CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Freezing and frozen storage is an excellent method of preserving muscle foods from spoilage caused by microorganisms. However, the muscle proteins undergo a number of changes which modify their structural and functional properties (Connell, 1960; Mackie, 1993). Depending on intrinsic factors such as species, season and technological factors such as handling practices prior to freezing, freezing rate, temperature of storage or the presence of protective barriers against oxidation, the practical storage life of frozen fish may vary substantially (Careche *et al*., 1999). Fish muscle deteriorates during frozen storage largely because the myofibrillar proteins undergo denaturation and aggregation (Huidobro *et al*., 1998). Dehydration and salt concentration are induced by the formation of ice crystals. Additionally, oxidation of lipids, formation of free fatty acids, and formation of formaldehyde (FA) contribute to denaturation and/or aggregation of protein and loss of functionality and texture (Shenouda, 1980; Careche *et al*., 1998a, 1998b).

Trimethylamine-*N*-oxide demethylase (TMAOase) is an enzyme found in the muscle and viscera of gadoids. It produces FA and dimethylamine (DMA) in equimolar amounts by the demethylation of trimethylamine oxide (TMAO) (Tejada *et al*., 2002). The rate of reaction depends on some factors such as storage temperature, species, muscle integrity and reducing conditions (Parkin and Hultin, 1982a). The level of TMAOase activity is characteristic of a given species, and it can vary widely between species (Castell *et al*., 1971; Rehbein and Schreiber, 1984). For the same species, different organs and tissues have different enzyme levels. Generally, TMAOase activity in viscera is higher than in muscle tissues (Rey-Mansilla *et al*., 2002). TMAOase is localized in very large quantities in the kidney and spleen (Hultin, 1992; Benjakul *et al*., 2004). Nevertheless, Parkin and Hultin (1982a) reported that the significant activity of TMAOase was found in the light muscle of red hake (*Urophycis chuss*). Additionally, TMAOase with an optimum pH of 7.0 was found in walleye pollack myofibrillar fraction (Kimura *et al*., 2000a, 2000b). TMAOase has been reported to be associated with the formation of FA and DMA

in the muscle of Pacific cod (*Gadus macrocephalus*), Alaska pollack (*Theragra chalcogramma*), and Japanese hake (*Lotella maximoniczi*) (Amano and Yamada, 1965; Tokunaga, 1980). This enzymatic activity has been considered to lower the quality of the gadoid species during frozen storage. The interaction of produced FA with muscle proteins is the principal cause of texture deterioration, causing quality loss and decreasing their storage life (Sotelo *et al*., 1995b).

Lizardfish (*Saurida* spp.) is another important species for surimi production in Thailand due to its high gel-forming ability with an appreciable whiteness (Benjakul *et al*., 2003a). Yasui and Lim (1987) suggested that the decrease in gelling property of lizardfish during iced storage was due to the formation of FA and DMA. The contamination of TMAOase from the internal organs, particularly from kidney, during fish processing such as mincing also induced protein aggregation caused by FA formed (Benjakul *et al*., 2004). However, the information regarding the contribution of lizardfish TMAOase on muscle protein alterations during extended frozen storage is scarce. Additionally, such a detrimental effect of this enzyme on quality of fish muscle can be minimized by using some inhibitor or selected treatment. The knowledge gained can be then transferred to the seafood processors for quality improvement of lizardfish, especially those with frozen storage, leading to full utilization of this species.

1.2 Literature reviews

Proteins of fish muscle

The proteins in fish muscle can be divided into the following three groups as follows (Borresen, 1995):

1. Sarcoplasmic proteins: Sarcoplasmic proteins are soluble in neutral salt solutions of low ionic strength (< 0.15 M). This fraction constitutes 25-30% of the total protein in muscle. Sarcoplasmic proteins or water soluble proteins, referred to as “myogen”, are soluble in the muscle sarcoplasm. They include a large number of proteins such as myoglobin, enzymes and other albumins. The content of sarcoplasmic proteins is generally higher in pelagic fish species as compared with demersal fish (Suzuki, 1981). Sarcoplasmic enzymes are responsible for quality deterioration of fish after death. These include glycolytic and hydrolytic enzymes (Shahidi, 1994).

2. Myofibrillar proteins: Myofibrillar proteins constitute 70-80% of the total protein content (compared with 40% in mammals). These proteins are soluble in neutral salt solutions of fairly high ionic strength ($\geq$ 0.5 M). The most important property of fish muscle is the ability of the myofibrillar proteins to form a three dimensional network or gel upon heating (Niwa, 1985; Doi, 1993). Gel forming ability of myofibrillar proteins depends on the fish species, type of muscle and freshness (Sikorski *et al*., 1976; Matsumoto, 1980; Shenouda, 1980). Myofibrillar proteins undergo changes during the rigor mortis, resolution of rigor mortis and extended frozen storage (Shahidi, 1994). The texture of fish products and the gel-forming ability of fish minces and surimi may also be affected by these changes (Shahidi, 1994).

Myosin is the most abundant myofibrillar fraction of fish muscles and contributes to 50-60% of total amount. The myosin molecule consists of two heavy chains (200 and 240 kD) associated non-covalently with two pairs of light chains (16 to 28 kD). In contrast to most proteins of mammalian origin, the loss of ATPase activity occurs at a faster rate in fish muscle (Kimura *et al*., 1980).

Paramyosin is a protein found in invertebrates and is present in quantities ranging from 0.1 to 10 times of myosin (Offer, 1987). Paramyosin molecules constitute a core of the thick myofibrils of invertebrate muscle, which is covered by a layer of myosin. Paramyosin is known to maintain the tension in the muscle tissues (Shahidi, 1994).

Actin, the second most abundant myofibrillar protein, constitutes approximately 20% of the total content of these proteins in fish muscles. G-actin is the monomeric form of the molecule and polymerizes to F-actin in the presence of neutral salts. When minced fish is extracted with neutral salt solutions, actin is co-extracted with myosin in the form of actomyosin. Other constituents of myofibrillar proteins include tropomyosin and troponins which account for 10% of the total amount (Shahidi, 1994).

3. Stroma proteins: Stroma proteins form connective tissue, representing approximately 3% of total protein content of fish muscle. It cannot be extracted by water, acid, or alkaline solution and neutral salt solution of 0.01-0.1 M. The components of stroma are either collagen, elastin or both (Suzuki, 1981).

Freeze-induced protein denaturation

During the freezing and frozen storage, fish muscle undergoes a number of changes, such as denaturation and aggregation of the myofibrillar proteins. This results in alteration of the functional properties of muscle proteins, loss of water-holding capacity and juiciness and unwanted changes in texture, which produces a hard, dry and fibrous product (Barroso *et al*., 1998). Fennema (1982) noted that all proteins would be expected to have an optimum stability at a temperature just above the freezing point of water. At higher temperatures, denaturation is accelerated as a result of destabilization of hydrogen bonds and increased molecular motion. At lower temperatures, intramolecular hydrogen bonding is enhanced (MacDonald and Lanier, 1991). However, hydrophobic interactions would be weaken to the point that a net destabilization may be expected to occur. This optimum temperature of stability might actually be below the freezing point, but the onset of ice crystal formation could induce changes in the protein environment that cause denaturation. Figure 1 summarizes some of the changes which may occur in a muscle protein system during freezing and frozen storage (Sikorski *et al*., 1976; Shenouda, 1980; Haard, 1990).

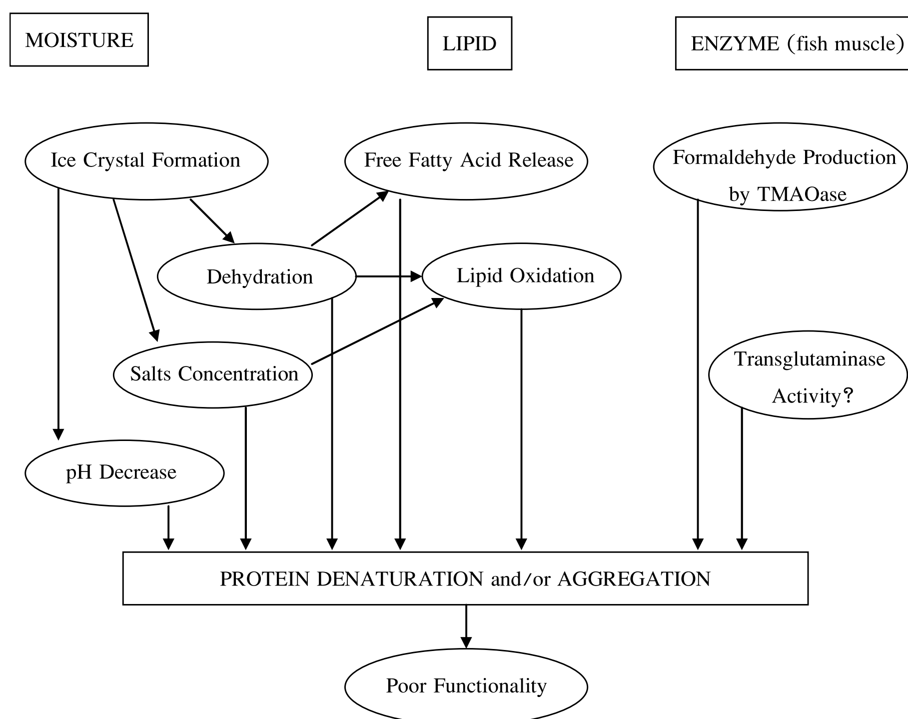

Figure 1. Factors affecting muscle protein denaturation and consequent loss of protein functionality during frozen storage.

Source: Adapted from Shenouda (1980)

Freezing is a physical process involving the transformation of water molecules from an amorphous state to highly structured ice crystals. The phase changes can lead to protein denaturation caused by alterations in the chemical and physical environment of the proteins. Freeze-induced damages of proteins are both mechanical and chemical. A conventionally accepted theory is that the damages involve three major alterations in the protein microenvironment: (1) changes in moisture; (2) changes in lipids; or (3) changes in certain cellular metabolites. According to Shenouda (1980), the changes in moisture can be subdivided into the following: (1) formation and accretion of ice crystals; (2) dehydration; and (3) increases in solute concentration. Table 1 lists the major causes of freeze-induced protein denaturation and some possible techniques to prevent the denaturation.

Table 1. Causes of freeze-induced protein denaturation and possible prevention methods

Cause	Mode of action	Method to minimize denaturation
Ice formation	Mechanical damage	Fast freezing rate
	Cell-wall disruption	Low thawing temperature
Recrystallization	Mechanical damage	Low temperature storage
		Prevent temperature fluctuation
Dehydration	Increased exposure of hydrophobic groups; unfolding	Cryoprotectants
Salt concentration	Modified electrostatic interaction; unfolding	Cryoprotectants
Oxidation	Modified amino acid side chains	Antioxidants
	Peptide bond cleavage	
Lipids	Protein-lipid complexes	?
Metabolites	Formaldehyde-methylene bridge between polypeptides	Inactivate trimethylamine demethylase

Source: Xiong (1997a)

Freeze-induced denaturation of proteins can be attributed to the different causes, both chemical and physical. Those changes take place during freezing or frozen storage and degree of denaturation is dependent on various factors, either intrinsic or extrinsic.

1. Mechanical damage

The formation of ice crystals from either intracellular or extracellular water can result in mechanical damage caused by irregular ice crystals protruding through and disrupting the cell walls (Xiong, 1997a). The size and location of ice crystals formed during freezing is greatly influenced by the freezing rate, storage time, and temperature fluctuations (Xiong, 1997a). At a slow freezing rate, the exterior fluid of cells cools more rapidly than the interior fluid. As the supercooled extracellular fluid reaches a critical temperature, water separates from the solute and forms ice crystals. As crystallization proceeds, extracellular salt becomes more concentrated, creating an osmotic pressure gradient across the cell membrane. In an attempt to balance the chemical potential, intracellular (hypoosmotic) moisture flows outward, leading to dehydration and an increase in the ionic strength of the cell. Water drawn from the interior of the cell will freeze onto the existing extracellular ice crystals, causing them to grow, thus distorting and damaging the membrane and proteins. In contrast, at a fast freezing rate, the interior moisture of the cell is cooled so rapidly and the small ice crystals, usually spearlike and separated by proteins, form inside the cell. Therefore, less dehydration and mechanical damage to proteins occurs (Love, 1968).

Thermodymatically, a small ice crystal is less stable than a large ice crystal. During frozen storage, especially when temperature fluctuates, ice crystals can undergo metamorphic changes referred to as recrystallization (Xiong, 1997a). In general, small ice crystals have a tendency to melt, recrystallize, or aggregate onto existing larger crystals (nuclei) because of differences in the surface energy. This phenomenon is often observed for food stored at close to the critical freezing zone (-0.8°C to -5°C). Because recrystallization involves enlargement in size, changes in shape and orientation, and movement of ice crystals, it usually causes tissue damage and accelerates protein denaturation (Xiong, 1997a).

Under normal commercial freezing regimes, ice nucleates not between the myofibrils but between the muscle cells (Goodband, 2002). As these ice crystals grow, water is abstracted from the myofibrils and the cells become condensed. The effect of this condensation of the muscle fibres on the myofibrillar lattice is shown in Figure 2. As a result of the condensation of the fibres, the myofilaments are in closer proximity to each other. If the fish is thawed soon after being frozen, the lattice recovers its original dimensions and a large proportion of the water returns to the muscle cells. In these circumstances, the textural quality of the fish is maintained. If however, fish is stored in the frozen state for a period of time, interactions between the myofilaments can occur. As a result, on thawing, the water is unable to return to the cells and remains in the extracellular spaces (Goodband, 2002). Some of this water may be lost as drip and further water may be lost on cooking. This redistribution or loss of moisture can result in the typical characteristics of frozen deteriorated fish. The fish may have a very high initial juiciness, but on further chewing, the fish is tough, fibrous and dry (Goodband, 2002).

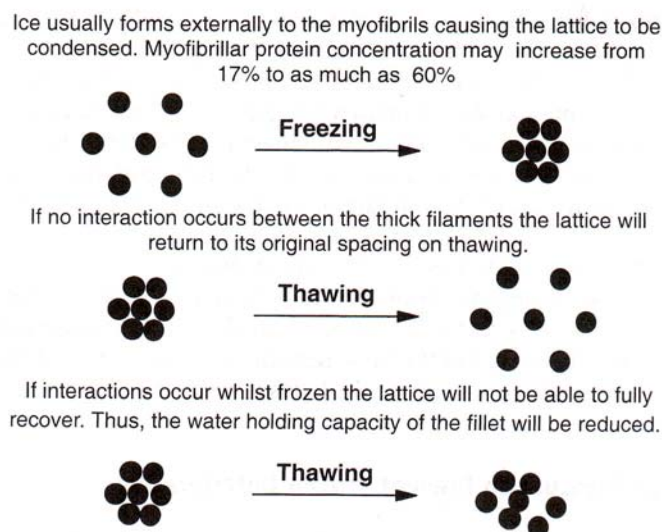

Figure 2. Effect of freezing on the myofibrillar lattice
Source: Goodband (2002)

2. Dehydration

Dehydration caused by ice formation can lead to protein denaturation, which can be explained in thermodynamic terms. In biological tissues, where moisture is the most abundant cellular constituent, proteins exposed to the aqueous environment have a

hydrophobic interior and a polar surface (i.e., most hydrophobic amino acid residues are occluded inside and most charged or polar side chains tend to stay outside). This structural assemblage is entropy driven, meaning it facilitates hydration of proteins by interacting with water molecules via hydrogen bonds and lowers the total free energy of the system (Privalov and Makhatadze, 1993), as can be expressed by the following equation:

$$\Delta G = \Delta H - T\Delta S$$

where ΔG, ΔH, and ΔS are changes in free energy, enthalpy, and entropy, respectively. The loss of entropy involved in the formation of ordered (folded) protein structure in its hydrated state is compensated by a large entropy gain of the surrounding water molecules ($\Delta S > 0$) owing to minimization of interactions between a polar groups of proteins and water. This results in a net reduction in global free energy ($\Delta G < 0$) and stabilization of proteins. However, in a dehydrated state, protein-solvent interactions are disrupted and protein molecules are exposed to an essentially "organic" environment (less polar than water). This will result in increased exposure of hydrophobic side chains and segments and, hence, changes in protein conformation. To maintain the lowest possible free energy status ($\Delta G < 0$) upon migration of water to form ice crystals, increased protein-protein interactions via hydrophobic and ionic forces occur, resulting in further denaturation of proteins and formation of protein aggregates. Hydrophobic interactions are generally weakened as the temperature decreases. Hence, lowering the temperature to subfreezing temperatures will destabilize proteins whose native structures are critically maintained by hydrophobic forces; this has been the basis for some recent theories put forth to explain cold-induced denaturation of proteins (Privalov *et al.*, 1986; Franks, 1995).

3. Concentration of salts

Water in biological tissues, cellular suspensions, or protein aqueous solutions can be divided loosely into free and bound types (Xiong, 1997a). During freezing to $-10^{\circ}C$ or below, as much as 10% water in muscle tissue could stay unfrozen and is more or less 'bound' to protein or other nonaqueous cellular components (Lawrie, 1991). However, the amount of unfreezable water depends on the concentration and type of solutes, including various ionic compounds or salts (Xiong, 1997a). Thus, as freezing progresses, proteins are exposed to increased concentrations of salts, and this process can

theoretically continue until the final eutectic temperature (at which all solutes are also crystallized out) is reached. Because electrostatic interactions are one of the major forces maintaining protein tertiary and quaternary structures, an abrupt increase in ionic strength or salt concentration in the nonfrozen phase can cause competition with existing electrostatic bonds, which in turn leads to extensive modification of the protein native structure (Xiong, 1997a). Disruption of energetically and entropically favorable native protein conformation usually results in denaturation, dissociation of subunits, and, ultimately, aggregation of denatured molecules (Xiong, 1997a). The extent of protein damage is a function of the type of salts, the freezing rate, the temperature and time of storage, and the characteristics of the protein involved in the interaction. For instance, freeze denaturation of carp myosin and myofibrillar proteins has been shown to increase with KCl and NaCl concentration, reaching maxima at about -11 to -13°C for KCl, and -8 to -18°C for NaCl (Inoue *et al.*, 1992; Takahashi *et al.*, 1993). Since these temperatures correspond to the eutectic points of the salts, the concentration effects by both salts are implicated (Xiong, 1997a).

4. Lipid oxidation

Oxidative processes also contribute to protein denaturation in many biological materials during frozen storage, particularly in muscle or plant tissues and cellular suspensions that contain lipids and pro-oxidants (Xiong, 1997a). The oxidation of unsaturated fatty acids in seafood involves the formation of free radicals and hydroperoxides (Haard, 1992). Non-enzymatic and enzymatic oxidation of fatty acids appears to require conversion of ground state (triplet oxygen) to singlet oxygen. However, superoxide dismutase or singlet oxygen quenchers can prevent oxidation (Khayat and Schwall, 1983). Non-enzymatic lipid oxidation is enhanced by metal ions like iron, cobalt, and copper, as well as heme compounds (Ladikos and Lougovois, 1990). Mincing of the meat before freezing, conditions that promote the oxidation of the tissue lipids, high frozen storage temperature, and long storage times lead to extensive deteriorative reactions (Sikorski and Kolakowska, 1990). Aubourg (1999) reported that blue whiting fillets stored at -40, -30 and -10°C up to 1 year underwent primary and secondary lipid oxidation. The highest rate of oxidation was found at -10°C.

Proteins exposed to oxidizing environments are very susceptible to chemical modifications, such as amino acids destruction, peptide scission, and formation of protein-

lipid complexes (Xiong, 1997a). Various oxidizing agents including enzymes, heme, and transition metals can be released from confined cellular organelles or derived from inactive compounds during freezing and frozen storage (Xiong, 1997a). These oxidizing agents can react with proteins either directly or indirectly through lipid and non-lipid radicals, leading to physical and chemical alterations in proteins. Many radicals and lipid degradation products such as malondialdehyde are also capable of cross-linking polypeptides and thus are responsible for the generation of insoluble protein aggregates (Figure 3) (Buttkus, 1970; Sikorski, 1978). The effect of oxidized lipids on protein aggregation was much more pronounced than that of free fatty acids. In fishery products, this type of interactions may be especially significant since fish lipids are rich in highly unsaturated fatty acids liable to oxidation, mainly in the phospholipids fraction (Sikorski, 1978; Haard, 1992). The rate of reaction of amino acids and myosin with malonaldehyde, a typical secondary product of lipid oxidation, is higher at -24°C than at 0°C (Lindelov, 1976). Toughening of frozen fish resulted from oxidation of lipids during frozen storage could be minimized by antioxidants such as BHA, BHT and natural antioxidants (Brand-Williams *et al.*, 1995). Badii and Howell (2002a) used vitamin C or vitamin C/E with citrate at different concentrations to preserve cod fillets stored at -10°C from oxidation. In the presence of antioxidants (vitamin C), toughening was lowering compared to the control. Vitamins C and E exhibited a protective effect against lipid oxidation and reduced protein aggregation in frozen fatty fish (Saeed *et al.*, 1999; Saeed and Howell, 2002).

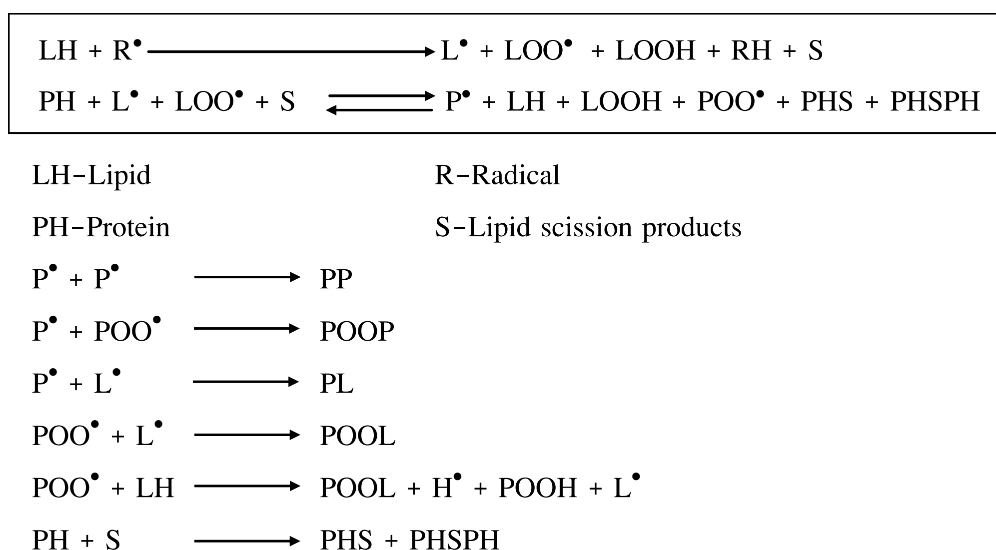

Figure 3. Potential reactions of proteins with lipid radicals and their oxidation products

Source: Sikorski (1978)

5. Cellular metabolites

Increased meat toughness and sponginess are common textural problems associated with freezing and frozen storage of fresh meat. The texture deterioration is a result of protein denaturation and loss of protein solubility. Some muscle-cellular metabolites, such as free fatty acid (FFA) and formaldehyde (FA), are directly responsible for such changes.

5.1 Hydrolysis of lipid

Extensive phospholipids hydrolysis in frozen fish indicated by the formation of free fatty acids and loss of phospholipids was reported (Dyer and Dingle, 1961; Shewfelt, 1981). Free fatty acids in frozen muscle are mostly derived from enzymatic hydrolysis of lipids, particularly the phospholipids, since many lipases and phospholipases remain active in frozen muscle tissue (Olley *et al.*, 1962). Simeounidou *et al.* (1997) reported that quality of whole fish and fillets of horse mackerel and Mediterranean hake was changed throughout 12 months of frozen storage at -18°C. The pH, expressible water, TMA, DMA, FA, TVB-N, TBA, PV, and FFA increased, while sensory attributes (odor, taste, and texture) decreased during the storage period. Fatty acid formation may contribute to texture toughening by influencing protein denaturation and to flavor deterioration by enhancing lipid oxidation (Haard, 1992). FFAs are able to bind to proteins and cause an increase in the hydrophobicity of proteins. Unless oxidation occurs, no covalent bonds are involved in the binding between FFA and proteins (Xiong, 1997a). However, some of the nonpolar amino acids or hydrophobic patches occluded in the native protein may be exposed because of the hydrophobic attraction by FFA. It is conceivable that a reduced polarity of the protein microenvironment brought about by dehydration would promote the formation of lipoprotein complexes (Xiong, 1997a). Subsequent conformational changes would then lead to insolubilization of the proteins through protein polymerization and aggregation. Myofibrillar proteins in frozen muscle are more susceptible than sarcoplasmic proteins to FFA-induced denaturation or loss in solubility (Xiong, 1997a). Among the myofibrillar proteins, myosin or actomyosin seems to be the major target. The rate of FFA-myofibrillar protein interaction and subsequent protein solubility loss are related directly to the degree of unsaturation of FFA and storage time and inversely related to the chain length of FFA

(Shenouda, 1980). Both oleic (C18:1) and myristic (C14:0) acids could bind to the hydrophobic regions of fish actomyosin during frozen storage, resulting in major losses of ATPase activity, solubility, and viscosity of the protein (Careche and Tejada, 1994). The denaturation of fish proteins during frozen storage could be caused by a complex interaction of all these factors, depending on the type of fish (Shenouda, 1980; Haard, 1992). Nevertheless, a reproducible correlation between the concentration of free fatty acids and the decrease in protein solubility was found for only a limited number of fish species (Sikorski, 1978).

5.2 Formaldehyde formation

In gadoid fish, TMAOase, an enzyme that remains active during both frozen storage and thawing, can catalyze the decomposition of TMAO to form DMA and FA (Figure 4) (Sotelo *et al.*, 1995b). This enzymatic activity has been considered as especially important for the quality of the gadoid species during frozen storage, because the interaction of produced FA with muscle proteins is the principal cause of protein-structure deformation, loss in functionality, and hardening of fish tissue (Haard, 1990; Careche and Reece, 1992; Sotelo *et al.*, 1995b).

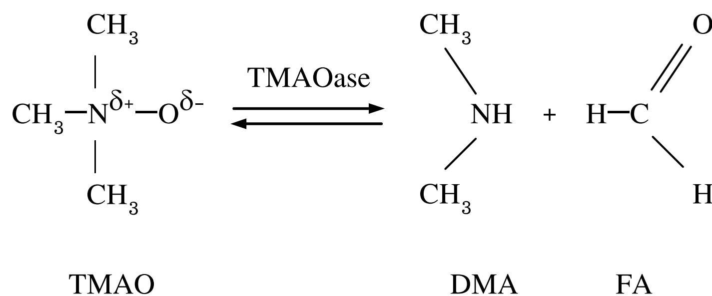

Figure 4. Decomposition of TMAO into DMA and FA by the action of TMAOase
Source: Sotelo *et al.* (1995b)

Differences in the rate of DMA and FA production among gadoid species depend on the amount of substrate, the amount of dark muscle, cofactors, temperature and the period of frozen storage (Kostuch and Sikorski, 1977; Shenouda, 1980). Depending on the initial concentration of TMAO and the pretreatment of the raw material as well as the conditions of freezing and storage, FA can be found to various extents and participates in promoting changes in frozen fish (Sikorski, 1978; Careche and Li-Chan, 1997). Those

species with higher rates of DMA and FA productions, such as red and silver hake, undergo a more rapid deterioration of texture during frozen storage (Dingle and Hines, 1975; Gill *et al.*, 1979). Changes in the muscle proteins of frozen cod fillets, which produce significant amounts of FA, and frozen haddock fillets, which produce negligible FA, were compared during storage at -10°C of storage temperature (Badii and Howell, 2001). FA production in cod was much higher (845 and 1065 nmol/g at 20 and 30 weeks of storage, respectively) than in haddock (93 and 101 nmol/g at 20 and 30 weeks of storage, respectively).

The degree of comminuting of the flesh affects the activity of TMAOase (Mackie and Thomson, 1974; Laird and Mackie, 1981). When comparing FA and DMA formation between minced flesh, fillets and whole fish frozen stored for the same periods of time, the greatest amounts of FA and DMA were produced in the minced sample (Babbit *et al.*, 1972; Crawford *et al.*, 1979). This could be explained by the fact that mincing helps to distribute the enzyme evenly. White muscle comes in contact with other tissues, e.g. dark muscle or viscera with high levels of TMAOase (Shenouda, 1980; Lundstrom *et al.*, 1982; Jahncke *et al.*, 1992).

The rate of freezing and thawing do not seem to influence FA and DMA production in red hake (*Urophycis chuss*) (Parkin and Hultin, 1982a), but the temperature of storage does play an important role. Above 0°C, TMA is preferentially produced by spoilage bacteria, whereas below 0°C DMA and FA are mostly produced, the greatest amount of FA and DMA was obtained at temperatures between -5°C and -10°C (Castell *et al.*, 1973). Sotelo *et al.* (1995a) reported that DMA and TMA in whole hake were both produced at -5°C. At -12°C, there was a small increase in DMA and none of TMA, while at -20°C, none of the amine was produced.

Being a very reactive compound, FA has the ability of reacting with many functional groups of proteins, with subsequent changes in native conformation or formation intra-and intermolecular cross-links via methylene bridges of adjacent polypeptides (Table 2) (Sikorski, 1978; Shenouda, 1980). FA was found to react in model systems and in fish muscles predominantly with the myofibrillar proteins at freezing temperatures (Figure 5). In model experiments, higher pH values favor the binding of FA to cod proteins (Sikorski, 1978). An increase in ionic strength promotes the desolubilizing effect of FA (Sikorski, 1978).

Table 2. Typical reactions of formaldehyde with functional groups of amino acids in aqueous solutions

Reactive group	Type of reaction		
Active hydrogen	$RH + CH_2O$	$\longrightarrow$	RCH_2OH
	$RH + RCH_2OH$	$\longrightarrow$	$RCH_2R + H_2O$
	$ArH + CH_2O$	$\longrightarrow$	$ArCH_2OH$
	$2ArCH_2OH$	$\longrightarrow$	$ArCH_2ArCH_2OH + H_2O$
Primary amine	$RNH_2 + CH_2O$	$\longrightarrow$	$RNHCH_2OH$
	$RNH_2 + CH_2O$	$\longrightarrow$	$RN{=}CH_2 + H_2O$
	$RNH_2 + 2CH_2O$	$\longrightarrow$	$RN(CH_2OH)_2$
	$RNH_2 + 3CH_2O$	$\longrightarrow$	CH_2-O RN $CH_2 + H_2O$ CH_2-O
Secondary amine	$R_2NH + CH_2O$	$\longrightarrow$	R_2NCH_2OH
Amide	$RCONH_2 + CH_2O$	$\longrightarrow$	$RCONHCH_2OH$
	$RCONH_2 + RCONHCH_2OH$	$\longrightarrow$	$RCONHCH_2NHCOR + H_2O$
Hydroxyl	$ROH + CH_2O$	$\longrightarrow$	$ROCH_2OH$
	$ROCH_2OH_2 + CH_2O$	$\longrightarrow$	$ROCH_2OCH_2OH$
	$2ROH + CH_2O$	$\longrightarrow$	$ROCH_2OR + H_2O$
Thiol	$RSH + CH_2O$	$\longrightarrow$	$RSCH_2OH$
	$2RSH + CH_2O$	$\longrightarrow$	$RSCH_2SR + H_2O$

Source: Sikorski (1978)

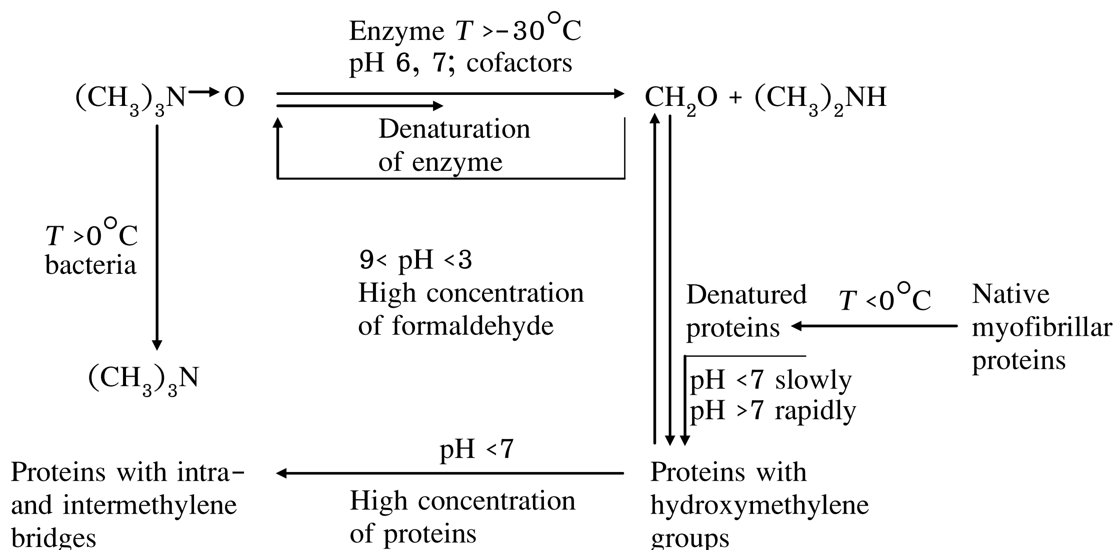

Figure 5. Possible interactions of FA with muscle proteins in frozen fish
Source: Sikorski (1978)

Regenstein *et al.* (1982) presented an interpretation of the series of changes occurring in the muscle of frozen stored fish, or in isolated muscle proteins where FA is produced and/or added. Three steps were envisaged as follows:

1. A series of "conformational-configurational" change of an unspecified nature that leads to a decrease in the amount of extractable protein nitrogen (EPN) which could be extracted by a high salt concentration.
2. Formation of new disulfide bond crosslinks.
3. Formation of a new conformation which requires both β-ME and urea to restore the original electrophoretic pattern. This suggests that all of the covalent crosslinks are disulfide bonds, or that the intermolecular FA crosslinks can be broken by either urea or β-ME.

One FA molecule is theoretically able to react with two amino groups to form crosslinks between protein chains and thus reduce the solubility of the protein in aqueous salt solutions (Connell, 1975; Shenouda, 1980). Addition of FA resulted in the loss of protein solubility of cod myosin during frozen storage at -18°C (Careche and Li-Chan, 1997). Furthermore, ANS fluorescence showed little change, possibly due to a balance between soluble and insoluble fractions differing in exposed hydrophobicity. SDS-PAGE showed irreversible insolubilization at increasing FA concentration, especially after

frozen storage. Careche *et al.* (1998a) proposed that the aggregation of natural actomyosin from hake (*Merluccius merluccius* L.) caused by FA during freezing and frozen storage resulted from the greater involvement of covalent bonds, leading to decreased protein solubility by the end of the storage at $-12^{\circ}C$ for 17 weeks. Additionally, the formation of aggregate in cod fillets stored at -20 and $-30^{\circ}C$ was compared (Careche *et al.*, 1998b). DMA levels increased with storage time at both temperatures and were significantly higher at the higher storage temperature. Loss of extractability in 0.6 M NaCl was slightly greater at $-20^{\circ}C$. The aggregate formed at $-30^{\circ}C$ was extractable in solutions which cleave secondary interactions, whereas at $-20^{\circ}C$ the role of non-disulfide covalent bonds was more important. The aggregates consisted largely of myosin and actin, but myosin was the most important constituent, contributing more to the formation of covalent bonds, particularly at the higher temperature.

Aggregation of minced hake (*Merluccius merluccius*) during frozen storage at $-20^{\circ}C$ was also studied (Torrejon *et al.*, 1999). An increase in DMA and FA indicated progressive deterioration of the minced hake muscle over frozen storage. The results showed a 75% decrease in 0.6 M NaCl extractability by the end of the storage period. The proteins involved in formation of the aggregate were myosin and actin. Neither of these proteins was fully recovered in the fractions extracted with 0.6 M NaCl, 2% SDS or 2% SDS plus 5% 2-mercaptoethanol. Therefore, it was inferred that some aggregates were stabilized by non-disulfide covalent bonds.

Physicochemical changes of muscle proteins during frozen storage

During freezing or frozen storage, muscle proteins undergo physicochemical changes, which are associated with quality losses. Additionally, those changes affected the functional properties of proteins.

1. Solubility

Solubility is a sensitive indicator and is widely used as an index for protein denaturation (Xiong, 1997a). When denaturation occurs, the hydrophobic amino acid side chains occluded in the protein molecules (except membrane proteins) will be exposed to the surface. To maintain the lowest free energy, the apolar groups tend to interact with one

another to form protein aggregates, thus decreasing water binding and solubility (Xiong, 1997a). The extractability of fish myosin decreased during frozen storage (Goodband, 2002). As the myosin heads protrude from the surface of the thick filaments, it is reasonable to presume that interaction between thick filaments is likely to occur via the head regions of the molecules (Figure 6) (Goodband, 2002).

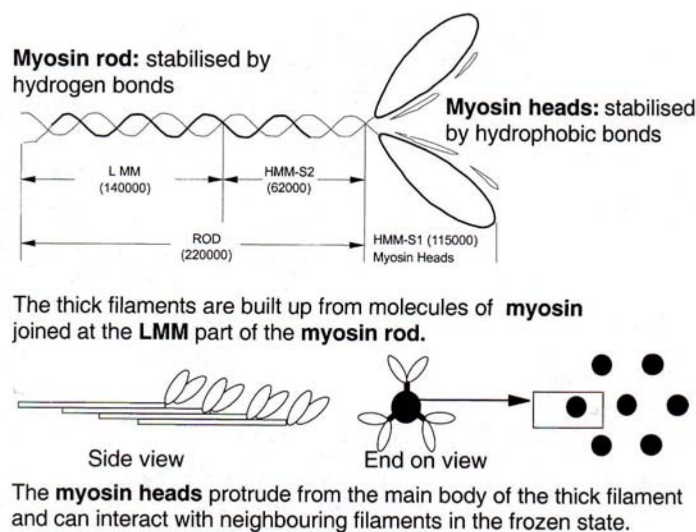

Figure 6. The myosin molecule
Source: Goodband (2002)

When muscle was stored between -1.5 and -20°C, actomyosin was readily insolubilized (Powrie, 1973). The process of freezing and thawing produced some insoluble myosin as well as small amount of protein with high-molecular weight (> 200 kDa). These changes are facilitated by the presence of FA (Ang and Hultin, 1989). Both myofibrillar and sarcoplasmic proteins are found to disappear with increasing FA concentrations. The myosin heavy chains and light chains and troponin are particularly susceptible (Xiong, 1997a). Huidobro *et al.* (1998) studied the aggregation of myofibrillar proteins in hake, sardine, and mixed mince during frozen at -20°C for 1 year. During 8 months of storage, the amount of protein linked by covalent bonds in hake increased with time while in sardine and mixed lots, a high percentage of proteins linked by covalent bonds were detected earlier. Furthermore, myosin and cod muscle became less extractable in 0.6 M KCl during frozen storage (Tejada *et al.*, 1996). The same evidence was observed with carp actomyosin (Oguni *et al.*, 1975; Tsuchiya *et al.*, 1975), milkfish actomyosin (Jiang *et al.*, 1987), trout myosin (Buttkus, 1970), cod actomyosin

(Jarenback and Lijimark, 1975), and tilapia myosin (Ramirez *et al.*, 2000). Badii and Howell (2001) observed that frozen storage of cod and haddock muscle at -10°C led to the denaturation of myosin causing a reduction in solubility and the selective precipitation of proteins.

2. Ca^{2+}-ATPase activity

During frozen storage, myofibrillar proteins, especially myosin, are susceptible to denaturation as indicated by the decrease in Ca^{2+}-ATPase (Benjakul and Bauer, 2000). Jiang *et al.* (1985) reported a decrease in Ca^{2+}-ATPase activity of mackerel and amberfish actomyosin during frozen storage. Del Mazo *et al.* (1999) found that Ca^{2+}-ATPase activity of natural actomyosin from hake fillet stored at -20 and -30°C for up to 49 weeks decreased as storage time increased. Ramirez *et al.* (2000) reported that after 15 days of frozen storage at -20°C, Ca^{2+}-ATPase activity of fish myosin obtained from *Tilapia nilotica* decreased markedly, losing 89% of its initial value, suggesting that myosin head could be involved in the fish myosin aggregation.

3. Sulfhydryl group/disulfide bond

The SH groups are known to be oxidized to disulfide bond during denaturation of proteins. Disulfide bridges are the important covalent bonds, occurring during frozen storage of muscle protein associated with the protein aggregation (Sikorski and Kolakowska, 1990; Makie, 1993). The formation of disulfide bonds via oxidation of SH groups or disulfide interchanges is coincidental with the decrease in total and surface SH contents (Hayakawa and Nakai, 1985). Oxidation of SH groups located in the head portion of myosin was found to play an important role in the loss in Ca^{2+}-ATPase activity (Benjakul *et al.*, 1997). Jiang *et al.* (1986) conjectured that protein denaturation of frozen mackerel and cod was mainly caused by the formation of disulfides. The milkfish actomyosin was denatured during frozen storage by formation of disulfide, hydrogen and hydrophobic bonds (Jiang *et al.*, 1988a). The addition of $NaNO_2$ and $NaBH_4$ to freeze-denaturated actomyosin resulted in the recovered actomyosin solubility in 0.6 M KCl and the total SH. Therefore, the formation of disulfides affected the denaturation of actomyosin during freezing and frozen storage (Jiang *et al.*, 1988a). More disulfides were formed than

other bonds in milkfish actomyosin stored at $-20^{\circ}C$ than $-35^{\circ}C$ (Jiang *et al.*, 1988b). Total SH content of fish muscle actomyosin decreased during frozen storage, suggesting that the formation of disulfides occurred at a much faster rate at $-20^{\circ}C$ than $-40^{\circ}C$ (Jiang *et al.*, 1989).

4. Surface hydrophobicity/hydrophobic interaction

In general, the native protein structure is stabilized by many forces, including hydrogen bonding, dipole-dipole interaction, electrostatic interactions, and disulfide linkages. Protein can undergo unfolding, which increases with temperature abuse, e.g. cyclic freezing and thawing (Srinivasan, 1997a, 1997b). Extended frozen storage caused the severe changes in tertiary conformation of actomyosin. As a result, an exposure of the hydrophobic portion localized inside molecule occurred, leading to an increase in surface hydrophobicity (Benjakul and Bauer, 2000). Moreover, ice crystallization may disturb the water structures surrounding the areas of hydrophobic interactions in proteins. It may also disrupt the water-mediated hydrophobic interactions, which participate in buttressing the native conformation of protein molecules (Sikorski and Kolakowska, 1990). Badii and Howell (2002b) reported the initial increase in surface protein hydrophobicity of cod muscle in the first month before decreasing during the frozen storage at -10 and $-30^{\circ}C$. The increase in surface hydrophobicity possibly caused the association of hydrophobic groups via hydrophobic interaction (Benjakul and Bauer, 2000). The surface hydrophobicity of muscle proteins was found to be inversely related to solubility (Owusu-Ansah and Hultin, 1987). In addition, frozen storage of cod and haddock fillets resulted in an increase in hydrophobicity of their muscle proteins. This could be attributed to the unfolding of proteins and exposure of hydrophobic aliphatic and aromatic amino acids. Hydrophobic interactions between the exposed groups resulted in a decrease in solubility of proteins and formation of aggregate. These changes were more pronounced in cod and haddock fillets stored at $-10^{\circ}C$, compared with fish stored at $-30^{\circ}C$ (Badii and Howell, 2001).

5. Thermal stability

Thermal stability is one of the most important properties of muscle proteins. Fish myosin is very unstable in comparison with that of mammal (Connell, 1961; Ogawa *et al.*, 1994). This dependence is more important for fish muscle than for mammalian muscle because of its low collagen content (Brown, 1986). Jonhston *et al.* (1973) found a relationship between the thermal stability of fish myosin and the environment temperature in which the species live. The myosin of cod (*Gadus morhua*) which lived in cold water was more labile, compared with that of snapper (*Lutjanus sebae*) which lived in tropical area (Davies *et al.*, 1988; Schubring, 1999). Prolonged frozen storage at -20°C had a marked effect on the myosin transition in fish. Sharp, narrow peaks became shallower and broader suggesting loss of cooperativity (Schubring, 1999). Herrera *et al.* (2001) reported that the transition I (T_m of myosin) of minced blue whiting muscle became less clearly defined and gradually flatted out over the storage period at -10°C. In contrast, transition II hardly changed during the whole storage period at this temperature, which reflects the high stability of actin.

6. Rheological behavior

The rheological characteristics of fish muscle are considered to be governed by both myofibrillar and connective tissue proteins, while the sarcoplasmic proteins contribute very little to the texture (Dunajski, 1979). An increase in storage modulus (G') of raw material such as fish fillets is related to protein-protein and protein-lipid interactions, causing the undesirable tough products (Badii and Howell, 2002a, 2002b; Dileep *et al.*, 2005). Dileep *et al.* (2005) found an increase in G' value of ribbonfish (*Trichiurus* spp.) meat along with the iced storage as a result of aggregation/denaturation of protein during iced storage. An increase in G' and loss modulus (G'') values, reflecting protein aggregation, was observed in Atlantic mackerel (*Scomber scombrus*) fillets stored at -20 and -30°C for 2 years. The G' values were higher in mackerel fillets stored at -20°C than that stored at -30°C, indicating a greater degree of protein denaturation and crosslinking in fillets, which was correlated with Ca^{2+}-ATPase activity loss and the decrease in protein solubility (Saeed and Howell, 2004).

7. Structural changes

Structural changes during frozen storage in cod myosin were induced by the addition of FA (Careche and Li-Chan, 1997). Raman spectral analysis indicated a change in secondary structure of cod myosin, from 95% α-helix in the control (no FA) to 60% after 12 mM FA treatment. Changes in vibrational modes assigned to aliphatic residues suggested the involvement of hydrophobic interactions after FA addition and/or frozen storage. Moreover, structural differences in proteins were found between fresh and frozen stored hake muscle after 10 months at temperatures known to render very different practical storage lives (-10 and -30 $^{\circ}$C) (Careche *et al*., 1999). Such structural changes mainly entailed an increase of β-sheets and the expense of α-helices. An increase of unordered protein structure was found only in samples stored at -10°C. Furthermore, exposure of buried tryptophan residues was observed at both storage temperatures. The decrease of the δCH_2 band upon storage suggested an increase of hydrophobic interactions of aliphatic residues. Badii and Howell (2002a) found that the denaturation of proteins caused by ice crystal formation and/or resultant solute concentration, and also by lipid oxidation products, involves hydrophobic groups and exposure of other polar groups due to the unfolding of the molecules, accompanied by changes in the secondary structure.

Trimethylamine-*N*-oxide demethylase (TMAOase; EC 4.1.2.32)

TMAOase is one of important enzymes in fish exhibiting an adverse effect on fish quality. TMAOase has been found at different degrees in various fish species. The inhibition or lowering the activity could be an effective approach to maintain the fish quality.

1. Distribution of TMAOase

Although TMAO is distributed among all kinds of marine fish and invertebrates, and in some fresh water fish, TMAOase activity has been identified in only 30 species of marine fish belonging to 10 families and 8 species of invertebrates, mainly mollusk (Sikorski and Kostuch, 1982). Postmortem TMAOase in different fish and invertebrate species is shown in Table 3. Accumulation of DMA and FA has been described

in species belonging to Gadidae, Merluccidae, and Myctophidae (Sikorski and Kostuch, 1982). Among the fish, Gadiform (families Gadidae and Merluccidae) is the group of fish having high TMAOase activity (Sotelo and Rehbein, 2000).

Table 3. TMAOase activity distribution in several marine organisms

Common name	Latin name
Gadoids	
Cod	*Gadus morhua*
Saithe	*Pollachius virens*
Haddock	*Melanogramus aeglefinus*
Ling	*Molva molva*
Blue ling	*Molva dypterygia*
Blue whiting	*Micromesistius poutassou*
Alaska pollack	*Theragra chalcogramma*
Cusk	*Brosme brosme*
	Coryphaenoides rupestris
Whiting	*Merlangus merlangus*
Red hake	*Urophycis chuss*
Nongadoids	
Herring	*Clupea harengus*
Cucumberfish	*Chlorophtalmus nigripinnis*
Crustaceans	
Blue crab	*Portunus trituberculatus*
Mollusk	
Squid	*Illex illecebrosus*
	Todarodes pacificus
	Sepia esculenta
	Loligo formosa

Source: Adapted from Sotelo and Rehbein (2000)

The level of TMAOase, in a particular species and tissue, has a large coefficient of variation (Rehbein and Schreiber, 1984) and seems to be influenced by intraspecific factors (Sotelo and Rehbein, 2000). Factors including gender, maturation stage, temperature of the habitat, and feeding status, or size can influence the actual level of TMAOase activity (Sotelo and Rehbein, 2000). Dark (red) muscle has a higher rate of activity than white muscle, whereas other tissues such as kidney, spleen, and gall bladder are extremely rich in the enzyme. Thus, it is important that minced fish is completely free of organ tissue such as kidney from gadoid species to prevent toughening during frozen storage. It is often difficult to ensure that the kidney is removed prior to mechanical deboning since this particular organ runs the full length of the backbone and is adherent to it (Gill, 1995). DMA content increased only in dark muscle upon storage at subfreezing temperatures ($0^{\circ}C$ up to $-6^{\circ}C$) (Tokunaga, 1970). Dark muscle content of Gadidae correlated with the degree of DMA accumulated during frozen storage (Castell *et al.*, 1971). Localization of FA production which was resulted from TMAOase during frozen storage of whole European hake (*Merluccius merluccius*) was studied (Rey-Mansilla *et al.*, 2001). Different DMA and FA contents were found among different anatomical locations, especially in those stored over one year. In general, the white muscle located right over the kidneys produced more DMA than other parts of the fish.

Depending on the source of the enzyme, the activity is located in the soluble fraction or in the particulate matter of homogenates of organs or tissues (Sotelo and Rehbein, 2000). The organs and tissues including viscera, muscle, and skin contain TMAOase. TMAOase was present both in the soluble and in the particulate fraction of red hake muscle homogenates (Phillipy, 1984; Phillipy and Hultin, 1993). Harada (1975) isolated TMAOase from the liver of lizardfish (*Saurida tumbil*). Distributions of TMAOase in various internal organs of lizardfish (*Saurida micropectoralis*) were studied (Benjakul *et al.*, 2004). Among internal organs tested, kidney contained the highest TMAOase activity, followed by spleen, bile sac, intestine and liver, respectively. Rey-Mansilla *et al.* (2002) studied the activity of TMAOase of several internal organs of hake (kidney, spleen, liver, heart, bile, and gall bladder). It was found that kidney and spleen showed the highest activities while liver, heart, bile, and gall bladder activities were much lower.

2. Characteristics and properties of TMAOase

2.1 Inhibitors and activators

Several substances affect the activity of TMAOase. Glutathione, Fe^{2+}, ascorbate, flavins, hemoglobin, myoglobin, and methylene blue were found to enhance the activity (Chen, 1992). However, iodoacetamide, cyanide, and azide showed the inhibitory activity on TMAOase (Sotelo and Rehbein, 2000). A heat-stable cofactor present in the viscera of several marine organisms was necessary for the activity of TMAOase from lizardfish liver (Harada, 1975). Reduced forms of NAD or NADP, flavins, and citric acid also enhanced TMAOase activity. The rate-limiting factor for TMAO breakdown to DMA and FA depended on the concentration of cofactors. A decrease in cofactor concentration can occur prior to frozen storage period (Reece, 1985). The muscle with decomposed co-factors, such as NAD or ascorbate, had the decreased rate of DMA and FA accumulation during frozen storage (Banda and Hultin, 1983). Inhibition of TMAOase is also caused by a variety of compounds. However, most of them are general inhibitors of redox reactions, being strong oxidants, such as H_2O_2, which inhibits the reaction (Phillippy and Hultin, 1993). The presence of oxygen or oxidants can promote inhibition of enzymatic TMAO breakdown in frozen red hake (*Urophycis chuss*) (Lundstrom *et al.*, 1983; Racicot *et al.*, 1984).

2.2 pH and temperature profile and stability

Although the enzyme is denatured by heat, its cold denaturation temperature seems to be rather low since the system is active at frozen storage temperatures. It has been demonstrated that TMAOase is active down to -29°C (Babbitt *et al.*, 1972; Castell *et al.*, 1973; Hiltz *et al.*, 1976). However, the activity decreases with decreasing frozen storage temperatures (Rehbein, 1988; Sotelo *et al.*, 1995b).

The heat denaturation of TMAOase activity was studied both in fish muscle systems and in partially purified extracts. Preheating Alaska pollack muscle at 40°C for 30 min before freezing provoked a 20% decrease in DMA formation after 4 weeks of frozen storage. The production of DMA and FA was totally inhibited when muscle was heated at 50°C (Tokunaga, 1964). Moreover, about 70% of activity in SDS extracts of TMAOase

from red hake muscle microsomal fraction is lost upon heating at 40°C for 10 min. The intact microsomal fraction was more heat resistant than the purified fractions (Parkin and Hultin, 1986a). An acetone powder containing TMAOase from pollack pyloric caecum lost most of its activity with the thermal treatment ranging from 40 to 50°C for 5 min (Tomioka *et al*., 1974). TMAOase activity in the myofibrillar fraction from walleye pollack muscle decreased sharply above 30°C, but was extracted and recovered from the heated myofibrillar fraction, suggesting that the activity seemed to be interrupted and apparently inactivated due to the thermal alteration of myofibrillar proteins or some unknown factors (Kimura *et al*., 2003). Benjakul *et al*. (2003a) found that partially purified TMAOase from lizardfish kidney was stable to heat treatment up to 50°C.

The optimum pH for the TMAOase *in vitro* seems to be around 5.0 (Tomioka *et al*., 1974; Harada, 1975; Gill and Paulson, 1982; Rehbein and Schreiber, 1984). However, the enzyme purified from Alaska pollack pyloric caecum (Tokunaga, 1980) and the enzyme from red hake muscle microsomal fraction (Parkin and Hultin, 1982b) had the optimum activity at neutral pH. Benjakul *et al*. (2003a) found that partially purified TMAOase from lizardfish (*Saurida micropectoralis*) kidney had optimum pH and temperature at 7.0 and 50°C, respectively.

3. Molecular characteristic

TMAOase was solubilized and partially purified from walleye pollack myofibrillar fraction by various treatments using detergents, acetone, and acid solution, followed by diethylaminoethyl (DEAE)-cellulose and gel filtration chromatography (Kimura *et al*., 2000b). The specific activity increased 13,000 fold and the yield was 13%, as compared with that in the starting myofibrillar fraction. The partially purified enzyme converted TMAO stoichiometrically to DMA and FA, and showed the optimum pH at 7.0. The K_m of the enzyme for TMAO was approximately 30 mM. The activation energy was 38.4 $kJmol^{-1}K^{-1}$ in the temperature range of 0-30°C. The partially purified TMAOase required Fe^{2+} alone for activity. Reducing agents, such as ascorbate, cysteine, and dithiothreitol, were required to maintain the iron in the active form, Fe^{2+}. The molecular weight of the enzyme was estimated to be 400,000 by the gel filtration chromatography and to be 25,000 on sodium dodecyl sulfate (SDS-PAGE). The

TMAOase activity was stable in the presence of SDS. Above 0°C, DMA formation increased, depending on the rise in temperature. At temperature below 0°C, it slightly increased at -20°C than at 0 and -4°C. The TMAOase activity in the myofibrillar fraction was depressed by the addition of NaCl at concentrations above 0.3 M (Kimura *et al.*, 2000a).

Benjakul *et al.* (2003a) purified TMAOase from lizardfish (*Saurida micropectoralis*) kidney by acidification followed by diethylaminoethyl (DEAE)-cellulose column chromatography, in which purification fold of 82 with a yield of 65.4% was obtained. The molecular mass of partially purified enzyme was estimated to be 128 kDa based on activity staining. Optimum pH and temperature were 7.0 and 50°C, respectively. The activation energy was calculated to be 30.5 $kJmol^{-1}K^{-1}$. Combined cofactors ($FeCl_2$, ascorbate and cysteine) were required for full activation. $FeCl_2$ exhibited a higher stimulating effect on TMAOase activity than $FeCl_3$. At concentration less than 2 mM, ascorbate was more stimulatory to the activity than cysteine. The activity was tolerated to salt at a concentration up to 0.5 M. The enzyme with K_m of 16.24 mM and V_{max} of 0.35 μmol/min was able to convert TMAO to DMA and FA.

Several kinetic studies performed in partially purified TMAOase showed that this activity follows Michaelis-Menten kinetics for the degradation of TMAO. The K_m for TMAO has been determined and it ranged from 3 mM up to 12 mM (Parkin and Hultin, 1982b; Joly *et al.*, 1992; Phillippy and Hultin, 1993). This low K_m for TMAO degradation suggested that TMAO is not the limiting factor for TMAO breakdown in white muscle, since TMAO concentration in gadoid muscle is higher (Rehbein, 1987; Hultin, 1992).

The concentration of TMAO in red hake muscle ranges between 70 and 100 mM, the K_m values for the TMAO demethylation system of this fish range from around 3 to 20 mM (Phillippy, 1984). In any case, the TMAO content of the muscle is always above its K_m value, suggesting that it should not be the limiting factor in the reaction unless it is physically separated from the catalysts of breakdown in the undisrupted tissue (Hultin, 1992). The addition of 500 or 1000 mg of TMAO per 100 g of minced cod did not increase the rate of accumulation of DMA and FA during frozen storage (Sikorski and Kostuch, 1982).

4. Retardation of demethylation of TMAO in fish muscle

TMAOase inhibition has been focused to prevent the quality loss of frozen fish, especially in some species (Dingle *et al.*, 1977; Owusu-Ansah and Hultin, 1987; Krueger and Fennema, 1989). The inhibition of the TMAO demethylation can be achieved using physical methods, chemical methods, or a combination method (Sotelo and Rehbein, 2000).

4.1 Physical methods

Among physical inhibition methods, the most important is increasing or lowering the temperature. Lower temperature (below -30°C) during frozen storage decreases the rate of DMA and FA formation (LeBlanc *et al.*, 1988). One of promising approaches to inhibit TMAOase is heat denaturation of the enzymatic system. A heat treatment of 50°C is needed for any significant reduction of activity (50%). A disadvantage is that this treatment, of course, will lead to some organoleptic changes in the product (Lall *et al.*, 1975).

Refrigeration prior to frozen storage, which depletes TMAO by bacterial growth, decreased the DMA and FA rate formation in frozen gadoids (Kostuch and Sikorski, 1977). TMAOase becomes less active both as result of the decrease in substrate concentration and the presence of TMA, which is produced by the enzymes spoilage bacteria and is able to inhibit TMAOase. Also, NADH depletion can influence the rate of DMA and FA formation since it is a cofactor of the enzymatic system involved in the reaction (Lundstrom *et al.*, 1982; Reece, 1985). Freeze-thaw cycles before the frozen storage period can decrease the cofactor concentration level, thus the rate of TMAO breakdown is lower (Reece, 1985). Also, addition of oxidized lipids to the mince causes inhibition of DMA formation but results in a loss of protein functionality during frozen storage (Careche and Tejada, 1990).

Removal of TMAOase rich tissues could be another approach to extend shelf life of frozen fish with TMAOase. The recommended practice is to eviscerate the fish prior to freezing process or not mince the fish muscle with TMAOase-rich tissues (e.g. red muscle or pieces of kidney), thus preventing the contact of TMAOase-rich tissues with the substrate rich white muscle (Sotelo and Rehbein, 2000). However, the localization of

kidney and peritoneum makes them difficult to remove when eviscerating. The extensive contact surface of kidney with muscle promotes the production of more DMA and FA in fish where kidney is not removed (Rey-Mansilla *et al.*, 1997). Also, red muscle removal will likely improve storage characteristic of gadoid fillets (Sotelo and Rehbein, 2000). Furthermore, the use of high-pressure treatments or heating of localized TMAOase-rich tissues can be used to maximize the inhibition of TMAOase in fish (Sotelo and Rehbein, 2000).

Lundstrom *et al.* (1982) reported that the minimal DMA production rates were obtained by storing fresh red hake fillets or mince exposed to air or to 100% oxygen. On the other hand, maximal DMA production rates were obtained by storage under vacuum or 100% nitrogen.

4.2 Chemical inhibition

Chemical inhibition consists of the addition of substances that can act as inhibitors or can reduce any of the components needed for the reaction to take place. TMAOase inhibitors have been described previously and most of them are not used as food additive (e.g., azide, cyanide, etc.) because of their toxicity. Sodium citrate and H_2O_2 have been found to slow the rate of DMA and FA formation in frozen gadoid mince (Parkin and Hultin, 1982b; Racicot *et al.*, 1984). Treating Alaska pollack (*Theragra chalcogramma*) fillets with sodium citrate and sodium pyruvate before freezing resulted in a less tough texture (Krueger and Fennema, 1989). Furthermore, the addition of 0.5% alginate could lower the DMA and FA formation in minced fillet of mackerel stored at -20°C for 2 months (Ayyad and Aboel-Niel, 1991).

Sotelo and Mackie (1993) found a protective role of different cryoprotectants (amino acids and sugars) in preventing FA-promoted aggregate formation of bovine serum albumin during frozen storage. Moreover, TMAO has also been found to exert a protective role over proteins. It has been shown that TMAO stabilizes the conformational changes of fish muscle proteins and some enzymes in frozen storage (Rodger and Hasting, 1984; LeBlanc and LeBlanc, 1989).

The binding of FA during frozen storage was dependent on protein rearrangements in the way that reactive groups become available. The constraints of cryostabilizers on molecular diffusion reduced the exposure of these groups (Herrera *et al.*,

1999). Herrera *et al.* (2000) also reported that the addition of maltodextrins to minced blue whiting muscle inhibited FA production during storage at -10 and -20°C. Sucrose, however, was effective only at -20°C. Herrera *et al.* (2002) studied the effects of various cryostabilizers on inhibiting FA production in frozen-stored minced blue whiting muscle. Several maltodextrins (dextrose equivalent 9, 12, 18 and 28) or sucrose retarded the decreases in protein solubility during storage at -20 and -10°C. These effects were greater at -20°C, and DE 18 maltodextrin seemed to be the most effective treatment at both temperatures. Conversely, sucrose was as effective as maltodextrins at -20°C, but showed hardly any effect at -10°C. A high correlation was found between the effectiveness of cryostabilizers in preventing protein alterations and their effectiveness in inhibiting FA production, particularly at -10°C. In addition, a sigmoidal relationship between protein solubility and FA content was found at this temperature, which supports the hypothesis of the cooperative nature of the effect of FA on protein alterations (Herrera *et al.*, 2002).

1.3 Objectives of study

1. To investigate the FA formation and physicochemical changes of lizardfish muscle proteins as affected by frozen storage and packaging atmosphere.
2. To study the inhibition of FA production in frozen-stored lizardfish mince by various inhibitors regulating TMAOase system.
3. To elucidate the effect of partially purified lizardfish TMAOase on aggregation of natural actomyosin (NAM) extracted from fish muscle during frozen storage in terms of biochemical, textural, and structural changes.
4. To study the impact of the use of TMAOase inhibitors in combination with antioxidants on the retardation of protein aggregation and denaturation of fish muscle protein in the presence of lizardfish TMAOase during frozen storage.