CHAPTER 3

RESULTS AND DISCUSSION

1. Chemical composition and property of white shrimp, bigeye snapper and lizardfish mince

1.1 Proximate composition

Proximate composition of mince from different species is shown in Table 6. White shrimp, bigeye snapper and lizardfish mince contained high moisture content, accounting for approximately 75.71%, 78.57% and 78.65%, respectively. Protein was found as the major constituent, indicating that the meat of all species could be the good source of amino acids. Protein contents of white shrimp, bigeye snapper and lizardfish mince were 20.71%, 18.62% and 18.10%, respectively, while trace amounts of ash and lipid were found. Depending on the species and the nutritional status of the animal, the muscle of marine fish and invertebrates consists of 50-80% water (Sikoroski *et al*., 1990a). In addition, the composition of muscle can vary with the season, size, sex, spawning and feeding condition (Pigott and Tucker, 1990).

Table 6 Proximate composition of mince from some marine fish and shrimp muscles

Compositions (% wet wt.)	White shrimp	Bigeye snapper	Lizardfish
Moisture	75.71 ± 0.32b	78.57 ± 0.05a	78.65 ± 0.01a
Protein	20.71 ± 0.12b	18.62 ± 0.49a	18.10 ± 0.11a
Fat	0.39 ± 0.08c	1.34 ± 0.02a	1.11 ± 0.04b
Ash	1.33 ± 0.03a	1.47 ± 0.01a	1.14 ± 0.03b

Values are given as mean ±SD from triplicate determinations.

Different letters in the same row indicate significant differences. ($p<0.05$)

1.2 Proteins and non-protein nitrogenous compounds

Fractionation of muscle proteins based on solubility was used to classify muscle proteins into five fractions (Table 7). Similar non-protein nitrogen content was observed between two fish species (2.97-2.98 mg N/g muscle), while the lower content was found in white shrimp

meat (1.28 mg N/g muscle). Non-protein nitrogen fraction included amino acid, imidazole, dipeptide, nucleotides, trimethylamine oxide, trimethylamine, urea and the products of postmortem changes (Sikorski, 1994; Foegeding *et al*., 1996). Meat of all species had myofibrillar protein as the major constituent, accounting for ~53.44-67.07% of total muscle proteins. Sarcoplasmic protein was found as the second predominant protein in the meat of all species. The result was in agreement with Hashimoto *et al.* (1979) who reported that myofibrillar and sarcoplasmic proteins are the major proteins in fish muscle. Among the sarcoplasmic proteins, myoglobin was dominant in dark muscle, while parvalbumin was prevalent in white muscle of sardine and mackerel (Hashimoto *et al*., 1979). From the result, both sarcoplasmic and myofibillar proteins varied with species. This might contribute to the different functional properties of meat from different species. For alkaline soluble protein, it was noted that bigeye snapper contained the highest content, whereas white shrimp had the lowest content. Conversely, white shrimp meat comprised the highest content of stromal protein (2.26). The high content of stroma was likely associated with the firmer or tougher texture of white shrimp, compared with fish meat. Stromal proteins consist not only of collagen and elastin, but also connectin and other proteins (Sikorski and Borderies, 1990). Teleost and elasmobranch species generally contain 3 and 10% stroma, respectively.

Table 7 Nitrogenous constituents in the mince from some marine fish and shrimp muscles

Composition (mg N/g muscle)	White shrimp	Bigeye snapper	Lizardfish
Non-protein nitrogen	1.28 ± 0.32b	2.97 ± 0.49a	2.98 ± 0.80a
Sarcoplasmic protein	7.62 ± 0.26a (31.31)	6.97 ± 0.24b (29.35)	6.26 ± 0.32b (23.53)
Myofibrillar protein	14.07 ± 0.84b (57.81)	12.14 ± 0.35c (53.44)	17.84 ± 0.25a (67.07)
Alkali-soluble protein	0.39 ± 0.05c (1.60)	2.67 ± 0.03a (11.75)	2.14 ± 0.03b (8.04)
Stromal protein	2.26a ± 0.17 (9.28)	1.24 ± 0.07b (5.45)	0.36 ± 0.03c (1.35)

Values are given as mean ±SD from triplicate determinations.

Different letters in the same row indicate significant differences ($p<0.05$).

Values in the parenthesis represent the percentage of each protein fraction based on total protein content.

Protein patterns of different fractions of fish are illustrated in Figure 7. For myofibrillar protein fraction (lane 1), two major protein bands, corresponding to MHC and actin, were observed. From the result, MHC band intensity was different among species and bigeye snapper meat showed the greatest MHC band intensity. Apart from MHC and actin, other regulatory proteins including troponin, tropomyosin, as well as myosin light chain were also found in this fraction from all species. Generally, low MW proteins were found in the sarcoplasmic protein fraction (lane 2).

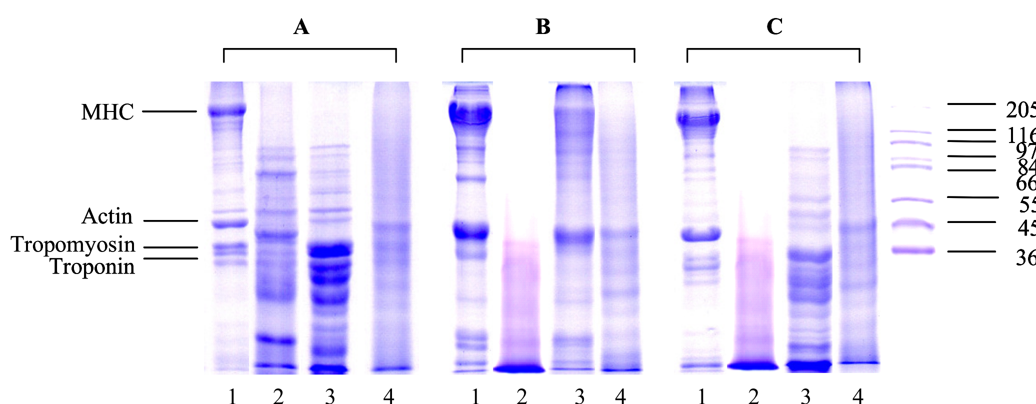

Figure 7 Electrophoretic pattern of various protein fractions of mince from some marine fish and shrimp. A: white shrimp, B: bigeye snapper and C: lizardfish. lanes 1, 2, 3, 4: myofibrillar, sarcoplasmic, alkali-soluble and stromal protein fractions, respectively.

The protein pattern of alkaline-soluble fraction of bigeye snapper was somehow similar to that of myofibrillar fraction. The results were in agreement with those of Hashimoto *et al*. (1979) who reported that alkaline-soluble fraction of sardine and mackerel muscles exhibited a similar protein pattern to myofibrillar fraction. Protein with MW higher than myosin heavy chain was found to a high extent in an alkaline-soluble fraction of bigeye snapper meat. Nevertheless, proteins with the MW lower than 40 kDa were observed as the major constituents in alkaline-soluble fraction of white shrimp and lizardfish (lane 3). Similar protein pattern was noticeable between stroma fractions of three species. The differences in protein compositions might result in the different properties and characteristics of fish and invertebrate. Since most of sarcroplasmic proteins and connective tissue are generally removed during surimi processing, myofibrillar proteins in muscle become concentrated and thus play an essential role in muscle functional

properties, especially gelling property (Foegeding *et al*, 1996). Due to different muscle compositions, especially the content and type of myofibrillar protein fraction, the differences in gelation of different fish species can be observed.

1.3 Thermal denaturation

Thermal transitions of marine fish and shrimp muscle proteins were determined using differential scanning colorimetry (Table 8). DSC thermogram of fish and shrimp meats revealed two major endothermic peaks with T_{max} of 50.78 and 71.11 °C for white shrimp, 51.44 and 72.44 °C for bigeye snapper, 49.55 and 70.94 °C for lizardfish. The observed T_{max} were similar to those found in various fish species, in which the first and second peaks were postulated to be the transitions of myosin and actin, respectively (Akahane *et al*., 1985). Enthalpies of the first and second peaks were 1.40 and 0.64 J/g; 1.10 and 0.35 J/g; 1.27 and 0.33 J/g, respectively. T_{max} of myosin (49.55-51.44°C) and actin (70.94-72.44°C) were in agreement with Poulter *et al*. (1985) who reported that T_{max} of the first and the second peak of fish were 41.70-52.70 and 72.6-73.8 °C, respectively. From the result, the slight differences in those values were found among three species. The differences in transition temperature of myosin might determine the setting phenomenon as well as gel property of fish meat or surimi from different fish species. Thermal gelation of meat paste was governed by how proteins behave or interact with each other during heating. A small quantity of heat is generally used for the easy-setting meat (Iso *et al*., 1991). Additionally, thermal stability of fish myosin varied with species. Species adapted to higher environmental temperature showed higher stability (Hastings *et al*., 1985).

Table 8 T_{max} and enthalpy (ΔH) of mince from some marine fish and shrimp muscles

Species	Peak I		Peak II	
	T_{max} (°C)	Δ H (J/g)	T_{max} (°C)	Δ H (J/g)
White shrimp	50.78 ± 0.67a	1.40 ± 0.10a	71.11 ± 0.67b	0.64 ± 0.09a
Bigeye snapper	51.44 ± 0.92a	1.10 ± 0.49b	72.44 ± 0.02a	0.35 ± 0.13b
Lizardfish	49.55 ± 0.25a	1.27 ± 0.26b	70.94 ± 0.42b	0.33 ± 0.06b

Values are given as mean ±SD from triplicate determinations.

Different letters in the same column indicate significant differences. (p<0.05).

2. Effect of setting condition and MTGase on gel forming ability of some marine fish and shrimp meats

2.1 Breaking force and deformation

The breaking force and deformation of mince gel from all species added with different MTGase levels and set under different conditions are shown in Figure 8. Different setting conditions used resulted in the varying gel properties. Without MTGase addition, gels of bigeye snapper and lizardfish meat set at high temperature (40°C for 30 min) prior to heating at 90°C for 20 min showed the higher breaking force and deformation than did gels with prior setting at medium temperature (25°C for 2 h) ($p<0.05$). On the other hand, white shrimp gel with prior setting at medium temperature (25°C for 2 h) prior to heating at 90°C for 20 min showed the higher breaking force than did that with prior setting at high temperature (40°C for 30 min) ($p<0.05$). No differences in deformation of white shrimp gels with different setting conditions were found ($p>0.05$). This result indicated that setting played an important role in cross-linking of gel network, especially by non-disulfide covalent bonds induced by endogenous transglutaminase (TGase). TGase was found to mediate the formation of myosin cross-linking via the ε-(γ-glutamyl-lysine) linkage (Seki *et al*., 1990). When comparing high and medium temperature setting in fish gels, the gels with high temperature setting showed the superior breaking force to those with medium temperature setting. This was probably because endogenous TGase in bigeye snapper and lizardfish muscle was much more active at 40°C than at 25°C. Furthermore, the alignment or aggregation of protein probably occurred to a higher extent at higher temperature. At sufficiently high temperature, both hydrophobic interaction and disulfide bond formation could occur (Niwa, 1992). Benjakul *et al*. (2003c,d) reported that the optimum conditions for setting of surimi sol from bigeye snapper and lizardfish were 40°C for 30 min. From the result, greater breaking force and deformation of gels set at medium temperature might be due to the greater activity of endogenus TGase in shrimp meat at 25°C as well as the lower autolysis at lower temperature. Lower breaking force of white shrimp gel prepared by one-step heating was observed, compared to gels with prior setting (40°C for 30 min or 25°C for 2 h) (data not shown). Heating process has been known to induce protein aggregation via various bonds including hydrophobic interaction and disulfide bond (Benjakul *et al*., 2001). Numakura *et al*. (1990) and Kamath *et al*. (1992) reported that walleye pollack surimi paste had the optimum

temperature of setting at 25°C and no setting occurred above 40°C. Gel properties of surimi were generally determined by the setting condition (Lanier, 1992; Jiang, 2000). The differences in setting response observed among gel from different species were possibly due to the differences in activity and thermal stability of endogenous TGase as well as the differences in thermal property of proteins.

From the result, the breaking force of all gels increased with increasing MTGase levels ($p<0.05$) regardless of setting conditions (Figure 8). Higher amount of MTGase added might induce the formation of non-disulfide covalent bond to a greater extent. As a result, the strength of gel matrix was enhanced. At the same MTGase level, it was noted that a greater breaking force of fish gels was observed with samples set at high temperature. It was noted that deformation of lizardfish gel increased continuously when MTGase levels were added up to 0.6 Units/g sample. However, MTGase levels did not show the pronounced effect on the increase in deformation of shrimp and bigeye snapper gels. From the result, shrimp gel set at medium temperature exhibited the higher breaking force than did that set at higher temperature. MTGase from *Streptoverticillium mobaraense* showed the maximal activity at 50°C (Motoki and seguro, 1998). Thus, the unfolding of muscle protein molecules at higher temperature might expose the reactive group for cross-linking induced by MTGase to a higher extent for fish gel. Thus, the conformation of muscle proteins, especially MHC in lizardfish and bigeye snapper, was possibly associated with the cross-linking efficacy of MTGase. When setting at 40°C for 30 min, both endogenous TGase and MTGase could induce the cross-linking with the sufficient time as evidenced by the increase in breaking force and deformation. However, for shrimp proteins, the unfolding of protein molecules at higher temperature might favor the aggregation via hydrophobic interaction. As a consequence, the reactive group for cross-linking induced by MTGase could be embedded. Therefore, glutamine or lysine residues were possibly masked and were not available for cross-linking mediated by MTGase. The effects of MTGase on surimi gel varied with fish species (Asagami *et al*., 1995). Jiang *et al*. (2000a) 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. Excessive amount of MTGase resulted in the decrease in breaking force of surimi gels (Seguro *et al*., 1995). The optimal 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). From the result, gel properties of all species were governed by both MTGase levels and setting condition.

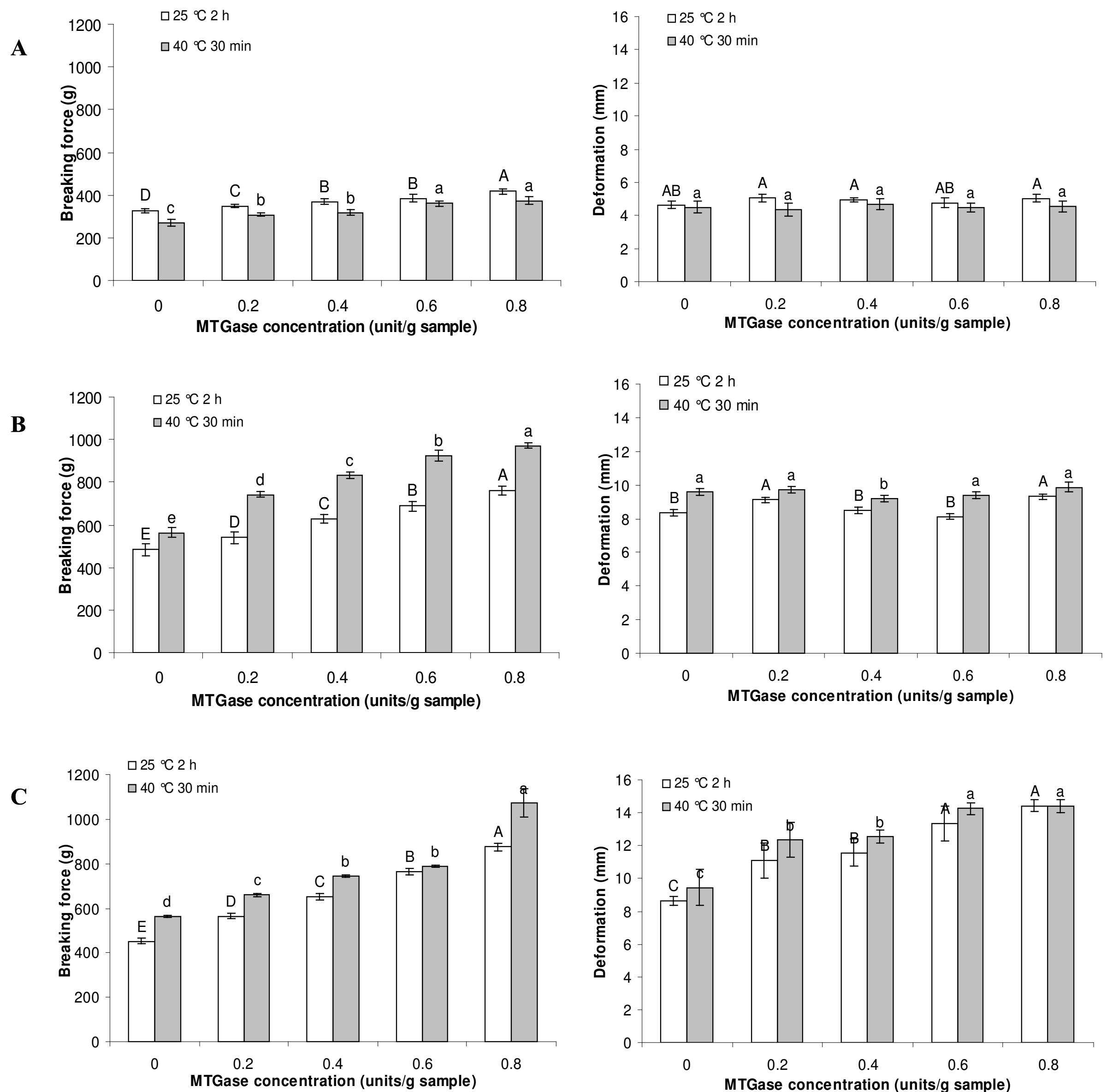

Figure 8 Breaking force and deformation of mince gels from some marine fish and shrimp added with various levels of MTGase and set under different conditions. A: white shrimp, B: bigeye snapper and C: lizardfish. Bars indicated the standard deviation from five determinations. Different letters within the same setting condition indicated significant differences ($p<0.05$).

2.2 Color and whiteness

L*, a*, b*-values and whiteness of mince gels from all species set under different conditions in the presence of various MTGase concentrations are shown in Tables 9, 10 and 11. The addition of MTGase caused the slight changes in color and whiteness of gels for all species. For white shrimp gels, L*-value (whiteness) increased slightly, but a* and b*-values slightly decreased. L* value of gels set at 25°C for 2 h was slightly higher than that of gels set at 40°C for 30 min for white shrimp gels. For the gels from bigeye snapper and lizardfish mince, higher L*-value was observed in gels with high temperature setting, compared with those with medium temperature setting. This was possibly due to the greater denaturation of pigments or proteins in muscle at higher temperature. Therefore, setting temperature directly affected lightness of shrimp gels. Shrimp gel generally showed the higher a*-value (redness) than fish gels. This was caused by the differences in pigments between fish and shrimp. Carotenoids including astaxanthin and canthaxanthin are the major pigments in shrimp muscle (Simpson, 1982; Okada *et al.*, 1998; Armenta-LÓpez *et al*., 2002). From the result, L*-value and whiteness slightly increased when higher amounts of MTGase were added. The increased whiteness might be caused by the maltodextrin used as the stabilizer in MTGase powder, particularly at the higher amount added. Thus, the use of MTGase is a potential approach for increasing gel strength without the marked changes in whiteness of resulting gel.

Table 9 L*, a*, b*-values and whiteness of gels from white shrimp mince added with various levels of MTGase and different setting conditions

Level of MTGase (unit/g of sample)	White shrimp							
	Setting at 25°C for 2 h				Setting at 40°C for 30 min			
	L*	a*	b*	Whiteness	L*	a*	b*	Whiteness
0	71.72 ± 0.46b	19.13 ± 0.22ab	16.19 ± 0.04a	62.21 ± 0.14b	70.73 ± 0.48a	19.00 ± 0.26a	16.18 ± 0.27b	61.54 ± 0.04b
0.2	71.85 ± 0.43b	19.13 ± 0.26ab	16.20 ± 0.10a	62.31 ± 0.23b	70.83 ± 0.36a	19.30 ± 0.25a	16.80 ± 0.11a	61.20 ± 0.05b
0.4	72.08 ± 0.27ab	19.38 ± 0.20a	16.07 ± 0.37a	62.41 ± 0.05b	71.12 ± 0.39a	19.25 ± 0.19ab	15.77 ± 0.26c	61.88 ± 0.09b
0.6	72.42 ± 0.24a	18.88 ± 0.41bc	16.00 ± 0.27a	62.44 ± 0.13b	71.85 ± 0.17a	18.92 ± 0.15a	15.73 ± 0.23c	62.61 ± 0.14a
0.8	72.42 ± 0.34a	18.61 ± 0.46c	15.91 ± 0.31a	63.12 ± 0.33a	71.60 ± 0.23a	18.56± 0.13b	16.19 ± 0.24b	62.41 ± 0.13a

Values are given as means ± SD from five determinations.

Different letters within the same column indicate the significant differences ($p<0.05$).

Table 10 L*, a*, b*-values and whiteness of gels from bigeye snapper mince added with various levels of MTGase and different setting conditions

Level of MTGase (unit/g of sample)	Bigeye snapper							
	Setting at 25°C for 2 h				Setting at 40°C for 30 min			
	L*	a*	b*	Whiteness	L*	a*	b*	Whiteness
0	75.01 ± 0.13a	-3.07 ± 0.05b	9.69 ± 0.11b	73.02 ± 0.10a	76.75 ± 0.18b	-2.92 ± 0.04a	9.99 ± 0.20a	74.53 ± 0.12b
0.2	75.27 ± 0.27a	-2.94 ± 0.16b	9.88 ± 0.39b	73.20 ± 0.02a	76.94 ± 1.11b	-3.01 ± 0.19a	9.42 ± 0.40b	74.90 ± 0.09b
0.4	75.31 ± 0.46a	-3.12 ± 0.06b	9.70 ± 0.27b	73.29 ± 0.03a	77.25 ± 0.35b	-2.80 ± 0.06a	9.96 ± 0.16a	75.00 ± 0.03b
0.6	75.33 ± 0.22a	-3.09 ± 0.07b	9.26 ± 0.16b	73.29 ± 0.04a	77.28 ± 0.22b	-2.87 ± 0.10a	9.95 ± 0.09a	75.03 ± 0.22b
0.8	75.49 ± 0.60a	-2.76 ± 0.09a	10.23 ± 0.31a	73.47 ± 0.24a	77.69 ± 0.16a	-2.99 ± 0.05a	9.38 ± 0.10b	75.61 ± 0.17a

Values are given as means ± SD from five determinations.

Different letters within the same column indicate the significant differences ($p<0.05$).

Table 11 L*, a*, b*-values and whiteness of gels from lizardfish mince added with various levels of MTGase and different setting conditions

Level of MTGase (unit/g of sample)	Lizardfish							
	Setting at 25°C for 2 h				Setting at 40°C for 30 min			
	L*	a*	b*	Whiteness	L*	a*	b*	Whiteness
0	73.25 ± 0.43b	-3.09 ± 0.23a	8.32 ± 0.07b	71.82 ± 0.38b	73.73 ± 0.20b	-3.10 ± 0.06a	7.88 ± 0.20b	72.40 ± 0.21b
0.2	73.30 ± 0.33b	-3.12 ± 0.09a	8.37 ± 0.13b	71.84 ± 0.34b	73.98 ± 0.19b	-2.89 ± 0.16a	8.65 ± 0.23a	72.43 ± 0.20b
0.4	74.06 ± 0.20a	-3.03 ± 0.19a	8.80 ± 0.23a	72.44 ± 0.15a	74.21 ± 0.76a	-2.87 ± 0.08a	8.28 ± 0.24a	72.76 ± 0.72ab
0.6	73.96 ± 0.34a	-3.14 ± 0.13a	8.20 ± 0.22b	72.52 ± 0.33a	74.28 ± 0.98a	-2.98 ± 0.04a	7.83 ± 0.15b	72.95 ± 0.89ab
0.8	74.00 ± 0.27a	-3.16 ± 0.03a	8.20 ± 0.19b	72.56 ± 0.31a	75.07 ± 0.16a	-2.90 ± 0.06a	8.87 ± 0.20a	73.38 ± 0.13a

Values are given as means ± SD from five determinations.

Different letters within the same column indicate the significant differences ($p<0.05$).

2.3 Expressible moisture content

Expressible moisture content of gel added with different levels of MTGase and setting conditions is shown in Table 12. In the absence of MTGase, the lowest expressible moisture content was obtained in gels from white shrimp meat set at medium temperature (25°C for 2 h). Among all gels set at high temperature (40°C for 30 min), gel from bigeye snapper had the lowest expressible moisture content. From the result, the decrease in expressible moisture content was observed with increasing MTGase levels ($p<0.05$). Gel added with 0.8 units/g sample and set at 40°C for 30 min prior to heating had the decrease in expressible moisture content by 73.67% and 53.94% for bigeye snapper and lizardfish mince gels, respectively. For shrimp gel added with 0.8 units/g sample and set at 2 h 25°C for 2 h prior to heating, expressible moisture content was decreased by 64.51%, compared with that without MTGase addition under the same setting condition. After the appropriate setting, heating is required to induce the aggregation of protein, in which gel matrix formed can imbibe the water. Alvarez and Tejada (1997) found that suwari gel had the lower water holding capacity than kamaboko gels. Different expressible moisture content suggested the differences in water holding capacity of gel network. Regardless of setting/heating condition, the lowered expressible moisture content was noticeable with addition of MTGase, especially with increasing MTGase amount. Low expressible moisture content of the gels suggested the more water retained in the gel network (Niwa, 1992). Therefore, the addition of MTGase in combination with the appropriate setting could be used to improve gel-forming ability via non-disulfide covalent bonds. As a consequence, the stronger gel network formed could hold water to a greater extent as evidenced by the lowest expressible moisture content.

Table 12 Expressible moisture content of mince gels from some marine fish and shrimp added with various levels of MTGase and different setting conditions

Level of MTGase (unit/g of sample)	Expressible moisture content (%)					
	Setting at 25°C for 2 h			Setting at 40°C for 30 min		
	White shrimp	Bigeye snapper	Lizardfish	White shrimp	Bigeye snapper	Lizardfish
0	3.10 ± 0.43a	4.69 ± 0.09a	9.15 ± 3.28a	3.44 ± 0.66a	3.00 ± 0.45a	8.88 ± 2.51a
0.2	2.95 ± 0.14b	3.97 ± 0.03b	9.11 ± 2.68a	3.10 ± 0.67a	2.85 ± 0.28ab	7.37 ± 1.81b
0.4	2.82 ± 0.08b	3.13 ± 0.15b	6.78 ± 2.21b	2.62 ± 0.11b	2.67 ± 0.12b	6.86 ± 2.52c
0.6	2.80 ± 0.16b	2.79 ± 0.05b	6.47 ± 1.24b	2.35 ± 0.22b	2.47 ± 0.15bc	4.97 ± 2.94d
0.8	2.00 ± 0.09c	1.98 ± 0.35c	5.60 ± 0.88c	2.10 ± 0.17b	2.21 ± 0.03c	4.79 ± 3.16d

Values are given as means ± SD from five determinations.

Different letters within the same column indicate the significant differences ($p<0.05$).

2.4 Protein patterns

The protein patterns of mince gel from all species added with different MTGase levels and set under different conditions are shown in Figure 9. Electrophoretic pattern revealed that MHC band intensity decreased as the concentration of MTGase increased. However, the degree of decrease in MHC band intensity varied with setting conditions, which most likely affected MTGase activity. This indicated that MTGase was very powerful for induction of MHC cross-linking. From the result, no marked changes in actin were obtained in the presence and the absence of MTGase. Nakahara *et al*. (1999) reported that MTGase and carp TGase could not cross-link actin molecules. The decrease in MHC band intensity was concomitant with the increased breaking force (Figure 8). This result indicated that MTGase effectively induced the polymerization of MHC, which is a major protein contributing to the gel network formation. As a consequence, the stronger gel network was formed. The result was in accordance with Jiang *et al*., (1998a) who reported that cross-linking of MHC occurred rapidly in mackerel surimi added with MTGase. At the same level of MTGase added, MHC band intensity decreased to a higher extent when the gels were set at 40°C for 30 min. This result indicates that MTGase effectively induced the polymerization of MHC at 40°C. MTGase has been reported to exhibit the greatest activity at

50°C (Motoki and Seguro, 1998; Ho et al., 2000). As a consequence, cross-linking of MHC was more pronounced at 40°C, which was close to the optimal temperature of MTGase. For gel set at 25°C for 2 h prior to heating, MHC was more retained in comparison with that found in gel set at 40°C for 30 min. This suggested the lower cross-linking of MHC in gel set at 25°C. This result was in agreement with Benjakul and Visessanguan (2003) who reported that the optimum condition for setting of surimi sol from *P. tayenus* was 40°C for 30 min. Benjakul *et al*. (2003c) also reported that lizardfish surimi gel prepared with high temperature setting had a higher breaking force and deformation than those with medium temperature setting. For the shrimp mince gels, though higher polymerization took place in gel set at 40°C, the lower breaking force was found in this gel (Figure 8), compared to the gel with prior setting at 25°C, in which lower cross-linking occurred. It was postulated that MHC might be polymerized intramolecularly as indicated by the decrease in MHC band intensity. As a consequence, inter-connection between dissociated MHC molecular was decreased and inter-molecular cross-links to form the well-organized network were limited. Additionally, the excessive cross-link might be associated with the poor gel quality. Gradual alignment of protein molecules at medium temperature contributed to more ordered-network, which could imbibe more water. From the result, the decrease in MHC via non-disulfide covalent cross-linking was more pronounced with increasing MTGase level added. This indicated that maximal polymerization of muscle proteins occurred at different temperatures, depending on species. Kamath *et al*. (1992) reported that optimum setting temperatures for Alaska pollock and Atlantic croaker surimi were 25 and 40°C, respectively. Jiang *et al*. (2000a) found that the gel strength of pollack surimi added with MTGase and set at 30°C for 90 min was improved more greatly than those set at 45°C for 20 min. The cross-linking reaction induced by MTGase occurs when protein molecules and the enzyme become associated in a highly oriented and conformation-dependent fashion at some stage of the catalytic process (Gorman and Folk, 1981). Furthermore, optimum temperature for setting among species may be determined by the heat stability of myosin and the rate of cross-linking may be dependent on the conformation of the substrate myosin at a given temperature rather than on the optimum temperature of MTGase (Kamath *et al*., 1992). Therefore, an appropriate temperature of setting for individual fish should be considered to obtain the highest gel quality.

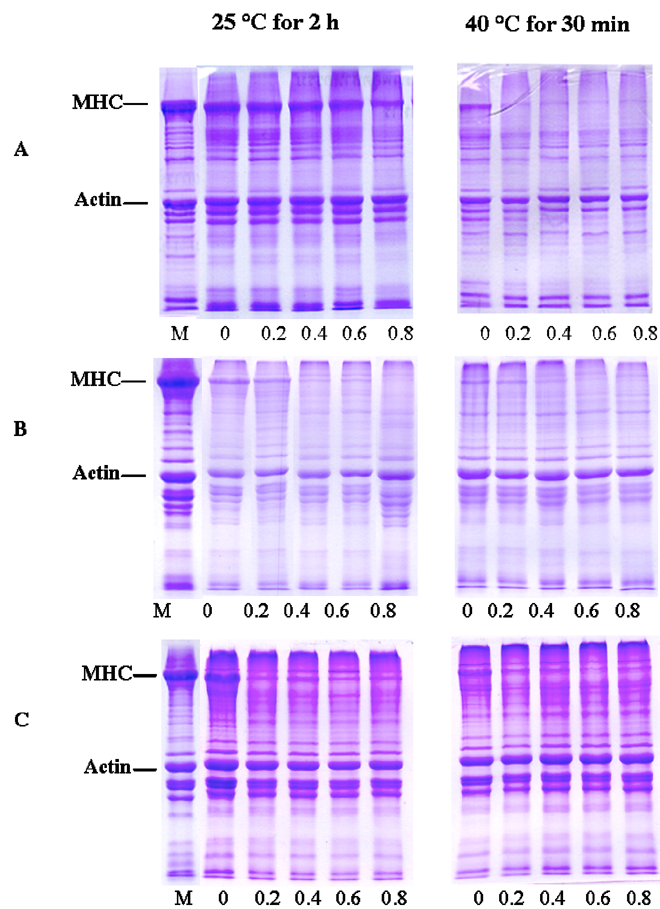

Figure 9 SDS-PAGE pattern of mince gels from some marine fish and shrimp added with various levels of MTGase and set under different conditions. A: white shrimp, B: bigeye snapper, C: lizardfish, M: mince. Numbers designate MTGase concentrations (units/g sample).

2.5 Microstructure

Microstructures of mince gels of different species without and with MTGase (0.6 units/g) and set under different conditions are illustrated in Figure 10. The gels with the addition of 0.6 units MTGase/g generally had the finer and longer strands than those without MTGase. These strands could form the network with more fibrillar structure. Regardless of setting condition, the gel possessed more ordered fibrillar structure when MTGase was added. MTGase was postulated to build up the network structure through intermolecular ε-(γ-glutamyl) lysine cross-linking in co-operation with protein aggregation via hydrophobic interaction, disulfide bonds and other interaction during heating process (Benjakul *et al*., 2003d). When comparing the microstructure of gel from different species, it was noted that shrimp gel had the coarser structure with the larger void. This was coincidental with the lower gel strength of this species. However, the addition of MTGase resulted in the finer network, which was associated with the improved gel strength. From the result, appropriate setting temperature in combination with MTGase addition could improve the gel-forming ability of gel by rendering the more ordered fibrillar structure.

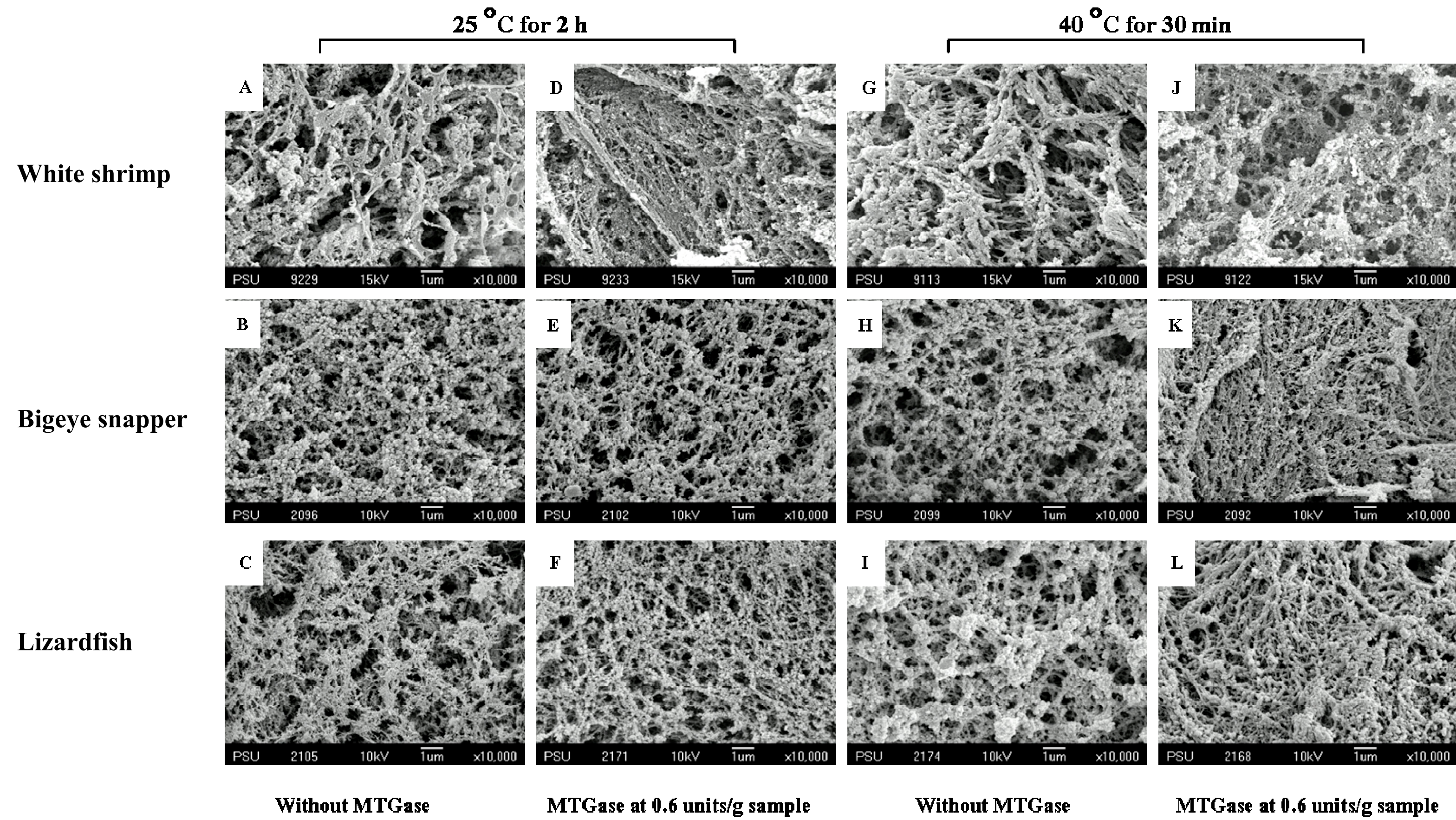

Figure 10 Microstructure of gels from some marine fish and shrimp added without and with MTGase (0.6 units/g) and set under different conditions.

3. Effect of post-mortem storage on protein cross-linking and gel enhancing ability of MTGase on mince from some marine fish and shrimp

3.1 Chemical changes during iced storage

3.1.1 Changes in pH

The increases in pH of muscle of all species were observed up to 10 days of iced storage as shown in Figure 11. The pH of white shrimp increased sharply within the first five days ($p<0.05$). For bigeye snapper and lizardfish, pH slightly increased up to 10 days of storage ($p<0.05$). The differences in pH changes of all species might be due to the differences in buffering capacity of muscle, which was presumably greater in lizardfish. The increase in pH was postulated to be due to an increase in volatile bases produced by either endogenous or microbial enzymes. Benjakul *et al.* (2002) reported that the decomposition of nitrogenous compounds caused an increase in pH in fish flesh. The increase in pH ranging from 0.1 to 0.2 units represented the first quality muscle with the acceptable quality and the increase higher than 0.2 units indicated the deteriorated samples (Riaz and Quadri, 1985). Rahman *et al.* (2001) and Benjakul *et al.* (2002) reported that the pH value of shrimp (*P. monodon*) and bigeye snapper (*P. tayenus*) varied from 6.63 to 7.95 and from 6.6 to 6.7, respectively, during 10 days of iced storage. Changes in pH might be different, depending on a variety of factors such as species, fishing ground, feeding, storage temperature and buffering capacity of meat (Pacheco-Aguilar *et al.*, 2000). Changes in pH also depended on the liberation of inorganic phosphate and ammonia due to the enzymatic degradation of ATP (Sikorski *et al.*, 1990b). From the result, the similar initial pH was obtained between white shrimp and bigeye snapper. However, white shrimp muscle had a higher increase in pH than did bigeye snapper and lizardfish muscle during the extended storage. Apart from the different buffering capacity, the activity of enzymes converting glycogen into lactic acid might be different among different species. Lactic acid, generated in anoxic conditions from glycogen, is the principal factor in lowering the post mortem pH in the fish muscles (Sikorski *et al.*, 1990b).

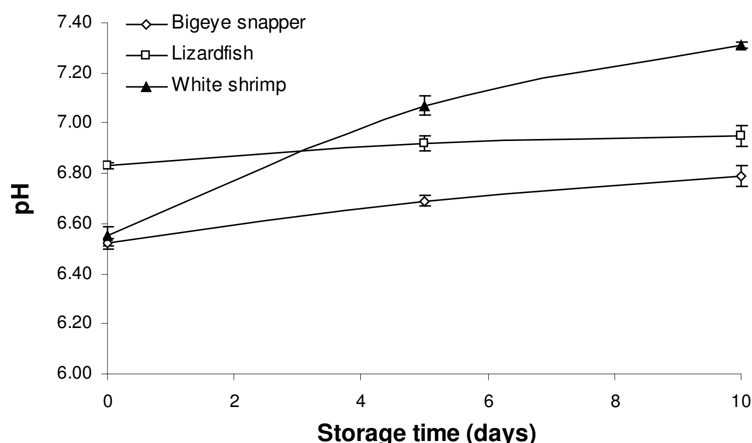

Figure 11 Changes in pH of some marine fish and shrimp during the iced storage. Bars represent the standard deviation from triplicate determinations.

3.1.2 Changes in TVB-N and TMA-N contents

TVB-N and TMA-N contents of all species were monitored during iced storage as depicted in Figure 12A and 12B, respectively. At day 0 of storage, the initial TVB-N content of white shrimp, bigeye snapper and lizardfish muscle were 10.72, 10.15 and 10.77 mg N/100 g, respectively. However, a much lower content of TMA-N was found at day 0. When storage time increased, both TVB-N and TMA-N contents increased ($p<0.05$). Riebroy *et al*. (2006) and Benjakul *et al*. (2003c) reported that the initial value of TVB-N in bigeye snapper and lizardfish samples were 5.53 and 10.01 mg N/100 g, respectively. The initial TVB-N of black tiger shrimp storage in ice was 5.88 mg N/100 g sample (Rahman *et al.,* 2001). The differences in TVB-N might be due to the varying handling process or storage condition prior to analysis. TVB-N content in albacore was reported to increase with increasing storage time (Perez-Villareal and Pozo, 1990). The presence of TVB-N and TMA-N content indicated that some changes in the nitrogenous compounds occurred prior to iced storage. The formation of TVB-N is generally associated with the growth of microorganisms and can be used as an indicator of spoilage (Benjakul *et al.,* 2002). From the result, TVB-N content of all species increased slightly during the first 5 days of iced storage ($p<0.05$). Thereafter, a sharp increase was found up to 10 days ($p<0.05$). Production of TMA-N in muscle during cold storage could be used as an indicator of bacterial activity (Gokodlu *et al.,*

1998). Generally, TVB-N comprises mainly TMA-N and ammonia, which are produced by both microbial and endogenous enzymes. A number of specific spoilage bacteria such as *Shewanella putrefaciens, Photobacterium phosphoreum* and *Vibrionaceae*, typically use TMAO as an electron acceptor in anaerobic respiration, resulting in off-odor and off-flavor due to the formation of TMA-N (Gram and Huss, 1996; Huss, 1995). Therefore, the formation of TVB-N and TMA-N in fish muscle has been used as the indication of fish spoilage (Ryder *et al*., 1984). TVB-N and TMA-N formed in fish during iced storage were probably mediated by psychotropic bacteria (Sasajima, 1973). TVB-N content of 30 mg N/100 g is generally regarded as the fish acceptability limit (Sikorski *et al*, 1990b). TVB-N of 30 mg N/100g and TMA-N of 5 mg N /100g were reported as the limit in shrimp (*Penaeus monodon*) (Cobb *et al.,* 1973). However, TVB-N content was not a good index of quality of black-skipjack during storage (Mazorra-Manzano, 2000). TVB-N of 25 mg N/100 g was suggested to be a limit level for sardine (Marrakchi *et al*., 1990), while a TVB-N value of 30-40 mg N/100 g was reported to be the limit for acceptability of cod and temperate water fish (Connell, 1975). Additionally, Bennour *et al* (1991) found a TMA-N of 5 mg/ 100 g as the rejection value for mackerel. From the result, the formation of TMA was coincidental with the occurrence of TVB, especially when the storage time increased. The increases in both TVB and TMA were also in accordance with the increases in pH (Figure 11).

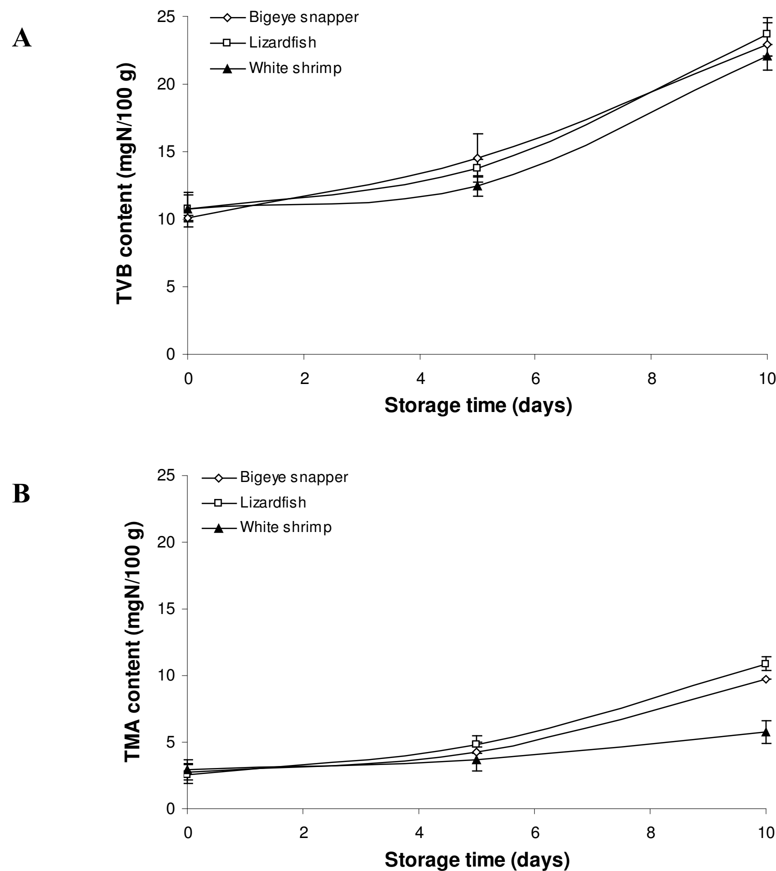

Figure 12 Changes in TVB-N (A) and TMA-N (B) contents of some marine fish and shrimp during the iced storage. Bars represent the standard deviation from triplicate determinations.

3.1.3 Changes in TBARS

Changes in TBARS value of meat from all species during iced storage are shown in Figure 13. The initial values of TBARS of white shrimp, bigeye snapper and lizardfish were 0.38, 0.32 and 0.35 mg malonaldehyde/kg meat, respectively. It was suggested that lipid oxidation occurred during post-mortem handling to some extent. TBARS of muscle increased slightly during storage, indicating that lipid oxidation occurred during the extended iced storage. TBARS is the most used indicator for advanced lipid oxidation (Nishimoto *et al.*, 1985). TBARS has been used to measure the concentration of relatively polar secondary reaction products, especially aldehydes (Nawar, 1996). The increase in

TBARS indicated the formation of secondary lipid oxidation products (Kolakowska, 2002). Fish muscle typically has a high content of polyunsaturated fatty acids and is consequently prone to oxidative reaction (Harris and Tall, 1994; Stamman *et al*., 1990). TBARS has been widely used to indicate lipid oxidation in meat and meat products (Jo and Anh, 2000). The lipid oxidation can be initiated and accelerated by various mechanisms including the production of singlet oxygen, enzymatic and non-enzymatic generation of free radicals and active oxygen (Kubow, 1992). From the result, the storage time in ice was an important factor determining the lipid oxidation in fish. At the same iced storage time, no marked differences in TBARS were found among species. All species had TBARS value not more than 0.5 mg malonaldehyde/kg sample during iced storage (10 days). Nishimoto *et al.* (1987) proposed the value of 3.0 mg malonaldehyde/kg sample for good quality fish. The result suggested that low temperature caused by icing could retard the lipid oxidation in all species effectively.

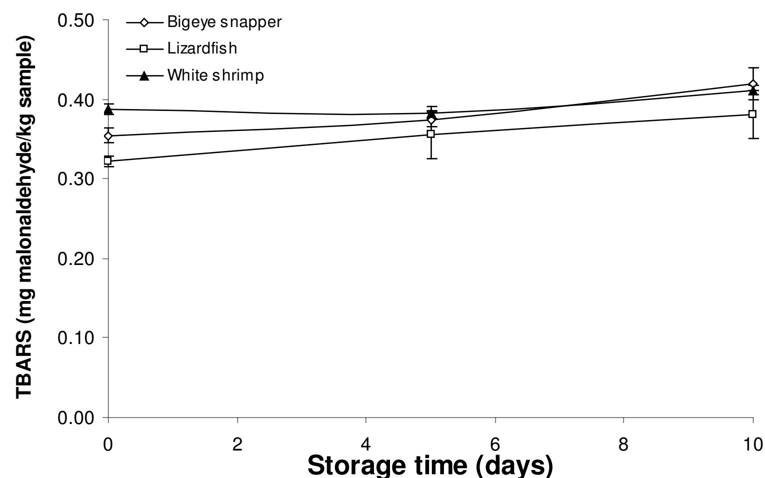

Figure 13 Changes in TBARS of some marine fish and shellfish during the ice storage. Bars represent the standard deviation from triplicate determinations.

3.1.4 Changes in formaldehyde content

Formaldehyde content of lizardfish increased continuously as the storage time increased ($p<0.05$) (Figure 14). Slight increases in formaldehyde contents were found in white shrimp and bigeye snapper during iced storage up to 10 days ($p>0.05$). For lizardfish, formaldehyde content increased markedly during iced storage ($p<0.05$). This result confirmed the work carried out by Benjakul *et al*. (2002) who reported the increase in formaldehyde in lizardfish during the extended iced storage. Formaldehyde is formed by demethylation of trimethylamine oxide (TMAO), a compound present in most marine species. Formaldehyde is able to react with protein, resulting in the conformational changes and insolubilization. The formaldehyde is known as an effective cross-linker via methylene bridge (Sikorski *et al*., 1990b). Trimethylamine-*N*-oxide demethylase (TMAOase) is capable of catalyzing the conversion of TMAO to dimethylamine (DMA) and formaldehyde (Gill and Paulson, 1982). This enzyme is concentrated in the internal organs and red muscle (Gill and Paulson, 1982; Rehbein and Schreiber, 1984; Benjakul *et al*., 2003a). This phenomenon is common for gadoid species (Hebard *et al*., 1982) and occurs during frozen storage, causing an alteration in the characteristics of fish muscle protein, particularly changes in functional properties (Careche *et al*., 1998). Formaldehyde accelerated the formation of high-molecular weight polymer in cod myosin (Ang *et al*., 1989; Careche and Li-Chan, 1997). Thus, formaldehyde might induce the conformational changes of myosin, especially the globular head region, leading to the decrease in Ca^{2+}-ATPase activity (Benjakul *et al*., 2002). The formation of formaldehyde was evident in lizardfish, suggesting that lizardfish had high contents of both TMAO and TMAOase (Benjakul et al., 2004a). Harada (1975) reported the enzymatic formation of DMA and formaldehyde in the muscle, viscera and liver. Among those samples, liver of lizardfish (*S. tumbil*) had the greatest ability to produce formaldehyde (Harada, 1975). Moreover, Dingle and Hines (1975) found that kidney tissue was responsible for the formation of formaldehyde in mince of gadoid fish. Recently, Benjakul et al. (2003a) partially purified TMAOase from the kidney of lizardfish with MW of 128 kDA. From the result, the lower formaldehyde contents in white shrimp and bigeye snapper might result from the lower TMAO as well as the lower TMAOase activity. Therefore, the formation of formaldehyde in lizardfish stored in ice can be impeded by removal of viscera and head.

However, formaldehyde was still formed at a lower rate in the muscle. Kimura et al. (2000) found that TMAOase in walleye pollack myofibrillar fraction caused the reduction of TMAO to DMA and formaldehyde. Thus, heading and eviscerating may retain the functionality of muscle protein, especially the gelation property of formaldehyde forming species.

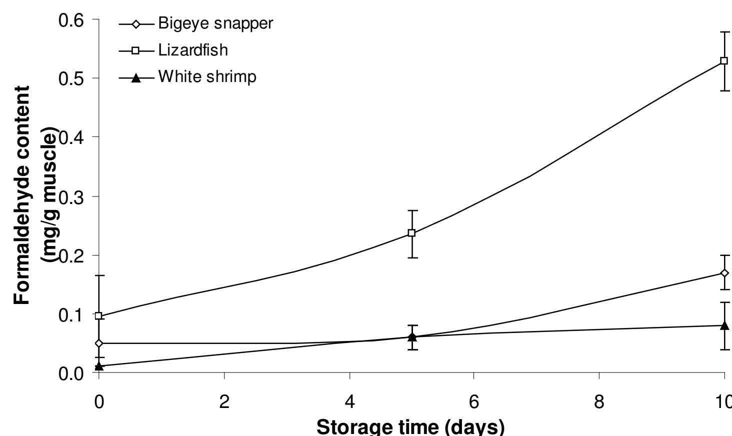

Figure 14 Changes in formaldehyde content of some marine fish and shellfish during the iced storage. Bars represent the standard deviation from triplicate determinations.

3.1.5 Changes in TCA-soluble peptide content

The TCA-soluble peptide content in muscle of all species increased slightly during iced storage ($p<0.05$), suggesting the occurrence of autolytic degradation of muscle proteins (Figure 15). At day 0, TCA-soluble peptide content in white shrimp, bigeye snapper and lizardfish muscle were 0.61, 0.75 and 0.97 μmol tyrosine/g sample, respectively. The TCA-soluble peptides detected at day 0 indicated the endogenous oligopeptides and free amino acids as well as degradation products accumulated during post-harvest handling (Benjakul *et al*., 1997). Varying TCA-soluble peptide contents among species were possibly due to the differences in susceptibility of proteins to hydrolysis. Furthermore, the proteases in the different muscles might belong to different groups having different hydrolytic property. The proteases have been recognized to hydrolyze the muscle during postmortem storage (Venugopal *et al*., 1983). At the beginning of storage in ice, endogenous enzymes are mainly involved in the gradual loss of fish freshness. Thereafter, bacterial enzymes predominate and lead to final spoilage (Pacheco-Aguilar *et al.,* 2000).

According to An *et al* (1994), the activity of cathepsin L was insignificant at 0–5 °C, whereas cathepsin B exhibited half of its maximal activity and cathepsin H retained about one-fifth of its maximal activity. In addition, microbial protease might also be a potential source of proteolytic degradation during iced storage. Protease from *Pseudomonas marinoglutinosa* was reported to hydrolyze actomyosin at 0-2 °C and the optimal pH was above 7.0 (Venugopal *et al*., 1983).

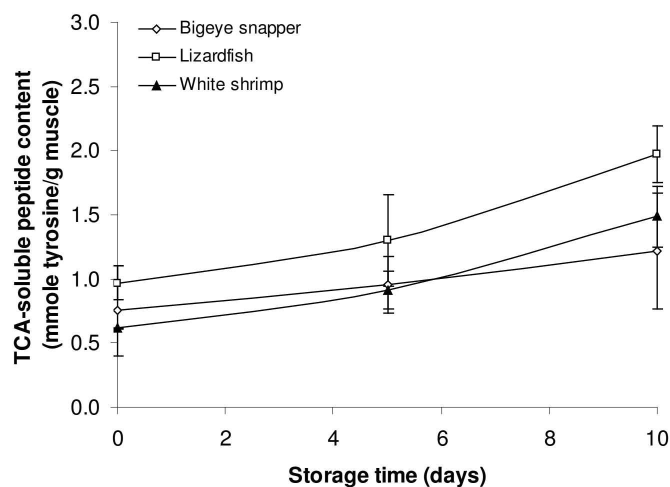

Figure 15 Changes in TCA-soluble peptide content of some marine fish and shellfish during the iced storage. Bars represent the standard deviation from triplicate determinations.

3.1.6 Changes in protein patterns

Protein patterns of muscle of all species kept in ice are shown in Figure 16. A decrease in MHC band intensity was observed in all species during iced storage of 10 days. This result corresponded with an increase in TCA-soluble peptide content during extended storage (Figure 15). Surprisingly, MHC of lizardfish meat tended to be degraded to a lower extent, particularly at day 10. It was suggested that cross-linked proteins induced by formaldehyde might be more resistant to proteolysis. As a result, MHC was more retained in this species. The result was in accordance with that reported by Benjakul *et al*., (2003c) who found the continuous decrease in MHC band intensity of lizardfish during ice storage. The differences might be caused by the different endogenous proteolytic activity governed by

varying reason, feeding period, etc. SDS-PAGE patterns revealed that MHC was much more susceptible to hydrolysis than was actin. However, actin of white shrimp was also degraded when the storage time increased. The result suggested the different susceptibility of actin to hydrolysis among different species. Proteolytic degradation of other cytosolic and cytoskeletal proteins present in the muscle caused by microbial growth and structural disintegration also occurred during ice storage of two species of bigeye snapper (Benjakul *et al.,* 2002). This result was in agreement with Benjakul *et al* (1997) who reported that MHC was more prone to proteolytic degradation than other muscle proteins including actin, troponin, and tropomyosin.

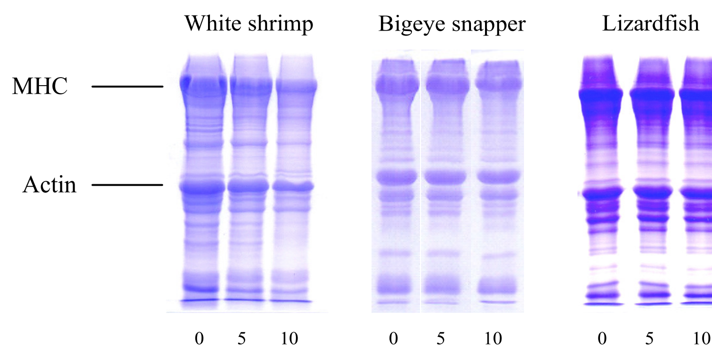

Figure 16 SDS-PAGE patterns of muscle proteins from some marine fish and shrimp during the iced storage. Numbers designate the storage time (days).

3.1.7 Changes in ATPase activity

The changes in Ca^{2+}-ATPase activity of natural actomyosin extracted during iced storage are shown in Figure 17. A slight decrease in Ca^{2+}-ATPase activity of natural actomyosin from all species was observed throughout the iced storage ($p<0.05$). Ca^{2+}-ATPase activity is considered to be a good indicator of myosin functionality in the actomyosin complex (Roura *et al*., 1990) and integrity of the myosin molecule (Roura and Crupkin, 1995). The loss in Ca^{2+}-ATPase was possibly associated with the proteolysis of myosin molecule (Ouali and Valin, 1981). Ca^{2+}-ATPase of ordinary muscle of sardine, during 6 days of iced storage, decreased to 50% with the concomitant degradation of MHC due to proteolysis (Seki *et al*., 1980). Moreover, denaturation of myosin was possibly caused by the

oxidation of SH groups or disulfide interchanges as well as changes in surface hydrophobicity during iced storage (Benjakul *et al*., 1997).

No changes in either Mg^{2+}-ATPase or Mg^{2+}-Ca^{2+}-ATPase activities of natual actomyosin from all species were found (Figure 17B, 17C). The result suggested no changes in actin-myosin complexes in the presence of both endogenous and exogenous calcium ions (Benjakul *et al*., 1997). Mg^{2+}-ATPase activity was found in the sarcoplasmic recticulum, near both ends of A band, mitochondria, and nuclease membrane (Tachibana *et al*., 1993). Mg^{2+}-ATPase activity functions through regulatory proteins such as troponin and tropomyosin, thus including muscle contraction between actin and myosin in the presence of endogenous Ca^{2+}ion (Roura *et al*., 1990). Roura and Crupkin (1995) reported that this enzyme activity was low in myofibrils from prespawned hake.

Mg^{2+}-EGTA-ATPase activity of natural actomyosin of all species is shown in Figure 17D. The activity increased significantly during storage ($p<0.05$). This result was in accordance with Benjakul *et al*. (1997) who reported the increase in Mg^{2+}-EGTA-ATPase activity of Pacific whiting myofibrillar proteins during 8 days of iced storage. The change in Mg^{2+}-EGTA-ATPase activity indicates the change of troponin-tropomyosin complex (Ebashi and Endo, 1968; Watabe *et al*., 1989). Additionally, proteinase in muscle could play a partial role in these changes. Mg^{2+}-EGTA-ATPase activity of myofibrils was reported to increase by treatment with lysosomal protease (Ouali and Valin, 1981). The changes in troponin C, a calcium binding protein, were possibly associated with the loss of Ca^{2+} sensitivity (Ebashi *et al*., 1986). Among all species, Mg^{2+}-EGTA-ATPase of natural actomyosin from white shrimp increased to a highest extent, compared to those of other two fish.

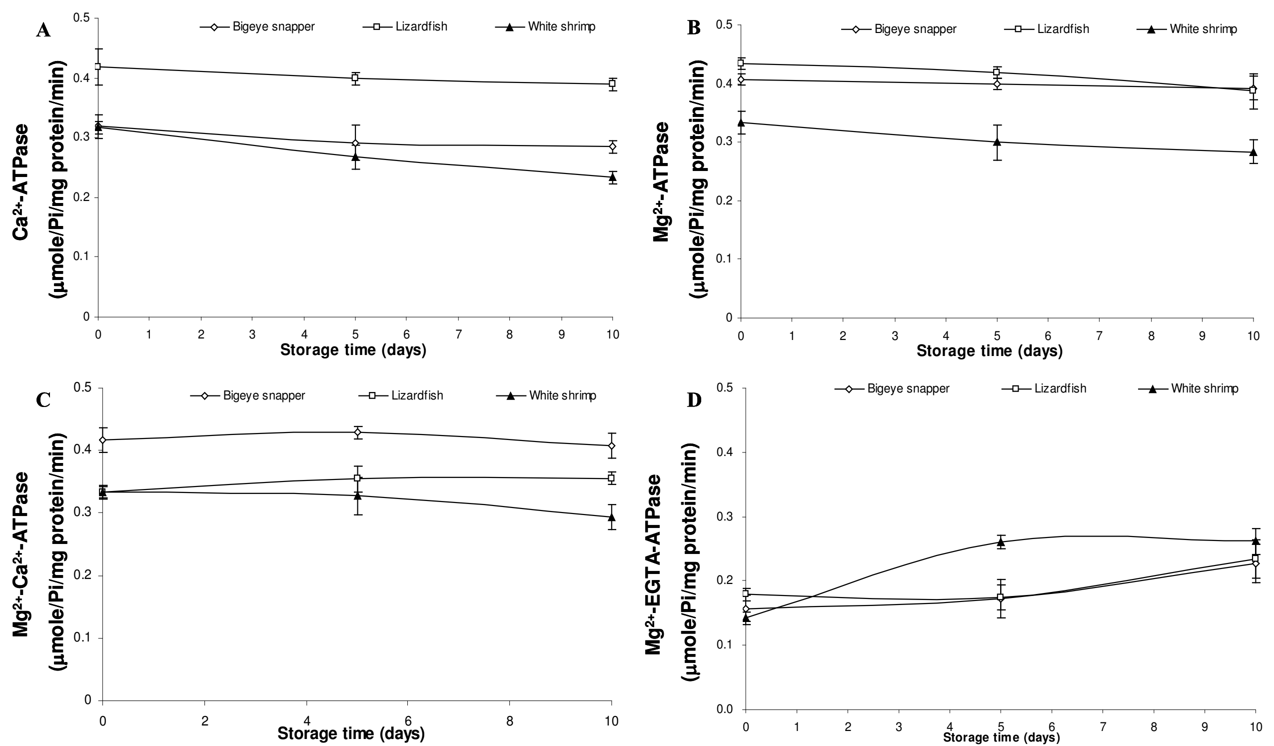

Figure 17 ATPase activities of natural actomyosin of some marine fish amd shrimp during the iced storage. Ca^{2+}-ATPase (A), Mg^{2+}-ATPase (B), Mg^{2+}-Ca^{2+}ATPase(C) and Mg^{2+}-EGTA ATPase (D). One unit activity was defined as that releasing 1 µmol Pi/mg protein/min. Bars represent the standard deviation from triplicate determinations.

Ca^{2+}-sensitivity of natural actomyosin sharply decreased with storage time ($p<0.05$) (Figure 18). Ca^{2+}-sensitivity was reported to be a good indictor of Ca^{2+}-regulation of myofibrillar proteins (Roura and Crupkin, 1995). The Ca^{2+}-sensitivity of the myofibrillar proteins was attributed to the activity of native tropomyosin (Ebashi *et al*., 1986). The decrease in Ca^{2+}-binding and Ca^{2+}-sensitivity was shown to be caused by proteolysis (Tokiwa and Matsumiya, 1969). Benjakul *et al*. (1997) postulated that the decrease in Ca^{2+}-sensitivity in Pacific whiting actomyosin during iced storage was caused by proteolysis. Therefore, the decrease in Ca^{2+}-sensitivity in all fish species probably resulted from the denaturation or degradation of troponin, especially troponin C. From the result, the sharp decrease in Ca^{2+}-sensitivity was noticeable in white shrimp, which was concomitant with the highest increase

in Mg^{2+}-EGTA ATPase activity during the prolonged storage. Therefore, the changes in troponin-tropomyosin complex were more pronounced in white shrimp, compared to those found in bigeye snapper and lizardfish.

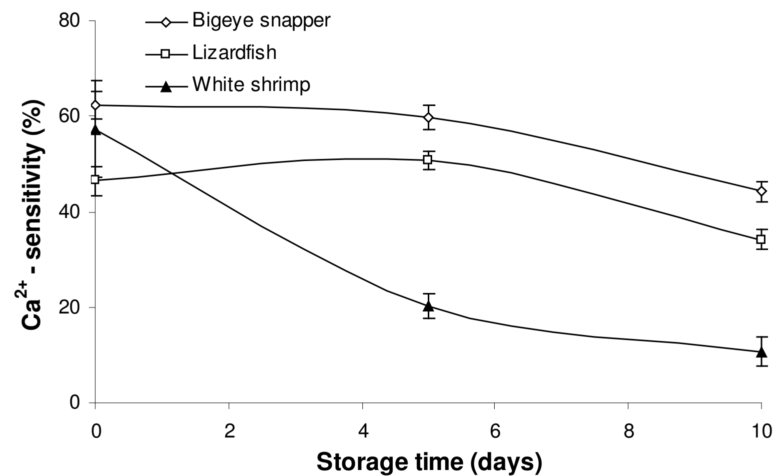

Figure 18 Ca^{2+}-sensitivity of natural actomyosin extracted from some marine fish and shrimp during the iced storage. One unit activity was defined as that releasing 1 μmol Pi/mg protein/min. Bars represent the standard deviation from triplicate determinations.

3.2 Effect of MTGase on gel-forming ability of some marine fish and shrimp stored in ice for different times

3.2.1 Breaking force and deformation

Breaking force and deformation of gels prepared from all species stored in ice for different times are shown in Figure 19. Breaking force of mince gels from all species decreased with increasing iced storage time, while the deformation increased ($p <0.05$). Without MTGase addition, breaking force of mince gel from lizardfish set at 25°C for 2 h and 40°C for 30 min decreased by 60.26 and 63.93% after 10 days of storage, respectively, compared to that of gel prepared at day 0. After 10 days of iced storage, breaking force of mince gel from bigeye snapper and white shrimp decreased by 50.29-59.42 and 54.46-57.73% when the gels were set at 25°C for 2 h and 40°C for 30 min, respectively. Generally, gels with prior setting at 40°C for 30 min had the higher breaking force and deformation than those with prior setting at 25°C for 2 h for bigeye snapper and lizardfish mince. Nevertheless, white shrimp gels had the hightest force and deformation when prior setting at 25°C for 2 h was used. With the extended storage time, the rate of decrease in breaking force varied with species. The differences in gel-forming ability of ice stored samples were postulated to be due to the differences in protein denaturation and degradation during storage. Among all samples, lizardfish showed the highest rate of decrease in breaking force. It was presumed that formaldehyde formed in lizardfish was a major contributor to protein denaturation or aggregation, leading to the poorer gel-forming ability. This result was in accordance with the marked decrease in Ca^{2+}-ATPase activity (Figure 18). Integrity of myosin is prerequisite for gelation of muscle protein (Benjakul *et al*., 2002). The continuous decrease in breaking force still occurred as storage time increased up to 10 days. This was presumed to be due to the increased denaturation of protein, as shown by the continuous decrease in Ca^{2+}-ATPase activity. The result was in agreement with Kurokawa (1979) who reported that gel strength of kamaboko made from lizardfish stored for 3 days in ice was less than 50% of that made from fresh fish. MacDonald *et al*. (1992) found that gel-forming ability of hoki muscle decreased with concomitant increase in formaldehyde as the storage time increased. Therefore, formaldehyde was crucial for protein danaturation, resulting in a lower gel forming ability (Careche and Li-Chan.,1997). The addition of formaldehyde to hake

natural actomyosin caused a reduction in gel strength (Careche *et al*., 1998). Apart from formaldehyde formed, the aggregation of protein via disulfide bond, hydrophobic interaction, etc. as well as lipid oxidation during storage also caused the denaturation of protein (Sikorski et al., 1976; Matsumoto, 1980). This led to the lower gel forming ability. The decreasing rate of gel-forming ability during ice storage could be also determined by stability of muscle protein, especially myosin. From the result, the decreasing rate of breaking force was smaller in bigeye snapper than other species. Apart from the denaturation of myosin, the loss in TGase activity induce by iced storage was also presumed. TGase has been known to induce the non-disulfide covalent bond formation, especially ε-(γ-glutamyl)lysine) linkage (Kumazawa *et al*., 1995). Accordingly, the setting was possibly hampered as a result of extended iced storage. Endogenous TGase might be inactivated by proteinase and formaldehyde formed in samples during iced storage, leading to the lowered TGase activity. The decrease in gel-forming ability of gel was concomitant with the increase in formaldehyde, TCA-soluble peptides, as well as the decrease in myosin heavy chain content. Myosin integrity is of paramount importance for gelation (An *et al*., 1996). The degradation of myosin resulted in an inferior gel network formation, causing a lower elasticity with poor water-holding capacity in gel matrix.

Effect of MTGase addition (0.6 units/g) on gel-forming ability of all species stored for different times was investigated (Figure 19). Under the same setting condition, breaking force increased but deformation decreased as MTGase was added ($p<0.05$). At day 0, breaking force of mince gel from white shrimp, bigeye snapper and lizardfish increased by 11.34, 21.30 and 13.71 %, respectively, when setting was performed at 25°C for 2 h and 5.82, 29.53 and 11.28 %, respectively, as setting was carried out at 40°C for 30 min, compared with the control (gel without MTGase). MTGase is known to catalyze the formation of ε-(γ-glutamyl)lysine) cross links, which is covalent and important for gel-forming and viscoelastic properties (Sakamoto *et al*., 1995; Seguro *et al*., 1995). Excessive formation of ε-(γ-glutamyl)lysine dipeptide would inhibit uniform development of the heat-induced protein network and the improvement of breaking strength or deformation or gel strength (Sakamoto et al., 1995). This behaviour might occur because an increment of protein-protein interactions decreases the water–protein interactions and consequently results

in a decrease in deformation. The effect of MTGase on gel varies with fish speices (Asagami *et al*., 1995). From the result, the addition of MTGase at levels 0.6 units/g was found to affect gelling properties of fish and shrimp stored in ice for an extended time differently. For white shrimp, the increase in breaking force and deformation of gels added with MTGase was presumably caused by the increased cross-linking induced by MTGase. After 5 days of iced storage, MTGase addition did not cause the increase in breaking force of lizardfish mince gel. Moreover, MTGase had no impact on gel enhancement of bigeye snapper and lizardfish stored in ice for 10 days. Therefore, the conformation of proteins might be altered and those proteins could not be cross-linked induced by MTGase. Some factors such as freshness, protein quality, and harvesting season were found to determine the cross-linking induced by MTGase (Asagami *et al*., 1995). For deformation, it was increased when fish and shrimp were stored in ice for 5 and 10 days. However, MTGase addition resulted in the decreases in deformation of resulting gel, regardless of storage time. The decrease in deformation suggested the lowered elasticity of gel. Non-disulfide covalent bond formed mainly contributed to the rigidity of gel matrix, which was associated with the loss in elasticity.

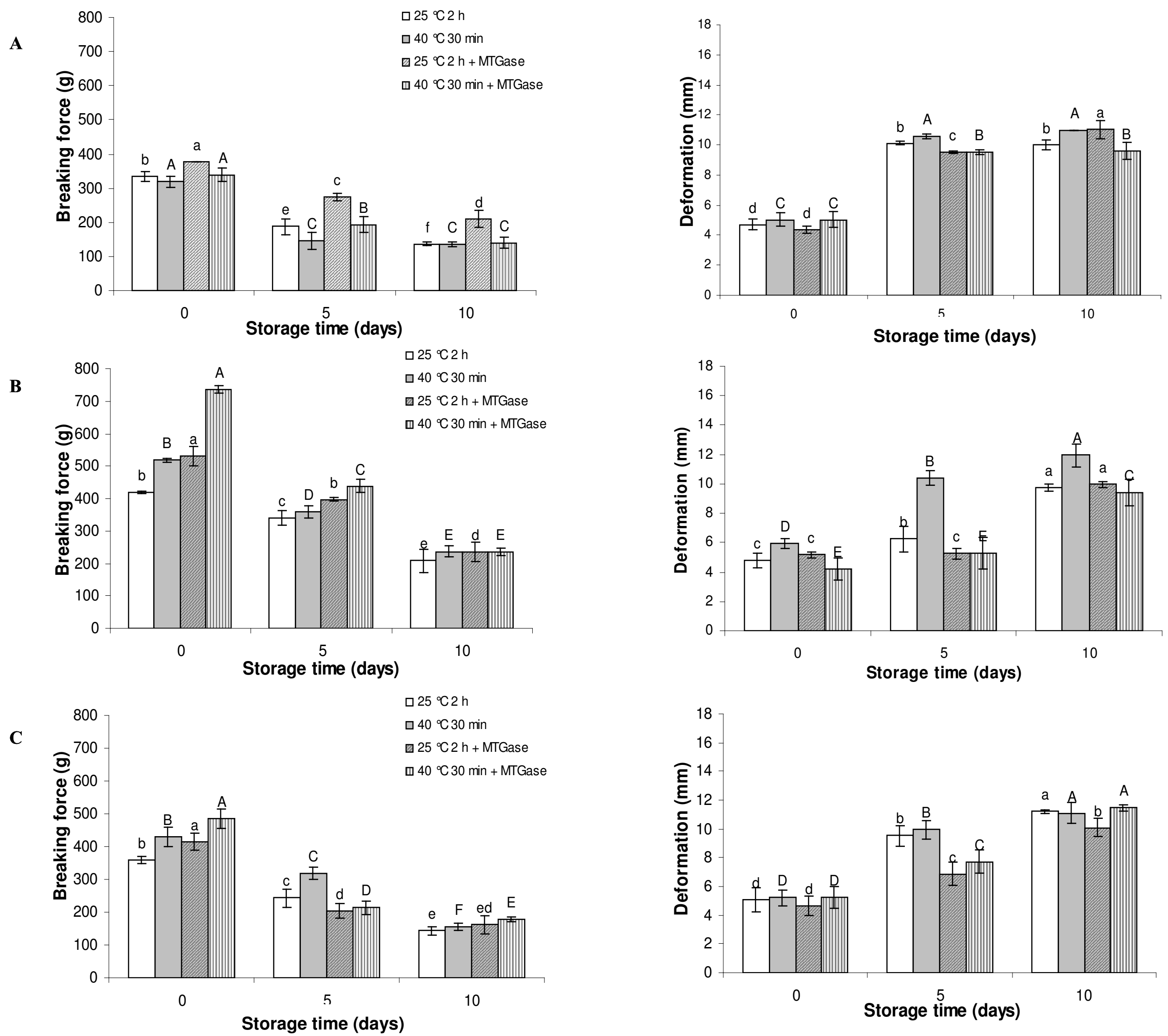

Figure 19 Breaking force and deformation of mince gels from some marine fish and shrimp stored in ice for different times without and with MTGase addition (0.6 units/g) and set under different conditions. A: white shrimp, B: bigeye snapper and C: lizardfish. Bars indicated the standard deviation from five determinations. Different letters within the same setting condition indicate significant differences ($p < 0.05$).

3.2.2 Color and whiteness

Color and whiteness of gels from all species stored in ice for different times are shown in Table 13, 14 and 15. L*-value and whiteness generally decreased as the storage time increased. Without MTGase addition, mince gel from bigeye snapper at day 0 rendered the highest L*-value and whiteness (78.51), compared to other species (62.38-76.20) at the same storage time. In general, the whiteness of gels set at 40°C for 30 min was greater than that of gels with setting at 25°C for 2 h. This was probably due to the fact that higher temperature caused the denaturation of protein, especially pigments remained in the muscle, leading to more turbidity as shown by higher whiteness. Park (1994) found that colors and whiteness of surimi gels were related to species, compositional and physical conditions during preparation and measurements. Decrease in whiteness was also probably due to the oxidation of pigment proteins, especially myoglobin and hemoglobin. Additionally, lipid oxidation in muscle during ice storage might induce cross-linking of pigment proteins and muscle proteins via the free radical process (Saeed *et al*., 1999). The oxidation product including aldehydes might undergo Maillard reaction, leading to the browner color of meat. During extended storage, blood and liquid from internal organs could penetrate into the muscle, especially when autolysis proceeded and caused a looser muscle structure. Surimi produced from fresh fish rendered the higher whiteness than that prepared from fish stored in ice for 10 days (Benjakul *et al*., 2003c). This was probably due to the extensive oxidation of myoglobin or hemoglobin during the extended storage, resulting in the more adduction of those pigments to the muscle proteins (Saeed *et al*., 1999). From the result, L*-value and whiteness gels increased with the addition of MTGase at a level of 0.6 units/g, particularly as storage time increased. The addition of MTGase had the profound effect on whiteness of surimi from all species. As discussed previously, maltodextrin used as MTGase stabilizer possibly exhibited the light scattering effect, resulting in the greater whiteness of surimi gels.

Table 13 L*, a*, b*-values and whiteness of mince gels from white shrimp stored in ice for different times without and with MTGase addition (0.6 units/g) and set under different conditions

Storage time (days)	Setting at 25°C for 2 h				Setting at 40°C for 30 min			
	L*	a*	b*	Whiteness	L*	a*	b*	Whiteness
Without MTGase								
0	71.32 ± 0.32a	19.24 ± 0.18a	15.72 ± 0.23c	62.06 ± 0.43b	71.97 ± 0.25a	19.38 ± 0.22a	15.93 ± 0.37b	62.38 ± 0.51b
5	67.80 ± 0.25b	17.51 ± 0.06b	15.56 ± 0.60c	60.18 ± 0.31c	70.75 ± 0.39b	18.47 ± 0.33b	17.30 ± 0.34a	61.32 ± 0.51c
10	66.23 ± 0.63b	19.31 ± 0.29a	18.41 ± 1.01a	56.96 ± 1.27e	67.99 ± 0.12c	18.10 ± 0.14b	16.26 ± 0.08b	59.80 ± 0.07d
MTGase 0.6 units/g								
0	71.76 ± 0.99a	17.42 ± 0.48b	14.19 ± 0.11d	63.91 ± 1.07a	71.42 ± 0.44a	16.41 ± 0.22c	13.73 ± 0.65c	64.30 ± 0.25a
5	71.44 ± 0.04a	18.62 ± 0.07b	16.38 ± 0.05b	62.17 ± 0.07b	71.80 ± 0.47a	19.14 ± 0.20a	16.20 ± 0.02b	62.26 ± 0.38b
10	71.42 ± 0.44a	16.41 ± 0.22c	13.73 ± 0.65d	59.11 ± 0.88d	67.85 ± 0.21c	18.75 ± 0.28b	17.71 ± 0.25a	58.79 ± 0.37e

Values are given as means ± SD from five determinations.

Different letters within the same column indicate the significant differences ($p<0.05$).

Table 14 L*, a*, b*-values and whiteness of mince gels from bigeye snapper stored in ice for different times without and with MTGase addition (0.6 units/g) and set under different conditions

Storage time (days)	Setting at 25°C for 2 h				Setting at 40°C for 30 min			
	L*	a*	b*	Whiteness	L*	a*	b*	Whiteness
Without MTGase								
0	80.14 ± 0.51b	-2.31 ± 0.07b	12.74 ± 0.01b	76.29 ± 0.53b	81.91 ± 0.41a	-2.28 ± 0.09b	11.37 ± 0.19b	78.51 ± 0.52a
5	74.22 ± 1.21e	-1.93 ± 0.11a	10.67 ± 0.17d	72.03 ± 1.41d	79.02 ± 0.72b	-1.93 ± 0.09a	13.71 ± 0.21a	74.86 ± 0.67b
10	73.18 ± 1.10f	-2.79 ± 0.09c	12.55 ± 1.17b	70.23 ± 1.24f	76.26 ± 0.59c	-2.55 ± 0.08b	13.14 ± 0.34a	72.74 ± 0.82c
MTGase 0.6 units/g								
0	82.09 ± 0.41a	-2.22 ± 0.34b	12.23 ± 0.33b	78.20 ± 0.59a	81.93 ± 0.58a	-2.24 ± 0.09b	11.28 ± 0.51b	78.59 ± 0.91a
5	77.29 ± 0.45c	-2.81 ± 0.08c	11.55 ± 0.22c	74.36 ± 0.61c	81.18 ± 0.13a	-1.77 ± 0.01a	11.97 ± 0.15b	78.46 ± 0.19a
10	75.26 ± 0.53d	-2.29 ± 0.17b	14.31 ± 0.35a	71.64 ± 0.72e	76.63 ± 0.10c	-2.80 ± 0.01b	11.79 ± 0.30b	73.68 ± 0.26b

Values are given as means ± SD from five determinations.

Different letters within the same column indicate the significant differences ($p<0.05$).

Table 15 L*, a*, b*-values and whiteness of mince gels from lizardfish meat stored in ice for different times without and with MTGase addition (0.6 units/g) and set under different conditions

Storage time (days)	Setting at 25°C for 2 h				Setting at 40°C for 30 min			
	L*	a*	b*	Whiteness	L*	a*	b*	Whiteness
Without MTGase								
0	74.13 ± 0.15b	-3.32 ± 0.37b	9.34 ± 0.05a	72.29 ± 0.16b	78.47 ± 0.23a	-2.16 ± 0.09b	9.90 ± 0.20a	76.20 ± 0.26a
5	73.48 ± 0.57c	-2.80 ± 0.07a	9.66 ± 0.63a	71.64 ± 0.91c	73.50 ± 0.58d	-2.80 ± 0.07b	9.66 ± 0.63a	71.66 ± 0.93d
10	68.28 ± 0.22d	-3.33 ± 0.06b	8.87 ± 0.29b	66.89 ± 0.31d	73.10 ± 0.35d	-3.29 ± 0.02c	8.10 ± 0.24c	71.71 ± 0.48d
MTGase 0.6 units/g								
0	78.48 ± 0.23a	-2.16 ± 0.09a	9.90 ± 0.20a	76.21 ± 0.18a	78.75 ± 0.39a	-2.14 ± 0.08b	10.13 ± 0.03a	76.36 ± 0.41a
5	73.41 ± 0.27c	-3.85 ± 0.07b	9.21 ± 0.09a	71.60 ± 0.33c	76.11 ± 0.52b	-1.66 ± 0.14a	9.28 ± 0.16b	74.32 ± 0.66b
10	73.56 ± 0.41c	-3.36 ± 0.10b	9.71 ± 0.21a	71.63 ± 0.55c	74.52 ± 0.41c	-2.85 ± 0.07b	10.21 ± 0.09a	72.41 ± 0.45c

Values are given as means ± SD from five determinations.

Different letters within the same column indicate the significant differences ($p<0.05$).

3.2.3 Expressible moisture content

The expressible moisture content in mince gels from all species stored in ice for various times is shown in Figure 20. Expressible moisture content of gels increased as the storage time increased ($p<0.05$). This indicated that less water was imbibed in the gel matrix. Denatured protein induced by extended ice storage had the low affinity for water. It was found that the degree of increase in expressible drip varied with species. After 10 days of iced storage, the expressible moisture contents of lizardfish mince gel with prior setting at 25°C for 2 h and at 40°C for 30 min increased by 84.20 and 85.54%, respectively, compared with that found at day 0. Among all samples, mince gel from lizardfish showed the highest expressible drip as the storage time increased ($p<0.05$). The expressible moisture content of gel from other species ranged from 41.89 to 66.28% when samples stored in ice for 10 days were used. The poor water holding capacity of gels from lizardfish was associated with the increased formaldehyde content, which caused the toughening in fish muscle by inducing the cross-linking of proteins (Connell, 1975). Formaldehyde is known to induce the aggregation of protein in the fish muscle and the loss in water holding capacity (Ang and Hultin, 1989). Water holding capacity is directly correlated with the myofibrillar protein (Smith, 1991). Furthermore, the increased expressible moisture of gels from all species was closely associated with the poor gel matrix. From the result, it was noted that setting condition had the influence on the expressible drip of resulting gels. Gels prepared from white shrimp stored for 10 days in ice with prior setting at medium temperature showed the lower expressible than those with high temperature setting. However, gels prepared from 10 days ice stored bigeye snapper and lizardfish showed the higher expressible moisture content when setting at medium temperature was used. This might be associated with the increased degradation of gel matrix induced by proteinases. From the result, it was suggested that different proteinases might be present in different species. Proteinases with different optimal temperature possibly contributed to degradation to different degrees. The degraded polypeptide could not form the strong three-dimensional matrix, which can imbibe water effectively.

Expressible moisture content of mince gel added with MTGase at a level of 0.6 units/g and subjected to different setting conditions is shown in Figure 19. From the result, the decrease in expressible moisture content was observed with the addition of MTGase ($p<0.05$). Low expressible moisture content of the gels suggested the more water retained in the gel network (Niwa, 1992). Therefore, the addition of MTGase in combination with the appropriate setting could be used to improve gel-forming ability via non-disulfide covalent bonds. As a consequence, the stronger gel network formed could hold water to a higher extent as indicated by the lowered expressible moisture content. However, the expressible moisture content increased with the increasing ice storage time, although MTGase was added, particularly for lizardfish mince.

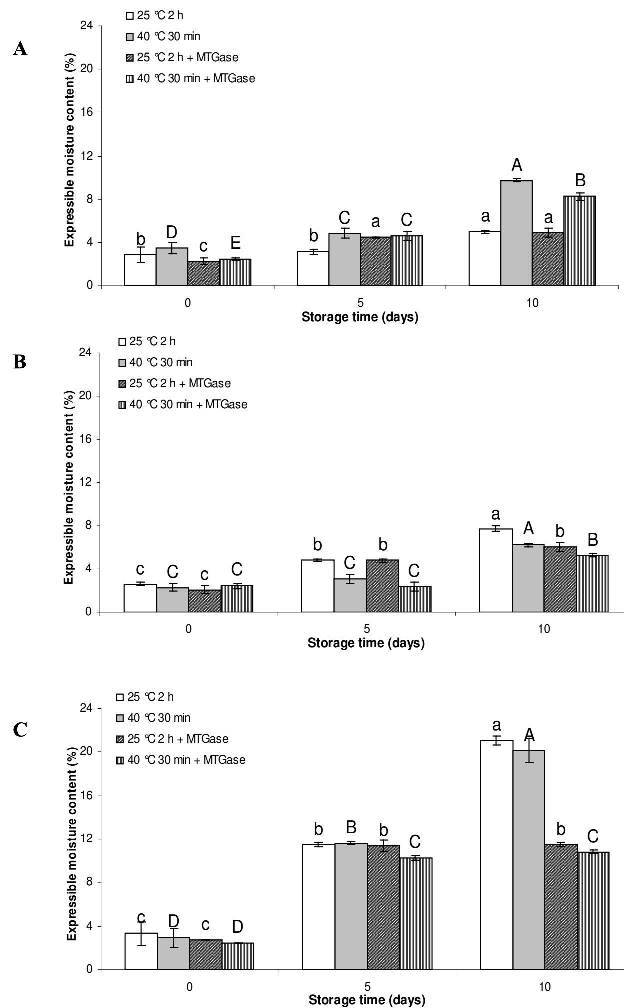

Figure 20 Expressible moisture content of mince gels from some marine fish and shrimp stored in ice for different times without and with MTGase addition (0.6 units/g) and set under different conditions. A: white shrimp, B: bigeye snapper and C: lizardfish. Bars indicated the standard deviation from five determinations. Different letters within the same setting condition indicate significant differences ($p<0.05$).

3.2.4 Protein patterns

SDS-PAGE patterns of mince gels from all fish species stored in ice for different times are shown in Figure21. In the absence of MTGase, band intensity of MHC of the gel decreased with an increasing storage time. This might be associated with the increase in degradation of muscle proteins during the prolonged storage. It was noted that gel from lizardfish mince had no MHC retained when the sample stored in ice for 5 and 10 days were used. When comparing the MHC band between gels with setting at high temperature and medium temperature, it was found that those gels with prior setting at 40°C had the lower MHC band remained. On the other hand, gel from lizardfish mince had MHC retained to a higher extent when prior setting at 40°C was applied. When MTGase at a level of 0.6 units/g was added, MHC in the gels from all species decreased. However, no marked changes in actins were observed for gels from white shrimp and bigeye snapper. Conversely, actin in lizardfish mince gel was polymerized with the addition of MTGase. The decrease in MHC was due to polymerization induced by MTGase (Seguro *et al*., 1995; Jiang *et al*., 1998a). Cross-linking rate as monitored by the disappearance of MHC varied, dependent upon species. MHC of bigeye snapper and lizardfish was more preferable for crosslinking reaction induced by MTGase. However, actin could be susceptible to cross-linking induced by MTGase. Protein with more integrity was a preferable substrate for both endogenous and microbial transglutaminase (Araki and seki, 1993). ε-(γ-glutamyl)lysine isopeptide formed induced by MTGase can not be dissociated by the mixture of SDS and mercaptoethanol during SDS-PAGE analysis (Jaing *et al*., 1998a). The polymerization was greater in the presence of MTGase as evidenced by lower myosin band intensities (Ashi and Lanier, 1999). The decreased MHC band intensity was in agreement with the increase in gel strength. From the result, MTGase induced the cross-linking effectively, regardless of iced storage time. The decrease in MHC was concomitant with the increased breaking force and deformation. Thus, MTGase could be used to improve the gel strength of low quality fish or shrimp via the induction of cross-linking. However, the efficacy could be varied with species.

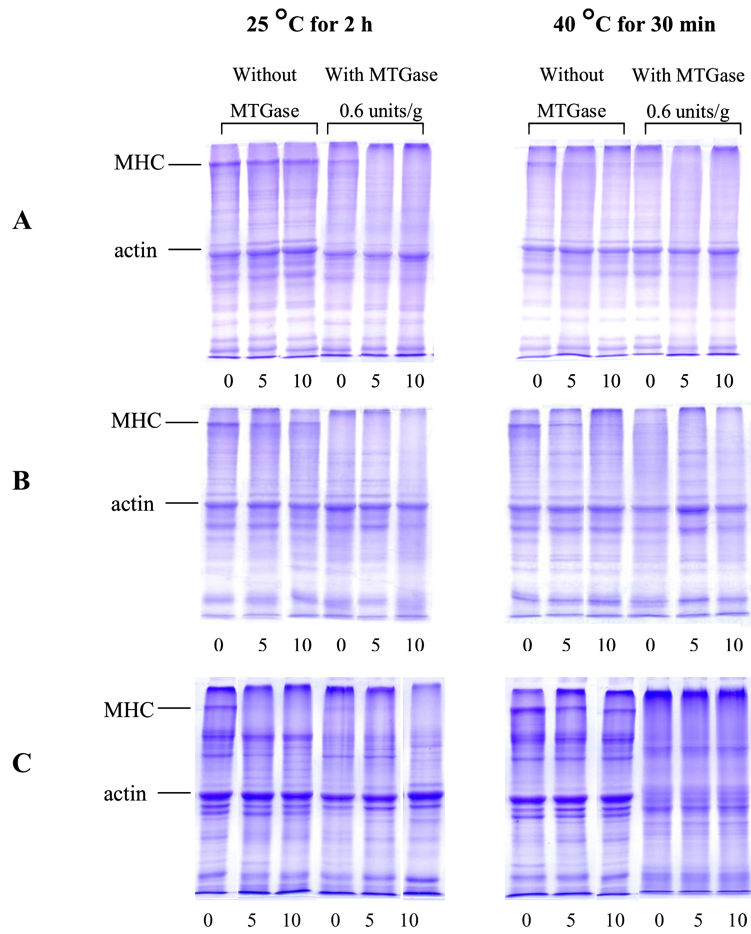

Figure 21 SDS-PAGE patterns of mince gels from some marine fish and shrimp stored in ice for different times without and with MTGase addition (0.6 units/g) and set under different conditions. A: white shrimp, B: bigeye snapper, C: lizardfish. Numbers designate storage time (days)

3.2.5 Microstructure

Microstructures of gels of all species stored in ice for 10 days without and with MTGase addition are shown in Figure 22. Regardless of setting condition, gels produced from all species stored in ice for 10 days added with MTGase (0.6 units/g) had more filamental structure. Among all samples, the gels from bigeye snapper possessed the most ordered fibrillar structure. MTGase added was postulated to build up network structure through intermolecular ε-(γ-glutamyl)lysine crosslinking in co-operation with protein aggregation via hydrophobic interaction, disulfide bonds and/or other interactions during heating process. From the result, it was suggested that during ice storage, proteins are prone to denaturation mainly due to the formation of hydrophobic interaction and covalent bonding among protein molecules. Formaldehyde formation could induce the protein aggregation (Jiang *et al*., 1986; Sotelo *et al.,* 1995). With the addition of MTGase, more ordered network was obtained, compared with the gels without MTGase. The improved gel matrix could hold water more potentially as evidenced by the lowered expressible moisture content with the addition of MTGase. From the result, it can be inferred that the addition MTGase effectively improved the gel-forming ability of ice-stored fish.

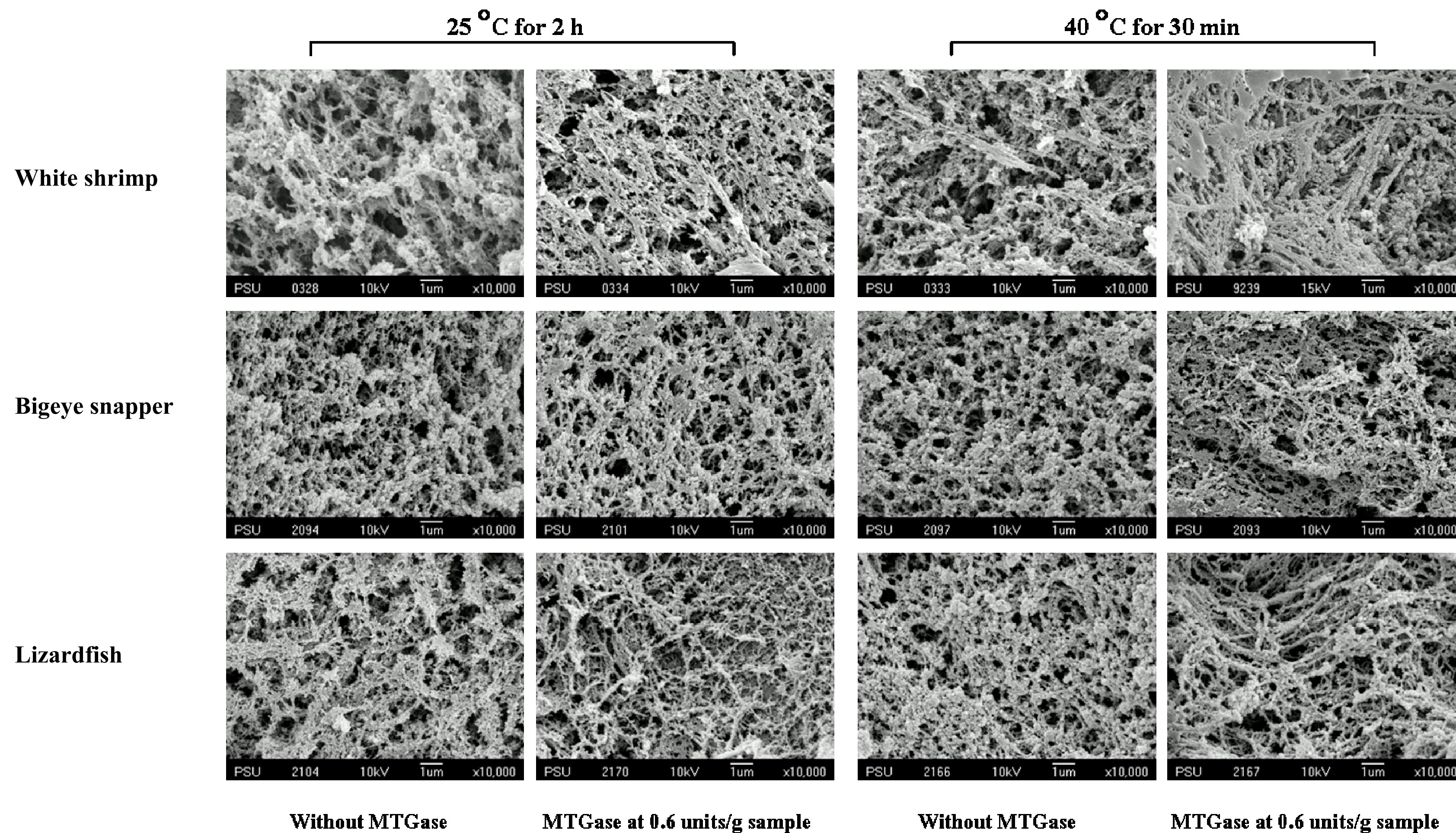

Figure 22 Microstructure of mince gels from some marine fish and shrimp stored in ice for 10 days without and with MTGase (0.6 units/g) addition and set under different conditions.

Table 9 L*, a*, b*-values and whiteness of gels from white shrimp mince added with various levels of MTGase and different setting conditions

Level of MTGase (unit/g of sample)	White shrimp							
	Setting at 25°C for 2 h				Setting at 40°C for 30 min			
	L*	a*	b*	Whiteness	L*	a*	b*	Whiteness
0	71.72 ± 0.46b	19.13 ± 0.22ab	16.19 ± 0.04a	62.21 ± 0.14b	70.73 ± 0.48a	19.00 ± 0.26a	16.18 ± 0.27b	61.54 ± 0.04b
0.2	71.85 ± 0.43b	19.13 ± 0.26ab	16.20 ± 0.10a	62.31 ± 0.23b	70.83 ± 0.36a	19.30 ± 0.25a	16.80 ± 0.11a	61.20 ± 0.05b
0.4	72.08 ± 0.27ab	19.38 ± 0.20a	16.07 ± 0.37a	62.41 ± 0.05b	71.12 ± 0.39a	19.25 ± 0.19ab	15.77 ± 0.26c	61.88 ± 0.09b
0.6	72.42 ± 0.24a	18.88 ± 0.41bc	16.00 ± 0.27a	62.44 ± 0.13b	71.85 ± 0.17a	18.92 ± 0.15a	15.73 ± 0.23c	62.61 ± 0.14a
0.8	72.42 ± 0.34a	18.61 ± 0.46c	15.91 ± 0.31a	63.12 ± 0.33a	71.60 ± 0.23a	18.56± 0.13b	16.19 ± 0.24b	62.41 ± 0.13a

Values are given as means ± SD from five determinations.

Different letters within the same column indicate the significant differences ($p<0.05$).

Table 10 L*, a*, b*-values and whiteness of gels from bigeye snapper mince added with various levels of MTGase and different setting conditions

Level of MTGase (unit/g of sample)	Bigeye snapper							
	Setting at 25°C for 2 h				Setting at 40°C for 30 min			
	L*	a*	b*	Whiteness	L*	a*	b*	Whiteness
0	75.01 ± 0.13a	-3.07 ± 0.05b	9.69 ± 0.11b	73.02 ± 0.10a	76.75 ± 0.18b	-2.92 ± 0.04a	9.99 ± 0.20a	74.53 ± 0.12b
0.2	75.27 ± 0.27a	-2.94 ± 0.16b	9.88 ± 0.39b	73.20 ± 0.02a	76.94 ± 1.11b	-3.01 ± 0.19a	9.42 ± 0.40b	74.90 ± 0.09b
0.4	75.31 ± 0.46a	-3.12 ± 0.06b	9.70 ± 0.27b	73.29 ± 0.03a	77.25 ± 0.35b	-2.80 ± 0.06a	9.96 ± 0.16a	75.00 ± 0.03b
0.6	75.33 ± 0.22a	-3.09 ± 0.07b	9.26 ± 0.16b	73.29 ± 0.04a	77.28 ± 0.22b	-2.87 ± 0.10a	9.95 ± 0.09a	75.03 ± 0.22b
0.8	75.49 ± 0.60a	-2.76 ± 0.09a	10.23 ± 0.31a	73.47 ± 0.24a	77.69 ± 0.16a	-2.99 ± 0.05a	9.38 ± 0.10b	75.61 ± 0.17a

Values are given as means ± SD from five determinations.

Different letters within the same column indicate the significant differences ($p<0.05$).

Table 11 L*, a*, b*-values and whiteness of gels from lizardfish mince added with various levels of MTGase and different setting conditions

Level of MTGase (unit/g of sample)	Lizardfish							
	Setting at 25°C for 2 h				Setting at 40°C for 30 min			
	L*	a*	b*	Whiteness	L*	a*	b*	Whiteness
0	73.25 ± 0.43b	-3.09 ± 0.23a	8.32 ± 0.07b	71.82 ± 0.38b	73.73 ± 0.20b	-3.10 ± 0.06a	7.88 ± 0.20b	72.40 ± 0.21b
0.2	73.30 ± 0.33b	-3.12 ± 0.09a	8.37 ± 0.13b	71.84 ± 0.34b	73.98 ± 0.19b	-2.89 ± 0.16a	8.65 ± 0.23a	72.43 ± 0.20b
0.4	74.06 ± 0.20a	-3.03 ± 0.19a	8.80 ± 0.23a	72.44 ± 0.15a	74.21 ± 0.76a	-2.87 ± 0.08a	8.28 ± 0.24a	72.76 ± 0.72ab
0.6	73.96 ± 0.34a	-3.14 ± 0.13a	8.20 ± 0.22b	72.52 ± 0.33a	74.28 ± 0.98a	-2.98 ± 0.04a	7.83 ± 0.15b	72.95 ± 0.89ab
0.8	74.00 ± 0.27a	-3.16 ± 0.03a	8.20 ± 0.19b	72.56 ± 0.31a	75.07 ± 0.16a	-2.90 ± 0.06a	8.87 ± 0.20a	73.38 ± 0.13a

Values are given as means ± SD from five determinations.

Different letters within the same column indicate the significant differences ($p<0.05$).

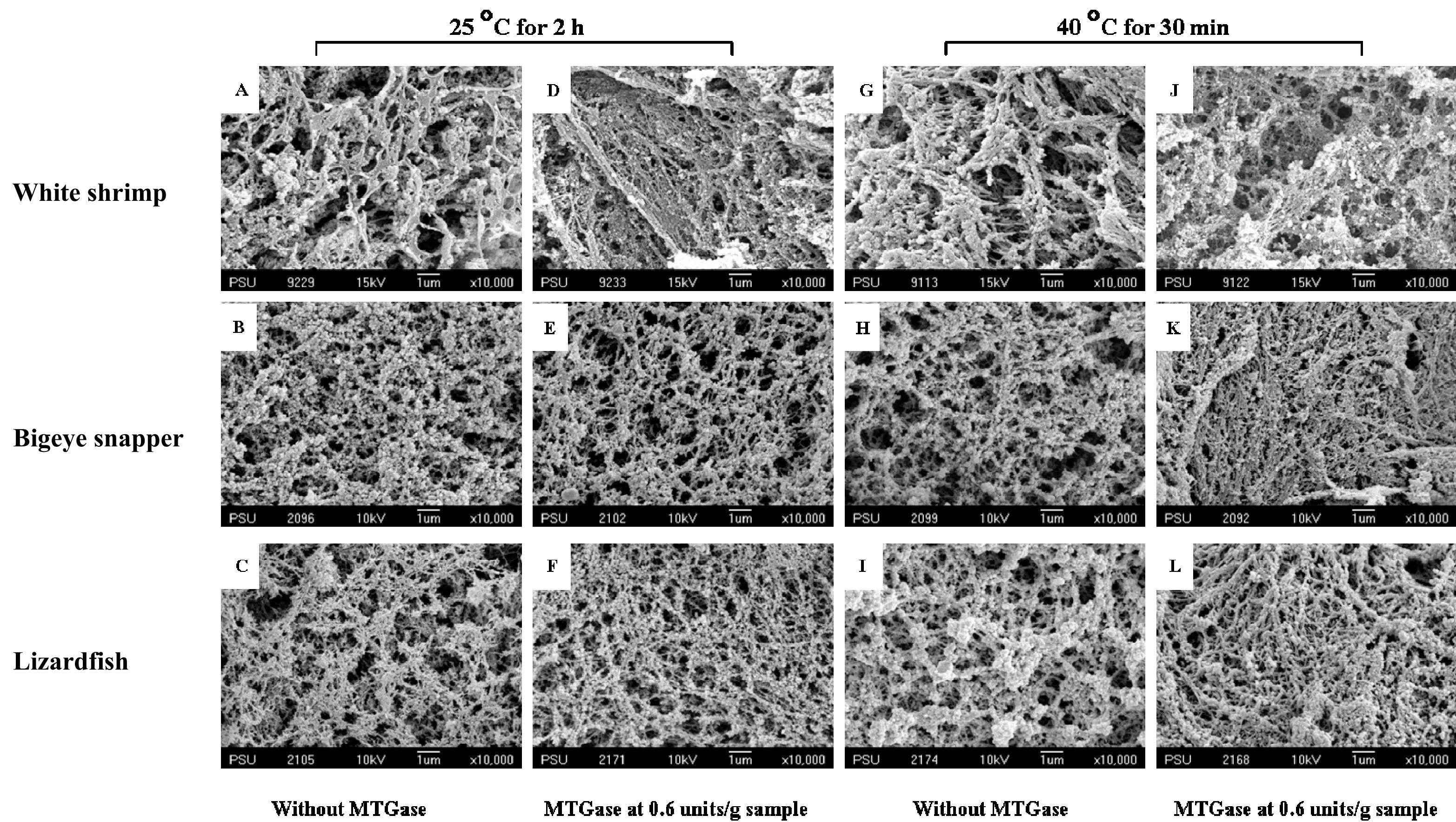

Figure 10 Microstructure of gels from some marine fish and shrimp added without and with MTGase (0.6 units/g) and set under different conditions.

Table 13 L*, a*, b*-values and whiteness of mince gels from white shrimp stored in ice for different times without and with MTGase addition (0.6 units/g) and set under different conditions

Storage time (days)	Setting at 25°C for 2 h				Setting at 40°C for 30 min			
	L*	a*	b*	Whiteness	L*	a*	b*	Whiteness
Without MTGase								
0	71.32 ± 0.32a	19.24 ± 0.18a	15.72 ± 0.23c	62.06 ± 0.43b	71.97 ± 0.25a	19.38 ± 0.22a	15.93 ± 0.37b	62.38 ± 0.51b
5	67.80 ± 0.25b	17.51 ± 0.06b	15.56 ± 0.60c	60.18 ± 0.31c	70.75 ± 0.39b	18.47 ± 0.33b	17.30 ± 0.34a	61.32 ± 0.51c
10	66.23 ± 0.63b	19.31 ± 0.29a	18.41 ± 1.01a	56.96 ± 1.27e	67.99 ± 0.12c	18.10 ± 0.14b	16.26 ± 0.08b	59.80 ± 0.07d
MTGase 0.6 units/g								
0	71.76 ± 0.99a	17.42 ± 0.48b	14.19 ± 0.11d	63.91 ± 1.07a	71.42 ± 0.44a	16.41 ± 0.22c	13.73 ± 0.65c	64.30 ± 0.25a
5	71.44 ± 0.04a	18.62 ± 0.07b	16.38 ± 0.05b	62.17 ± 0.07b	71.80 ± 0.47a	19.14 ± 0.20a	16.20 ± 0.02b	62.26 ± 0.38b
10	71.42 ± 0.44a	16.41 ± 0.22c	13.73 ± 0.65d	59.11 ± 0.88d	67.85 ± 0.21c	18.75 ± 0.28b	17.71 ± 0.25a	58.79 ± 0.37e

Values are given as means ± SD from five determinations.

Different letters within the same column indicate the significant differences ($p<0.05$).

Table 14 L*, a*, b*-values and whiteness of mince gels from bigeye snapper stored in ice for different times without and with MTGase addition (0.6 units/g) and set under different conditions

Storage time	Setting at 25°C for 2 h				Setting at 40°C for 30 min			
(days)	L*	a*	b*	Whiteness	L*	a*	b*	Whiteness
Without MTGase								
0	80.14 ± 0.51b	-2.31 ± 0.07b	12.74 ± 0.01b	76.29 ± 0.53b	81.91 ± 0.41a	-2.28 ± 0.09b	11.37 ± 0.19b	78.51 ± 0.52a
5	74.22 ± 1.21e	-1.93 ± 0.11a	10.67 ± 0.17d	72.03 ± 1.41d	79.02 ± 0.72b	-1.93 ± 0.09a	13.71 ± 0.21a	74.86 ± 0.67b
10	73.18 ± 1.10f	-2.79 ± 0.09c	12.55 ± 1.17b	70.23 ± 1.24f	76.26 ± 0.59c	-2.55 ± 0.08b	13.14 ± 0.34a	72.74 ± 0.82c
MTGase 0.6 units/g								
0	82.09 ± 0.41a	-2.22 ± 0.34b	12.23 ± 0.33b	78.20 ± 0.59a	81.93 ± 0.58a	-2.24 ± 0.09b	11.28 ± 0.51b	78.59 ± 0.91a
5	77.29 ± 0.45c	-2.81 ± 0.08c	11.55 ± 0.22c	74.36 ± 0.61c	81.18 ± 0.13a	-1.77 ± 0.01a	11.97 ± 0.15b	78.46 ± 0.19a
10	75.26 ± 0.53d	-2.29 ± 0.17b	14.31 ± 0.35a	71.64 ± 0.72e	76.63 ± 0.10c	-2.80 ± 0.01b	11.79 ± 0.30b	73.68 ± 0.26b

Values are given as means ± SD from five determinations.

Different letters within the same column indicate the significant differences ($p<0.05$).

Table 15 L*, a*, b*-values and whiteness of mince gels from lizardfish meat stored in ice for different times without and with MTGase addition (0.6 units/g) and set under different conditions

Storage time (days)	Setting at 25°C for 2 h				Setting at 40°C for 30 min			
	L*	a*	b*	Whiteness	L*	a*	b*	Whiteness
Without MTGase								
0	74.13 ± 0.15b	-3.32 ± 0.37b	9.34 ± 0.05a	72.29 ± 0.16b	78.47 ± 0.23a	-2.16 ± 0.09b	9.90 ± 0.20a	76.20 ± 0.26a
5	73.48 ± 0.57c	-2.80 ± 0.07a	9.66 ± 0.63a	71.64 ± 0.91c	73.50 ± 0.58d	-2.80 ± 0.07b	9.66 ± 0.63a	71.66 ± 0.93d
10	68.28 ± 0.22d	-3.33 ± 0.06b	8.87 ± 0.29b	66.89 ± 0.31d	73.10 ± 0.35d	-3.29 ± 0.02c	8.10 ± 0.24c	71.71 ± 0.48d
MTGase 0.6 units/g								
0	78.48 ± 0.23a	-2.16 ± 0.09a	9.90 ± 0.20a	76.21 ± 0.18a	78.75 ± 0.39a	-2.14 ± 0.08b	10.13 ± 0.03a	76.36 ± 0.41a
5	73.41 ± 0.27c	-3.85 ± 0.07b	9.21 ± 0.09a	71.60 ± 0.33c	76.11 ± 0.52b	-1.66 ± 0.14a	9.28 ± 0.16b	74.32 ± 0.66b
10	73.56 ± 0.41c	-3.36 ± 0.10b	9.71 ± 0.21a	71.63 ± 0.55c	74.52 ± 0.41c	-2.85 ± 0.07b	10.21 ± 0.09a	72.41 ± 0.45c

Values are given as means ± SD from five determinations.

Different letters within the same column indicate the significant differences ($p<0.05$).

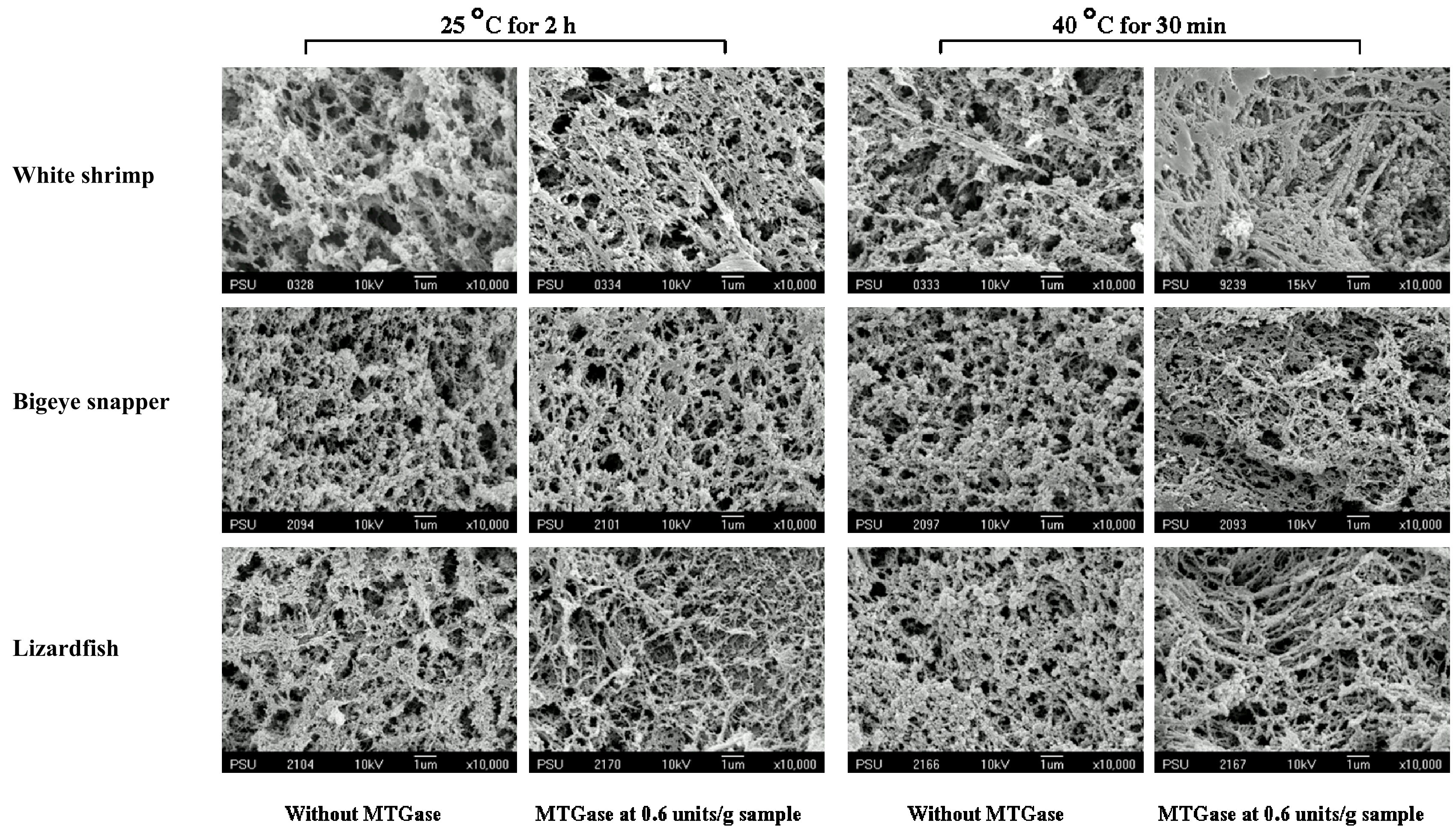

Figure 22 Microstructure of mince gels from some marine fish and shrimp stored in ice for 10 days without and with MTGase (0.6 units/g) addition and set under different conditions.

4. Effect of protein substrate denaturatuion on cross-linking activity of MTGase

4.1 Thermal stability

The inactivation rate constants (K_D value) of natural actomyosin (NAM) from all species at different temperatures are shown in Table 16. The inactivation rate constant or K_D value of actomyosin and myosin Ca^{2+}-ATPase activity has been generally used to evaluate the thermal stability of fish proteins (Tsai *et al.* 1989; Jiang *et al.* 1989). At temperatures below 20°C, very low K_D was observed for natural actomyosin from bigeye snapper and lizardfish but K_D of 3.14 was observed for natural actomyosin from white shrimp (Table 16). K_D values of white shrimp natural actomyosin increased substantially at temperature above 20°C. However, K_D of both fish increased gradually when heated up to 40°C and the sharp increase in K_D was found at temperature above 40°C. The results implied that thermal stability of natural actomyosin from different species was varied. Tsai *et al.* (1989) reported that K_D values of different fish species determined at definite temperature were different, indicating the different stability of muscle protein from different species. At the same temperature, natural actomyosin from white shrimp had a higher K_D value, compared to other species. From the results, it was most likely that muscle proteins of white shrimp were more susceptible to thermal denaturation than those of fish. Similar changes in K_D were noticeable between two fish species. However, slightly higher K_D was observed in lizardfish natural actomyosin. Thus, the stability of muscle proteins from white shrimp was lower than that of both fish. From the results, the loss in Ca^{2+}-ATPase activity was used to monitor the K_D. MHC has been reported to possess Ca^{2+}-ATPase activity, which can be used as the indicator of MHC integrity (Benjakul *et al.,* 1997). Actin was suggested to play a protective role in the stability of myosin (Jiang *et al.,* 1989). The differences in stability of all species possibly resulted from the different intrinsic properties, amino acid composition as well as actin/myosin ratio. K_D of natural actomyosin was significantly lower than that of myosin (Jiang *et al.* 1989).

Table 16 Thermal inactivation rate constant (K_D x 10^{-5} S^{-1}) of NAM from some marine fish and shrimp meats

Temperature (°C)	Species		
	White shrimp	Bigeye snapper	Lizardfish
0	0.17 ± 0.24gA	0.10 ± 0.16fA	0.19 ± 0.14fA
10	3.14 ± 0.49fA	0.20 ± 0.12fB	0.38 ± 0.11fB
20	7.98 ± 1.23eA	0.48 ± 0.39eC	1.26 ± 1.03eB
30	40.67 ± 1.58dA	4.47 ± 1.32dB	5.16 ± 0.05dB
40	43.72 ± 1.53cA	6.51 ± 1.11cB	7.26 ± 0.05cB
50	59.22 ± 0.39bA	29.28 ± 0.43bB	30.11 ± 0.69bB
60	62.45 ± 2.24aA	49.57 ± 0.28aC	57.63 ± 0.33aB

Values are given as mean ±SD from triplicate determinations.

Different letters in the same column indicate significant differences. ($p<0.05$).

Different capital letters in the same row indicate significant differences. ($p<0.05$).

4.2 Effect of MTGase on cross-linking and physico-chemical properties of NAM with different degrees of denaturation

4.2.1 Changes in free amino group content

Figure 23 shows the changes in free amino group content of NAM from all species pre-heated for different times in the absence and the presence of MTGase at different levels. TNBS-reactive amino groups of NAM decreased to some extent as pre-heating time increased ($p<0.05$). This indicated that the amino group might undergo changes during pre-heating either via cross-linking or unfolding of proteins which made the amino groups masked for TNBS reaction. The rate of loss in free amino groups was generally higher in samples added with different MTGase. The decrease in the free amino group content was possibly associated with cross-linking induced by MTGase during incubating at 40°C for 30 min. The decrease in TNBS-reactive amino groups was used as a measure of the reaction of MTGase towards proteins. Lysine is one of the most reactive side chain groups for MTGase reaction. Moreover, Ang and Hultin (1989) proposed that a relatively small number of side chain modifications can markedly affect the stability of some proteins, especially when they are subjected to stress conditions. At the same level

of MTGase added, the loss of free amino groups was more pronounced with NAM pre-heated for a longer time (30 min). This might be due to the higher reactivity of MTGase towards more denatured proteins. Proteins with the greater denaturation might have the altered conformation, in which the reactive groups, mainly glutamine and lysine were more exposed and ready for cross-linking reaction induced by MTGase. Also, the loss of free amino groups was found to be different, depending upon species, when NAM with the same pre-heating time was used. This indicated that the cross-linking activity of MTGase was governed by the conformation of protein substrates. Also, partial denaturation might favor the cross-linking activity induced by MTGase. The lower free amino group in NAM without MTGase addition after incubated at 40 °C for 30 min indicated that the cross-linking induced by endogenous TGase could occur. Seki et al. (1998) also reported that endogenous TGase activity involved in cross-linking of fish protein during setting.

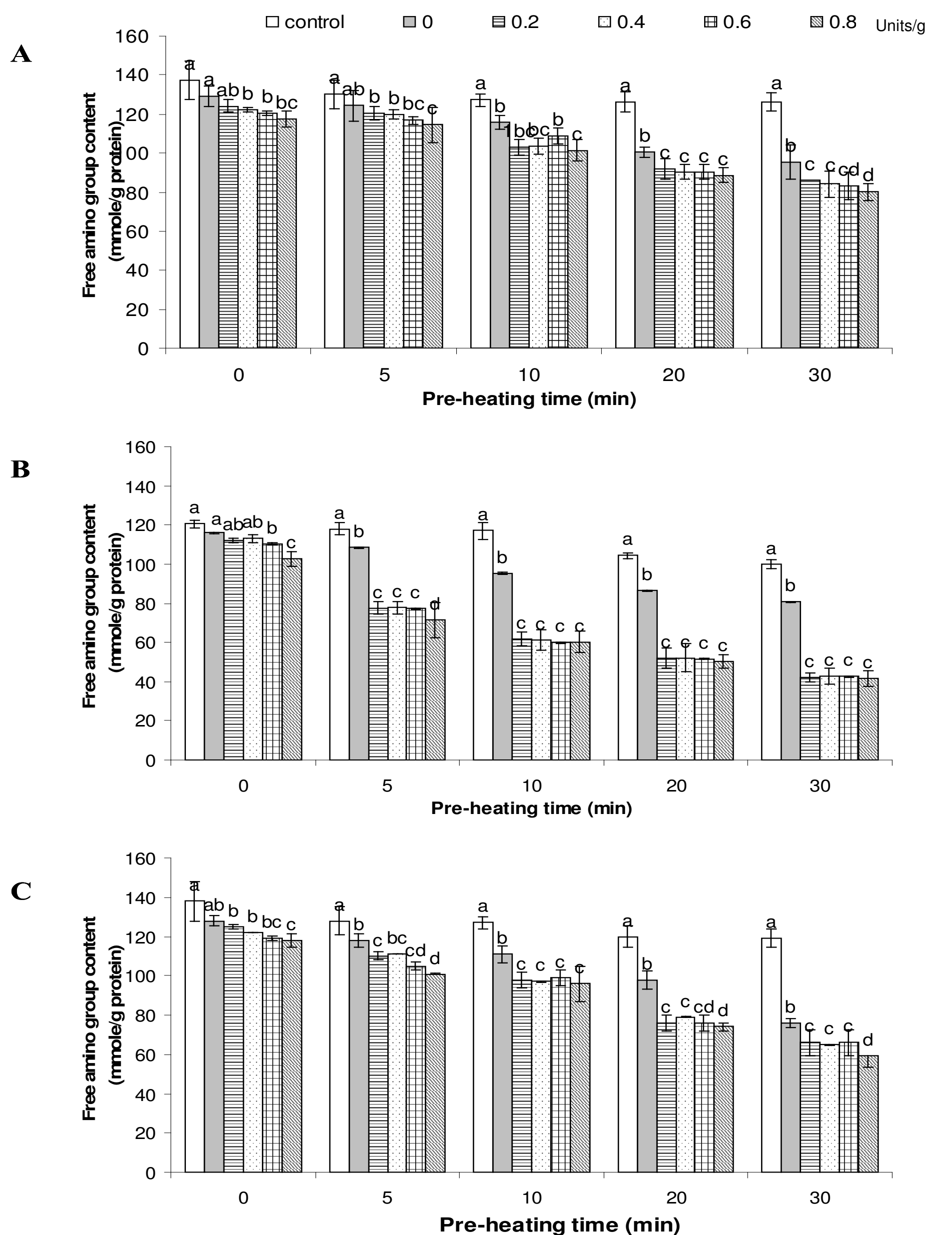

Figure 23 Changes in free amino group content of some marine fish and shrimp NAM with pre-heating at 50°C for different times as influenced by MTGase addition at different levels. After pre-heating, the sample was added with MTGase at different levels and incubated at 40°C for 30 min. A: white shrimp, B: bigeye snapper, C: lizardfish and Control: NAM preheated at 50°C for different times without subsequent incubation at 40°C for 30 min. Bars represent the standard deviation from triplicate determinations. Different letters within the same incubation time indicate significant differences ($p < 0.05$).

4.2.2 Changes in Solubility

The solubility of NAM from all species in 0.6 M KCl pH 7.0 after pre-heating at 50°C and added without and with MTGase at different levels is shown in Figure 24. Slight decrease in solubility was observed in NAM subjected to preheating up to 30 min without subsequent incubation at 40°C for 30 min. The decrease in solubility was in accordance with the increase in surface hydrophobicity (Figure 28). The loss in salt soluble protein suggested that protein denaturation was induced by the thermal process. The decrease in solubility was most likely associated with the formation of disulfide bond (Figure27). The decrease in solubility of protein has been used as an index of oxidative deterioration of muscle protein (Decker *et al.,* 1993; Srinivasan and Hultin, 1997; Xiong and Decker, 1995). Thermodynamically, a decrease in protein solubility is the result of a shift from a balance of protein intermolecular interaction and protein–water interaction, resulting in a situation, where protein intermolecular interaction is forced, while protein water interaction is weakened (Vojdani, 1996). As a result of loss of ordered tertiary structure, the cross-linkages are formed among proteins easily as evidenced by the decrease in solubility. Free radical attack is also a major cause of decreased protein solubility (Decker *et al.,* 1993). Most of the hydrophobic and cysteine amino acid residues are located in the interior of the globular protein. Heating causes protein unfolding, exposure of the interior amino acids and increased protein-protein interactions, with resulting loss of solubility. At temperatures lower than 65°C, the hydrophobic effect is dominant; whereas at higher temperatures, the configurational entropy dominates (Albert 1989; Bryant and McClements, 1998). From the result, when pre-heating time increased, protein solubility decreased. The disruption of the native 3-dimensional structure of the proteins as heat denaturation time increases, exposing SH groups formerly buried inside the molecules, enables the formation of covalent intermolecular disulfide bonds. Disulfide bonds are the strongest bonds stabilizing the protein-protein interaction, and these bonds are not affected by changes in temperature (Bryant and McClements, 1998).

Solubility of NAM decreased when pre-heating time increased and MTGase levels increased. However, the effect of MTGase on solubility depended on species. The lowest solubility was found in NAM from all species added with 0.8 units/g protein. With the addition of MTGase, the decrease in solubility of NAM suggested the increased

non-disulfide covalent bond formation as evidenced by the decrease in free amino group (Figure 23). Therefore, preheating resulted in the conformational changes as well as the increased aggregation mainly by hydrophobic interaction and disulfide bond. This protein substrates were still reactive and available for cross-linking induced by MTGase. The decrease in solubility in the absence of MTGase with the subsequent incubation at 40°C for 30 min might be due to the role of endogenous TGase associated with NAM in cross-linking. The larger aggregate likely caused the loss in solubility.

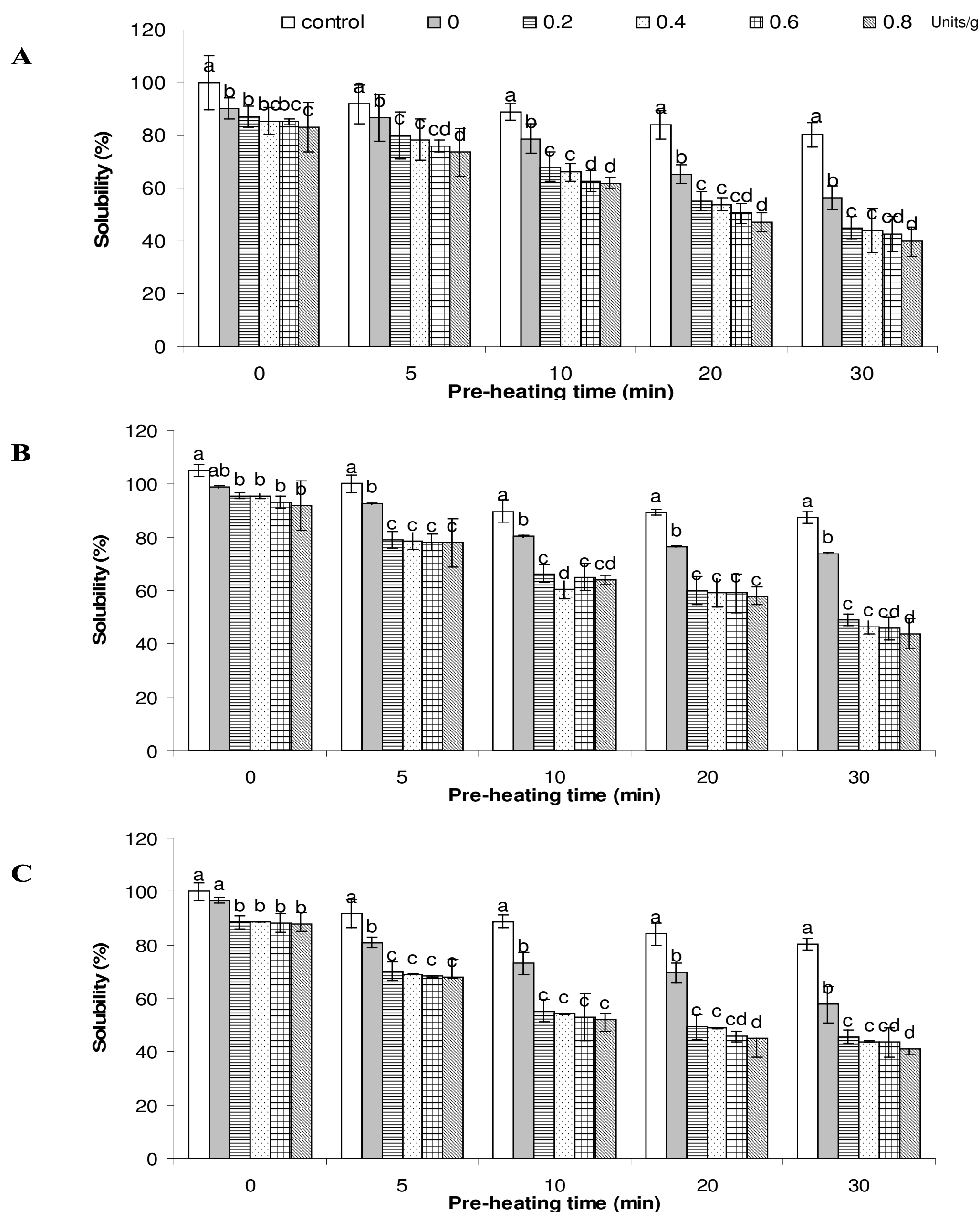

Figure 24 Changes in solubility of some marine fish and shrimp NAM with pre-heating at 50°C for different times as influenced by MTGase addition at different levels. After pre-heating, the sample was added with MTGase at different levels and incubated at 40°C for 30 min. A: white shrimp, B: bigeye snapper, C: lizardfish and Control: NAM preheated at 50°C for different times without subsequent incubation at 40°C for 30 min. Bars represent the standard deviation from triplicate determinations. Different letters within the same incubation time indicate significant differences ($p<0.05$).

4.2.3 Changes in protein patterns

The effects of MTGase addition on the cross-linking of NAM from all species at 50°C for different times in the presence and the absence of MTGase were determined by SDS-PAGE (Figure 25). NAM from different species contained different MHC band intensity. White shrimp NAM had the highest MHC band intensity, while lizardfish NAM comprised the highest actin band intensity. During incubation at 40°C for 30 min, MHC and actin band intensity of white shrimp NAM decreased to a high extent as shown by the lower retaining bands of those two proteins. This suggested that NAM of white shrimp under went aggregation via non-disulfide covalent bond during incubation at 40°C. However, no marked changes in protein pattern of NAM from both bigeye snapper and lizardfish were noticeable after incubation at 40°C. Thus, it was most likely that NAM contained a negligible activity of TGase or TGaase could not react well with native NAM.

After NAM was preheated for different time, followed by incubation at 40°C for 30 min. It was noted that MHC of both fish, bigeye snapper and lizardfish was decreased to some extent with the concomitant occurrence of the polymerized proteins on the stacking gel. Pre-heating time did not show the pronounced effect on the cross-linking of NAM. For white shrimp NAM, no differences in proteins patterns between those with and without pre-heating were observed. Also, pre-heating time showed no effect on the protein patterns. Endogenous TGase play a crucial role in ε-(γ-glutmyl)lysine linkage formation (Kamath *et al*., 1992). The cross-link formed during heating was dependent on fish species, suggesting the differences in TGase activity as well as different protein conformation. White shrimp was found to undergo cross-link most effectively, as observed by the lowest myosin band remained. Araki and seki (1993) reported that when the same amount of carp TGase was used in reaction with different actomyosins, the rates of polymerization were different.

When MTGase at 0.6 units/g proteins was added into NAM preheated at 50°C for different times, no MHC was remained in NAM from white shrimp and lizardfish. Very low MHC band intensity was observed in NAM from bigeye snapper. Furthermore, actin intensity band of NAM from bigeye snapper was lowered. From the result, cross-linking induced by MTGase occurrenced regardless of preheating time. Thus, MTGase

could induce the polymerization of NAM irrespective of the denaturation of protein substrates. Similar results have also been reported in beef heart myofibrillar protein isolates (Ramirez-Suarez *et al*., 2001). Large molecular size polymers formed by MTGase gave an extra band at the top of the gel because they could not migrate well on the gel. These extra bands were denser in the sample containing MTGase than in the samples without MTGase. It has been shown that with increasing polymer content, density of myosin band was decreased (Kumazawa *et al*., 1993; Lee *et al*., 1997). Similar results on reduction of myosin and actin have been reported previously when myofibrillar protein isolates were incubated with MTGase (Ramirez-Suarez and Xiong, 2003). A reduction in myosin content is an indicator of cross-linking of myosin catalyzed by MTGase (Nishimoto *et al*., 1987; Ramirez-Suarez and Xiong, 2002). Cross-linking of proteins results in formation of dimers, trimers and larger protein polymers (De Jong and Koppelman, 2002). The efficiency at which a protein can be utilized as a substrate by MTGase, seems to be influenced by the amino acid sequence around the reactive glutamines. Thus, the primary structure of a protein is of greater importance in assessing its ability to act as a substrate than its absolute lysine and glutamine contents (Kurth and Rogers, 1984). Seguro *et a1*., (1995) reported that MHC apparently decreased with increasing MTGase concentration, regardless of the grade and setting conditions. Asagami *et al*., (1995) found that the effect of MTGase on quality improvement of surimi depended not only on the fish species but also on the freshness of the fish used.

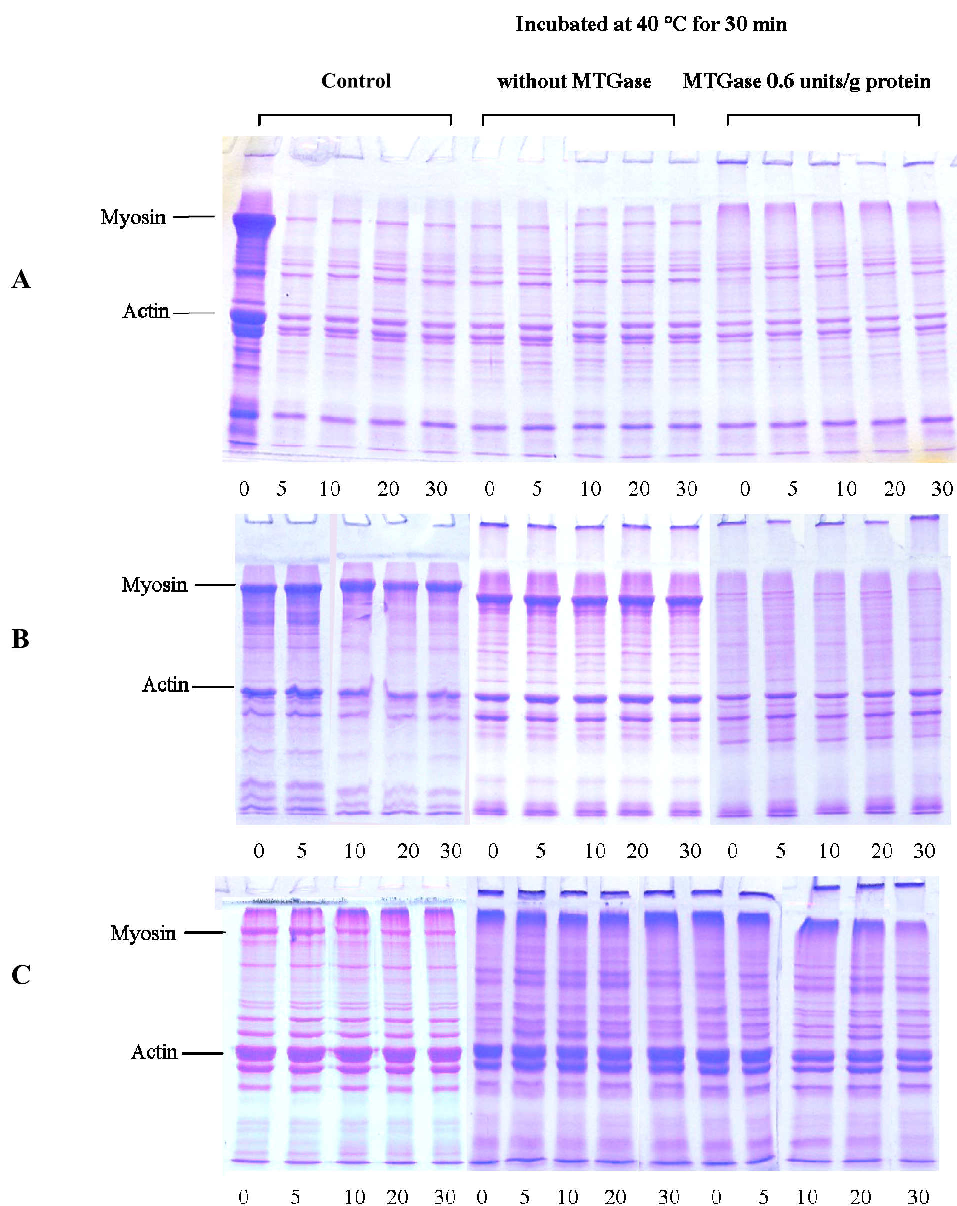

Figure 25 Changes in protein patterns of some marine fish and shrimp NAM with pre-heating at 50°C for different times as influenced by MTGase addition. After pre-heating, the sample was added without and with MTGase at 0.6 units/g protein and incubated at 40°C for 30 min. A: white shrimp, B: bigeye snapper, C: lizardfish and Control: NAM preheated at 50°C for different times without subsequent incubation at 40°C for 30 min. Numbers designate pre-incubation time (min).

4.2.4 Changes in sulfhydryl group content

Total sulfhydryl group content of all samples decreased gradually when the pre-heating time at 50°C increased up to 30 min ($p<0.05$) (Figure 26). The decrease in sulfhydryl group generally resulted from the formation of disulfide bonds through oxidation of sulfhydryl groups or disulfide interchanges (Hayakawa and Nakai, 1985). Sulfhydryl groups on the myosin head portion, named SH_1 and SH_2, were reported to involve in ATPase activity of myosin (Kielley and Bradley 1956; Sekine *et al.* 1962). SH_a, another sulfhydryl group on the light meromyosin region of myosin molecule, was susceptible to oxidation during thermal denaturation of myosin (Smyth *et al*., 1998). From the result, total sulfhydryl group content decreased with increasing pre-heating time. Therefore, it was postulated that both sulfhydryl groups localized on either head or tail portions of myosin molecules underwent oxidation, leading to less available free sulfhydryl groups. The oxidation of sulthydryl groups on the head portion presumably involved in denaturation of myofibrillar proteins during heat treatment. From the result, NAM from different species underwent oxidation with different degrees. Among all species, NAM from lizardfish was more prone to oxidation as indicated by the higher rate of SH group decrease. This might be associated with the higher sulhydryl group content. Additionally, protein molecules of this species might undergo conformation change in the way, which enchanced the oxidation of SH group and the formation of disulfide bonds. Jiang *et al.* (1988) suggested that sulfhydryl groups on F-actin might compete with those on myosin molecules for the oxidation into disulfides. As a result the denaturation of myosin may also be dependent on actin/myosin ratio, which may be different with species. Samejima *et al*. (1981) proposed that heat-induced gelation rabbit skeletal myosin involved two reactions: (1) irreversible aggregation of S-I at 45°C, which was associated with the oxidation of SH groups, and (2) network formation resulting from the unfolding and noncovalent interactions in the rod throughout the gelation process. The globular region of myosin, which includes S-1, contains 24-26 of the 42 SH groups in myosin (Buttkus, 1971). From the result, slightly higher decrease in total sulfhydryl content was observed in pre-heated NAM added with different levels of MTGase, especially in white shrimp NAM. It was noted that high amount of MTGase did not cause more decrease in sulhydryl group content,

particularly when pre-heating time was greater than 5 min for NAM from bigeye snapper and lizardfish. However, the decrease in sulfhydryl group content was observed in NAM from all species preheated for 0-5 min with increasing MTGase level. Therefore, NAM denaturated by pre-heating for a longer time might undergo denaturation and disulfide bonds to a higher extent. However, cross-linking induced by MTGase still occurred as indicated by the decrease in free amino group content. However, those cross-links might hamper or enhance the oxidation of sulhydryl group, possibly governed by configuration alteration of protein substrates.

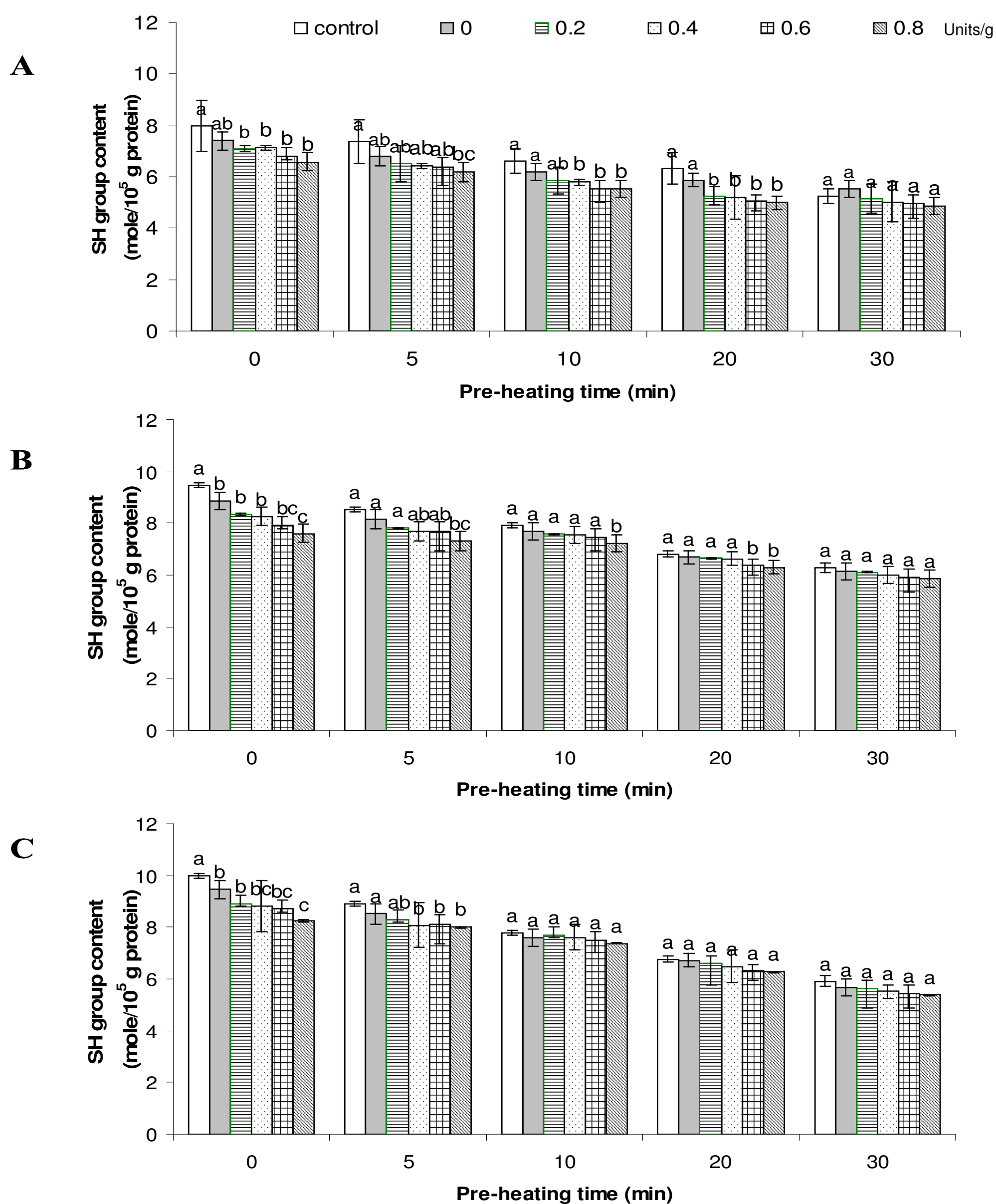

Figure 26 Changes in sulhydryl group content of some marine fish and shrimp NAM with pre-heating at 50°C for different times as influenced by MTGase addition at different levels. After pre-heating, the sample was added with MTGase at different levels and incubated at 40°C for 30 min. A: white shrimp, B: bigeye snapper, C: lizardfish and Control: NAM preheated at 50°C for different times without subsequent incubation at 40°C for 30 min. Bars represent the standard deviation from triplicate

determinations. Different letters within the same incubation time indicate significant differences ($p<0.05$).

4.2.5 Changes in disulfide bond content

Changes in disulfide bond content of NAM from all species after pre-incubation at 50°C for different times with and without MTGase at different levels are shown in Figure 27. Disulfide bond content of all samples increased gradually as the pre-heating time increased. NAM from all species had the increased content of disulfide bonds which was generally coincidental with the decreased sulfhydryl groups when the pre-heating time increased. It was suggested that the oxidation of sulfhydryl groups to disulfide bonds took place during pre-heating. Degree of sulfhydryl oxidation/reduction were different, depending on species (Benjakul *et al.,* 2003b). Therefore, heat-treatment might induce the conformational changes of proteins in the fashion which sulfhydryl groups were oxidized and disulfide bonds could be formed. Lanier (2000) reported that disulfide bond is the only covalent cross-links found naturally in proteins. An intermolecular disulfide bond is formed by the oxidation of two cysteine molecules on neighboring protein chains (Lanier, 2000). From the result, disulfide bond content of NAM extracted from all species without and with pre-heating for different time without MTGase had the lower value than that those added with MTGase at different levels. However, no changes in disulfide bond contents were observed in NAM from white shrimp preheated for 5-20 min, regardless of MTGase concentrations. The result suggested the reactivity of MTGase on cross-linking in different species, which might influence the formation of disulfide bonds differently.

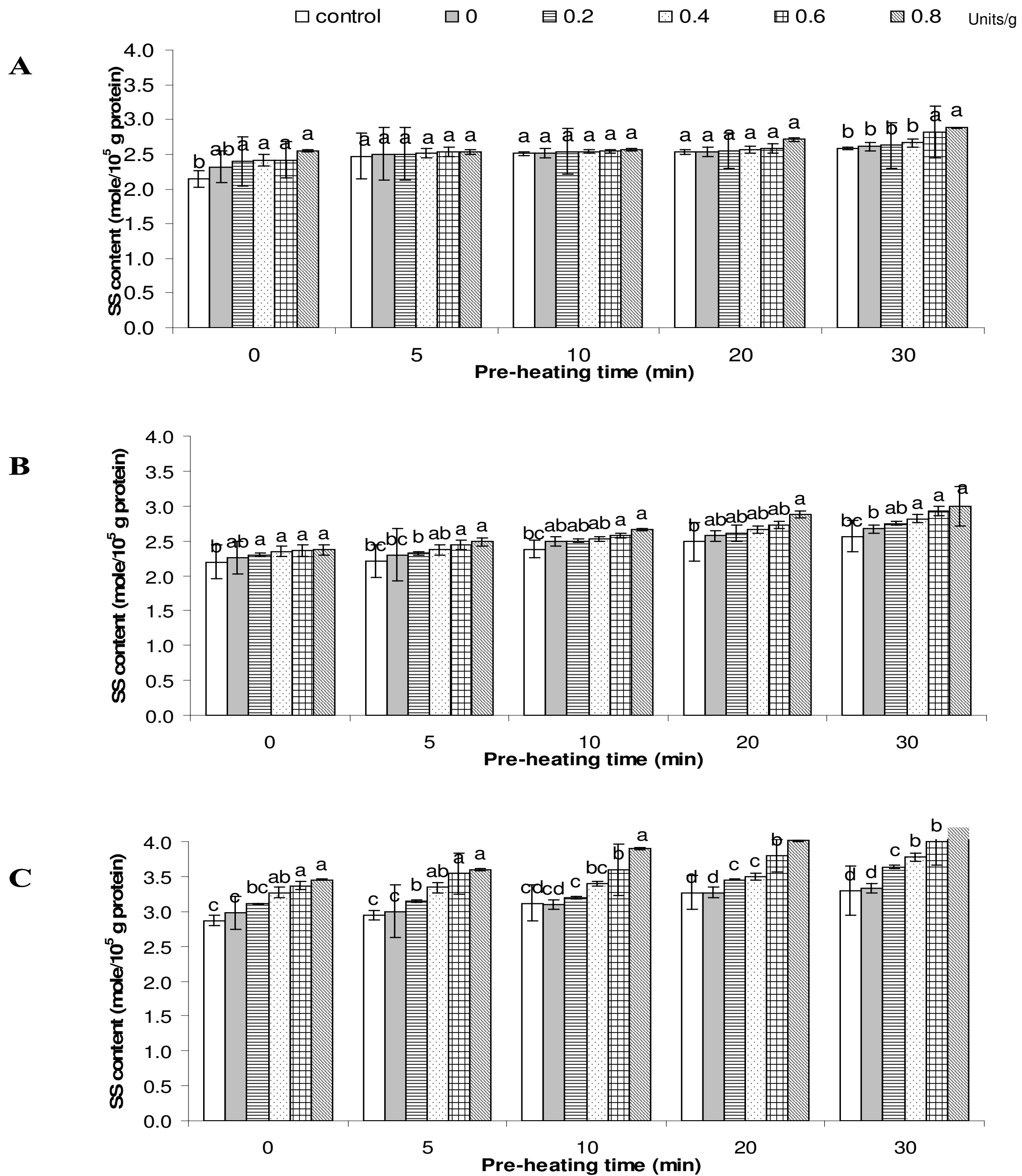

Figure 27 Changes in disulfide bond content of some marine fish and shrimp NAM with pre-heating at 50°C for different times as influenced by MTGase addition at different levels. After pre-heating, the sample was added with MTGase at different levels and incubated at 40°C for 30 min. A: white shrimp, B: bigeye snapper, C: lizardfish and Control: NAM preheated at 50°C for different times without subsequent incubation at 40°C for 30 min. Bars represent the standard deviation from triplicate determinations.

Different letters within the same incubation time indicate significant differences (p <0.05).

4.2.6 Changes in surface hydrophobicity

Changes in surface hydrophobicity of NAM from all species after pre-heating at 50°C and added without and with MTGase at different levels are shown in Figure 28. As the pre-heating time increased, surface hydrophobicity of NAM from all species increased (p<0.05). The increased surface hydrophobicity indicated the structural and conformational changes of myofibrillar proteins. The differences in the exposure of hydrophobic portion caused by heating were possibly caused by the differences in amino acid compositions as well as the different stability of protein from different species. From the result, pre-heating presumably resulted in an exposure of the interior of molecule, where the hydrophobic portion was located. Exposed hydrophobic amino acids containing an aromatic ring, e.g. phenylalanine and tryptophan, were able to bind to ANS (Kato and Nakai 1980). Hydrophobic interaction between amino acids and oxidation of sulthydryl groups affected surface hydrophobicity (Hill *et al.* 1982). Therefore, natural actomyosins from all species were susceptible to conformational changes during pre-heating, as indicated by the increased surface hydrophobicity. During the denaturation processes, the hydrophobic and hydrogen bonds buried in the interior of the protein molecule become exposed and broken from their native arrangement with the following conformational changes in coiled or helical sections of the peptide chain (Morawetz, 1972). Finley (1988) reported the effects of heating may appear in two different ways with regard to hydrophobicity: (1) unfolding of molecules, thus exposing hydrophobic sites, and (2) heat-induced aggregation with resulting decrease in the exposure of hydrophobic sites and loss of solubility.

Generally, NAM without pre-heating from fish added with MTGase had the higher surface hydrophobicity than that without MTGase (p<0.05). This result indicated that MTGase might induce the conformational changes of proteins to some extent as evidenced by the increase in surface hydrophobicity. However, MTGase addition caused the decreased in hydrophobicity of NAM from white shrimp as MTGase concentration increased (p<0.05). Generally, pre-heating of NAM for a longer time had the marked effect on changes in surface hydrophobicity when the same level of MTGase was used. At the

same pre-heating time, MTGase at higher levels increased surface hydrophobicity of NAM from all species. Nevertheless, the degree of increase was different among species. Thus, it was suggested that after proteins underwent thermal denaturation, MTGase still showed the impact on the conformational changes, particularly the exposure of hydrophobic residues. This was coincidental with the greater cross-linking induced by MTGase.

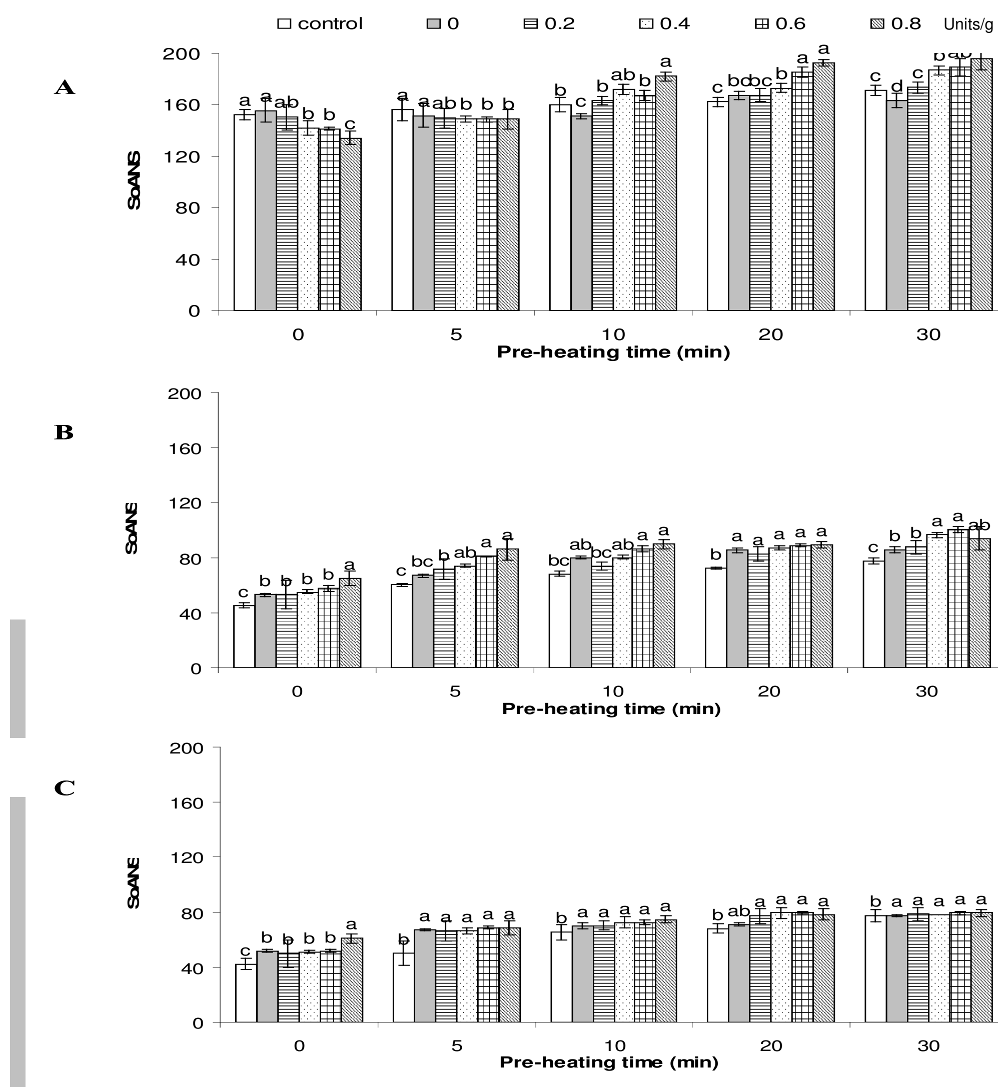

Figure 28 Changes in surface hydrophobicity of some marine fish and shrimp NAM with pre-heating at 50°C for different times as influenced by MTGase addition at different levels. After pre-heating, the sample was added with MTGase at different levels and incubated at 40°C for 30 min. A: white shrimp, B: bigeye snapper, C: lizardfish and Control: NAM preheated at 50°C for different times without subsequent incubation at 40°C for 30 min. Bars represent the standard deviation from triplicate determinations.

Different letters within the same incubation time indicate significant differences ($p < 0.05$).

5. Effect of MTGase on cross-linking and physicochemical properties of NAM added with different levels of formaldehyde

5.1 Changes in free amino group content

Figure 29 shows the changes in free amino group content of NAM from all species added with different levels of formaldehyde as affected by MTGase addition at different levels. With the addition of formaldehyde, free amino group content decreased. Higher formaldehyde concentrations caused the more decrease in free amino content ($p<0.05$). This indicated that the cross-linking took place via amino groups. Formaldehyde addition resulted in a decrease in the number of free amino groups in NAM, indicating the occurrence of polymerization. Formaldehyde is known to react with a wide spectrum of amino side chains in proteins (Ang & Hultin, 1989). The decrease in TNBS-reactive amino groups was used as a measure of the reaction between formaldehyde and proteins (Ang & Hultin, 1989). Lysine is one of the more reactive side chain groups with formaldehyde (Tome *et al.,* 1985). Moreover, Ang and Hultin (1989) proposed that a relatively large number of side chain modifications can markedly affect the stability of some proteins. From the results, the rates of loss in free amino groups was generally higher in samples added with MTGase and incubate at 40°C for 30 min. Decrease in free amino group content was not much affected by the increasing concentration of MTGase. The result suggested that aggregation of protein from different species possibly occurred in different fashion, in which amino group could be used in MTGase mediated reaction differently. Therefore, the reactivity of MTGase towards NAM treated with formaldehyde varied, depending on fish species. Therefore, MTGase could induce the cross-linking of proteins in the presence of formaldehyde but at the lower degress.

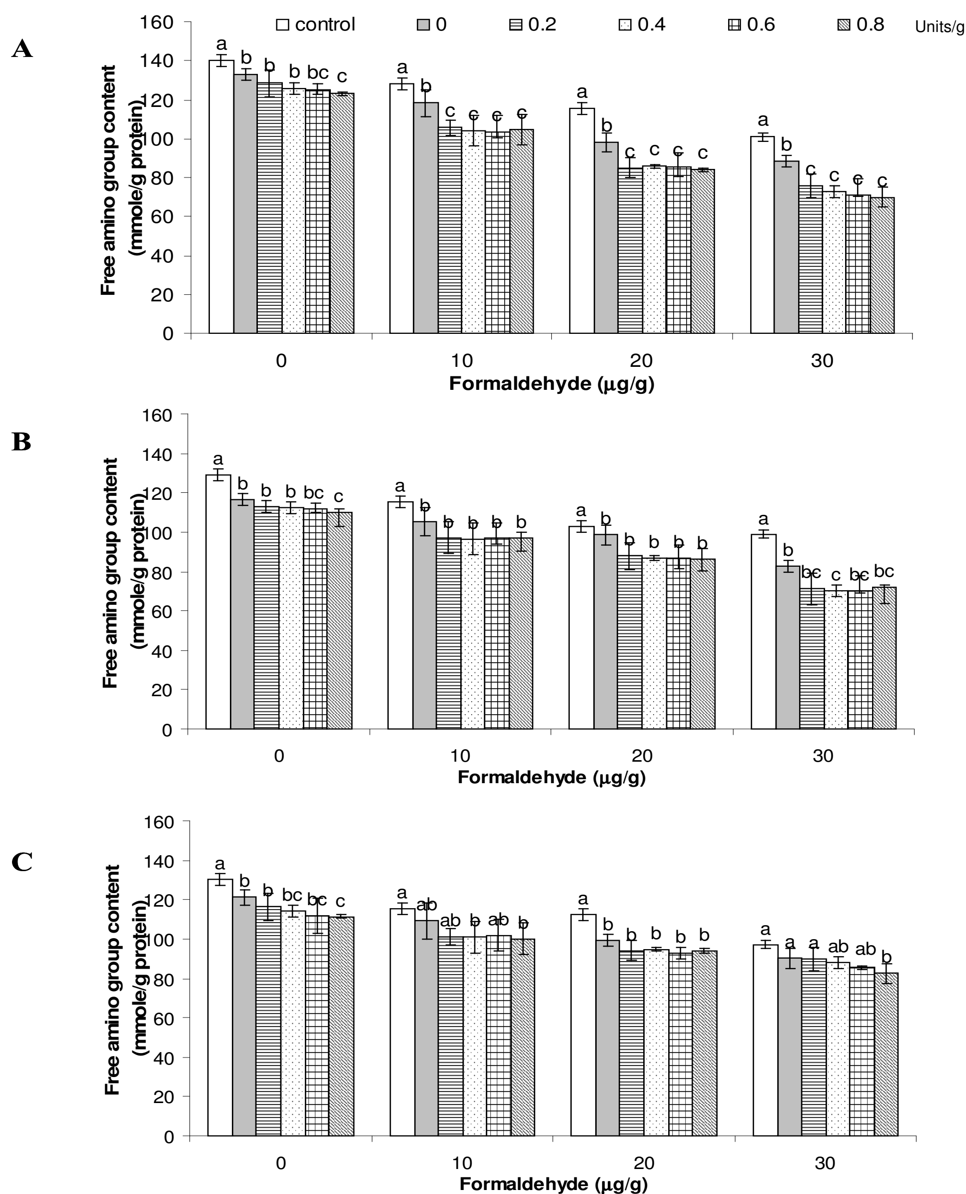

Figure 29 Changes in free amino group content of some marine fish and shrimp NAM added with different levels of formaldehyde as influenced by MTGase addition at different levels. After addition of formaldehyde, the sample was added with MTGase at different levels and incubated at 40°C for 30 min. A: white shrimp, B: bigeye snapper, C: lizardfish and Control: NAM pretreated with formaldehyde at different levels without subsequent incubation at 40°C for 30 min. Bars represent the standard

deviation from triplicate determinations. Different letters within the same formaldehyde level indicate significant differences ($p<0.05$).

5.2 Changes in solubility

Solubility of NAM from all species in 0.6 M KCl decreased continuously after addition of formaldehyde as shown in Figure 30. Generally, the greater content of formaldehyde resulted in the higher losses in solubility ($p<0.05$). After setting for 30 min at 40 ^{o}C, lower solubility of NAM regardless of formaldehyde addition was observed. Nevertheless, the degree of decrease was different, suggesting the role of endogenous TGase in cross-linking. In gadoid fish, losses of protein solubility and water binding capacity in the muscle have been attributed to cross-linking between adjacent polypeptide by formaldehyde derived from trimethylamine oxide (Sotelo *et al.,* 1995). Formation of disulfide, hydrogen and hydrophobic bonds was known to cause the aggregate when the formaldehyde was present (Ang and Hultin, 1989). NAM aggregates formed in the presence of formaldehyde are mostly linked by secondary interaction and disulfide bridges (Careche and Li-Chan, 1997; Ragnarsson and Regenstein, 1989). The denaturation and aggregation of protein during frozen storage started from the formation of disulfide bonds, followed by a rearrangement of hydrophobic and hydrogen-bonded regions on an intra- and inter-molecular basis (Buttkus, 1974). Careche and Li-Chan (1997) found the changes in cod myosin structure, especially exposure of the hydrophobic portion, after formaldehyde addition, leading to the formation of covalent cross-links responsible for insolubility of proteins. Hydrophobic interactions between the exposed groups results in a decrease in solubility of proteins (Buttkus, 1974). From the result, the decreased solubility indicated the aggregation as well as denaturation of proteins caused by formaldehyde. From the result, the losses in solubility of NAM from all species were more intense when MTGase at higher levels were added. At higher level of MTGase, the reactivity of MTGase was lower as evidenced by the lower decrease in solubility. Nevertheless, the degree of decrease varied dependent upon species. From the results, the decrease in solubility among different was possibly governed by the differences in protein conformation as well as the amino acid composition. From the result, MTGase could induce the cross-linking of NAM subjected to formaldehyde modification to some extent. Available amino groups ramaining after formaldehyde modification were able to undergo polymerization induced by MTGase. Therefore, the addition of MTGase improved the formation of cross-linking of NAM modified by

formaldehyde. However, the reactivity of those modification proteins to MTGase was dependent on fish species.

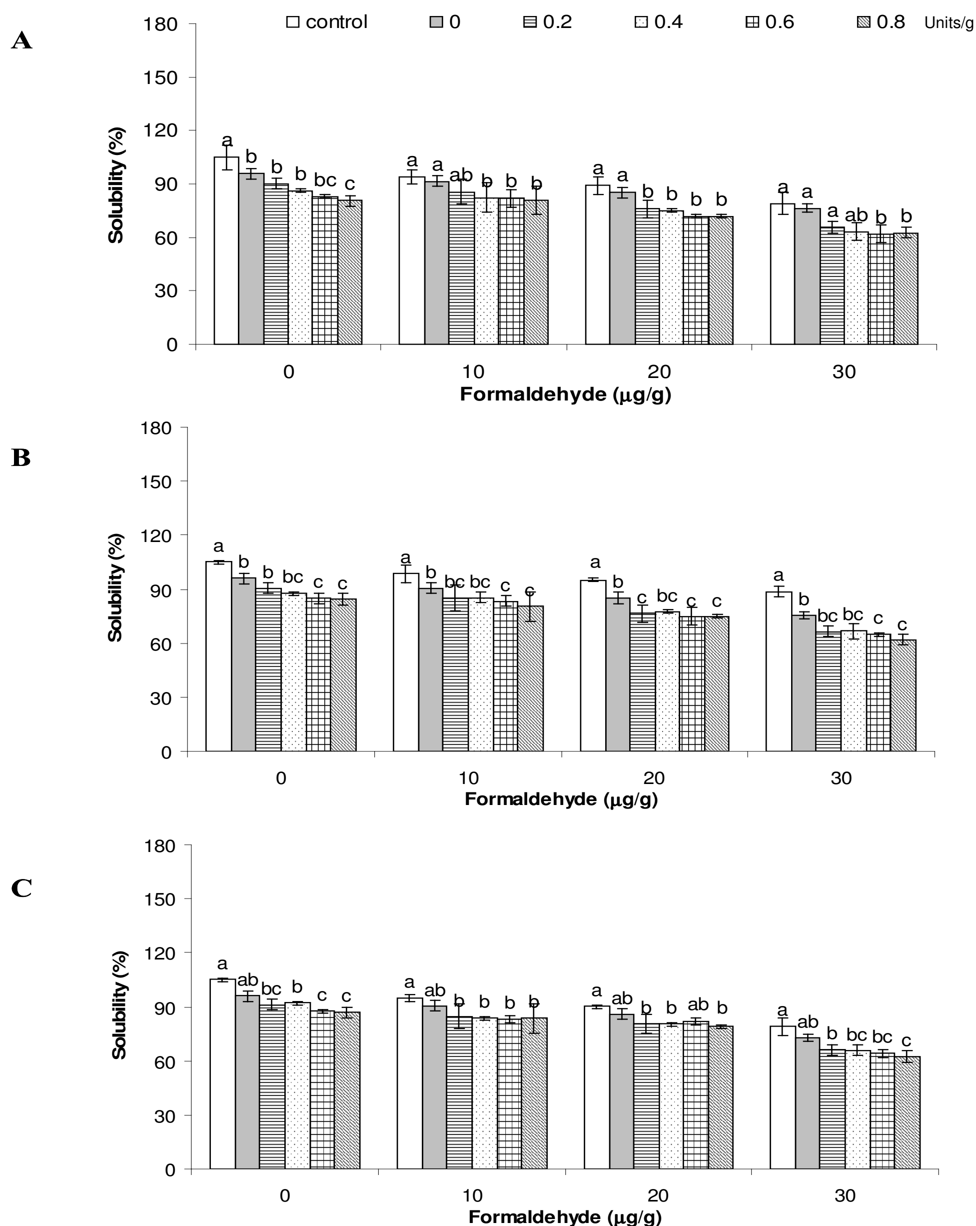

Figure 30 Changes in solubility of some marine fish and shrimp NAM added with different levels of formaldehyde as influenced by MTGase addition at different levels. After addition of formaldehyde, the sample was added with MTGase at different levels and incubated at 40°C for 30 min. A: white shrimp, B: bigeye snapper, C: lizardfish and Control: NAM pretreated with formaldehyde at different levels without subsequent incubation at 40°C for 30 min. Bars represent the standard deviation from triplicate

determinations. Different letters within the same formaldehyde level indicate significant differences ($p<0.05$).

5.3 Changes in protein patterns

Figure 31 shows the protein patterns of NAM from different species added with different levels of formaldehyde followed by the addition of MTGase at different levels and incubation at 40°C for 30 min. With the addition of formaldehyde, it was noted that MHC band intensity decreased slightly as the formaldehyde content increased. The rate of decrease was found to be slightly different. During incubation at 40°C for 30 min, MHC band intensity decreased to some extent, indicating the role of endogenous TGase in cross-link of protein molecules. Endogenous TGase induced the non-disulfide covalent bond formation, especially ε-(γ-glytamyl)lysine linkage (Kumazawa *et al*, 1995). Thus, the loss in MHC during incubation at 40°C for 30 min without MTGase was possibly a result of endogenous TGase. This result reconfirmed the role of endogenous TGase in protein cross-linking during setting temperature (40°C). The result was in agreement with the higher decrease in solubility. In the presence of MTGase, a much higher decrease in MHC was obtained in all samples regardless of formaldehyde addition. Seguro *et al.* (1995) reported that MHC apparently decreased with increasing MTGase concentration, regardless of the grade and setting conditions. Therefore, MTGase still exhibited the cross-linking activity toward muscle proteins irrespective of the modification by formaldehyde. However, Asagami *et a1*. (1995) found that the effect of MTGase on quality improvement of surimi depended not only on the fish species but also on the freshness of the fish used. The result suggested that formaldehyde induced the aggregation of protein substrate, but did not much decrease the substrate reactivity for MTGase.

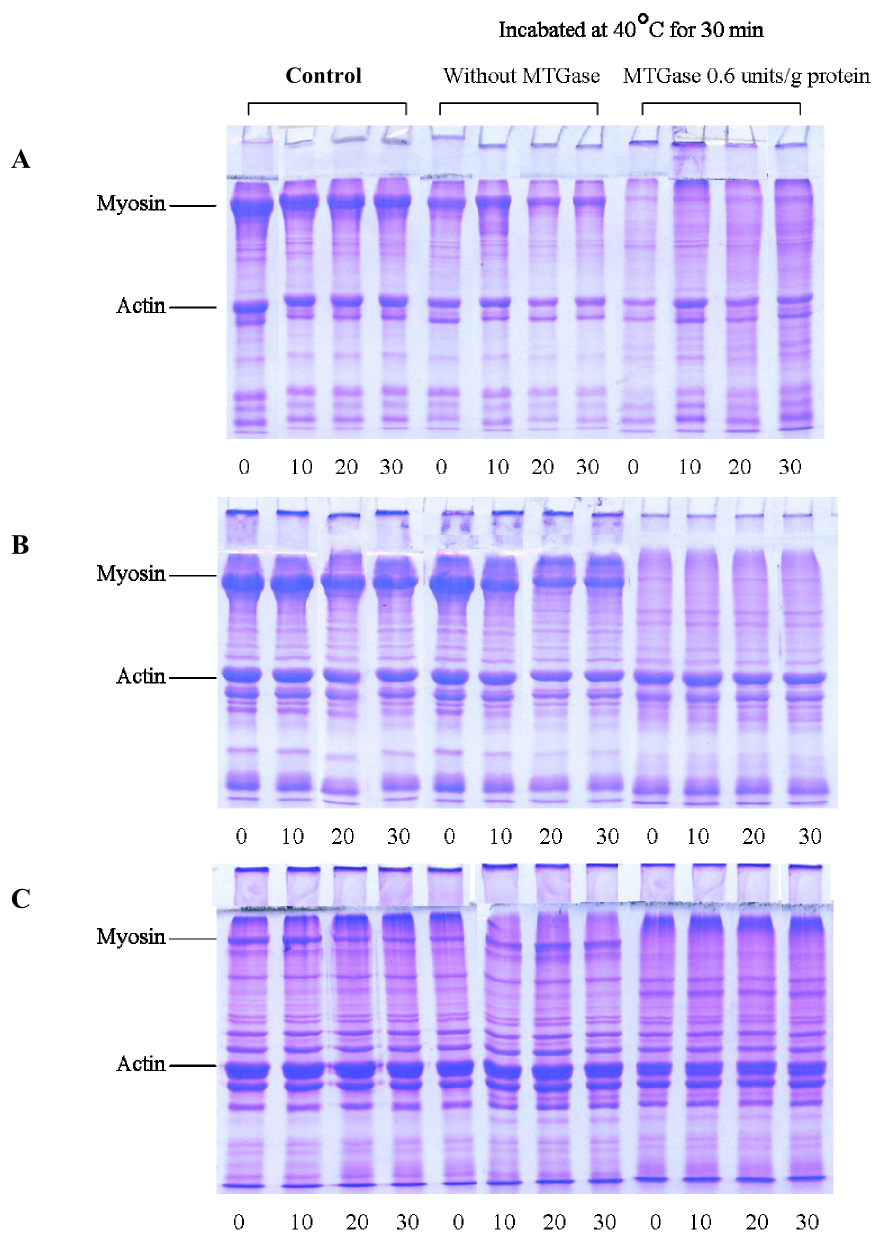

Figure 31 Protein patterns of some marine fish and shrimp NAM added with different levels of formaldehyde as influenced by MTGase addition at different levels. After addition of formaldehyde, the sample was added without and with MTGase at a level of 0.6 units/g protein and incubated at 40°C for 30 min. A: white shrimp, B: bigeye snapper, C: lizardfish and Control: NAM pretreated with formaldehyde at different

levels without subsequent incubation at 40°C for 30 min. Numbers designate levels of formaldehyde (μg/g).

5.4 Changes in sulfhydry group content

Total sulfhydryl group content of NAM from all species decreased when formaldehyde was added (Figure 32). The decreases in sulfhydryl content were more pronounced as the concentration of formaldehyde increased. Similar results were found for all species. The decrease in sulfhydryl groups generally resulted from the formation of disulfide bonds through oxidation of sulfhydryl groups or disulfide interchanges (Hayagawa and Nakai, 1985). The decrease in the sulfhydryl group content might be explained by the following two reasons: the sulfhydryl groups of inter-or intra-proteins formed cross-linkage (Huidobro *et al.*, 1998), or the exposed sulfhydryl groups in protein interacted with additives or small molecular weight compounds (e.g., peptides) in the water-soluble protein fraction (Owusu-Ansah and Hultin, 1987). Therefore, it is postulated that conformational changes of myosin, especially in the head region, occurred with the addition of formaldehyde. A higher content of formaldehyde could induce the conformational changes of proteins, in which SH groups were exposed to oxidation. Nevertheless, the aggregation induced by formaldehyde might mask the reactive SH group for determination. Formaldehyde is known to bind to various groups of proteins, including primary amino and imidazole groups (Ohba, *et al.*, 1979). The formaldehyde has been proposed to form cross-links with proteins, thus resulting in aggregation and toughening of fish muscle. After incubation at 40°C for 30 min, all NAM samples with and without formaldehyde addition had the decrease in SH group content. From the result, a greater decrease in sulfhydryl groups tended to be found in NAM from all species added with MTGase at higher levels and incubated at 40°C for 30 min. It was shown that MTGase preferentially cross-linked connectin, followed by MHC, troponin T and actin respectively (Nakahara *et al.*, 1999). In addition to the polymerization of myosin, MTGase produced highly polymerized forms of myofibrillar proteins, such as heteropolymers between connectin and actin. Therefore, MTGase not only induced the cross-linking of proteins, but also enchanced the oxidation of SH groups, irrespective of formaldehyde modification. This might be due to the remaining reactive SH groups could undergo the cross-linking reaction induce by MTGase.

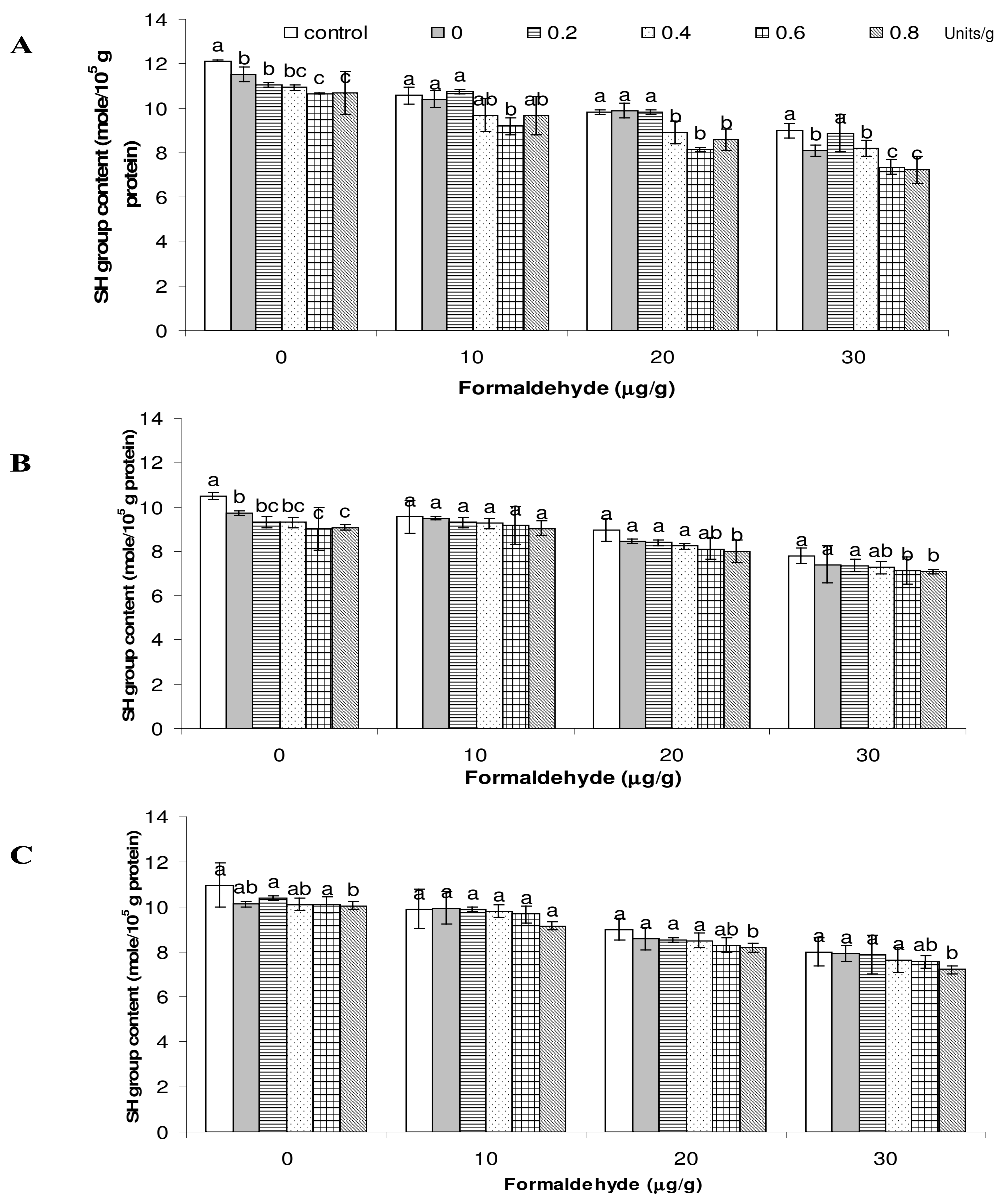

Figure 32 Changes in sulhydryl group content of some marine fish and shrimp NAM added with different levels of formaldehyde as influenced by MTGase addition at different levels. After addition of formaldehyde, the sample was added with MTGase at different levels and incubated at 40°C for 30 min. A: white shrimp, B: bigeye snapper, C: lizardfish and Control: NAM pretreated with formaldehyde at different levels without subsequent incubation at 40°C for 30 min. Bars represent the standard

deviation from triplicate determinations. Different letters within the same formaldehyde level indicate significant differences ($p<0.05$).

5.5 Changes in disulfide bond content

The disulfide bond content of NAM from all species increased as the formaldehyde was added (Figure 33). The formation of disulfide bond increased when the concentration of formaldehyde increased ($p<0.05$). It was noted that lizardfish contained the highest content of disulfide bond, followed by bigeye snapper and white shrimp, respectively. Addition of formaldehyde possibly accelerated aggregation/denaturation of protein molecules, leading to the conformational changes, in which the reactive sulfhydryl groups were exposed to oxidation easily. This might result in the increased disulfide bond formation. From the result, disulfide bond content of NAM from bigeye snapper increased with increasing MTGase and incubated at 40°C for 30 min, regardless of formaldehyde addition. The highest content of disulfide bond was found in NAM added with MTGase at the highest concentration. The addition of MTGase increased the formation of disulfide bond in bigeye snapper NAM to a higher extent, compared to that of other species. No marked changes in disulfide bond content were noticeable in NAM from white shrimp and lizardfish as MTGase added increased ($p>0.05$). The results suggested that the configuration of protein molecules was essential for MTGase induced reaction. The reaction with MTGase proceeds through an acyl-enzyme intermediate, in which an active site sulthydryl group forms a thio-ester with the glutamyl group of the substrate, and the active acyl intermediate then reacts with an adjacent amino group (Finch, 1983). Thus, the enzyme functions as a zero-length cross-linker between the amino groups involved, and can fail to induce any residue due to the enzyme modification between the inter-linked amino groups (Wong, 1991). The results indicated that reaction of MTGase in NAM might induce the formation of disulfide, regardless of formaldehyde. However, this effect varied with the source of NAM.

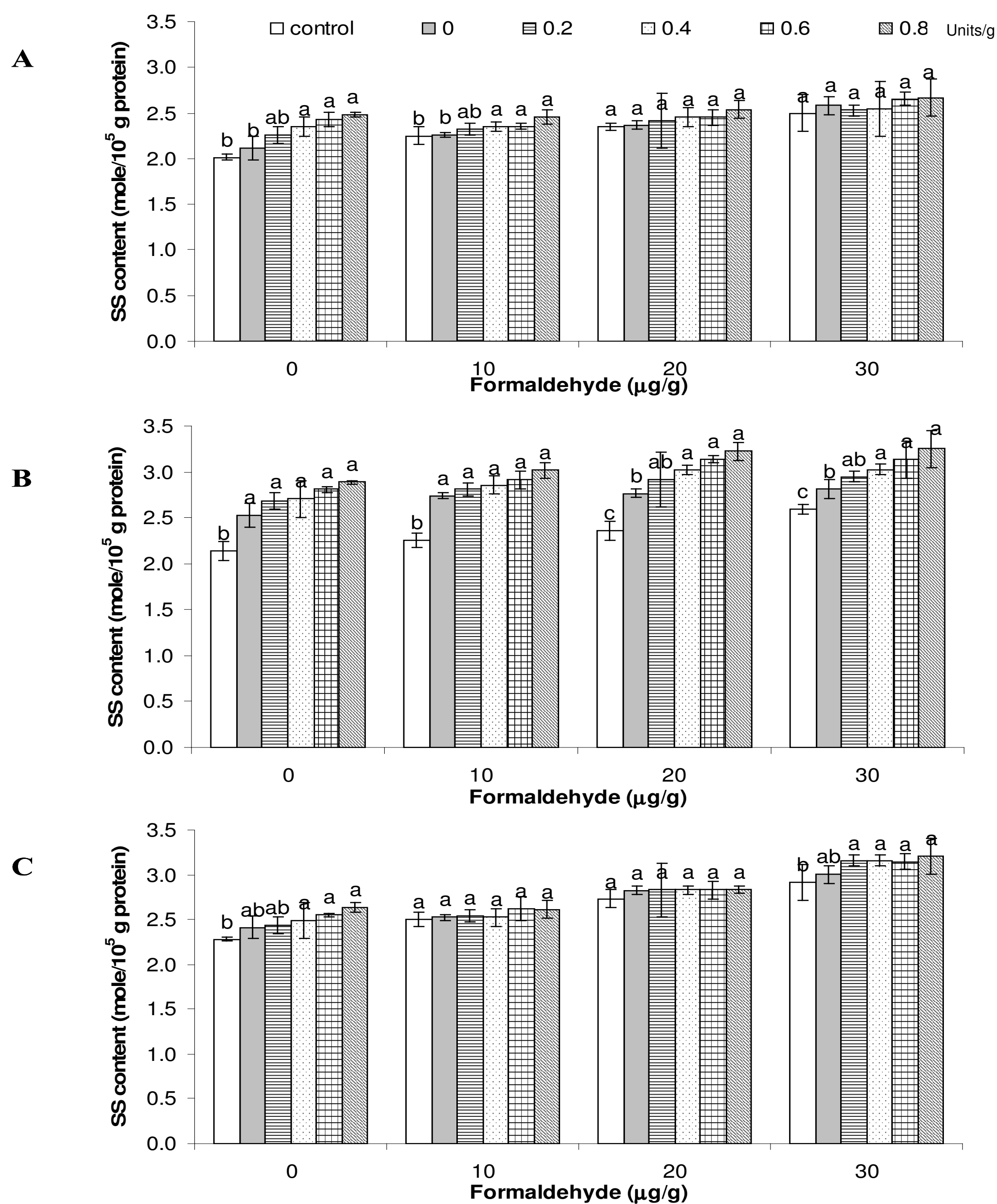

Figure 33 Changes in disulfide bond content of some marine fish and shrimp NAM added with different levels of formaldehyde as influenced by MTGase addition at different levels. After addition of formaldehyde, the sample was added with MTGase at different levels and incubated at 40°C for 30 min. A: white shrimp, B: bigeye snapper, C: lizardfish and Control: NAM pretreated with formaldehyde at different

levels without subsequent incubation at 40°C for 30 min. Bars represent the standard deviation from triplicate determinations. Different letters within the same formaldehyde level indicate significant differences ($p<0.05$).

5.6 Changes in surface hydrophobicity

Surface hydrophobicity of NAM from all species added with formaldehyde as influenced by MTGase addition is shown in Figure 34. A slight increase in surface hydrophobicity was observed in NAM added with different levels of formaldehyde. Higher surface hydrophobicity was found with increasing formaldehyde concentration added. Increased surface hydrophobicity indicates an exposure of the interior of the molecule due to denaturation or degradation (Multilangi *et al.,* 1996). Increase in surface hydrophobicity of proteins added with different levels of formaldehyde can be attributed to the unfolding of proteins and the exposure of hydrophobic aliphatic and aromatic amino acids. With the addition of MTGase and incubate at 40°C for 30 min, no marked increase in surface hydrophobicity was observed. Careche and Li-Chen (1997) suggested that in the presence of formaldehyde during frozen storage of cod myosin, more extensive protein denaturation results in more interactions between hydrophobic groups and formation of covalent bonds. From the result, the addition of MTGase generally caused no changes in surface hydrophobic of NAM from all species, irrespective of formaldehyde formation.

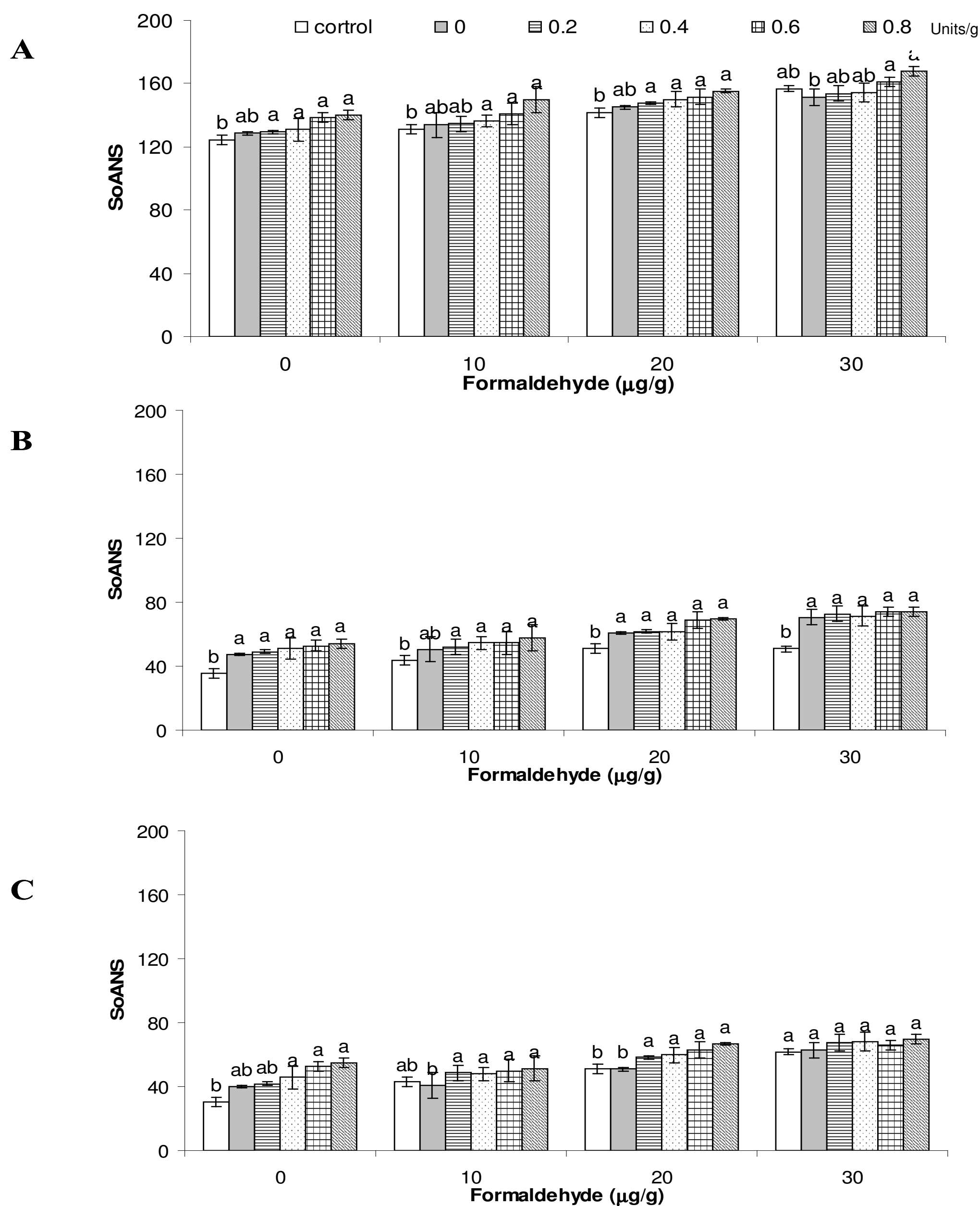

Figure 34 Changes in surface hydrophobicity of some marine fish and shrimp NAM added with different levels of formaldehyde as influenced by MTGase addition at different levels. After addition of formaldehyde, the sample was added with MTGase at different levels and incubated at 40°C for 30 min. A: white shrimp, B: bigeye snapper, C: lizardfish and Control: NAM pretreated with formaldehyde at different

levels without subsequent incubation at 40°C for 30 min. Bars represent the standard deviation from triplicate determinations. Different letters within the same formaldehyde level indicate significant differences ($p<0.05$).

6. Effect of MTGase on cross-linking and physicochemical properties of NAM with different degrees of hydrolysis

6.1 Changes in physicochemical properties of NAM with different degrees of hydrolysis

6.1.1 Changes in surface hydrophobicity

No changes in surface hydrophobicity of NAM from white shrimp with different degrees of hydrolysis (DH) were found (Figure 35). However, slight increase was noticeable with NAM from bigeye snapper and lizardfish when DH increased from 0 to 10%. Partial hydrolysis might contribute to the alteration of protein molecules, in which hydrophobic portions might be more exposed. Nevertheless, Mahmoud *et al*. (1992) reported that enzymatic hydrolysis decreased the hydrophobicity of casein protein. Thus, the hydrolysis caused by enzymatic reaction could affect the conformation differently, depending on species. This was most likely caused by the differences in composition of amino acid as well as structure of proteins from different sources.

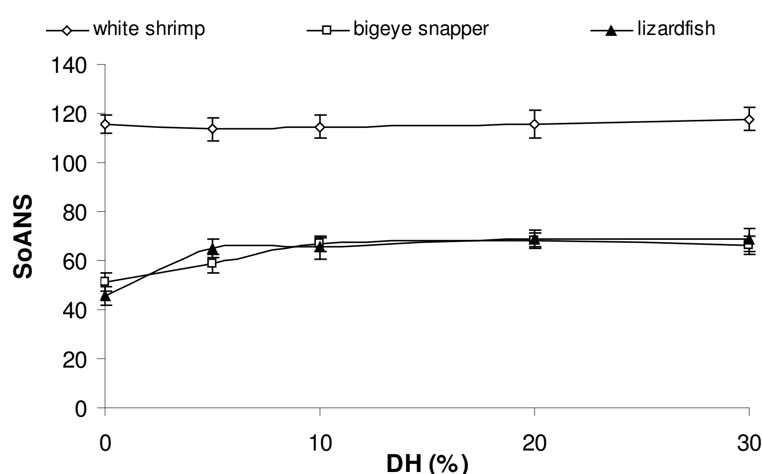

Figure 35 Surface hydrophobicity of NAM from some marine fish and shrimp with different degrees of hydrolysis. The hydrolytic reaction by trypsin was run at 37°C. Bars represent the standard deviation from triplicate determinations.

6.1.2 Changes in free amino group content

Figure 36 shows the free amino group content of NAM from all species with different DHs. Free amino group content increased gradually with increasing DHs up to 30%. However, the extended hydrolysis was not found as the amount of trypsin or reaction time increased (data not shown). Trypsins specifically hydrolyzes proteins and peptides at the carboxyl side of arginine and lysine residues and plays the major role in biological process including digestion (Cao *et al*., 2000). Wu *et al.* (2003) reported different levels of free amino acids, anserine and carnosine in mackerel hydrolysates derived from different enzymes during hydrolysis.

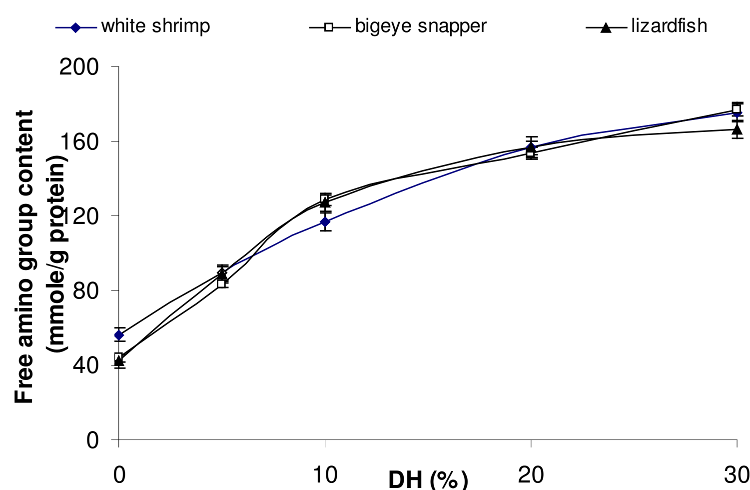

Figure 36 Free amino group content of NAM from some marine fish and shrimp with different degrees of hydrolysis. The hydrolytic reaction by trypsin was run at 37°C. Bars represent the standard deviation from triplicate determinations.

6.1.3 Changes in protein patterns

Protein patterns of NAM from all species subjected to hydrolysis at different degrees are shown in Figure 37. NAM, extracted from all species, contained MHC and actin as the major constituents. MHC and actin were susceptible to hydrolysis by trypsin used. Those proteins were degraded rapidly at 5% DH by trypsin. Total disappearance of MHC was observed after 5% DH. For actin, the degradation increased as DHs increased. From the result, NAM from bigeye snapper and lizardfish in this study contained the low content of MHC, suggesting the degradation took place before NAM extraction. Generally, myofibrillar proteins are susceptible to degradation by lysosomal enzymes and calcium-activated neutral proteinases (Ouali & Valin, 1981). Yamashita and Konagaya (1991) also reported that three myofibrillar components (α-actinin and troponinT and -I) were markedly degraded by salmon cathepsin B and L, along with the disappearance of myosin heavy and light chains. From the result, trypsin hydrolyzed myofibrillar proteins effectively, particularly MHC which is the dominant protein in fish muscle.

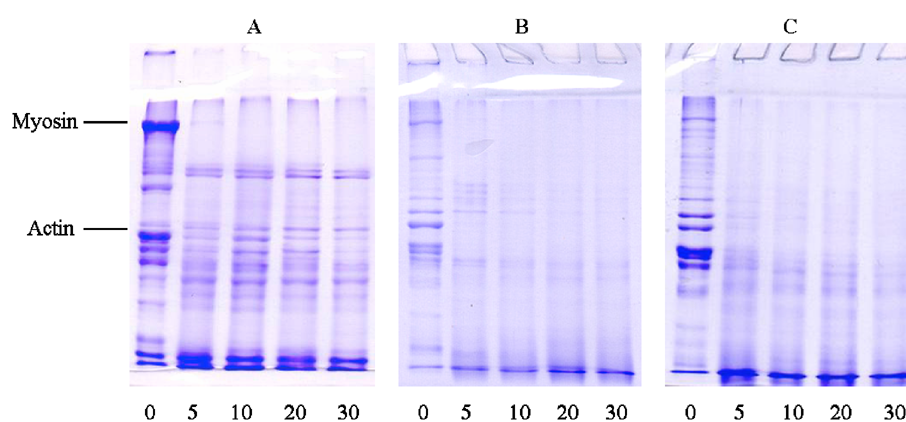

Figure 37 SDS-PAGE of NAM from some marine fish and shrimp with different degrees of hydrolysis. The hydrolytic reaction by trypsin was run at 37°C. A: white shrimp, B: bigeye snapper, C: lizardfish. Numbers designate the degrees of hydrolysis (%).

6.2 Effect of MTGase on cross-linking and physico-chemical properties of NAM with different degrees of hydrolysis

6.2.1 Changes in free amino group content

For all NAM with different DHs, free amino group content was not affected by the addition of MTGAse even at the higher levels. However, the decrease in free amino group content was found in NAM without hydrolysis, especially with increasing MTGase added. Therefore, the chain length and conformation of myofibrillar proteins determined the reactivity of MTGase. The addition of MTGase could not improve the cross-linking of hydrolyzed NAM. The results clearly indicated that hydrolysis substantially decreased the ability of MTGase in cross-linking NAM. The results suggested that the presence of the native myosin structure with sufficient chain length was essential for MTGase to maximize the setting response. Therefore the degraded NAM could not develop an elastic matrix even with an excessive amount of MTGase added. The cross-linking and setting response were directly governed by the addition of MTGase.

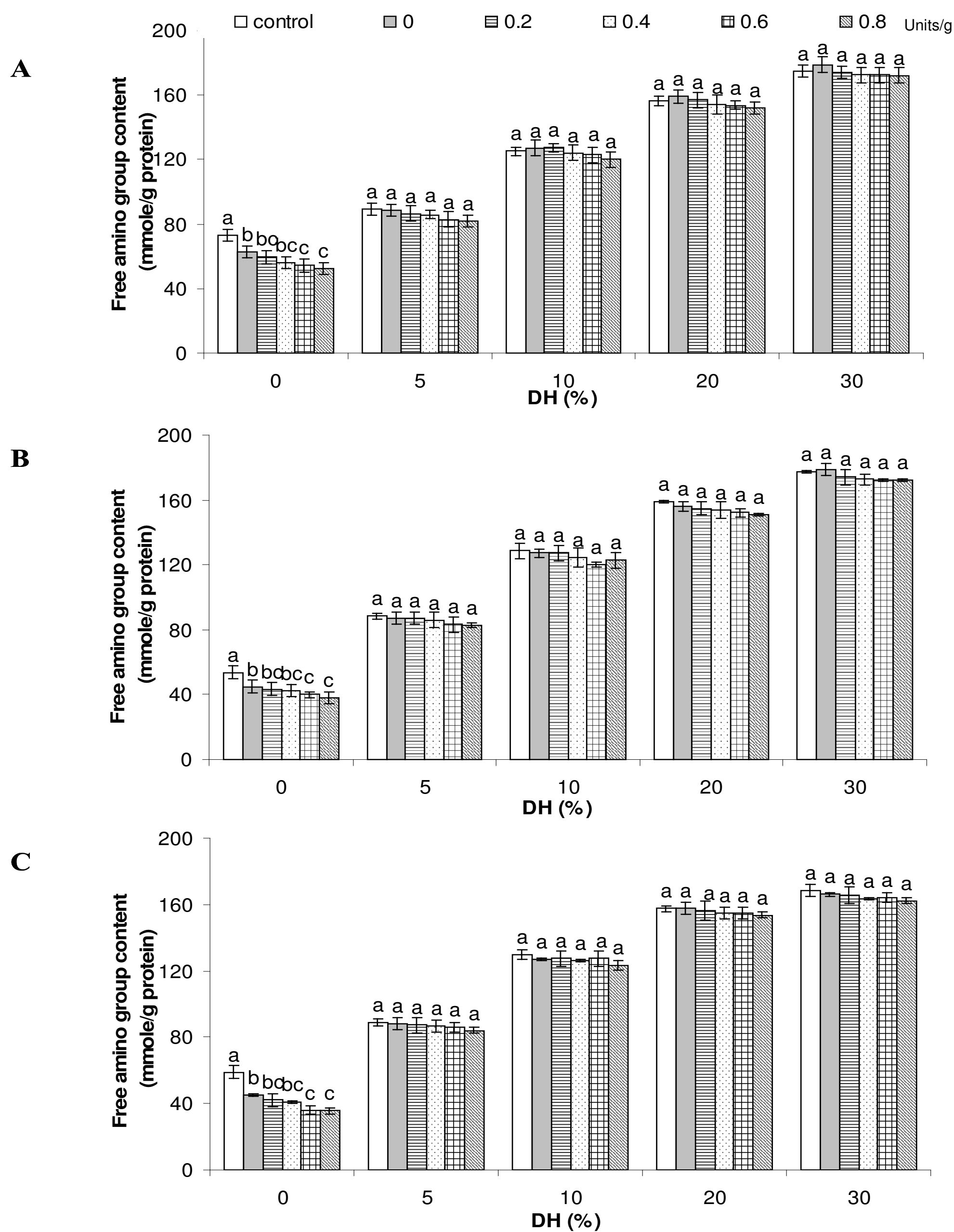

Figure 38 Changes in free amino group content of NAM from some marine fish and shrimp as influenced by different degrees of hydrolysis. After hydrolysis, the sample was added with MTGase at different levels and incubated at 40°C for 30 min. A: white shrimp, B: bigeye snapper, C: lizardfish and Control: NAM with different degrees of hydrolysis without subsequent incubation at 40°C for 30 min. Bars represents the

standard deviation from triplicate determinations. Different letters within the same degrees of hydrolysis indicate significant differences ($p<0.05$).

6.2.2 Changes in solubility

Solubility of NAM from all species in 0.6 M KCl increased continuously as the degrees of hydrolysis increased regardless of MTGase addition as shown in Figure 39. The proteins with the shorter chain length normally are more soluble than the longer chain. Hydrolysis produced the lower MW peptides, particularly with the higher degree of hydrolysis. The enhanced solubility of the hydrolysates could be attributed to: (1) demolishment of the tight structure of the proteins and large peptides by enzymatic hydrolysis and heat treatment; (2) decrease in the molecular size of polypeptide chains; and (3) exposure of more charged and polar groups to the surrounding water (Guan et al., 2006). It has been also reported that trypsin-hydrolyzed oat protein concentrate exhibits a broad solubility curve with greatly improved solubility (Ma, 1985). With the native protein (DH=0%), the lower solubility was found when MTGase at the higher levels was added. However, no changes in solubility were noticeable with hydrolyzed NAM, even with the higher amount of MTGase. The result indicated that the addition of MTGase could not apparently induce a setting effect in proteins, which underwent hydrolysis. Normally, MTGase catalyzes a reaction to incorporate a variety of primary amines covalently into reactive glutamine residues on the surface of substrate proteins, as well as the cross-linking of polypeptides through the formation of isopeptides between lysine and glutamine residues (Folk, 1970). The rate and extent of the incorporation were elevated with increasing MTGase concentration and incubation time (Maruyama *et al*., 1995). From the result, the polymerization of hydrolyzed NAM by MTGase was impeded. The enzyme-induced polymerization was likely dependent on the cahin length and conformation of protein substrates. Generation of peptide fragments increases the exposure of hydrophilic amino acid residues and the exposure of amino and carboxyl groups may account for the observed increase in solubility of the hydrolyzed samples (Hayakawa and Nakai, 1985; Panyam and Kilara, 1996). Thus, MTGase might not be able to increase the cross-linking of hydrolyzed NAM, particularly those with the greater extent of hydrolysis.

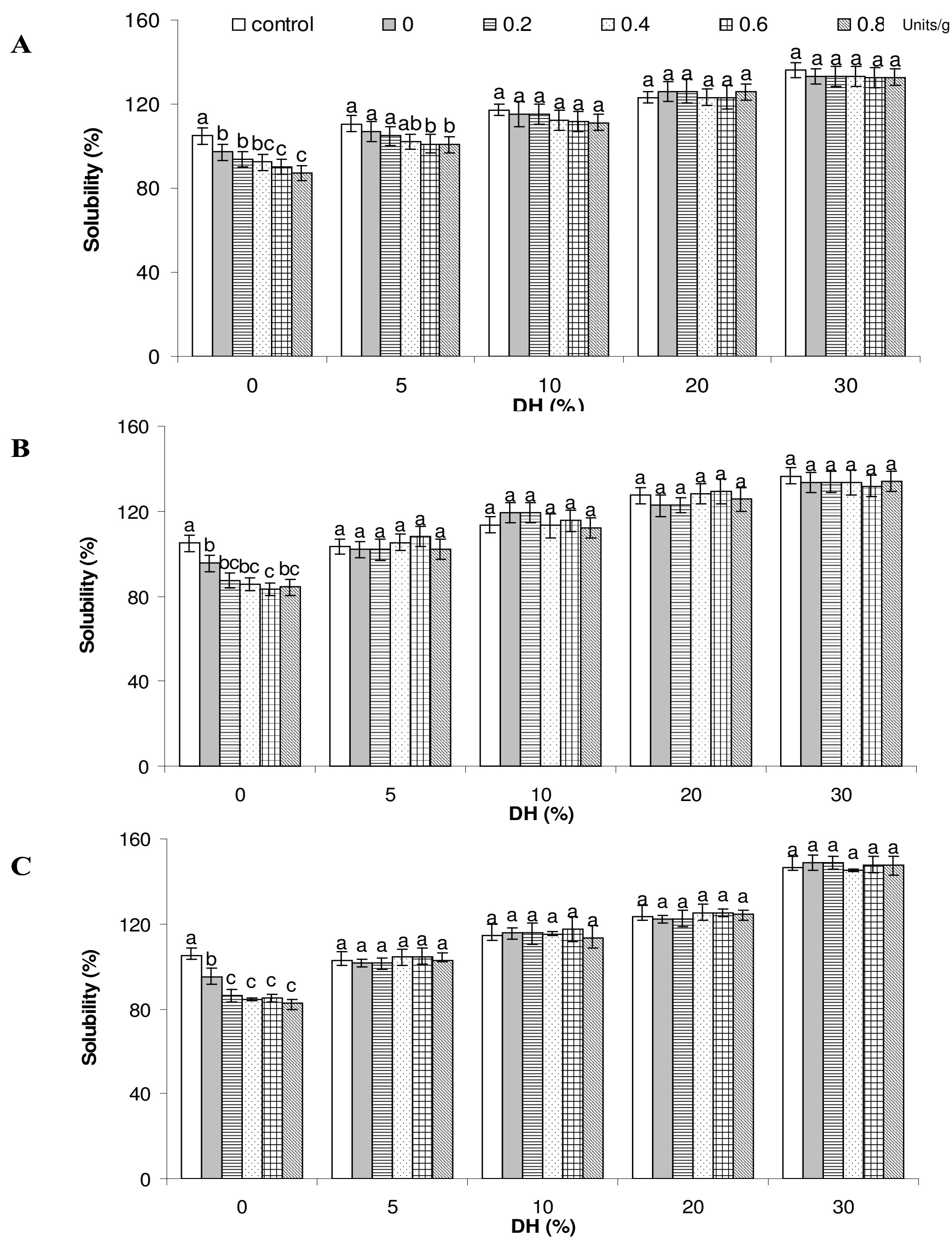

Figure 39 Changes in solubility of NAM from some marine fish and shrimp as influenced by different degrees of hydrolysis. After hydrolysis, the sample was added with MTGase at different levels and incubated at 40°C for 30 min. A: white shrimp, B: bigeye snapper, C: lizardfish and Control: NAM with different degrees of hydrolysis without subsequent incubation at 40°C for 30 min. Bars represents the standard deviation

from triplicate determinations. Different letters within the same degrees of hydrolysis indicate significant differences ($p<0.05$).

6.2.3 Changes in protein patterns

Protein patterns of NAM from all species with different degrees of hydrolysis with and without MTGase addition (0.6 units/g proteins) are shown in Figure 40. It was observed that hydrolysis markedly change the protein patterns of NAM. After incubation at 40°C for 30 min, MHC and actins of all NAM with no hydrolysis decreased markedly, suggesting the role of endogenous TGase in cross-linking during setting at 40°C. With addition of MTGase, no changes in protein patterns of NAM with different DHs were observed. This indicated that the small peptides and amino acids were not the good substarte for MTGase. From the result, NAM without hydrolysis and added with MTGase showed the disappearance of bands corresponding to the myosin along with the coincidented appearance of high molecular mass polymerized proteins on the stacking gel. Additionaly, actin also underwent cross-linking induced by MTGase. The results indicated that MTGase catalyzed the transfer reaction between an amide group in a protein-bound glutamine and an ε-amino group in a protein-bound lysine side-chain, resulting in cross-links between NAM. Thus, it was confirmed that peptide chain length and conformation of protein substrate were essential for cross-linking activity of MTGase.

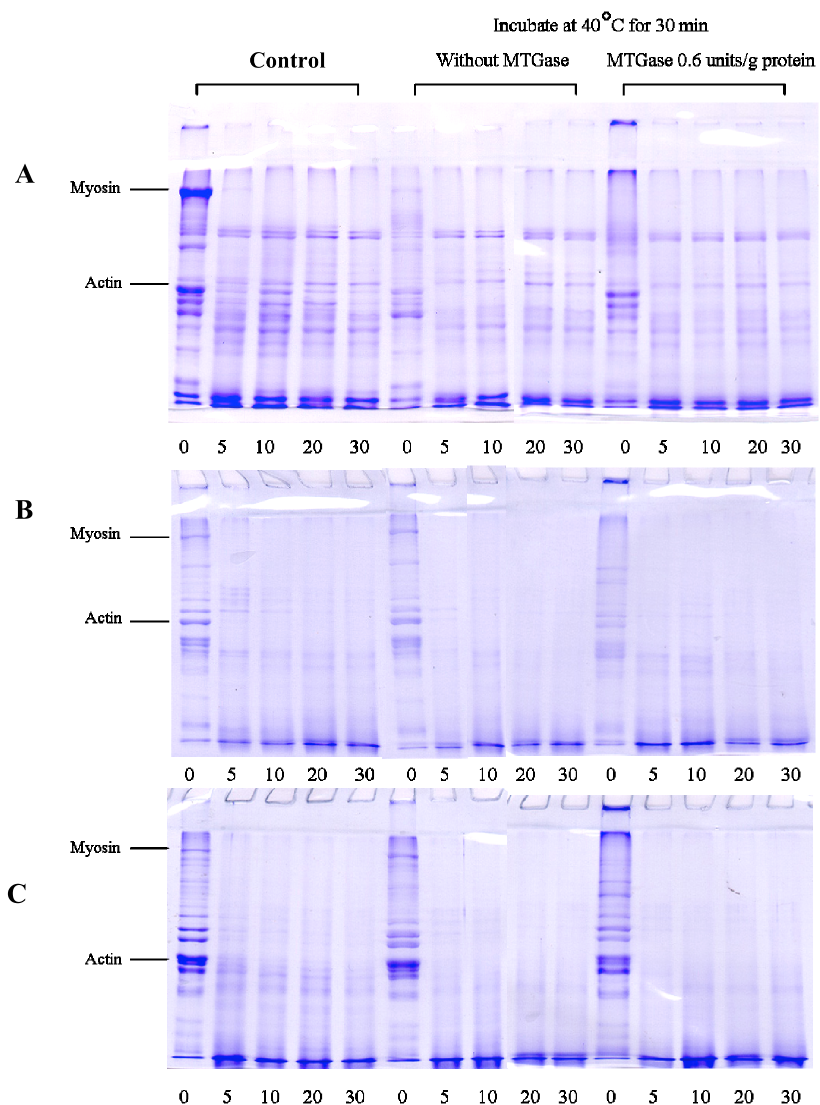

Figure 40 Protein patterns of NAM hydrolysate from some marine fish and shrimp with different degrees of hydrolysis added without and with MTGase at different levels and incubated at 40°C for 30 min. A: white shrimp, B: bigeye snapper, C: lizardfish and Control: NAM with different degrees of hydrolysis without subsequent incubation at 40°C for 30 min. Numbers designate the degrees of hydrolysis (%).

6.2.4 Changes in sulfhydryl group content

Figure 41 depicts the sulfhydryl group content in NAM with different DHs added with different levels of MTGase and incubate at 40°C for 30 min. From the result, slight decreases in sulfhydryl group content were observed with increasing DHs. This suggested the oxidation of sulfhydryl groups tended to be increased after hydrolysis. The cleaved peptides might have more flexible structure and the oxidation of reactive sulfhydryl groups could take place easily. However, the resultant position and number of active groups involved in the oxidation of sulfhydryl groups could be varied with species. With addition of different levels of MTGase, sulfhydryl group contents decreased slightly. Since MTGase could not induce the cross-linking of hydrolyzed NAM, the decrease in sulfhydryl group content might result from the oxidation during setting at 40 °C for 30 min.

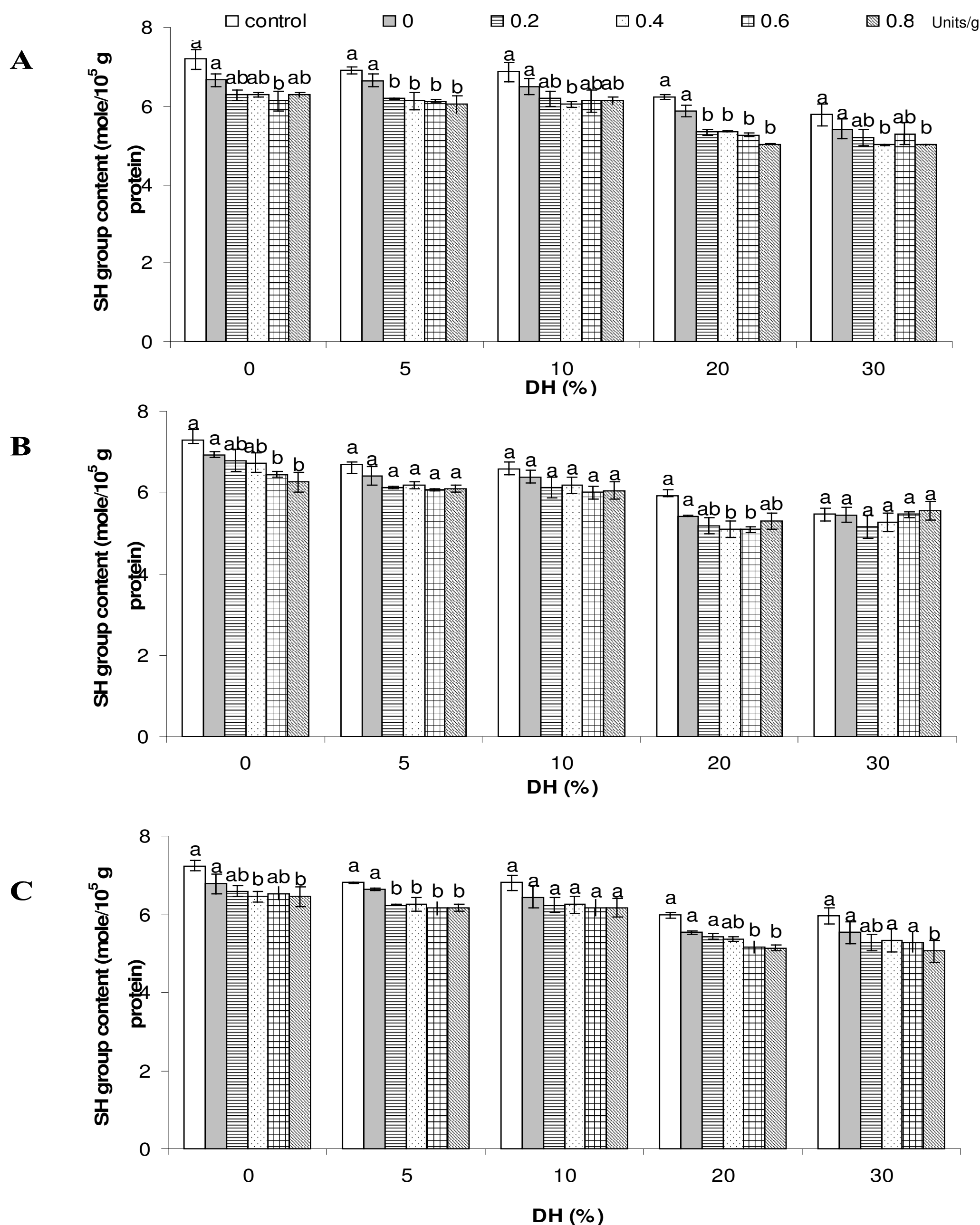

Figure 41 Changes in sulfhydryl group content of NAM from some marine fish and shrimp as influenced by different degrees of hydrolysis. After hydrolysis, the sample was added with MTGase at different levels and incubated at 40°C for 30 min. A: white shrimp, B: bigeye snapper, C: lizardfish and Control: NAM with different degrees of hydrolysis without subsequent incubation at 40°C for 30 min. Bars represents the

standard deviation from triplicate determinations. Different letters within the same degrees of hydrolysis indicate significant differences ($p<0.05$).

6.2.5 Changes in disulfide bond content

The disulfide bond content in NAM with different DHs added with different levels of MTGase and incubate at 40°C for 30 min is shown in Figure 42. With increasing DHs, disulfide bond formation tended to occur to a greater extent. Accelerated oxidation of sulhydryl group via some changes of protein conformation induced by hydrolysis might take place and free sulhydryl group in protein molecule could be oriented in the appropriate position for disulfide formation. At the same DH, disulfide bond content of NAM slightly increased with addition of higher level of MTGase. The increase in disulfide bond content was coincidental with the decrease in sulfhydryl group content (Figure 41). The decrease in total sulhydryl group was reported to be due to the formation of disulfide bonds through oxidation of sulhydryl groups or disulfide interchanges (Hayakawa and Nakai, 1985).

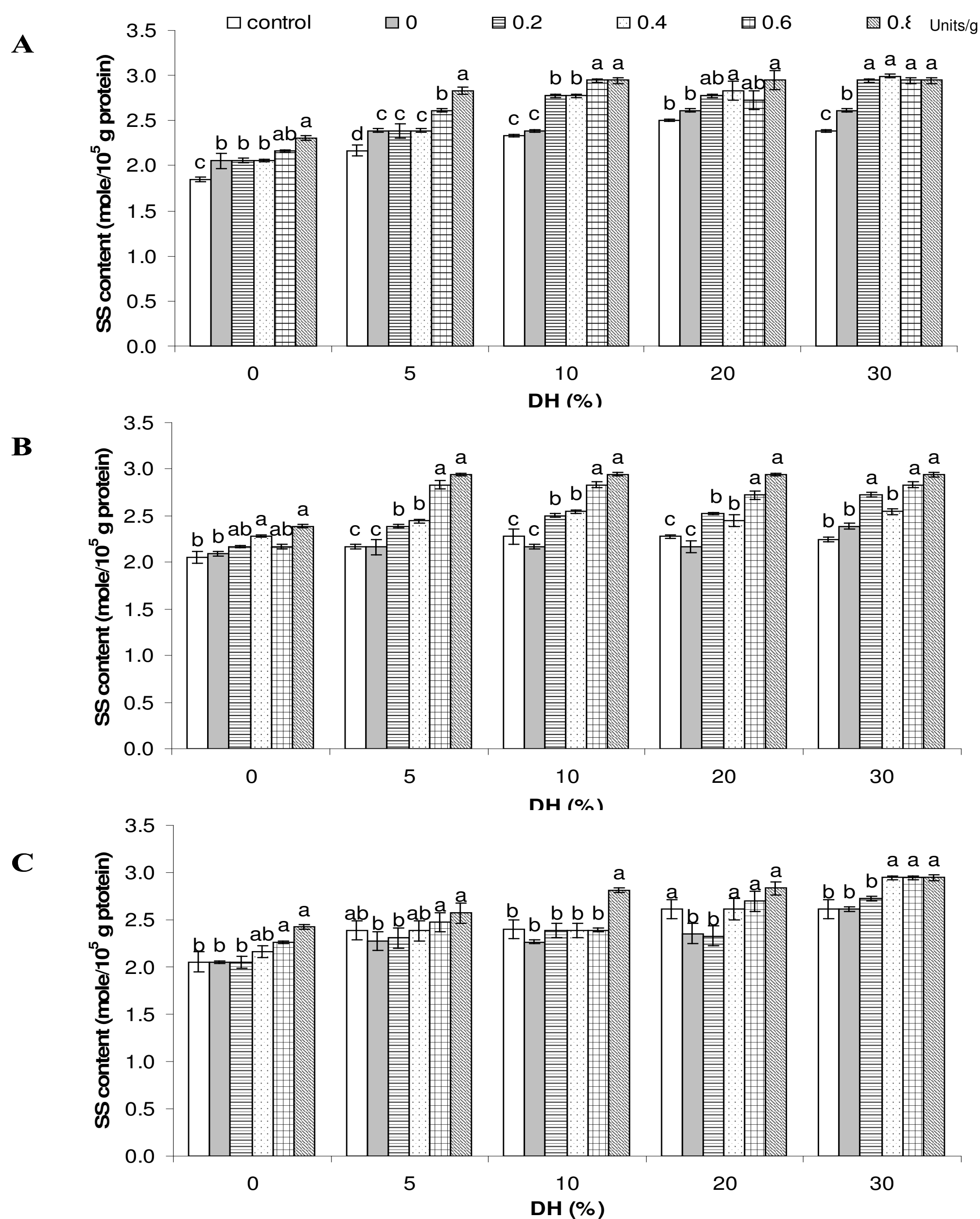

Figure 42 Changes in disulfide bond content of NAM from some marine fish and shrimp as influenced by different degrees of hydrolysis. After hydrolysis, the sample was added with MTGase at different levels and incubated at 40°C for 30 min. A: white shrimp, B: bigeye snapper, C: lizardfish and Control: NAM with different degrees of hydrolysis without subsequent incubation at 40°C for 30 min. Bars represents the

standard deviation from triplicate determinations. Different letters within the same degrees of hydrolysis indicate significant differences ($p<0.05$).

6.2.6 Changes in surface hydrophobicity

The surface hydrophobicity in NAM with different DHs added with different levels of MTGase and incubate at 40°C for 30 min is shown in Figure 43. NAM of all species had a slight increase in surface hydrophobicity when DHs increased. White shrimp NAM possessed higher surface hydrophobicity than NAM from other species at all DHs tested. From the result, MTGase at all concentrations used had no impact on the changes in surface hydrophobicity of all NAM, regardless of DHs.

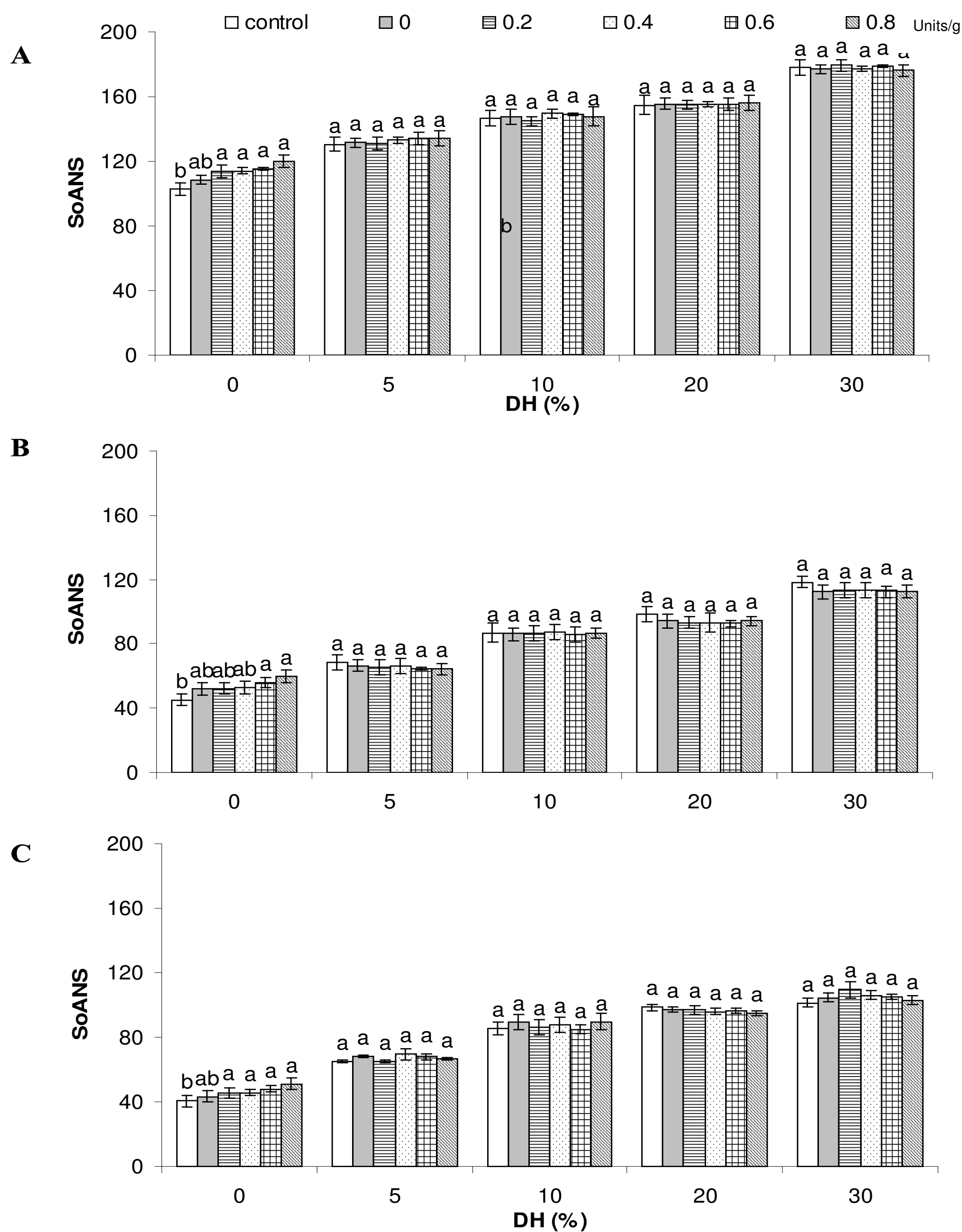

Figure 43 Changes in surface hydrophobicity of NAM from some marine fish and shrimp as influenced by different degrees of hydrolysis. After hydrolysis, the sample was added with MTGase at different levels and incubated at 40°C for 30 min. A: white shrimp, B: bigeye snapper, C: lizardfish and Control: NAM with different degrees of

hydrolysis without subsequent incubation at 40°C for 30 min. Bars represents the standard deviation from triplicate determinations. Different letters within the same degrees of hydrolysis indicate significant diff erences ($p<0.05$).