Review of Literature
II. REVIEW OF LITERATURE

Fish proteins are recognized as highly functional in nature and gelation is one such important functional property. Interest in gel forming ability of fish muscle has arisen from the potential use of washed mince for production of fabricated products. The addition of cryoprotectants to the washed fish mince and then subsequent freezing have gained popularity all over the world and is known as ‘surimi’. Surimi has been used as a raw material for texturized and formulated fish products like seafood analogue and fish sausage. The gelling ability of surimi is considered as one of the important attribute in defining the overall quality of surimi. The gel imparts the appropriate viscoelastic property, juiciness and texture (Kinsella, 1982). Fish muscle protein has unique ability to set when ground with salt and incubated below 40°C, a gel like structure will form. This is known as ‘setting’ or ‘suwari’ in Japanese language. The endogenous transglutaminase (TGase) enzyme, which catalyses the formation of $\varepsilon$-(γ-glutamyl) lysine (EGL) cross-links has been implicated in the setting process. In this section a detailed account of setting mechanism, properties of transglutaminase enzyme and thermal gelation characteristics of set meat have been presented.

2.1. Setting

Formation of fish meat gel has been characterized, with respect to changes in three-dimensional structure, by three stages as heating proceeds: ‘suwari’, ‘modori’, and ‘kamaboko’ (Suzuki, 1981). After being mixed with salt, fish meat sols incubated below 40°C from most fish species have a unique ability to form a translucent, highly deformable gel. This process is called ‘setting’ or ‘suwari’. A stronger gel results by heating at higher temperatures than by cooking directly (Lanier, 1986). Modori describes the partial disruption of a loose protein network as the temperature is further increased to 50$^\circ$-60°C. Modori is associated with the action of endogenous heat activated proteinases (Niwa, 1992). Kamaboko refers to the formation of an ordered, strong and elastic gel at temperatures above 65$^\circ$-70°C.
2.1.1. Species specificity of setting process

Setting is considered to be the sole characteristic of fish protein (Matsumoto and Noguchi, 1992). However, setting response has been found to vary among different fish species (Benjakul and Visessanguan, 2003; Morales et al., 2001; Shimizu et al., 1981). Shimizu et al. (1981) examined 52 species for setting ability, of which 49 species of fish and cartilaginous fish, squid, prawn, chicken and rabbit were included. Based on setting, the authors have divided these species into four categories.

1. Difficult-setting and difficult-disintegrating group, in which sharks, needlefish and marlin were included together with chicken and rabbit

2. Difficult setting and easy-disintegrating group, to which the greater part of the red meat fish other than sardine, belonged

3. Easy setting and easy-disintegrating group, in which sardines, croaker and cold-water fish such as Alaska pollack and ice fish were included and,

4. Easy setting and difficult-disintegrating group, in which flying fish, barracuda and grub were the typical species.

Chan et al. (1992) observed the different cross-linking ability of myosin heavy chain (MHC) from herring, cod and silver hake. The herring MHC formed only small polymers but cod and silver hake MHC formed both small and large polymers. Decrease in body temperature of fish results in decrease of setting temperature (Katoh et al., 1984; himizu, 1985). It was found that the actomyosin pastes from easily setting species gave more elastic gels than those from hard setting species (Niwa et al., 1993).

2.1.2. Mechanism and role of myosin during setting

It is widely acknowledged that myosin molecules are involved in setting process. It has been postulated that setting process brings about change in conformation of the myosin molecule accompanied by various interactions. Chan et al. (1995) reported that there are three types of protein-protein interactions involved in the formation of myosin polymers during setting at 10°C and / or subsequent heating at 90°C. The interactions included non-disulfide interactions, disulfide bonds and noncovalent interactions (Niwa et al., 1981; Lanier et al., 1982; Kim et al., 1987;
Kamath, 1990). Disulfide bonds and ε-(γ-glutamyl)-lysine cross-link represent the covalent bonds influencing the gel characteristics (Niwa, 1992).

Setting can be achieved within a short period (2-4 h) near 40°C (high-temperature setting), or following an extended period (12-24 h) at lower temperatures of 0-40°C (low temperature setting) (Wu et al., 1985). Although low temperature setting is related to TGase activity, high-temperature setting is related to the transition in rheological properties at 36°-38°C as measured in Atlantic croaker actomyosin using a thermal scanning rigidity monitor (Wu et al., 1985). Lanier (1986) reported that preincubation of surimi gels from Atlantic croaker and sand trout at 40°C greatly increased their strength (stress at failure) and to a smaller extent, elasticity (strain at failure). The α-helical structure of myosin, prevalent in the tail portion of the molecule, unfolded at 30°-40°C corresponding to high-temperature setting (Ogawa et al., 1993). The participation of the α-helix in the setting of fish actomyosin and myosin among different fish species has been studied using circular dichroism measurements (Ogawa et al., 1993, 1995). It was found that the gel strength was highly correlated with the decrease in α-helicity (r = 0.85) initiated by unfolding of α-helices during setting.

2.1.2.1. Chemical bonds involved in setting

Non-disulfide covalent bonding

The unique setting ability of fish meat paste is thought to mainly result from enzymatically catalyzed formation of non-disulfide covalent bonds between protein molecules (Lanier, 2000). Several authors have attributed the increased textural properties of set meat to the endogenous transglutaminase enzyme (TGase) (R-glutamyl-peptide; amine γ-glutamyl transferase; EC 2.3.2.13) that catalyses the cross-linking action of muscle proteins, especially myosin (Seki et al., 1990; Kimura et al., 1991; Kishi et al., 1991; Kuraishi et al., 2001). TGase has been reported to catalyze an acyl transfer reaction between γ-carboxamide groups of glutamyl residues in proteins as the acyl donors and a variety of primary amine and water as the acyl acceptor (Folk, 1980; Kumazawa et al., 1996). When the amino group of lysine acts as acyl acceptor, cross-linking of proteins is mediated through the formation of ε-(γ-glutamyl) lysine linkages. In the absence of amine substrates, TGase is capable of catalyzing the deamidation of glutamine residues (Motoki et al., 1986). The resulting
ε-(γ-glutamyl) lysyl isopeptide bonds are stronger than hydrogen bonds and hydrophobic interactions (Yongsawatdigul et al., 2002). This three-dimensional network formed subsequently acts as the backbone of the final gel (Niwa et al., 1995). Such inter and intra molecular covalent cross-linking of myosin molecules result in higher gel elasticity (Lee et al., 1997b). The cross-linking of myosin polymers during setting resulted with a concurrent decrease in myosin heavy chain monomers (Benjakul et al., 2003; Ramirez-Suarez and Xiong, 2003 a).

Cross-linking seems to occur between actomyosin and other myofibrillar protein constituents of the muscle to yield huge aggregates (Funatsu et al., 1996). Consequently, the content of ε-(γ-glutamyl)-lysine dipeptides cross-links generally correlates with the level of increase in gel strength (Imai et al., 1996). However, Lee et al. (1997a) found that the rate at which the cross-linking reaction proceeds is an important factor in the ultimate strength attained. Also, a correlation between cross-link type, number and the ultimate gel mechanical properties would not be expected in all cases. This is because other factors, such as the distribution of the cross-links, the distribution of the proteins being cross-linked, the geometry of the gel matrix formed, and the relative lengths of non cross-linked proteins between points of cross-linking all likely contribute to the ultimate mechanical / fracture properties of protein gels (Lanier, 2000).

Seki et al. (1990) reported the presence of TGase in soluble extract of Alaska pollack muscle and surimi, which catalyzed the cross-linking reaction between myosin heavy chains of carp myosin B when incubated at 25°C. Presence of TGase was also reported in walleye pollack muscle and surimi and plays a major role in strengthening surimi gels by modifying thermal gelation of myosin molecules through ε-(γ-glutamyl) lysine (EGL) cross-linking (Kimura et al., 1991; Wan et al., 1994; Takeda and Seki, 1996; Seki et al., 1998). Tsukamasa et al. (1993) studied the relationship between ε-(γ-glutamyl) lysine cross-link content and gel-strength of salt-ground myofibril sol from sardine (Sardinops melanostictus) during incubation at 25°C. They concluded that, the EGL cross-links formed by TGase are important in the setting of sardine meat sol at temperature less than 30°C. Wan et al. (1995) found that the poorer gel forming ability of chum salmon surimi was primarily attributable to lower TGase activity as well as the lower contents of myosin and calcium ion,
compared with walleye pollack surimi. This TGase-mediated protein cross-linking creates drastic changes in physical attributes such as texture in protein-rich foods.

It was found that the myosin heavy chain (MHC) band disappeared in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) pattern of surimi from Alaska pollack with the progress of setting; consequently, the bands due to macromolecular cross-linked myosin heavy chain (CMHC) appeared (Numakura et al., 1985; Lee et al., 1990). The participation of myosin heavy chain in the cross-link formation was also established based on solubilization results (Careche et al., 1995). The solubility of set meat prepared from Alaska pollack surimi at 20°C or 30°C in a medium containing SDS, urea and mercaptoethanol decreased with the prolonged setting and concomitant disappearance of MHC was noticed with the appearance of CMHC (Numakura et al., 1985). They proposed that, cross-linking of myosin during setting was responsible for texture of the final gel. The non-appearance of MHC band on SDS-PAGE pattern clearly indicated insolubilization of cross-linked myosin heavy chain. A similar observation was made by Careche et al. (1995) in set sardine surimi (35° or 40°C), where solubility of protein was reduced considerably, suggesting that a covalent cross-linking has taken place during setting.

Nowsad et al. (1993) investigated the electrophoretic behavior of cross-linked myosin heavy chain (CMHC) in suwari gel from Alaska pollack surimi set at 30°C. They reported that as the setting time prolonged, MHC band became weak with the appearance of CHMC band at the top of acrylamide gel. The formation of CMHC during setting of Alaska pollack surimi was attributed to the aggregation of MHC. The CMHC could be solubilized in 8M urea and the PAGE pattern revealed more of MHC and 66 kDa component. The results suggested that the kDa proteins might play an important role in cross-linking of MHC.

The increased strain and stress values were observed due to non-disulphide covalent cross-links in pre-incubated gels reducing myosin heavy chain content and the concurrent appearance of cross-linked higher molecular weight band on SDS-PAGE pattern (Kamath et al., 1992). Based on turbidimetric study and SDS-PAGE pattern of myosin solution from cod, herring and silver hake set at 40°C in 0.6 M NaCl, pH 6.5, Chan et al. (1992) reported that fish myosin exhibited different thermal aggregation pattern and cross-linking ability. The turbidity of myofibrillar solution of three species, heat-treated at 40°C for 40 min were significantly different from one
another. These species specificity in turbidity were attributed to the size and/or rate of aggregation. The same authors reported that, in SDS-PAGE pattern, around 90-100% of the MHC from several species were cross-linked during setting (40°C for 50 min). The differences in cross-linking ability of myosin solution of three species during heat treatment at 40°C for 40-50 min were attributed to inherent properties of MHC. They suggested cross-linking ability of MHC molecules accounted for differences in aggregation for three species, which in turn accounted for difference in gel forming ability of three species at 40°C.

High variability in the TGase activity of surimi is common and is due to a combination of factors (Lanier, 2000). First, TGase is a water-soluble enzyme and thus its content can vary greatly with the type and extent of purification process used during surimi manufacture. Nowsad et al. (1995) showed that the sarcoplasmic protein fraction of fish could actually enhance the gelling ability when added back to surimi because of its higher TGase activity. Second, it is likely that different fish species, and perhaps different individuals within species, could vary in natural content of the enzyme, possibly affected by habitat, feed, and physiological conditions. This possibility has not been well-established and investigated to date. A third possible contributing factor is that the α2-macroglobulin component of fish blood plasma has the ability to form ε-(γ-glutamyl) lysine cross-links in fish protein (Wang and Lanier, 1999). Last, it has been shown that in certain species, such as salmon, the water soluble fraction of muscle also contains factors that inhibit TGase activity (Wan et al., 1994).

Kimura et al. (1991) investigated the cross-linking of myosin by crude suwari promoting enzyme extracted from hoki surimi. They found that cross-linking reaction induced by crude enzyme was calcium dependent. The same authors detected ε-(γ-glutamyl) lysine cross-link by high performance liquid chromatography (HPLC) analysis in suwari kamaboko which gave the direct proof of presence of TGase enzyme in crude extract from hoki surimi. Cross-linking seems to occur between actomyosin and the other myofibrillar protein constituents of the muscle to yield huge aggregates (Funatsu et al., 1996). Consequently the content of ε- (γ-glutamyl) lysine isopeptide cross-links generally correlates with the level of increase in gel strength (Imai et al., 1996).
As the TGase mediated cross-linking of proteins produces a covalent modification between the side chains of glutamine and lysine, the resulting ε-(γ-glutamyl) lysine isopeptide bonds are not susceptible to cleavage by proteases that hydrolyze ordinary peptide bonds (Miller and Johnson, 1999). Thus, isolation and detection of ε-(γ-glutamyl) lysine is generally accomplished using exhaustive proteolysis of the cross-linked proteins, followed by chromatographic purification of the isopeptide (Loewy, 1984). Several chromatographic methods have been used to isolate the isopeptide, including paper, thin-layer, and ion-exchange chromatography (Loewy, 1984). However, as the TGase mediated cross-link generally occurs in a low stoichiometry, the most reliable method of isolation involves reverse phase high performance liquid chromatography (RP-HPLC) (Loewy, 1984; Tarcza and Fesus, 1990).

Fish TGase has been found to be Ca\(^{2+}\)-dependent; however, the requirement varies among fish species (Nozawa et al., 1997, 1999; Lee and Park, 1998). Sufficient Ca\(^{2+}\) must be present for the endogenous TGase to be active and induce setting (Ofstad et al., 1993; Seki, 1996; Lee and Park, 1998). The optimal CaCl\(_2\) concentration for TGase also varied with the source of the enzyme (Yasueda et al., 1994; Kumazawa et al., 1996). Calcium could be a limiting factor during the setting of warm water fish species. Therefore, the use of CaCl\(_2\) during processing could improve the mechanical properties of the surimi obtained. Stronger gel could be obtained with the addition of calcium compounds to enhance TGase-mediated setting (Kuraishi et al., 2001; Benjakul and Visessanguan, 2003). The addition of CaCl\(_2\) at 0.2\% level significantly increased the breaking force of threadfin bream surimi gel when set at 40\(^\circ\)C for 2hr (Yongsawatdigul et al., 2000). Lee and Park (1998) found that addition of 0.2\% calcium compounds improved shear stress of Pacific whiting whereas the lower concentrations of 0.05 to 0.1\% effectively increased gel texture of Alaska pollack. Species dependence of optimum CaCl\(_2\) was attributed to the inherent varied concentrations of Ca\(^{2+}\) in muscle among species (Lee and Park, 1998). Wan et al (1994) reported that the gel strength of salted meat paste increased during setting in the presence of Ca\(^{2+}\) ions, and that the major effect of calcium ions on gelation was to activate intrinsic TGase.

Several researchers have reported on the Ca\(^{2+}\)-dependent catalytic action of TGase from different fish species; hoki (Kimura et al., 1991), red sea bream (Yasueda
et al., 1994), the egg envelop of rainbow trout (Ha and Iuchi, 1997) and scallop (Nozawa et al., 1999; Nozawa and Seki, 2001). Reduction of MHC reached 90% of its content when 0.06% CaCl$_2$ was added in to hoki surimi incubated with crude hoki TGase (Kimura et al., 1991). Cross-linked MHC of Alaska pollack surimi was higher in presence of 0.02% Ca$^{2+}$, and a further increase in Ca$^{2+}$ concentration did not increase MHC cross-links (Wan et al., 1994).

Hossain et al. (2001) found that addition of dithiothreitol (DTT) increased the gel strength of surimi gels from walleye pollack. They suggested that DTT enhances gel formation not by the oxidation of sulphydryl groups to disulphide bonds but by other bonds, such as cross-linking by TGase, which is known to be a cysteine enzyme. It has been reported that reducing agents have a similar effect on the setting of sardine paste (Roussel and Cheflel, 1990). Reducing agents appear to promote the setting of fish meat paste (Hossain et al., 2001). However, Niwa et al. (1995) reported that setting of meat paste was observed even under conditions when the oxidation of sulphydryl groups occurred.

In certain processes, where, endogenous TGase activity is not desired, such as in the holding of paste before extrusion or forming, a chelating agent can be added, such as sodium citrate or EDTA. This will bind calcium and prevent the TGase from cross-linking of proteins. Complete suppression of myosin cross-linking of walleye pollack surimi gel was associated with the inhibition of endogenous TGase by EDTA and ammonium chloride (Takeda and Seki, 1996; Kumazawa et al., 1995).

Disulfide covalent bonding

Another type of covalent bonding, intermolecular disulfide bonding during setting is a result of oxidation of sulphydryl groups in the presence of oxidants or metal ions (Okada, 1959; Itoh et al., 1979a; Kishi et al., 1995). In the setting process the $\alpha$-helical structure of myosin and actomyosin unfold markedly in the temperature range of 30$^\circ$-40$^\circ$C (Ogawa et al., 1993, 1995). Sulphydryl group present on the surface and inner side of the myosin molecule exposed during setting are oxidized to form disulfide bonds.

$$\text{Protein-SH + HS-protein } \rightarrow \text{ Protein-S-S-Protein + H}_2\text{O}$$

The polymericsation of molecules of carp actomyosin (AM), when heated at 40$^\circ$C could be suppressed by treating with dithiothreitol (Itoh et al., 1980). Further,
the rigidity modulus of suwari gel from AM decreased by the addition of p-chloromercuribenzoate (PCMB) or n-ethyl maleimide (NEM) which suppressed the formation of S-S bonds of proteins (Itoh et al., 1979a). From the finding, it was presumed that S-S bonds contributed to the elasticity of the suwari gel. Considering that TGase is inactivated by SH reagents, the above decrease in the modulus might have been induced not by the suppression of S-S bond formation but by the inactivation of remaining TGase. Infact, TGase contained in the fish flesh could not easily be removed with the extraction of AM (Nowsad et al., 1994 a). Therefore, the contribution of the S-S bonds to the elasticity of the gel cannot be proved only from the above finding. In order to confirm the contribution of the S-S bonds, Niwa et al. (1995) investigated the contribution of S-S bond to the elasticity of suwari gel from various actomyosins in which TGase was inactivated. TGase was inactivated by addition of 8 M urea. Suwari gel prepared by setting –SH unblocked actomyosin paste for 1 hr at 40°C had higher gel breaking force than the gel prepared from –SH blocked actomyosin. The same author reported some decrease in the –SH group content in the –SH unblocked actomyosin during setting while –SH content was unchanged in the -SH blocked actomyosin gel.

Samejima et al. (1981) showed that the total number of free sulphhydryl groups in the myosin head decreased as temperature increased. The authors also reported that the rigidity of myosin S-1 (globular head) sub fragment was decreased by adding the sulphhydryl-blocking reagent, dithiothreitol. From this evidence, they postulated that the cross-links that initiate the formation of a myosin-actomyosin gel network are formed through disulfide bonds. Disulfide bonding is also involved in the further development of the gel network during heating and the S-1 (globular head) and rod (fibrillar tail) fractions of myosin being primarily involved in the dimerisation and polymerization of myosin respectively (Kishi et al., 1997)

Hydrophobic interaction

Covalent linkages are not the only protein-protein interactions that stabilize the low temperature-induced set gel (Lanier, 2000). Several experiment findings suggest that intermolecular hydrophobic interactions contribute to the low temperature setting reaction as well (Wicker et al., 1986; Gill and Conway, 1989; Akahane and Shimizu, 1990; Chan et al., 1992; Niwa, 1992; Stone and Stanley, 1992). Many studies have demonstrated the importance of hydrophobic interactions in
isolated myosin or actin fractions, actomyosin, meat sols or salt extracts (Nakai et al., 1985). Also, substitution of hydrophobic groups onto the surface of proteins from poorly setting species greatly enhanced their gelling ability at low temperature (Niwa et al., 1981). Involvement of hydrophobic interactions in setting was also observed in the Raman spectroscopy of set surimi gels, resulting in the decreased intensity of a band near 2930 cm\(^{-1}\), assigned to C-H stretching vibrations (Bouraoui et al., 1997). The requirement of salt addition to surimi paste to induce setting also supports the role of hydrophobic interactions in setting because certain salts, such as sodium chloride, act with water molecules to strengthen the hydrophobic interactions between proteins (Wicker et al., 1989; Niwa, 1992). The formation of intermolecular or intramolecular hydrophobic interactions result from the thermodynamic response of protein surfaces exposed to the water in which they are dispersed or solubilized. The interior of the folded protein has a greater density of hydrophobic amino acid residues. Conversely, the amino acid residues on the surface of the denatured protein are largely hydrophilic.

Sharp and Offer (1992) postulated that head-to-head aggregation of myosin was actually due to the hydrophobic interactions resulting from the removal of certain light chains from the head region during heating, which revealed hydrophobic patches on the surface. The hydrophobic interaction between proteins is a logical consequence of the general increase in surface hydrophobicity, which accompanies heat-induced unfolding and seems to precede the gelation of myofibrillar proteins during heating (Wicker et al., 1986).

Use of ANS (1-anilino-naphthalene-8-sulphonate) and cis parinaric acid as fluorescent probe has been successfully used to demonstrate the role of hydrophobic residues during setting process. In the presence of a hydrophobic probe, the fluorometric intensity of actomyosin solutions from setting flat fish increased with heating (Niwa, 1992). Wicker et al. (1986) demonstrated with tilapia myosin, fluorescent intensity decreased with increase in temperature due to quenching, therefore, any increase in fluorescence intensity of myosin ANS complex must indicate a change in the hydrophobic character in the protein (Freifelder, 1982). During cooking (kamaboko-making or gel strengthening) it has been postulated that more protein unfolding and interactions between exposed hydrophobic sites result (Bouraoui et al., 1997).
Herring surimi held at 10°C for 0 to 24 hr showed ANS induced surface hydrophobicity of myofibrillar protein slightly increased during first 3 hr but increased to a maximum by a setting time of 12 hr (Chan et al., 1995). Further, after 24 hr of incubation, the surface hydrophobicity dropped to initial value. The increase in surface hydrophobicity of surimi during 10°C setting was attributed to opening up of myosin molecules. Hence, herring myosin molecule underwent some conformational changes during setting at 10°C and rate of these changes appeared to be slow initially and then increased when setting time was extended.

The addition of calcium chelating agents, like EDTA or citrate (Takeda and Seki, 1996; Wang and Lanier, 1999) or addition of specific TGase inhibitors, like certain ammonium salts (Shoji et al., 1994) almost totally eliminate the setting phenomenon in surimi pastes. On the other hand, the addition of beef plasma has a strengthening effect on gels subjected to low temperature setting before cooking (Kang and Lanier, 1999). Plasma in addition to being a gelling adjunct and protease inhibitor is high in fibrinogen, a natural substrate for TGase.

These data suggest that covalent cross-linking by endogenous TGase is the main contributor to gelation induced by low temperature setting of salted surimi pastes (Lanier, 2000). However, intermolecular hydrophobic interactions and covalent disulfide bonds must also form and reinforce the gel matrix. Hydrogen bonds will additionally affect the strength of these gels, particularly at colder temperatures (Howe et al., 1994).

2.1.3. Transglutaminase (TGase)

Several forms of TGase with distinctive specificity are widespread in plants (Del Duca et al., 2000), vertebrates (Folk and Cole, 1966), invertebrates (Noguchi et al., 2001) and microorganisms (Ando et al., 1989). Plant TGase as component of various cell compartments such as chloroplasts, mitochondria, cytoplasm and cell walls are related to growth, differentiation, programmed cell death and stress (Della Mea et al., 2004). Animal TGase are Ca^{2+}-dependent enzymes, which are widely distributed in body tissues such as liver, lung, intestine, hair follicles, epidermis, prostate, placenta and body fluids such as blood. The enzymes originating from marine organisms, for example, red sea bream liver (Yasueda et al., 1994, 1995), oyster (Kumazawa et al., 1997), limulus hemocyte (Tokunaga et al., 1993), lobster
muscle and sea urchin eggs (Myhrman and Bruner-Lorand, 1970) have been reported. The distribution of TGase in different organs of oyster was investigated by Kumazawa et al. (1997) and reported that gill and mantle comprised of 73% of the total activity. TGase activity has been found in the muscle of tilapia, common carp, rohu, small scale mud carp, silver carp, giant cat fish, spotted feather back, grey feather back, threadfin bream, giant gourami, striped snakehead fish (Worrratao and Yongsawatdigul, 2003), sardine, mackerels (Pacific, horse and Atka), red sea bream, ayu, silver eel, white croaker, walleye pollack, chum salmon and rainbow trout (Tsukamasa and Shimizu, 1990; Araki and Seki, 1993). Since 1960, the isolation, purification and characterization of TGase from mammal body fluids, such as human plasma factor XIIIa or thrombin-activated blood coagulation factor XIII (Gorman and Folk, 1980 b; Traore and Meunier, 1992) have been performed. TGase from mammal tissue, namely guinea pig liver TGase has been widely characterized (Folk and Cole, 1966; Case and Stein, 2003). Tissue TGase has been ascribed to several processes including stabilization of extracellular matrices, formation of cross-linked cell envelopes, cell matrix assembly, wound healing and cellular adhesive processes (Leblanc et al., 2001). Industrial applications of animal TGase have been intensively studied (Traore and Meunier, 1991; Oh et al., 1993), but its scarce source and complicated separation and purification procedures made the enzyme very expensive and limit its application in the industry.

TGase form isopeptide bonds between glutamine and lysine residues in proteins, thus introducing both inter and intra molecular covalent cross-links. However, two other important reactions can be catalyzed by TGase as well. In the presence of primary amines, TGase can cross-link the amines to the glutamines of a protein (acyl-transfer reaction). In the absence of lysine residues or other primary amines, water will act as a nucleophile, resulting in deamidation of glutamines (Ohtsuka et al., 2001). TGase exhibits high specificity towards L-glutamine residues as the acyl-donor substrate and lower specificity towards the acyl-acceptor, for this reason the ε-amino group of lysine as well as many primary amines, polyamines and ammonia can act as substrates (Griffin et al., 2002). The mechanism of reaction of TGase enzyme is given in Fig.1.

The purification of TGase is known to be difficult, since they have a propensity to form irreversible aggregates under native conditions (Wilhelm et al.,
Fig. 1: General reaction catalyzed by transglutaminase enzyme (De jong and Koppelman, 2002)

A: Cross-linking reaction
B: acyl transfer reaction
C: deamidation reaction
TGase itself contains 38 glutamyl and 42 lysyl residues, which allow autocatalytic cross-linking (homoaggregates) during purification when calcium ions are present. In addition, the TGase can also catalyze the aggregation of various hetero aggregates in crude extracts. To avoid the purification of the homo or hetero aggregates, it is essential to use complexing reagents such as EDTA to prevent irreversible cross-linking during separation (Wilhelm et al., 1996). Although all types of TGase share functional and structural mutuality, they differ in their molecular and immunological characteristics, demanding different purification procedures. In most separation protocols, combinations of ion exchange and size-exclusion chromatography have been described. Furthermore, other chromatographic methods such as hydrophobic-interaction, affinity, adsorption, and metal chelating chromatography as well as electrophoretic separation methods like preparative isoelectric focusing, SDS-PAGE and zone electrophoresis have been applied.

2.1.3.1. Purification of TGase

TGase preparations can be derived from either animal or microbiological sources (Motoki and Seguro, 1998). The main sources for TGase are guinea-pig liver (Motoki et al., 1984), Streptoverticillium S-112 (Ando et al., 1989), S. mobaraense (Sakamoto et al., 1995), Streptomyces lydicus (Faergemand et al., 1997) and S. ladakanum (Ho et al., 2000). Production of TGase from animal sources appears to be industrially uneconomical as yields are low (Zhu et al., 1999). The production of TGase for industrial use was made possible by the isolation and purification of a bacterial TGase from a microorganism taxonomically classified as a variant of Streptoverticillium mobaraense, which has got a higher activity than animal (Nonaka et al., 1989; Ando et al., 1989). Because the enzyme is secreted into the cultural broth, its purification is relatively straightforward, and so its production for food use is feasible on a commercial scale (Seguro et al., 1996). The microbial TGase (MTGase) is a simple monomeric protein containing 331 amino acid residues (molecular weight of 3.8 x 10^4, isoelectric pH of 8.9) (Ando et al., 1989; Kanaji et al., 1993). The substrate specificity of MTGase is lower than that of guinea-pig liver TGase, whereas the thermal stability is greater (Gerber et al., 1994). TGase generally require Ca^{2+} for expression of enzymatic activity; however, MTGase is totally independent of Ca^{2+} (Motoki and Seguro, 1998). Such a property is very useful in the modification of the functionality of food proteins, such as casein and myosin, because
they are easily precipitated in the presence of Ca$^{2+}$ and become less sensitive to MTGase (Motoki and Seguro, 1998). In addition, microbial fermentation makes it possible to achieve mass production of TGase at a low cost, which is advantageous for its industrial application in food technology, cosmetics as well as pharmaceutical products and medical treatment (Zhu et al., 1995). Motoki et al. (1989) reported, that other Streptoverticillium strains, such as S. griseocarneum and, S. cinnamoneum also have the ability to produce TGase. Intracellular MTGase was found in Bacillus subtilis (Ramanujam and Hageman, 1990) and in the spherules of Physarum polycephalum (Klein et al., 1992).

In general, there are three approaches to develop industrially useful TGase. The first approach is to extract and purify the enzyme from the tissues or body fluids of food-use animals, such as cattle, swine, and fish such as cod, salmon and flounder. In Europe, factor XIII, a certain type of TGase is extracted commercially from the blood of cattle and swine at slaughter (Wilson, 1992). The blood enzyme is however, rarely utilized in food manufacture since thrombin, a specific protease, is required to activate the enzyme and the red pigmentation is often detrimental to product appearance. The second approach is to obtain the enzyme by means of genetic manipulation using host of microorganisms, such as E. coli, Bacillus, yeast, and Aspergillus. Combination of genetic engineering and fermentation technology has been attempted by many researchers to get higher level of TGase enzyme production. Guinea pig liver TGase in E. coli (Ikura et al., 1988), human factor XIIIa in yeast (Bishop et al., 1990), Streptoverticillium TGase in E. coli (Takehana et al., 1994), Streptoverticillium TGase in Streptomyces sp.(Washizu et al., 1994) and fish TGase in E. coli (Yasueda et al., 1995) have been attempted to obtain TGase in commercial quantity at low price. However, none of these TGase has been commercialized due to factors such as food regulations and consumer acceptability. The third approach is to screen for TGase producing microorganisms. If an appropriate microorganism which produces TGase could be found, it would be possible to mass produce TGase by fermentation technology.

Several methods have been described for the purification of tissue TGase from guinea pig liver (Folk and Cole, 1966; Connellan et al., 1971; Brookhart et al., 1983; Ikura et al., 1985) and rat liver (Chang and Chung, 1986; Croall and DeMartino, 1986; Knight et al., 1990; Wong et al., 1990). The starting point for each TGase
purification was the supernatant of centrifuged liver homogenate, which was subjected first to anion-exchange chromatography with DEAE cellulose (Folk and Cole, 1966; Chang and Chung, 1986; Connellan et al., 1971), QAE-Sephadex (Brookhart et al., 1983), DEAE Sepharose (Wong et al., 1990) or Mono Q (Knight et al., 1990). Tissue TGase was eluted with 0.25 M-0.45 M NaCl using a Tris buffer containing EDTA and DTT at pH 7.5. For the subsequent purification steps several alternative methods are described below:

(a) TGase was enriched by protamine precipitation and then rechromatographed on DEAE-cellulose. Both steps were repeated once (Folk and Cole, 1966).

(b) After enrichment by protamine precipitation, proteins were separated using cation-exchange chromatography with CM-cellulose. TGase was precipitated by ammonium sulfate and was finally isolated by size-exclusion chromatography using agarose gel (Connellan et al., 1971).

(c) TGase-containing fractions were chromatographed using hydroxyapatite on Biogel-HTP (Wong et al., 1990) and finally purified by gel filtration chromatography using Superdex G 100 or affinity chromatography with phenylalanin-Sepharose 4B (Wong et al., 1990).

(d) TGase-containing fractions were subjected to immunoaffinity chromatography using an antibody against cytosolic TGase (Knight et al., 1990).

(e) TGase was isolated using size-exclusion chromatography on Biogel A 0.5 m (Chang and Chung, 1986).

Brookhart et al. (1983) reported purification of guinea pig liver TGase by phenylalanine affinity chromatography. Purification of red sea bream liver TGase was achieved by anion-cation exchange chromatography and heparin affinity chromatography (Yasueda et al., 1994). One-step purifications of guinea pig TGase (Ikura et al., 1985) and rat liver TGase (Croall and DeMartino, 1986) have also been described. In these procedures, the supernatants of guinea pig liver homogenates were applied to an affinity column utilizing a monoclonal antibody against guinea pig liver TGase (Ikura et al., 1985). Extracts from rat liver were purified by calcium-dependent affinity chromatography using casein-Sepharose (Croall and DeMartino, 1986). Using the methods described above, 1-5 mg of pure TGase could be isolated from 100 g
liver. Tissue TGase of liver origin has a relative molecular mass of approximately 85,000 as determined by SDS-PAGE (Wilhelm et al., 1996). Testicular tissue TGase has been purified from rat (Seitz et al., 1991) and bovine (Bergamini and Signorini, 1992) testis. TGase was obtained in an enriched form from rat testis homogenates by fractionated ammonium sulfate precipitation (50-60%) and subsequent anion-exchange chromatography on a Mono Q column. TGase was finally purified by hydrophobic-interaction chromatography using phenyl-Superose and size-exclusion chromatography using Superdex 200. The purified enzyme has a relative molecular mass of 82,000 using SDS – PAGE and has an isoelectric point of 5.25 (Seitz et al., 1991). TGase of bovine origin was isolated by anion-exchange chromatography using DEAE-cellulose and Q-Sepharose as well as hydrophobic-interaction chromatography with phenyl-Sepharose. Approximately 0.07 mg of protein was isolated from 50 g of bovine testis. The purified enzyme migrated as a single band on SDS-PAGE with a relative molecular mass of 80,000 (Bergamini and Signorini, 1992). Tissue-type TGase was also isolated from human erythrocytes (Brenner and Wold, 1978; Lee et al., 1986; Ando et al., 1987; Signorini et al., 1988; Weber, 1993). First, the supernatant of centrifuged human erythrocyte lysates was adsorbed in batches to an anion-exchange matrix of DEAE-cellulose and subjected to further purification steps. These included one or different steps as listed below.

(a) After size-exclusion chromatography using Biogel A 0.5 m, TGase was finally purified by preparative SDS-PAGE with a total acrylamide concentration of 6 or 8.5% (Brenner and Wold, 1978).

(b) The fraction containing TGase was applied to a size-exclusion AcA 44 column, followed by heparin-Sepharose chromatography. For final purification, TGase was chromatographed by size-exclusion HPLC using a TSK 125 column (Signorini et al., 1988).

(c) TGase was isolated using size-exclusion chromatography with Sephacryl S-300, affinity chromatography on Blue-Sepharose CL-6B and finally anion-exchange chromatography using DEAE Biogel A (Ando et al., 1987).

(d) TGase was enriched using ammonium sulfate precipitation and finally purified by immuno affinity chromatography (Lee et al., 1986).
TGase was finally purified by anion-exchange chromatography on Mono-Q (Weber, 1993). Approximately 1-2 mg of pure TGase could be isolated from hemolysate (20,000 mg protein) using the described methods. The pure enzyme has a relative molecular mass of 82,000, as established by SDS-PAGE. In addition, a tissue type TGase was isolated from cultured human A431 tumor-cells. After cell homogenization, the next step used was anion-exchange chromatography on DEAE-cellulose. Subsequently, affinity chromatography on heparin-Agarose and on casein-Agarose was performed. A 0.15 mg amount of pure TGase could be isolated from a cytosol fraction containing 650 mg total protein. The purified enzyme showed a single band on SDS-PAGE with a relative molecular mass of 83,000 (Dadabay and Pike, 1989).

2.1.3.2. Structure of TGase

Enzyme and structural properties of the TGase have been well characterized for mammalian origins (Nakanishi et al., 1991). Plasma TGase (factor XIIIa subunit) (Grundman et al., 1986; Takahashi et al., 1986; Ichinose and Davie, 1988) and keratinocyte TGase (Phillips et al., 1990; Yamanishi et al., 1992) are typical examples of well-characterized extra cellular and membrane-associated TGase, respectively. Among the tissue-type or cytosolic TGase, guinea pig liver TGase (Ikura et al., 1988) and the bovine and human endothelial cell TGase (Nakanishi et al., 1991; Gentile et al., 1991) have also been well characterized structurally. Although the exact physiological role of the tissue-type enzyme remains unclear, it has been postulated to participate in regulating the growth, differentiation and apoptosis of various types of cells (Folk, 1980; Lorand and Conrad, 1984; Greenberg et al., 1991). In lower vertebrates, little is yet known about the structural characteristics and the physiological functions of TGase (Yasueda et al., 1995). In fish, TGase have been studied in relation to analysis of the molecular mechanisms underlying gel formation in fish mince sols, but little structural analysis of these enzymes has been carried out (Tsukamasa and Shimizu, 1991; Kimura et al., 1991; Wan and Seki, 1992; Wan et al., 1994). Since terrestrial vertebrates originally diverged from fish ancestors, structural information on the fish-derived TGase could be valuable for analysis of the biological functions and molecular evolution of this class of enzymes.
TGase from guinea pig liver has been purified, and its kinetic properties were investigated (Folk, 1969; Connellan et al., 1971; Folk and Chung, 1973). The enzyme consists of 690 amino acid residues corresponding to a MW of 76,620 Da. An essential cysteine is located at position 276 and the region is highly homologous to that of the sulfhydryl proteases (Folk and Cole, 1966; Ikura et al., 1987, 1988, 1989, 1990). The active site is within a highly hydrophobic region near the N-terminal end. Most studies have been conducted on the soluble cytosolic TGase of guinea pig liver, which is non-glycosylated. Recent work on chondrosarcoma cells of rat suggests that the TGase associated with the particulate fractions are glycoproteins (Chang and Chung, 1986). Because of its unique cross-linking function, considerable interest has focused on the application of TGase for protein labelling (Dutton and Singer, 1975; Gorman and Folk, 1980 a), linking of fluorescent probes (Pober et al., 1978), immobilization of coenzymes (Yoshikawa et al., 1982), and modification of the structural and physical properties of proteins, including soy, casein, gluten, p-lactoglobulin and myosin, for food applications (Kurth and Rogers, 1984; Nio et al., 1985; Tanimoto and Kinsella, 1988).

Yasueda et al. (1994) performed the purification and characterization of a tissue-type TGase from the red sea bream, Pagrus major, and found that the amino acid sequence of this fish TGase was similar to guinea pig liver TGase. The same authors (1995) performed molecular cloning of a cDNA encoding the fish-derived TGase and characterized its entire primary structure in comparison to those of guinea pig liver TGase. In addition, functional expression of the fish TGase in E. coli was achieved, and formation of ε-(γ-glutamyl)-lysine bonds by the recombinant enzyme was confirmed. A cDNA clone encoding a tissue-type TGase was isolated from a cDNA library prepared from the liver of red sea bream. The cDNA sequence had an open reading frame coding for a protein of 695 amino acids and showed 43% identity to the sequence of guinea pig liver TGase, revealing a relatively low overall similarity. However, the 25-amino-acid sequence containing the putative active site (Cys272) of the enzyme was completely conserved between the two species, and was also identical to the corresponding regions of human and bovine endothelial cell TGase. In addition, the critical residues (His 332 and Asp 355) thought to form the catalytic-center triad together with Cys272, were found in the highly conserved region. The red sea bream TGase had an extension of 11 amino acids in the C-
terminal region and some differences in the N-terminal region when compared with guinea pig liver TGase. From the cloned cDNA, a semi-synthetic TGase gene suitable for over expression in *Escherichia coli* was constructed (pTTG2-22). At a reduced temperature (28°C), *E. coli* cells transformed with pTTG2-22 could produce soluble TGase which exhibited catalytic activity in the presence of calcium. *E. coli* extracts containing the recombinant red sea bream TGase induced gelation of actomyosin solutions, accompanied by a significant increase of ε-(γ-glutamyl)-lysine bonds, which are predominantly derived from the cross-linking of myosin heavy chains.

The crystal structure of the tissue-type TGase from red sea bream liver (fish-derived TGase, FTG) was determined at 2.5-Å resolution using the molecular replacement method, based on the crystal structure of human blood coagulation factor XIII, which is a TGase zymogen (Noguchi et al., 2001). The Fish-derived TGase showed 33% sequence homology to human factor XIII. The model contains 666 residues of a total of 695 residues, 382 water molecules, and 1 sulfate ion. FTG consists of four domains, and its overall and active site structures are similar to those of human factor XIII. However, significant structural differences are observed in both the acyl donor and acyl acceptor binding sites, which account for the difference in substrate preferences. The catalytic Cys-272 hydrogen-bonded to Tyr-515 is thought to be displaced upon acyl donor binding to FTG. It is postulated that the binding of an inappropriate substrate to FTG would lead to inactivation of the enzyme because of the formation of a new disulfide bridge between Cys-272 and the adjacent Cys-333 immediately after the displacement of Tyr-515. Also, Cys-333 and Tyr-515 are important in strictly controlling the enzymatic activity of FTG. If the Tyr covering the catalytic Cys were simply removed, then it is predicted that the Cys would form a disulfide bridge with the adjacent Cys, and then the enzyme would lose the catalytic activity immediately. In other words, the formation of an acyl-enzyme intermediate is essential to prevent the formation of the disulfide bridge.

The crystal structure of a microbial TGase from *Streptoverticillium mobaraense* has been determined at 2.4 Å resolution (Kashiwagi et al., 2002). The protein folds into a plate-like shape, and has one deep cleft at the edge of the molecule. The catalytic residue, Cys64, exists at the bottom of the cleft. Asp255 resides at the position nearest to Cys64 and is adjacent to His274. The structure accounts well for the catalytic mechanism, in which Asp255 is considered to be enzymatically
essential, as well as for the causes of the higher reaction rate, the broader substrate specificity, and the lower deamidation activity of this enzyme.

2.1.3.3. TGase assays

*Fluorometric and radioactive assays*

TGase activity assays are based on the enzymatic function of the enzyme, i.e. the covalent binding of a primary amine to a peptide-bound glutamine in a calcium-dependent/independent reaction. In most assays used, a lysine analogue (radiolabeled putrescine $^{14}$C or $^{3}$H) (Lorand et al., 1972; Miraglia and Greenberg, 1985) or fluorescent-labeled primary amine derivatives) is incorporated into a protein acceptor (Lorand et al., 1972; Fink et al., 1992).

*Fluorometric chromatographic and electrophoretic assays*

A fluorescent amine such as monodansylcadaverine (MDC) is incorporated into casein or a synthetic peptide-like benzylxocarbonyl-L-glutamyl glycine (CBZ) (Lorand et al., 1972; Fink et al., 1992). The fluorescent cross-linked product is separated from the initial substrate by electrophoresis, ion-exchange chromatography, thin-layer chromatography, size-exclusion chromatography (Lorand et al., 1972) or reversed-phase HPLC (Fink et al., 1992). In addition, activity staining on agarose gels has been described. After electrophoresis filter papers soaked with calcium, monodansylcadaverine and N, N-dimethylcasein were applied on the gel. After fixation of the proteins with ethanol-acetic acid and subsequent gel drying, the incorporation of the dansyl group was visualized with an UV lamp (Lorand et al., 1979).

*Radioactive assays*

Radio labeled putrescine ($^{14}$C or $^{3}$H) is incorporated into a protein substrate, e.g. casein. The separation of unreacted amine and cross-linked protein is conveniently done by precipitation. The increase of radioactivity is measured as TGase activity (Lorand et al., 1972; Miraglia and Greenberg, 1985)

*Photometric assays*

Photometric assays have been described using ethylamine (Muszbek et al., 1985) or glycine-ethyl ester as substrates for the incorporation into modified β-casein (Muszbek et al., 1985) or a specific synthetic peptide (Fickenscher et al., 1991; van
Wersch, 1993). The released ammonia is detected in a combined reaction: catalyzed by glutamate dehydrogenase, ammonia is incorporated into ketoglutarate under NADH consumption. The decreasing NADH concentration is monitored at 340 nm.

Different amines have pH optima varied from 7.8 for glycinamide, 8.3 for cadaverine (Clarke et al., 1959), to 6.0 for hydroxylamine (Folk and Cole, 1966). The reactivity of the amine depends on its nucleophilicity, that is, the relative concentration of the unprotonated form (Mycek and Waelsch, 1960). The pKa, values of the amines used in cross-linking therefore determine largely the efficiency of incorporation at specific pH values. Therefore the pH of the reaction should be above the pKa of the amine over the pH range where the enzyme is stable.

Comparison of different methods

The most sensitive methods for accurate quantification of TGase activity are radioactive and fluorometric assays (Wilhelm et al., 1996). However, to date many laboratories try to avoid working with radioisotopes. Furthermore, fluorometric assays require special instruments for the detection of the fluorescent products. Colorimetric and photometric assays are an alternative to radioactive or fluorometric assays. These assays are fast, reproducible and sensitive. Also, all substrates are commercially available and special equipment is not necessary.

2.1.3.4. Substrate for TGase enzyme

It has been suggested that TGase from different origins recognize different peptide sequences over the same protein substrates (Matsumura et al., 2000). TGase is known to catalyse both inter and intra molecular cross-linking reactions (Ohtsuka et al., 1996). Intramolecular ‘zero length’ cross-linking between Gln-41 and Lys- 50 in intact actin by TGase has been reported (De Jong and Koppelman, 2002) TGase from Streptoverticilium was demonstrated to react with a large number of substrate proteins than the plasma and erythrocyte TGase from mammalian blood (De Jong et al., 2001). Quantitative analysis of the β-casein cross-linking revealed that bacterial TGase has a higher activity compared to plasma TGase and erythrocyte TGase.

Primary amino groups can replace the lysil residue as it is applied in activity measurement methods (Slaughter et al., 1992). In order to clarify the physiological role of TGase, numerous techniques were used for detection of protein substrates with the help of labeled primer amines. In case of tissue TGase and mammalian organisms
the presence of some intracellular protein substrates were identified in vitro, among them there are β-crystallines (Berbers et al., 1984; Lorand et al., 1992; Groenen et al., 1992), lipocortin I (Ando et al., 1991), fructose-1, 6- bisphosphate aldolase (Lee et al., 1992), actin (Takashi, 1988) in addition to several extracellular proteins (Aeschlimann and Paulsson, 1994) which can be glutamine donor substrates. In contrast to the limited donor substrate specificity, TGase possesses a broad specificity for acceptor substrates (Matheis and Whitaker, 1987). Actin was demonstrated to be a TGase substrate during programmed cell death using a new substrate detection procedure in living cells (Nemes et al., 1997). The number of proteins acting as glutamyl substrates for TGase is restricted. Both primary structure and conformation of proteins appear to determine whether a glutamine residue can be reactive (Folk, 1983; Gorman and Folk, 1980 a; Aeschlimann et al., 1992). A considerable number of reactive glutamine residues have been identified in various substrate proteins for TGase (Aeschlimann et al., 1992; Coussons et al., 1992). Accessibility of these glutamines in solvent-exposed surface regions or flexible extensions of the protein is of primary importance (Grootjan et al., 1995). A common notion is that TGase are much less selective toward amine donor lysine residues in proteins than they are to the glutamine substrates (Greenberg, 1991; Aeschlimann and Paulsson, 1994). Additionally, the modes of interaction of TGase with small amines and with native protein substrates are likely to be different (Folk, 1983; Hettasch and Greenberg, 1994).

A prerequisite for the cross-linking reaction with TGase is sufficient exposure of lysine and glutamine residues of substrate proteins (Matsumura et al., 1996). Some proteins, such as casein and gelatin are easily cross-linked by bacterial TGase because lysine and glutamine residues are readily available. In contrast, many other food proteins have more rigid structure that prevents cross-linking. Various methods can be applied to destabilize the protein structure and make more of glutamine and lysine residues available to the enzyme. Native α-lactalbumin is, for example, hardly susceptible to cross-linking by TGase. However, the protein contains a bound Ca$^{2+}$, removal of which destabilizes the native structure and induces a transition into a molten globule state (Matsumura et al., 1996).

The most described method for improvement of the accessibility of glutamine and lysine residues is addition of a reducing agent, for example, dithiothreitol (DTT)
(Nielsen, 1995). Because of the addition of DTT, disulfide bridges are reduced, the protein structure is opened and the TGase induced cross-linking can occur. An alternative for the reduction of disulfide bridges is heating of the protein to temperatures, where disulfide bridges are broken. Increase of pH may also affect protein structure and increase cross-linking. A successful combined use of heating and increased pH has been reported for cross-linking of ovalbumin (Lim et al., 1998).

The rate of cross-linking by TGase is more dependent on the macromolecular structure of each protein substrate (Dickinson, 1997) than their absolute protein content (Kurth and Rogers, 1984). Reactive glutamine residues reside in flexible regions of the polypeptide chains or in regions with reverse turn (Berbers et al., 1983). The flexible caseins are therefore good substrates (Nio et al., 1986). On the other hand, it has been widely reported (Ikura et al., 1984; Traore et al., 1992) that globular food proteins such as ovalbumin and β-lactoglobulin are not attacked by TGase in their native states. The susceptibility of globular proteins to TGase-induced cross-linking may be increased in several ways: by chemical modification (Larre et al., 1992), by disruption of intermolecular disulphide bonds (Abourmahmoud and Savello, 1990), by conversion into the molten globule state (Matsumura et al., 1996) or by adsorption at the oil-water interfaces (Faergemand et al., 1997). Other factors affecting the TGase reaction rate are temperature, pH and calcium ion content (Nielsen, 1995).

The TGase-mediated setting reaction is mostly affected by the conformation of the substrate (An et al., 1996). The extent of MHC cross-linking by crude tilapia TGase seemed to depend on the conformation of both myosin and TGase (Worratao and Yongwatdigul, 2003). Carp TGase polymerized actomyosins from various fish species at different rates; these ranged from 0.1 units/hr to 13.5 units/hr (1 unit of activity is defined as the amount of enzyme required to incorporate 1 nM of monodansylcadaverine into 1 mg of acetylated casein per minute at 25°C), with the highest rate being obtained with walleye pollack actomyosin (Araki and Seki, 1993). The authors suggested that the TGase-mediated cross-linking reaction was mainly regulated by the conformation of actomyosin, which varied among fish species. Joseph et al. (1994) reported that the maximum storage modulus and MHC polymerization occurred at the same pH and temperature conditions optimal for the setting of surimi paste, even with the addition of TGase from guinea pig liver,
implying that substrate conformation is more important than the level of enzyme activity. Incubation of surimi at temperatures above 10°C induces the denaturation of myosin in the presence of salts (Toyoda et al., 1992), thereby exposing more glutamine and lysine residues, which can be utilized by TGase (Tsukamasa and Shimizu, 1991).

Nonaka et al. (1989) showed that rabbit myosin was polymerized by a catalytic reaction of the microbial TGase (MTGase), but actin was not affected under the same conditions. On the other hand, globular proteins, such as β-lactoglobulin, α-lactalbumin and ovalbumin, have proven to be poor substrates because of their compact structures, which limit the accessibility of the TGase to the target glutamine and lysine residues (Nio et al., 1985; Sakamoto et al., 1994). Furthermore, ovalbumin and conalbumin were found only to be modified by MTGase when a reducing agent like dithiothreitol was used, which is undesirable for food manufacturing (Nonaka et al., 1989). Subsequent investigations have shown that pre-treatment or simultaneous application of high pressure at 400–600 MPa can induce structural changes in the native protein, making it accessible to the acyl binding site of MTGase (Nonaka et al., 1997). Other reports of high-pressure effects on various biomolecules indicate that this may be a suitable denaturing treatment for enhancing TGase activity (Ashie and Lanier, 2000; Gilleland et al., 1997).

TGase has been used to introduce cross-links to the myofibril proteins, actin and myosin (De Backer-Royer, 1992), actomyosin of turkey (Akamittath and Ball, 1992) and beef (Kim et al., 1993), and chicken myosin heavy chain (Tseng et al., 2002). Myosin molecules react readily with TGase contains high amounts of glutamic acid/glutamine and lysine, which are the amino acid residues acting as counterparts in TGase-catalyzed isopeptide bonds. Presumably, the rod-like shape and better accessibility of the target amino acids explain the observed susceptibility of myosin to cross-linking with both enzymes studied (Tseng et al., 2002).

Motoki and Nio (1983) used guinea pig liver TGase with various proteins (αs1-casein, k-casein, β-lactoglobulin, soybean 11S and 7S globulin) to form intermolecular and intramolecular ε-(γ-glutamyl)-lysyl cross-links. Some workers have used guinea pig liver TGase with mechanically deboned poultry meat (Akamittath and Ball, 1992) or beef (Kim et al., 1993) for the polymerization of

Apart from their substrate specificity, the TGase also shows differences in characteristics like required cofactors and reaction conditions (De Jong and Koppelman, 2002). The microbial TGase is a calcium independent enzyme (Ando et al., 1989), whereas, the requirement of mammalian and fish TGase for calcium ion for optimum activity has been thoroughly established (Folk and Chung, 1985). For guinea pig liver TGase, the Ca$^{2+}$ requirement could be replaced by Sr$^{2+}$ and to a lesser extent, by Mn$^{2+}$, Ba$^{2+}$ and Mg$^{2+}$ (Folk and Chung, 1985). Inactivation of the enzyme by Cu$^{2+}$ may have been caused by the metal-catalyzed oxidation of the cysteine, and the inactivated enzyme was shown to contain two new disulfide bonds (Boothe and Folk, 1969). Both Mg$^{2+}$ and Sr$^{2+}$ have been shown to cause slight activation in TGase isolated from particulate fractions of rat chondrosarcoma cells (Chang and Chung, 1986), carp muscle (Kishi et al., 1991) and scallop (Nozawa and Seki, 2001). Kinetic studies using both Ca$^{2+}$ and Sr$^{2+}$ suggested that the divalent cation complexes with the enzyme after substrate binding (Folk et al., 1967). Cross-linking of myosin in beef heart myofibrillar protein isolate has been achieved with Na$^+$ ions, when suspended in 0.6 M NaCl (Ramirez-Suarez et al., 2001). Nozawa et al. (1999, 2001) reported that the activities of TGase from marine invertebrates such scallop (Patinopecten yessoensis), squid (Todarodes pacificus) and shrimp (Pandalus nipponensis) are dramatically enhanced in the presence of NaCl and Ca$^{2+}$ at concentrations found in normal seawater compared to that found in muscle enzymes. The NaCl induced activity was also reported for TGase obtained from Japanese oyster (Crassostrea gigas) (Kumazawa et al., 1997).

The TGase enzyme is quite sensitive to temperature of incubation. The optimum temperature of TGase activity varies with the source (Yongsawatdigul et al., 2002). The activity of guinea pig liver TGase using cadaverine incorporation showed rapid decrease at temperatures above 40°C (Clarke et al., 1959) and rat liver TGase activity decreased significantly at 44°C (Wong et al., 1990). For several cold water fish species, it was found that endogenous TGase has an optimal temperature in the range 25–30°C, with a minimum effect at 20 °C (Nowsad et al., 1996). Yasueda et al. (1994) reported that the optimum temperature of TGase purified from red sea bream (Pagrus major) was 55°C. Purified TGase from Japanese oyster shows optimum
activity at 25°C and 40°C (Kumazawa et al., 1997), while optimum activity for walleye Pollack liver was at 50°C (Kumazawa et al., 1996). The presence of an endogenous TGase in striped mullet with optimal activity at 30–35 °C and pH 7.5–7.7 has been reported (Lee et al., 1998).

2.1.3.5. Tools to follow TGase reaction

The intermolecular cross-linking of proteins by TGase can be followed with SDS-PAGE or size exclusion chromatography (De Jong and Koppelman, 2002). Monomeric proteins will form polymers of increasing size that can be distinguished according to their molecular weights. Intramolecular cross-linking is more difficult to observe, however, changes in hydrodynamic size as a result of intramolecular cross-linking can be visualized with SDS-PAGE. Acyl transfer reactions can be observed on SDS-PAGE in case the substrate protein is small and the ligand is relatively large (De Jong and Koppelman, 2002).

In order to obtain more quantitative data on the amount of cross-linking, analysis of ammonia production (De Backer Royer et al., 1992) is a useful tool. This analysis, which follows the ammonia that is formed by the TGase reaction, can provide information on the rate of cross-linking even after the point were the SDS-PAGE is no longer accurate. A disadvantage of the ammonium measurement is that this analysis is not able to distinguish between cross-linking, acyl transfer and deamidation (De Jong and Koppelman, 2002). Moreover, in processes with high concentrations of substrate proteins, cross-linking may lead to increased viscosity or even gelation. This hampers the ammonia measurement because of problems with accurate sampling. Small increases in molecular weight, as in the case of acyl transfer or deamidation, can be studied with MALDI-TOF MS (Matrix-Assisted Laser Desorption / ionization- Time of Flight Mass Spectrometry). These analyses, however, are limited to small proteins and will be disturbed by protein cross-linking (De Jong and Koppelman, 2002).

2.1.3.6. Perspectives

To date all current popular chromatographic purification methods (ion-exchange, size-exclusion, hydrophobic interaction, affinity, and adsorption chromatography) were described for TGase isolation. Nevertheless, during recent years protein purification procedures have improved remarkably. One or two decades...
ago it took a long time and a lot of effort to purify proteins, because conventional chromatography columns usually ran for several hrs or days. Using recent methods for protein purification, such as FPLC or Smart systems and modern chromatography media, it is possible to run columns in 1 or 2 hr with a high reproducibility. Since column size, solvent consumption, as well as the void volume of the instruments is small, it is possible to separate proteins even in the micro molar range. Short purification times and improved chromatography matrices with low self absorption capacities have meant better yields for TGase, since self-aggregating and cross-linking of contaminating proteins is diminished. At present, new electrophoretic methods are being developed, such as automated capillary electrophoresis. However, at the moment this method is only usable for analytical and not for preparative applications. At present, manufacturers are trying to improve this method for preparative applications also. Purified TGase can be applied to the following three major areas:

1. Clinical aspects: factor XIIIa is an important protein for wound healing. Patients with factor XIIIa deficiency have a pathological hemostasis and insufficient wound healing. Substitution with factor XIIIa isolated from placenta guarantees normal blood coagulation, wound healing and placenta retention (De Jong and Koppelman, 2002).

2. Tissue TGase can be used as a cross-linker to bind glutamine-containing peptides or polypeptides to NH$_2$-columns, in order to get new affinity matrices, or for the covalent attachment of antigen and antibody after blotting procedures. In addition, it can be utilized as an inductor of cell adhesion in cells cultured on substrates (e.g. fibronectin or collagen). Furthermore, proteins can be labeled with dansyl- or radioactive-residues for direct analytical demonstration.

3. TGase is now widely used in seafood, surimi products, meat products, noodles / pasta, dairy products, baked goods, and so on. It has great potential to improve the firmness, elasticity, viscosity, heat stability, and water-holding capacity of prepared foods through the mild enzyme reaction. Enzymes that stabilize proteins by forming additional covalent cross-links may be used to fabricate meat products with better texture despite low salt or low protein content. Currently, TGase that originated from the *Streptomyces* bacterium
genus are the only commercially available enzymes for cross-linking proteins and for improving the texture of meat products. This microbial TGase (MTGase) is not calcium sensitive; therefore, neither chelating agents nor calcium salts have any marked effects on its activity. Recently, it was found that endogenous fish and pig TGase have higher deamidation activities than MTGase (Ohtsuka et al., 2001).

The effects of TGase in food protein matrixes have been reviewed by Kuraishi et al. (2001). The potential of TGase has been intensively studied in meat processing to improve texture and gelling (De Backer-Royer, 1992). In cooked products, consistency can be positively affected by TGase (Dimitrakopoulou, 2005), although with a concomitant increase in cooking loss. The covalent cross-linking of proteins catalyzed by TGase can cause dramatic changes in the size, conformation, stability, and other properties of proteins (Van-Den Truong et al., 2004). The enzyme has been used for modifying the functionalities of various proteins including soy proteins, myosin, gluten, globulin, casein, and whey proteins (Abourmahmoud and Savello, 1990; Ikura et al., 1992; Sakamoto et al., 1994; Nielsen, 1995; Zhu et al., 1995; Faergemand et al., 1997; Motoki and Seguro, 1998; Siu et al., 2002; De Jong and Koppelman, 2002).

TGase is now widely used in seafood, surimi products, meat products, noodles and pasta, dairy products and baked goods (Kuraishi et al., 2001). The effects of MTGase from Streptoverticillium mobaremense, Streptoverticillium sp. and Streptoverticillium ladakanum on protein gels have been extensively studied (Nonaka et al., 1989, 1992, 1994; Tanaka et al., 1990; Sakamoto et al., 1994, 1995; Seguro et al., 1995; Tsai et al., 1996; Jiang et al., 1998). The effect of the MTGase is well documented for raw and restructured meats (Nielsen, 1995; Kuraishi et al., 1997; Tsao et al., 2002; Lee and Park, 2002; Serrano et al., 2004). The increase in mechanical properties of low-salt restructured products, by adding 3 units/kg of MTGase, has been also reported for silver carp (Hypophthalmichthys molitrix) (Tellez-Luis et al., 2002).

The improving of mechanical properties of surimi gels from striped mullet at regular-salt levels by using MTGase (3 unit/kg), or endogenous TGase has been reported (Ramirez et al., 2000; Ramirez et al., 2003; Ramirez et al., 2007). A shear stress of 146 KPa and shear strain of 1.59 KPa was achieved by employing a MTGase
concentration of 8.8 g/kg of surimi at 9.6°C for 1 hr. Jiang et al. (2000) reported that the gel strength of pollack surimi added with MTGase and set at 30°C for 90 min improved to a higher extent than those set at 45°C for 20 min. Cross-linking of MTGase on mackerel and hair tail myosin heavy chain was reported by Jiang et al. (1998). An experimental fungal TGase has also been commercially developed, which, like the endogenous tissue enzyme, is calcium sensitive (Lanier, 2000). It was demonstrated that the fungal enzyme was as effective in surimi as MTGase, provided a small amount of calcium was also added (Wang and Lanier, 1999).

Joseph et al. (1994) showed that temperature and pH effects on substrate (myosin) conformation had effects on the characteristics of TGase-catalyzed myosin gels. Other studies have shown that modification of various globular proteins including bovine and human serum albumin, conalbumin and the 11S seed protein of pea legumin by dithiothreitol and pH changes greatly increased TGase activity (Nielsen, 1995). It is therefore conceivable that alteration of substrate conformation by processing conditions could render protein substrates more accessible to TGase catalysis and related modification of product characteristics. Reports of high-pressure effects on various biomolecules indicate that this might be a suitable denaturing treatment for enhancing TGase activity (Masson, 1992; Gilleland et al., 1997; Nonaka et al., 1997; Ashie and Lanier, 2000).

In an investigation by Hojbjerg (1993), it was concluded that the gelling of cooked gels from cod meat was dependent on the temperature of setting prior to cooking. Gel elasticity was especially influenced, where gel strength and hardness were unaffected. Further it was found that addition of TGase (human FXIIIa) was efficient when the raw material quality was low. The utilization of frozen fish as raw material was markedly improved with addition of TGase enzyme. When the fish is of prime quality the level of endogenous TGase activity may be adequate to ensure good setting ability. The endogenous enzyme activity in poor quality fish can be significantly reduced. Also, TGase activity is affected by size of fish, stage of maturity and season of harvest (Lanier, 2000).

Although some reports have indicated that benefits from MTGase addition are mainly noticeable in products from lower quality surimi, there is also a substantial increase in the strength of gels made with high-quality Pollack surimi (Lanier, 2000). Only 5 units/g of surimi protein is effective in increasing the gel strength during setting. However, there are reports that added MTGase leads to a harder, less natural texture in surimi gels than the endogenous enzyme (Abe et al., 1996; Yasunaga et al.,
In Pacific whiting gels, which normally exhibit lower gel strength than gels from Alaska pollack surimi, the addition of MTGase has even more pronounced effects, especially when combined with beef plasma (1%), which inhibits the parasite-related heat-stable protease content. In addition, the fibrinogen from beef plasma is an excellent substrate for the enzyme (Kang and Lanier, 2000).

In milk products, MTGase has been used to increase firmness in yoghurt. Whey protein isolate was effectively cross-linked using enzyme/substrate ratios of 0.12-10 units of activity/g whey protein isolate (Van-Den Truong, 2004). Lauber et al. (2000) found that formation of isopeptide cross-links from skimmed milk incubated with MTGase had direct relation with the yoghurt consistency. Gelation and water-holding capacity were improved in skimmed milk powder after TGase treatment, indicating that it could be used for low fat yoghurt manufacture to reduce syneresis (Imm et al., 2000). TGase-whey protein gelation depends on glutamine residues in proteins, α-lactoalbumin showing more cross-links than β-lactoglobulin because of glutamine residue exposure (Fargemand et al., 1997). The effectiveness of TGase in improving emulsifying properties of whey and soy protein has been demonstrated. This has been attributed to an improved viscoelasticity (rheology) at the oil–water interface as well as to a reduced coalescence of the oil-droplets because of the presence of a cross-linked membrane structure (Babiker, 2000). Mean droplet diameter of bovine serum albumin (BSA) emulsion is not significantly different due to TGase incubation, but BSA polymers are more adsorbed at the oil–water interface than monomers (Chanyongvorakul et al., 1994). Solubility of casein and soy globulin (11S and 7S) treated with TGase is higher than in native proteins, enhancing emulsions activity and stability (Motoki et al., 1984). Incubation time and temperature affect the rate of cross-linking (Kurth and Rogers, 1984).

The rheological properties of TGase-set globular protein gels are rather different from those of heat-set globular protein gels (Dickinson, 1997). Experiments indicate (Dickinson and Yamamoto, 1996) that gels can be produced at lower protein content by enzyme cross linking than by thermal treatment, and that the elastic moduli and breaking strengths of enzyme-cross-linked gels are greater than those of heat set gels made under similar conditions. The storage modulus for the viscoelastic gel made by thermal processing was found to be strongly frequency dependent, whereas, that for the elastic gel made by TGase treatment (2 hr at 55°C) was found to be nearly
independent of frequency. Protein gels containing both covalent and physical cross-links can be prepared by combining the enzyme and thermal treatments. Although the formation of intramolecular covalent bonds may actually impede heat induced unfolding and gelation of native molecules, once sufficient intermolecular covalent cross-links have been introduced to make an enzyme-induced protein gel, any subsequent thermal processing can act to substantially strengthen the network (Dickinson and Yamamoto, 1996). This glutamine–lysine cross-linking increases with increasing TGase concentration, with a correlation between breaking strength and glutamine–lysine cross-links (Sakamoto et al., 1995). The presence of additional covalent cross-links in protein gels formed by heating, pH change or high-pressure treatment could also be beneficial in terms of the inhibition of post-gelation structural rearrangements and associated syneresis (Dickinson, 1997).

Kamat et al. (1992) suggested utilization of immobilized TGase for commercial application. The enzyme was absorbed on to an ion-exchanger followed by incubation with TGase. The concept was demonstrated by experiments with immobilized trypsin and amylases. They found this method of immobilizing more advantageous than using glutaraldehyde as the loss of enzyme activity was less. The use of large-scale bioreactors for processing proteins in order to obtain better functionality was suggested by Swaisgood et al. (1997). A limited hydrolysis step followed by cross-linking by immobilized TGase may be useful for large-scale bioprocessing. They also reported remarkable stability of the immobilized TGase.

The setting process can be considered as a useful tool to modify the protein functionality for better utilization. There is no doubt that both endogenous fish TGase and exogenous TGase can improve the functionality of proteins by increased cross-linking. When an excess of TGase is used, the texture becomes too firm and less pliable which is generally less acceptable (Dondero et al., 2006). The controlled use of the enzyme TGase is a potential new tool for generating emulsion droplet networks and for stabilizing protein-coated interfaces in food colloids (Ikura et al., 1992; Tsukamasa et al., 1993). Protein modification by TGase apparently does not lead to the retention of toxic side products or undesirable off-flavors, or to significant loss of essential nutrients (Nielsen, 1995). There is no problem of bitter peptide production, which is a disadvantage that is commonly attributed to proteolytic enzymes. The ε-(γ-glutamyl) lysine cross-link occurs naturally in animal and plant tissues and in
processed foods (Sakamoto et al., 1995), and its formation is enhanced by
conventional cooking (Hurrel and Carpenter, 1977). Although it is resistant to
digestion by mammalian gastrointestinal enzymes, the cross-link is apparently broken
down readily by kidney enzymes (Seguro et al., 1996). Bioavailability experiments
with rats fed on heavily cross-linked caseins have demonstrated (Seguro et al., 1996)
that the ε-(γ-glutamyl) lysine moiety is fully metabolized and that the lysine becomes
fully integrated into animal tissues. TGase treatment therefore provides exciting new
opportunities for extending the range of functional properties of proteins in food
colloid systems. Covalent cross-links tend to enhance elasticity (‘chewiness’),
whereas weaker physical cross-links are more associated with pseudoplastic flow
(‘creaminess’). By combining enzymic cross-linking with other processing operations
such as heating or high-pressure treatment, it may be possible for food manufacturers
to generate high-quality novel products with both of these desirable textural attributes.

2.2. Gelation

Gelation of proteins is a process, which involves protein unfolding and
ordered aggregation. During heating, the proteins unfold, exposing reactive surfaces
of neighboring protein molecules, which then interact to form intermolecular bonds.
When sufficient bonding occurs, a three-dimensional network is formed, resulting in a
gel (Lanier, 2000). This gelation process affects the overall quality and acceptability
of surimi gel products. The ability of surimi to form an elastic gel is largely derived
from myosin (Sano et al., 1988). Myosin comprises 55-60% of the myofibrillar
proteins. It is a multidomain protein with two large heavy chains and four light chains
arranged into an asymmetrical molecule with two globular heads attached to a long α-
helical rodlike tail (Lopez-Lacomba et al., 1989). Myosin has been shown exclusively
to possess gelling ability, in contrast to other protein components. In surimi, myosin is
present in a complex form with actin and other proteins, collectively called
actomyosin, which could form an elastic gel (Niwa, 1992). Because actin itself could
not form a strong gel on heating, it is thought that the gelling characteristics of
actomyosin are derived from the myosin portions. The binding with actin has been
shown to modify the gelling characteristic of myosin (Yasui et al., 1980). However,
gels prepared from myosin alone have higher gel strength and elasticity than those
prepared from natural actomyosin (Sano et al., 1988). According to the studies on
physicochemical changes using myosin and its subfragments from several species of
fish, namely, flying fish (Taguchi et al., 1987), carp (Sano et al., 1990), croaker, cod, and herring (Chan et al., 1992; Gill et al., 1992; Chan and Gill, 1995), it is agreed that heat-induced gelation of myosin under high-salt conditions involves two processes including denaturation and aggregation (Stone and Stanley, 1992). In denaturation, myosin undergoes conformational changes, exposing functional groups, such as hydrogen bonds and hydrophobic groups. In aggregation, denatured myosin molecules align themselves and interact with each other to form a three-dimensional network. The interactions involved in gelation have been shown to be dependent on the species of fish employed (Taguchi et al., 1987; Sano et al., 1990; Chan et al., 1993). Because myosin heavy chain is the main subunit involved in gelation (Samejima et al., 1989), differences in the composition and structure of myosin heavy chain among various fish species may be responsible for species-to-species differences in the cross-linking ability and gelation properties of fish muscle under the same conditions.

The changes in the patterns of dynamic moduli (including the storage modulus, \(G'\) and loss modulus, \(G''\)) or phase angle (\(\tan \delta\)) can reflect the gelation process and the development of formed gels (Tabilo-Munizaga and Barbosa-Canovas, 2005). Observations of dynamic viscoelastic behavior and differential scanning calorimetric analysis revealed that the gelation of carp actomyosin occurred in two stages: at temperature ranges of 30°C-41°C and 51°C-80°C (Sano et al., 1988). Differential shear modulus studies also revealed two transitional temperature ranges for Argentinean hake (\(Merluccius hubbsi\)) natural actomyosin, with the first at 36-38°C and the second at 48°C. Sano et al. (1988) have proposed that the first stage of gel elasticity development was due to interactions among the tail portions of myosin molecules. The second stage was attributed to hydrophobic interactions among the head portions of myosin, because the protein conformation changes during heating such that the hydrophobic amino acids, which are found mainly in the head portion, become exposed on the surface. Ziegler and Foegeding (1990) summarized that myosin from mammals generally undergoes gelation by losing its non-covalently stabilized \(\alpha\)-helical structure as a result of heating, followed by increased turbidity caused by intermolecular association. Myosin then forms a rigid structure that is stabilized by covalent disulfide bonds and non-covalent interactions.
The gel strength of myofibrillar proteins can be influenced by factors that affect myosin structure. The gelling property of myosin is highly related to the length of the double-stranded α-helical tail. Myosin rod (140 nm long) showed higher rigidity than light meromyosin (80 nm long) at all salt concentrations studied (Ishioroshi et al., 1983). Accordingly, proteolysis of myosin has been shown to lower surimi gel strength (Morrissey et al., 1995). The native conformation of myosin is of primary importance for proper gelation. Maximum gel strength cannot be obtained if myosin is denatured before gelation is initiated (Niwa, 1992). Myofibrillar proteins from fish are more susceptible to heat denaturation than those from warm-blooded animals (Ogawa et al., 1993). The hydrophobic amino acid residues of actomyosin exposed on freezing surimi were mainly contained in the myosin component; freezing had minimal effect on the actin component. Thus, it was observed that repeated freezing and thawing of surimi made from Alaska pollack and sand trout denatured myosin resulting in a substantial decrease in gel strength (Kim et al., 1986).

Some of the sarcoplasmic proteins of fish muscle are enzymes, the biological catalysts of the chemical reactions which muscle cells carry out in life. Nakagawa et al. (1988) observed that the gelling properties of surimi were negatively related to the residual aldolase activity, a glycolytic enzyme. Some other studies, however suggested that sarcoplasmic proteins might actually contribute positively to gel strength (Morioka and Shimizu, 1990: Karthikeyan et al., 2004). Ko and Hwang (1995) reported that the addition of sarcoplasmic protein fraction to surimi improved the heat-induced gelling properties by exhibiting a restrictive effect on the proteolytic softening of the gels. Siang and Miwa (1992) examined the sarcoplasmic fraction of fish and found that the presence of a cross-linking enzyme (TGase) also had a gel-enhancing effect.

From the review of literature on transglutaminase enzyme, it is well understood that TGase can catalyse formation of the ε-(γ-glutamyl)-lysine bond in many food proteins; the resulting crosslink drastically alters protein functionalities. Applications are emerging in the development of novel foods and non-food processing methods. There may be many applications in the incorporation of various amines, amino acids, lysine-containing peptides, glutamine-containing peptides and heterologous polypeptides. There is no doubt that TGase technology will be an
essential tool for protein modification in both food processing and non-food processing in the future.