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Studies on the protein changes in skeletal muscles of Gulf Coast finfish species associated with frozen storage, formaldehyde addition and freeze-thawing cycles

Nisperos-Carriedo, Myrna O., Ph.D.

The Louisiana State University and Agricultural and Mechanical Col., 1987
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UMI
STUDIES ON THE PROTEIN CHANGES IN SKELETAL MUSCLES OF GULF COAST FINFISH SPECIES ASSOCIATED WITH FROZEN STORAGE, FORMALDEHYDE ADDITION AND FREEZE-THAWING CYCLES

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Department of Food Science

by

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Lastly, the author wishes to thank God for "in Him, all things are possible."
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ABSTRACT

The effects of frozen storage, formaldehyde addition and freeze-thawing cycles were determined using three finfish species from the Gulf of Mexico, spotted seatrout (Cynoscion nebulosus), black drum (Pogonias cromis) and mullet (Mugil cephalus). Frozen storage of spotted seatrout fillets at -20°C for 6 weeks led to reductions in pH, water-binding capacity and solubility of salt-extractable proteins. SDS-Polyacrylamide gel electrophoretic examination of the salt-extractable proteins showed that as time of storage increased, aggregation of high-molecular weight components occurred. The addition of 300 ppm formaldehyde prior to frozen storage did not produce significant changes. However, muscles containing 500 ppm formaldehyde showed decreases in pH, water-binding capacity, solubility of salt-extractable proteins and extensive diminution of high-molecular weight proteins on electrophoretic gels after 6 weeks of storage.

Frozen storage of black drum fillets at -20°C for 6 weeks led to a loss in solubility of salt-extractable proteins, decrease in water-binding capacity and increase in expressible moisture. Electrophoretic gel patterns of salt-extractable proteins showed complete insolubilization of high-molecular weight proteins. Protein extractions using 4% SDS and 5% NaCl in 0.02 M NaHCO₃ (pH 7.2) with or
without 5% 2-mercaptoethanol revealed that protein aggregation during frozen storage was due the formation of hydrogen, hydrophobic and disulfide bonds. The reduction of proteins in SDS solutions suggests that intermolecular covalent crosslinks could also be formed.

Black drum and mullet muscles with 3 freeze-thaw cycles (done by freezing at -20°C for 24 hrs and thawing in a refrigerator at 4-5°C overnight) did not show significant changes in protein properties. Continuing to 5 freeze-thaw cycles in black drum resulted in an increase in expressible moisture and extensive reductions in salt-extractable protein, water-binding capacity and protein components on electrophoretic gels occurred. Differential protein extraction revealed that hydrophobic and disulfide bonds were involved during protein denaturation with increased freeze-thaw cycles.

Elution profiles of salt-extractable proteins from spotted seatrout by high-performance gel permeation chromatography showed that low-molecular weight degradation products are formed during frozen storage. The solubility of these proteins during frozen storage decreased as formaldehyde concentration increased. No significant changes were observed in mullet with 0, 1 and 3 freeze-thaw cycles.
CHAPTER 1
INTRODUCTION

Fish is an important part of the human diet. In the world as a whole, fish represent a major source of animal protein. It is an excellent source of protein and compares favorably with meat and poultry in nutritional value. It has been estimated that the world needs an extra 20 million metric tons of protein per annum (Bligh, 1981). World population continues to increase, so the search for protein must continue and expand. In view of this current situation, experts agree that the development of non-conventional fishery products, together with the utilization of non-traditional or underexploited species would offer immediate potential for increasing the world’s production of protein.

The development of products and processes from fish is not without problems. One major problem is the extreme perishability of seafoods compared with other sources of proteins. Fish is generally considered to be more prone to bacterial and biochemical spoilage than meat products and accordingly, quality suffers during processing and distribution. Fish fats are generally sensitive to oxidation and are associated with the production of rancidity and other off-flavors that are difficult to remove from fish. Fish proteins are less stable than those of mammals or vegetables. They tend to lose their important functional properties during freezing and drying.
Fish research revolves around the basic, systematic understanding of properties and behavior of fish for use as food and for more efficient production of fish products. Numerous studies center on understanding the cause and ultimate prevention of deteriorative reactions in fish during frozen storage. These reactions result in a variety of undesirable changes such as deterioration in texture and loss in functionality. These properties are very important in that they are closely related to commercial use. Research in these areas requires a thorough understanding of muscle and protein structures and of protein reactions (Connell, 1982). Although it seems apparent that conformational changes play a crucial role in deteriorative processes, the reasons for their occurrence as well as their exact nature and effect on seafood products require further elucidation.

In this regard, studies were conducted to determine the effects of frozen storage, formaldehyde addition and freeze-thaw cycles on protein properties of spotted seatrout, black drum and mullet from the Gulf of Mexico. These investigations were designed to develop needed information on protein stability and potential adverse biochemical changes in the flesh of these important finfish species.
CHAPTER 2
REVIEW OF LITERATURE

2.1 STRUCTURE AND COMPOSITION OF FISH MUSCLE

2.1.1 General Chemical Composition

The skeletal muscle of fish is made up of bundles of muscle fibers embedded in connective tissues (Matsumoto, 1980). A muscle fiber is composed of bundles of striated myofibrils surrounded by sarcoplasmic reticulum, mitochondria and other organelles. The myofibrils are the long thin contractile elements that give the characteristic striated pattern of the muscles (Bechtel, 1986). The unique structure of the striated myofibrils, composed of thin (actin)-filaments and thick (myosin)-filaments, is common to all striated muscles of vertebrates including fish (Love, 1970).

There are two major classifications of fish muscle, red and white (Spinelli and Dassow, 1982). Red muscle is distinguished, not only by its higher myoglobin content (1 to 3%), but also by its characteristic sarcoplasmic proteins (Hamoir and Konusu, 1965). A higher content of succinic dehydrogenase and respiratory enzymes has also been found in red muscles from fish (Fukuda, 1958; Lawrie, 1953). Fatty acid oxidation and lecithinase activity are greater in the red muscles than in white muscle of trout (Bilinski, 1963). The red meat fraction varies from a low
of 1-2% in lean fish such as whiting, pollack, cod and flounder to a high of 10% or more in fat species such as salmonids. Although it is the light muscles of the fish that are primarily used as foods, red muscle is very important to the physiology and biochemistry of fish (Buttkus and Tomlinson, 1966). The compounds present in red muscles may contribute to postmortem spoilage problems.

The main constituents of fish flesh are water, protein and fat, but while the relative amounts of these components are generally within the range found for mammals, the nature and properties of both fats and proteins are markedly different (Mackie, 1983). The approximate composition of fish muscle is: 15-24% protein; 0.1-22% lipid; 1-3% carbohydrate; 0.8-2% inorganic matter and 66-84% water (Forrest et al., 1975; Jaquot, 1961). In comparison, muscle from livestock species is: 16-22% protein; 1.5-13% lipid; 0.5-13% carbohydrate; 1% or less inorganic matter and 65-80% water (Forrest et al., 1975).

Fish lipids, in general, are highly unsaturated phospholipids, occurring largely as cell membrane constituents. Much of the lipid in fatty species, like herring and mackerel, is triglyceride and tends to occur in depots. This explains why some regions of fillets are much more fatty than others. It is well known that these fats are more highly unsaturated than those of meat and poultry, therefore, they are more susceptible to oxidative rancidity, which lead to rancid flavors and odors (Bligh,
These lipids are believed to interact with proteins to produce a loss of functional properties during frozen storage (Shenouda, 1980; Sikorski et al., 1976; Connell, 1968). Fish proteins are of high nutritional value and are essentially equivalent to those from other flesh foods such as beef and poultry (Howgate, 1977; 1979). Fish muscle, however, has a lower iron content than most meat.

Trimethylamine oxide is a natural constituent of all marine fish, but it is absent in freshwater species. The bacterial production of trimethylamine from this substance, even at temperatures as low as 0°C, is the principal cause of marine fishy odors. This breakdown can be minimized by proper handling and care of the fish, prompt cooling and maintenance of storage at ice temperatures.

2.1.2 Component Proteins of Fish Muscle

Vertebrate muscles contain similar types of proteins, although some differences exist in their relative amounts (Table 2-1) and in the properties of each protein.

Proteins in fish are classified into three groups based on solubility (Spinelli and Dassow, 1982; Hamoir, 1955). These are: sarcoplasmic proteins, extractable at low ionic strength ( <0.15 ); myofibrillar proteins, extracted by salt solutions of high ionic strength ( >0.5); and stroma proteins, the residual group that is not extracted by salt solutions or dilute alkaline or acid solutions.
Table 2-1. Protein composition of muscles.

<table>
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<th>Sarcoplasmic proteins</th>
<th>Myofibrillar proteins</th>
<th>Stroma proteins</th>
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<tr>
<td></td>
<td>(% of total proteins)</td>
<td>(% of total proteins)</td>
<td>(% of total proteins)</td>
</tr>
<tr>
<td>Beef</td>
<td>17</td>
<td>68</td>
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<tr>
<td>Rabbit</td>
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Sarcoplasmic proteins include many kinds of soluble proteins called myogen (Suzuki, 1981), which can be obtained by pressing fish meat, or by extracting with low ionic strength salt solutions. This group of proteins is composed of many soluble proteins (mainly enzymes) of the sarcoplasmic fluid, other globular proteins of organelles and proteins attached on the sarcolemma. The content of sarcoplasmic proteins in fish meat varies with fish species, but is generally higher in pelagic fish such as sardine and mackerel and lower in demersal fish like plaice and snapper (Shimizu et al., 1976; Suzuki, 1976).

Myofibrillar protein is the protein that forms myofibrils, which contain myosin, actin and regulatory proteins such as tropomyosin, troponin and actinin. This protein group encompasses 66-77% of the total protein in
fish meat, and plays an important role in coagulation and gel formation when fresh meat is processed. Fish meat contains a larger percentage of myofibrillar proteins than does mammalian muscle (Suzuki, 1981).

Stroma is the protein that forms connective tissue. The stroma protein component is made up of either collagen, elastin or both. Connectin has also been found to exist in the elastic fibers of the muscle cell (Suzuki, 1981). The amount of stroma protein is less in fish muscles (3-5%) than in beef or rabbit muscles (Matsumoto, 1980). Tenderness of fish products is generally related to collagen content, while toughness and water-holding capacity are associated with myofibrillar proteins (Fennema, 1973).

2.1.3 Rigor Mortis

The contractile system of the muscle is made up of fibrils with diameters of about 1 micron. Electron microscopic examination reveals the existence of a finer structure consisting of two types of filaments arranged in a hexagonal array (Eskin et al., 1971). The thick filaments are molecules of myosin, arranged head to tail. The thin filaments consist of helical molecules of another protein, actin. Myosin accounts for about 35% of muscle protein and has the important feature of being an ATPase. Contraction and relaxation of striated muscle appear to be related to the interaction between actin, myosin and adenosine triphosphate (ATP). The ATPase activity of
myosin, in the presence of magnesium and calcium ions, catalyzes the splitting of ATP. Most of the energy liberated from the decomposition of ATP is used as energy for the generation of work. In living tissue, ATP is quickly regenerated using the energy of the main fuel system of the body, aerobic oxidation of sugar. The myosin and actin filaments slide passively past each other since the presence of the ATP-Mg\(^{+2}\) complex plasticises the muscle by preventing reaction between these proteins (Dunajski, 1979). Mention should be made of creatine, which serves as a reservoir of high energy phosphate necessary for the regeneration of ATP. Since fish muscle is composed of two types, red and white muscle, rigor contraction has been shown to differ with the particular muscle under investigation. The proportion of these two muscles varies between species, although red muscle never exceeds 10% of the total muscle for any one species, e.g., tuna. Red muscle is characterized by a high myoglobin content as well as other characteristic proteins (Hamoir and Konusu, 1965). The rigor contraction of red muscle was shown to be considerably greater than that for white muscle and corresponds more closely with that reported for mammalian muscle (Buttkus, 1963).

When a fish dies, the transport of oxygen to its muscle stops and hence, aerobic oxidation of sugar ceases (Berk, 1976). ATP can no longer be regenerated and the muscle becomes gradually depleted of ATP. For sometime
after death, the ATP level is maintained in the muscle by an active creatine kinase. The adenosine diphosphate (ADP) formed as a result of ATPase activity is rephosphorylated to ATP while free creatine is produced (Lawrie, 1966; Newbold, 1966). During early postmortem, the concentration of ATP remains more or less constant, whereas that of creatine phosphate falls rapidly. Mammalian muscle is capable of maintaining its ATP level for as long as several hours postmortem, while fish skeletal muscle generally exhibits a rapid decline in ATP concentration (Tomlinson and Geiger, 1962). ATP is particularly important since it forms a cushion between the actin and myosin filaments, keeping the muscle soft and pliable. As the concentration of ATP falls, actin and myosin gradually associate to form inextensible actomyosin, resulting in the rigid and stiff condition referred to as rigor mortis. Actin and myosin undergo important changes closely linked with the development of rigor mortis. For instance, in the pre-rigor stages, actin and myosin are dissociated, myosin being extractable in solutions of high ionic strengths. Fish actin and myosin are also dissociated during the pre-rigor phase, but are far more labile, associating together at the slightest injury (Partmann, 1963). This has tended to render the isolation of pure fish myosin an extremely difficult operation. Another important change in the flesh is a marked drop in pH after death, from nearly neutral to acidic (pH 5.6-5.8). This increase in acidity is due to
the fact that, with the absence of oxygen, only anaerobic
glycolysis can occur, the end product of which is lactic
acid. In meat, it is certainly advantageous to attain as
low a pH as possible, since in addition to retarding the
growth of spoilage bacteria, it helps to impart a more
desirable color to the meat. This differs with frozen
fish since a high pH is desirable to prevent toughness
(Eskin et al., 1971). In general, most fish exhibit a
higher postmortem pH than do warm-blooded animals, attaining a value of around 6.2-6.6 even in full rigor. When
struggling has occurred, the glycogen store in fish is
deprecated considerably. Consequently, the pH at rigor is
higher, around 7.0, giving rise to a condition referred to
as "alkaline rigor". This condition has been found to
develop in cod as well as in a number of other species
(Fraser et al., 1961).

The length of time which elapses between the death of
the animal or fish and the onset of rigor mortis is
ultimately determined by the relative activities of the
enzymic systems involved in the synthesis and breakdown of
ATP. This, in turn, is controlled by the levels of
creatine phosphate, ATP and glycogen within the muscle
tissue at the moment of death. Any subjection of the
animal or fish to starvation or struggling results inevitantly in a much shorter delay period, producing an
inferior product.
2.2 CHANGES IN FISH PROTEIN DURING FROZEN STORAGE

Fish muscle tissues stored in the frozen state progressively decline in quality due to deteriorative changes. These changes are considered to be due to severe alterations of the muscle proteins, usually referred to as denaturation or denaturation-aggregation (Acton et al., 1983, Noguchi, 1982, Andou et al., 1980; Shenouda, 1980; Dyer, 1951). The term "protein denaturation" describes a complex phenomenon involving alterations of the secondary and tertiary structure of protein. Structural changes come about due to a disruption of the forces that contribute to the stability of the native protein conformation without the rupture of covalent linkages between carbon atoms in the polypeptide chains (Kinsella and Melachouris, 1976; Sikorski et al., 1976). Denaturation can be induced by various physical treatments, depending upon their intensity and duration, i.e., heating, freezing, radiation, extreme dilution, exposure to air-water or oil-water interfaces. Investigations of deteriorative changes that take place in such complex mixtures of proteins must consider the solutes that are originally present in the tissues. These solutes can induce aggregation that results in loss of solubility and alterations in the technological properties of the product.

Early investigations on cod and other fish showed that the overall decrease in protein extractability during
frozen storage was due to alterations in the myofibrillar proteins (Dyer et al, 1951, 1956a, 1956b). No significant change was observed for sarcoplasmic proteins. In view of this finding, most studies that followed focused mainly on myofibrillar proteins, particularly on the myosin-actomyosin system (Matsumoto, 1980; Sikorski et al., 1976).

2.2.1 Myosin

Myosin is the principal protein in the thick filament. Rabbit myosin is a long thin molecule with a molecular weight of 480,000 (Tonomura, 1972). It is composed of two heavy chains and four light chains as demonstrated by SDS-Polyacrylamide Gel electrophoresis. On tryptic digestion, myosin is split into subunits, H-meromyosin (HMM) and L-meromyosin (LMM) (Matsumoto, 1980). HMM is further split into S-1 and S-2 subunits. While LMM is a rod of about 90% -helical content, the -helical content for HMM, S-1 and S-2 fragments is 46%, 33% and 87%, respectively. The ATPase activity is localized in the S-1 subunit (Love, 1962; Love and Mackay, 1962). Rabbit myosin is easily obtained from skeletal muscle by short-period extraction with a Guba-Straub solution (0.3 M KCl - 0.15 M Phosphate buffer, pH 6.5) (Suzuki, 1981). With fish myosin, however, extraction presents a problem because actin combines immediately with the myosin forming the actomyosin complex, thus hindering the isolation of myosin. Generally speaking, the main component in salt-soluble protein of
fish is actomyosin, even when obtained from a 1-3 minute extraction (Suzuki et al., 1969; Suzuki and Kanna, 1966; Migita and Matsumoto, 1957). Earlier studies on myosin extraction indicated that the extraction periods varied from 6 to 30 minutes depending on fish species (Takashi et al., 1970; Chung et al., 1967). Normally, it is difficult to obtain highly purified myosin from fish meat because denaturation occurs easily and forms irreversible aggregates (Suzuki, 1981). A procedure using DEAE-Sephadex Gel Chromatography was successfully employed for the isolation of myosin from tuna, striped bass and skipjack (Chung et al., 1967; Richards et al., 1967) as well as tilapia and carp (Takashi, 1973).

Fish myosin and rabbit myosin have been found to exhibit similar molecular weights, sedimentation coefficient and amino acid composition. However, striking differences in the ATPase activity, stability, solubility and viscosity has been found between these myosins (Connell, 1964, 1963, 1958a, 1958b, 1954; Dyer and Dingle, 1961). Connell (1961) showed that myosins of several fish species are less resistant to treatment with urea or guanidine-HCl than myosin of rabbit, beef and chicken, as determined by ultracentrifugal analyses, specific rotation, free -SH groups and ATPase activity. He also noted that fish myosins were more readily digested by trypsin than those of warm blooded animals.
Because of the difficulty of isolating pure myosin from fish, studies on the behavior of this protein during frozen storage have been delayed (Matsumoto, 1980). Connell (1959) was the first to conduct such studies. Solutions of cod myosin in 0.6 M KCl were frozen and stored at different temperatures ranging from -70 to -78°C and the thawed solutions were examined by ultracentrifugal analysis. The progressive aggregation of myosin monomers to dimers, trimers and other larger polymers was demonstrated. Neither the specific rotation nor the number of -SH groups changed appreciably during polymerization. Aggregation was ascribed to bonding of an unknown nature rather than to disulfide bonding. He suggested that myosin molecules aggregated side-to-side without unfolding or undergoing any change in intramolecular conformation. He also found that the amount of aggregated myosin in myosin preparations from frozen, stored cod increased with time of storage (Connell, 1962). Changes in the solubility, ultracentrifugal behavior, number of -SH groups and electron microscopic profiles in frozen myosin were also noted by Buttkus (1970, 1971).

Fish myosin is unstable compared to that of rabbit (Connell, 1961; 1960). When extracted fish was stored, aggregation occurred faster than in rabbit myosin. The degree of aggregation also differed greatly depending on the species of fish. The speed of aggregation of fish
myosin was found to be: haddock > flounder > sole > halibut > herring (Connell, 1960).

2.2.2 **Actomyosin**

In muscle, actomyosin exists in the form of actin and myosin (Suzuki, 1981). Actin is the main constituent of the thin filament. Rabbit muscle G-actin is globular with a molecular weight of $4.2 \times 10^4$ (Matsumoto, 1980). In the presence of salts, it is polymerized into F-actin (Ebashi and Nonomura, 1973). The principal properties of fish actin, including amino acid composition, are similar to those of rabbit actin. However, fish actin is more readily extracted from wet muscle by salt solutions as a viscous solution of actomyosin (Connel, 1958, 1954; Roth, 1948).

At high salt concentrations, (e.g. 0.6 M KCl), actin and myosin combine to form actomyosin filaments giving a highly viscous solution (Matsumoto, 1980). Actomyosin retains the ATPase activity of myosin, and demonstrates "super-precipitation" with the addition of ATP (Ebashi and Nonomura, 1973). Differences exist between actomyosin of rabbit and fish with respect to solubility, viscosity and ultracentrifugal behavior (Suzuki et al., 1964; Dyer and Dingle, 1961; Migita and Suzuki, 1959; Connell, 1958, 1954). Fish muscle actomyosin ATPase activity was lost more readily on heat treatment than those of mammals (Arai et al., 1973; Takashi, 1973). Since actomyosin is the most readily available form of myofibrillar proteins from fish
muscle, its behavior relative to deterioration during frozen storage has been most frequently studied.

The change in amount of soluble actomyosin is regarded as the primary criterion of freeze denaturation (Matsumoto, 1980). Studies on actomyosin have revealed that actomyosin forms various aggregated states during frozen storage (Jarenback and Liljemark, 1975a, 1975b; Oguni et al., 1975; Buttkus, 1971; Buttkus, 1970; Noguchi and Matsumoto, 1970). Electron microscopic analyses of an isolated preparation of fish actomyosin denatured by frozen storage showed that actomyosin filaments with arrowhead structures aggregated side-to-side and crosswise when thawed immediately after freezing (Ohnishi et al., 1978a, 1978b; Tsuchiya et al., 1975). This aggregation led to formation of network structures with increased storage. In addition to aggregation, dissociation of F-actomyosin also occurred (Matsumoto, 1980). It appeared that the dissociated F-actin, as thin filaments, became entangled and aggregated and that the dissociated monomers folded into a globular form. At advanced stages of denaturation, large masses with diffuse outlines were frequently found, suggesting complex aggregation of actin and myosin (Matsumoto, 1980). This hypothetical mechanism is given in Fig. 2-1.

The data reported concerning the properties of myosin and actomyosin indicate that fish proteins are fragile in structure and highly susceptible to denaturing factors including storage at low temperature.
2.3 FACTORS CAUSING FISH PROTEIN DENATURATION DURING FROZEN STORAGE

The tertiary native structure of protein is maintained by several different forces, namely, electrostatic and hydrophobic interactions, hydrogen-bonding and van der Waals forces (Wynn, 1973). Among these, hydrophobic interactions are regarded as being of primary importance. However, during frozen storage, the strength of these interactions is weakened. The lowering of temperature during freezing therefore produces structural changes in those protein molecules that are dependent on hydrophobic interactions for maintenance of native structure (Taborsky, 1979; Oakenfull and Fenwick, 1977).

For fish proteins, the simple relationship between hydrophobic interactions and temperature can not account
for all of the structural protein changes that are observed at low temperatures. Many hypotheses have been advanced to explain the structural changes of fish protein during frozen storage. These include: 1. the effects of inorganic salts concentrated into the liquid phase of the frozen system; 2. water-activity relations; 3. reaction with lipids; 4. reactions with formaldehyde derived from trimethylamine oxide; 5. surface effects at the solid-gas interface; and 6. effects of other water-soluble proteins (Matsumoto, 1980; Sikorski et al., 1976; Fennema, 1973; Love, 1966; Dyer and Dingle, 1961). Among these hypotheses, the effects of inorganic salts, lipids and formaldehyde appear to be most important as shown in Fig. 2-2.

2.3.1 Effect of Inorganic Salts

Suzuki (1981) and Linkko and Nikkila (1961) reported that freeze denaturation can be caused by increase in salt concentration following removal of water by ice formation. Model system studies of myosin preparations show that, at high ionic strength, myosin molecules exhibit a rapid, reversible monomer-dimer equilibrium (Godfray and Harrington, 1970). With an extended period of exposure to a concentrated solution, myosin molecules dissociate into subunits: a heavy core and light components (Dreizen and Gershman, 1970). Furthermore, with prolonged salt treatment, the light chains undergo irreversible aggregation and the heavy chain core also forms insoluble aggregates, accompanied by conformational changes.
Sikorski (1978) explained that at low temperatures, the effect on proteins of inorganic salts present in the tissues may be based on the capacity of different ions for
hydration, leading to alterations in the equilibrium of various associations in the bulk water. In solutions of comparatively low concentrations, corresponding to ionic strengths of 0.5 to 1.0, many salts have a solubilizing effect on proteins by rupturing existing ionic linkages and creating hydration of the newly formed associations. Increasing the concentration of the salt may lead to salting-out by reducing the number of hydrophilic protein groups associated with water molecules. Furthermore, a proportional increase in the surface tension value caused by the salts favors the formation of new intra- and intermolecular linkages. The inorganic ions can exhibit specific effects by forming cross-linking bridges between adjacent peptide chains. They may also influence the activity of muscle enzymes, participate in formation of lipid-protein complexes, and promote lipid oxidation.

2.3.2 Effect of Lipids

The accumulation of unesterified fatty acids and oxidation products during frozen storage can denature proteins (Castell, 1971). The amount of free fatty acids, derived from enzymatic and non-enzymatic hydrolysis of lipids, particularly the phospholipids, has been found to increase with prolonged storage time at elevated frozen temperatures (Dyer and Dingle, 1961). The free fatty acids are believed to associate primarily with the myofibrillar proteins (Shenouda, 1980). Their binding to the sarcoplas-
mic proteins is not excluded, but apparently this occurrence is less effective in making them insoluble. Actomyosin was considered by many researchers to be the primary proteinaceous reactant and was reported to be inextractable in the presence of free fatty acids. The effect of free fatty acids in the myofibrillar structure, as revealed by electron microscopy, showed that low levels of free fatty acids induced aggregation in the extracted proteins, but the fibrils retained much of their original shape (Jarenback and Liljemark, 1975b). With higher levels of free fatty acids, however, micelles were found adhering to the actomyosin filaments. When these filaments were extracted with a high ionic-strength buffer, most of the thick filaments disappeared from the residual myofibrils. The extracted proteins showed fewer actomyosin filaments, but were still rich in myosin, which indicated that myosin extraction was not severely affected during the treatment. Sikorski and co-workers (1976) postulated that the free fatty acids attach themselves hydrophobically or hydrophilically to an appropriate site on protein surfaces. Consequently, they may create more hydrophobic regions in place of polar or charged groups and surround the protein surface with a more hydrophobic microenvironment. Thus, the end result is a decrease in protein solubility in aqueous buffers, or further intermolecular linkages extensive enough to decrease extractability.
Polymerization and aggregation of proteins, leading to loss of solubility, can also be brought about by oxidized lipids. It has been demonstrated that during frozen storage, the products of lipid oxidation cause the fish protein to become harder and more elastic, forming insoluble complexes (Takama, 1974a; Takama et al., 1972). It is believed that the mechanism of the reaction involves unstable free radical intermediates of lipid peroxidation that can abstract hydrogen from labile side-chain groups such as SH-. Polymerization follows, which could result in water-soluble polymers where lipid is not incorporated, or in water-insoluble polymers that are formed by cross-linking of proteins through an additional reaction involving lipid peroxy radicals (Shenouda, 1980). The free radicals could initiate various reactions such as cross-linking with other proteins or lipids, forming protein-protein and protein-lipid aggregates (Fig. 2-3) (Sikorski, 1978). Kuusi and co-workers (1975) and Buttkus (1967) reported that the functional groups that are susceptible to attack by oxidized lipid products are: cysteine -SH, the -amino groups of lysine or the N-terminal amino groups of aspartic acid, tyrosine, methionine and arginine.

The effect of fish lipids on protein denaturation during frozen storage varies according to the state of the lipid (Andou et al., 1981a, 1981b; Shenouda, 1980). The conceptualization of the role of intact lipid on fish protein stability has been vague. There is some evidence
Fig. 2-3. Potential reactions of proteins with lipid radicals and their oxidation products (Sikorski, 1978).

that their presence, particularly in biological systems, is essential and plays a protective role for the proteins involved (Andou et al., 1979; Taguchi and Ikeda, 1968a, 1968b; Dyer and Dingle, 1961; Dyer, 1951).

2.3.3 Effect of Formaldehyde

Another factor that can bring about denaturation of fish muscle proteins is formaldehyde. Several species of fish, especially those of the Gadoid and elasmobranch families, as well as squid and clams, contain an enzymic system for the breakdown of trimethylamine oxide (TMAO) to dimethylamine (DMA) and formaldehyde (FA) (Regenstein et al., 1982; Crawford et al., 1979; Dingle and Hines, 1975; Mackie and Thomson, 1974; Babbit et al., 1972; Castell et
al., 1971; Tokunaga, 1970; Yamada and Amano, 1968). The DMA and FA are produced in equimolar amounts at temperatures below freezing (Amano and Yamada, 1965). This reaction may be affected by storage temperature, physical condition of the fish and inclusion of red muscles or viscera in the fish (Gill et al., 1979; Dingle et al., 1977; Castell et al., 1971). Formaldehyde is a very reactive compound, capable of interacting with protein groups thereby inducing intra- and inter-methylene bridges of the molecule, as shown in Fig. 2-4 (Gill et al., 1979; Kostuch and Sikorski, 1977; Sikorski et al., 1976; Castell et al., 1973; Castell, 1971; Tokunaga, 1964; Walker, 1964; Frankel-Conrat and Olcott, 1948a). These molecules are

![Diagram](image-url)
believed to be covalent in nature, but evidence presented by Connell (1975) showed that the proteins could cross-link through non-covalent binding. Subsequent work by other investigators has presented evidence that FA can cause polymerization of proteins as well as react with small molecular compounds in muscle tissue (Owusu-Ansah and Hultin, 1984; Ohnishi and Rodger, 1979).

2.3.4 Nature of Aggregation and Crosslinking

There is considerable experimental evidence for the existence of different types of intermolecular cross-links in myofibrillar proteins of fish. Connell (1959, 1960) argued against a role for disulfide bond formation in the intermolecular aggregation of myosin since he did not detect any significant change in the number of free -SH groups in the macerate of frozen stored cod. The addition of 1% SDS solubilized almost all of the myofibrillar proteins of cod flesh stored for up to 29 weeks at -14°C. Therefore, Connell (1965) attributed the crosslinkages in severely toughened cod flesh to the formation of non-covalent bonds. In contrast, involvement of -SH groups in the process of denaturation of trout and rabbit myosins during frozen storage has been emphasized by Buttkus (1970, 1971). He successfully resolubilized aggregated myosin by using a solution containing both 6 M guanidine-HCl and either 0.5 M mercaptoethanol, 0.3 M sodium sulfite or 0.3 M sodium cyanide. From these results, he attributed the
crosslinkages involved in aggregation of myosin during frozen storage to disulfide bonds, hydrophobic interactions and hydrogen bonds. It is possible that free $-\text{SH}$ groups might be oxidized first to disulfide bonds. However, only a small decrease was found in the number of free $-\text{SH}$ groups during frozen storage. Therefore, the changes appear to be a result of rearrangement of disulfide bonds from intramolecular to intermolecular through a sulfhydryl disulfide interchange reaction. Buttkus (1974) also raised the concept of concurrent action of different denaturing factors, stating that "a molecular alteration brought about by the formation of disulfide bonds followed by a rearrangement of hydrophobic and hydrogen bonded regions on intra- and intermolecular basis during denaturation and aggregation could not be reversed by $-\text{S-S-}$ bond reducing agents alone, but would have to include conditions which also break intermolecular and hydrogen bonds." The overall result of the concurrent action of different factors depends on the contribution of purely conformational changes and chemical interactions leading to cross-linking at different levels of organization of the proteins and muscle structures.

Tsuchiya and co-workers (1980) utilized different extraction solutions in order to test for the different types of bonds in crosslinked muscle protein. It was concluded that denaturation and/or insolubilization of actomyosin and myosin during frozen storage was a result of
aggregation caused by a progressive increase in inter-molecular linkages. Apparently, hydrogen bonds, ionic bonds (electrostatic), non-polar (hydrophobic) bonds and disulfide (S-S) bonds were responsible for the aggregates.

2.4 CHANGES IN FUNCTIONAL CHARACTERISTICS OF FISH FLESH DURING FROZEN STORAGE

2.4.1 Quality Changes in Fish Muscles During Frozen Storage

The effects of denaturation on protein functionality are significant in actual and potential food applications, and hence should be studied. During frozen storage, functional properties such as emulsifying capacity, lipid-binding capacity, water-holding capacity and gel forming ability are much lower than in fresh fish (Suzuki, 1981). These quality changes are attributed to denaturation of protein, especially myofibrillar protein.

Studies by Connell (1960a, 1960b) compared the solubilities of actomyosin, actin and myosin from cod fillets stored at -14°C. Results indicated no change in the amount of extracted actin regardless of the storage period. However, the solubilities of actomyosin and myosin decreased in accordance to the length of storage. This decrease in salt-extractable protein with increasing frozen storage has been reported for other fish species, such as blue whiting stored at -15°C (Afolabi et al., 1982), rose fish stored at -12°C (Dyer et al., 1956), red hake stored at -7°C (Owusu-Ansah and Hultin, 1986), haddock stored at
-15°C (Laird and Mackie, 1981), spotted sand bass stored at
-10°C (Young and Tableros, 1981) and rainbow trout stored
at -20°C (Borderias et al., 1982). The change in the
amount of soluble actomyosin is regarded as the primary
criterion of freeze denaturation. It must be noted,
however, that solubility data do not indicate precisely how
much protein is denatured or how much is native, rather it
provides a relative measure of denaturation (Matsumoto,
1980).

The viscosity of isolated actomyosin or salt-soluble
protein from stored fish meat decreases with increased
storage (Oguni et al., 1975; Noguchi and Matsumoto, 1970;
Ueda et al., 1962). This suggests that the actomyosin
filaments have become less rod-like or less filamentous,
either by folding of individual molecules or by aggregation
of the filaments (Matsumoto, 1980).

Prolonged storage, especially at temperatures around
-18°C, brings about significant deterioration in the
texture of frozen fish described as having increased
toughness, chewiness, rubberiness or stringiness (Connell,
1964). In investigations by Bremner (1977), sensory
properties of frozen minced fish after steaming were
defined by members of their taste panel as cardboardy,
chewy, crumbly, dry, fibrous and spongy. According to
Connell (1962), development of toughness and loss of water-
holding capacity were caused by formation of additional
linkages and by greater strength of existing linkages
between myofibrillar proteins. The deterioration of proteins during frozen storage is also reflected in a drastic decrease in their gel-forming ability (Acton et al., 1983; Suzuki, 1981; Sikorski, 1979), fat emulsifying capacity (Grabowska and Sikorski, 1976), a reduction in water-holding capacity (Laird and Mackie, 1981) and formation of low molecular weight products (Shenouda, 1980; Gill et al., 1979; Frankel-Conrat and Olcot, 1948a, 1948b).

Dyer and Morton (1956) studied the relationship between solubilities of myofibrillar proteins and sensory qualities of meats from frozen cod and halibut. No change was observed in solubility of sarcoplasmic proteins, but the amount of extracted actomyosin decreased with storage periods, which also correlated with lower sensory scores. Another experiment with three different storage temperatures, -12, -18 and -23°C, showed that sensory evaluation scores and speed of denaturation of actomyosin were related to storage temperature. The quality of fish meat stored at -23°C was highest. Comparable results were obtained for plaice and rosefish (Dyer et al., 1956).

Luijpen (1957) and Cowie and Little (1966) in their work on cod reported that toughness, as determined by sensory evaluation, did not correlate with the degree of decreased solubility of myofibrillar proteins. Bremner (1977) reported that in spiny flathead and ocean perch, a slight softening occurred during storage, although protein extractability dropped markedly.
The rate of texture change varies markedly between fish species. Connell (1975) reported that hake, Alaskan pollack and cod were less stable and flat fish as a group were more stable in this regard than the generality of fish that live in temperate waters. The muscle proteins of frozen mackerel were found to be most unstable, followed by amberfish, mullet and carp by Jiang and Lee (1985).

Other factors that influence degree of damage to fish protein during freezing include season, nutritional and spawning status, storage temperature, storage time and conditions of freezing and thawing (Fennema, 1982; Suzuki, 1981; Love, 1962).

2.4.2 Methods Used for Measuring Protein Denaturation during Frozen Storage

The most popular tests used to study changes that occur in fish proteins during frozen storage are related to the loss in solubility or loss in extractability of total proteins, or of particular groups of proteins (myofibrillar, sarcoplasmic or the actomyosin group), or even of specific protein, such as myosin, tropomyosin or actin (Shenouda, 1980). Owusu-Ansah and Hultin (1986) determined the solubility of salt-extractable proteins in 4% SDS with and without 2-mercaptoethanol and in 0.02 M NaHCO₃ (pH 7.2) containing 5% NaCl with and without 2-mercaptoethanol. This differential extraction procedure can help identify some of the bonds involved in protein insolubilization during frozen storage.
The extracting conditions usually employed in these tests are not standardized. For example, the extracting solution may vary in the type of salts used, ionic strengths, concentration of divalent cations, incorporation of detergents such as sodium dodecyl sulfate (SDS), and pH or buffering capacity. Variations also exist in the ratio of muscle to solution, and in the duration and speed of blending. All of these variables are rarely duplicated in the literature, in spite of their importance in determining the type and degree of solubility of various proteins.

Measurement of the value of drip-thaw and determining the change in water-holding capacities of muscles are among the simple tests used to reflect decreases in the capacity of fish muscle proteins to reabsorb water of melted ice crystals during thawing. This decrease is attributed to surface dehydration of protein or, to a lesser extent, to physical damage in the cells or cell membranes. In addition to sensory evaluations, which are the major determinations of quality, objective textural measurements are now recognized as powerful tools in studying changes in rheological properties of tissues during frozen storage.

Visual examination of the structural arrangement of fish muscles under a light microscope, a transmission electron microscope (TEM), or a scanning electron microscope (SEM) has proven useful in detecting disturbances or damage to the macro- or microstructures of tissues or cells during frozen storage.
Biochemical parameters such as changes in enzymatic activity of fish muscles, or in the susceptibility of fish proteins to various proteolytic enzymes, are sensitive indicators for monitoring protein denaturation as a consequence of conformational changes that may occur during frozen storage. Several endogenous enzymes exhibit a correlation between freeze damage and storage time, among them adenosine triphosphatase (ATPase), aldolase, malic enzyme and glycerophosphate dehydrogenase (Matsumoto, 1980; Gould and Peters, 1971; Connell, 1960a).

Changes in the physical and chemical properties of extracted fish proteins give more extensive information on changes that have occurred at the molecular level during frozen storage. Simple tests, such as those that determine emulsifying capacity, viscosity, gel-forming properties show the general condition of proteins. Other tests monitor changes in the more susceptible functional groups of proteins, such as free sulfhydryl groups, reactable SH groups and available -amino groups on the lysine residue. These tests can reveal the existence of protein cross-linking, or allow prediction of deformation and explain aggregation phenomena.

The mobility of protein groups subjected to external forces, as indicated by ultracentrifuge sedimentation, isoelectric focusing, or electrophoretic patterns, would reflect changes that occur in the shape, size, charge and weight (aggregation or dissociation) of protein molecules.
Electrophoresis is a general term that describes separation of charged molecules through a porous matrix when an electric field is applied (Wasserman, 1985). Commonly used matrices are polyacrylamide and agarose gels. Electrophoresis in polyacrylamide gels in the presence of the anionic detergent, SDS, has proven to be a useful tool for separation of protein subunits and determination of their molecular weights (Sigma, 1986). This method has undoubtedly become the most rapid and sensitive method for determining protein composition, monitoring protein purification and for assessing modifications in protein structure, e.g., those due to cross-linking or proteolysis.

Another method used to monitor the mobility and fractionation of fish proteins under an external force is by chromatographic separation. Gel filtration chromatography is an established method for determining the size and molecular weight of proteins (Sigma, 1986). Fractionation is based on differential diffusion of various molecules into the gel pores. Proteins having high molecular weights do not enter the gel pores, but pass through the fluid volume of a column of porous gel particles faster than those having low molecular weights. The protein molecules are eluted from the column in order of decreasing molecular weight. This method provides a relatively high resolving capacity, but typically requires hours or even days because of the inability of the gel matrix to withstand the high pressures required for increased flow rates (Montelaro et
al., 1981). With the recent development of rigid hydrophobic bonded resins as macroporous inorganic supports, the rapidity, sensitivity and high-resolution properties of high-performance liquid chromatography (HPLC) are now available for gel permeation chromatography of proteins (HPGPC). In fact, during the past several years, numerous reports have described HPLC procedures for separation of complex protein mixtures, isolation of enzymes and antigens, and determination of protein molecular weights (Regnier and Gooding, 1980; Gruber et al., 1979; Ui, 1979; Mathes and Engelhardt, 1977). High-Performance Gel Permeation Chromatography is increasingly being used for the separation of proteins in the lower molecular weight range (Rivier, 1980). By chemical modification of the silica gels, a higher mechanical stability and excellent recovery has been achieved (Regnier and Gooding, 1980; Engelhardt and Mathes, 1979).
CHAPTER 3
PROTEIN CHANGES IN SPOTTED SEATROUT MUSCLES
AS INFLUENCED BY FROZEN STORAGE AND
FORMALDEHYDE ADDITION

3.1 INTRODUCTION

Freezing is considered to be one of the best methods for fish preservation. It is being employed extensively on shore and on board fishing vessels to prevent deterioration caused by microbial action, putrefaction and autolysis. Freezing, therefore, provides a satisfactory means of preserving fish from the hygienic point of view.

However, after prolonged storage, even at temperatures as low as -20°C, undesirable textural changes take place in fish. In frozen and thawed fish, there is an increased loss of water with a corresponding softening of the tissue. When frozen and thawed fish is cooked, the succulence and water-holding capacity is decreased and texture becomes progressively drier, firmer, tougher and more rubbery. Compared with unfrozen fresh fish, functional properties such as emulsifying capacity, lipid-binding properties, water-holding capacity and gelation properties are decreased in frozen fish.

There is a wealth of information available concerning the aforementioned deteriorative changes during frozen storage. Most of the studies suggest that these changes are due to severe alterations in muscle proteins. This
alteration, usually termed denaturation or denaturation-aggregation, occurs in the myofibrillar proteins, particularly the myosin-actomyosin system.

Several factors influence this denaturation including denaturation caused by an increase in salt concentration or change in pH after removal of water by ice formation (Shenouda, 1980). Interaction with lipids can denature proteins due to their surfactant effects, while lipid oxidation products can change protein conformation by modifying side groups or by inducing cross-linking by a free radical process (Karel et al., 1975). Gadoid fish contain an enzymic system for the breakdown of trimethylamine oxide to dimethylamine and formaldehyde (Crawford et al., 1979; Dingle et al., 1977; Yamada et al., 1969; Amano and Yamada, 1964). Earlier studies indicated that formaldehyde cross-links the proteins in the muscle tissue, forming a three-dimensional network that causes textural toughness (Gill et al., 1979; Castell et al., 1973). Formaldehyde can cause polymerization of some proteins (Owusu-Ansah and Hultin, 1984; Ohnishi and Rodger, 1979) and can also react with the small molecular components of the muscle tissue (Banda and Hultin, 1983).

The following study was designed to determine the potential adverse effects of frozen storage on protein properties of spotted seatrout muscles. A model experiment involving the addition of formaldehyde was also conducted to determine its involvement in protein changes, compared
with frozen storage.
3.2 MATERIALS AND METHODS

3.2.1 MATERIALS

Spotted seatrout (*Cynoscion nebulosus*) fillets were purchased from a local store and transported to the Food Science Department laboratory on ice. The fillets were randomly divided into two lots, one for the frozen storage study and the other for the combined effects of formaldehyde addition and frozen storage. The fillets for the frozen storage study were washed, vacuum packaged (150 g each) in polyethylene bags and frozen in an air blast freezer at -20°C. During sampling and testing, three bags of fish were thawed by holding in a refrigerator at 4-6°C overnight prior to analyses. Determinations for the different properties were conducted at 0, 3 and 6 weeks.

For the formaldehyde study, samples of spotted seatrout were treated with formaldehyde (FA) at 0, 300 and 500 ppm concentrations. Seventy ml of chilled formaldehyde in the form of reagent grade formalin (37-38%) was added to 70 g fish. The mixture was blended for 1 min using a Galaxie osterizer. Samples were vacuum packaged (150 g each) in polyethylene bags and frozen in an air blast freezer maintained at -20°C. During sampling and testing, three bags of frozen fish were thawed by holding in a refrigerator overnight prior to analyses. Determinations for the different muscle properties were conducted at 0, 3 and 6 weeks.
3.2.2 METHODS

pH

The pH was determined by homogenizing 10 g of muscle with 20 ml of distilled water using a Microprocessor Ionalyzer (Orion Research, Model 901) calibrated with buffers at 3.2 and 7.0.

Viscosity

The procedure of Borderias et al. (1985) was used for viscosity determination. A 100 g sample was mixed with 400 ml of chilled 5% NaCl solution. The pH of the mixture was adjusted to 6.5-7.0 using either conc. NaOH or HCl. The mixture was homogenized for 1 min using a Galaxie os- terizer. After homogenization, the mixture was filtered through cheesecloth and the filtrate was allowed to stand for 1 hr in a refrigerator to permit the release of air bubbles formed during blending. Viscosity was measured by a Brookfield viscometer using either spindle No. 2, 3 or 4 at 20 rpm.

Water-binding capacity

The water-binding capacity of the fish muscle was determined by the method of by Gierhart and Potter (1978). One gram of minced sample was added to a test tube. After obtaining the weight of the test tube and the sample, 10 ml of distilled water was added. The mixture was vortexed for
1 min and held at room temperature for 1 hr. The sample was centrifuged at 3,000 rpm for 20 min in an IEC centrifuge (Model CU-5000). Water was poured off and the tube was inverted at a 45° angle for 30 min to drain excess water onto an absorbent paper. The tube was reweighed and water-binding was reported as the weight in g of water per g sample.

Salt-extractable protein

Salt-extractable protein was determined by extracting one gram of sample with 15 ml of ice-cold 15% NaCl solution. The mixture was vortexed for 5 min and the pH was adjusted to 7.0-7.5 using 2N NaOH or HCl. The mixture was further vortexed for another 30 min before centrifuging at 3,000 rpm for 20 min using an IEC centrifuge (Model CU-5000) at room temperature.

The supernatant was analyzed for total protein using the Micro-Biuret method (Itzhaki and Gill, 1964). Two milliliters of 0.21% CuSO₄·5H₂O in 30% NaOH was added to 1 ml of the protein extract. To another ml of the same extract, 2 ml of 30% NaOH was added. The mixtures were shaken vigorously. After 5 min, the optical density was obtained at 310 nm against the alkaline copper sulfate blank with a Gilford Response UV-VIS Spectrophotometer. Corrections for absorption due to the protein alone, and for any other ultraviolet-absorbing material present, were made throughout by measuring the optical density of the
protein solution in alkali (against an alkali blank) and subtracting this value from the optical density in the presence of copper. Concentrations of the protein extracts were calculated from a standard curve prepared by plotting the corrected absorption readings against known protein concentrations (0, 0.1, 0.5, 1.0, 1.5 and 2.0 mg/ml bovine serum albumin). Purified bovine serum albumin was purchased from Sigma Chemical Co. (St. Louis, Mo.).

Electrophoresis

Reagents. Electrophoresis purity grade acrylamide, N,N'-methylene-bis-acrylamide (bis), N,N,N',N'-tetramethylenediamine (TEMED), Tris (hydroxymethyl aminomethane), ammonium persulfate, 2-mercaptoethanol, bromophenol blue and Coomassie Brilliant Blue R250 were purchased from Bio-Rad (Richmond, CA). Sodium phosphate (monobasic) was obtained from Mallinckrodt Inc. (Paris, KY) while sodium phosphate (dibasic), methanol and glacial acetic acid were obtained from Baker Chem Co. (Milwaukee, NJ).

Gel preparation. The procedure outlined by Weber and Osborn (1969) was used for SDS-Polyacrylamide gel electrophoresis. For the 7.5% acrylamide gel system, a stock solution was prepared by dissolving 22.2 g acrylamide and 0.6 g Bis in deionized water and bringing the final volume of the solution to 100 ml. To prepare 20 gels, 20.2 ml stock acrylamide solution, 6.8 ml water and 30.0 ml gel
buffer (0.5 M Na phosphate buffer with 0.2% SDS) were mixed in an ice bath and degassed for 30 min. Immediately before loading the gel onto the tubes, 3.0 ml of ammonium persulfate (50 mg/4 ml water) and 0.09 ml N,N,N',N'-tetramethylelenediamine (TEMED) were added. The gels were loaded onto the tube to the 11.0 cm mark allowing the solution to flow down the sides of the tubes. The top layer of the tubes was layered with distilled water to prevent meniscus formation. After polymerization was completed (approximately one hour), the water layer was removed and the tubes were placed into the electrophoresis apparatus. A Buchler 3 - 1500 constant power supply with 0 - 1000 volts and 0 - 200 mA capacity was used. The electrophoresis cell (Bio-Rad Model 155) compartments were filled with gel buffer diluted with distilled water (1:1) before the samples were introduced into the tubes.

Sample preparation. Extracts obtained from salt-extractable protein were used for electrophoretic studies. One ml of each extract was diluted with 2.0 ml sample buffer (prepared by mixing 0.8 ml of 0.5 M NaH₂PO₄·H₂O, 1.2 ml of 0.5 M Na₂HPO₄, 1.0 g SDS, 1.0 ml of 2-mercaptoethanol and diluting to a final volume of 100 ml with deionized water). The mixtures were heated at 100°C for 5 min and dialyzed overnight against 25 mM Tris/HCl buffer (pH 7.4) containing 0.2% SDS and 0.1% 2-mercaptoethanol. Glycerol (25 ul), 2-mercaptoethanol (5 ul) and bromophenol blue (5
ul of 2.5 mg/ml solution) were added to the dialyzed samples (60 ul) prior to electrophoresis.

**Molecular weight markers.** A mixture of carbonic anhydrase, egg albumin, bovine plasma albumin, phosphorylase b, β-galactosidase and myosin with molecular weights of 29,000, 45,000, 66,000; 97,000; 116,000 and 205,000, respectively, was used in the study. The reconstituted standard mixture (1 mg/ml sample buffer) was heated to 100°C for 5 min prior to electrophoresis.

**Electrophoresis.** Fish protein samples and protein standards (85 ul) were loaded onto the gel. Electrophoresis was carried out at 5 mA/gel until the marker dye was 1 cm from the anodic end of the gel (about 10 hrs). The gels were removed from the tube and the center of the dye front was marked with a piece of fine wire.

**Staining and Destaining.** The gels were stained overnight in Coomassie Brilliant Blue R250 solution (1.25 g/500 ml of 45.4% methanol : 9.2% glacial acetic acid ). The gels were destained by diffusion against several changes of 7.5% glacial acetic acid : 15.0% methanol solution at 40°C during the first day. Destaining was carried out on the second day at room temperature until the background was clear. The gel length and distance migrated
by each protein band were recorded. Gels were stored in 7.5% acetic acid solution.

**Scanning.** The destained gels were scanned colorimetrically at 590 nm in a Gilford Response UV-VIS Spectrophotometer.

### 3.2.3 STATISTICAL ANALYSIS

Data were analyzed by Analysis of Variance using the General Linear Model (GLM) procedure, a package program of the Statistical Analysis System (SAS Institute Inc., Cary, NC). Specific differences between treatments were determined by Tukey's Studentized Range (HSD). All comparisons were made at 5% level of significance.
3.3 RESULTS

3.3.1 Effects of Frozen Storage

Changes in muscle pH, water-binding capacity (WBC), viscosity and salt-extractable protein (SEP) of spotted seatrout as influenced by storage at -20°C are presented in Table 3-1. A downward trend in pH was observed as frozen storage increased (P < .01). Water-binding capacity significantly decreased during frozen storage. The viscosity of the fresh and frozen muscles were nearly the same. A slight reduction in SEP was observed as frozen storage increased, however, this reduction was not found to be significant.

The salt-soluble protein extracts of the muscles frozen for varying storage periods were subjected to electrophoresis on SDS-Polyacrylamide gels. The results are given in Fig. 3-1. Myosin appeared as a heavy chain sub-unit with a molecular weight of 205,000 daltons (D). Other sub-units of myosin that appeared were three light chains of 25,000; 18,000; and 15,000. Actin appeared as a whole protein with a molecular weight of 42,000. By comparing their electrophoretic mobility with known protein markers, it was observed that the relative abundance of the high-molecular weight proteins increased with frozen storage. Only a slight decrease in the extractability of myosin (MW = 205,000) was observed. Slight reduction in the low-molecular weight components occurred only after
Table 3-1. Some changes in muscle properties of spotted seatrout muscles during frozen storage at -20°C.¹

<table>
<thead>
<tr>
<th>Storage period (weeks)</th>
<th>pH*</th>
<th>WBC* (g H₂O/g sample)</th>
<th>Viscosity (cps)</th>
<th>SEP* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.73a ± 0.03</td>
<td>0.76a ± 0.05</td>
<td>6,400</td>
<td>3.34a ± 0.04</td>
</tr>
<tr>
<td>3</td>
<td>6.35b ± 0.00</td>
<td>0.52b ± 0.02</td>
<td>6,800</td>
<td>1.73a ± 1.07</td>
</tr>
<tr>
<td>6</td>
<td>6.10c ± 0.00</td>
<td>0.39b ± 0.08</td>
<td>6,400</td>
<td>2.66a ± 0.05</td>
</tr>
<tr>
<td>HSD</td>
<td>0.04</td>
<td>0.14</td>
<td>-</td>
<td>3.47</td>
</tr>
</tbody>
</table>

¹Values represent averages for 3 determinations ± standard deviation
²Means followed by a common letter are not significantly different (P ≤ .05).

storage for 6 weeks. Two components, actin and tropomyosin, were still readily extractable from the muscles stored for 6 weeks.

3.3.2. Effects of Formaldehyde Addition and Frozen Storage

The effects of formaldehyde (FA) on pH, water-binding capacity and salt-extractable proteins are given in Table 3-2. A progressive decline in pH was observed during frozen storage with all FA treatments (Fig. 3-2). The pH values for the untreated samples were slightly (NS) lower than those with FA at each storage period. The use of FA at increasing levels gave a slight but non-significant increase in the pH of the frozen muscles. It was noted
Fig. 3-1. SDS-Polyacrylamide gel scans of salt-extractable proteins of spotted seatrout during frozen storage at -20°C for: a) 0; b) 3; and c) 6 weeks.
Fig. 3-2. Effects of formaldehyde and frozen storage on pH.
Table 3-2. Changes in muscle properties of spotted seatrout as influenced by formaldehyde addition and frozen storage.1

<table>
<thead>
<tr>
<th>FA (ppm)</th>
<th>Storage (weeks)</th>
<th>pH</th>
<th>WBC (g H₂O/ g sample)</th>
<th>SEP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>6.73 ± 0.03</td>
<td>0.76 ± 0.05</td>
<td>3.34 ± 0.04</td>
</tr>
<tr>
<td>0</td>
<td>3</td>
<td>6.35 ± 0.00</td>
<td>0.52 ± 0.02</td>
<td>1.73 ± 1.07</td>
</tr>
<tr>
<td>0</td>
<td>6</td>
<td>6.10 ± 0.00</td>
<td>0.39 ± 0.08</td>
<td>2.66 ± 0.05</td>
</tr>
<tr>
<td>300</td>
<td>0</td>
<td>6.73 ± 0.03</td>
<td>0.76 ± 0.05</td>
<td>3.34 ± 0.04</td>
</tr>
<tr>
<td>300</td>
<td>3</td>
<td>6.43 ± 0.03</td>
<td>0.28 ± 0.07</td>
<td>2.55 ± 0.07</td>
</tr>
<tr>
<td>300</td>
<td>6</td>
<td>6.12 ± 0.00</td>
<td>0.46 ± 0.06</td>
<td>2.15 ± 0.05</td>
</tr>
<tr>
<td>500</td>
<td>0</td>
<td>6.73 ± 0.03</td>
<td>0.76 ± 0.05</td>
<td>3.34 ± 0.04</td>
</tr>
<tr>
<td>500</td>
<td>3</td>
<td>6.45 ± 0.00</td>
<td>0.30 ± 0.09</td>
<td>2.63 ± 0.06</td>
</tr>
<tr>
<td>500</td>
<td>6</td>
<td>6.16 ± 0.01</td>
<td>0.41 ± 0.03</td>
<td>2.52 ± 0.07</td>
</tr>
</tbody>
</table>

1Values represent averages for 3 determinations ± standard deviation

that the differences in pH for the different fish muscles varied with storage time. This indicates that an interaction existed between the FA treatments and the storage periods, which complicates an understanding for the cause of the pH decline.

The decrease in the water-binding capacity of the muscles was influenced by FA and frozen storage (P ≤ .01), as shown in Fig. 3-3. The untreated samples exhibited
Fig. 3-3. Effects of formaldehyde and frozen storage on WBC.
higher water-binding properties than those with FA at 3 weeks, but there were no differences after 6 weeks. Water-binding capacity for the untreated samples declined progressively during frozen storage while those with FA exhibited a decrease on the 3rd week and a slight increase on the 6th week. The interaction between formaldehyde and frozen storage also contributed to the decline in WBC, making it difficult to conclude which factor actually caused the decline.

The addition of FA reduced the amount of salt-extractable proteins in the frozen muscles (Fig. 3-4). A progressive decline in SEP was observed for the muscles treated with 300 ppm FA. A decline in SEP was also observed for the muscles with 500 ppm FA although the value remained nearly constant after 3 weeks of storage. For the untreated muscle, an abrupt decrease in SEP was observed after 3 weeks of storage that resulted in lower SEP than either FA treatment, however, this property increased markedly after storage for 6 weeks. There was, however, considerable sampling error as indicated by a high standard deviation (1.07).

The addition of 300 ppm FA to the muscles resulted in a slight decrease in the extractability of the higher molecular weight protein components after 3 weeks of storage (Fig. 3-5). A further decrease in high-molecular weight proteins was observed as FA increased from 300 to 500 ppm (Fig. 3-6). The stability of the actin, tropo-
myosin and the lower molecular weight myosin sub-units are clearly depicted by the electrophoretic patterns. The extractability of these proteins was not affected by the addition of 300 ppm FA and only a slight decrease was observed when 500 ppm FA was added. Under conditions of high formaldehyde concentration and prolonged frozen storage (i.e., muscle stored for 6 weeks with 500 ppm FA), the reduction in extraction of the higher molecular weight proteins became more evident and the lower molecular weight proteins started to show slight decreases.
Fig. 3–4. Effects of formaldehyde and frozen storage on SEP.
Fig. 3-5. SDS-Polyacrylamide gel scans of salt-extractable proteins of spotted seatrout stored at -20°C for 3 weeks with the addition of: top) 0 , middle) 300 and bottom) 500 ppm FA.
Fig. 3-6. SDS-Polyacrylamide gel scans of salt-extractable proteins of spotted seatrout stored at -20°C for 6 weeks with the addition of: top) 0, middle) 300 and bottom) 500 ppm FA.
3.4 DISCUSSION

3.4.1 Effects of Frozen Storage

Results obtained in this study confirm earlier reports that changes in finfish proteins are manifested mainly by a loss in solubility or extractability of myofibrillar proteins (Owusu-Ansah and Hultin, 1986; Borderias et al., 1982; Shenouda, 1980; Sikorski et al., 1976), formation of aggregates (Sikorski et al., 1976) and loss of functional properties such as water-holding capacity (Acton et al., 1983; Laird and Mackie, 1981; Suzuki, 1981; Grabowska and Sikorski, 1976). Results obtained for viscosity were contrary to earlier findings of Ueda and co-workers (1962), Noguchi and Matsumoto (1970), Matsumoto and Noguchi (1971) and Oguni et al. (1975).

The progressive decline in pH of frozen stored seatrout muscles indicated production of lactic acid via anaerobic glycolysis. Production of lactic acid in fish muscles reduces the postmortem pH to between 6.2 and 6.6. Unlike meat, where it is advantageous to attain as low a pH as possible, a high pH is desirable in frozen fish to prevent toughness (Eskin et al., 1971). This relationship has been clearly shown in the present study where the decrease in pH corresponded to a decrease in water-binding capacity, in that at higher pH levels, muscles exhibited greater water-binding properties (r = .96). The acidity is probably the most important factor affecting the rheologi-
cal properties of a given muscle (Dunajski, 1979). The tightening or loosening of the muscle structure due to changes in net protein charge under different concentrations of hydrogen ion account for major changes in the texture of the flesh. It has been reported that an exceptionally large effect of pH occurs at 5.7-6.7. In this range, a decrease by one unit resulted in up to a 2.5 fold increase in toughness in terms of the required shear force. Water-binding capacity refers to the ability of muscle to retain its bulk phase water during application of various stresses such as pressure, heating, or grinding, etc. Muscle contains about 75% water, of which 5% is bound water, with almost all of the remainder entrapped bulk phase water. Water-binding capacity is related to juiciness, taste, tenderness and color and is influenced greatly by a variety of processing and handling conditions (Hamn, 1970). In this particular study, water-binding capacity of the fish muscles was greatly decreased by frozen storage.

Previous studies on viscosity changes during frozen storage were centered on actomyosin. Viscosity of soluble actomyosin fractions decreased with increasing time of storage, suggesting that the actomyosin filaments became less filamentous either by individual molecular folding or by aggregation of the filaments. Viscosity results for this study did not show a similar trend, possibly due to differences on the measurement being conducted on the fish
muscle. Other protein compounds that remained fairly stable during frozen storage would have played a major role in the maintenance of viscosity.

The observed rate of decrease in the salt-extractable protein may be partly explained on the basis of formation of aggregated myofibrillar proteins, particularly myosin. King (1966), on the basis of ultracentrifugal analysis, suggested that during frozen storage of cod, there is a rapid dissociation of F-actomyosin into G-actomyosin followed by a slower rate of dissociation of G-actomyosin into components, which then aggregate to form inextractable protein. He also suggested the possibility of direct aggregation of F-actomyosin macromolecules. Earlier studies have also shown that fish myosin, upon standing in unfrozen and frozen solution, had a tendency to aggregate by side-to-side attachment of the monomers (Matsumoto, 1980). Several theories have been advanced to explain this "denaturation-aggregation" phenomenon (Shenouda, 1980). Among these are the denaturation or aggregation caused by the increase in salt concentration or change in pH following removal of water by ice formation. Interactions with lipids can also denature proteins due to their surfactant properties, while lipid oxidation can change protein conformation by modifying side groups or by inducing cross-linking of proteins through a free radical process (Karel et al., 1975). It has also been suggested that formaldehyde, produced from the breakdown of
trimethylamine oxide, induces cross-links between protein molecules in the muscle tissue, forming a three-dimensional network (Gill et al., 1979; Castell et al., 1973; Amano and Yamada, 1965).

SDS-Polyacrylamide gel electrophoretic examination of the various fractions obtained by salt extraction procedures was conducted to gain information about the nature of the changes at the molecular level. The myofibrillar proteins of unfrozen seatrout muscle were readily extractable in salt solutions and gave a characteristic distribution of protein sub-units on the SDS electrophoresis gels. These proteins were completely extractable from fresh unfrozen flesh in high concentrations of salt, the inextractable residue being presumed to be the connective tissue proteins. With increasing time of frozen storage, protein denaturation may have occurred which induced the lower yield of proteins obtained by salt extraction. The gels also showed that the amount of material at the top, which signifies exclusion from the gel, increased with increased frozen storage. This indicates that very high molecular weight components ( > 205,000 D) were accumulating. This change suggests that changes associated with a decrease in solubility and loss in functional properties could be attributed to aggregation of myofibrillar proteins. The electrophoretic patterns also showed that actin and tropomyosin were still extractable after 6 weeks of storage although a slight disappearance of myosin
occurred, possibly due aggregation as a result of covalent bond formation.

These results suggest that as the length of frozen storage increases, some proteins are being denatured, and the formation of aggregates, possibly due to creation of new bonds, lead to a loss of solubility and deterioration of functional properties.

3.4.2 Effects of Formaldehyde and Frozen Storage

Numerous studies have indicated that fish species that produce large amounts of formaldehyde (FA) deteriorate rapidly during frozen storage. Because of this, a model experiment involving the addition of formaldehyde to unfrozen muscle was carried out to determine its effects on frozen spotted seatrout muscle and to relate those effects to those of frozen storage.

During frozen storage for 3 weeks, formaldehyde treated muscles exhibited higher values in pH and salt-extractable proteins but lower water-binding capacity. The ability of the frozen muscle to retain bulk phase water was greatly affected by formaldehyde, however, no significant change in this value occurred between muscles with 300 and 500 ppm FA. Upon storage for 6 weeks, values for the three properties seem to converge, but all were lower than those obtained from fresh samples. It is apparent that a majority of the functional changes associated with frozen storage is due to some factors other than FA. Thus, the
role played by formaldehyde alone in the diminution of these properties is difficult to confirm.

An obvious change can be seen from the electrophoretic profiles of the 'high salt' proteins obtained from untreated and FA-treated muscles. At increasing formaldehyde levels, muscles exhibited extensive reductions in extractability of the high-molecular weight components. Reductions in lower molecular weight proteins were observed only with increased frozen storage and higher formaldehyde concentration.

From these results, it is seen that a level of 300 ppm FA does not produce a noticeable effect on the extractability of myofibrillar proteins in fish muscles. With an increased level up to 500 ppm FA, the extractability of the high-molecular weight proteins is greatly impaired. The most striking observation is that treatment of spotted seatrout muscle with FA extensively decreases the extractability of the high-molecular weight proteins including myosin. With untreated muscles frozen for 6 weeks, only a slight reduction in the extractability of myosin was detected. Reductions in the lower molecular weight proteins were also being detected under the conditions of high formaldehyde concentration and prolonged storage. These changes in protein extractability did not appear to impact functional properties of the fish muscle.

The mechanism by which FA affects proteins at the molecular level was postulated from the fact that FA has
the ability to bind to various functional groups in the proteins (Connell, 1964). This binding causes a deformation accompanied by cross-linking between the protein peptide chains via methylene bridges. The molecules formed are believed to be covalent in nature but evidences presented by Connell (1964, 1975) showed that the proteins could cross-link through non-covalent binding. Subsequent studies have also indicated that FA can cause polymerization of proteins as well as react with small molecular compounds in the muscle tissue (Owusu-Ansah and Hultin, 1984; Ohnishi and Rodger, 1979).

Results obtained from frozen storage and formaldehyde addition studies revealed that loss in solubility and reduction in other properties were mainly due to frozen storage. The adverse effects of formaldehyde were not detected in muscles frozen for 6 weeks, although a reduction in protein extractability especially higher molecular weight components did occur. The significance of these changes must be determined. Nevertheless, it appears that a longer storage period is required before major changes can be detected in the functional properties of spotted seatrout muscles.
3.5 SUMMARY AND CONCLUSIONS

The experimental evidence presented here leads to the conclusion that during frozen storage, the myofibrillar proteins of spotted seatrout muscles undergo a series of conformational changes. These changes included a reduction in water-binding capacity and solubility of salt-extractable proteins, possibly due to a reduction in pH. Results gathered from electrophoretic examination of the salt-extractable proteins show that the amount of very high molecular weight components increases with increased time of storage. It is speculated that high-molecular weight proteins are the result of aggregation, possibly through the creation of new bonds leading to loss of solubility and deterioration of functional properties.

Results obtained from the formaldehyde study indicate that frozen storage was primarily responsible for reduction in pH, water-binding capacity and salt-extractable proteins. Under conditions of prolonged storage and high concentration of formaldehyde, extensive diminution in extractability of high-molecular weight components including myosin and slight reductions in the amount of lower molecular weight proteins occurred. Formaldehyde has been shown to be an effective agent in the reduction of protein solubility, but the significance of those changes on functional properties was not evident.
4.1 INTRODUCTION

Muscle tissue stored in the frozen state undergoes progressive deterioration in organoleptic and other properties. There is considerable evidence that this deteriorative change is mainly the result of alterations in the myofibrillar proteins, particularly the myosin-actomyosin system (Sikorski, 1978; Connell, 1975 Dyer et al., 1951, 1956a, 1956b). This protein change is seen mainly by a loss of extractability of myofibrillar proteins and loss of functional properties such as water-holding capacity, ease of fat emulsification, and gel-forming ability (Sikorski, 1978). The set of reactions leading to this protein alteration or denaturation-aggregation is complex but are believed to involve intermolecular aggregation. Many hypothesis have been proposed to explain denaturation of muscle proteins during frozen storage. These include the effect of inorganic salts, water-activity relations, reactions with lipids, reactions with formaldehyde, surface effects at the solid-gas interface and effects of water-soluble proteins (Fennema et al. 1973, Sikorski et al., 1973).

The rate of this change varies markedly between species. The muscle proteins of fish and other aquatic
animals have been found to be less stable than those of beef, pigs and poultry (Matsumoto, 1980). Because of the complexity of the denaturation-aggregation phenomenon, and because of the diversity in the degree of damage between fish species, considerable research is being conducted with the objective of elucidating the nature of protein interactions. Our previous work (Chapter 3) with spotted seatrout indicated that frozen storage could affect functional properties of Gulf coast finfish species. These changes did not appear to be related to the production of formaldehyde.

In this regard, an investigation was conducted to determine the potential adverse effects of frozen storage and freeze-thaw cycles on protein properties of black drum and mullet muscles. These two underutilized species have been identified as having potential commercial importance to the Gulf coast fishing industry. This study was designed to develop background information on protein stability to ultimately aid processors in development of products and processes from these fish species. Furthermore, an evaluation of mechanistic aspects of protein denaturation-aggregation was conducted using differential protein extraction.
4.2 MATERIALS AND METHODS

4.2.1 MATERIALS

Two finfish species from the Gulf of Mexico, black drum (*Pogonias cromis*) and mullet (*Mugil cephalus*), were used in the study. The fish were purchased from commercial sources and transported to the Food Science Department laboratory on ice. Black drum were obtained as fillets while mullet was obtained in-the-round. Upon receipt at the laboratory, black drum was randomly divided into two groups, one for frozen storage study and the other for the freeze-thaw cycle study. Black drum fillets for the frozen study were washed, vacuum packaged (150 g each) in polyethylene bags and frozen in an air blast freezer at -20°C. During sampling and testing, three bags of fish were thawed by holding in a refrigerator at 4-6°C overnight prior to analyses. Determinations of the different properties were conducted at 0, 3 and 6 weeks. For the freeze-thaw study, the fillets were washed, vacuum packaged (150 g each) in polyethylene bags and frozen in an air blast freezer at -20°C. The muscles were subjected to different freeze-thaw cycles (0, 1, 3, 5, 7) by freezing for 24 hrs and thawing by holding in a refrigerator at 4-6°C overnight. Determinations of the different properties were conducted after each freeze-thaw cycle.

Mullet for the freeze-thaw study were eviscerated, beheaded, filleted by hand, washed, vacuum packaged (150 g
each) in polyethylene bags and frozen in an air blast freezer at -20°C. The muscles were subjected to different freeze-thaw cycles (0, 1 and 3) by freezing for 24 hours and thawing in a refrigerator at 4-6°C overnight. Determinations of the different properties were conducted after each freeze-thaw cycle.

4.2.2 METHODS

pH

The pH was determined by homogenizing 10 g of muscle with 20 ml of distilled water using a Microprocessor Ionalyzer (Orion Research, Model 901) calibrated with buffers at 3.2 and 7.0.

Water-binding capacity

Water-binding capacity of the fish muscle was determined by the method of Gierhart and Potter (1978). One gram of minced sample was added to a test tube. After obtaining the weight of the test tube and the sample, 10 ml of distilled water was added. The mixture was vortexed for 1 min and held at room temperature for 1 hr. The sample was centrifuged at 3,000 rpm for 20 min in an IEC centrifuge (Model CU-5000). Water was poured off and the tube was inverted at a 45° angle for 30 min to drain excess water onto an absorbent paper. The tube was
reweighed and water-binding was reported as the weight in g of water per g sample.

**Expressible Moisture**

Expressible moisture was determined by the method of Jauregui et al. (1981). Three pieces of Whatman #3 filter paper, 5.5 cm in diameter and one piece of Whatman #50 filter paper, 7.0 cm in diameter were shaped into a thimble using the bottom of a 16 X 150 mm test tube. The #50 filter paper was used as the internal surface of the thimble. The filter paper was weighed before a 1.5 ± 0.3 g sample was added, after which the sample was centrifuged in a 50-ml polycarbonate centrifuge tube at 16,000 rpm for 15 min using a refrigerated centrifuge (Sorvall RC2-B) at 4°C. The filter paper and the sample were removed from the tube using a forcep. The meat "cake" was removed from the filter paper and the filter paper was reweighed. The expressible moisture was reported as percent weight loss from original sample.

**Salt-extractable protein**

Salt-extractable protein was determined by extracting one gram of sample with 15 ml of ice-cold 15% NaCl solution. The mixture was vortexed for 5 min and the pH adjusted to 7.0-7.5 using 2N NaOH or HCl. The mixture was further vortexed for another 30 min before centrifuging at
3,000 rpm for 20 min using an IEC centrifuge (Model CU-5000) at room temperature.

The supernatant was analyzed for total protein using the Micro-Biuret method (Itzhaki and Gill, 1964). Two milliliters of 0.21% CuSO$_4$$\cdot$5H$_2$O in 30% NaOH was added to 1 ml of the protein extract. To another ml of the same extract, 2 ml of 30% NaOH was added and the mixtures shaken vigorously. After 5 min, the optical density was obtained at 310 nm against the alkaline copper sulfate blank with a Gilford Response UV-VIS Spectrophotometer. Corrections for absorption due to protein alone, and for any other ultraviolet-absorbing material present, was made throughout by measuring optical density of the protein solution in alkali (against an alkali blank) and subtracting this value from the optical density in the presence of copper. Concentrations of the protein extracts were calculated from a standard curve prepared by plotting the corrected absorption readings against known protein concentrations (0, 0.1, 0.5, 1.0, 1.5 and 2.0 mg/ml bovine serum albumin).

**Differential Extractable Protein**

Four extracting solvents were used for determination of differential extractable protein as outlined in the method of Owusu-Ansah and Hultin (1986). Six grams of minced samples was extracted with ice-cold a) 5% NaCl in 0.02 M NaHCO$_3$ (pH 7.2), b) 5% NaCl in 0.02 M NaHCO$_3$ (pH 7.2) with 0.5% 2-mercaptoethanol (ME), c) 4% SDS and d) 4%
SDS with 0.5% ME using a Galaxie osterizer for 5 min. Protein contents of the extracts were determined by the modified Biuret procedure of Pelley et al. (1978).

A quantity of 2.0 ml of Biuret reagent (prepared by dissolving 3.9 g of CuSO$_4$·5H$_2$O and 6.7 g of Na$_2$EDTA in a final volume of 700 ml distilled water and then adding 200 ml of 20% NaOH, giving a total volume of 900 ml) was added to the protein extract (0.2 ml) and incubated for 5 min at 60°C. Absorbance was measured at 545 nm against a blank containing all components except protein.

**Electrophoresis**

**Reagents.** Electrophoresis purity grade acrylamide, N,N'-methylene-bis-acrylamide (bis), N,N,N',N'-tetramethylenediamine (TEMED), Tris (hydroxymethyl aminomethane), ammonium persulfate, ME, bromophenol blue and Coomassie Brilliant Blue R250 were purchased from Bio-Rad (Richmond, CA). Sodium phosphate (monobasic) was obtained from Mallinckrodt Inc. (Paris, Kent.) while sodium phosphate (dibasic), methanol and glacial acetic acid were obtained from Baker Chem. Co. (Milwaukee, NJ).

**Gel preparation.** The procedure outlined by Weber and Osborn (1969) was used for SDS-Polyacrylamide gel electrophoresis. For the 7.5% acrylamide gel system, a stock solution was prepared by dissolving 22.2 g acrylamide and 0.6 g Bis in deionized water and bringing the final volume
of the solution to 100 ml. To prepare 20 gels, 20.2 ml stock acrylamide solution, 6.8 ml water and 30.0 ml gel buffer (0.5 M Na phosphate buffer with 0.2% SDS) were mixed in an ice bath and degassed for 30 min. Immediately before loading the gel onto the tubes, 3.0 ml of ammonium persulphate (50 mg/4 ml water) and 0.09 ml N,N,N',N'-tetramethylenediamine (TEMED) were added. The gels were loaded onto the tube to the 11.0 cm mark allowing the solution to flow down the sides of the tubes. The top layer of the tubes was layered with distilled water to prevent meniscus formation. After polymerization was completed (approximately one hour), the water layer was removed and the tubes were placed into the electrophoresis apparatus. A Buchler 3-1500 constant power supply with 0 - 1000 volts and 0 - 200 mA capacity was used. The electrophoresis cell (Bio-Rad Model 155) compartments were filled with gel buffer diluted with distilled water (1:1) before the samples were introduced into the tubes.

**Sample preparation.** Extracts obtained from salt-extractable protein and differential protein determinations were used for electrophoretic studies. One milliliter of each of the extracts were diluted with 2.0 ml sample buffer (prepared by mixing 0.8 ml of 0.5 M NaH₂PO₄·H₂O, 1.2 ml of 0.5 M Na₂HPO₄, 1.0 g SDS, 1.0 ml of ME and diluting to a final volume of 100 ml with deionized water). The mixtures were heated at 100°C for 5 min and dialyzed overnight.
against 25 mM Tris/HCl buffer (pH 7.4) containing 0.2% SDS and 0.1% ME. Glycerol (25 ul), ME (5 ul) and bromophenol blue (5 ul of 2.5 mg/ml solution) were added to the dialyzed samples (60 ul) prior to electrophoresis.

Molecular weight markers. A mixture of carbonic anhydrase, egg albumin, bovine plasma albumin, phosphorylase b, B-galactosidase and myosin with molecular weights of 29,000, 45,000, 66,000; 97,000; 116,000 and 205,000, respectively, was used in the study. The reconstituted standard mixture (1 mg/ml sample buffer) was heated to 100°C for 5 min prior to electrophoresis.

Electrophoresis. Fish protein samples and protein standards (85 ul) were loaded onto the gel. Electrophoresis was carried out at 5 mA/gel until the marker dye was 1 cm from the anodic end of the gel (about 10 hrs). The gels were removed from the tube and the center of the dye front was marked with a piece of fine wire.

Staining and Destaining. The gels were stained overnight in Coomassie Brilliant Blue R250 solution (1.25 g/500 ml of 45.4% methanol : 9.2% glacial acetic acid ). The gels were destained by diffusion against several changes of 7.5% glacial acetic acid : 15.0% methanol solution at 40°C during the first day. Destaining was carried out on the second day at room temperature until the
background was clear. The gel length and distance migrated by each protein band were recorded. Gels were stored in 7.5% acetic acid solution.

Scanning. The destained gels were scanned colorimetrically at 590 nm in a Gilford Response UV-VIS Spectrophotometer.

4.2.3 STATISTICAL ANALYSIS

Data were analyzed by Analysis of Variance using the General Linear Model (GLM) procedure, a package program of the Statistical Analysis System (SAS Institute Inc., Cary, NC). Specific differences between treatments were determined by Tukey's Studentized Range (HSD). All comparisons were made at 5% level of significance.
4.3 RESULTS

4.3.1 Effects of frozen storage on black drum muscles.

Changes in water-binding capacity (WBC), expressible moisture (EM) and salt-extractable protein (SEP) of black drum muscles are presented in Table 4-1. A general decrease in water-binding capacity was observed as frozen storage increased, with a significant ($P < 0.05$) difference after 6 weeks. Expressible moisture increased slightly with increasing frozen storage times, although not significant. A slight non-significant reduction in salt-extractable protein with prolonged frozen storage was also observed.

Table 4-1. Effects of frozen storage on some properties of black drum muscles.

<table>
<thead>
<tr>
<th>Storage (g H$_2$O/g sample)</th>
<th>WBC ($\pm$ standard deviation)</th>
<th>Express. Moist. ($%$ wt. loss)</th>
<th>SEP ($%$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$0.32^a \pm 0.08$</td>
<td>$46.22^a \pm 0.45$</td>
<td>$6.24^a \pm 0.50$</td>
</tr>
<tr>
<td>3</td>
<td>$0.23_{ab} \pm 0.03$</td>
<td>$48.92^a \pm 1.44$</td>
<td>$6.18^a \pm 0.40$</td>
</tr>
<tr>
<td>6</td>
<td>$0.15^b \pm 0.01$</td>
<td>$50.97^a \pm 0.93$</td>
<td>$6.16^a \pm 0.58$</td>
</tr>
</tbody>
</table>

Values represent averages for 3 determinations

*Means followed by a common letter are not significantly different ($P \leq .05$).
The SDS-Polyacrylamide gel scans of the salt-extractable proteins revealed the disappearance of most components, particularly the high-molecular weight proteins. The occurrence of a component with MW approximately 100,000 D was observed along with the reduction in the high-molecular weight components (200,000 D) after 6 weeks of storage (Fig 4-1). Actin, tropomyosin remained extractable after 6 weeks of storage but with slight reductions.

The extractability of the muscle proteins in various extraction solutions is shown in Fig. 4-2. The solubility of the proteins in all solutions decreased slightly with increasing frozen storage. Among all extractants, extractability of the proteins was highest in 4% SDS with 0.5% ME, followed by that of 4% SDS, then by 5% NaCl in 0.02 M NaHCO₃ (pH 7.2) with 0.5% ME and finally by 5% NaCl in 0.02 M NaHCO₃ (pH 7.2). The percent extractable protein in solutions containing 0.5% ME were 50% higher than those extracted without this disulfide splitting reagent. Percent extractable protein was further increased by extracting with 4% SDS. The addition of the disulfide reagent to the SDS solution increased the amount of extractable protein to a greater degree in SDS than that obtained in the buffered salt solution.

Proteins extracted by the different reagents from the fresh muscles were subjected to electrophoresis. Results are given in Fig 4-3. Solubility of the proteins was increased by using ME or SDS. The use of the disulfide
Fig. 4-1. SDS-Polyacrylamide gel scans of salt-extractable proteins from black drum muscles during frozen storage at -20°C for: a) 0; b) 3; and c) 6 weeks.
Fig. 4-2. Percent extractable protein of black drum muscles in different solutions during frozen storage at -20°C: Solvent 1 = 5% NaCl in 0.02 M NaHCO₃ (pH 7.2); Solvent 2 = 5% NaCl in 0.02 M NaHCO₃ (pH 7.2) with 0.5% 2-mercaptoethanol; Solvent 3 = 4% SDS; Solvent 4 = 4% SDS with 0.5% 2-mercaptoethanol.
Fig. 4-3. SDS-Polyacrylamide gel scans of extractable proteins from fresh black drum muscles in a) 5% NaCl in 0.02 M NaHCO₃ (pH 7.2); b) 5% NaCl in 0.02 M NaHCO₃ (pH 7.2) with 0.5% 2-mercaptoethanol; c) 4% SDS; and d) 4% SDS with 0.5% 2-mercaptoethanol.
reagent in the SDS solution did not produce a significant increase in the extractability of the proteins. The electrophoretic profiles of the extractable proteins in different reagents at varying storage times are shown in Figs. 4-4 to 4-7. Solubility of the protein components in the buffered salt solution decreased only after 6 weeks of frozen storage. Extensive reduction in the levels of the high-molecular weight components was very evident. The addition of 0.5% ME increased the solubility of the proteins but as time of storage increased, the yield of the proteins obtained by the solution decreased. Extraction of proteins was not increased by ME in 4% SDS solution as was observed with the buffered salt solution. Similar results were obtained when the disulfide splitting reagent was added to the 4% SDS. These results clearly show that a considerable reduction in the extractability of the different protein components is occurring with frozen storage.

4.3.2 **Effects of freeze-thawing cycles on black drum muscles.**

The effects of freeze-thawing cycles on certain functional properties of black drum muscles are shown in Table 4-2. A significant (P ≤ 0.05) decrease in water-binding capacity was not observed until after the seventh freeze-thaw cycle. Values for this property progressively declined with increasing freeze-thaw cycles. Expressible moisture generally increased with increasing freeze-thaw cycles. Salt-extractable protein showed a downward trend
Fig. 4-4. SDS-Polyacrylamide gel scans of proteins extracted by 5% NaCl in 0.02 M NaHCO₃ (pH 7.2) from black drum muscles during frozen storage at -20°C for: top) 0; middle) 3; and bottom) 6 weeks.
Fig. 4-5. SDS-Polyacrylamide gel scans of proteins extracted by 5% NaCl in 0.02 M NaHCO₃ (pH 7.2) with 0.5% 2-mercaptoethanol from black drum muscles during frozen storage at -20°C for: top) 0; middle) 3; and bottom) 6 weeks.
Fig. 4-6. SDS-Polyacrylamide gel scans of proteins extracted by 4% SDS from black drum muscles during frozen storage at -20°C for: top) 0; middle) 3; and bottom) 6 weeks.
Fig. 4-7. SDS-Polyacrylamide gel scans of proteins extracted by 4% SDS with 0.5% 2-mercaptoethanol from black drum muscles during frozen storage at -20°C for: top) 0; middle) 3; and bottom) 6 weeks.
Table 4-2. Effects of freeze-thaw cycles on some properties of black drum muscles.  

<table>
<thead>
<tr>
<th>No. freeze-thaw cycle (g H₂O/g sample)</th>
<th>WBC</th>
<th>Exp. Moist. (% wt. loss)</th>
<th>SEP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.32b ± 0.08</td>
<td>46.22a ± 0.45</td>
<td>6.24a ± 0.50</td>
</tr>
<tr>
<td>1</td>
<td>0.29a ± 0.07</td>
<td>54.19a ± 1.89</td>
<td>6.00a ± 0.79</td>
</tr>
<tr>
<td>3</td>
<td>0.22a ± 0.04</td>
<td>50.88ab ± 1.50</td>
<td>6.16a ± 0.58</td>
</tr>
<tr>
<td>5</td>
<td>0.24a ± 0.08</td>
<td>53.74ab ± 0.23</td>
<td>5.11a ± 0.10</td>
</tr>
<tr>
<td>7</td>
<td>0.09a ± 0.04</td>
<td>54.68b ± 1.03</td>
<td>4.42a ± 0.35</td>
</tr>
</tbody>
</table>

1Values represent averages of 3 determinations ± standard deviation.

*Means followed by a common letter are not significantly different at 5% level of significance.

with increasing freeze-thaw cycles, although these decreases were not significant.

The salt-soluble protein extracts of black drum muscles were subjected to SDS-Polyacrylamide gel electrophoresis. The electrophoretic patterns are shown in Fig. 4-8. Extractability of the different protein components decreased with increasing freeze-thaw cycles.

The solubility of the extractable proteins in various reagents with increasing freeze-thaw cycles is shown in Fig. 4-9. Percent extractable protein was highest in 4% SDS with 0.5% ME, followed by that in 5% NaCl in 0.02M NaHCO₃ (pH 7.2) with 0.5% ME and by 4% SDS and lastly by
Fig. 4-8. SDS-Polyacrylamide gel scans of salt-extractable proteins from black drum muscles with varying freeze-thaw cycles: a) 0; b) 1; c) 3, d) 5; and e) 7 freeze-thaw cycles.
Fig. 4-9. Percent extractable protein of black drum muscles in different solutions at varying freeze-thaw cycles:
Solvent 1 = 5% NaCl in 0.02 M NaHCO$_3$ (pH 7.2);
Solvent 2 = 5% NaCl in 0.02 M NaHCO$_3$ (pH 7.2) with 0.5% 2-mercaptoethanol; Solvent 3 = 4% SDS; and Solvent 4 = 4% SDS with 0.5% 2-mercaptoethanol.
5% NaCl in 0.02 M NaHCO₃ (pH 7.2). The use of the disulfide splitting reagent in the buffered salt increased the extractable protein concentration by about 60%, a concentration similar to that obtained for the 4% SDS solution. Extractability of the proteins was further increased by addition of disulfide splitting reagent to the 4% SDS solution. Percent extractable proteins in the buffered salt solution with or without the disulfide splitting reagent decreased slightly with increasing freeze-thaw cycles. In the SDS solutions, there was no change in the amount of proteins as freeze-thaw cycles increased.

The proteins extracted by the different reagents were subjected to SDS-Polyacrylamide gel electrophoresis. Results are given in Figs. 4-10 to 4-13. Solubilities of the proteins in buffered salt solution, with or without the disulfide reagent, decreased with increasing freeze-thaw cycles. A greater amount of high-molecular weight proteins was extracted initially with ME but the difference was not evident after 3 freeze-thaw cycles. No apparent changes were observed as freeze-thaw cycle increased for either of the SDS solutions. These results agree with those presented for the percent extractable proteins determined by the Biuret method (Fig. 4-9).
Fig. 4-10. SDS-Polyacrylamide gel scans of proteins extracted by 5% NaCl in 0.02 M NaHCO₃ (pH 7.2) from black drum muscles with varying freeze-thaw cycles: a) 0; b) 1; c) 3; d) 5; and e) 7 freeze-thaw cycles.
Fig. 4-11. SDS-Polyacrylamide gel scans of proteins extracted by 5% NaCl in 0.02 M NaHCO_3 (pH 7.2) with 0.5% 2-mercaptoethanol from black drum muscles with varying freeze-thaw cycles: a) 0; b) 1; c) 3; d) 5; and e) 7 freeze-thaw cycles.
SDS-Polyacrylamide gel scans of proteins extracted by 4% SDS from black drum muscles with varying freeze-thaw cycles: a) 0; b) 1; c) 3; d) 5; and e) 7 freeze-thaw cycles.
Fig. 4-13. SDS-Polyacrylamide gel scans of proteins extracted by 4% SDS with 0.5% 2-mercaptoethanol from black drum muscles with varying freeze-thaw cycles: a) 0; b) 1; c) 3; d) 5; and e) 7 freeze-thaw cycles.
4.3.3 Effects of freeze-thawing cycles on mullet muscles.

Results for the effect of freeze-thawing on mullet properties are presented in Table 4-3. Values for pH remained nearly constant with increased freeze-thaw cycles. The muscle water-binding capacity decreased with increasing freeze-thaw cycles. A slight increase in expressible moisture was observed with increased freeze-thaw cycle. No significant change was found for the salt-extractable protein. The SDS-Polyacrylamide gel scans of the different components remained virtually unchanged after three freeze-thaw cycles (Fig. 4-14).

Table 4-3. Effects of freeze-thaw cycles on some properties of mullet muscles.¹

<table>
<thead>
<tr>
<th>Freeze-thaw cycle</th>
<th>pH</th>
<th>WBC (g H₂O/ g sample)</th>
<th>Exp. Moist. (% wt. loss)</th>
<th>SEP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.06 ± 0.01</td>
<td>0.33 ± 0.01</td>
<td>50.00 ± 0.99</td>
<td>8.26 ± 0.20</td>
</tr>
<tr>
<td>1</td>
<td>6.07 ± 0.01</td>
<td>0.25 ± 0.02</td>
<td>50.87 ± 0.12</td>
<td>8.62 ± 0.33</td>
</tr>
<tr>
<td>3</td>
<td>6.07 ± 0.00</td>
<td>0.20 ± 0.03</td>
<td>64.23 ± 0.77</td>
<td>8.20 ± 0.41</td>
</tr>
</tbody>
</table>

¹Values represent averages for 3 determinations ± standard deviation
*Means followed by a common letter are not significantly different (P ≤ .05).
Fig. 4-14. SDS-Polyacrylamide gel scans of salt-extractable proteins from mullet muscles at varying freeze-thaw cycles: top) 0; middle) 1; and bottom) 3 freeze-thaw cycles.
4.4 DISCUSSION

4.4.1 Effects of frozen storage on black drum muscles.

A decrease in water-binding capacity, increase in expressible moisture and loss in solubility of the salt-extractable proteins are indications of the type of protein alterations that occur in fish muscles during frozen storage. This type of protein change, which follows freezing and frozen storage, has been regarded by most investigators as denaturation. Denaturation describes a complex phenomenon that involves alterations of secondary and tertiary structures of the proteins without rupturing the covalent linkages between carbon atoms in the polypeptide chains (Kinsella and Melanchouris, 1976; Sikorski et al., 1976). Water-binding capacity, which refers to the ability of the muscle to retain its bulk phase water during application of stress, is closely related to its juiciness, taste, tenderness and color. Hamn (1975) reported that this property is influenced greatly by a variety of processing and handling conditions including rigor mortis, storage, heating, drying and freezing. Black drum muscles stored for 6 weeks showed a marked decline in this property.

Expressible moisture refers to the amount of liquid squeezed from a protein system by application of force, and measures the amount of loose water released under the test conditions (Jauregui et al., 1981). This property in-
creased with prolonged storage and a strong correlation was found to exist between this variable and the muscle water-binding capacity ($r = - .99$). An increase in expressible moisture corresponded to a decrease in water-binding capacity.

The loss in protein solubility of black drum muscles during frozen storage could be caused by aggregate formation (Sikorski, 1978). Early observations have shown that rabbit and fish myosin, upon standing in unfrozen and frozen solutions, have a tendency to aggregate by side-to-side attachment of the monomers (Matsumoto, 1980). King (1966), on the basis of ultracentrifugal analysis, suggested that during frozen storage of cod there is a rapid dissociation of F-actomyosin into G-actomyosin into components, which then aggregate to form inextractable protein. He also suggested the possibility of direct aggregation of F-actomyosin macromolecules.

Results obtained for black drum confirm earlier reports that denaturation during frozen storage is accompanied by a loss in solubility of myofibrillar proteins (Owusu-Ansah and Hultin, 1986; Borderias et al., 1982; Shenouda, 1980; Sikorski et al., 1976), and loss of functional properties (Acton et al., 1983; Laird and Mackie, 1981; Suzuki, 1981; Grabowska and Sikorski, 1976).

SDS-Polyacrylamide gel patterns of black drum salt-extractable proteins give a basic understanding of molecular events occurring in proteins during frozen storage.
With increasing time of storage, the myofibrillar proteins that were readily extractable in salt solution decreased, indicating that protein alterations have occurred. The most obvious change was the disappearance of the high-molecular proteins. Protein complexes arising from the aggregation of the high-molecular weight components that were too large to enter the polyacrylamide gels, might have been formed. A different pattern was obtained for the proteins extracted by the salt solution from frozen spotted seatrout (Chapter 3, Fig. 3-1). The patterns showed that as the time of frozen storage increased, the amount of material at the top of the gel increased, indicating accumulation of very high molecular components. The same phenomenon would have occurred with frozen black drum muscles, however the aggregates probably became inextractable in the salt solution. This reduction in solubility of the myofibrillar proteins is associated with the decrease in solubility and loss in functional properties of the muscles during frozen storage.

Differential protein extraction was conducted to identify some of the bonds involved in protein insolubilization during frozen storage. Before attempting to discuss the role of the reagents in protein extraction, it is helpful to look at the nature of the bonds that stabilize protein structures. The covalent peptide bond is the important bond in primary structure of protein. This bond is not broken in protein denaturation. The secondary
structure of a protein is stabilized by hydrogen, hydrophobic and electrostatic bonds. Individual hydrophobic bonds are weak, but collectively the numerous hydrophobic bonds are an important stabilizing factor in maintaining the secondary, tertiary and quaternary structures of a protein. The types of bonds involved in maintaining the tertiary structures of proteins are the covalent disulfide bonds. The quaternary structures of proteins are stabilized by the same types of bonds as for the tertiary structure. It has been postulated that during frozen storage, various forms of cross-linking reactions, both inter- and intramolecular, occur in myofibrillar proteins, particularly in the myosin. These crosslinks are believed to be non-covalent but evidences presently available indicate that covalent bonds could be involved. If the crosslinks formed during frozen storage are of non-covalent origin, then the proteins from the frozen muscles will completely dissolve in solutions of hydrogen and other secondary-bond breaking substances such as sodium dodecyl sulfate (SDS). Data presented in Fig. 4-2 show that with both 3 and 6 week frozen storage periods, a slight decrease in proteins extracted with SDS alone occurred. The inference drawn from these results could be that intermolecular covalent crosslinks are present in the frozen stored muscle. This observation was also reported by Laird and Mackie (1981) who, in their investigation of deteriorative changes during frozen storage of cod,
indicated that some covalent bond formation can occur. Sikorski (1978) also revealed that the formation of different types of new covalent crosslinks in the proteins of frozen stored fish cannot be ruled out, as the creation of a small number of SDS-resistant intra- or intermolecular crosslinks may not necessarily lead to significant desolubilization. The observed increase in the solubility of the proteins in solutions containing 0.5% ME also gives an indication that disulfide bonds are involved during aggregation of the proteins in frozen stored muscles. This observation confirmed the results presented by Buttkus (1970, 1971) in which he successfully resolubilized aggregated myosin with a solution containing both 6 M guanidine-HCL and either 0.5 M mercaptoethanol, 0.3 M sodium sulfite or 0.3 M sodium cyanide. In another experiment by Tsuchiya and co-workers (1980), different extraction solutions were used to test for the different types of bonds in crosslinked muscle protein. It was concluded that denaturation and/or insolubilization of actomyosin and myosin during frozen storage was a result of aggregation caused by hydrogen, electrostatic, hydrophobic and disulfide bonds. The disulfide splitting compound ME had a greater effect when the primary extractant was SDS than when buffered salt solution was used. An explanation for this occurrence could be that in the presence of SDS, either the proteins disaggregated or individual proteins unfolded such that the ME had access to the disulfide
bonds. With the splitting of the disulfide bonds, protein extractability was therefore, further increased.

Electrophoretic profiles for the proteins extracted by the different reagents gave similar results. Decreases occurred in the protein components with increasing time of frozen storage. Comparison of Figs. 4-5 and 4-6 suggest that a decrease in protein extractability in the salt solutions may be due to formation of non-covalent bonds. These non-covalent linkages are dissolved in SDS solutions as revealed by the relative increase in protein components extracted from stored muscles.

4.4.2 Effects of freeze-thawing cycles on black drum muscles.

Results for water-binding capacity, expressible moisture and salt-extractable protein during freeze-thawing cycles are indicative of the conformational changes that occurred in black drum muscles during freeze-thawing processes.

The flesh of fresh fish was soft to the touch and the tissue was able to retain water. With subsequent freezing and thawing, the flesh became firmer and the tissue was unable to retain water to the same degree as the original fresh samples. The excess fluid was released as 'drip'. During freezing, ice crystals are formed in the tissue and their size, shape and extra- or intracellular position depend on rate of freezing and whether or not the flesh is frozen pre- or post-rigor (Howgate, 1979). Howgate (1979)
further indicated that on thawing, the fibers resorbed the melt water completely or nearly so, regaining their original shapes, though fissures were left as evidence of the existence of ice crystals. Observations gathered in this study indicated that with increased freeze-thaw cycles, certain functional properties were greatly reduced. The muscles lost their ability to retain their bulk phase water during the application of stress.

Reductions in the solubility of the proteins in salt solution with increased freeze-thaw cycles are indicative of protein alterations. Sikorski (1978) reported that freezing-induced alterations of muscle proteins are manifested not only by formation of intra- and intermolecular crosslinks, but also by modification of enzyme activity. Freezing and thawing of bovine, porcine, poultry and fish muscles has been found to liberate into the extract several isozymes. The liberated enzymes subsequently increase the rate of alteration of the myofibrillar proteins during freeze-thawing cycles. The extensive reduction of salt-extractable proteins and the different protein components on electrophoretic gels (Fig. 4-8) with increased freeze-thaw cycles could therefore be attributed to both aggregation of the myofibrillar proteins and to increased enzymatic activity. Muscles frozen for 6 weeks did not show such extensive reduction in salt-extractable protein components due to the fact that long-term frozen
storage results in significant decrease in activity of many enzymes.

Extraction of proteins using different solvents revealed a decreasing trend of extractability in the buffered salt solutions as freeze-thaw cycles increased. The increase in protein yield with 0.5% ME indicates that disulfide bonds are probably involved in protein denaturation during freeze-thawing. The use of SDS did not significantly increase protein solubility. This could be attributed to the fact that most of the bonds formed during subsequent freezing and thawing have been dissolved by the disulfide breaking reagent. The effectiveness of ME in improving protein solubility has been demonstrated if used in combination with SDS solution. SDS has the ability to disaggregate or unfold individual proteins by acting on their non-covalent bonds. With this unfolding or disaggregation, the ME would have a greater access to the disulfide linkages. With the splitting of the disulfide bonds, protein extractability was markedly increased. The same results were gathered when the protein extracts were subjected to SDS-Polyacrylamide gel electrophoresis. The solubility of the proteins in buffered salt solutions slightly decreased with increasing freeze-thaw cycles. However, solubility in the SDS solutions showed a very slight decrease, or none at all, with increased freeze-thawing cycles. Results from this investigation suggest that both hydrophobic interactions and disulfide bond
formation are involved in the loss of protein extractability during freeze-thawing cycles. It appears, however, that disulfide bond formation has a greater effect, which is in contrast to frozen storage where hydrophobic interactions have greater impact.

4.4.3 Effects of freeze-thawing on mullet muscles.

Results for the freeze-thawing study on mullet muscles revealed that with three freeze-thaw cycles, functional properties, such as water-binding capacity and expressible moisture, are significantly affected. The pH, solubility of the salt-extractable proteins and extractability of different protein components on electrophoretic gels were not significantly different. Furthermore, a greater quantity of protein was extractable with mullet than with black drum. These observations demonstrate that proteins of mullet muscles are fairly stable for up to three freeze-thaw cycles. Although electrophoretic patterns were similar to black drum, it appeared that mullet was more stable to freeze-thaw cycles than black drum. This apparent stability of mullet muscles proteins to freezing-induced alterations may be attributed to the presence of intact lipids. The effect of fish lipids varies according to the state of the lipid. It has been demonstrated that the products of lipid oxidation cause the fish protein to become harder and more elastic, forming insoluble complexes (Takama, 1974a; Takama et al., 1972). There has been some
evidence, however, that the presence of intact lipid is essential and could play a protective role for the proteins (Andou et al., 1979; Taguchi and Ikeda, 1968a, 1968b; Dyer and Dingle, 1961; Dyer, 1951). The effect of oxidized lipid was minimized during the course of the experiment since the muscles were vacuum packed in polyethylene bags with high oxygen barrier properties and thawing was conducted in a refrigerator. Thus, perhaps the higher lipid content of mullet (data not shown) contributes to freeze stability. Further confirmation of this possibility is needed in future studies.
4.5 SUMMARY AND CONCLUSIONS

The possibility for adverse changes brought about by frozen storage and freeze-thawing cycles have been demonstrated by this study. Denaturation of proteins during frozen storage is accompanied by loss in solubility of myofibrillar proteins, decrease in water-binding capacity and increase in expressible moisture. The loss in the extractability of the protein components with increased frozen storage were caused by the disappearance of the high-molecular weight components, probably due to aggregation. Inferences drawn from differential protein extraction procedures revealed that aggregation of proteins during frozen storage was potentially due to the formation of hydrogen, hydrophobic and disulfide bonds. The diminution of the proteins in the SDS solutions suggests that intermolecular covalent crosslinks could also be formed during frozen storage. Results from this study would indicate that hydrophobic interactions are more important in frozen storage, whereas disulfide linkages have greater influence as a result of increased freeze-thaw cycles.

The freeze-thawing experiments on black drum and mullet muscles showed marked alterations in the water-holding capacity, expressible moisture, protein solubility and extractability of the different protein components on electrophoretic gels. No significant changes were observed
in protein extractability after three freeze-thaw cycles, however, increasing the number of cycles to five or greater resulted in an extensive loss in this property. The extensive reduction in solubility of the different protein components with increased freeze-thaw cycles can be attributed to both aggregation of the myofibrillar proteins as well as to the increased activity of the enzymes liberated into the extract during the thawing process. The type of bonds involved in protein denaturation during freeze-thawing operations were identified during the differential protein extraction as hydrophobic and disulfide bonds.

Changes in the proteins of black drum muscles during frozen storage and freeze-thawing cycles did not appear to differ significantly in nature from other finfish species. Such changes probably comprise both partial deconformation of the native molecules and creation of new bonds leading to the formation of aggregates, loss of solubility and deterioration of functional properties.

Mullet, under the conditions of this experiment appeared to have greater freeze stability than other finfish species.
CHAPTER 5

CHARACTERIZATION OF SALT-EXTRACTABLE PROTEINS DURING FROZEN STORAGE, FORMALDEHYDE ADDITION AND FREEZE-THAW CYCLES IN FINFISH BY HIGH-PERFORMANCE GEL PERMEATION CHROMATOGRAPHY

5.1 INTRODUCTION

There is a progressive deterioration in organoleptic and other physical and chemical properties of fish muscles during frozen storage. Deterioration is due to conformational changes in proteins leading to a loss of extractability in the myofibrillar proteins, changes in enzyme activity and loss of functional properties such as water-holding capacity, ease of emulsification and the ability to form gels (Laird and Mackie, 1981; Suzuki, 1981; Sikorski, 1978; Connell, 1964).

The most popular tests used to study the changes in muscle proteins during frozen storage are related to the loss in solubility or the loss in extractability of total proteins or of particular groups of proteins (myofibrillar, sarcoplasmic or the actomyosin group), or even of specific protein, such as myosin, tropomyosin or actin (Shenouda, 1980). Changes in the chemical properties of the extracted fish proteins give more extensive information on the changes that have occurred at the molecular level during frozen storage. These are accomplished by methods such as ultracentrifugal sedimentation, isoelectric focusing, electrophoresis or chromatographic separation. It is with
the application of chromatography that the present study was conducted.

The development of the high-performance liquid chromatography (HPLC) during the past decade has brought a surge of new discoveries in food chemistry and biochemistry. Separation of proteins is one of the most challenging areas in modern liquid chromatography and is currently receiving great attention (Laurent et al., 1983). In fact, during the past years, numerous reports have described HPLC procedures for separation of complex protein mixtures, isolation of enzymes and antigens and determination of protein molecular weights (Regnier and Gooding, 1980; Gruber et al., 1979; Ui, 1979; Mathes and Engelhardt, 1977). HPLC methods have been successfully employed in determination of native and denatured milk proteins (Gupta, 1983), separation of major milk proteins (Bican and Blanc, 1982), separation of wheat gluten proteins (Bietz, 1984) and analysis of histidine dipeptides in fresh meat (Carnegie et al., 1983). However, HPLC methods have not yet been developed for separation of salt-extractable fish proteins.

The purpose of this work was to investigate the feasibility of using high-performance gel permeation chromatography for separation of salt-extractable proteins from spotted seatrout and mullet muscles during frozen storage following formaldehyde addition and freeze-thaw cycles.
5.2 MATERIALS AND METHODS

5.2.1 TEST SAMPLES

Finfish species from the Gulf of Mexico, spotted seatrout (*Erisson nebulosus*) and mullet (*Mugil cephalus*), were used in the study. The fish were purchased from commercial sources and transported to the laboratory on ice. Spotted seatrout were obtained as fillets while mullet was obtained in-the-round.

Upon receipt at the laboratory, spotted seatrout was randomly divided into two groups, one for the frozen storage study and the other for the formaldehyde addition study. Spotted seatrout fillets for the frozen storage study were washed, vacuum packaged (150 g each) in polyethylene bags and frozen in an air blast freezer at \(-20^\circ\text{C}\). During sampling and testing, two bags of fish were thawed by holding in a refrigerator at 4-6°C overnight prior to analyses. Extractions of salt-extractable protein for chromatographic separation were conducted at 0, 3 and 6 weeks.

Mullet for the freeze-thaw study were eviscerated, beheaded, filleted by hand, washed, vacuum packaged (150 g each) in polyethylene bags and frozen in an air blast freezer at \(-20^\circ\text{C}\). The muscles were subjected to different freeze-thaw cycles (0, 1, 3) by freezing for 24 hours and thawing in a refrigerator at 4-6°C overnight. Extractions
for salt-extractable protein were conducted after each freeze-thaw cycle.

5.2.2 METHODS

Salt-extractable protein extraction

Salt-extractable protein was determined extracting one gram of sample with 15 ml of ice-cold 15% NaCl solution. The mixture was vortexed for 5 min and the pH was adjusted to 7.0-7.5 using 2N NaOH or HCl. The mixture was further vortexed for another 30 min before centrifuging at 3,000 rpm for 20 min using an IEC centrifuge (Model CU-5000) at room temperature. The supernatant was filtered through a 0.45 um Millipore filter prior to injection.

High-Performance Gel Permeation Chromatography

Chromatography. The chromatographic equipment consisted of a Liquid Chromatograph (Varian, Model 5000) with two solvent reservoirs, a reciprocating pump (processor controlled, single-piston), solvent-delivery system and a sample injector. Detection of the proteins was performed at 280 nm using a UV/VIS Detector (Varichrom Model VUV-10) interfaced with a portable chromatography data system (CDS 1111L). Chromatograms were printed on a strip chart recorder (Varian, Model 9176).

A Bio-Sil TSK 250 (300 X 7.5 mm) gel permeation column packed with hydrophobic bonded phase silica purchased from
Bio-Rad (Richmond, CA) was used for the separation of the proteins.

**Materials.** A mixture of Vitamin B-12, myoglobin, ovalbumin, gamma globulin and thyroglobulin (Bio-Rad, Richmond, CA) with molecular weights of 1,350; 17,000; 44,000; 158,000 and 670,000, respectively, was used as molecular weight markers. Water for buffer preparation was treated with ion-exchange resins and carbon filters after distillation. The buffer solution was filtered and degassed through a 0.45 μm cellulose acetate filter prior to use with an all-glass filtration unit (Millipore).

**Separation of proteins.** The standard mixture was dissolved in 2 ml HPLC-grade water. The column was equilibrated with the mobile phase (0.05 M Na₂SO₄ - 0.02 M NaH₂PO₄, pH 6.8) for 2 hrs, after which 10 ul of the standard mixture was introduced into the column. The extracts obtained from the salt-extractable protein determination were filtered using a 0.45 μm filter prior to injection. The flow rate used was 1.0 ml/min with an accompanying pressure of 28-34 atm. Retention time and areas under each peak were determined by the computing integrator.
Results for the chromatographic separation of the standard protein markers are presented in Fig. 5-1. The elution profiles of the salt-extractable proteins obtained from spotted seatrout muscles under varying frozen storage times are shown in Fig. 5-2. The protein components detected were below the molecular weight range of the protein standards. It was observed that the amount of lower molecular weight components (MW < 12,000) increased with prolonged storage at -20°C. No proteins of higher molecular weights were detected. The elution profiles of the salt-extractable proteins extracted from spotted seatrout muscles with varying FA levels indicate that formaldehyde slightly decreased the extractability of the lower molecular weight protein components in the fish muscles stored for 3 weeks (Figs. 5-3). Storage of the FA-treated muscles at -20°C for 6 weeks resulted in more pronounced reductions in solubility of the different protein components (Fig. 5-4). Fig. 5-5 shows that no apparent change occurred in the lower molecular weight protein components of mullet muscles even after three freeze-thaw cycles.
Fig. 5-1. Retention times of standard protein markers as detected by high-performance gel permeation chromatography. Peaks: a & b) protein aggregates and thyroglobulin, MW 670,000; c) gamma globulin, MW 158,000; d) ovalbumin, MW 44,000; e) myoglobin, MW 17,000; and f) Vit. B-12, MW 1,350.
Fig. 5-2. Elution profiles of salt-extractable proteins from spotted seatrout during frozen storage at -20°C for: top) 0; middle) 3; and bottom) 6 weeks as determined by HPGPC.
Fig. 5-3. Elution profiles of salt-extractable proteins from spotted seatrout during frozen storage at -20°C for 3 weeks with the addition of: top) 0; middle) 300; and bottom) 500 ppm FA as detected by HPGPC.
Fig. 5-4. Elution profiles of salt-extractable proteins from spotted seatrout during frozen storage at -20°C for 6 weeks with the addition of: top) 0; middle) 300; and bottom) 500 ppm FA as detected by HPGPC.
Fig. 5-5. Elution profiles of salt-extractable proteins from mullet muscles at varying freeze-thaw cycles: top) 0; middle) 1; and bottom) 3 freeze-thaw cycles.
5.4 DISCUSSION

The elution profiles obtained from high-performance gel permeation chromatography (HPGEC) of the salt-extractable proteins showed an increase in low molecular weight components (< 12,000) with increasing storage period. No proteins of higher molecular weights were detected. These results cannot be compared with those obtained for electrophoresis (Chapter 3) since the proteins were of different molecular weight range. Further experiments would be necessary before attempting to draw any firm conclusion from these figures. However, they do indicate that during frozen storage, low molecular weight degradation products are formed. Numerous studies have been conducted on protein changes of skeletal fish muscles during frozen storage, however, no work has been reported on the measurement of low-molecular weight degradation products. This test can give an indirect indication of certain reactions that occur in other protein components during protein denaturation.

The insolubilizing effect of formaldehyde on proteins was clearly shown in this study. The mechanism by which formaldehyde affects proteins at the molecular level was postulated from the fact that formaldehyde has the ability to bind covalently and/or non-covalently to various functional groups in the protein and hence would cause a conformational change accompanied by cross-linking between
the protein peptide chains via methylene bridges (Shenouda, 1980; Gill et al., 1979; Kostuch and Sikorski, 1977; Sikorski et al., 1976; Castell et al., 1976; Castell, 1971; Tokunaga, 1964; Walker, 1964; Frankel-Conrat and Olcott, 1948). The reaction of formaldehyde with lower molecular weight proteins supports earlier work which indicated that formaldehyde can cause polymerization as well as react with small molecular compounds in muscle tissue (Owusu-Ansah and Hultin, 1984; Ohnishi and Rodger, 1979).

The mullet muscles subjected to various freeze-thawing cycles did not show significant changes in the lower molecular weight protein components. This suggests that the proteins were stable up to three freeze-thaw cycles, with the muscles probably retaining their important functional characteristics. This apparent stability could be ascribed to the pH which was greater than 6 for mullet (Chapter 4). A higher pH is desirable in maintaining muscle texture (Eskin et al., 1971). The presence of intact lipids may also play a protective role against muscle denaturation.

High-performance gel permeation chromatography (HPGPC) of proteins is frequently used for separation and molecular weight determination. Theoretically, HPGPC is the simplest and most predictable chromatographic method. Solute are separated by size with the large excluded molecules eluting first and the small, totally included molecules eluting last. In practice, however, many extraneous mechanisms
such as adsorptive, hydrophobic and ionic effects may affect the retention of a solute (Regnier and Gooding, 1979). During the chromatographic separation of the salt-extractable proteins, only a small number of peaks were obtained. This could have been caused by the interaction between the higher molecular weight proteins and the chromatographic packing material allowing only the lower molecular weight components to pass through the column. Hydrophobic binding predominates when the mobile phase is of high ionic strength, whereas electrostatic interactions are maximal at low ionic strength. The use of sodium chloride, potassium chloride, SDS or guanidine hydrochloride in the mobile phases can minimize such effects, however, these substances may interfere with subsequent analytical procedures and are often difficult to remove (Regnier, 1983). Natural polypeptides are structurally very complex. With all the possible combinations of amino acids and sequences, molecular shape and hydrodynamic volume may vary widely between molecular species of the same molecular weight. It is, therefore, inevitable that some proteins will interact with any given support since the physical and chemical properties vary so widely. Because of the problems that arose during the course of the chromatographic separation, it is important to identify the mode of interaction and possibly to eliminate it. A search for a more suitable mobile phase is also recommended in order to attain an ideal size-exclusion process where there
is minimal interaction occurring between the macromolecules and the chromatographic packing material.
5.5 SUMMARY AND CONCLUSIONS

Elution profiles obtained by high-performance gel permeation chromatography indicate that during frozen storage, low molecular weight degradation products are formed. Formaldehyde renders the low molecular weight proteins less extractable causing a loss in the solubility of these components. Significant change was not detected in the amount of the lower molecular weight protein components after three freeze-thawing cycles.

High-performance gel permeation chromatography proved to be a useful tool for the preliminary characterization of proteins from fish skeletal muscles. However, interaction between the macromolecules and the packing material is inevitable because of the complex structural nature of polypeptides. It would therefore be beneficial to identify the mode of interaction and possibly eliminate it in order to attain an ideal size-exclusion chromatography system for the analysis of muscle proteins.
CHAPTER 6
GENERAL SUMMARY AND CONCLUSIONS

The changes observed in solubility and functional properties of proteins in spotted seatrout, black drum and mullet during frozen storage, formaldehyde addition and freeze-thawing operations appear not to differ from other finfish species. Muscle proteins are composed of natural polypeptides that are structurally complex. With all the possible combinations of amino acids and sequences, interactions between proteins and with other factors are inevitable. Several factors may influence these deteriorative changes that comprise both partial deconformation of the native molecules and creation of new bonds leading to formation of aggregates, loss of solubility and reduction of functional properties. The formation of hydrophobic, ionic, disulfide and hydrogen bonds in denatured muscle proteins are suspected. The formation of covalent cross-links in the proteins of frozen stored fish also cannot be ruled out as shown by the observed loss of extractability in SDS solutions.

In such complex structures as muscle foods, it is not possible to precisely predict the course of events that take place under given conditions. However, with the experimental evidence presented in this study, it is feasible to predict the extent and intensity of the changes, knowing the state of the product and the para-
meters of freezing and storage. By applying proper procedures, much of the deteriorative changes caused by different factors can be avoided.

Further knowledge of the effect of muscle protein changes on textural properties is needed. The action of intact and oxidized lipids requires thorough investigation. Further work should be conducted to effectively separate the salt-extractable proteins using high-performance gel permeation chromatography. The significance of these results could aid in understanding the complex nature of the reactions leading to protein denaturation-aggregation and in the application of the most suitable protective measures for fish products preserved by freezing.


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Major Field: Food Science

Title of Dissertation: Studies on the Protein Changes in Skeletal Muscles of Gulf Coast Finfish Species Associated with Frozen Storage, Formaldehyde Addition and Freeze-Thawing Cycles

Approved:

Major Professor and Chairman

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