Chapter 1

Introduction

Surimi is minced fish washed with water, rinsed and dewatered to remove sarcoplasmic proteins and to increase concentration of myofibrillar proteins (Sych *et al.*, 1991). Surimi can be used for the manufacture of the traditional fish gel products, such as kamaboko, tempura, and chikuwa, which are very popular in Asia markets because of their unique textural properties (Jiang *et al.*, 2000). During frozen storage of fish muscle, denaturation and/or aggregation of myofibrillar proteins occur, causing a loss of functional properties (Sultanbawa and Li-Chan, 1998) such as gel-forming ability, water holding capacity, and solubility (Sych *et al.*, 1991; Park *et al.*, 1988). Therefore, freezing of surimi is done commercially by incorporation of 4% sucrose, 4% sorbitol, and 0.2% polyphosphates to protect fish myofibrillar proteins during extended periods of frozen storage (Sultanbawa and Li-Chan, 1998).

However, sucrose and sorbitol imparts a sweet taste to surimi product which may be undesirable to the consumer. So far, consumers have been conscious of caloric content, and low caloric cryoprotectants in surimi are more preferable (Sych *et al.*, 1990; Sultanbawa and Li-Chan, 1998). Thus, interest has focused on identifying other cryoprotectants with reduced sweetness. Many efforts to find non-sweet additives with cryoprotective protects equivalent to commercial cryoprotectant or can be incorporated in surimi at equal or less than 8% (w/w) have been intensively paid (Auh *et al.*, 1999; Sultanbawa and Li-Chan, 2001). Trehalose is about 45% as sweet as sucrose (Neta *et al.*, 2000) and has been regarded to stabilize proteins and biological cells under freezing condition. Thus, trehalose would be one of effective cryoprotectants for surimi, which does not cause the

sweetness problem in surimi and its products. However, no information regarding the use of trehalose in surimi has been reported.

Literature Review

Fish muscle is functionally close correspondence in the relative amounts of the main proteins of skeletal muscle. Myofibrillar proteins in white muscle of fish represent 65-75% of total proteins (Mackie, 1984) and are the largest fraction of proteins of muscle tissue. They influence the meat's culinary and commercial properties because of their high water binding capacity and emulsifying capacity. Properties and possible implications of the structure and composition of myofibrillar proteins are related with meat quality (Pomeranz, 1991). The interactive properties of myosin and actin are of particular importance in determining functional properties of protein from fish, especially gel-forming ability (Mackie, 1994).

1. Myofibrillar proteins

- Myosin

Myosin is the major protein with the thick filaments and comprises 45% of the myofibrillar proteins. It is an elongated protein molecule about 160 nm in length with a molecular mass of approximately 480,000 D. Myosin contains a total of six polypeptide chains, two heavy chains and four light chains. Myosin heavy chains have "head" and "tail" regions, reflecting the respective globular and rod portions of the molecules (Figure 1) (Foegeding *et al.*, 1996).

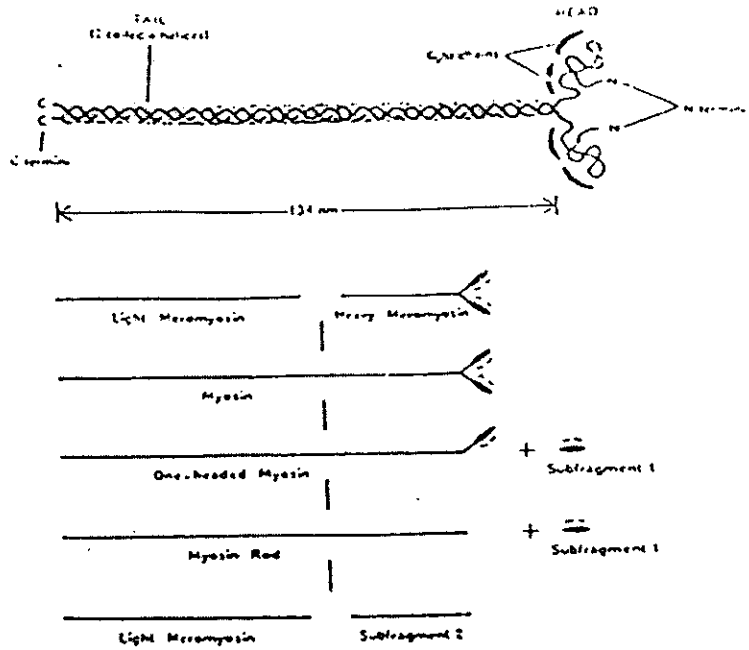

Figure 1. Structure of myosin.

Source: McCormick (1994)

The six polypeptide chains of myosin are assembled in a quaternary structure that resembles a stick (tail) with two pear-shaped heads. The tail regions consists of two alpha-helical heavy chains coiled together into a coiled-coil alpha-helical supersecondary structure. This structure terminates at the head region. The main secondary structure in the head is alpha-helix, accounting for approximately 48% of amino acid. The myosin head contains the actin binding site, ATPase site, alkali light chain site, and DTNB [(5,5-dithiobis)-2-(nitrobenzoic acid)] light chain site. The light chains bind to the alpha-helical regions of the heavy chain. The tail portion of the heavy chain molecule is responsible for its association into thick filaments. (Foegeding *et al.*, 1996)

Myosin ATPase activity is stimulated by Ca^{2+}. The activity reaches its maximum with 3-5 mM Ca^{2+}. This activity is solely due to myosin alone, and thus is not essentially affected by the presence of actin (Ochiai and Chow, 2000). Ca^{2+}-

ATPase activity is a good parameter to estimate the quality or the extent of deterioration of protein in muscle food (Matsumoto, 1980; Huidobro and Tejada, 1994). Myosin ATPase is also largely affected by chemical modification of reactive SH residues (SH1, SH2). Modification of SH_2 results in inactivation of Ca^{2+}-ATPase (Ochiai and Chow, 2000).

- Actin

The major protein of the thin filaments is actin, which comprises 20% of myofibrillar protein of muscle. Actin's shape can be described as two peanut-shaped domains of equal size lying side-by-side. Actin monomers, called globular actin or G-actin, are assembled in a double-helical structure called fibrous actin, or F-action (Figure 2). G-actin has a molecular mass of 42,000-48,000 D. It is stable in water, where it can also exist as a dimer (Xiong, 1997). Globular actin binds ATP very firmly and, in the presence of Mg^{2+}, spontaneously polymerizes to form F-actin. It also polymerizes in the presence of neutral salts at a concentration of approximately 0.15 M. Filaments of F-actin interact with the head portion of myosin (Foegeding *et al.*, 1996).

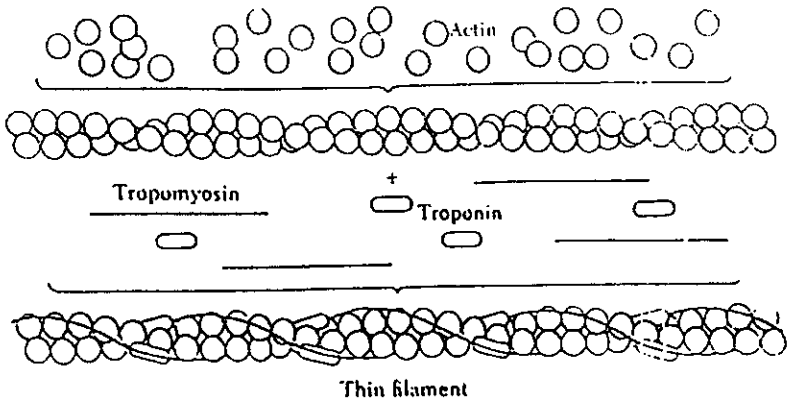

Figure 2. Structure of actin, troponin and tropomyosin.

Source: Foegeding *et al.* (1996)

- Actomyosin

When actin and myosin are mixed *in vitro*, a complex, called actomyosin, is formed. This complex can be dissociated by addition of ATP. Actomyosin is the main state of actin and myosin in postmortem muscle because ATP is depleted by postmortem metabolism (Ochiai and Chow, 2000). However, unlike prerigor muscle, simple addition of ATP and other solubilizing compounds such as Mg^{2+} to meat does not dissociate all the myosin from actin, and extraction of myosin from postrigor meat is therefore difficult. Myosin and actomyosin are found in extracts of postmortem muscle (Foegeding *et al.*, 1996).

The dissociation constant for actin and myosin is 10^{-8} to 10^{-7} M. The actomyosin specifically dissociates with ATP, pyrophosphate, and other polyanions. The higher the ionic strength, the less ATP is required. Mg^{2+} is also required for the dissociation (Ochiai and Chow, 2000).

- Tropomyosin

Tropomyosin, representing 5% of myofibrillar protein, is composed of two alpha-helical polypeptides wound together into a two-stranded, coiled-coil supersecondary structure. It resembles the tail or rod portion of the myosin molecule. In skeletal muscle two polypeptides, alpha- and beta-tropomyosin, can combine to form a tropomyosin dimer, The alpha-and beta-tropomyosin polypeptides have molecular masses of 37,000 and 33,000 D, respectively. Tropomyosin aggregates end-to-end and binds to actin filaments along each groove of the actin double helix such that each molecule interacts with seven G-actin monomer (Figure 2) (Foegeding *et al.*, 1996).

- Troponin

Troponin, accounting for 5% of myofibrillar protein, consists of three subunits designated troponin C (for calcium binding), troponin I (for inhibitory), and troponin T (for binding with tropomyosin). Each subunit of troponin has distinct functions. Troponin C is a calcium binding protein and confers calcium regulation to the contractile process via the thin filament (Figure 2) (Foegeding *et al.*, 1996).

2. Functional properties of fish proteins

Functionality is an expression of the physicochemical properties of proteins as modified by environmental conditions (Tanabe and Saeki, 2001). Fish meat is an abundant protein resource that is widely used in food product. Fish myofibrillar protein has excellent functional characteristics such as emulsifying properties, gel-forming ability, and water holding capacity (Tanabe and Saeki, 2001; Lin and Park, 1996). Many kinds of seafood products, and as surimi-based products, take advantage of the functional properties of fish myofibrillar protein. However, fish myofibrillar protein is thermally and chemically less stable than that of other

vertebrates (Yamashita *et al.*, 1978; Hashimoto *et al.*, 1982) and its functional properties are lowered when protein denaturation occurs (Regenstein *et al.*, 1983). Since the solubility in high ionic strength media of the protein is impaired with the progress of protein denaturation, the solubility of muscle proteins in salt solutions has been used to assess the quality of fish meat (Regenstein *et al.*, 1983; Akahane *et al.*, 1984). The relative importance of each functional property varies with the product, processing method, and stage of processing (Xiong, 1997; Smith, 1988). Additionally, the functional properties of proteins are governed by primary, secondary, tertiary and quaternary structural components of the molecules (Pomeranz, 1991).

- Water holding capacity

Myofibrils are composed of 25% protein and 75% water. Therefore, the majority of water in meat is confined within the myofibrils in the spaces between the myosin and actin (Xiong, 1997). Myofibrils are the primary site for intracellular water. Other cellular components may also contribute to water-binding in the meat. Entrapment and mobility of the water present outside the cell could be affected by many environmental factors, and water content varies with different processing treatments (Xiong *et al.*, 2000). There are two major types of forces that contribute to water retention in meat: polarity, including surface charges, and capillary effects. Binding of water to the surface of protein through hydrogen bonds between water molecules and charges and dipolar amino acid residues seems to be insignificant for water retention in meat. Any change in the surroundings of myofibrils that results in increased protein charges or dipoles (high concentrations of salt and pH away from the protein isoelectric point) would lead to increase water retention in meat (Xiong, 1997).

- Solubility

Solubility of proteins is of a primary importance for the manufacture of processed muscle foods, including comminuted, restructured, and formed meats (Xiong, 1997). Most functional properties of muscle proteins are related to protein solubility (Lin and Park, 1996). The solubility of a protein under a given set of environmental conditions is the thermodynamic manifestation of the equilibrium between protein-protein and protein-solvent interactions (Lawrence *et al.*, 1986). It is related to the net free energy change arising from the interactions of hydrophobic and hydrophilic residues of the protein with the surrounding aqueous solvent. The lower the average hydrophobicity and higher the charge frequency, the higher the solubility is obtained (Damodaran, 1996). Decrease in solubility during frozen storage and loss of ATPase activity was described for myosin in frozen fish (Li-Chen *et al.*, 1985). The textural properties depend largely on protein solubility, especially in the case of fish (Colmenero and Borderias, 1983).

- Gel-forming ability

Gelation of myofibrillar proteins has been shown to be largely responsible for the textural properties of processed fish product (Xiong and Brekke, 1989). Generally, myosin alone forms excellent gels. Actin has a synergistic or antagonistic effect on myosin gelation, depending upon the myosin/actin ratio in the gelling system (Grabowska and Sikorski, 1976; Matsumoto, 1979). Gel-forming ability of frozen surimi is the most important functional requirement of imposing good quality on surimi-based products (Saeki *et al.*, 1995). Myosin has been reported to be an important protein mainly responsible for surimi gel formation (Samejima *et al.*, 1981). Differences in cross-linking of myosin heavy chain contribute to the differences in gel-forming ability among the muscles of various fish (Benjakul *et al.*, 2001). Protein gels may be visualized as three-dimensional matrixes or network in which water is entrapped (Pomeranz, 1991). According to Benjakul *et al.*, (2001),

thermal gelation of fish muscle has been reported to occur in a three-step process including:

1. Dissociation of myofibril structures by protein solubilization in the presence of salt.
2. Partial unfolding of protein structure caused by heat treatment.
3. Aggregation of unfolded protein via both covalent and non-covalent bonds to form a three-dimensional network.

A gel network is formed by cross-linking of actomyosin with the aid of both hydrogen and hydrophobic bonds, and water is retained within the network. Hydrogen bonding is primarily involved in setting at low temperature, while hydrophobic bonding dominates in subsequent gel heating at high temperature (Lee, 1984).

Surimi is highly concentrated with myofibrillar proteins, primarily actomyosin, which is solubilized by salt during chopping. The solibilized protein sol, or paste, gels upon heating (Lanier, 2000). The gel-forming ability, measured by the water-binding capacity of the comminuted tissue (protein solubilization) and gel strength are determined by the level of functional actomyosin (Morrissey *et al.*, 1993). The level of functional actomyosin, measured as extractable actomyosin or ATPase activity increase in the number of washing cycle and decrease as the freshness of the fish decreases (Lee 1984). In addition, the quality of surimi during frozen storage is also affected by storage temperature, storage period, the level of remaining moisture, and the type and level of cryoprotectants used (Reppond and Babbitt, 1997). Regenstein (1986) reported the use of various sugars, salts of organic acids, and other compounds to prevent the loss of gelling properties in the frozen fish or fish product.

3. Denaturation of surimi proteins during freezing/ frozen storage

Freezing is generally the best method for achieving long-term preservation of fish. After prolonged storage of fishery products at -20^{0}C or above, significant undesirable sensory changes take place (Tomaniak *et al.*, 1998). During frozen storage of fish and fish products, the losses of quality generally occur. The growth of ice crystals causes osmotic removal of water, denaturation and/or conformational changes of myofibrillar proteins (Benjakul and Bauer, 2000), resulting in a loss of functional properties and deteriorative changes of frozen stored fish muscle (Sych *et al.*, 1990b). During freezing process and frozen storage, fish muscle can undergo a number of changes, such as denaturation and aggregation of muscle proteins. Those changes an associated with a hard, dry and fibrous texture (Reynolds *et al.*, 2002; Barroso *et al.*, 1998). The degree of protein denaturation is influenced, by many factors such as treatments before freezing, freezing rate, storage temperature and thawing method (Jiang and Lee, 1985).

- Effect of freezing and frozen storage on muscle proteins

Freezing causes certain unfavorable change in meat quality. Ice occupies a greater volume than water, and the exclusion of solutes from ice-crystals causes an increase in the ionic strength in unfrozen water. These phenomena cause a loss in tissue structure and a partial denaturation of some muscle proteins, in turn reducing protein solubility and gelation capacities (Sultanbawa and Li-Chan, 2001). The solubility of protein was decreased after 25 weeks of frozen storage at -18^{0}C by 50% in pork and by 36% in beef (Tomaniak *et al.*, 1998).

Frozen-storage induced myosin aggregation and consequent loss of gelling capacity of fish muscle. The globular heads of myosin are responsible for its enzymatic (ATPase) activity, which is sensitive to changes in the configuration of the molecule around the enzymatic site (Sultanbawa and Li-Chan, 2001). However, loss

in ATPase activity is not necessarily synonymous with frozen-storage-induced aggregation of myosin (Ramirez *et al.*, 2000). Sulfhydryl (SH) groups are considered to be the most reactive functional group in proteins, being easily oxidized to disulfide (SS) groups especially during frozen storage of fish (Benjakul *et al.*, 2003). Ramireze *et al.*, (2000) suggested that frozen storage of myosin in suspension results in aggregation involving side-to-side interactions of the rod with low formation of disulfide bonds; on the other hand, when myosin is solubilized prior to frozen storage, mainly head-to-head interactions with a higher formation of disulfide bonds are implicated in the aggregations. Extrinsic fluorescence probes such as 1-anilinonaphthalene-8-sulfonic acid (ANS) and *cis*-parinaric acid (CPA) have been widely used to study surface hydrophobicity in fish proteins (Careche and Li-Chen, 1997). During extended frozen storage, increase in surface hydrophobicity was observed, suggesting the conformation changes in fashion which hydrophobicity protein was exposed to an aqueous environment (Benjakul *et al.*, 2003). Several hypothesis have been advanced to explain alterations that fish muscle proteins undergo during frozen storage, and these include: (1) partial dehydration of proteins during freezing, (2) changes in the environment of the proteins due to freeze-concentration of inorganic salts, (3) interaction of lipids, free fatty acids and / or lipid oxidation products with proteins, and (4) the action of trimethylamine oxide demethylase (TMAOase) (Parkin and Hultin, 1982). The last mechanism is believed to be particularly important in fish that belong to the gadoid family. TMAO is converted to equimolar quantities of formaldehyde (FA) and dimethylamine (DMA) by TMAOase (Amano and Yamada, 1963; Crawford *et al.*, 1979; Parkin and Hultin, 1982). FA is believed to cause cross-linking of muscle proteins and thereby promote toughening (Ang and Herbert, 1989; Krueqer *et al.*, 1989; Chen *et al.*, 1997). The addition of cryoprotectants such as sucrose, sorbitol, or polydextrose has been proved to reduce the level of FA produced (Herrera *et al.*, 2000; Simpson *et al.*, 1994).

- Effect of freezing and frozen storage on gel-forming ability

Frozen storage, an essential step in the process of surimi production, can bring about detrimental changes in functionality of the surimi material (Wang and Xiong, 1998; Lee and Park, 1998). Most of the surimi-based products are prepared in the cooked form and stored refrigerated or frozen. However, some molded products are frozen uncooked. Consequently, during frozen storage, the products will undergo freeze deterioration in terms of drip loss and development of a spongy texture unless a proper type of cryoprotectant is used (Yoon and Lee, 1990). Sensory changes of frozen fish are due to the formation of secondary oxidation products such as volatile aldehydes and ketones (Refsgaard *et al.*, 1998). The drip occurs, particularly when the products are improperly stored and thawed. Generally, molded products are more susceptible to freeze-thaw changes than fiberized products since the molded ones have a greater matrix volume for ice crystal growth than the fiberized ones. Uncooked products are less stable to freezing than cooked. Thermal gel setting stabilizes the matrix by tightening the protein gel network preventing ice crystal growth from damaging the matrix (Lee, 1986).

- Effect of freeze-thawing on muscle proteins

Freezing and thawing also affect the membrane structure of muscle cells (Refsgaard *et al.*, 1998). Thawing method was found to play an important role in membrane integrity as well as the sensory attributes. Fast thawing rendered a superior quality compared to slow thawing. The thermal property was affected by the rate of thawing. Rapid thawing using a combination of microwaves and tap water resulted in lower thermal stability of prawn protein compared to slow (refrigeration) or moderately fast (tap water) thawing methods (Srinivasan *et al*, 1997). Benjakul and Bauer (2000) reported that the freeze-thaw cycle affected the physicochemical and enzymatic properties of cod muscle protein. The greater the

number of freeze-thaw cycles, the more detrimental effect was found. Protein solubility decreased when the freeze-thaw cycles increased. The solubility was reduced to approximately 60% after five cycles of the freeze-thaw process. (Benjakul and Bauer, 2000).

4. Cryoprotectants for surimi

Cryoprotectants are compounds that extend the shelf-life of frozen foods. The term "cryoprotectant" can be interpreted broadly to include all compounds that aid in preventing change induced in foods or food ingredients by freezing, frozen storage, or thawing (MacDonald *et al.*, 1996). For this reason, surimi requires the addition of a cryoprotective component prior to freezing to ensure long-term stability of the proteins in frozen storage. This in turn assures good functionality of surimi, expressed primarily as gel-forming potential with its manifestations of texture formation and water-binding properties (MacDonald and Lanier, 1991). Besides a variety of carbohydrate compounds, including most of the mono-and disaccharides evaluated and several low-molecular-weight polyols, many amino acids and carboxylic acids were also found to be cryoprotective (Jiang *et al.*, 1987).

- Sugar and polyhydric alcohols

Sugar and polyhydric alcohols have been used for many years as stabilizing agents for the maintenance of the biological activity of macromolecules (Table 1) (Arakawa and Timasheff, 1982). Arakawa and Timasheff (1982) showed that polyhydric alcohols and sugars increased the transition temperature of some proteins in aqueous solution, and the stabilizing action of these substances is due to their induction in water of a decrease in hydrogen bond rupturing potency.

Table 1. Characteristics of sugars and polyalcohols

Name	Sugar type	Subunits	MW (g)	Formula	Total OH number per molecule
Glucose	Monosaccharide	-	180.2	$C_6H_{12}O_6$	5
Sucrose	Disaccharide	Glucose Fructose	342.3	$C_{12}H_{22}O_{11}$	8
Trehalose	Disaccharide	Glucose Glucose	342.2 ([a]378.3)	$C_{12}H_{22}O_{11}$	8
Glycerol	Polyalcohol	-	92.09	$C_3H_8O_3$	3
Xylitol	Polyalcohol	-	152.1	$C_5H_{12}O_5$	5
Mannitol	Polyalcohol	-	182.2	$C_6H_{14}O_6$	6
Sorbitol	Polyalcohol	-	182.2	$C_6H_{14}O_6$	6

[a] Hydrated form $C_{12}H_{22}O_{11}.2\ H_2O$.

Source: Turner *et al.* (2001)

The effects of sugars and polyols on the thermal stability of proteins can be considered in terms of their effects on various forces and interaction (Back *et al.*, 1979).

1. Hydrogen bonding. The endothermic transitions observed in the scanning calorimeter are indicative of the "melting" of a cooperative hydrogen-bonded structure, in which water successfully competes as both donor and acceptor with backbone and side-chain groups in the protein (Arakawa and Timasheff, 1982). The individual hydroxyl groups of sugars and polyols may also compete, but no more effectively than water, as indicated by the minor stabilizing effect of glycerol compared with sorbitol at the same concentration of hydroxyl groups. It is also unlikely that new cooperative structures are formed since transition heats appear to remain the same as in the absence of stabilizer. Specific cooperative bonding with

groups on the outside of the protein molecule, displacing water, has been suggested (Back *et al.*, 1979).

2. Electrostatic interactions. Those sugar or polyol solutions have lower dielectric constants than pure water. Thus, electrostatic interactions should be stronger in these solutions than in water. However, this contribution to the stabilizing effect must be relatively small. Stabilizing effects of sucrose or glycerol on lysozyme are almost the same at pH 7 as at pH 3 suggests that any electrostatic contribution to the stabilizing effect must be minimal (Back *et al.*, 1979).

Hydrophobic interactions. Hydrophobic interactions are generally considered to be the major single factor in stabilizing the three-dimensional structure of proteins. In aqueous-organic mixed solvents, hydrophobic interaction depends on solvent structure. With maximum hydrophobic interaction occurring in those solvent mixtures, the three-dimensional hydrogen-bonded structure of water was developed (Back *et al.*, 1979). The effects of sugars and polyols on hydrophobic interaction and consequently on the thermal stability of proteins should also depend upon how they affect the structure of water (MacDonald and Lanier, 1991). Hydrophobic interactions between pairs of hydrophobic groups are stronger in sucrose or glycerol solutions than in pure water. It seems likely therefore that this is the mechanism by which sugars and polyols in general may stabilize proteins to heat denaturation.

Sugars and polyols interact with water to an extent which depends upon their molecular structure. Glucose behaves differently from sucrose and mannitol behaves differently from sorbitol. Dominant mechanism by which sugars and polyols stabilize proteins to heat denaturation is through their effect on the structure of water, which, in turn, determines the strength of hydrophobic interactions. The differences in response between proteins are most likely related to the protein structure involving contacts between non-polar side chains (Back *et al.*, 1979).

Sugar is one of the main ingredients in surimi-based, not only as a sweetener, but also more importantly as a cryoprotectant to protect fish protein during frozen storage (Ziegler and Foegeding, 1992). When stored at frozen storage conditions, the functional properties of water holding and gel-forming ability become lower than in the fresh fish. These quality changes are due to protein denaturation (Arakawa and Timasheff, 1982).

Noguchi *et al.*, (1976) found that sugars exhibiting marked effect were sucrose, lactose, glucose, fructose, glycerol, and sorbitol. Among the mono-saccharides, pentoses (sylose and ribose) gave a little less protective effect than hexoses (glucose and fructose), and these differences seem to be related to the number of –OH groups on the molecules (Konno *et al.*, 1997). It was also noted that additives that react with or bind to the protein molecules strongly or have high molecular weight (e.g., starch) have no protective effect (MacDonal and Lanier, 1991). The cryoprotective effect of sugar can be enhanced by adjusting pH to about 7.5 and by adding polyphoshpate. The level of sugar required is dependent on the quality of surimi and the amount of salt present. High-quality surimi needs little sugar, whereas surimi containing more salt requires higher levels of sugar (Lee, 1990). The use of cryoprotectant, which can eliminate the undesirable sweetness and /or browning has been paid more attention in surimi manufacture.

- **Mechanisms of protein stabilization by cryoprotectant**

The most commonly used cryoprotectants in the food industry are low molecular weight sugars and polyols, such as sucrose and sorbitol used in surimi manufacture. They not only are cryoprotectant, but are also known to stabilize proteins to the denaturing effects of heat (Back *et al.*, 1979, Park and Lanier, 1987). Sugars have frequently been used for protecting both proteins and cells during freezing (Conrad and de-Pablo, 1999). A number of mechanisms have been suggested for their effectiveness. These include their ability to form glasses, to

mimic the hydrogen bonding character of water, to increase the surface tension of water as well as the amount of bound water preventing ice crystal growth and migration of water molecules from protein (Conrad and Pablo, 1999; Arakawa and Timasheff, 1982; Yoon and Lee, 1990). Lee and Timasheff, (1981) and Arakawa and Timasheff, (1982) reported that denaturation of proteins is thermodynamically less favorable in sugar solution than in water. Although the free energy (G) of protein denaturation in water and in sugar solution cannot be measured directly, measurement of the ΔG of transfer of the native and denatured (unfolded) forms of proteins from water to sugar solution were made with a high-precision densimeter (Figure 3). Since $\Delta G_2 > \Delta G_3$, and $\Delta G_1 + \Delta G_2 = \Delta G_3 + \Delta G_4$, it follows that $\Delta G_4 > \Delta G_1$. They calculated that the equilibrium constant of the denaturation reaction in sugar solution decreased by a factor of 10^3 relative to that in water.

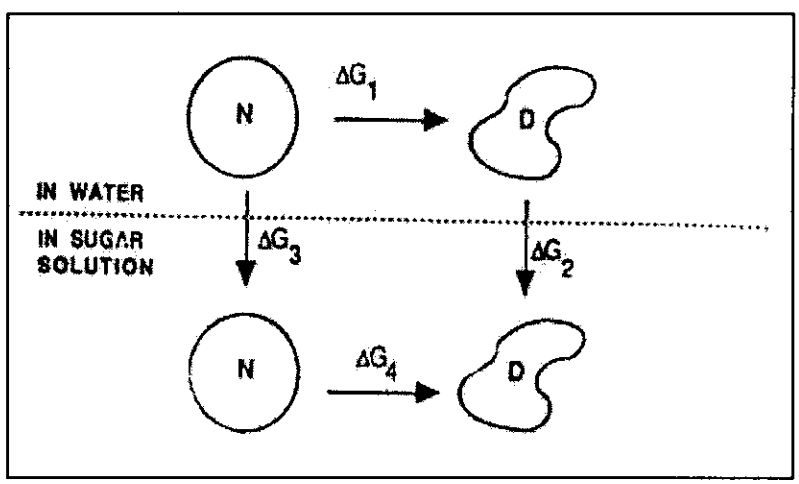

Figure 3 Conversion of a protein native state (N) to the denatured state (D) in water or sucrose solution.

Source: Arakawa and Timasheff (1982)

Arakawa and Timasheff (1982) proposed that the stabilizing solute molecules (sugar, low-MW polyols) were excluded from the surface of the protein molecule, thus "preferentially hydrating" the protein (Figure 4). The preferential hydration of the protein has sometimes been identified as the primary protective effect of solute exclusion, implying that the protein is thus protected against surface dehydration during freezing. The true protective solutes results in a positive (unfavorable) free energy change because the sugar is excluded from the protein surface. The magnitude of this unfavorable free energy shift is assumed to be in proportion to the surface area of the protein, especially the volume of the "cavity " occupied by the protein and its hydration shell. Since the protein cavity is assumed to be greater when the protein is unfolded, this means that the native state of the protein is thermodynamically favored in a sugar-low-MW polyol solution (MacDonald and Lanier, 1991).

Cryoprotectant sugars and polyols increase the surface tension of water, which may be important in other ways to protein stabilization (MacDonald and Lanier, 1991). Sugar protects protein from freeze denaturation by increasing the surface tension of water as well as the amount of bound water. This prevents withdrawal of water molecules from the protein, thus stabilizing the protein. One of the main causes of water migrating from between proteins to form ice crystals is caused by disruption of hydrogen bonding and bonds between proteins (Matsumoto and Noguchi, 1992). Back *et al.* (1979) from careful measurements note that hydrophobic interactions between pairs of hydrophobic groups are stronger in sucrose than in pure water. It may concluded that those polyols and sugars which increase the surface tension of water may act to stabilize proteins dually by favoring solute exclusion from the protein surface and by enhancing the strength of intramolecular hydrophobic interactions. The latter effect arises from the unfavorable decrease in entropy that occurs when water molecules experience a decreased mobility (enhanced hydrogen bonding) in the vicinity of exposed

hydrophobic side chains (Figure 5) (MacDonald and Lanier, 1991; Back *et al.*,1979).

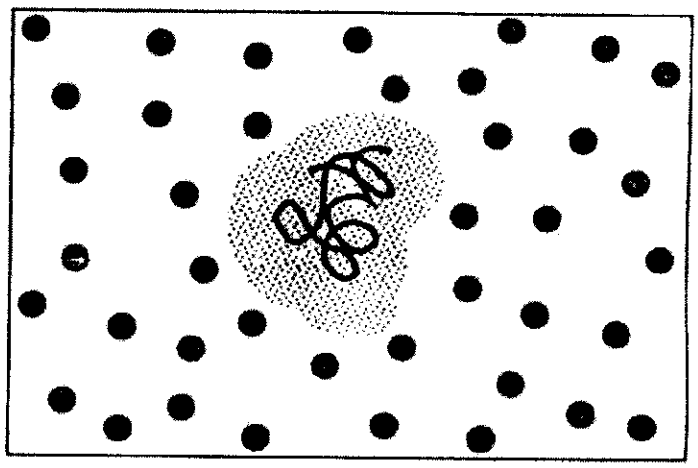

Figure 4 Solute exclusion from the cavity occupied by the protein and its hydration shell (shaded area), the black dots signifying solute molecules.

Source: MacDonald and Lanier (1991)

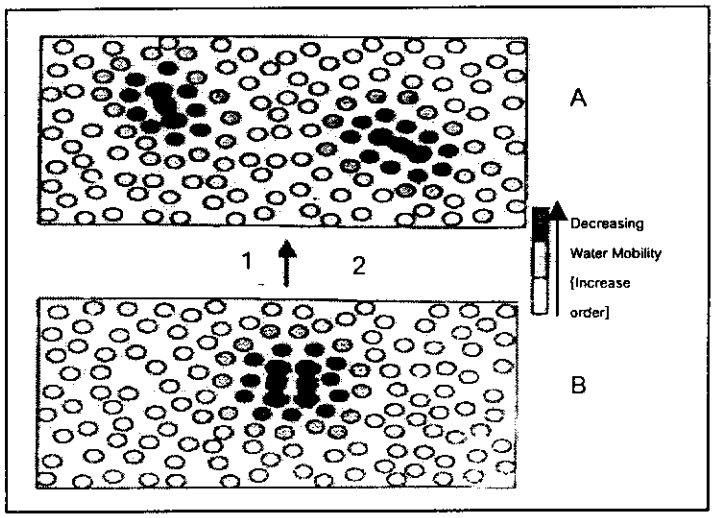

Figure 5 Hydrophobic interaction in an aqueous environment. Hydrophobic groups (dark bodies) dispersed in water (A) destabilize the system by decreasing the entropy (randomness; S) because the mobility of neighboring water molecules is decreased by their presence. Although the enthalpy (heat content, H) of the system is slightly increased when the hydrophobic group associate closely (ie., $\Delta H_1 > \Delta H_2$) as in (B), entropy is greatly increased ($\Delta S_1 > \Delta S_2$). Because ΔG (free energy of the system)= $\Delta H - T\Delta S$, where T = temperature, then the free energy of the system is decreased when hydrophobic groups associate as in (B) (ie., $\Delta G_1 < \Delta G_2$. Systems of the lowest free energy are the most stable.

Source: MacDonald and Lanier (1991)

- Application of cryoprotectants

Sucrose

Sucrose (Figure 6) is cryoprotectant commonly used in surimi. The cryoprotective role of sucrose is to prevent actomyosin denaturation during frozen storage. Initially a high level (8%) of sucrose was used, but this made the surimi too sweet and caused a brown color change during frozen storage. The U.S. market is particularly turned off by sweet fish products (Turner *et al.*, 2001; Kulp *et al.*, 1991).

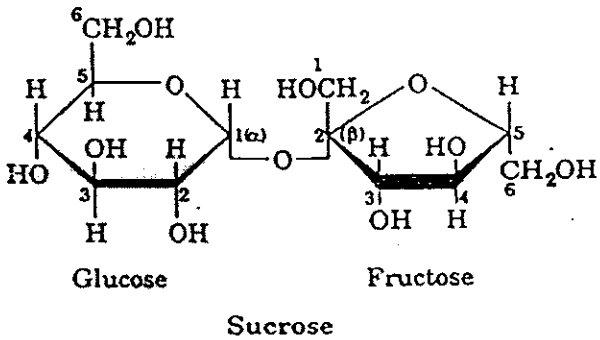

Figure 6. Structure of sucrose

Source: Turner *et al*. (2001)

Sucrose can affect macromolecular structure by direct interaction with the macromolecule, by indirect action through effects on the structure and properties of sucrose or, by a combination of both of these mechanisms. The stabilizing action of sucrose is related to its effect on the surface free energy of cavity formation. Sucrose is known to increase the surface tension of water (Back *et.al.*, 1979; Carpenter and Crowe, 1988). Generally, sucrose has high sweetness, compare to then sugars or sugar alcohols (Table 2) (Pomeranz, 1985; Kulp *et al.*, 1991)

Table 2. Relative sweetness of different sweeteners[a]

Sugar	Index
Sucrose	100
Dextrose (glucose)	70-80
Levulose (fructose)	140
70-DE corn syrup	70-75
High conversion corn syrup	65
Regular conversion corn syrup	50
Maltose	30-50
Lactose	20
High fructose corn syrup	
90%	120-160
55%	>100
42%	100
Invert sugar	>100
Sorbitol	50
Xylitol	100
Galactose	32
Mannitol	40

[a]Based on sucrose as 100. Syrup sweetness based on dry solids.

Source: Pomeranz, (1985); and Kulp *et al.* (1991).

Sorbitol

Sorbitol has been used to reduce the level of sucrose in surimi (Figure 7). Even though sorbitol has a little less protective than sucrose, it in less as sweet and does not cause discoloration. Surimi gel containing sorbitol alone, however, gives a "harder" texture than that containing sucrose. Yoon and Lee (1990) studied the relative cryoprotective effects of liquid sorbitol alone and in combination with

sucrose in surimi, extruded uncooked and cooked products. Addition of 8% sorbitol resulted in best water-holding ability, gel strength and least ice crystal formation.

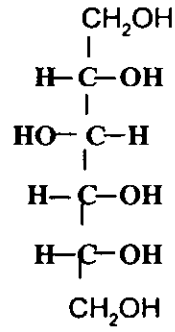

Figure 7. Structure of sorbitol

Source: Turner *et al.* (2001)

Sucrose and sorbitol are the most commonly used in frozen surimi processing (Arai *et al.*, 1970). It is well known that 5-10% sucrose and sorbitol used in frozen surimi results in an oversweet taste, resulting a limitation of the utilization of these compounds (Jiang *et al.*, 1987). Sucrose imparts a sweet taste to surimi products which in undesirable to the consumer (Sultanbawa and Li-Chan, 2001; Auh *et al.*, 1999; Sych *et al.*, 1990). Therefore, interest on identifying other cryoprotectants with reduced sweetness and with cryoprotective effects equal to commercial cryoprotectants and/or incorporated in surimi at 8% (w/w) or less than 8% (w/w) have been intensively paid (Sultanbawa and Li-Chan, 2001; Auh *et al.*, 1999; Sych *et al.*, 1990).

Auh *et al.* (1999) used HBOS (highly concentrated branched oligosaccharids mixture) as cryoprotectant in a fish protein. Addition of HBOS resulted in the remained Ca^{2+}ATPase activity of actomyosin extracted from Alaska pollock after freeze-thawing and the best stabilization of actomyosin with HBOS concentration was observed at a level of 8%.

Park *et al.* (1988) reported that Polydextrose® appeared to substitute for the sucrose/sorbitol in surimi manufacture without changes in cryoprotective effect. The maltodextrin adversely affected gel-forming properties, although it maintained the salt-soluble protein extractability nearly as well as did sucrose/sorbitol or Polydextrose®.

Sych *et al.* (1990a) studied the cryoprotective effects of lactitol dihydrate, Polydextrose® and Palitinit (Isomalt) at 8% w/w in cod-surimi in comparison with an industrial control containing a sucrose/sorbitol 1:1 mixture and a control without additive. Salt extractable protein and myosin peak enthalpy for surimi were maintained at the same level as the industrial control. The best cryoprotective effect was achieved from sorbitol, glucose syrup (DE=60), sucrose and sucrose/sorbitol 1:1 w/w mixture at 8% in surimi (Sych *et al.*, (1990b). Sensory charteristics of several cryoprotectants including sucrose, sorbitol, maltodextrin DE 24-38 and synthetic Polydextrose® was evaluated. Polydextrose® was least sweet and its taste was distinctly suppressed by meat (Tomaniak *et al.*, (1998).

Sultanbawa and Li-Chen (1998) studied cryoprotectant blends for their potential to stabilize ling cod surimi during frozen storage at –18 ^{0}C for 4 months. Twenty five blends containing lactitol, Litesse®, sucrose, and sorbitol, at final total concentration of 4-12% were used. All blends gave surimi and cooked gels comparable to those obtained with commercial mix (4%sucrose+ 4%sorbitol), with the 4% blend containing 1% of each cryoprotectant being the most economical and the lowest in calorie content. Sultanbawa and Li-Chen (2001) studied gel–forming ability of surimi and natural actomyosin (NAM) from ling cod subjected to frozen storage in the absence or presence of cryoprotectant (sorbitol, sucrose, lactitol and Litesse®). The highest gel strength of unfrozen NAM was observed for treatments containing 8% lactitol, 8%sorbitol and commercial blend was maintained after frozen storage.

Trehalose

Trehalose is a non-reducing disaccharide which consists of two molecules of glucose bound to glucose by an α,α-1,1 linkage (Figure. 8) (Neta *et al.*, 2000; Lambruschini *et al.*, 2000). It occurs in a large range of organisms, such as bacteria, fungi, nematodes and crustaceans. (Aeschbacher *et al.*, 1999; Conrad and de-Pablo., 1999; Muller *et al.*, 1999; Gallo *et al.*, 2002). Trehalose is about 45% as sweet as sucrose and is also slightly hygroscopic (Neta *et al.*, 2000; Branca *et al.*, 1999).

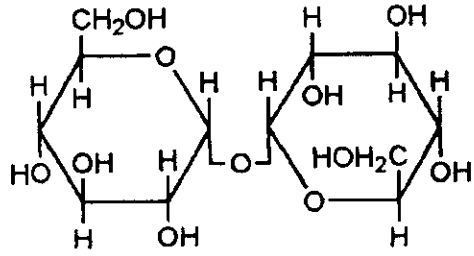

Figure 8. Structure of trehalose.

Source: Wingler (2002)

Trehalose has been shown to stabilize proteins and membranes under stress conditions, especially during desiccation (Lambruschini *et al.*, 2000). By replacing water through hydrogen bonding to polar residues, trehalose prevents the denaturation and aggregation of proteins and the fusion of membranes (Bardos-Nagy *et al.*, 2001). In addition, trehalose forms glasses (vitrification) in the dry state, a process that may be required for the stabilisation of dry macromolecules. Furthermore, trehalose remains stable at elevated temperatures and at low pH and does not undergo Maillard browning with proteins (Brance *et al.* (1999). These protective properties of trehalose are clearly superior to those of other sugars, such as sucrose, making trehalose as an ideal stress protectant (Wingler, 2002). Brance

et al. (1999) suggested that trehalose does not caramelize at normal emulsion processing temperatures, and on heating unlike other sugars, it does not undergo reaction with proteins or other reactive molecules.

Luzardu *et al.* (2000) suggested that trehalose appears to be able to intercalate between the phospholipid head groups, as suggested by the interaction with the carbonyls. Trehalose can act as a spacer between the lipids, affecting the water permeability of the bilayer and increasing the area per molecule. McGarvey and Craigd (2001) reported that trehalose protects chicken egg white lysozyme to a greater extent than sucrose immediately post lyophilization and after 1-month storage at room temperature in a dessicator. The freeze preservation of plant cells is desired for efficient storage. Successful recovery of viable tissues usually requires the use of a cryoprotectant which itself can be very toxic. The use of naturally occurring compounds like proline or trehalose, which may not be toxic, would be advantageous (Bhandal *et al.*, 1985). Bhandal *et al.* (1985) reported that supension cultures of carrot, and tobacco frozen under controlled conditions with trehalose had maximal post-thaw viability. Carrot cells with a 24 hours pretreatment with 5 or 10% trehalose and with 40% trehalose as the cryoprotectant during freezing showed 71-74% viability. Tobacco cells pretreated for 24 hours with 10% trehalose and cryoprotected with 40% trehalose during freezing showed 47% viability.

Trehalose is known to provide effective anti-freezing and anti-dehydration protection to biological cells, and for this reason, it is extensively used in food processing, and increasingly exploited in pharmacology and biomedical applications (Ballon *et al.*, 2000; Sussich *et al.*, 1998). Trehalose-water system (in the crystal, glassy, and liquid solution phases) is now well characterized (Figure 9), and can serve as a crucial benchmark for the modeling of this molecule (Engelsen and Perez, 2000). Sastry and Agmon (1997) suggested that the ability of the sugar to from a glass is essential for the macromolecule stabilization.

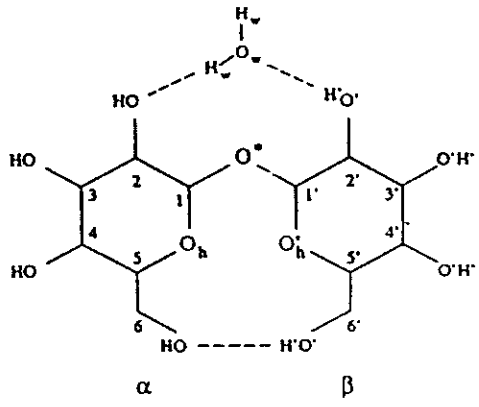

Figure 9. Structure of trehalose-water complex.

Source: Ballone *et al.* (2000)

Trehalose may specifically protect delicate biological molecules, as follows: (Murray and Liang, 1999)

1. The glass state explanation. With the formation of a glassy state around a protein, all physical and chemical transformations will be enormously slow as result of the very high viscosity of the system. Work has therefore highlighted the special properties of trehalose in relation to its ability to form a glass, rather than a crystalline state (Murray and Liang, 1999).

2. Water replacement explanation. The water content of some anhydrobiotic organisms in their dry state is so low. Thus, the water of hydration of proteins must be at least partially disrupted or removed (Sastry and Agmon, 1997). Crowe *et al.* (1984) and Luzardo *et al.* (2000) proposed a water replacement hypothesis, by which, in the absence of water, the hydroxyl groups of trehalose (and other saccharides) form hydrogen bonds with polar residues of biological structures. Iannilli and Tettamanti (2001) suggested the ability of trehalose to strongly interact with water molecules, thus breaking its H-bonded structure and affecting the friction coefficient of the solvent. A drastic temperature induced conformational change of

the sugar molecule, which passes from a closed to an open structure through the rotation around the glycosidic linkage.

3. Water exclusion explanation. Arakawa and Timasheff (1982) explained the stabilizing effects of sugars as being due to a higher (positive) free energy difference between the native and unfolded state when sugars are present in aqueous solution, which is exhibited by the preferential exclusion of sugar molecules from the surface of proteins and the increase the surface tension of water when sugars are present.

4. Maillard browning explanation. Trehalose is a non-reducing sugar and so does not readily undergo non-enzymatic browning with amino-containing compounds, such as proteins. It is also relatively stable to heat and slow to caramelize. The resistance to browning must be another aspect of the success of trehalose protecting macromolecules during drying (which also concentrates reactants) and heating.

Objectives

1. To study cryoprotective effect of trehalose on fish muscle proteins subjected to different freeze-thaw cycles
2. To study effect of trehalose on physico-chemical properties and gel forming ability of surimi subjected to multiple freeze-thaw cycles.
3. To study effect of trehalose on physico-chemical and gel-forming ability of surimi during frozen storage.