Discussion
V. DISCUSSION

The gel forming ability of fish meat is influenced by intrinsic and extrinsic factors. Gel forming ability is highly species specific and many fish species, which lack this ability, is not commercially favored for preparation of heat-processed fish mince based products. Many attempts have been made to improve the gel forming ability of fish either by physical or chemical means. To improve the gel forming ability one of the most widely used techniques is setting process, wherein, the fish mince is incubated at a temperature below 40°C, after mixing with common salt to get a soft elastic gel. Upon further heat processing the set meat yields a gel, which is further elastic in nature. This process is termed as setting and an enzyme transglutaminase (TGase) has been identified as the causative agent. The presence of TGase in muscle cell is relatively species specific and it is of interest to study the properties of TGase from different commercial fish species and its role in enhancing the gel forming ability. The present investigation was undertaken to evaluate in detail the setting ability of two marine and two fresh water fish and further its gelling ability. In order to understand the mechanism of setting reaction the TGase enzyme has been isolated, purified and characterized. The addition of isolated TGase to other fish species has been attempted to evaluate the setting and gelling ability. In this section, the results obtained have been discussed for its mechanism of interaction in setting and gelling process with activators and inhibitors.

5.1 Proximate composition

The proximate composition of four fish species revealed lower moisture content in the oil sardine. The crude fat content of bigeye snapper, tilapia and common carp were less than 3% and that of oil sardine was 16.15%. The moisture content of fish muscle and invertebrates varies between 50-80% and depends on physiological stage of the animal and environmental factors (Sikorski et al., 1990). It is well documented that fat content of fish varies with season and inversely related to moisture content (Mukundan et al., 1981; Aguilera et al., 1992; Gopakumar, 1997). The fat content of fresh water fish like tilapia and common carp are greatly influenced by age, size, feed and season of harvest (Gopakumar, 1997).
5.2. Isolation and properties of transglutaminase (TGase) enzyme

The isolation of TGase from four different fish species have been carried out. The crude enzyme activity was higher from tilapia muscle and was lowest from bigeye snapper. Upon different steps of purification, mainly anion-exchange chromatography, protamine sulphate precipitation and ammonium sulphate extraction, the specific activity of the enzyme increased in all the four fish species. The extensive dialysis of extracted enzyme was carried out so as to remove salts and to get pure enzyme. Mammalian endogenous tissue-type TGase, such as guinea pig liver and rabbit TGase, has been purified by anion-exchange chromatography, protamine extraction and gel filtration technique (Connellan et al., 1971; Abe et al., 1977). It was reported that guinea pig liver and rat liver TGase was adsorbed to anion-exchange resin strongly (Brookhart et al., 1983; Wong et al., 1990). Physicochemical properties of fish TGase are considered to be similar to that of the mammalian TGase, such as guinea pig liver TGase (Nozawa et al., 1999). Hence the procedure followed for the extraction of guinea pig liver TGase was adapted for purifying fish TGase with slight modification. Table 3 summarizes the activity of TGase in each purification step. TGase could be eluted between 0.2 - 0.4 M NaCl and the maximum specific activity was observed at 0.3 M NaCl concentration. In the present study, ion-exchange chromatography was carried out by batch process. Though the standard protocol is anion-exchange chromatography to follow gradient elution (either ionic strength or pH), the present study was mainly focused on batch process so as to obtain higher yield and ease of operation. Since the activity of enzyme was found in the fractions eluted from 0.2 – 0.4 M NaCl, the different fractions were pooled and used for further purification. The enzyme obtained from dorsal muscle of carp, rainbow trout and atka mackerel were eluted at near 0.3 M NaCl concentrations and squid scallop and oyster TGase at 0.16, 0.12 and 0.15 M respectively (Nozawa et al., 1997; Kumazawa et al., 1997). The TGase activity assay is based on the extent of covalent binding of primary amine to a peptide bound glutamine, which is a Ca\(^{2+}\)- dependent reaction. In most assays lysine analogue (radio labeled putrescence or fluorescent labeled primary amine derivative) is incorporated into a protein acceptor. A fluorescent amine such as monodancyl cadaverine is incorporated in to casein or a synthetic peptide like benzyloxy carbonyl-L- glutaminyl-glycine (CBZ) (Lorand et al., 1971; Fink et al., 1992). The fluorescent cross-link produced is separated from the initial substrate by
electrophoresis, ion-exchange chromatography, thin layer chromatography, size exclusion chromatography or reverse phase HPLC. In the present investigation the substrate used was CBZ. The formation of \( \gamma \)-glutaminyl-monohydroxamate from CBZ (catalysed by TGase) is taken as an index of activity of the enzyme or simply ‘units’.

The yield obtained for TGase from different species varied between 14-39% with the highest yield value for bigeye snapper and the lowest value for oil sardine. The difference in the yield was mainly aroused after the protamine sulphate precipitation and further extraction with ammonium sulphate solution. The final recovery yield of purified tilapia TGase was 22.64%, which was considerably higher than that reported for tropical tilapia by Worratao and Yongsawatdigul (2005). Specific activity of TGase from scallop striated muscle was 38.0 U/ mg and the yield and fold during chromatography were 9.2% and 55 respectively (Nozawa et al., 1999).

### 5.2.1. Characterization of fish TGases

In order to determine the purity of the isolated TGase enzyme gel filtration and electrophoresis (SDS-PAGE) technique was followed. The gel filtration profile indicated a major peak in all the four TGase samples indicating nearly 90% homogeneity of the sample (Fig. 4). There were some minor peaks in the gel filtration profile, which could be either contaminant or an unresolved component. The SDS-PAGE pattern did not reveal any bands, however, the TGase from common carp showed two prominent bands in the molecular weight range of 66-99 kDa. Despite using higher concentration of TGase loaded on the gel no clear band was observed for TGase from other three fish species.

The molecular weight of TGases as determined by gel filtration technique was in the range of 73-95 kDa. This is similar to that of other fish, including walleye pollack liver TGase (77 kDa) (Kumazawa et al., 1996), red sea bream liver TGase (80 kDa) (Kishi et al., 1991) and tropical tilapia TGase (85 kDa) (Worratao and Yongsawatdigul, 2005). Partially purified TGases from ordinary carp and Alaska pollack muscles showed molecular weights of 80 kDa and 85 kDa, respectively (Kishi et al., 1991; Seki et al., 1990). The molecular weight of TGase from scallop, botan shrimp, squid, carp, rainbow trout and atka mackerel was found to be in the range of
80-100 kDa (Nozawa et al., 1997). In contrast, the molecular weight of Ca\(^{2+}\)-independent microbial TGase was reported to be only 38 kDa (Ando et al., 1989). It can be suggested that fish TGase is monomeric in structure and composed of a single polypeptide chain similar to TGase from other marine organisms (Kumazawa et al., 1996; Tokunga et al., 1993; Yasueda et al., 1994).

**Optimum temperature of fish TGase**

The TGase activities were measured at various temperatures. The temperature profile of the activity is shown in Fig. 6. TGase from different species had different optimum temperatures. The optimum temperature of the purified fish TGase for the catalytic reaction of CBZ ranged from 37 to 55°C (Table 6 and Fig. 6).

TGase of bigeye snapper showed maximum activity around 37°C and nearly 89% activity was retained at 50°C. In contrast, TGase of oil sardine showed the highest activity at 37°C and 62% of which was shown at 25°C. The oil sardine TGase was found to be less stable at a temperature higher than 37°C than TGase from Bigeye snapper. Optimum temperature of TGase from tilapia was found to be at 50°C and nearly 65% of the specific activity was retained even at 60°C, whereas, at 25°C, only 18% of total activity was exhibited. TGase from common carp showed the highest activity from 37°C, but it rapidly decreased to 29% of the highest activity at 60°C. At 25°C, 57% of the activity was detected. The TGase activity was not detected at a temperature beyond 70°C indicating conformational changes / denaturation.

The optimum temperature of the purified TGase varied with sources. The optimum temperature of the purified TGase from mullet (*Mugil cephalus*) was 35°C (Lee et al., 1998); from Alaska pollock, mackerel and carp pastes between 25° and 30°C (Nowsad et al., 1996); from scallop, Japanese oyster, and pollack liver between 35° and 50°C (Kumazawa et al., 1996; Kumazawa et al., 1997; Nozawa and Seki, 2001); from red sea bream liver TGase between 55° and 60°C (Yasueda et al., 1994). The optimum temperature of the purified tilapia TGase for the catalytic reaction of MDC ranged from 37° to 50°C while that of crude tilapia TGase was at 50°C (Worratao and Yongsawatdigul, 2003). The difference in optimum temperature of TGase from different species is probably affected by the habitat temperature. The variation in optimum temperature within one species may be due to the presence of
water-soluble interfering protein that may fluctuate with the season of harvest (Lee et al., 1998). Also, purity of the TGase plays an important role in determining the optimum temperature for its reaction.

**Effect of CaCl$_2$ concentration on fish TGase**

Purified TGase from four different fish species showed an absolute requirement for calcium ions to catalyze cross linking reaction (Table 7, Figure 7), which is characteristic of mammalian and fish TGase (Yongsawatdigul et al., 2002). The concentration of CaCl$_2$ to be incorporated in the reaction mixture of TGase is 100 mM as reported in the standard assay protocol (Folk and Cole, 1966). The present study demonstrated that the optimum concentration of CaCl$_2$ for TGase activity varied between four different fish species. The TGase from bigeye snapper required 50mM CaCl$_2$ for maximum activity, while for that from oil sardine the activity almost remained constant in the concentration range from 20-100 mM (Table 7, Fig.7). Tilapia and common carp TGase showed its maximum activity at 50 mM CaCl$_2$. Optimum Ca$^{2+}$ concentrations for TGase from red sea bream liver, Japanese oyster, scallop, and pollack liver were at 0.5, 25, 50, and 3 mM, respectively (Kumazawa et al., 1996; Kumazawa et al., 1997; Nozawa et al., 1997; Yasueda et al., 1994). Kishi et al. (1991) reported that partially purified TGase from carp muscle required 5 mM Ca$^{2+}$ for full activation whereas, Nozawa et al. (1997) have reported a value of 0.8 mM CaCl$_2$. The purified enzymes of limulus hemocyte (Tokunga et al., 1993) and guinea pig liver (Ando et al., 1989) required 8 and 10 mM Ca$^{2+}$ respectively. The difference in CaCl$_2$ requirement within the same species may be due to the difference in the extent of purity. On the other hand, the difference between different species may be explained by the dissimilarity in specific interaction between calcium ion and various TGase (Nozawa et al., 1997). It is postulated that the calcium ion induced the conformational changes of the enzyme, which consequently exposed the cysteine residues located at the active site to a substrate (Jiang and Lee, 1992). In red sea bream, Nogushi et al. (2001) reported that the calcium ion bound to a binding site of TGase molecule, resulting in conformational changes. Subsequently, tyrosine covering the catalytic cysteine was removed and the acyl donor bind with the cysteine at the active site, forming an acyl-enzyme intermediate. The present study indicated, the optimum concentration of Ca$^{2+}$ ions for enzyme activity is species dependent.
Effect of inhibitors on TGase activity

The effect of various TGase inhibitors like EDTA, ammonium chloride and lysine-HCl was assessed on the purified fish TGase enzyme. The activity of TGase from four fish species towards different inhibitors was varied depending on the nature of inhibitors and the concentration used. The Ca\(^{2+}\)-chelating agent EDTA, at a concentration of more than 20 mM completely inhibited bigeye snapper TGase activity. In case of oil sardine 8.3% of TGase activity was detected even at 60 mM concentration. Tilapia and common carp TGase could be totally inhibited by the addition of EDTA at concentration of 60 mM. The results confirmed that the TGase from all the four fish species were Ca\(^{2+}\)-dependent (Table 8). Worratao and Yogsawatdigul (2005) reported that the tropical tilapia TGase was completely inhibited by EDTA at a concentration of 10 mM.

The effect of ammonium chloride (NH\(_4\)Cl) in reducing the activity of TGase from four fish species was higher at 1.0 M concentration. NH\(_4\)Cl is a specific inhibitor for TGase while EDTA is a calcium-chelating agent. The presence of NH\(_4\)Cl could suppress the activity of enzyme by higher quantity of ammonia liberated during catalysis (Takagi et al., 1986; Ashie and Lanier, 2000). The formation of cross-linked myosin heavy chain will be suppressed in presence of ammonium chloride (Lee et al., 1997b). Increase in ε-(γ-glutamyl)-lysine and gel strength was suppressed by the addition of EDTA or NH\(_4\)Cl, which are known as TGase inhibitors (Kumazawa et al., 1995).

Free lysine-HCl was chosen as a competitive inhibitor of TGase due to its high solubility in water and minimum effect on pH of the system. It has been reported that lysine-HCl inhibit cross-linking of myosin associated with peptide-bound ε-amino groups (Kim et al., 1984). The effect of lysine-HCl on the inhibition of TGase from four different fish species was not uniform. For instance, at 0.25 M, no activity was detected in TGase obtained from oil sardine, tilapia and common carp. The TGase from bigeye snapper showed a specific activity of 8.82 units / mg at a concentration of 0.25 M lysine-HCl. For the reactive groups (glutamine and lysine) to participate in the cross-linking reaction via TGase, the two substrate protein molecules and the enzyme must be associated in a highly oriented and conformation-dependent fashion at some stage of the catalytic process (Folk, 1983). The methylene
group of the glutamine residue is necessary to confer substrate properties and this hydrophobic methylene group is essential for interaction with a hydrophobic region near the active site of the enzyme. It was also suggested that hydrophobic amino acids proximate to the glutamine residue may have a strong influence on TGase activity. This hydrophobic enzyme-substrate interaction requirement of the enzyme TGase fits well with the theory visualizing initial unfolding of protein molecules to expose hydrophobic residues as a primary step in the setting mechanism (Niwa, 1992; Wicker et al., 1986, 1989). It may be presumed that the unfolding (denaturation) of protein molecules may lead to a greater exposure of both hydrophobic and glutamine residues, the latter being made available for cross-linking with lysine residues (Kamath et al., 1992).

5.3. Gel forming ability of four different fish species

The gel forming ability of mince from four different fish species was assessed by small strain (DVB) test using Controlled Stress Rheometer (CSR). The gel forming ability of oil sardine was assessed both in washed and unwashed mince, while for other fishes it was unwashed mince.

The thermal gelation profile of bigeye snapper revealed, an increase in storage modulus (G’) values with increase in temperature and reached a maximum value of 378.6 KPa at 63.6°C. The loss modulus (G”) value also increased during heating; however, the magnitude was less than that of G’ values indicating the formation of a viscoelastic gel network. The temperature at which the phase transition from sol-gel occurred as revealed by Tan δ values was found to be at 43.3° and 63.3°C. Thermal gelation patterns of natural actomyosin from bigeye snapper (Priacanthus tayenus and Priacanthus macracanthus) indicated formation of gel network at 35°C and a maximum G’ at 38°- 40°C (Benjakul et al., 2001). Heating of natural actomyosin at temperature less than 40°C generally resulted in dissociation of some myofibrillar components, for example, tropomyosin from the F-actin backbone and F-actin from its super helix structure (Ziegler and Acton, 1984). However, conformational changes of these proteins had no significant contributions on the changes of gel modulus (Xiong, 1997). Although it is present in a complex form with actin and other proteins, myosin is responsible for the gel elasticity development of natural actomyosin during the thermal gelation (Sano et al., 1988; Sano et al., 1989a). It is postulated that partial
unfolding of the protein structure initiated by the dissociation of myosin light chain subunits from the heavy chains may lead to an interfilamental association of myosin and form a three-dimensional structure. The temperature at which unfolding of myosin molecule occurred in myosin molecule from bigeye snapper and white shrimp as measured by Differential Scanning Calorimeter was found to be at 47.72°C and 50.78°C respectively (Benjakul et al., 2002).

The dynamic viscoelastic behavior of unwashed oil sardine meat in the temperature range of 30°C-90°C revealed a maximum G' value of 213.5 KPa at 63.3°C. This is more than the previously reported value for the same species (Karthikeyan et al., 2004). The storage modulus (G') values increased with increase in temperature and the maximum structure build up reaction occurred in between 43.3°C and 56.7°C. The temperature at which the phase transition from sol to gel occurred was at 36.7°C and 56.8°C.

The gelation profile of washed oil sardine, meat did show a marked increase in the G' values compared to that of unwashed meat. The G' maxima recorded was 406.4 KPa at 63.3°C which was higher by 200 KPa in the unwashed meat. There were two transition points as revealed by Tan δ values at 36.7°C and 56.8°C similar to unwashed meat. The rate of increase in G' values were higher between 43.5°C-63.7°C. The higher G' values in washed meat indicates the effect of washing process in concentration of myofibrillar proteins as well as removing sarcoplasmic protein fractions which may inhibit gelation process (An et al., 1994; Chen et al., 1997). In the present study the sardine meat was subjected to washing process (one washing) so as to remove lipid fraction and pigments.

The dynamic viscoelastic behavior (DVB) of tilapia meat revealed good gel forming ability with the maximum G' value of 594.6 KPa at 56.7°C, indicating myofibrillar proteins from tilapia has the ability to form ordered network. The maximum increase in G' value was recorded between 36.7°C-56.7°C. There was a sharp increase in G' values at 56.7°C, which also happens to be the temperature at which transition from sol to gel occurred.

The DVB profile of common carp meat was comparable to that of bigeye snapper. The maximum G' value of 365.6 KPa was attained at 63.3°C. This value is
higher than the reported value for the same species by Ganesh et al. (2006). The maximum rate of increase in G’ values were found in the temperature range of 43.3-56.7°C. The gel forming ability of common carp actomyosin was reported as inferior (Ni et al., 1988). From the DVB profile it can be inferred that the gel forming ability of common carp was moderate. The temperature at which the phase transition from sol-gel occurred was 36.7° and 49.9°C. Sano et al. (1988, 1989a) have reported that the carp actomyosin showed considerable increase in G’ value in the 32°-43°C and 52°-80°C ranges. The rheological behavior of carp actomyosin sol exhibited two peaks at 36°C and 40°-45°C (Ni et al., 1998). The dynamic rheological test and Differential Scanning Calorimetry of cod muscle revealed three transitions viz. 36°C, between 48°–52°C and 65°C, corresponding to myosin, sarcoplasmic proteins and actin respectively (Badii and Howell, 2002). The transition in the temperature range of 30°-40°C is assigned to the tail portion of myosin and 51°-80°C to the head portion of the molecule (Sano et al., 1988). The number of transition in gelation process is related to nature of polypeptide structure. Wicker et al. (1989) postulated mechanism of fish myosin denaturation and subsequent gelation involves minimum of three steps. The first involves a change in the conformation of myosin, followed by aggregation and gelation occurs due to intermolecular hydrophobic interaction at the newly exposed sites. Such interactions of myosin may be head to head as proposed by Samejima et al. (1981), but a tail-to-tail or tail to head interaction cannot be excluded (Wicker et al., 1989).

Gelation of muscle proteins results from transformation of an amorphous viscous solution to a three-dimensional network. Hence, changes in stress-strain relationships during gelation process could be monitored by rheological parameters (Egelandsdal et al., 1995). The dynamic rheological test or small strain gel rigidity test has been widely used to study the heat-induced gelation of myofibrillar proteins (Visessanguan et al., 2000). The small deformation test is very useful for monitoring sol-gel transition and for characterizing the viscoelastic behavior of the gels in which amplitude of the stress and strain is in the linear region (Visessanguan et al., 2000). Changes in storage modulus (G’) have been used to monitor gelation of proteins including structural proteins (Venugopal et al., 2002). Gelation of myofibrillar proteins is a process, which involves protein unfolding and 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).

The viscoelastic nature/strength of the heat set gel (after DVB experiment) were assessed by torque sweep. The stress at fracture was taken as critical stress value. This value was higher for gels from tilapia followed by common carp, bigeye snapper and oil sardine (unwashed mince). The value for gel from washed sardine mince was 2017 Pa which was higher than that of bigeye snapper. By taking into consideration the G’ maxima during DVB profile and torque sweep data it can be said that the gel forming ability of the four fish species has the following order – Tilapia > Common carp > Oil sardine (washed) > Big eye snapper.

The differences in gel forming ability exhibited between species might have resulted from the differences in protein integrity and bonding formed during thermal process (Benjakul et al., 2001). Since oil sardine contains considerable quantity of dark muscle, it is difficult to remove the dark muscle with a meat separator (Ochiai et al., 2001). The gel-forming ability of dark muscle has been known to be lower than that of ordinary muscle. Hence, the washing process is necessary for color improvement and gel strengthening of surimi produced from whole muscle (Chaijan et al., 2004). This apparently resulted in the differences in the unfolding abilities and thermal stability of myosin from different species (Lo et al., 1991). The presence of sarcoplasmic proteins may also change the rheological properties of the fish gels (Chaijan et al., 2004). Some sarcoplasmic proteins may be bound to the myofibrils during the heat treatment, thus decreasing the strength of the gel (Sikorski, 1994). The content of sarcoplasmic protein is generally high in pelagic fish, such as sardine and mackerel (Haard et al., 1994; Hashimoto et al., 1979). Small quantities of sarcoplasmic proteins can have an adverse effect on the strength and deformability of myofibril protein gels (Haard et al., 1994; Hultin and Kelleher, 2000 a, b). These proteins may interfere with myosin cross-linking during gel matrix formation because they do not form gels and have poorer water holding capacity according to Sikorski (1994). Knowledge on protein thermal denaturation and aggregation will be a critical input so as to manipulate the gel-forming ability of fish actomyosin. Chan et al. (1992) reported that herring, cod and silver hake aggregated in different fashions, accounting for differences in gel elasticity between the three species.
5.4. Setting ability of various fish species

The setting ability of the fish species have been assessed at two different temperatures, viz., 25°C and 40°C for 30 min of duration. The setting process was monitored by dynamic rheological testing. The setting ability of washed and unwashed oil sardine meat at 25°C and 40°C has been assessed. At the end of 30 min, the mince incubated at 40°C exhibited higher G’ value in all the samples indicating the formation of network leading to soft elastic gel. The setting temperature had no influence with unwashed sardine mince, while for that of washed sardine mince higher temperature setting could increase the G’ values significantly. The increase in G’ value was always associated with reduction in Tan δ value. This shows that there exists a transition from sol to soft elastic gel during incubation below 40°C. From the dynamic rheological testing it can be said that tilapia meat had higher setting ability followed by washed oil sardine, common carp and bigeye snapper mince.

Benjakul et al. (2003) suggested that setting at different temperatures might lead to different gel characteristics, especially with different fish species. Gel strength of surimi can be increased by subjecting surimi sol to setting below 40°C prior to cooking (An et al., 1996). Gelation of fish mince during setting has been reported to have a close relationship to the formation of cross-linking between myosin heavy chains induced by endogenous TGase (Kumazawa et al., 1995; Benjakul et al., 2003). It has been reported that optimum setting temperature for surimi from cold water fish species is 25°C and for warm water fish species is 40°C (Kamath et al., 1992; Lee et al., 1998). Higher setting temperature (40°C) typically reported in warm water species is due to high thermal stability of myosin / actomyosin (Lanier, 2000). Therefore, to estimate the setting effect on thermal gelation, the viscous paste was incubated at 25° and 40°C for 30 minutes and the viscoelastic behavior was assessed.

The DVB profile in the temperature range of 25°/ 40°C – 90°C of the set meat were assessed to know whether setting can improve the gel network formation during heating process. The DVB profile clearly demonstrated setting at 40°C for 30 min could enhance the dense network formation during heating. From the DVB profile, it can be noted that the G’ maxima values recorded was almost same in tilapia, oil sardine (washed) and common carp mince while it was lower in bigeye snapper. It can be concluded that setting at a temperature of 40°C could significantly enhance gel network formation during heating regime. If we look at the temperature profile of
TGase from four different species (Table 6 and Fig.6), it is evident that at 37°C the activity was higher. The temperature of setting in the present study was 40°C which was closer to 37°C. The TGase from tilapia had temperature optima at 50°C and at 37°C the activity exhibited was 90% of maximum activity at 50°C. Hence the TGase could catalyze the reaction during setting leading to higher network formation. The reason for not getting good gel network when the meat was set at 25°C is because of temperature optima of the TGase enzyme.

Bigeye snapper (*Priacanthus* sp.) has been known as an easy-set species, possibly due to endogenous TGase (Benjakul *et al.*, 2004). So far, setting of sol from surimi including that from bigeye snapper prior to heating is a common practice to improve gel quality in the surimi industry (Benjakul and Visessanguan, 2003). Benjakul and Visessanguan (2003) confirmed that endogenous TGase played an essential role in setting of two species of bigeye snapper, *Priacanthus tayenus* and *Priacanthus macracanthus*. Washing of fish mince is recognized to improve the general quality of gel, especially gel strength and whiteness (Benjakul *et al.*, 2004). As the washing process was reported to remove some proteinases, it may wash out a cross-linking enzyme to some extent (Benjakul *et al.*, 2001). Nowsad *et al.* (1995) showed that the sarcoplasmic fraction of fish could actually enhance the gelling ability when added back to surimi because of its higher TGase activity.

The effect of setting on the strength of heat induced gel for all the four fish species have been assessed. Based on the torque sweep data it is evident that the stress at fracture was higher in gels obtained by setting at 40°C. Based on stress at fracture value it could be said that the set tilapia meat when set at 40°C could lead to dense network during thermal gelation process.

From the above discussion it is evident that setting of mince from bigeye snapper, oil sardine (washed), tilapia and common carp meat at 40°C could enhance the viscoelastic properties and thereby higher gel strength in heat-induced gel. This was also supported by isolated TGase enzyme activity studies at different temperatures.

### 5.5. Effect of TGase activators and inhibitors on setting and gel forming ability of mince

The activity of TGase enzyme could be enhanced in the presence of activators or inhibited in the presence of inhibitors as revealed with studies on isolated enzyme.
Hence, it will be of interest to study the effect of activators and inhibitors in the meat system on its setting and gelling ability. The temperature of setting chosen was 40°C for duration of 30 min as the earlier study revealed the setting at 40°C could lead to higher elastic network in the final gel. Hence, the effect of activators and inhibitors on setting was carried out at 40°C and further the setting ability of the set meat was assessed.

**Effect of CaCl\(_2\)**

The concentration of CaCl\(_2\) used for meat system was varied depending on the fish species. Bigeye snapper mince mixed with 10 mM and 20 mM CaCl\(_2\) gave a higher G’ value after 30 min of setting at 40°C. Further, heating the set meat could yield a G’ maxima value of 738.8 KPa. The difference between 10 and 20 mM CaCl\(_2\) was marginal both in terms of setting ability and gelling ability. For washed oil sardine mince two concentrations of CaCl\(_2\) namely, 1 and 10 mM were tried and 1mM was found to be optimum in enhancing setting and gelling ability. Optimum concentration of CaCl\(_2\) for tilapia and common carp mince for setting and gelling ability was found to be 10 and 20 mM respectively. Hence, the gels obtained from set meat treated with 10 mM CaCl\(_2\) were subjected to torque sweep to assess the strength of the gel. The data revealed the gels were very strong as indicated by higher stress value at fracture.

The optimum concentration of Ca\(^{2+}\) for isolated TGase from four different species was different than that obtained for meat system. This is mainly because while assaying the activity of TGase, the reaction mixture will comprise of 20mM of EDTA which is known to chelate the added Ca\(^{2+}\) ions. Hence the requirement of Ca\(^{2+}\) ions will be higher considering the chelating action of EDTA. In the meat system there will be no EDTA and hence the requirement of Ca\(^{2+}\) for TGase activity will be lower.

Yongsawatdigul and Sinsuwan (2007) reported that setting of tilapia actomyosin occurred at 4°C in the presence of Ca\(^{2+}\) ions above 10mM concentration. This was attributed to the activation of endogenous TGase activity by Ca\(^{2+}\) ions by increasing hydrophobic interactions and enhancing the formation of \(\varepsilon-(\gamma\text{-glutamyl})\)-lysine cross-links among actomyosin molecules. Addition of 10 mM Ca\(^{2+}\) ions induced formation of large actomyosin aggregates at 40°C in tilapia actomyosin (Yongsawatdigul and Sinsuwan, 2007). The endogenous TGase is calcium-dependent
and its activity can be optimized during the setting phenomenon by adding calcium to fish paste before incubation at 25°C–40°C. It was found that the shear stress property of surimi gels from cold water fish species, such as Pacific whiting and Alaska pollack, was increased by adding 0.2% calcium chloride (Lee and Park, 1998). The shear stress of surimi gels from warm-water fish species, such as, Mexican flounder (*Cycloptetta chittendeni*) and Northern kingfish (*Menticirrhus saxatilis*) was also improved by adding 0.2% calcium chloride (Morales et al., 2001). They found that although endogenous TGase is present in surimi obtained from most species of fish, the endogenous calcium level was a limitation in obtaining maximum shear stress in warm-water fish species studied (Ramirez et al., 2003). Formation of non-disulfide covalent cross-linking in Alaska pollack and Atlantic croaker surimi subjected to the optimum setting condition was also noted (Kamath et al., 1992). Covalent cross-linkings of myosin heavy chain induced by Ca²⁺ has been reported in Alaska pollack (Wan et al., 1994) and threadfin bream surimi (Yongsawatdigul et al., 2002). Endogenous TGase has been reported to be responsible for the formation of these cross-linked polymers.

The present investigation demonstrated that Ca²⁺ ions salt at 10 mM level could significantly modify the setting and gelling ability of the fish species under study. If Ca²⁺ can activate the enzyme, can inhibitors of TGase like EDTA, NH₄Cl and lysine-HCl modify the setting and gelling ability of fish mince?

*Effect of inhibitors*

The addition of EDTA to the mince from all the four fish species suppressed the setting phenomenon and was found to be concentration dependent. EDTA is a known chelator for divalent metal ions like Ca²⁺ and Mg²⁺. Once a complex of Ca²⁺ ions and EDTA is formed the TGase enzyme will be lacking the Ca²⁺ (activator) and hence will not be able to catalyze the cross linking of myosin heavy chain during setting. The subsequent heating of set meat with EDTA revealed lower gelling ability as given by G’ maximum value. The data on the torque sweep of heat induced gel obtained from mince containing different concentration of EDTA further supported the view that setting and gelling have been suppressed.

The setting process of four different fish species in presence of 1 M ammonium chloride was considerably suppressed. Heating the set meat further did not
improve the thermal gelling ability over that of control indicating the inhibitory effect of ammonium chloride over control. Ammonium chloride suppressed the setting ability of Alaska pollack surimi as assessed by the formation by reduction of the \( \varepsilon-(\gamma\text{-glutamyl}) \) lysine isopeptide (Kumazawa et al., 1995). Therefore, ammonium chloride added was presumed to inhibit TGase activity, resulting in improper cross-linking and network formation. The torque sweep of the heat set gel revealed lower critical stress value where the fracture occurred in all the samples. The lower stress value at fracture has led to higher Tan \( \delta \) values in all the samples indicating the nature of gel as more viscous than elastic.

The addition of lysine-HCl inhibited the setting effect of mince from all the four fish species. The concentration required to inhibit the setting process was different for different species. For bigeye snapper 0.3 M could suppress the setting and gelling ability significantly; for oil sardine 0.04 M; for tilapia 0.2 M and for common carp 0.5 M. The effect of lysine-HCL in suppressing setting process was concentration dependent. The viscoelastic behavior of set meat during heating also indicated weak network formation as given by \( G' \) maxima at any given temp. Kamath et al. (1992) used free lysine-HCl as a competitive inhibitor for the TGase enzyme. The effect of free lysine-HCl addition on setting was evaluated at 0.3, 0.6 and 1.2 M in pollack surimi and at 1.2 and 1.5 M in croaker surimi. They found that free lysine-HCl acts as a competitor of reactive group lysine and inhibited the cross-linking during setting at 25\(^\circ\)C and 40\(^\circ\)C. The gel strength and extent of cross linking was also affected considerably. The authors proposed that unfolding or denaturation of protein molecules may lead to a greater exposure of both hydrophobic and glutamine residues, the latter being made available for the cross linking with lysine residue. The strength of the gel network in presence of 0.25 M lysine-HCl was assessed by torque sweep measurement. There was significant reduction in stress at fracture from all the gel samples studied in presence of lysine-HCl.

Incorporation of activators like CaCl\(_2\) has increased the setting ability and elastic structure of heat induced gel. Incorporation of inhibitors decreased the setting ability and elastic structure of heat induced gel. While considering the effect of activators or inhibitors on setting and further gelation, it should be borne in mind that rheological parameters have been compared to that of control (set without activators
or inhibitors) and conclusions have been drawn. The TGase enzyme, which has been isolated and purified from four different species, revealed activation process in presence of CaCl$_2$ and inhibition to various degrees in presence of inhibitors. The same mechanism also exhibited in the meat system from different species validating in vivo study. It should be noted that setting of meat without activators or inhibitors also yielded network, which was different from the treated samples. This indicates higher elastic gels could be obtained by using appropriate concentration of activator.

During setting the properties of proteins are likely to get altered, as there is evidence of cross-linking. Hence, it is of interest to study the changes in solubility profile in different solvents, changes in free sulfhydryl group content and electrophoretic mobility under reduced condition as affected by setting. The changes in the properties of proteins in presence of activators and inhibitors during setting of mince from four different fish species have been studied.

### 5.6. Effect of activators and inhibitors on the properties of proteins from four different fish species

**Protein solubility**

The solubility of proteins as related to setting process has been assessed in two solvents viz., EB (EB – phosphate buffer, 50 mM, pH 7.5 containing 1.0 M sodium chloride) and tris buffer containing 1% SDS. It was found that setting process itself without activators or inhibitors decreased the solubility in EB by more than 50% in all the fish species studied. When tris buffer containing SDS was used as the solvent it was found that a higher solubility value was obtained in comparison to EB as solvent from all the fish species. After setting the solubility in SDS also reduced to more than 50%, but, the absolute values were higher than that obtained for EB. Incorporation of CaCl$_2$ during setting did affect the solubility both in EB and tris buffer containing SDS significantly in all the four fish species. A higher solubility was obtained in the meat set with inhibitor like EDTA, NH$_4$Cl and lysine-HCl. A reduction in protein solubility is an indication of aggregation process. An aggregated protein will have lesser solubility in any given solvent, as the nature of linkages in the complex is normally strong. In the present study, the setting process *per se* has reduced the solubility in both EB and tris buffer containing SDS considerably. Since, the meat
was set at 40°C formation of hydrophobic interaction, ionic interaction and possibly disulfide will lead to an ordered aggregation (Niwa, 1992; Ziegler and Foegeding, 1990). The solubility of proteins from set meat with inhibitors was relatively higher than that of set meat alone. The higher solubility in SDS buffer is mainly due to detergent action wherein, the charge of the protein molecule is masked by SDS which in turn interacts with aqueous phase to increase hydration. Setting of meat with CaCl$_2$ has reduced the solubility indicating enhancement of aggregation process. A reduction in solubility of suwari gels from bigeye snapper (*Priacanthus tayenus*), threadfin bream (*Nemipterus bleekeri*), barracuda (*Sphyraena jello*) was observed at CaCl$_2$ concentration up to 20 mM (Benjakul et al., 2004).

Two conclusions can be drawn from the above results. One, higher activation of TGase in presence of CaCl$_2$ has cross-linked the protein molecule thereby reducing the solubility. Two, meat with inhibitors when subjected to setting has inhibited the TGase activity and thereby minimizing the cross-linking process results in higher protein solubility values. Another distinct feature of the study is that all the four fish species behaved in a similar pattern in presence of activator and inhibitors.

*Electrophoretic mobility (SDS-PAGE pattern)*

Since the reduction in solubility of proteins during setting is attributed to cross-linking, electrophoretic mobility under reduced condition (SDS - PAGE pattern) of set meat from four fish species has been assessed. The pattern clearly demonstrated a reduction in 205 kDa components after setting at 40°C. Setting with CaCl$_2$ also indicated significant reduction in 205 kDa component indicating that cross-linking of myosin heavy chain during setting process. This pattern was almost similar in all the four fish species studied with minor variations. Some of the low molecular weight components like 45 kDa and 20 kDa were also affected by setting without and with CaCl$_2$.

The SDS-PAGE pattern of set meat with inhibitors revealed a different pattern wherein, 205 kDa components was prominent as in unset meat. It is reasonable to expect reduced TGase activity in presence of inhibitors, thereby minimizing cross-linking. The data on solubility and SDS-PAGE pattern clearly demonstrates that the TGase enzyme activity has been reduced significantly. Hence, a higher solubility
value and emergence of 205 kDa component in SDS-PAGE pattern has been observed.

*Free sulphhydryl (-SH) content*

An increase in the free sulphhydryl content of all the fish species has been observed after setting at 40°C. Setting at 40°C for 30 min will facilitate the opening up of light meromyosin subfragment of myosin molecule, wherein, nearly 8-17 SH residues have been accommodated (Sano et al., 1990). Hence, a higher free SH content was observed in all four fish species as the meat was set at 40°C. Addition of CaCl₂ did enhance the free sulphhydryl content both in bigeye snapper and oil sardine while in that of tilapia and common carp there was no change in the value. The free sulphhydryl content of set meat in presence of inhibitors showed a lower value than the control sample. It is difficult to ascertain the role of inhibitors during setting in the reduction of free sulphhydryl group. However, this reduction was marginal and may not have any consequence in elastic structure development.

5.7. Effect of addition of purified TGase on the setting and viscoelastic behavior of flat fish (*Cynoglossus sp.*) mince

Addition of purified TGase from four different fish species to the mince of flat fish was attempted to study its setting and viscoelastic properties. The quantity of TGase added was 2 units / g mince. CaCl₂ at a concentration of 10 mM was also added to the flatfish mince and the setting ability was assessed at 40°C for a duration of 30 min. Further, the viscoelastic properties of set meat in the temperature range of 40-90°C was assessed. Appropriate controls were maintained during the experiment.

The setting ability and the viscoelastic nature of heat-induced gel of flat fish mince improved considerably by the addition of TGase from all the fish species. TGase from oil sardine was found to be better than the other three fish species in promoting the setting process and improving the viscoelastic nature of heat induced gel. The torque sweep of the heat induced gel with TGase revealed a higher shear stress at fracture values indicating viscoelastic nature. The gel strength measurement of the gel revealed a higher value indicating strong viscoelastic nature. Gel strength of lizard fish surimi was significantly increased when 0.6 units / g surimi of microbial
transglutaminase (MTGase) was added and pre-incubated at 25°C (Yongsawatdigul, 2003). Jiang et al. (2000) reported that gel strength of surimi from threadfin bream and Alaska pollack increased when MTGase was added up to 0.3 and 0.2 units / g surimi, respectively. The optimum level of MTGase for minced mackerel was 0.34 unit / g (Tsai et al., 1996). The amount of MTGase added depended on types of fish as well as other factors such as freshness, protein quality and harvesting season (Asagami et al., 1995). Sakamoto et al. (1995) observed a decrease in gel strength with longer incubation time, with surimi from Alaska pollack and MTGase. They hypothesized that excessive formation of ε-(γ-glutamyl)-lysine cross-link would inhibit uniform development of the protein networks affecting breaking strength, deformation or gel strength. Tammatinna et al. (2007) studied the properties of white shrimp (Penaeus vannamei) gel added with different levels of microbial TGase (MTGase) and subjected to setting at 25 °C for 2 hr or 40°C for 30 min, prior to heating at 90°C for 20 min. They concluded that gels prepared by setting at 25°C exhibited the greater breaking force than those set at 40°C, possibly associated with the appropriate protein structure for cross-linking at 25°C and greater degradation at 40°C.

The addition of TGase from four different fish species to the flat fish mince during setting process clearly demonstrated polymerization of myosin heavy chains as revealed by SDS-PAGE pattern. The addition of endogenous TGase had significant beneficial effect in improving the gel network, thereby textural properties. The TGase from sardine mince and tilapia was found to have higher myosin heavy chain cross-linking ability than other species.