total nitrogen and non protein nitrogen in the muscle and non protein nitrogen in the extracts were estimated as described by Dyer (1950). Extractable protein nitrogen was calculated and expressed as percentage of total protein nitrogen.

6.3 Results and Discussion

Results of the experiments with prawns (*M. affinis*) and oil sardines (*Sardinella longiceps*) are given in tables 18 and 19 respectively. Experiments with each set of

buffers using one particular phosphate were done separately. Solubility in the control samples therefore shows some variations depending on the freshness of the raw material used each time. One set of experiments was always done using the same raw material.

In the case of prawns, as seen from table 18, sodium dihydrogen orthophosphate and tetrasodium pyrophosphate increased the extraction marginally when the ionic strength contributed by these ions was between 0.06 to 0.1. In sardines also, the pattern was the same. But while the increase in extractability was marginal in prawns, it was somewhat more pronounced in the case of oil sardines. This is to be expected since in prawns almost all the soluble protein are extracted by Dyer's buffer itself. In sardines however, tetra sodium pyrophosphate had a distinctly beneficial effect by way of increased extraction. The effect of dihydrogen orthophosphate was nominal in sardines also. Hexameta phosphate decreased the extractability steadily with increasing concentration, in both cases (Tables 18 and 19).

In these experiments, the ionic strength of the buffers was kept constant and there was the inevitable change in pH values. Buffers containing sodium hexametaphosphate had an acidic pH in all cases, pH values varying from 4.8 to

4.95. This is near to the isoelectric point of the major contractile proteins. Therefore the decreased extraction is to be expected. Buffers containing tetra sodium pyrophosphate had pH values between 8.9 and 9.4. This high pH along with the ability of pyrophosphate to dissociate actomyosin explains the increased extraction in these cases. Buffers containing disodium hydrogen orthophosphate, also had an alkaline pH (8.4 - 8.8). But this alkaline pH, alone does not increase the solubility to any significant extent as can be seen from Table 1g. Tetra sodium pyrophosphate of course had a still higher pH. But in that case in addition to the effect of pH, the effect of pyrophosphate on the actomyosin complex is also apparent.

Fukazawa et al. (1951) have also reported similar changes in solubility. Dyer (1950) also found that extraction of muscle proteins was good between pH 7 and 9 whereas near to pH 5 extraction was considerably reduced in presence of 5% sodium chloride. Working with sodium hexametaphosphate Spinelli & Koury (1970) also observed its precipitating action at pH values near 4.

These results thus show that except for tetra sodium pyrophosphate, other phosphate tried do not have any special beneficial effect in increasing protein extractability.

Since extraction at neutral pH is preferable for routine comparative studies and since the increase in extraction at pH 9.4 (tetra sodium pyrophosphate buffer) is only marginal, Dyer's buffer itself is found to be the ideal extractant for following protein denaturation in frozen stored fish.

Table 17. EFFECT OF DIFFERENT PHOSPHATES IN THE EXTRACTION BUFFER, ON THE EXTRACTABILITY OF MUSCLE PROTEINS OF PRAWNS (M. AFFINIS)

Ionic strength of the salts in the buffer		Total ionic strength of the buffer	Extractable protein as % of total protein		
NaCl	Phosphate		Na_2HPO_4	$(NaPO_3)_6$	$Na_4P_2O_7$
0.8547 (Dyer's buffer)	-	0.8747	68.69	74.87	87.94
0.8647	0.01	0.8747	67.47	74.39	88.69
0.8547	0.02	0.8747	67.89	-	88.69
0.8447	0.03	0.8747	68.34	73.35	88.69
0.8347	0.04	0.8747	67.47	66.37	88.69
0.8247	0.05	0.8747	67.89	58.38	89.28
0.8147	0.06	0.8747	70.21	56.43	91.31
0.7947	0.08	0.8747	74.89	54.91	89.91
0.7747	0.10	0.8747	76.89	41.43	89.28
0.7497	0.125	0.8747	76.05	34.43	-
0.7247	0.150	0.8747	68.69	31.95	89.86

Table 18. EFFECT OF PHOSPHATES ON THE EXTRACTABILITY OF MUSCLE PROTEINS OF OIL SARDINES

Ionic strength contributed by sodium chloride	Ionic strength contributed by the phosphate	Extractable protein as % of total protein nitrogen		
		Na_2HPO_4	$(NaPO_3)_6$	$Na_4P_2O_7$
Control Dyer's Buffer 0.8547 + 0.02 $NaHCO_3$	-	39.67	41.74	50.97
0.8647	0.01	39.33	33.64	-
0.8547	0.02	39.67	22.94	52.89
0.8447	0.03	39.20	22.44	53.34
0.8347	0.04	40.07	-	49.89
0.8247	0.05	42.91	20.39	57.97
0.8147	0.06	41.54	19.88	58.48
0.7947	0.08	41.07	18.35	59.40
0.7747	0.10	42.44	-	63.60
0.7497	0.125	39.67	18.87	64.07
0.7247	0.150	36.86	17.34	63.97

7. CHAPTER V

7.1 Introduction

For short term preservation of fish, iceing is the preferred method. Changes in the muscle proteins of fishes and shell fishes during storage in ice are already reported in chapter 1. For long term preservation, quick freezing and storage at -18°C (or below) is the only satisfactory method. The main deteriorative changes during this type of preservation is the toughening of muscle due to denaturation of proteins. Toughening is attributed to denaturation of the myofibrillar proteins. As seen from the results

reported in chapter III, solubility in Dyer's buffer is by far the best criterion available for following this denaturation during frozen storage of fish. Results reported in chapter I support the view expressed by Dyer & Dingle (1961) that residue after extraction of sarcoplasmic protein is not a suitable material for estimating the soluble myofibrillar proteins. Thus for following the protein changes in fish muscle during frozen storage, sarcoplasmic and total salt soluble proteins are to be extracted separately. Studies on the protein changes during iced storage (chapter I) revealed some interesting differences between prawns and different fishes. In this chapter, results of a comparative study on the changes in muscle proteins of prawns and fishes during frozen storage at -18°C are reported. Prawns (P. indicus), oil sardine (Sardinella longiceps) and mackerel (Rastrelliger kanagurta) were used for this study. Changes in the sarcoplasmic proteins, total salt soluble proteins, and stroma proteins of these species during quick freezing and subsequent storage at -18°C are presented.

Dyer (1950) and Sawant & Magar (1961) have reported results on the changes in the protein fractions during frozen storage of fish. Awad et al. (1969) studied the deteriorative changes in frozen stored freshwater white fish. Changes in the protein fractions of the red and white

meats of Japanese sardine and mackerel during frozen storage were reported by Hashimoto et al. (1979). In general, these workers found that sarcoplasmic proteins are not affected to any significant extent during frozen storage. Myofibrillar proteins got rapidly insolubilised during frozen storage. However, as observed by Dyer (1967) different species of fishes and shell fishes may differ greatly from one another in their changes during frozen storage. Hak-Kyoonkim, et al. 1977, observed that Lemon sole fish frozen and stored could be kept for over 17 years in good condition whereas cod could be kept for 15 weeks only in such a good condition.

7.2 Materials and Methods

Fresh prawns (P. indicus), oil sardine (Sardinella longiceps) and mackerel (Rastraliger kanagurta) obtained from the vessels of Integrated Fisheries Project, Cochin. The fishes were kept under ice immediately after capture and brought to the laboratory. The fish were washed well and quick frozen at -40°C. Prawns were frozen as blocks of one pound. The slabs were given water glaze, wrapped in polythene paper and kept in paper cartons and stored at -18°C. Samples were analysed immediately after freezing and thereafter at regular one month intervals for following protein changes.

Sarcoplasmic proteins were extracted from 10 gm portions of well minced muscle by blending with 100 ml potassium chloride-phosphate buffer μ = 0.05 pH 7.2 at 0°C for 2 minutes. The extract was centrifuged at 5000 r.p.m. at 0°C for 30 minutes and nitrogen content in the supernate determined to estimate sarcoplasmic proteins. Salt soluble proteins were determined by the method of Dyer (1950). Stroma was estimated by removal of all soluble proteins from 10 gm muscle of minced muscle by repeated extraction with 0.1 N sodium hydroxide and direct digestion of the insoluble reside with concentrated sulphuric acid. Total nitrogen in the muscle was estimated by the micro kjeldahl procedure (A.O.A.C. 1960). Total non-protein nitrogen was estimated by precipitating all proteins from a water extract of muscle with 10% trichloroacetic acid and estimating the nitrogen in the filtrate. Content of different protein fractions are expressed as percentage of total protein nitrogen.

7.3. Results and Discussion

Changes in the sarcoplasmic, total salt soluble (soluble in Dyer's buffer) and stroma protein fractions of mackerel, oil sardine and prawns (*P. indicus*) during frozen storage at -18°C are given Tables 19, 20 and 21 respectively.

In the case of prawns, during storage at -18°C for a period of 20 weeks, the content of sarcoplasmic proteins did not change to any significant extent. But the salt soluble proteins showed a regular fall insolubility (from 78.62 to 48.68%). The pattern of changes was the same in oil sardine also. But as observed in studies reported in previous chapters, the percentage of salt soluble proteins from oil sardines was much lesser compared to prawns even in the fresh condition. Subsequent changes were more pronounced in prawns. As in the ice storage studies here also mackerel differed in its protein composition and changes during frozen storage. Mackerel had a slightly higher percentage of sarcoplasmic proteins compared to sardines. In this respect it resembled prawns. But unlike prawns and sardines, in mackerel the percentage of soluble sarcoplasmic proteins registered a regular decrease with progressive frozen storage (from 29.86 to 20.42%). The salt soluble protein fractions showed greater decrease in solubility in this case also. However, the sarcoplasmic proteins of mackerel appears to be different from the same fraction of other species in its easy susceptibility to denaturation due to possible reasons already discussed in chapter I. Working with Japanese sardine and mackerel, frozen stored at -80°C. Hashimoto et al. (1979) has observed some other interesting phenomenon. In these cases the sarcoplasmic protein in the red meat

increased during storage at -80°C whereas white meat did not show any such phenomenon. They attributed this unusual behaviour to easy denaturation of myofibrillar proteins like myosin and troponin. These denatured myofibrillar proteins then appear in the sarcoplasmic extract. In the present study, red and white meats were not studied separately. Storage temperature was also different.

Stroma did not register any significant change during storage in any case. The results thus are in general agreement with those reported by other workers for other species of fishes (Sawant & Magar, 1961; Awad et al. 1969; Dyer, 1953, 1956).

Cowie & Mackie (1968) reported that the type of homogeniser used determined the content of soluble protein extracted with Dyer's buffer. Variations in the centrifugal force used for removing the insoluble material also can introduce considerable variations in the content of soluble proteins. In these studies, the conditions used were throughout the same. As such, the changes were reliable. However, the lower content of salt soluble proteins in our fishes compared to values reported for cod etc. may be due to variations in these conditions.

Connell (1960, 1962) have observed that during frozen storage, cod myosin as well as actin got insolubilised

slowly. But the rate of insolubilization was much faster for the actomyosin complex. This suggested that rather than denaturation, some sort of crosslinking or aggregation is happening to the actomyosin complex leading to its reduced extraction, during storage. Lipids or oxidied lipids may be playing a part in bringing about these protein-protein interactions (Desai et al. 1963; Roubal et al. 1966). Such changes may be naturally more pronounced in fatty fishes like oil sardine and mackerel in which cases solubility is lower even in the initial stages, compared to prawns. At the high ionic strength of Dyer's buffer (0.87) actomyosin is dissociated to myosin to a greater extent than at μ = 0.6. Myosin needs greater amount of free fatty acid for initiating protein-protein interactions, than actomyosin. This may be a factor causing the more pronounced (Dyer, 1967) insolubilising effect reported in chapter I when μ = 0.6 buffer was used for extracting myofibrillar proteins.

Table 19. CHANGES IN PROTEINS OF FROZEN MACKERELS DURING STORAGE AT -18°C

Storage time in weeks	Sarcoplasmic protein as % of total protein	Total salt soluble protein as % of total protein (Dyer's buffer)	Stroma as % of total protein
0	29.86	60.42	5.86
4	28.26	56.28	6.08
8	24.38	43.62	5.64
12	22.96	33.53	6.23
16	20.18	28.38	6.23
20	20.42	20.64	5.72

Tables 20. CHANGES IN PROTEINS OF FROZEN OIL SARDINES DURING STORAGE AT -18°C

Storage time in weeks	Sarcoplasmic protein as % of total protein	Total salt soluble protein as % of total protein (Dyer's Buffer)	Stroma as % of Total protein
0	26.28	56.74	5.04
4	25.86	53.48	4.76
8	24.72	55.62	5.22
12	25.34	53.15	5.81
16	23.71	49.42	5.93
20	23.15	44.28	—

Table 21. CHANGES IN PROTEINS IN FROZEN PRAWNS DURING STORAGE AT -18°C (P. INDICUS)

Storage time in weeks	Sarcoplasmic protein as % of total protein	Total salt soluble protein as % of total protein (Dyer's Buffer)	Stroma as % of total protein
0	29.26	78.62	4.70
4	27.38	72.80	4.82
8	28.59	56.40	5.04
12	26.46	54.32	4.91
16	26.86	49.64	4.22
20	25.84	48.68	4.91

8. CHAPTER VI

8.1 Introduction

In this chapter, the changes in the protein fractions of six major species of Indian freshwater fishes, during storage at -18°C as fillets are presented.

Comparatively very little information is available on the proteins of freshwater fishes of India. Baliga et al. (1962 a,b) and Moorjani et al. (1962) made some pioneering studies on the protein fractions of Barbus carnaticus, Barbus dubius, Labeo sp. and Wallago attu. Protein nitrogen distribution in these species showed considerable species

differences, as per their results. Barbus species had a high content of sarcoplasmic proteins (about 35% of total proteins) whereas, Wallago attu had only 22%. Labeo was intermediate with about 29% sarcoplasmic protein. Stroma content was almost the same in all. Species having a higher sarcoplasmic protein had a correspondingly lower content of myofibrillar proteins. The same authors further studied the pre rogor changes in the nitrogen distribution in these fishes during storage in ice. Extraction of the sarcoplasmic proteins with salt solutions of different ionic strengths and pH and the influence of pyrophosphate containing buffers on the extractability were also studied by them. Baliga et al. (1969) later studied the solubility characteristics of muscle proteins of Labeo sp. Cirrhina fulungee, Ophicephalus sp. and Barbus dubius by a serial dilution method. They found that actomyosin precipitated completely at an ionic strength of 0.175, when a high ionic strength extract was diluted. In these species, about 90% of the total protein was soluble in buffers of ionic strength 0.55. Ice storage characteristics of some fresh water fishes have been reported by Nair et al. (1971, 1974). But little has been reported so far on the frozen storage of these fishes with the increase in reservoir fish culture in India in recent years freshwater fish landings have gone up. Freezing of

freshwater fishes is also gradually gaining popularity. But very few studies have been reported on the freezing preservation of Indian freshwater fishes. Shenoy & James (1972) studied the changes in protein solubility during frozen storage of tilapia. They showed that tilapia from freshwater had a better shelf life compared to the same species caught from brackishwaters. Freshwater carps like Labeo rohita, Catla catla, Cirrhina mrigala and Labeo kalbasu and species like Mullago attu and Mystus seenghala are perhaps the most important varieties in our river waters. These fishes grow to a fairly big size weighing 3-5 kg per fish on an average. As such, in retail marketing as frozen products, they will preferably go as fillets. So the protein changes when these fishes are frozen as fillets are more relevant from the practical point of view. So unlike in the marine fishes and shell fishes, these fishes were frozen as fillets to study the changes in their proteins during storage.

8.2 Materials and Methods

Samples of the six species of freshwater fishes were procured from the Machu II reservoir in Morbi, Gujarat and these studies were conducted at the Veraval Centre of Central Institute of Fisheries Technology. The fish, immediately after capture were washed well and packed in insulated boxes with crushed ice and transported to the laboratory. Within

18 hours, the fish were filleted, quick frozen at -40°C and the frozen fillets packed in thick polythene bags were stored in a deep freezer at -18°C. Samples of fillets from all species were analysed before freezing. Immediately after freezing and thereafter at regular monthly intervals. Sample fillets of all species were thawed and analysed to estimate the water soluble and salt soluble protein. Changes in the total nitrogen and non-protein nitrogen and α-amino nitrogen were also followed during frozen storage of these fillets.

Total nitrogen was determined by the method of A.O.A.C. (AOAC 1960). Total non-protein nitrogen was estimated by precipitating all protein from 10 gm of minced muscle with 30% trichloroacetic acid by blending in a high speed blendor and estimating the nitrogen content of the filtrate.

Water soluble nitrogen: (WSN)

10 gm of well minced fish muscle was blended for two minutes in a high speed blendor with 100 ml double distilled water at 0°C. The extract was then centrifuged at 5000 r.p.m. in a high speed refrigerated centrifuge at 0°C for 30 minutes. The nitrogen content of the supernatant was determined by the microkjeldahl method (A.O.A.C. 1960) and percentage of water soluble nitrogen was calculated. Salt soluble nitrogen (SSN) was determined as per the method of Dyer (1950) using

Dyer's buffer. α-amino nitrogen was estimated by the method of Pope & Stevens (1939).

8.3 Results

Proximate composition of the six species are given in Table 22. Changes in the total nitrogen, total non-protein nitrogen, water soluble nitrogen (WSN) salt soluble nitrogen (SSN) and α-amino nitrogen in fillets of the six species of freshwater fishes studied, during frozen storage at -30°C, are given in tables 23-28. TNPN, WSN, SSN and α-amino nitrogen values are expressed as % of total nitrogen. Fillets showed considerable variations in their composition. Representative values are given in Table 29.

8.4 Discussion

All the six species were comparable in their total protein content (about 18-19%). Proximate composition of the species were similar to that reported by Baliga et al. (1962b) for other freshwater fishes. Labeo calbasu and Wallago attu had a slightly higher fat content. But in general all species could be classified as lean. Cirrhena mrigala had a lower fat content.

All species were found to have a high content of non protein nitrogen. Values for the species examined are found to be higher than those reported by Baliga et al. (1962) for Barbus, Labeo and Wallago attu.

Water soluble nitrogen was found to be high in all cases. Baliga has reported lower content of sarcoplasmic proteins for Wallago attu. But in these studies, this species also was found to have a comparably high content of myogens.

In the case of salt soluble nitrogen, consistantly high values were reported by earlier authors. 90-92% of the total proteins were reported to be soluble in many species of freshwater fishes. According to Baliga et al. (1962a) in Wallago attu 79-80% of the protein was soluble in Dyer's buffer. But in these studies 62-73% of the proteins only were found to be soluble. This may be due to differences in the centrifuging speed and to the fact that no baffle plate was used during blending in these experiments. Foaming during blending might have rendered part of the proteins inextractable.

About 28% of the total nitrogen was soluble in water in Labeo rohita (Table 23). During the storage of 24 weeks at -18°C, this part of the protein remained more or less unaffected. But during the same period salt soluble nitrogen decreased from the original 62.28% to about 40%. Non-protein nitrogen showed a clear decreasing trend. α -amino nitrogen also showed a slight decrease. This is to be expected, since during thawing "drip" will carry some of these compounds. This is especially so, when fish is stored as frozen

fillets, as exposed area is more in fillets.

Labeo calbasu has a higher content of water soluble protein (around 30-32% of the total nitrogen)(Table 24). Salt soluble nitrogen was also higher compared to Labeo rohita (73% of the total nitrogen). After storage at -18°C, this value decreased to about 52% whereas the percentage of water soluble protein was not affected significantly. Non protein nitrogen and α -amino nitrogen showed the same trend in this case also.

In Catla catla and Cirrhena mrigala (Tables 25-26) also water soluble nitrogen accounted for about 30% of the total nitrogen. This fraction was unaffected during frozen storage. The e two species had 62% and 67% of the total nitrogen soluble in Dyer's buffer. After 24 weeks at -18°C, these values decreased to nearly 48% and 50% respectively.

Mystus [illegible] had a high content of water soluble nitrogen which appeared to decrease slightly during frozen storage. Only 62% of the total nitrogen of this species was soluble in Dyer's buffer. After 24 weeks at -18°C this came down to around 50%.

Wallago attu had a slightly lower content of water soluble nitrogen (around 29% of the total nitrogen) compared to M. seenghala. Here also WSN decreased slightly during frozen storage. But salt solubility was higher than

M. seenghala for the protein of this species (about 73% of the total nitrogen). After 24 weeks only 53% of the total nitrogen was soluble in Dyer's buffer.

Fillets from different parts of the body of each fish and fillets from different fishes of the same species showed considerable variation. Representative values are given in the tables. Results in general are similar to those reported by Awad et al. (1969) and Moorjani et al. (1960). As in the case of marine fishes, in freshwater fishes also, sarcoplasmic proteins are relatively unaffected during frozen storage. It is the myofibrillar proteins which are affected by denaturation (Sawant & Magar, 1961; Connell, 1962).

Freshwater fishes thus resemble prawns in their high content of sarcoplasmic proteins, and also in the relatively high salt solubility of their proteins. Compared to fatty marine fishes like oil sardine or mackerel, they are lean. The low fat content and thereby low free fatty acid content can be the explanation for this higher solubility as in prawns.

Table 22. PROXIMATE COMPOSITION OF FILLETS OF SIX SPECIES OF FRESHWATER FISHES

Species	Average weight kg	Moisture %	Fat (DMB %)	Protein % (TN x 6.25)	Ash %
Labeo rohita	3.00	79.71	1.879	18.60	1.316
Catla catla	6.50	79.28	1.325	19.60	0.932
Cirrhina mrigala	2.50	79.47	0.808	19.19	1.140
Labeo calbasu	1.65	79.26	2.898	18.72	1.021
Mystus seenghala	2.20	80.83	1.191	18.34	0.9105
Wallago attu	5.00	79.11	1.191	18.34	0.9274

Table 23. CHANGES IN FROZEN LABEO ROHITA FILLETS DURING STORAGE AT -18°C

	Storage time in weeks						
	0	4	8	12	16	20	24
TN% (gm/100 gm muscle)	2.97	2.882	2.786	2.780	-	-	-
TNPN% (gm/100 gm muscle)	0.56	0.595	0.535	0.455	0.435	0.450	0.495
WSN (as % of TN)	28.68	28.72	31.73	27.19	30.86	29.85	30.64
SSN (as % of TN)	62.28	57.99	46.06	41.30	41.97	39.39	40.68
α-amino N_2 (mg/100 gm)	35.00	42.00	46.20	36.40	40.00	28.60	24.20

TN: Total nitrogen gm/100 gm muscle; TNPN: Total Non protein nitrogen

WSN: Water soluble nitrogen; SSN: Salt soluble nitrogen

Table 24. CHANGES IN FROZEN FILLETS OF LABEO CALBASU DURING STORAGE AT -18°C

	Storage time in weeks						
	0	4	8	12	16	20	24
TN %	2.996	3.298	3.088	3.142	-	3.152	3.068
TNPN %	0.56	0.70	0.508	0.49	0.455	0.490	0.455
WSN (% of TN)	31.119	32.50	30.86	25.84	-	26.28	28.64
SSN (% of TN)	72.90	-	57.86	52.64	56.84	53.06	51.76
α-amino N_2 (mg/100gm)	46.50	39.00	34.80	40.40	27.20	37.44	24.86

TN: Total nitrogen; TNPN: Total non protein nitrogen

WSN: Water soluble nitrogen; SSN: Salt soluble nitrogen

Table 25. CHANGES IN FROZEN FILLETS OF CATLA CATLA DURING STORAGE AT -18°C

	Storage time in weeks						
	0	4	8	12	16	20	24
TN %	3.136	2.944	2.918	-	2.773	-	-
TNPN %	0.49	0.455	0.475	0.315	0.330	-	0.360
WSN (as % of TN)	32.14	28.19	28.88	30.73	28.74	27.86	26.98
SSN (as % of TN)	62.82	53.21	52.86	49.54	48.27	53.26	48.27
α-amino N_2 (mg/100 gm)[2]	42.00	42.00	44.80	36.80	38.00	27.20	28.60

TN: Total nitrogen; TNPN: Total non protein nitrogen

WSN: Water soluble nitrogen; SSN: Salt soluble nitrogen

Table 26. CHANGES IN FROZEN FILLETS OF CIRRHINA MRIGALA DURING STORAGE AT -18°C

	Storage time in weeks						
	0	4	8	12	16	20	24
TN %	3.07	2.839	2.966	2.734	2.826	2.684	2.598
TNPN %	0.665	0.560	0.490	0.490	0.518	0.546	0.490
WSN (% of TN)	-	28.42	33.97	30.73	28.72	27.96	29.64
SSN (% of TN)	67.22	62.77	59.75	56.82	58.26	52.74	49.86
α -amino N (mg/100 gm)	38.50	42.00	36.40	30.40	37.60	26.20	32.86

TN: Total nitrogen; TNPN: Total non protein nitrogen

WSN: Water soluble nitrogen; SSN: Salt soluble protein nitrogen

Table 27. CHANGES IN FROZEN FILLETS OF MYSTUS SEENGHALA DURING STORAGE AT -18°C

	Storage time in weeks						
	0	4	8	12	16	20	24
TN %	2.666	3.095	2.598	2.60	-	-	-
TNPN %	0.485	0.455	0.390	0.46	0.46	0.39	0.39
WSN (as % of TN)	31.96	27.14	28.62	27.68	26.92	27.06	26.92
SSN (as % of TN)	62.03	66.28	64.86	56.24	54.46	52.28	49.36
α -amino N_2 (mg/100 gm)	35.00	42.00	42.00	36.40	29.40	30.60	26.28

TN: Total nitrogen; TNPN: Total non protein nitrogen

WSN: Water soluble nitrogen; SSN: Salt soluble protein nitrogen

Table 28. CHANGES IN FROZEN FILLETS OF MALLAGO ALTU DURING STORAGE AT -18°C

	Storage time in weeks						
	0	4	8	12	16	20	24
TN %	2.936	2.722	2.749	2.909	1.824	-	2.623
TNPN %	0.525	0.525	0.490	0.455	0.420	0.490	0.420
WSN (% of TN)	29.04	28.68	27.86	28.02	24.68	22.74	26.46
SSN (% of TN)	73.33	69.86	67.64	65.82	58.76	56.28	52.46
α-amino N_2 (mg/100 gm)	35.50	35.50	39.20	36.00	28.80	27.40	28.80

TN: Total nitrogen; TNPN: Total non protein nitrogen

WSN: Water soluble nitrogen; SSN: Salt soluble nitrogen

9. CHAPTER VII

9.1 Introduction

Denaturation of fish muscle proteins during drying, freezing, heat processing etc is different from each other. Luipjen (1957) observed that denaturation of muscle proteins caused by freezing is basically different from that happening during heat processing. Differences between the mechanism of denaturation during freeze drying and heat treatment were noted by Hamm & Deatherage (1960). But in contrast, many workers have reported the basic similarity between denaturation during freezing and that during salting. They suggested that denaturation during freezing is caused by the

concentration of tissue salts during the freezing process (Love, 1958; Simidu & Hibiki, 1951 and Luipjen, 1957).

Duerr & Dyer (1952) and Fougers (1952) reported that if cod fillets are kept in sodium chloride solutions at 0-20°C, the muscle proteins are denatured when the salt concentration in the muscle reaches 8 - 10%. But Nikkila & Linko (1954b) observed that in the case of Baltic Herring, this threshold value was much lower namely, 1.7%. Similar critical salt concentration values for different species of fishes have been reported by different workers (Simidu & Hibiki (1951) and Simidu & Simidu (1957). All these works further proved that it is the myosin fraction which gets denatured as a result of the action of sodium chloride and that denaturation of myosin by salt is similar to the denaturation of proteins during freezing. Shimizu & Simidu (1953) and Nikkila & Linko (1954b) compared the denaturing effect of different inorganic salts. Linko & Nikkila (1961) in a later study reported that various alkali phosphates and citrates could prevent the denaturation of myosin by salt. They studied this protective effect of phosphates and citrates at various pH values and discussed possible mechanisms of this phenomenon also (Nikkila et al. 1967). Love & Abel (1966) applied the cell fragility technique for following protein denaturation in phosphate treated cod fillets.

Arai et al. (1970) reported that sugars also have a protective effect against denaturation in frozen fish actomyosin. Yasui & Hashimoto (1966) observed that sucrose inhibited the denaturation of proteins in solution during freeze drying. Protective effect of sugars varied from sugar to sugar. Glucose, fructose and sucrose were found to be very effective (Putnam, 1955; Haurowitz, 1963), but the mechanisms of this protective effect is not known. Denaturation of fish muscle proteins by solutions of sodium chloride is an important aspect to be studied in fish preservation, since chilled brine and refrigerated sea water are often used for short term preservation of fish. In view of the findings cited, a systematic study on this aspect of protein denaturation and its possible prevention by phosphate, citrates, sugars etc in tropical fishes and shell fishes was undertaken. Preliminary studies were made on model protein solutions. Proteins were extracted from prawns and sardines with salt solutions of two concentrations namely, 2% and 5% and stored at 4°C to see the extent of insolubilisation during storage in contact with sodium chloride. To aliquots of these extracts, different phosphates, sodium citrate, sodium acetate, glucose etc were added at two levels (namely, 1% and 3%) and stored along with the control samples at 4°C, to study the protective or insolubilising effect of each, on the extracted protein. The protein

solutions were stored for one week at 4°C and at regular intervals the concentration of the protein remaining in solution was estimated. Disodium hydrogen orthophosphate, sodium hexameta phosphate, sodium tripolyphosphate and tetra sodium pyrophosphate were the phosphates used. Sodium acetate, sodium citrate and glucose were also tried to ascertain their effect.

9.2 Experimental

Prawns and sardines used in these experiments were procured in fresh condition from the boats of the Institute. Muscle from these, were minced well. Minced muscle were extracted with 2% sodium chloride (1:10 w/v) at 0°C in a high speed blendor, fitted with a baffle plate for minimising air mixing and foaming. Blending was continued for two minutes (with a break of 30 seconds after the first minute). The extract was centrifuged at 5000 r.p.m. for 30 minutes at 0°C in an IEC model refrigerated centrifuge. Nitrogen content of the supernatant was determined (as mg/ml) by the microkjeldahl method (A.O.A.C. 1960).

To portions of the supernatant, different salts (Disodium hydrogen phosphate, sodium hexameta phosphate, sodium tripolyphosphate, tetra sodium pyrophosphate, sodium citrate, sodium acetate) and also glucose were added at 1% and 3% levels. All salts/glucose were dissolved well and

these extracts were then stored at 4°C along with the control extract with no added salt or glucose in it. Each solution was divided into three equal parts and stored in 3 flasks for convenience. At definite intervals, -1, 3 and 7 days (in some cases 5 or 6 days) - one set of extracts containing each salt at each concentration and one control sample was taken out and centrifuged at 5000 r.p.m. at 0°C for 30 minutes. The nitrogen content of the supernatant in each case was determined by the microkjeldahl procedure.

The same experiment was repeated using all the salts and glucose with a 5% sodium chloride extract of the muscle also (using both prawn and oil sardine) to see the effect of a higher concentration of sodium chloride.

Along with these, another experiment to study the effect of sodium chloride solutions at two concentration, on the proteins of fish fillets dipped in it for several hours was also taken up. For this, fresh oil sardines were filleted and kept immersed in sodium chloride solutions of two concentrations namely, 2% and 5%. 2% sodium chloride solutions containing the sodium phosphates (dihydrogen orthophosphate, hexameta phosphate, tripolyphosphate and tetra pyrophosphate) at 1% level and 5% sodium chloride containing the same phosphates at 3% level were also prepared along with these. All solutions were cooled to 4°C

in plastic bottles. Sardine fillets were kept dipped in these solutions for 3 hours. After that they were taken out and stored in polythene bags, under crushed ice. At intervals of 1 day, 3 days and 5 days, salt solubility of the muscle proteins of these fillets was estimated by the standard method of Dyer (1950).

9.3 Results and Discussion

Results of the experiments are given in tables 29-37.

Results of the experiments on the effect of different phosphates on the proteins of oil sardine and prawns (P. indicus) extracted with 2% sodium chloride are given in table 29 and 30. Tables 31 and 32 give results of similar experiments with proteins extracted with 5% sodium chloride. Effects of sodium citrate, sodium acetate and glucose on the proteins of oil sardine and prawns extracted with 2% sodium chloride are reported in tables 33 and 34. Tables 35 and 36 give the results of similar experiments using proteins extracted with 5% sodium chloride. The effect of dipping sardine fillets in sodium chloride solutions of two concentrations and sodium chloride solutions containing different phosphates, citrates, acetate and glucose at two different levels, on the salt solubility of muscle proteins is shown in table 37.

Results of the experiments on the effect of phosphates,

citrate, acetate and glucose on the proteins extracted by 2% and 5% sodium chloride (Table 28-30) show that the changes follow the same pattern in oil sardine as well as prawns in the case of salt solutions of both concentrations.

Proteins extracted into solution got gradually insolubilised during storage at 4°C in both cases. Added dihydrogen orthophosphate and hexameta phosphate enhanced the rate of insolubilisation. But tripoly and tetra sodium pyrophosphates protected the proteins from getting insolubilised. Oil sardines extracted with 2% sodium chloride had an initial protein concentration of 0.3248 mg/ml. After 7 days storage at 4°C, this came down to 0.2241 mg/ml. Dihydrogen phosphate when added at 1% level reduced the solubility further to 0.1580 mg/ml in the same period. At 3% level, it brought down the protein concentration to 0.1120 mg/ml. Corresponding figures for hexameta phosphate were 0.1960 mg/ml and 0.1792 mg/ml respectively. Pyrophosphate and sodium tripolyphosphates on the other hand inhibited the insolubilisation of proteins in solution. When soluble protein nitrogen in the control sample (2% sodium chloride) decreased from 0.3248 mg/ml to 0.2241 mg/ml in seven days, the solutions with added tripoly and tetra sodium pyrophosphate could hold 0.2856 mg/ml nitrogen in solution. But increasing the concentration from 1% to 3% did not give any added protective effect. The trend of the results was

the same in the case of proteins extracted with 5% sodium chloride also, as is clear from the figures in the concerned tables (Tables 29-32).

Acetates clearly promoted insolubilisation. In this case also increasing the concentration from 1% to 3% had little effect. Citrate and glucose did not offer any noticeable protection against insolubilization in the concentration used in this work in the case of prawns as well as sardines (Tables 33-36).

Results of the study, on the effect of these phosphates when sardine fillets were kept immersed in salt solutions containing these phosphates, also followed the same pattern (Table 37). Sodium chloride at 2% concentration itself is found to denature proteins of sardine fillets as measured by salt solubility. After 3 hours dipping in 2% sodium chloride, salt solubility decreased from the initial 65.96% to 57.63%. When 5% sodium chloride was used solubility decreased further to 49.02% of the total protein nitrogen.

When fillets were further stored in ice, solubility predictaby decreased. Solubility showed regular fall in the case of samples of both salt concentrations. Sodium dihydrogen orthophosphate and hexameta phosphate increased the denaturation at both concentrations whereas tripoly and

pyrophosphate could effectively prevent denaturation by sodium chloride. When concentration of sodium chloride was raised to 5%, denaturation also showed a corresponding increase as measured by decrease in salt solubility. For a comparable protection against denaturation concentration of pyro and tripolyphosphates had to be increased to 3%.

The pH of the solutions were not adjusted in these studies. In the case of proteins extracted and stored at 4°C, the addition of different phosphates, acetates, citrates etc changed the pH considerably which may account partially for the observed changes in solubility. Hexameta phosphate when added to 2% sodium chloride extract at 1% and 3% level gave pH values 5.10 and 5.02 respectively. Values were in the same range in the case of 5% sodium chloride extracts also. Dihydrogen orthophosphate changed the pH to 8.94 - 8.96 under the same conditions. Corresponding figures for tripoly and pyrophosphate samples were 9.72 and 9.74. Citrate solutions had pH values in the range 8.5 - 8.6. Acetate solutions had pH 8.33 and 8.61 at 2% and 3% concentrations in 2% sodium chloride. Glucose did not change the pH of sodium chloride significantly. At 1% level pH was 6.86 and at 3% level it was 6.44. The pronounced acidic pH coming near to the isoelectric point of contractle proteins, explains the severe insolubilization observed in the case of hexameta phosphate solutions.

Spinelli & Kouri (1970) have found the same effect in their studies on the protein precipitating efficiency of condensed phosphates.

Polyphosphates are generally known to increase the water holding capacity of proteins. The mechanism of their action is not yet established beyond controversy. Phosphate may be taken up by the protein molecule thereby increasing the polar groups in the protein. Condensed phosphates may bind to actomyosin increasing its negative charge, thus leading to its dissociation and increased solubility (Bendall, 1954). Hamm & Grau (1958) suggested that phosphates may remove Ca^{++} and Zn^{++} ions from proteins resulting in an increase in the polar groups. But Inklaar (1967) proved that phosphates do not complex with Ca^{++} and Mg^{++} ions in meat. Moreover EDTA (Ethaylene Diamine tetra acetic acid disodium salt) which can remove Ca^{++} and Mg^{++} ions do not possess a denaturation-inhibiting effect (Nikkila & Linko, 1961). So this theory does not seem to be correct. Love & Abel (1966) have suggested that tripolyphosphates interact with proteins to produce a surface film on the treated fillets. This film, according to them prevents drip formation in frozen fillets and increases the water holding capacity.

Table 29. EFFECT OF DIFFERENT PHOSPHATES ON THE SOLUBILITY OF MUSCLE PROTEINS OF OIL SARDINES, EXTRACTED WITH 2% NaCl AND STORED AT 4°C

Initial nitrogen content of the extract = 0.3248 mg/ml

No. of days of storage at 4°C	Nitrogen content of the extracts expressed as mg/ml								
	Control No phosphate	Disodium hydrogen orthophosphate		Sodium hexameta phosphate		Tetra sodium pyrophosphate		Sodium tripolyphosphate	
		1%	3%	1%	3%	1%	3%	1%	3%
1	0.2296	0.1624	0.1456	0.1680	0.1636	0.2745	0.2856	0.2687	0.2860
3	0.2241	0.1512	0.1344	0.1680	0.1568	0.2576	0.2856	0.2664	0.2820
7	0.2241	0.1380	0.1120	0.1660	0.1192	0.2545	0.2856	0.2576	0.2856

Table 30. EFFECT OF DIFFERENT PHOSPHATES ON THE SOLUBILITY OF MUSCLE PROTEINS OF PRAWNS (P. INDICUS) EXTRACTED WITH 2% NaCl AND STORED AT 4°C

Initial nitrogen content of the extract = 0.906 mg/ml

No. of days of storage at 4°C	Nitrogen content of the extract expressed as mg/ml								
	Control No phosphate	Disodium hydrogen orthophosphate		Sodium hexameta phosphate		Tetra sodium pyrophosphate		Sodium tripolyphosphate	
		1%	3%	1%	3%	1%	3%	1%	3%
1	0.8664	0.5028	0.4826	0.5286	0.5168	0.8756	0.8826	0.8756	0.8784
3	0.8628	0.4684	0.4564	0.5286	0.5168	0.8628	0.8628	0.8248	0.8626
6	0.8583	0.4286	0.3978	0.5028	0.4826	0.8612	0.8656	0.8648	0.8648

Table 31. EFFECT OF DIFFERENT PHOSPHATES ON THE SOLUBILITY OF MUSCLE PROTEINS OF OIL SARDINES EXTRACTED WITH 5% NaCl AND STORED AT 4°C

Initial nitrogen content of the extract = 0.3634 mg/ml

No. of days of storage at 4°C	Nitrogen content of the extracts expressed as mg/ml								
	Control (No phosphate)	Disodium hydrogen orthophosphate		Sodium hexameta phosphate		Tetra sodium phyrophosphate		Sodium tripolyphosphate	
		1%	3%	1%	3%	1%	3%	1%	3%
1	0.3248	0.1848	0.0784	0.1960	0.1400	0.3360	0.3528	0.3360	0.3305
3	0.3080	0.1288	0.1176	0.1568	0.1288	-	0.3305	0.3193	0.3348
5	0.3080	0.1368	0.0840	-	0.1264	0.3080	0.3305	0.3248	0.3305

Table 32. EFFECT OF DIFFERENT PHOSPHATES ON THE SOLUBILITY OF MUSCLE PROTEINS OF PRAWNS (P. INDICUS) EXTRACTED WITH 5% NaCl AND STORED AT 4°C

Initial nitrogen content = 1.148 mg/ml

No. of days of storage at 4°C	Nitrogen content of the extract expressed as mg/ml								
	Control	Disodium hydrogen orthophosphate		Sodium hexameta phosphate		Tetra sodium pyrophosphate		Sodium tripolyphosphate	
		1%	3%	1%	3%	1%	3%	1%	3%
1	1.126	0.8068	0.6824	0.8246	0.7956	1.160	1.188	1.126	1.138
3	1.132	0.7658	0.6832	0.7856	0.5642	1.126	1.126	1.138	1.138
6	1.064	0.7128	0.5624	0.6436	-	1.084	1.132	1.168	-

Table 33. EFFECT OF ADDED ACETATE, CITRATE AND GLUCOSE ON THE SOLUBILITY OF MUSCLE PROTEINS OF OIL SARDINES EXTRACTED WITH 2% NaCl AND STORED AT 4°C

Initial nitrogen content: 0.4256 mg/ml

Days of storage at 4°C	Nitrogen content as mg/ml solutions						
	Control	1% sodium acetate	3% acetate	1% citrate	3% citrate	1% glucose	3% glucose
1	0.3753	0.2968	0.3080	0.3360	0.3416	0.3360	0.3360
3	0.3416	0.2912	0.3080	0.3416	0.3360	0.3360	0.3360
5	0.3416	0.3080	0.3136	0.3360	0.3380	0.3248	0.3472
7	0.3360	0.2800	0.2800	0.3248	0.3248	0.3192	0.3192

Table 34. EFFECT OF ADDED ACETATE, CITRATE AND GLUCOSE ON THE SOLUBILITY OF MUSCLE PROTEINS OF PRAWNS (P. DOBSONI) EXTRACTED WITH 2% NaCl AND STORED AT 4°C

Initial nitrogen content: 0.868 mg/ml

Days of storage at 4°C	Nitrogen content as mg/ml						
	Control	1% sodium acetate	3% sodium acetate	1% citrate	3% citrate	1% Glucose	3% glucose
1	0.8482	0.7682	0.7286	0.8064	0.8216	0.8216	0.8168
3	0.8268	-	0.6886	0.8316	0.7926	0.8264	0.8168
7	0.7886	0.6632	0.6754	0.7926	0.8268	-	0.8064

Table 35. EFFECT OF ADDED ACETATE, CITRATE AND GLUCOSE ON THE SOLUBILITY OF MUSCLE PROTEINS OF OIL SARDINES EXTRACTED WITH 5% NaCl AND STORED AT 4°C

Initial nitrogen content 0.4984 mg/ml

Days of storage at 4°C	Nitrogen content as mg/ml solution						
	Control	1% sodium acetate	3% acetate	1% sodium citrate	3% citrate	1% Glucose	3% Glucose
1	0.4984	0.4536	0.4480	0.4760	0.4760	0.4704	0.4760
3	0.4816	0.4200	0.4224	0.4704	0.4760	0.4760	0.4704
5	0.4200	0.3696	0.3808	0.4088	0.4144	0.4256	0.4256
7	0.4088	0.3640	0.3752	0.4144	0.4032	0.4144	0.4200

Table 36. EFFECT OF ADDED ACETATE, CITRATE AND GLUCOSE ON THE SOLUBILITY OF MUSCLE PROTEINS OF PRAWNS (M. DOBSONI) EXTRACTED WITH 5% Nacl AND STORED AT 4°C

Initial nitrogen content: 1.126 mg/ml

No. of days storage at 4°C	Nitrogen content as mg/ml solution						
	Control	1% sodium acetate	3% acetate	1% citrate	3% citrate	1% Glucose	3% Glucose
1	1.126	0.9864	0.8986	1.086	0.9968	-	0.9864
3	0.9864	-	0.8684	0.9628	0.9628	0.9864	-
7	0.8286	-	0.8064	0.7926	0.8864	0.8864	-

Table 37. EFFECT OF DIPPING SARDINE FILLETS IN SOLUTIONS OF SODIUM CHLORIDE (2% AND 5%) AND SODIUM CHLORIDE CONTAINING DIFFERENT PHOSPHATES, AT 4°C FOR 3 HOURS, AND STORING THE DIPPED FISH IN ICE, ON THE SOLUBILITY OF MUSCLE PROTEINS

INITIAL SOLUBILITY IN DYER'S BUFFER = 65.96% OF TOTAL PROTEIN

Dipping solution	Solubility of muscle proteins as % of total protein		
	1 day in ice	3 days in ice	5 days in ice
2% Sodium chloride	57.63	55.88	41.45
2% Sodium chloride + 1% orthophosphate	52.80	49.71	38.13
2% Sodium chloride + 1% hexametaphosphate	-	50.84	-
2% Sodium chloride + 1% tetra sodium pyrophosphate	62.80	62.64	47.28
2% Sodium chloride + 1% tripolyphosphate	64.57	59.81	46.86
5% Sodium chloride	49.02	38.96	33.00
5% Sodium chloride + 3% orthophosphate	47.10	36.65	25.01
5% Sodium chloride + 3% hexametaphosphate	38.96	31.35	31.44
5% Sodium chloride + 3% tetra sodium pyrophosphate	55.12	49.02	39.41
5% Sodium chloride + 3% tripolyphosphate	55.59	46.65	38.64

SUMMARY

This thesis is an attempt to make a comparative study of the composition of the muscle proteins of some commercially important species of fishes and shell fishes of our coast and their changes during preservation and processing.

As a part of this, the distribution of the major protein nitrogen fractions in several species of fishes (oil sardine, mackerel and lactarius) and shell fishes (different species of prawns) was studied in detail. The changes in these fractions during storage of fish/prawn

in ice were also followed. When extracted without blending, sarcoplasmic proteins accounting for 25-30% of the total proteins, could be easily extracted from all fishes and shell fishes. Prawns and mackerels had a slightly higher content of sarcoplasmic proteins compared to oil sardines and lactarius. From the residue after extraction of sarcoplasmic proteins a fairly good percentage of the myofibrillar proteins could be extracted in the case of shell fishes. But in fishes, this extraction was inhibited to a considerable extent. Extraction of sarcoplasmic proteins appeared to modify the myofibrillar proteins in some way, rendering them inextractable. Sarcoplasmic proteins were not affected to any great extent when fish/prawns were stored in ice. But the extractable myofibrillar proteins showed a steady decrease resulting in a corresponding increase in alkali soluble proteins extracted from the residue after extraction of myofibrillar proteins. Stroma remained unaffected throughout, in all cases. In the case of mackerel, the sarcoplasmic fraction was higher than other fishes. Unlike in the case of other fishes this fraction in mackerels showed a rapid decrease during storage in ice presumably due to the presence of some easily denatured minor myofibrillar protein components getting extracted alongwith the sarcoplasmic proteins, as reported in the case of squid and octopus by other workers.

The decreased extraction of myofibrillar proteins in fishes could be due to appreciable amounts of free fatty acids present in the muscle of fishes, as different from prawns. So a detailed study on the effect of free fatty acids on protein solubility was undertaken, using different fishes and shell fishes. Commonly occuring C_{18} unsaturated fatty acids (Oleic and linoleic) were externally added to the extractant (5% sodium chloride containing 0.02 M sodium bicarbonate) at different levels, during extraction, to see their effect on protein solubility. In the case of fishes addition of fatty acids reduced the extractability, the reduction increasing with the amount of added fatty acids. Fatty acids probably get deposited on protein micelles providing a more hydrophobic surface, thereby reducing the solubility. But, in prawns, the insolubilising effect was apparent only after a critical level of free fatty acid concentration. This might be due to the extremely low content of free fatty acids in prawn muscle, whereas fish muscle generally have this minimum free fatty acids in the muscle. This difference was apparent in the total extractable proteins from fishes and shell fishes also. While about 85% of the total protein can be extracted with 5% sodium chloride in 0.02 M sodium bicarbonate, from fresh prawn muscle, 55-70% only is extractable from fish muscle, even in fresh condition. The insolubilising

effect of free fatty acids questions the reliability of solubility as a criterion of protein denaturation.

A study was therefore taken up to compare the salt solubility and cell fragility techniques as methods for measuring protein denaturation in frozen fish, using prawns and several species of fishes. The two methods were comparable. But cell fragility did not offer any special advantage over salt solubility. Moreover, in fatty fishes, cell fragility also had problems as a result of emulsions formed during blending and consequent cloudiness of the extracts. Solubility was found to be a more convenient, though more time consuming, method showing minimum variations due to experimental conditions.

Solubility with all its limitations, was thus found to be the best criterion for protein denaturation. Solubility is affected by the ionic strength of the extractants, its pH and nature of the ions. Dyer's buffer (Ionic strength 0.8747) was found to be the best extractant for muscle proteins. But in the case of fatty fishes Dyer's buffer does not extract all soluble proteins. Fatty acid binding to proteins is minimum at high ionic strength, since actomyosin is dissociated at high ionic strength. This may be the explanation for the high extraction in the case of Dyer's buffer. Condensed phosphates are also known to

dissociate actomyosin. So a study was taken up to see if extraction can be improved by substituting sodium bicarbonate with some phosphates in Dyer's buffer. Sodium dihydrogen phosphate, hexameta phosphate and tetra sodium pyrophosphate were tried for this purpose. The ionic strength was kept constant at 0.8747 by adjusting the concentration of sodium chloride in all cases, varying the concentration of each phosphate from 0.00 to 0.15. Using these buffers, solubility of proteins was measured in prawns as well as sardines. In the case of prawns, since Dyer's buffer itself extracts most of the protein, enhancement of solubility was not significant in any case. However, in the case of sardines, tetra sodium pyrophosphate showed a significant enhancement of solubility. Hexameta phosphate decreased solubility in all cases. Dihydrogen orthophosphate caused a marginal increase in solubility. The increase by these phosphates may be partly due to the alkaline pH of the buffer when ionic strength is kept constant. Hexameta phosphate alone brought the pH near to the isoelectric point of the major contractile proteins (pH near 5) which explains its insolubilising effect.

Changes in the muscle protein fractions of oil sardine, mackerel and prawns during quick freezing and subsequent storage at -18°C were also followed by direct extraction

with appropriate buffers. With progressive storage, total salt soluble proteins registered a regular fall. But sarcoplasmic proteins remained relatively unaffected except in mackerel. As in the case of iced storage, the myofibrillar proteins appear to be affected more, during frozen storage. In the case of mackerel, some minor myofibrillar protein component appeared to get extracted into the sarcoplasmic extract, in these studies also.

Six species of freshwater fishes namely, Labeo rohita, Catla catla, Cirrhina mrigala, Labeo calbasu, Wallago attu and Mystus seenghala were also quick frozen as fillets and the changes in their protein fractions during frozen storage at -18°C were followed. Freshwater fishes were found to have a high content of water soluble proteins. Compared to marine fishes the percentage of salt soluble proteins were also high, probably due to their low fat content. In these respects they resembled proteins of prawns. Water soluble proteins in these cases also, remained relatively unaffected whereas salt soluble proteins showed regular decrease with progressive storage. Changes in the α-amino nitrogen in these fillets during frozen storage, were also followed.

The effect of phosphates, sodium citrate, sodium acetate and sugars like glucose etc on the solubility of proteins was also studied. For this, proteins extracted

by sodium chloride solutions of two strengths namely, 2% and 5% were used. In these extracts, different phosphates (sodium hydrogen phosphate, hexameta phosphate, tripoly phosphate and tetra sodium pyrophosphate) sodium citrate, sodium acetate and glucose were added at 1% and 3% levels and the protein solutions were stored at 4°C. Acetates, orthophosphate and hexameta phosphate enhanced insolubilisation of the protein considerably whereas citrates and glucose did not have any significant effect. Sodium tripoly and tetra sodium pyrophosphate could prevent insolubilisation to a considerable extent. The effect was the same at both concentrations of sodium chloride and in the case of prawns as well as sardines.

Denaturing effect of salt on fish muscle proteins and its possible prevention by alkali phosphates was also studied in this connection. Sardine fillets were dipped in sodium chloride solutions of two concentrations namely, 2% and 5% for three hours. Alongwith these, sodium chloride solutions containing sodium dihydrogen orthophosphate, hexameta phosphate, tripolyphosphate and tetra sodium pyrophosphate at 1% level (in the case of 2% sodium chloride) and at 3% level (in the case of 5% sodium chloride) were also prepared. Sardine fillets dipped in all these solutions for three hours were stored separately in polythene

bags under crushed ice. Sodium chloride at 2% concentration itself was found to cause considerably denaturation of proteins as measured by salt solubility. Sodium dihydrogen orthophosphate and hexameta phosphates further enhanced this denaturation, whereas tripoly and tetra sodium pyrophosphate could prevent denaturation considerably. In these cases also the pH of the different phosphate solutions might have played a deciding role. Other possible mechanisms are also discussed.

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