CHAPTER 1

INTRODUCTON

Gel forming ability and viscoelastic property are important for meat and meat products (Hamann, 1992; Lanier, 1992). Quality of gel product depends on the intrinsic and extrinsic factors such as species, season, harvesting etc. Gelation of fish proteins is the most important step in forming desired textures in many seafood products, particularly those from surimi. Various physical conditions and chemical additives can affect surimi gelling property. Freshness of fish is considered as the crucial factor determining the surimi quality (Benjakul *et al*., 2002; MacDonald *et al.,* 1990). Fresh or ice-stored fish are commonly used for surimi production worldwide. Due to over exploitation and the lack of raw material, fish fleet has to go farther, leading to the poorer quality of raw material. This is mainly caused by degradation mediated by endogenous proteinase or microorganism.

Gel strength is largely dependent on interaction or bonds stabilizing the network. Gel strengthening can be achieved by subjecting sols to setting at the temperatures ranging from 0 to 40 $^{\circ}$C prior to heating (Alvarez and Tejada, 1997; An *et al*., 1996; Kamath *et al*., 1992). During setting, myosin heavy chain (MHC) undergoes polymerization via formation of non-disulfide covalent cross-links catalyzed by endogenous TGase (Kumazawa *et al*., 1995; Kimura *et al*., 1991). Setting response has been found to vary among fish species (Benjakul and Visessangaun, 2003; Morales *et al*., 2001; Shimizu *et al*., 1981). However, the rate of TGase cross-linking may be primarily dependent on the protein substrate myosin (Kamath *et al*., 1992). To improve the setting of surimi and strengthen the gel, microbial TGase has been used to induce the polymerization of proteins. Microbial TGase, which is capable of introduction covalent cross-linking between protein molecules, has become more popular for surimi industry (Seguro *et al.,* 1995; Jiang *et al.,* 2000a). However, the efficiency of MTGase in strengthening the gel is determined by some intrinsic factors, especially the freshness of raw material, which is associated with the integrity, denaturation and degradation of proteins. To maneuver the gel improvement of surimi, those influencing factors should be investigated to gain more understanding in using MTGase in surimi.

Literature Review

1. Chemical composition of fish and shellfish

The main constituents of fresh fish are water (65-80%), protein (15-24%), fat (0.1-22%), carbohydrate (1-3%) and inorganic substances (0.8-2%) (Suzuki, 1981). The composition of fish meat varies with species (Table 1). Age, part of body, pre- or post-spawning season and the food condition also affect the fish composition (Suzuki, 1981). The relative amounts of these components are generally within the range found in mammals (Mackie, 1994). Protein is a major composition of fish muscle with the range of 15-20% (wet weight) depending upon muscle type, feeding period and spawning, etc. Generally, protein content is reduced in spawning period (Almas, 1981). Shrimp meat consists of high protein content (50% dw) (Barelay *et al.,* 1983). However, chemical compositions of shrimps were reported to change seasonally (Yanar *et al.,* 2004). Karakoltsidis *et al.* (1995) reported that the changes in chemical compositions of shrimp (*Aristeus antennatus*) and Norway lobster were influenced by seasons (Karakoltsidis *et al.*, 1995).

Table 1 Composition of fish and shellfish meat

Fish species	Average amounts (%)		
	Moisture	Crude fat	Crude protein
Anchovy	74.4	6.0	17.5
Round herring	71.9	4.6	21.3
Frigate mackerel	62.5	16.5	19.8
Carp	75.4	6.0	18.0
Black sea bream	75.7	1.7	21.2
Sole	77.7	1.2	19.5
King crab	80.0	1.3	15.9
Lopster (Norway)	78.0	2.0	17.0

Source: Adaped from Suzuki (1981); Karakoltsidis *et al.* (1995)

There are different proteins in fish muscle. These proteins perform different tasks and have varying properties (Sikorski *et al.,* 1990a). The proteins can be classified into three groups based on solubility as follows:

1.1 Myofibrillar protein

Myofibrillar proteins are the major proteins in fish muscle. Normally, these proteins account for 65-75% of total protein in muscle, compared with 52-56% in mammals (Mackie, 1994). These proteins can be extracted from the muscle tissue with neutral salt solutions of ionic strength above 0.15, usually ranging from 0.30 to 0.70. The myofibrillar proteins are related with the water holding capacity and the other functional properties of proteins such as gelation, etc (McCormick, 1994). Contractile proteins, which are different in size and location in the muscle, are listed in Table 2 (Ashie and Simpson, 1997).

Table 2 Contractile proteins in food myosystems

Protein	Relative Abundance (%)	Size (kDa)	Location
Myosin	50-60	470	Thick filaments
Actin	15-30	43-48	Thin filaments
Tropomyosin	5	65-70	Thin filaments
Troponins	5		Thin filaments
Troponin-C		17-18	
Troponin-I		20-24	
Troponin-T		37-40	
C-protein	-	140	Thick filaments
α-Actin	-	180-206	Z-disc
Z-nin	-	300-400	Z-disc
Connective/Titin	5	700-1,000	Gap filaments
Nebulin	5	~600	N_2-line

Source: Adapted from Ashie and Simpson (1997)

Myofibrillar protein can be further divided into three subgroups as follows:

- **Myosin and Paramyosin**

Myosin is the protein, which forms the thick filament (Suzuki, 1981). A molecular weight is about 500,000 daltons (Ogawa *et al.,* 1994). It is the most abundant myofibrillar component, constituting approximately 40-60% of total protein content (Bechtel, 1986). Myosin consists of six polypeptide subunits, two large heavy chains and four light chains arranged into an asymmetrical molecule with two pear-shaped globular heads attached to a-helical rod-like tail (McCormick, 1994; Xiong, 1997) (Figure 1). Myosin is a protein possessing ATPase activity. The globular head regions of myosin bind and hydrolyze ATP to ADP. 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. Modification of SH_2 results in inactivation of Ca^{2+}-ATPase (Ochiai and Chow, 2000). When myosin is digested by trypsin or chymotrypsin for a short period, myosin is divided into two components, a rapid sediment component called H-meromyosin (HMM), and a slow sediment called L-meromyosin (LMM). When HMM is treated with papain, it is divided into a head and a neck part. A head is called S-1 and the neck part is S-2 (Suzuki, 1981). The S-1 globular heads in myosin have ATPase activity and actin binding ability. A helical rod forms a filament (Ogawa *et al.,* 1994). The myosin head contains the actin binding site, ATP site, alkali light chain site, and DTNB [(5,5 dithiobis)-2-(nitrobenzoic acid)] light chain site (McCormick, 1994). 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).

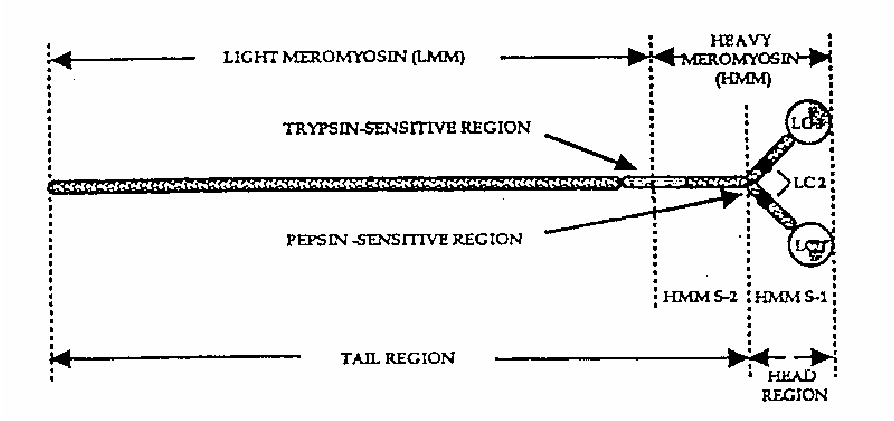

Figure 1 Model of myosin molecule

Source: Xiong (1997)

Paramyosin is one of major muscle proteins found in mollusks (Sikorski, 1994). It consists of high basic amino acid and amide content such as glutamine (20 to 23.5%), aspartic acid (12%), argenine (12%) and lysine (9%), but low in proline content. Paramyosin, a rod shaped alpha-helical chains, consists of 2 subunits, which are 120 nm long with a molecular weight ranging from 95,000 to 125,000 dalton per subunit (Foegeding *et al.,* 1996).

- Actin

Actin constitues about 22% of myofibrillar mass with a molecular weight of 42,000 daltons. Normally, actin in muscle tissue is associated with troponin and tropomyosin complex. It also contains a myosin binding site, which allows myosin to form temporary complex with it during muscle contraction or the permanent myosin-actin complex during rigor mortis in postmorterm (Xiong, 1997). Monomer form of actin is called G- actin and after polymerization, actin filaments are formed and referred to as F-actin. Two F-actin in helix form is called super helix (Foegeding *et al.,* 1996) (Figure 2a). Two F-actins wrap about each other, forming a double helix, called thin filament or I-band, which is associated with tropornyosin and troponin (McCormick, 1994).

- Tropomyosin

Tropomyosin represents approximately 8-10% of myofibrillar protein. It has two subunit chains (Suzuki, 1981). In skelatal muscle, two polypeptides, alpha and beta-tropomyosin can combine to form a tropomyosin dimer. 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 monomers (Foegeding *et al.,* 1996). Tropomysin, a rod-like molecule, consists of two polypeptide chains, each with a molecular weight range of 34,000-36,000 dalton, which associate to form a coiled helix. Each tropomyosin molecule is about 385 A° long and associates in head-to-tail fashion to form a filament that follows and associates with the coil of the F-actin filament (McCormick, 1994) (Figure 2a).

- Troponin

Troponin and tropomyosin regulate muscle contraction. Troponin, accounting for 8-10% of myofibrillar proteins, consists of three subunits including troponin C; which is a calcium binding protein and confers calcium regulation to the contraction process via the thin filament, troponin I; which strongly inhibits ATPase activity of actomyosin, and troponin T, which provides a strong association site for binding of tropomyosin (Foegeding *et al.,* 1996) (Figure 2b).

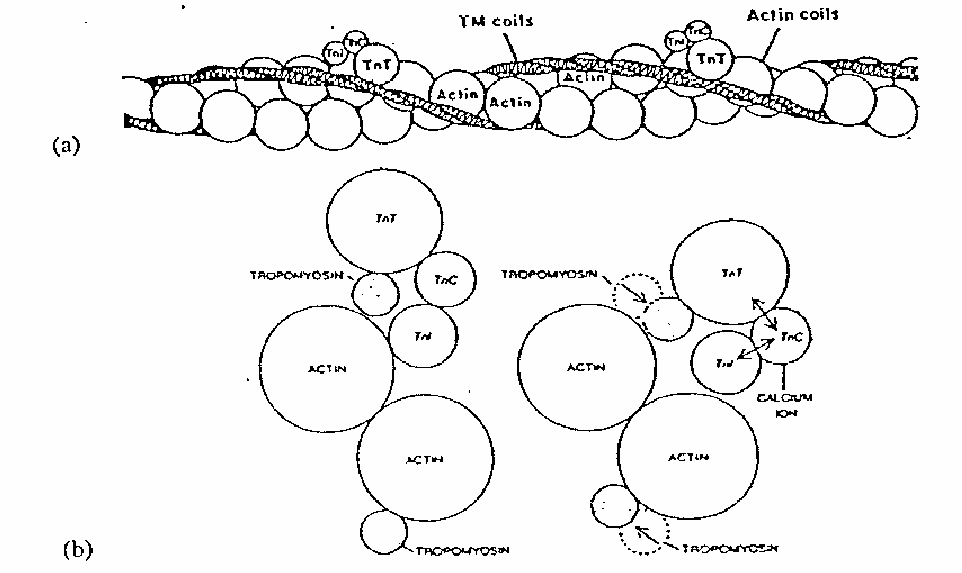

Figure 2 (a) A thin filament of muscle formed by the filament of tropomyosin molecules wound in each of the two grooves of the actin helix. (b) Proposed model for configuration of actin, tropomyosin and troponin (Tn) subunits.

Tn T = troponin-tropomyosin subunit

Tn I = troponin-inhibitory subunit

Tn C = troponin-calcium-binding subunit

Source: McCormick (1994)

1.2 Sarcoplasmic proteins

The sarcoplasmic proteins usually refer to the proteins of the sarcoplasm as well as the components of the extracellular fluid and the sarcoplasm. The sarcoplasmic proteins comprise about 20-35% of the total muscle proteins and are commonly called myogens (Mackie, 1994; Pearsons and Young, 1989). Despite their diversity, sarcoplasmic proteins share many common physicochemical properties. Most are of relatively low molecular weight, high isoelectric pH, and have globular or rod-shaped structures. The sarcoplasmic proteins are extracted by homogenizing the muscle tissue with water or solutions of neutral salts of ionic strength below 0.15. Among the sarcoplasmic enzymes influencing the quality of fish, the enzymes of the glycolytic pathway and the hydrolytic enzymes of the lysosomes are found to be important (Sikorski *et al.,* 1990a).

1.3 Stroma protein

Stroma is the protein, which forms connective tissue, representing approximately 3% of total protein content of fish muscle. It can not be extracted by water, acidic, or alkaline solution and neutral salt solution of 0.01-0.1 M concentration. The components of stroma are collagen, elastin or both (Suzuki, 1981). Elastin is very resistant to moist heat and cooking. Normally, it is a reflection of the different structural arrangements of muscle cells in fish, compared to mammals (Mackie, 1994).

2. Post mortem changes of fish

2.1 Changes of proteins

2.1.1 Degradation of proteins

Post mortem tenderization is one of the most unfavorable quality changes in fish muscle. A proteolytic degradation of myofibrillar and connective tissue components is observed during the extended storage. Myofibrillar proteins fraction in muscle of Monterey sardine was unstable during ice storage (Pacheco-Aguilar *et al*., 2000). The participation of various proteinases in autolytic processes of ice-stored fish depends on location of the enzymes in cytosol and/or factors affecting tissue compartmentization, the presence of activators or inhibitors and the susceptibility of the proteins responsible for muscle integrity to cleavage by the respective enzymes (Ladrat *et al*., 2003). Among post-harvest changes, degradation of fish muscle caused by endogenous proteases is a primary cause of quality losses during cold storage or handling (Haard *et al.,* 1994). The decrease in the relative amount of myosin heavy chain (MHC) and a concomitant increase in the number and intensity of bands of molecular size about 100 kDa were found in *Penaeus borealis* after 24 h and the appearance of a band of slightly less than 50 kDa after 5 h of iced storage (Martinez *et al*., 2001). Proteases are able to hydrolyze the muscle proteins differently. An *et al.* (1994) reported that among the Pacific whiting proteins, MHC was the most extensively hydrolyzed, followed by troponin-T, α- and β-tropomyosin. Microbial proteases may also be a potential cause of proteolytic degradation. Protease from *Pseudomonas marinoglutinosa* was reported to hydrolyze actomyosin at 0-2 $^{\circ}$C and the optimal pH was above 7.0 (Venugopal *et al.,* 1983). Benjakul *et al.* (1997) showed that MHC of Pacific whiting

muscle was hydrolyzed continuously throughout iced storage. MHC decreased to 45% of the original content within 8 days, whereas no changes in actin were observed on SDS-PAGE. Eckhoff *et al.* (1998) reported that insoluble collagen in salmon (*Salmo salar,* L) decreased gradually during 15 days of the storage in ice. The relationship between collagen content and texture was further confirmed by Hatae *et al.* (1986), who showed that a high collagen content resulted in a firm meat. Raw fish meat softened rapidly during chilled storage and histological examinations were shown to be caused by disintegration of collagen (Sato *et al.*, 1991). The initial steps in deterioration of raw fish during its storage on ice consist of hydrolytic reactions catalyzed by endogenous enzymes, which produce nutrients that allow bacteria proliferation (Busconi *et al.*, 1989). A change in microstructure of *Macrobrachium rosenbergii* during storage in ice was observed (Nip and Moy, 1988). Rowland *et al.* (1982) reported that substantial morphological changes due to proteolysis in the tails of M. *rosenbergii.*

2.1.2 Denaturation of proteins

Denaturation of muscle proteins during postmortem storage is another phenomenon causing the changes of fish quality and the functional properties. Pacific whiting muscle protein underwent denaturation during iced storage (Benjakul *et al.*, 1997). ATPase (E.C.3.6.1.8, ATP pyrophosphohydrolase) is associated with the postmortem disappearance of ATP in fish muscle, leading to rigor mortis (Nambudiri and Gopakumar, 1992). Ca^{2+}-ATPase activity is a good indicator of the integrity of the myosin molecule (Roura and Crupkin, 1995). Mg^{2+}-ATPase and Mg^{2+}-Ca^{2+}-ATPase activities are indicative of the integrity of the actin-myosin complex in the presence of endogenous or exogenous Ca^{2+} ions, respectively. Mg^{2+}-EGTA-ATPase activity indicates the integrity of the tropomyosin-troponin complex (Ouali and Valin, 1981; Watabe *et al.*, 1989). Kamal *et al.* (1991) reported that myofibrillar ATPase activities of sardine ordinary and dark muscles decreased during extended iced storage of 10 days. During iced storage of Pacific whiting muscle, no changes in Ca^{2+}-ATPase, Mg^{2+},-Ca^{2+}-ATPase or Mg^{2+}-ATPase were observed, but Mg^{2+}-EGTA-ATPase activity gradually increased during iced storage (Benjakul *et al.*, 1997). Roura and Crupkin (1995) reported the enzymatic activities of myofibrils from pre- and post-spawned hake during iced storage. Mg^{2+}ATPase, Mg^{2+}-Ca^{2+}-ATPase, and Ca^{2+}-

ATPase activities of myofibrils were higher in post-spawned fish than those in pre-spawned fish. ATPase activities of myofibrils from post-spawned hake were not changed during iced storage for 10 days. For pre-spawned myofibrils, Mg^{2+}-ATPase was retained after 7 days of iced storage, while enzymatic activities of the others gradually decreased during iced storage. Seki and Narita (1980) found that EGTA-ATPase activity of the myofibrils from minced carp muscle decreased rapidly, while Ca^{2+}-ATPase and Mg^{2+}-ATPase activities decreased gradually during iced storage with the exception that Mg^{2+}-ATPase activity in the presence of EGTA increased. The increase in this ATPase activity was accompanied with a loss of Ca^{2+}-sensitivity of myofibrils during iced storage for 16 days. Chalmers *et al.* (1992) showed that the apparent viscosity and Ca^{2+}-ATPase activity of actomyosin isolated from cod (*Gadus morhua*) tended to decrease slightly during aging of the fish in ice.

Ca^{2+}-sensitivity was reported to be a good indicator of Ca^{2+} regulation of myofibrillar proteins (Roura and Crupkin, 1995) and was dependent upon the affinity of the troponin molecule for Ca^{2+} ion (Ebashi and Endo, 1968). The decrease in both Ca^{2+}-binding capacity and Ca^{2+}sensitivity was shown to be caused by proteolysis (Tokiwa and Matsumiya, 1969). Additionally, oxidation of thiol groups of myosin has been shown to reduce Ca^{2+}-sensitivity and modify actin-myosin interactions (Seki *et al.*, 1979). Therefore, loss of Ca^{2+}-sensivity in myofibrils from pre-spawned fish could be related to an increment in proteinase activity, which selectively degrades myosin during gonadal maturity (Ruara and Crupkin, 1995).

The denaturation of muscle proteins is caused by the oxidation of thiol (SH) groups of muscle. Hamada *et al.* (1977) reported the oxidation of carp and rabbit actomyosin during 14 days of storage at 4 °C. The rabbit actomyosin had little changes in SH content and disulfide bond contents. On the other hand, carp actomyosin had the marked decreased in SH content with an increase in disulfide bond. Benjakul *et al.* (1997) found that total SH content of actomyosin increased slightly after 2 days of iced storage, followed by a gradual continued decrease up to 8 days. A decrease in total SH group was reported to be due to formation of disulfide bonds through oxidation of SH groups or disulfide interchanges (Hayakawa and Nakai, 1985). Chan *et al.* (1995) reported that myosin contained

42 SH groups. Two types of SH groups on the myosin head portion (SH_1 and SH_2) have been reported to be involved in ATPase activities of myosin (Yamaguchi and Sekine, 1966). Another SH group (SH_a) was localized in the light meromyosin region of myosin molecule and was responsible for oxidation of MHC (myosin heavy chain) and its dimer formation resulted in an increase in Mg^{2+}-EGTA-ATPase activity of carp actomyosin during iced storage (Sompongse *et al.*, 1996a).

2.1.3 Cross-linking of proteins

During storage of fish, quality is lost due to deterioration of texture, flavor and color, especially after long period of storage, and when poor freezing practices are employed or when the initial quality of fish is low (Matsumoto, 1980; Shenouda, 1980). The development of drier and firmer texture of fish muscle after storage has been frequently attributed to protein denaturation (Castell *et al.*, 1980). Both formaldehyde (FA) and dimethylamine (DMA) are products of the enzyme-catalyzed reaction, which has trimethylamine oxide (TMAO) as its substrate (Benjakul *et al*., 2004a). TMAOase is located in the viscera (Benjakul *et al*., 2003a) and is commonly found in certain gadoid species (Krueger and Fennema, 1989). The effect of FA on the loss of protein functionality was studied (Careche and Tejada, 1990; Tejada *et al*., 2002). Formaldehyde is a reactive substance that can rapidly bind to free amino groups of protein. One formaldehyde 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 (Badii and Howell, 2002; Leelapongwattana *et al*., 2005). Addition of formaldehyde resulted in changes in the secondary structure of cod myosin, causing the exposure of the hydrophobic aliphatic groups, and eventually leading to the appearance of covalent cross-links (Careche and Li-Chan, 1997). Oxygen or potential oxidants, such as oxidized lipids, have been reported to be inhibitors of TMAOase (Lunstrom et al., 1982a) along with cyanide, riboflavin, sodium azide and oxaloacetate (Hamada *et al.*, 1981), while glutathione, Fe^{2+}, ascorbic acid increase the rate of TMAO breakdown. Herrera *et al.* (2000) reported that addition of maltodextrins to minced blue whiting muscle inhibited formaldehyde production during storage at -10 °C and -20 °C. Addition of oxidizing agent such as H_2O_2, NaOCI and $KBrO_3$ to red hake mince reduced the rate of DMA and FA formation, while reducing agents accelerated their formation (Racicot et al.,

1984). Lundstrom *et al.* (1982b) and Leelapongwattana *et al.* (2005) found that packaging should be used in choice to prevent possible textural alterations cause by degrading TMAO to DMA and FA.

3. Functional properties of fish protein

Fish myofibrillar protein has the excellent functional properties such as emulsifying properties, gel-forming ability, and water holding capacity (Tanabe and Saeki, 2001; Lin and Park, 1996). Generally, 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 generally lowered when protein denaturation occurs (Regenstein *et al.,* 1983). The functional property of muscle protein 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).

3.1 Water holding capacity

Myofibrils are composed of 25 % protein and 75 % water. Majority of water in meat is confined within the myofibrils in the spaces between the myosin and actin (Xiong, 1997). Myofibrils are the primary sites 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 (Xiong, 1997). 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).

3.2 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, 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). Lizardfish was a species susceptible to protein denaturing during frozen storage, most likely due to the formation of formaldehyde induced by TMAO demethylase (Benjakul *et al*., 2003b). 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 textural properties depend largely on protein solubility, especially in the case of fish (Colmenero and Borderiad, 1983).

3.3 Gel-forming ability

Gelation of myofibrillar proteins has been shown to be largely responsible for the textural properties of processed fish products (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 of surimi-based products (Saeki *et al.,* 1995). Differences in cross-linking of MHC contribute to the differences in gel-forming ability among the muscles of various fish (Benjakul *et al.,* 2001a). Protein gels are three-dimensional matrixes or network, in which water is entrapped (Pomeranz, 1991). Table 3 gives a summary of changes, which may occur during the heat denaturation of actomyosin.

Table 3 Conformational changes which may occurring during the thermal denaturation of natural actomyosin

Temperature (°C)	Protein(s) or segment involved	Description of events
30-35	Native tropomyosin	Thermally dissociated from the F-actin backbone
38	F-actin	Super helix dissociates into single chains
40-45	Myosin	Dissociates into light and heavy chains
	Head	Possibly some conformational change
	Hinge	Helix to random coil transformation
45-50	Actin, myosin	Actin-myosin complex dissociates
50-55	Light meromyosin	Helix to coil transformation and rapid aggregation
>70	Actin	Major conformational changes in the G-actin monomer

Source: Ziegler and Aton (1984)

The process of protein gelation involves two steps; protein denaturation and aggregation (Ziegler and Aton, 1984).

3.3.1 Protein denaturation

Addition of salt in combination of heating is two major factors involved in denaturation and gelation of muscle proteins. The addition of salt shifted the denaturation transitions to lower temperatures and decreased the enthalpies of heat denaturation. The addition of salt might cause a partial unfolding of proteins and increased sensitivity to denaturation (Park and Lanier, 1989). Gill *et al*. (1992) concluded that thermal aggregation of herring myosin showed little dependence on salt concentration (0.6-1.4 M NaCl, pH 6.5) but salt enhanced aggregation of cod myosin at heating temperatures higher than 50 °C. Alvarez *et al*. (1995) found that the maximal gel strength of sardine surimi was obtained at NaCl concentrations of 2.24%. The formation of a firm and elastic kamaboko gel from sardine surimi requires the addition of 2-4% NaCl (Roussel and Cheftel, 1990).

Heat denaturation mainly contributes to the aggregation of protein molecules. Rate of denaturation is governed by the stability toward heat applied. Thermal stability is one of the most important properties of muscle proteins. Fish myosin is very unstable in comparison with that of mammal (Connell, 1960; Ogawa *et al*., 1994). The transition due to denaturation is often referred to in term of its peak maximum temperature (T_{max}). Peak transition temperatures (T_{max}) for the proteins present in different fish muscle were reported by Poulter *et al*. (1985). T_{max} of the first peak is assumed to correspond to myosin denaturation. Peak 2 is the small peak usually seen at a temperature intermediate between the myosin and actin denaturation peaks. Peak 3 is the most stable transition observed in the thermograms and is assumed to correspond to actin denaturation. The T_{max} of myosin was different among fish species (Ogawa *et al.,* 1993). Thermal denaturation of hake (*Merluccius hubbsi*) myofibrillar proteins of whole muscle showed two T_{max}, 46 °C and 75 °C (Beas *et al.,* 1990).

Thermal denaturation of the myosin was dependent on pH and ionic strength (Stabursvik and Martens, 1980; Wright and Wilding, 1984). Davies *et al.* (1988) found that snapper myosin showed the distinct peak with relatively high T_{max}. As the pH was increased, the transition peak became distinguishably less stable, as shown with a sharp decrease in T_{max}.

3.3.2 Aggregation

Denatured proteins begin to interact noncovalently to form a fine elastic network when surimi sol is subjected to heating process. Samejima *et al.* (1981) proposed the heat-induced gelation of myosin involving of two reactions as follows:

1. Aggregation of the globular head segments of the myosin molecule, which is closely associated with the oxidation of sulfhydryl groups. This aggregation process, ultimately responsible for the formation of a three dimensional structure, requires the participating molecules to interact at specific point. At least three cross-links (bonds) per polymer segment are formed (Figure 3c). A network cannot be formed without three cross-links (Figure 3a, b) (Niwa, 1992). The resultant molecular orientation necessary to facilitate these interactions may proceed at a relatively slow rate (Hermansson, 1979).

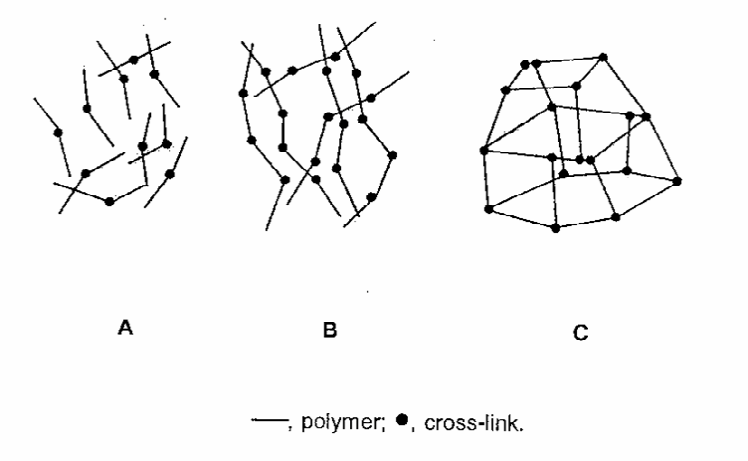

Figure 3 Formation of a gel network structure

Source: Niwa (1992)

2. Network formation resulting from the unfolding of the helical tail segment. The head portions also associate to form "super-junctions" which provide extra cross-linking to the gel network. Since agents that block formation of disulfide bonds did not retard its onset, Samejima *et al.* (1981) concluded that another type of aggregation, perhaps due to intermolecular association of side chains, superimposes on the sulfhydryl-dependent reaction. Niwa (1992) reported that various bonds involve in gel network formation. The factors determining the number and kind of interactions or bonds include not only the species from which the surimi is derived (Suzuki, 1981; Shimizu, 1985) but also the heat conditions in which the gel is made (Ishikawa, 1978; Akahane and Shimizu, 1990; Lee *et al.,* 1990; Yamazawa, 1990). Chan *et al.* (1992) reported that thermal aggregation of fish myosin was coincidental with an increase in the surface hydrophobicity of the unfolded domains of myosin molecules and was affected by the temperature at which these domains unraveled. Temperature and ionic strength have a profound effect on the hydrophobic interaction of proteins. Chan and Gill (1994) compared the denaturation and aggregation behaviors of cod and herring myosins and reported that the inferior gel forming ability of herring muscle proteins was related to the unfolding profile of interior hydrophobic domains when heated. Extent of aggregation for fish myosin seems to depend on the amount of hydrophobic surface exposed on the heated molecules (Chan *et al.,* 1992; wicker et *al.,* 1986). Therefore, it is

reasonable to assume that the extent of aggregation for poor gel forming species, i.e. herring, would be improved by finding a strategy to increase the surface hydrophobicity of heated myosins. Gill *et al.* (1992) reported that myosins from different fish species aggregated to different extents as temperature increased. Thus, the decrease in viscosity was probably due to thermal induced aggregation of myosin sub-fragments. Heating actomyosin solution at 40 and 85 °C resulted in reduction of ATPase activity. Similar trend was observed by Sano *et al.* (1994) who found that reactive SH increased from 20 to 50 °C, suggesting that SH groups inside the actomyosin molecule emerged to the surface as a result of unfolding, thereby causing a gradual decrease in ATPase activity with the increase in temperature. A rapid loss in ATPase activity was found from 40 to 50 °C, indicating conformational changes in active sites in actomyosin. Calcium ions can form salt linkages between negatively charged sites on two adjacent proteins (Wan *et al.,* 1994). However, the addition of calcium salts to improve gelling properties of surimi may actually be more due to their effects on a crosslinking enzyme, transglutaminase (TGase), in the muscle than from ionic linkages between proteins. TGase catalyzes the polymerization and crosslinking of proteins through the formation of covalent bonds between protein molecules. This link enhances the physical strength (hardness and cohesiveness) of surimi gels. Seki *et al.* (1990) isolated TGase from Alaska pollock and found that it could induce the gelation of minced fish. Tsukamasa and Shimizu (1990) further reported that the strong gel forming ability of sardine was due to the formation of the non-disulfide bond which later was shown to be due to the action of TGase (Tsukamasa *et al.,* 1993). Benjakul et al. (2001a) reported that TGase played a role in setting of bigeye snapper surimi. TGase from different species determines the setting condition owing to the differences in their optimal temperatures (Benjakul and Visesanuguan, 2003).

As the temperature is increased over 45-50 °C, gel network (suwari) is partially disrupted to form a broken network (modori). This process is species dependent (Shimizu *et al.,* 1981). The action of proteases has been found to promote this gel weakening (Lanier *et al.,* 1981; An *et al*, 1994; Benjakul *et al*., 2003c). However, in systems without enzymes or containing enzyme inhibitors, softening still occurred (Iwata *et al.,* 1974; Lee, 1984). Once the temperature is increased above 65-70 °C, the gel becomes

ordered and nontransparent. This stage is referred to as kamaboko (Suzuki, 1981). In this final stage, the cohesiveness and elasticity of the gel is enhanced.

4. Suwari (setting)

Meat paste left for a period of time at temperatures lower than 50-60 °C after grinding loses its plasticity and turns into gel, called "suwari" or setting. The degree of suwari forming depends on the fish species (Table 4). Different species had different degree of surface hydrophobicity and this was suggested to be related to the differences in setting (Niwa *et al.*, 1981). Suwari gel is formed by retaining water in the molecule linkage formed by hydrophobic bond and hydrogen bond.

During setting, the protein molecules can align and interact each other. Gill and Conway (1989) identified the HMM S-2 and LMM regions of the tail as those engaged in the interactions. It was noted that at 30-44°C only the LMM formed gels, while HMM formed a curd-type structure (Sano *et al.*, 1990).

Itoh *et al.* (1979) and Niwa *et al.* (1982) concluded that sulfhydryl groups were prominent in gelation at 40 °C of fish actomysin and myosin. Taguchi *et al.* (1987) reported that at setting temperatures (30-40 °C), HMM S-1 fragments were thermally aggregated, whereas only LMM displayed a similar behavior when heated above 50 °C. Other forms of interaction such as hydrogen bonding are also present during setting. Like disulfide bond, they are not thought to regulate the suwari structure, but instead impart elasticity to the system (Niwa *et al.*, 1982).

TGase has been known to involve in setting of surimi via inducing the formation of non-disulfide covalent bond, TGase may be varied between species and contributes to the different gelation characteristics. Benjakul and Visessanguan (2003) found that setting of surimi paste with and without subsequent heating resulted in the increase in both breaking force and deformation of suwari and kamaboko gel from bigeye snapper surimi, respectively. Suwari and kamaboko gels with the setting at 25 °C had a lower solubility, when the setting time increased. The decrease in solubility indicated the formation of non-disulfide cross-links induced by endogenous TGase (Benjakul and Visessanguan, 2003). Seki *et al.* (1990, 1998); Kimura *et al.* (1991); Kamath *et al.* (1992); Wan *et al.* (1994) reported that an endogenous

TGase was largely responsible for the setting phenomenon. Wan *et al.* (1995) confirmed the essential role of TGase in the setting of walleye pollack surimi paste, compared with that of salmon, non-setting fish.

Table 4 Suwari forming capacity of different fish species

Suwari forming capacity	Fish species
Easy	Sardine *(Sardinops melanosticta)*
	Anchovy *(Engraulis japonica)*
	Alaska pollack *(Theragra chalcogramma)*
	Lizard fish *(Saurida undosquamis)*
	Cutlass fish *(Trichiurus lepturus)*
	Horse mackerel *(Trachurus japonicus)*
	Mackerel *(Scomber japonicus)*
Normal	Sea bass *(Lateolabrax japonicus)*
	Skipjack *(Katsuwonus pelamis)*
	Cod *(Gadus morhua macrocephalus)*
	Bigeye tuna *(Parathunnus sibi)*
Difficult	Sharks
	Carp *(Cyprinus carpio)*
	Black marlin *(Makaira mazara)*
	Sharp-toothed eel *(Muraenesox cinereus)*
	Croaker *(Argyrosomus argentatus)*
	Squids *(Todarodes pacificus* etc.)

Source: Okada (1992)

The quality of directly cooked gel is in most case poorer than those with prior setting. The lower quality of such directly cooked gels is thought to be due to the rapid formation of disulfide and hydrophobic protein-protein bonds in the absence of the conditions required for the protein to orient to form a network. Protein coagulation becomes more prevalent, compared to set gels (Niwa, 1975; Alvarez *et al.*, 1999).

5. Transglutaminase (TGase)

TGase is a transferase, having the systematic name as proteinglutamine γ-glutamyltransferase (EC 2.3.2.13). It catalyzes the acyl transfer reaction between γ-carboxyamide groups of glutamine residues in proteins, peptides, and various primary amines (Motoki and seguro, 1998). When the ε-amino group of lysine acts as acyl acceptor, it results in polymerization and inter- or intra-molecular cross-linking of protein via formation of ε-(γ-glutamyl) lysine linkages (Motoki and seguro, 1998). This occurs through the exchange of the ε-amino group of the lysine residue for ammonia at the carboxyamide group of a glutamine residue in the protein molecule(s) (Figure 4). In the absence of primary amines, water may act as the acyl acceptor, resulting in deamination of γ-carboxyamide groups of glutamine to form glutamic acid. Formation of covalent cross-links between protein is the basis of the ability of TGase to modify the physical properties of protein foods (Ashie and Lanier, 2000).

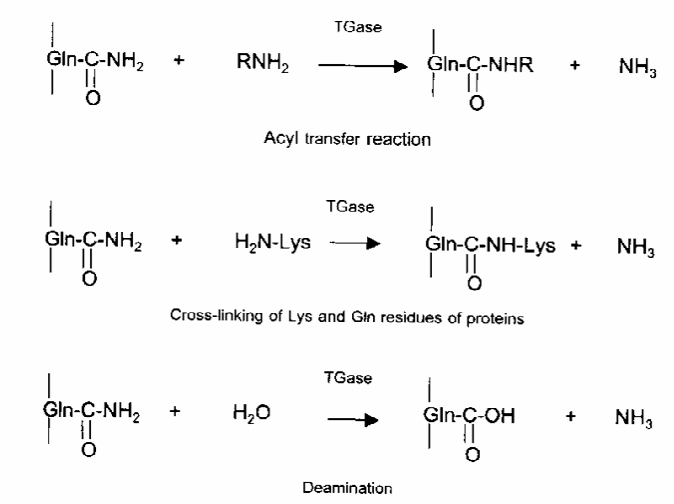

Figure 4 Reaction catalyzed by TGase

Source: Ashie and Lanier (2000)

TGase has been found in tissues of various species including mammals, birds, fish and shellfish, microorganisms, and plants (Table 5).

Table 5 Source and characteristics of some TGases

Sources	Mol. Wt. (kDa)	Optimum Temp. (°C)	Optimum pH
Mammal			
Human plasma factor XIII	300-350[t]		8.0
Bovine factor XIIIa			
Guinea pig liver	75-85[m]		
Rabbit liver	80[m]		
Plant			
Pea seedlings			
Alfalfa	39[m]		
Microbial			
Physarum polycephalum	77[d]		
Streptoverticillium ladakanum	37.5	50	6.0
Streptoverticillium mobaraense	40[m]	50	6.0-7.0
Seafoods			
Red sea bream liver	78	55	9.0-9.5
Carp muscle	80		
Walleye pollack liver	77	50	9.0
Lobster muscle	200		
Japanese oyster	84/90	40/25	8.0
Limulus hemocyte	86		
Scallop	80		
Botan shrimp	80		
Squid	80		
Rainbow trout	80		
Atka mackerel	80		

[t] tetramer; [m] monomer; [d] dimer

Source: Adapted from Ashie and Lanier (2000)

TGase has a broad specificity for primary amine acceptors (peptidebound lysine or polyamines) (Folk and Finlayson, 1977). However, the cross-linking capability is influenced by the amino acid sequence (including charge) surrounding the susceptible glutamine residue and local secondary structures that are not well defined (Folk, 1980). Proteolysis of a nonreactive protein can convert it to a transglutaminase substrate (Greenberg *et al.,* 1991).

TGase activity in tissues may be determined by one of several mechanisms including: amine incorporation into substrates using monodansylcadaverine, hydroxamate or radioactive putrescine (Folk and Chung, 1985; Lorand *et al.,* 1969), disappearance of amino groups by trinitrobenzenesulfonate or fluorescence intensity methods (Ikura *et* al., 1980); increase in molecular weight of substrate by sodium dodecylsulfatepolyacylamide gel electrophoresis (SDS-PAGE) (Ikura *et al.,* 1980); release of ammonia (Backer-Royer *et al.,* 1992); or measurement of functional effects such as viscosity and gel strength (Sakamoto *et al.,* 1994). Ohtusuka *et al.* (1996) have also developed an enzyme-linked immunosorbent assay (ELISA) for specifically estimating microbial TGase activity in surimi-based products.

6. Microbial transglutaminase

TGases have been found in some microorganisms. Ando *et al.* (1989) isolated microorganism (*Streptoverticillium mobaraense*) that produced a TGase, which did not require calcium ions for activity. This microbial TGase (MTGase) has been applied to polymerize rabbit myosin, carp myosin, beef myosin and actin (Nonaka *et al.,* 1989; Muguruma *et al.,* 1990; Kato *et* al., 1991). MTGase has been shown to be useful in strengthening surimi gels during the setting reaction (Seguro *et al.,* 1995; Sakamoto *et al.,* 1995a). The gel-forming and viscoelastic properties of surimi-based products were improved by a treatment with MTGase (Seguro *et al.,* 1995). Tsukamasa and Shimizu (1990) reported that the strong gel-forming ability of sardine was due to the formation of the non-disulfide bond, which was shown to be due to the action of TGase (Tsukamasa *et al.,* 1993). Ca^{2+}-independent microbial TGase from *Streptoverticillium mobaraense* (Nonaka *et al.,* 1989; Huang *et al.,* 1992; Gerber *et al.,* 1994) or from *Streptoverticillium ladakanum* (Tsai *et al.,*

1995,1996) has shown potential to increase the gel strength of fish surimi.

Since MTGase is excreted into the culture medium, cell disruption is unnecessary. Its purification thus proves to be rather easy. Consequently, its commercialization has been accelerated (Kanaji *et al*., 1993). Physicochemical properties, such as molecular weight and secondary structures, and enzymatic properties have already been reported (Ando *et al.,* 1989; Nonaka *et al*., 1989; Kanaji *et al*., 1993). The isoelectric point of MTGase was approximately 8.9. The molecular weight of MTGase was previously determined to be 40,000 on both SDS-polyacrylamide electrophoresis (SDS-PAGE) and gel-permeation chromatography (Kanaji *et al*., 1993). MTGase has a molecular weight of 38,000 and comprises of 331 amino acid residues (Figure 5) (Kanaji *et al*., 1993). The overall sequence data indicate that MTGase has a single cysteine residue. The molecular weight calculated from the amino acid composition (331 residues) is 37,842, which is similar to the experimentally obtained value of 38,000. MTGase is, therefore, considered to be a monomeric and simple protein (not a glycoprotein or lipoprotein), although there are two potential glycosylation sites (-Thr-Xxx-Asn-) in the primary structure.

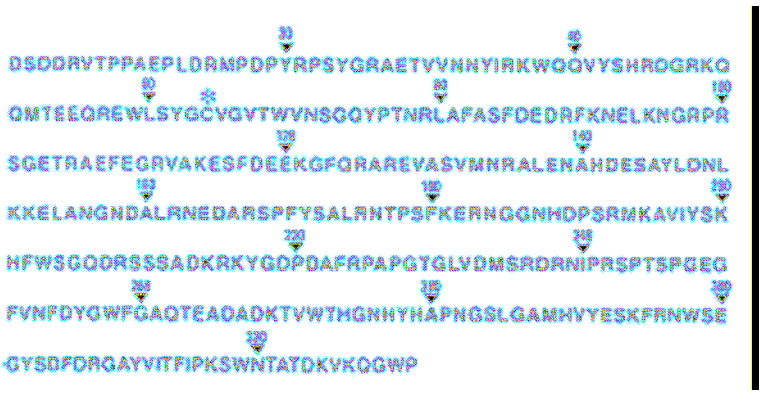

Figure 5 Primary structure of microbial transglutaminase (MTGase): all amino acids are denoted by the three letter codes; *, indicates the possible active cysteine residue.

Source: Motoki and Seguro (1998)

The pH optimum of MTGase was around 5 to 8. However, even at pH 4 or 9, MTGase still expresses some enzymatic activity (Motoki and Seguro, 1998). MTGase is thus considered to be stable over a wide pH range. The optimum temperature for enzymatic activity was 50 °C, and MTGase fully sustained its activity even at 50 °C for 10 min (Motoki and Seguro, 1998). On the other hand, it lost activity within a few minutes on heating to 70 °C. MTGase still expressed activity at 10 °C, and still retained some activity at temperatures just above the freezing-point (Motoki and Seguro, 1998). TGases, including the well-characterized guinea pig liver enzyme, require Ca^{2+} for expression of enzymatic activity. However, MTGase from a variant of *Streptoverticillium mobaraense is* totally independent of Ca^{2+}. In this aspect, MTGase is quite unique from other mammalian enzymes. Such a property is very useful in the modification of functional properties of food proteins, because many food proteins, such as milk caseins, soybean globulins and myosins, are susceptible to Ca^{2+}. They are easily precipitated in the presence of Ca^{2+} and become less sensitive to MTGase (Nonaka *et al*., 1997; seguro *et al*., 1995).

The sensitivity of MTGase toward other cations in the absence of reducing agents was investigated (Seguro *et al*., 1996). Cu^{2+}, Zn^{2+}, Pb^{2+} and Li^{+} significantly inhibited MTGase. Heavy metals such as Cu^{2+} Zn^{2+} and Pb^{2+} bind the thiol group of the single cysteine residue, and this strongly supports the idea that the cysteine residue could be part of the active site of MTGase (Tsai et al., 1996).

Addition of microbial TGase to surimi significantly increases its gel strength, particularly when the surimi has lower natural setting ability (presumably due to lower endogenous TGase activity) (Lee *et al.,* 1997; Seguro *et al.,* 1995; Kumazawa *et al.,* 1993). An increase in non-disulfide polymerization and formation of ε-(γ-glutamyl) lysine dipeptides was found with increase in setting time and microbial TGase concentration (Tsukamasa and Shimizu, 1990). At equal levels of ε-(γ-glutamyl) lysine content, gels prepared with added microbial TGase displayed higher gel stress (Lee *et al.,* 1997). However, the rate of myosin polymerization may also be a factor influencing gel strength, not isopeptide content alone. Yasunaga *et al.* (1996) observed that increased isopeptide content was concomitant with increased gel strength and increased microbial TGase added. The relatively higher gel

strength with an increasingly lower gel strain produced gels that differed in texture from those produced by setting without additives (Abe, 1994). Jiang *et al.* (2000a) investigated the properties of surimi gels from threadfin bream and pollack surimi set at 30 °C or 45 °C with MTGase from *Streptoverticillium ladakanum*. The optimal amounts of MTGase and setting conditions were 0.3 unit/g surimi either at 30 °C for 90 min or at 45 °C for 20 min for threadfin bream. For pollack surimi, MTGase at a level of 0.2 unit/g surimi with setting at 30 °C for 60 min was found to be the optimum condition. MTGase catalyzed MHC cross-linking of both pollack and threadfin bream surimi and increased the gel forming ability of surimi. Ramirez *et al.* (2000) reported that optimal condition for the addition of MTGase to improve gel quality from striped mullet (*Mugil cephalus*) involved: a concentration of MTGase of 9.3 g/kg of surimi, and setting at 37 °C for 3.9 h. Under these conditions, the maximum shear strain was observed. However, the addition of MTGase at 5 g/kg of surimi in combination with setting at 34.5 °C for 1 h rendered the maximum shear stress. Concentration of microbial TGase, temperature and time were optimized to improve the mechanical properties of surimi from silver carp. Optimal predicted properties were obtained by employing the following setting conditions: a concentration of microbial TGase of 8.8 g/kg of surimi, at 39.6 °C for 1 h. Under these conditions, a surimi from silver carp with shear stress of 146 kPa and shear strain of 1.59 was obtained. Shear stress was strongly affected by temperature and time, while shear strain was moderately affected (Ramirez *et al.*, 2000).

To increase the efficacy of MTGase in gel strengthening, many approaches have been reported. Jiang *et al.* (1998a) investigated the effect of combining MTGase from *Streptoverticillium ladakanum* with ultraviolet (UV) irradiation on the gelation of minced mackerel. The gel strength of minced mackerel with MTGase alone at a concentration of 0.47 unit/g reached 1789 g.cm, which was greater than the control. When MTGase supplemented minced mackerel was exposed to UV light for the optimal irradiation time of 20 min, the gel strength could be further increased by 25%. MTGase causes the cross-linking of MHC of mackerel actomyosin and UV irradiation enhances this polymerization of MHC by MTGase. Accordingly, the gel strength of minced mackerel increased significantly when MTGase and UV irradiation were used in combination. Jiang *et al.* (2000b) reported that a combination of MTGase, reducing agent and protease inhibitor was employed to improve

the quality of underutilized fish surimi. MTGase could catalyze the MHC cross-linking and increase the gel forming ability of hairtail surimi. The texture degradation caused by the endogenous proteases could be inhibited by the addition of inhibitor. The best solution to improve gel-forming ability of frozen hairtail surimi was the combination of 0.35 units MTGase/g, 0.1% sodium bisulfite and 0.01 mM E-64. Addition of exogenous TGase could possibly offset somewhat the effects of the heat-stable proteases because the isopeptide bonds formed by TGase are supposedly resistant to proteolytic attack (Lorand, 1983). Generally, a protease inhibitor substance, such as beef plasma or egg white, is added to eliminate gel weakening by proteases and thus maximize the impact of TGase cross-linking on gel texture.

OBJECTIVES

1. To investigate the influence of MTGase in combination with setting on the gel properties of some marine fish and shrimp.
2. To elucidate the efficiency of MTGase on the quality improvement of gel produced from some marine fish and shrimp stored in ice for different times.
3. To study the effect of MTGase on cross-linking of proteins from some marine fish and shrimp modified by different methods.