Chapter 3

RESULTS AND DISCUSSION

1. Characterization of acid soluble collagen from skin and bone from bigeye snapper

1.1 Compositions of skin, bone and their collagens

The compositions of bigeye snapper skin, bone and their collagen are shown in Table 5. Skin and bone contained a high moisture content (62.3-64.1%). Ash and fat contents of bone were greater than those of skin. Conversely, a lower protein content was observed in bone, compared with that in skin. Skin comprised the higher contents of hydroxyproline and collagen than bone. Sadawska *et al.* (2003) reported that hydroxyproline content of cod skin was 14.62 mg/g sample, which was lower than that of bigeye snapper skin (19.53 mg/g sample). Different hydroxyproline content between two species might depend upon the species, environmental and body temperature of fish (Kijowski, 2001; Sikorski *et al*., 1990; Rigby, 1968). When collagen was extracted from the skin and bone, it was noted that higher yield (10.9% wet wt.) was obtained for skin, compared with that from bone (1.6% wet wt.). The yield of collagen extracted was coincidental with hydroxyproline and collagen contents found in the skin or bone. Extracted collagen from both skin and bone had a low content of ash and fat, indicating the efficacy of inorganic matters and fat removal, respectively. Collagen samples had the low moisture content with protein content ranging from 84.2 to 94.0%. Increase in hydroxyproline and collagen contents

in extracted collagen samples were generally accompanied with the increased protein content.

Table 5 Chemical compositions of skin, bone and their collagen from bigeye snapper.

Sample	Compositions* (% wet wt.)				Hydroxy proline (mg/g sample)	Collagen (mg/g sample)
	Moisture	Ash	Fat	Protein		
Descaled skin	64.08 ± 0.05	3.23 ± 1.41	0.98 ± 0.23	31.99 ± 0.19	19.53 ± 0.41	287.16 ± 6.06
Bone	62.27 ± 0.29	14.40 ± 0.68	8.77 ± 0.46	13.28 ± 0.43	5.71 ± 0.23	83.93 ± 3.35
Descaled skin collagen	7.06 ± 0.58	0.68 ± 0.09	0.33 ± 0.07	93.99 ± 0.75	58.45 ± 0.85	859.18 ± 12.52
Bone collagen	11.57 ± 0.43	0.88 ± 0.07	0.48 ± 0.11	84.17 ± 1.63	42.53 ± 0.94	625.15 ± 13.86

* Average ± SD from triplicate determination
** The conversion factor for calculating the collagen content from hydroxyproline was 14.7 (Sadowska *et al*., 2003).

From Table 5, the established conversion factor for calculating the collagen content from hydroxyproline was 14.7 (Sadowska *et al*., 2003). However, the conversion factor should be obtained from the amino acid composition of individual sample and used for precise calculation of collagen content in each sample. Therefore, the values presented might not be the true values.

1.2 Amino acid composition of skin and bone collagens

The amino acid composition expressed as residues per 1,000 total residues is shown in Table 6. Skin and bone collagens had glycine as the major amino acid. Relatively high contents of alanine, proline, glutamic acid and hydroxyproline in the descending order were observed. Amino acid compositions of skin collagen were

slightly different from those of bone collagen. Compared to bone collagen, it is noted that skin collagen contained higher amount of hydroxyproline, proline and arginine but lower content of glycine and hydroxylysine Glycine content in skin and bone collagens was approximately 30% of total amino acids. Generally, glycine represents nearly one-third of the total residues and occurs as every third residue in collagen except first 14 or so amino acid residues from the N-terminus and the first 10 or so from the C-terminus (Wong, 1989; Pearson and Young, 1989; Foegeding *et al*., 1996; Burghagen, 1999). The imino acid content of skin and bone collagens were 193 and 163 residues/1,000 residues, which were lower than that of porcine dermis collagen (220 residues/1,000 residues) (Ikoma *et al*., 2003). Foegeding *et al*. (1996) reported that fish collagens contain less imino acid content than mammalian collagens. The imino acid content of animal collagens correlated with their habitat (Rigby, 1968; Foegending *et al*., 1996). The degrees of hydroxylation of proline in skin and bone collagens were 39.9% and 41.1%, respectively. Skin and bone collagens had the degree of lysine hydroxylation of 24.4% and 44.4%, respectively. Oxidation of proline and lysine to their hydroxylated residues is catalyzed by proline hydroxylase and lysine hydroxylase, respectively (Wong, 1989; Pearson and Young, 1989; Foegeding *et al*, 1996; Burghagen, 1999). From the result, hydroxylation of proline and lysine in bone collagen was slightly greater than that in skin collagen. Hydroxylated proline plays a role in stabilizing the triple helix (Ramachandran, 1988) and hydroxylated lysine contributes to the formation and stabilization of cross-links, giving rise to complex, nonhydrolysable bonds (Stimler and Tanzer, 1977; Asghar and Henrickson, 1982). The results suggested that collagen from bone might had

slightly complicated structure than that from skin as evidenced by higher degree of hydroxylation.

Table 6 Amino acid composition of skin and bone collagens from bigeye snapper (residue/1,000 residues).

Amino acids	Skin collagen	Bone collagen
Aspartic acid + Asparagine	51	47
Hydroxyproline	77	68
Threonine	29	25
Serine	36	34
Glutamic acid + Glutamine	78	74
Proline	116	95
Glycine	286	361
Alanine	136	129
Valine	22	17
Methionine	12	8
Isoleucine	5	5
Leucine	24	25
Tyrrosine	4	2
Phenylalanine	15	12
Hydroxylysine	10	20
Lysine	31	25
Histidine	10	6
Arginine	60	46

Total	1000	1000

1.3 SDS-polyacrylamide gel electrophoresis patterns of skin and bone collagens

The electrophoretic patterns of skin and bone collagens under reducing and non-reducing conditions are shown in Figure 17. No differences in electrophoretic patterns between skin and bone collagens were observed. Both skin and bone collagens comprised at least two different α chains, α1 and α2. The α3 chain, if present, could not be separated under the electrophoretic conditions employed because α3(I) migrates electrophoretically to the same position as α1(I) (Kimura and Ohno, 1987; Kimura, 1992; Matsui *et al*., 1991). The result was in agreement with those of skin and bone from other fish species (Piez, 1965; Kimura, 1985; Kimura and Ohno, 1987; Matsui *et al*., 1991; Ciarlo *et al*., 1997; Nagai and Suzuki, 2000a; Nagai and Suzuki, 2000b). The electrophoretic patterns of skin and bone collagens under non-reducing and reducing conditions were similar, indicating the absence of the disulfide bonds in those collagens. The result was in accordance with that previously reported from hake and trout collagens (Montero *et al*., 1990). Generally, type I collagen consisted of a low amounts of cysteine (~0.2%) and methionine (~1.24-1.33%) (Owusu-Apenten, 2002), which play an essential role in disufide bond formation. However, type III and IV collagens contain oxidizable cysteine residues (Foegeding *et al*., 1996). Based on electrophoretic mobility and subunit composition, it was suggested that collagens from skin and bone of bigeye snapper were type I collagens. Type I collagen consists of two identical α1 (I) chains and one α2 chain

(Wong, 1989; Pearson and Young, 1989; Foegeding *et al*., 1996; Burghagen, 1999). Fish skin and bone have been reported to contain type I collagen as the major collagen (Kimura and Ohno, 1987; Montero *et al*., 1990; Ciarlo *et al*., 1997; Nagai and Suzuki, 2000b).

From the result, both collagens were rich in inter- and intra-molecular cross-linked components, β and γ components. Starving fish contain more collagen with a greater degree of cross-linking than do fish that are well fed (Regenstein and Regenstein, 1991; Foegeding *et al*., 1996). Moreover, Love *et al*. (1976) reported that myocommata are thickened during starvation with a mechanically stronger collagen than was present when the fish were adequately nourished. Such collagen contains more intermolecular cross-links. The number of cross-links in mammalian animal collagen increases with increasing age. However, most connective tissue in fish is renewed annually and highly cross-linked protein is not generally found in fish (Foegeding *et al*., 1996; Zayas, 1997).

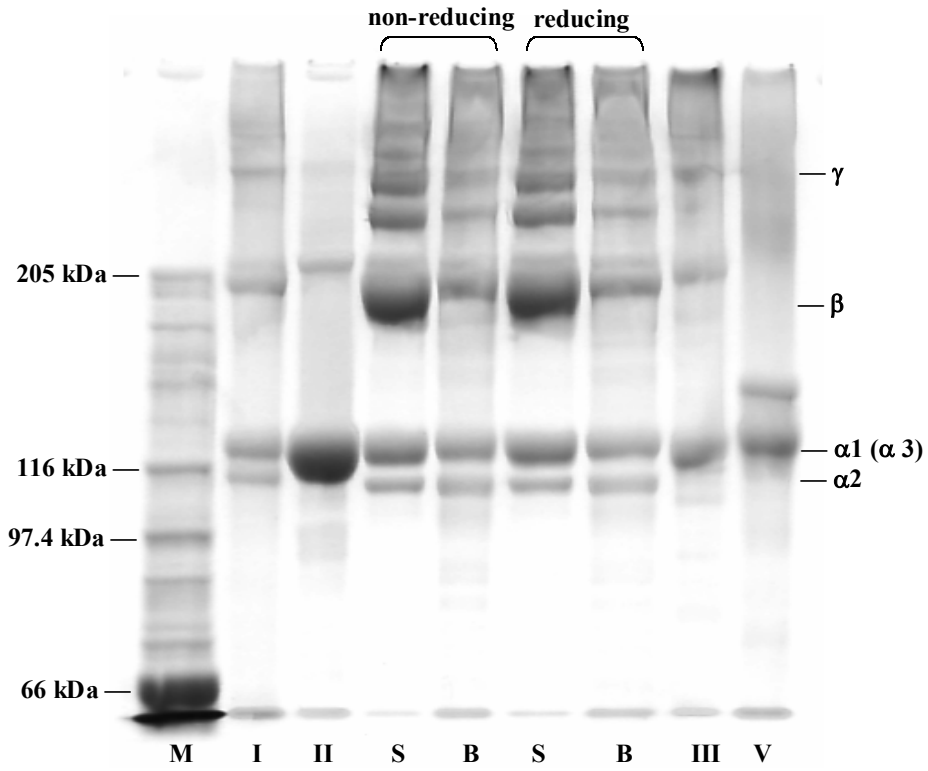

Figure 17 SDS-PAGE patterns of collagen from bigeye snapper skin (S) and bone (B) under reducing and non-reducing conditions. M, I, II, III and V denote high molecular weight protein markers, collagens type I, II, III and V collagen, respectively.

1.4 Peptide mapping of skin and bone collagens

The peptide maps of bigeye snapper skin and bone collagens digested by V8 protease and lysyl endopeptidase in comparison with calf skin collagen type I are shown in Figure 18. For peptide maps of collagens digested by V8 protease (Lane 5-7), the band intensity of molecular cross-linked components, β- and γ- components, of calf skin collagen slightly decreased with the appearance of 144.8 kDa peptide fragment. Additionally, α-components, α1 and α2, of calf skin collagen was hydrolyzed to some extent. For bigeye snapper collagens, α- and molecular cross-

linked components of bigeye snapper skin and bone collagens were markedly degraded after digestion with V8 protease. The result suggested that α- and molecular crosslinked components of calf skin collagen were more tolerant to digestion by V8 protease than bigeye snapper skin and bone collagens. V8 protease exhibits a high degree of specificity for glutamic acid and aspartic acid residues of peptides and proteins (Vercaigne-Marko *et al*., 2000). Thus, calf skin collagen might contain lower glutamic acid and aspartic acid residues than bigeye snapper skin and bone collagens. After digestion, bigeye snapper skin collagen was degraded into 3 major peptide fragments with MW of 53.3, 36.9 and 33.8 kDa, while bone collagen was degraded into 7 major peptide fragments with MW of 85.1, 77.9, 65.3, 59.0, 53.3, 36.9 and 33.8 kDa. When comparing peptide maps between bigeye snapper skin and bone collagens, similar peptide fragments with MWs ranging from 53.3 to 33.8 kDa were observed. However, fragments with MW larger than 59.0 kDa disappeared in bigeye snapper skin collagen.

For peptide maps of collagens digested by lysyl endopeptidase (Lane 8-10), all components of calf skin collagen were more hydrolyzed, compared to the hydrolysis with V8 protease as evidenced by the lower original band intensity of each components remained with the concomitant increase in lower MW peptide fragments. Molecular cross-linked components and α-components of calf skin collagen and bigeye snapper skin and bone collagens were susceptible to hydrolysis by lysyl endopeptidase especially, calf skin collagen and bigeye snapper bone collagen. The result suggested that those components of bigeye snapper skin collagen were more tolerant to digestion by lysyl endopeptidase than those of calf skin collagen and bone collagen. After digestion, peptide fragments of bigeye snapper skin and bone

collagens were quite similar, especially with MW ranging from 186.5 to 69.5 kDa. Nevertheless, calf skin collagen showed distinct peptide map from those of bigeye snapper skin and bone collagens. Thus, it was presumed that primary structure of bigeye snapper skin and bone collagens were quite similar in term of amino acid sequence. However, accessibility of susceptible bonds to the proteinase might be different, leading to varying degree of hydrolysis between collagens from bone and skin.

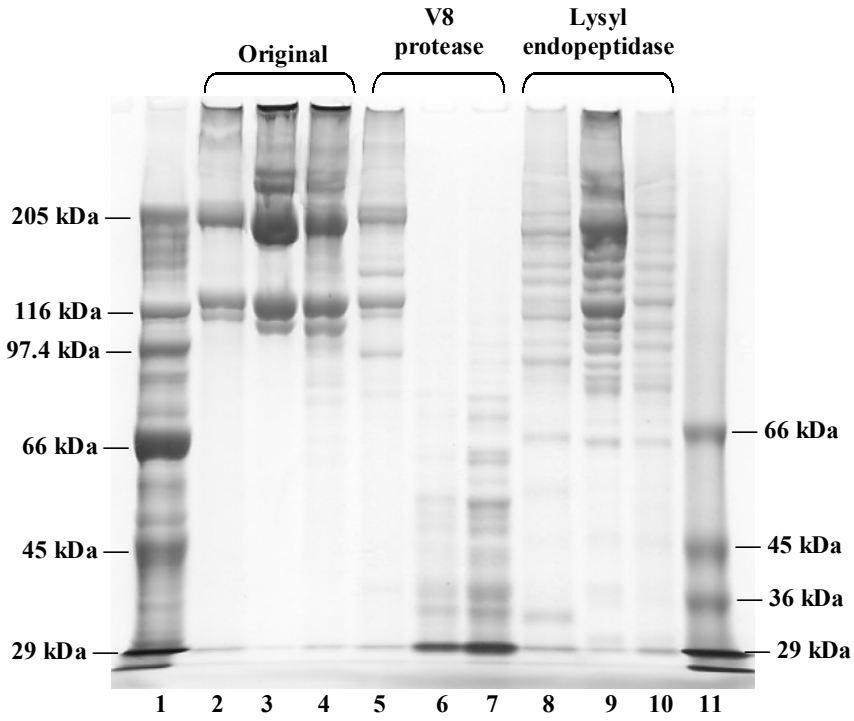

Figure 18 Peptide mapping of skin and bone collagens from bigeye snapper digested by V8 protease and lysyl endopeptidase. Lane 1 and 11: High and Low MW protein markers, respectively; lane 2-4: collagen type I, skin and bone collagens; lane 5-7: peptide fragments of collagen type I, skin and bone collagens with V8 protease digestion, respectively; lane 8-10: peptide fragments of collagen type I, skin and bone collagens with lysyl endopeptidase digestion, respectively.

1.5 Thermal stability of skin and bone collagens

Differential scanning calorimetry (DSC) thermograms of bigeye snapper skin and bone collagens rehydrated in 0.05 M acetic acid and deionized distilled water are shown in Figure 19. An endothermic peak with peak maximum temperature (T_{max}) of 31.0 and 31.5°C was observed for skin and bone collagens rehydrated in water, respectively. For collagens rehydrated in acetic acid, it was noted that T_{max} shifted to a lower temperature, 28.68 and 30.80°C for skin and bone collagens, respectively. Though T_{max} was generally higher for collagen from bone, either rehydrated in water or acetic acid, lower enthalpy was observed, compared with those of skin collagen. Calf skin collagen had an endothermic transition with T_{max} of 40.8°C and enthalpy of 2.83 J/g (Komsa-Penkova *et al*., 1999). The different T_{max} of transitions among collagen from animal species (fish and calf) seems to be correlated with the content of imino acids (proline and hydroxyproline). The higher the imino acid content, the more stable the helices are (Wong, 1989). The imino acid content of calf skin collagen is higher than fish skin collagen (Foegending *et al*., 1996). Moreover, collagen stability is correlated with environmental and body temperature (Rigby, 1968). Collagen from cold water fish has a low imino acid content (Piez and Gross, 1960). The increasing imino acid (proline and hydroxyproline) content resulted in increased denaturation temperature of collagen (Kimura, 1992; Yamaguchi *et al*., 1976; Gustavson, 1955; Piez and Gross, 1960).

From the result, transition temperature of collagen from skin and bone was similar. However, acetic acid played an essential role in the change in thermal properties of collagen, especially that from skin. Skin and bone collagens rehydrated in acetic acid had the decrease in T_{max} and enthapy by 2.32 and 0.68°C, respectively

and decreased in transition enthalpy (ΔH) about 0.07 and 0.33 J/g, respectively, when compared to those rehydrated in water. Acetic acid can cleave hydrogen bonds (Gustavson, 1956), which stabilizes collagen structure, triple-helical structure (Xiong, 1997). So collagen structure was broken, leading to the decreased thermal stability of collagens as shown by the lowered T_{max} and enthalpy.

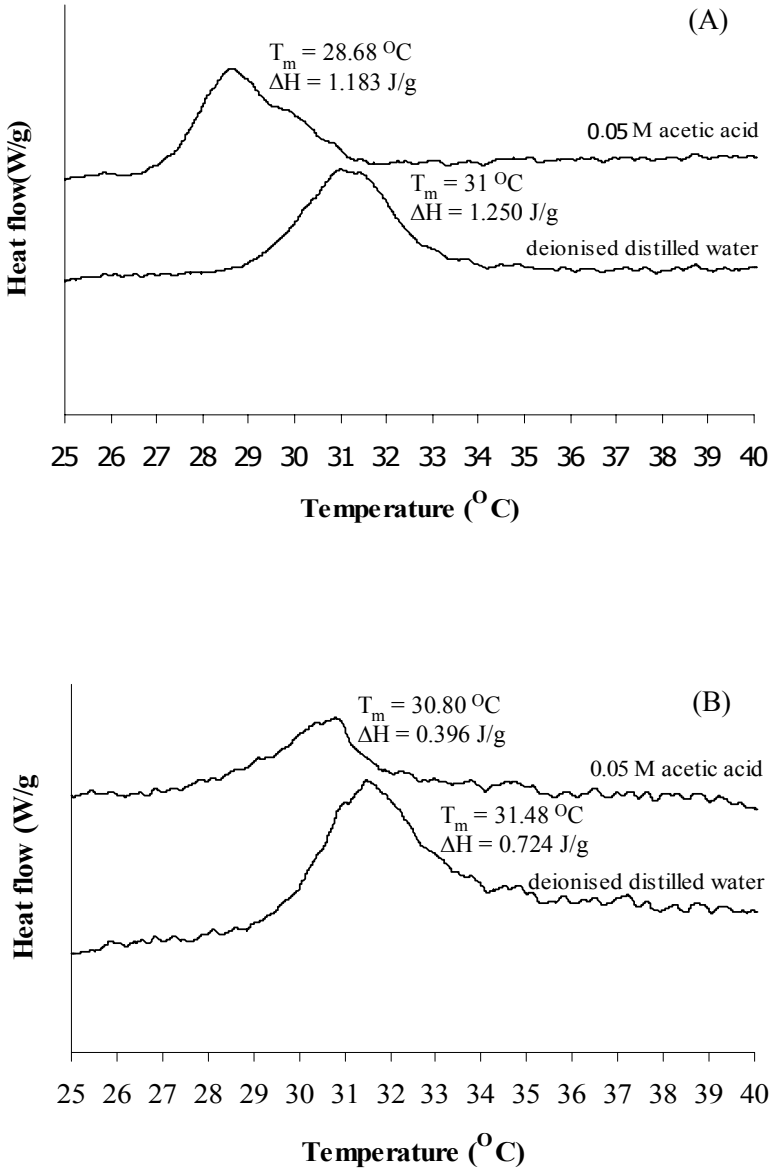

Figure 19 Thermograms of bigeye snapper skin (A) and bone (B) collagens rehydrated in 0.05 M acetic acid and deionized distilled water.

1.6 Viscosity of skin and bone collagen solutions

Relative viscosity of collagen in acetic acid subjected to heat treatment at different temperatures is depicted in Figure 20. Relative viscosity decreased continuously when heated up to 30°C. Rate of decrease was retarded in the temperature of range of 35-50°C. Heat treatment at high temperature can break down the hydrogen bonds, which stabilized collagen structure (Wong, 1989). Collagen denatures at temperatures above 40°C to a mixture of random-coil single, double and triple strands (Wong, 1989). From the result, similar changes in viscosity of skin and bone collagens caused by heat treatment were observed. Denaturation of collagen structure caused by heat treatment is associated with the changes in viscosity (Nagai *et al*., 1999; Nagai and Suzuki, 2000a; Nagai & Suzuki, 2002). Denaturation temperature of collagens from skin of Japanese sea-bass, chub mackerel, bullhead shark and ocellate puffer fish ranged from 25.0 to 28.0°C and that of collagen from bone of Japanese sea-bass, skipjack tuna, ayu, yellow sea bream, and horse mackerel was 29.5-30.0°C (Nagai and Suzuki, 2000a; Nagai and Suzuki, 2002).

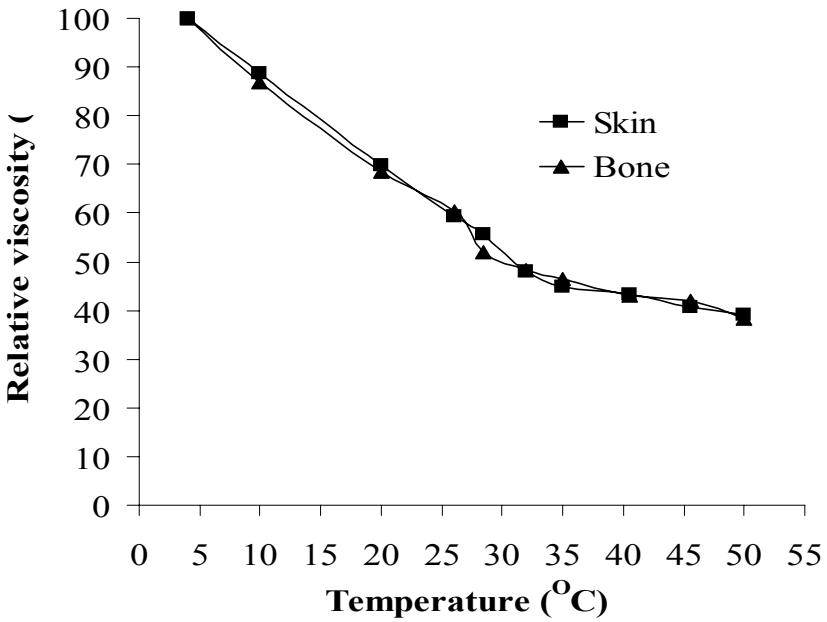

Figure 20 Relative viscosity of bigeye snapper skin and bone collagens solution at different temperatures.

1.7 Solubility of skin and bone collagens

The effects of pH and NaCl concentrations on collagen solubility are depicted in Figure 21 and 22, respectively. Highest solubility of skin and bone collagens was found at pH 2 and 5, respectively. Generally, both collagens could be more soluble in the acidic pH ranges (Foegending *et al*., 1996). The sharp decrease in solubility was observed in the neutral pH. However, solubility was slightly decreased at very acidic pH. When pH values are above and below pI, a protein has a net negative or positive charge, respectively (Vojdani, 1996). As a consequence, more water interacts with the charged proteins (Vojdani, 1996). Nevertheless, slight increase in solubility was found with the alkali pHs ranges. Charge repulsion contributes to greater protein solubility (Vojdani, 1996). The differences in pH maxima for solubility between skin and bone collagens suggested the differences in molecular properties and

conformation between both collagens. Skin collagen showed higher solubility than bone collagen at pH > 6 (Figure 21). This results indicated that skin collagen might possess a lower degree of cross-linking. The predominance of weaker bonds in skin collagen was also presumed in comparison with bone collagen.

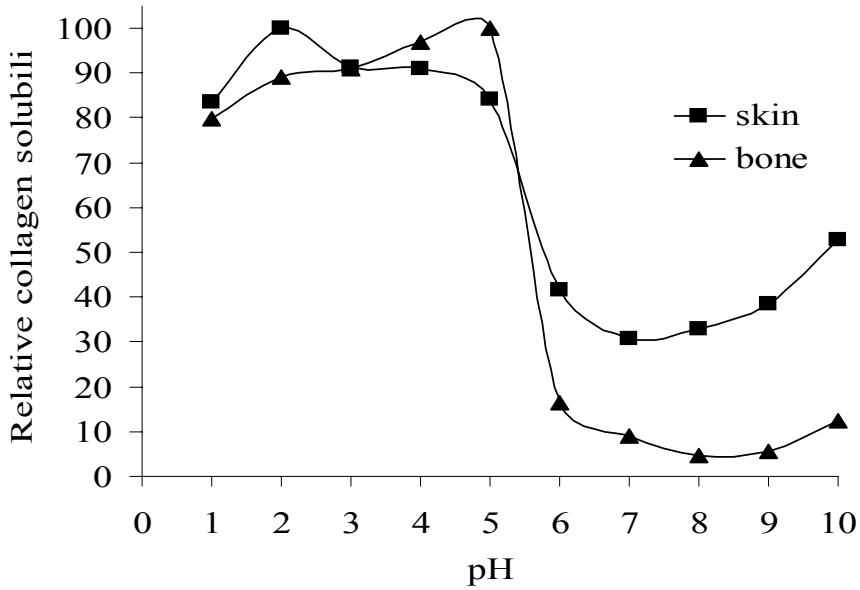

Figure 21 Relative collagen solubility (%) of bigeye snapper skin and bone collagens at different pHs.

The solubility in 0.5 M acetic acid of collagens from both skin and bone was maintained in presence of NaCl up to 3%. A marked decrease in solubility was observed with an increasing NaCl concentration, especially, at concentration above 3%. NaCl at a higher concentration might result in decreasing protein solubility (salting out effect) by increasing hydrophobic interaction and aggregation, and competing with the protein for water, thereby causing the protein to precipitate (Vojdani, 1996). From the result, bone collagen was more tolerant to salt than skin collagen as evidenced by the greater solubility in the presence of NaCl at level of 4-

6% (Figure 22). Thus, collagen from different sources might have the different molecular properties, leading to the varied characteristics of both collagens.

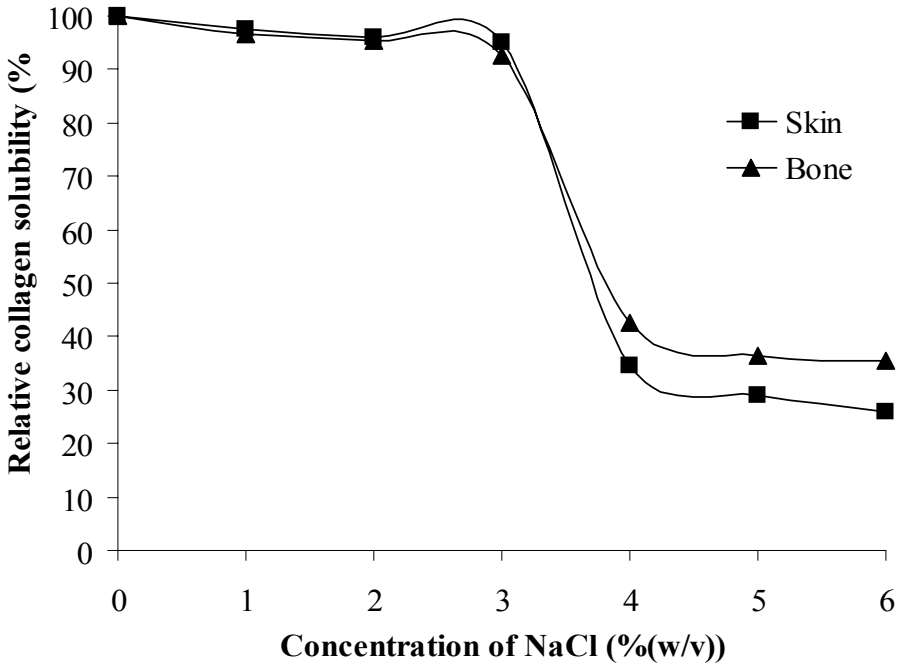

Figure 22 Relative collagen solubility (%) in 0.5M acetic acid of bigeye snapper skin and bone collagens in presence of NaCl at different concentrations.

2. Extraction of gelatin from skin and bone from bigeye Snapper

2.1 Deproteinization for gelatin extraction

Deproteinization of skins was optimized by varying the concentration of NaOH, soaking time and repetition. Accumulative protein and hydroxyproline contents in deproteinizing solutions are shown in Figure 23A and 23B, respectively. No differences in accumulative protein contents of deproteinizing solution were observed when concentration of NaOH solution and soaking time increased, while accumulative hydroxyproline content increased with increasing soaking time, concentration of NaOH solution and repetitions ($p<0.05$). NaOH at high concentration not only dissolved non-collagenous constituents, but also solubilized the collagen from the skin. As a result, some collagen was leached out from the skin, causing a lower yield. Also, NaOH at high concentration might cause the disintegration of collagen molecules, possibly affecting its functionality (Sato *et al*., 1987). Under the same condition used for deproteinization, the accumulative protein content increased with increasing repetition ($p<0.05$). From the results, optimum condition for deproteinzation of skin was 0.025 N NaOH for 1 h with 2 repetitions due to the mildest condition and shortest time. Under such condition, the loss of collagen as monitored by hydroxyproline content in deproteinizing solution was reduced, while non-collagenous protein removal was maximized. Sato *et al.* (1987) reported that the removing non-collagenous proteins with high concentration of NaOH solution modified the polypeptide chains of collagen, thus increasing the solubility of collagen.

For bone deproteinization, accumulative protein and hydroxyproline content in deproteinizing solution varied with conditions used (Figure 24A and 24B). At both concentrations of NaOH solution, accumulative protein content in alkali solution increased with increasing soaking time and repetition ($p<0.05$). Accumulative hydroxyproline content also increased as soaking time, concentration of NaOH solution and repetition increased ($p<0.05$). From the results, optimum condition for bone deproteinization was 0.025 N NaOH for 1 h with 2 repetitions. The lowest hydrxyproline content in deproteinizing solution was obtained under this condition, suggesting the great portion of collagen retained and effective removal of non-collagenous proteins.

From the result, non-collagenous protein removed into deproteinizing solution between skin and bone was different (Figure 23A and 24A). Protein content in deproteinizing solution of skin was much lower than that of bone. The result suggested that the bones had the high proportion of flesh attached to it, compared with the skin (Muyonga *et al*., 2003).

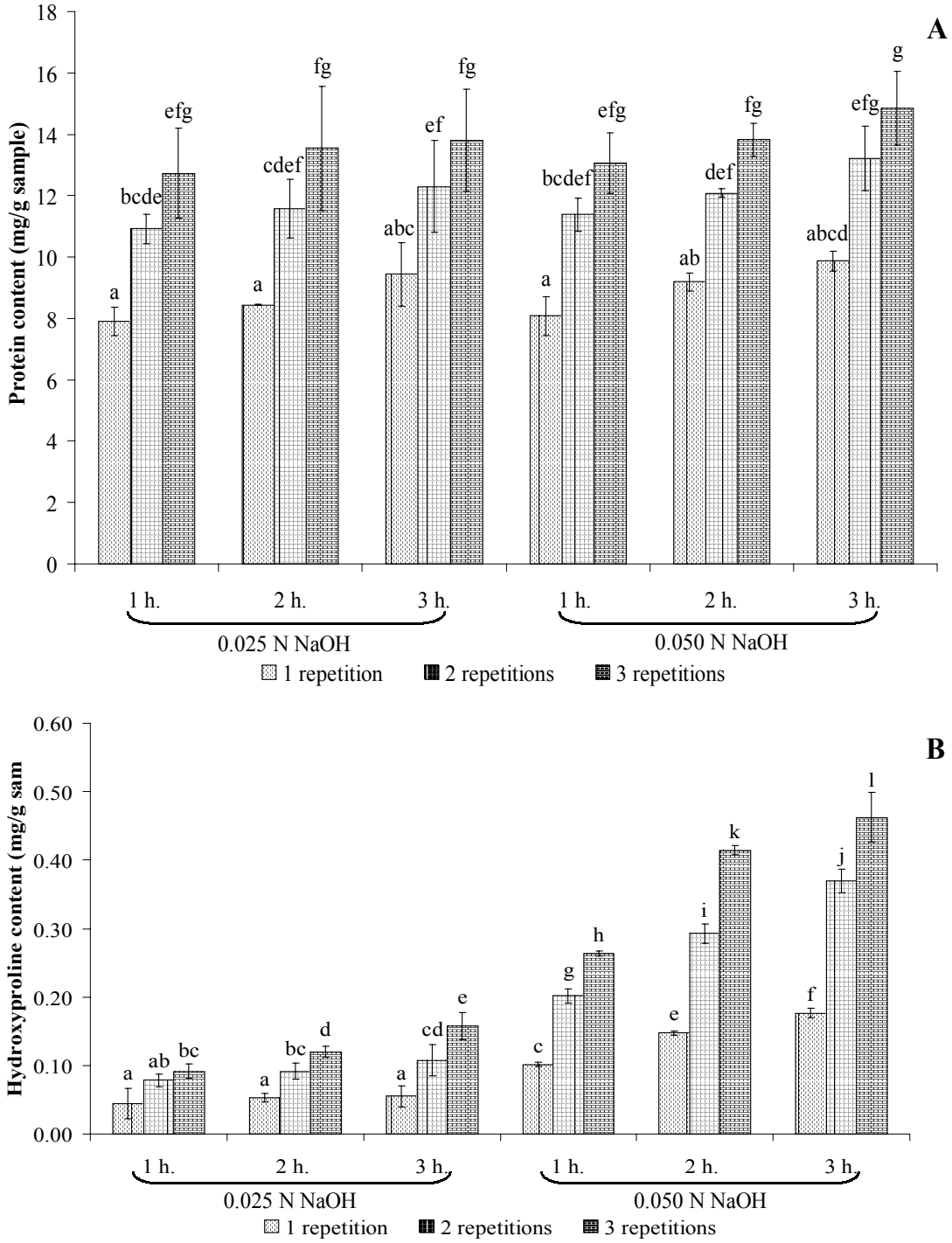

Figure 23 Accumulative protein content (A) and hydroxyproline content (B) in deproteinizing solution of bigeye snapper skin.

Different letters on the bars denote the significant differences ($p<0.05$).

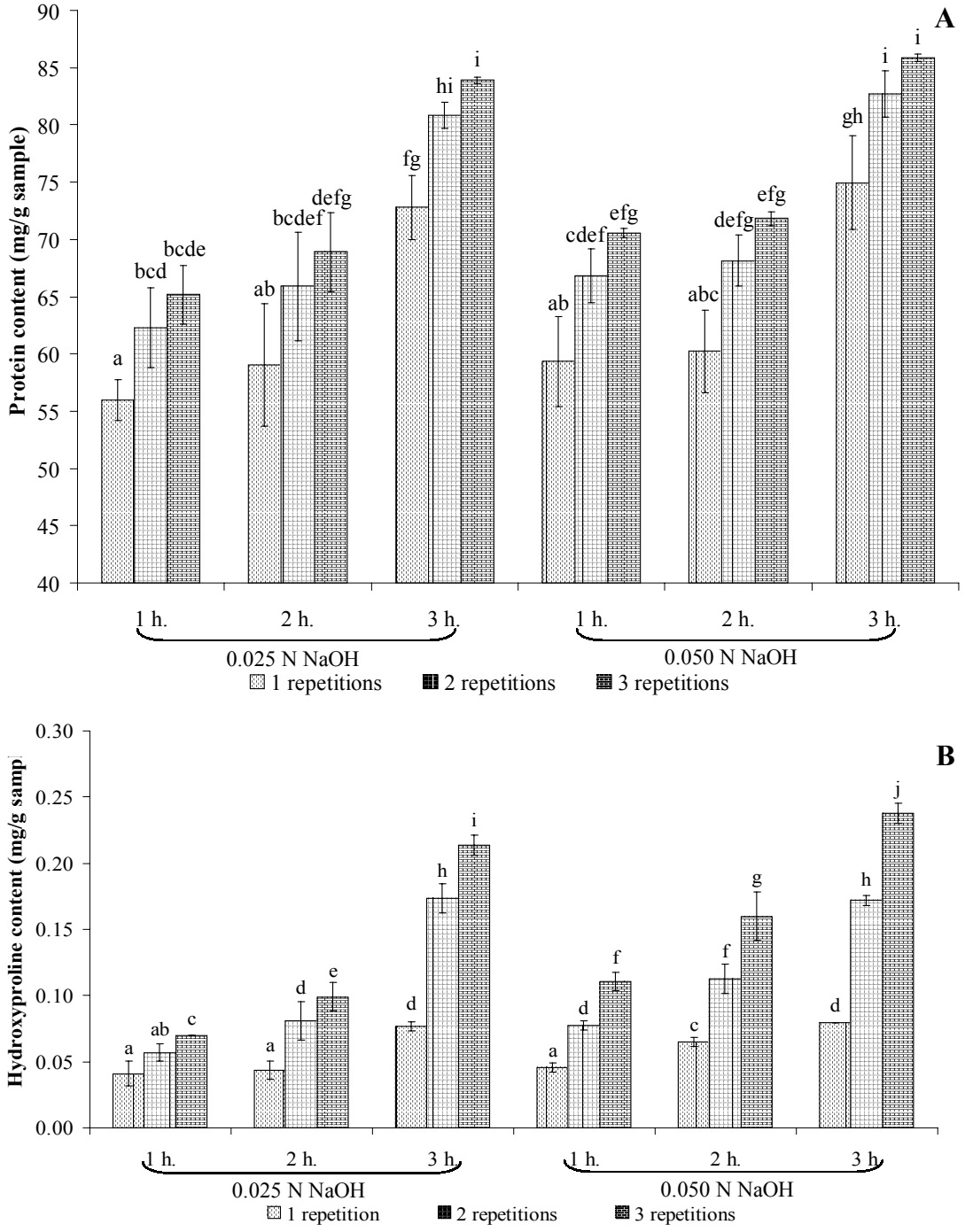

Figure 24 Accumulative protein content (A) and hydroxyproline content (B) in deproteinizing solution of bigeye snapper bone.

Different letters on the bars denote the significant differences ($p<0.05$).

2.2 Demineralization for gelatin extraction from bone

Due to the high content of ash in deproteinized bones, demineralization using HCl and citric acid at the concentration of 0.6 and 1.2 M was carried out. Ash content in bones after demineralization under different conditions was used as an index for demineralization efficiency (Table 7). Generally, the removal of inorganic substances was increased with increasing repetitions. However, HCl was shown to be more effective in dissolving the inorganic matters at the same concentration used. Ash content in samples demineralized with 1.2 M HCl was considerably low after first demineralization. Demineralization with 0.6 M HCl resulted in the similar ash content to that observed in bone demineralized using 1.2 M HCl with one repetition. To obtain the lowest ash content and to avoid the harsh condition, demineralization either with 1.2 M citric acid for 1 h with 4 repetitions or 0.6 M HCl for 1 h with 2 repetitions were selected. Although demineralization with 1.2 M HCl could remove ash effectively after second repetitions, this condition was very harsh, which might affect the properties of gelaitn. HCl removed the inorganic substances, especially calcium carbonate, by dissolving it into soluble calcium chloride (Muzzarelli, 1997). Citric acid has been reported to chelate various ion, leading to the lower ash content (Lindsay, 1996).

Table 7 Ash content in residues of deproteinized bone from bigeye snapper after demineralization process under different conditions.

Repetition	Citric acid		HCl	
	0.6 M	1.2 M	0.6 M	1.2 M
0	51.84 ± 0.17^{Ei}	51.84 ± 0.17^{Ei}	51.84 ± 0.17^{Eg}	51.84 ± 0.17^{Dg}
1	16.77 ± 0.10^{Dh}	4.60 ± 0.15^{Df}	3.48 ± 0.24^{Df}	0.80 ± 0.08^{Ce}
2	9.80 ± 0.15^{Cg}	3.89 ± 0.05^{Ce}	0.98 ± 0.14^{Ce}	0.30 ± 0.08^{Bc}
3	3.78 ± 0.17^{Be}	3.10 ± 0.07^{Bd}	0.86 ± 0.13^{Ce}	0.13 ± 0.04^{Aabc}
4	2.31 ± 0.13^{Ac}	1.73 ± 0.12^{Aa}	0.56 ± 0.03^{Bd}	0.07 ± 0.01^{Aab}
5	2.06 ± 0.27^{Ab}	1.60 ± 0.05^{Aa}	0.25 ± 0.03^{Abc}	0.01 ± 0.01^{Aa}

Different superscripts under the same acid indicate the significant difference ($p<0.05$).
Different capital superscripts in the same column indicate the significant difference ($p<0.05$).

2.3 Swelling for gelatin extraction from skin and bone

The yield of skin and bone gelatin increased with increasing concentration of swelling solution. Skin and bone, which were swollen with acetic acid, showed the lower yield of gelatin when compared with that swollen with citric acid (Table 8). The increasing yield was in agreement with increased swelling (Figure 25). Skin fiber was swollen in acidic solution, especially with increasing acid concentration. Stainsby (1987) reported that swelling is important for gelatin extraction because it can favor protein unfolding by disruption of non-covalent bonding and predispose the collagen to subsequent extraction and solubilization. Although a low pH can favor a maximum extraction rate, it may be detrimental to the physical properties because it produces more degradation and proliferation of lower-molecular weight peptides (Johnston-Banks, 1990). From the result, higher yield (6.29-7.76%) was generally obtained from skin, compared with that of bone (1.19-2.25%). The yield of gelatin from bone

demineralized with citric acid was lower than that with HCl. However, the yield of bone gelatin was very low. Thus, gelatin from bone was not used for analysis and further study. Moreover, it was unworthy to extract that gelatin for commercial purposes.

Table 8 Effect of swelling on the yield of gelatin from bigeye snapper skin and bone with different demineralization conditions.

Gelatin types	Demineralization condition	Swelling condition*	Yield** (% wet wt.)
Bone gelatin	1.2 M Citric acid for 1 h (repeat 4 times)	0.05 M acetic acid	1.19 ± 0.13^{a}
		0.10 M acetic acid	1.24 ± 0.17^{ab}
		0.20 M acetic acid	1.36 ± 0.09^{abc}
		0.05 M citric acid	1.57 ± 0.14^{bcd}
		0.10 M citric acid	1.66 ± 0.13^{cd}
		0.20 M citric acid	2.17 ± 0.26^{f}
	0.6 M HCl for 1 h (repeat 2 times)	0.05 M acetic acid	1.49 ± 0.08^{abcd}
		0.10 M acetic acid	1.52 ± 0.15^{abcd}
		0.20 M acetic acid	1.56 ± 0.20^{bcd}
		0.05 M citric acid	1.75 ± 0.17^{de}
		0.10 M citric acid	2.02 ± 0.08^{ef}
		0.20 M citric acid	2.25 ± 0.09^{f}
Skin gelatin	None	0.05 M acetic acid	6.29 ± 0.65^{A}
		0.10 M acetic acid	6.71 ± 0.33^{AB}
		0.20 M acetic acid	6.88 ± 0.55^{AB}
		0.05 M citric acid	7.34 ± 0.77^{AB}
		0.10 M citric acid	7.68 ± 0.30^{B}
		0.20 M citric acid	7.76 ± 0.18^{B}

Different superscripts in the same column of bone gelatin indicate the significant difference ($p \leq 0.05$).
Different capital superscripts in the same column of skin gelatin indicate the significant difference ($p \leq 0.05$).
* Raw materials were swollen with acid solutions for 40 min with 3 repetitions.
** Average $\pm$ SD from duplicate determinations.

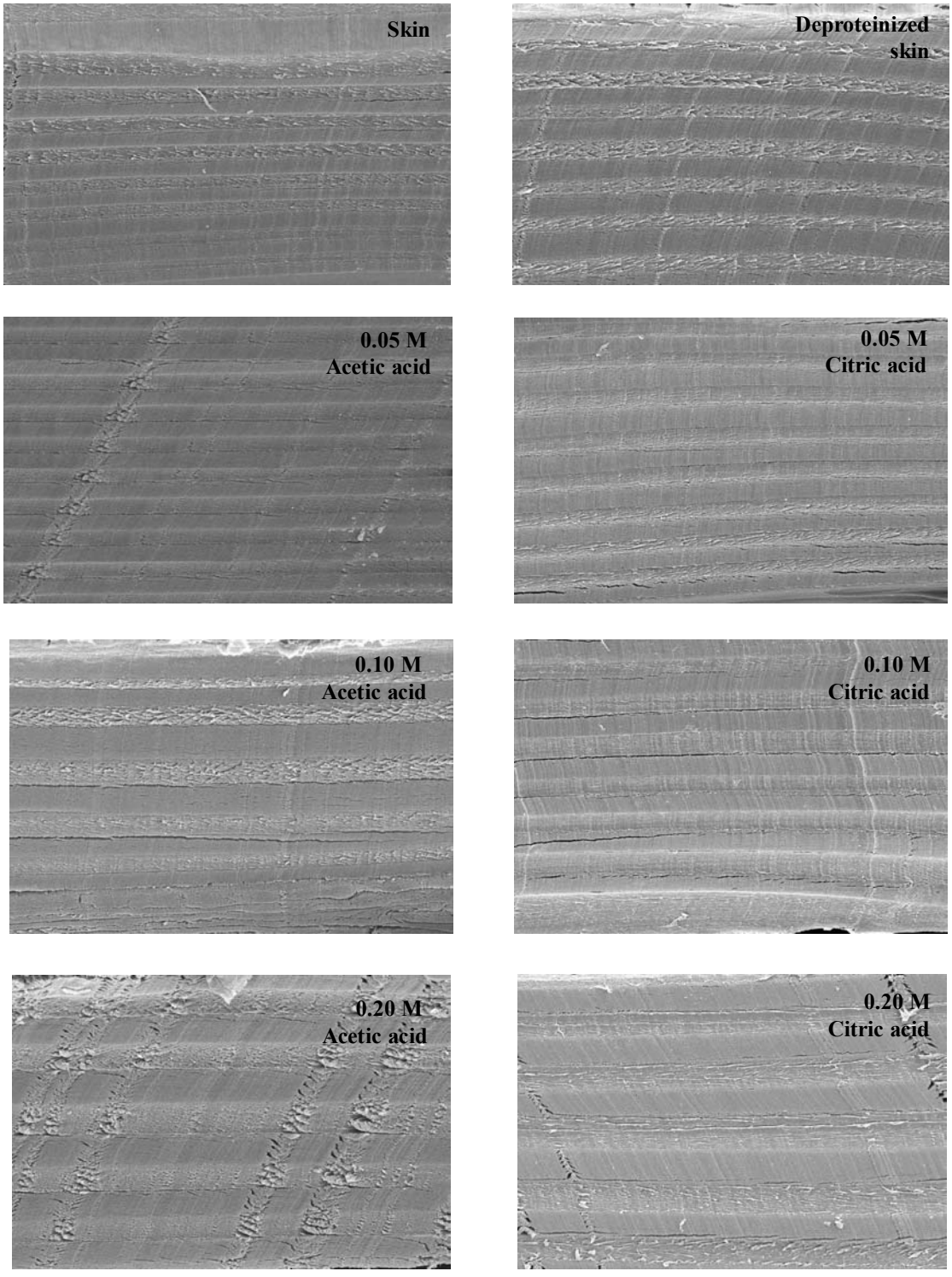

Figure 25 Microstructure of skin, deproteinized skin and skin swollen with acetic acid and citric acid at different concentrations.

The bloom strength of skin gelatin prepared by swelling the skin with acetic acid or citric acid at various concentrations (0.05, 0.1 and 0.2 M) is shown in Figure 26. The bloom strength of gelatin prepared from skin swollen with acetic acid increased as acid concentration increased. Conversely, bloom strength of gelatin from skin swollen with citric acid decreased with increasing acid concentration. Those changes related with pH of gelatin gel solution and pI of gelatin, about 6-9 for type A gelatin (Johnston-Banks, 1990). At pH near pI (swelling with 0.05 and 0.1 M acetic acid, pH of 4.54 and 4.23, respectively), the gelatins easily formed a coagulum as a result of the rapid and random aggregation. At pH far away from pI (swelling with 0.05, 0.1 and 0.2 M citric acid, pH of 3.04, 2.58 and 2.09, respectively), the repulsive forces predominated because of the greater net charge, and so the aggregation of gelatin did not occur and at the intermediate regions between pI and extreme pH (swelling with 0.2 M acetic acid, pH of 3.93), the repulsive electrostatic forces and attractive forces (mainly hydrophobic interaction) were well-balanced to form the gel network (Matsumura and Mori, 1996). From the result, skins swollen with acetic acid had greater bloom strength than that swollen with citric acid, though the higher yield was obtained when swollen with citric acid (Table 8). With the lower pH of citric acid (Figure 26), the higher repulsion was presumed, leading to the higher extraction of collagen from the matrix. Although the lower yield of collagen prepared by swelling with acetic acid, it had the greater bloom strength. This was postulated that the long chains of collagen were extracted with the skin swollen with acetic acid. Gomez-Guillen and Montero (2001) reported that the smaller molecular size of acid may constitute a lesser obstacle in the correct annealing of protein chains during gel network formation. From the results, skin gelatin which obtained from skin swollen

with 0.2 M acetic acid had the greatest bloom strength. Therefore, swelling condition had the influence on gelatin gel strength.

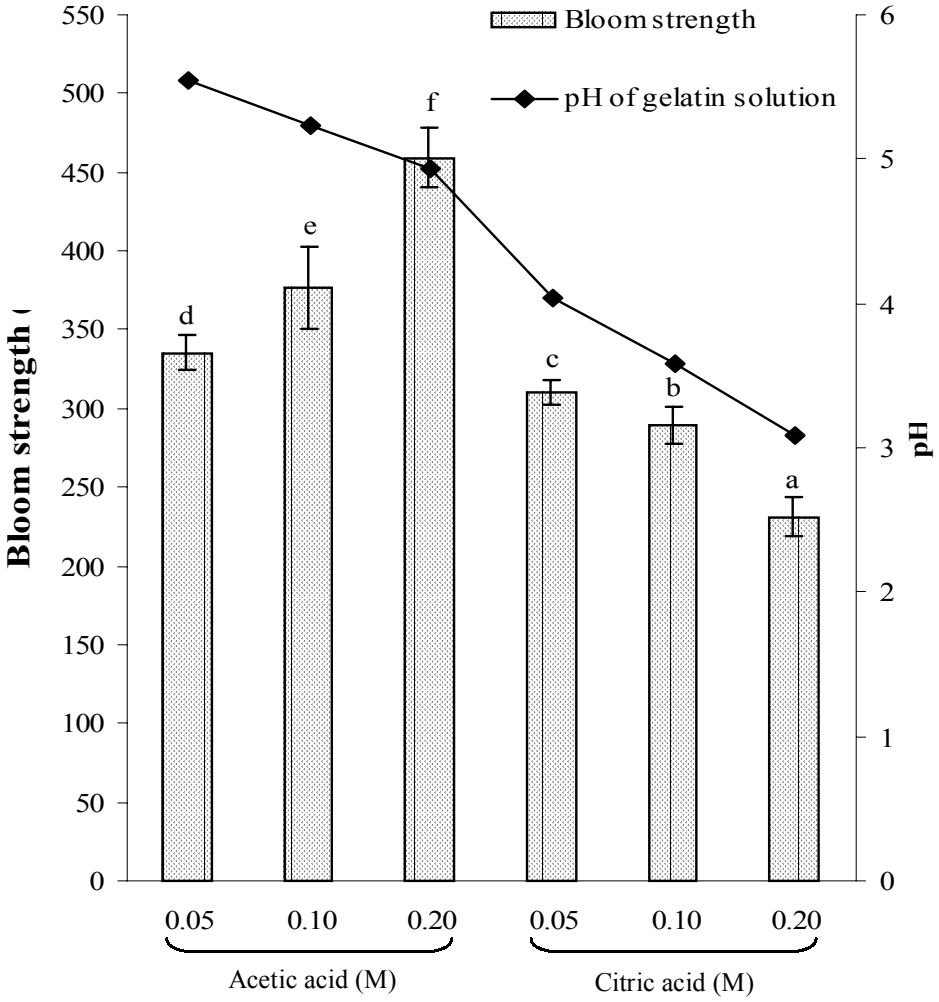

Figure 26 Bloom strength of gelatin gel from skins swollen with acetic acid or citric acid at different concentrations. Bars represent the standard deviation from five determinations.

Different letters on the bars denote the significant differences ($p<0.05$).

3. Characterization and functionality of gelatin from skin of bigeye snapper

3.1 Proximate compositions of bigeye snapper skin and its gelatin

Proximate compositions of bigeye snapper skin and its gelatin are shown in Table 9. Bigeye snapper skin constituted water (65.36%) as the major component, followed by protein (21.44%), ash (11.55%) and trace amount of fat (0.26%). Moisture, protein, fat and ash contents of the Nile perch skin were 68.4%, 21.6%, 6.8%, and 6.0%, respectively (Muyonga *et al*., 2003). The compositions of skin between two species were slightly different. When gelatin was extracted from skin, it was noted that gelatin contained high protein content (84.23%) with the low moisture content (8.94%). High ash content (5.98%) was observed when compared with the gelatin from other fish species, Nile perch skin gelatin (0.5-1.4%) (Mugonga *et al*., 2003) and commercial fish gelatin form Norland Inc., USA. (0.82%) (Haung, 2004). Moreover, it was higher than the recommended maximum of 2.6% (Jones, 1977). The result suggested that the high ash in bigeye snapper skin gelatin might be from the scales. Thus, the removing scales from skin with the appropriate demineralization prior to gelatin extraction should be done to reduce the ash content. The hydroxyproline and collagen contents in skin were slightly higher than descaled skin (data shown in Table 5). The result suggested that the higher hydroxyproline and collagen contents in skin might be from the scales. From the results, the collagen constituted approximately 95% of dry matter, indicating that gelatin obtained had high purity with the negligible contaminating proteins.

Table 9 Proximate compositions of skin and skin gelatin from bigeye snapper.

Samples	Compositions* (% wet wt.)				Hydroxy proline (mg/g sample)	Collagen** (mg/g sample)
	Moisture	Ash	Fat	Protein		
Skin (include scale)	65.36 ± 0.47	11.55 ± 1.07	0.26 ± 0.04	21.44 ± 0.37	20.53 ± 0.48	301.86 ± 7.02
Skin gelatin	8.94 ± 0.61	5.98 ± 0.48	0.66 ± 0.09	84.23 ± 1.27	64.73 ± 2.27	951.59 ± 33.38

* Average ± SD from triplicate determinations.
** The conversion factor for calculating the collagen content from hydroxyproline was 14.7 (Sadowska *et al*., 2003).

3.2 SDS-polyacrylamide gel electrophoretic (SDS-PAGE) patterns of skin gelatin

The electrophoretic patterns of skin gelatin in comparison with its collagen under reducing condition are shown in Figure 27. The skin gelatin had β-component as the major constituent. In general, protein pattern of collagen and gelatin was very similar. Protein bands with the molecular weight less than α2 were observed in gelatin. Coincidentally, the cross-link components decreased to some extent. Those proteins might be the degradation peptides produced during extraction with hot water (45°C). Heat activated proteinases, especially collagenase, might involve in the degradation. Muyonga *et al*. (2003) reported that gelatin extraction at high temperature was found to contain more peptides (molecular weight less than α-chains) and lower proportion of high molecular weight (greater than β-chain) fractions than low temperature extraction. Degradation of major components into lower molecular weight fragments might result in the changes in properties of gelatin such as bloom strength, viscoelastic properties, melting point setting point, setting time, etc. According to Tavernier (1989), high incidence of low molecular weight peptides was associated with low viscosity, melting point, setting point and high

setting time. However, there was no correlation between gelatin gel strength and molecular weight distribution for high gel strength gelatin (Graesser, 1985; Koepff, 1984).

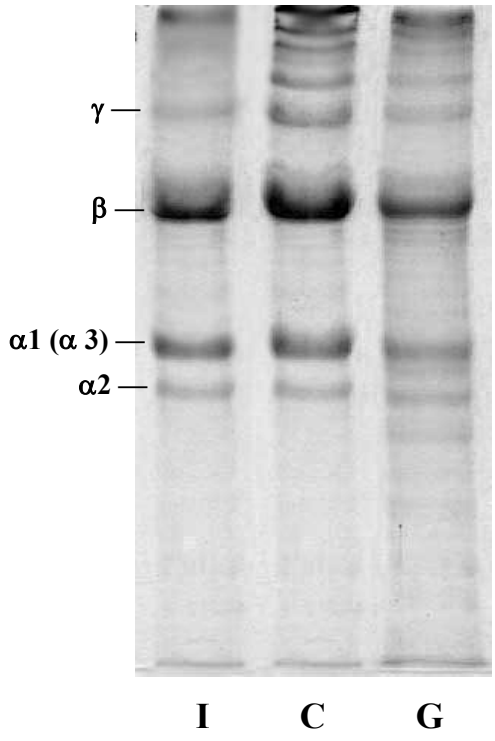

Figure 27 SDS-PAGE pattern of gelaitn from bigeye snapper skin under reducing condition. I, C and G denote type I collagen, collagen and gelatin from bigeye snapper skin, respectively.

3.3 Gelation of gelatin from bigeye snapper skin

3.3.1 Turbidity of gelatin solution

The turbidity of gelatin solution at different pHs is shown in Figure 28. The turbidity of gelatin solution increased when pH of those solutions increased ($p<0.05$). At pH 5, the turbidity of gelatin was not different from that of control, which had the pH of approximately 5. The highest turbidity of gelatin was observed at pH 7 and 9,

while the gelatin solution at pH 3 had the lowest turbidity. From the result, it was suggested that pI of gelatin was in the range of 7-9, type A gelatin (Zayes, 1997). At pH was below pI, protein molecules had the net positive charge. As such, molecules underwent repulsion and were effectively solvated by water molecules, and were thus more soluble (Milewski, 2001). Accordingly, the gelatin solution at this pH was very clear. At pH near pI, protein molecules tend to from the aggregate and less water interacts with the protein molecules (Vojdani, 1996).

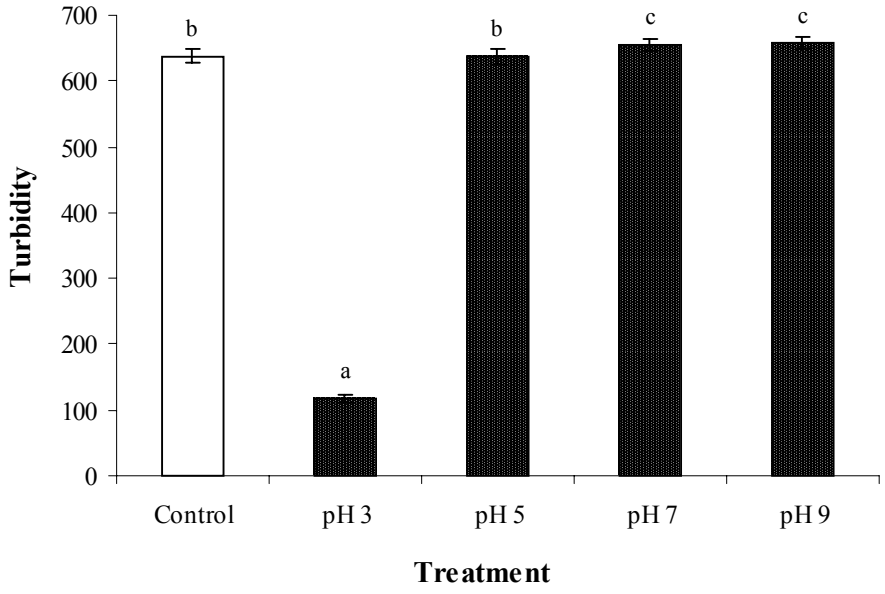

Figure 28 Turbidity of gelatin solution from bigeye snapper skin at different pHs. Bars represent the standard deviation from five determinations.
Different letters on the bars denote the significant differences ($p<0.05$).

3.3.2 Bloom Strength

The effect of pH on bloom strength of gelatin gel is depicted in Figure 29. The bloom strength increased with increasing pH, especially at pH 9. Bloom strength might be dependent on isoelectric point (pI) and might also be controlled to some

extent by adjusting pH (Gudmundsson and Hafsteinsson, 1997). More compact and stiffer gel can be formed by adjusting the pH of the gelatin close to its pI, where the proteins will be more neutral and thus the gelatin polymers are closer to each other (Jamilah and Harvinder, 2002; Gudmundsson and Hafsteinsson, 1997). Moreover, the bloom strength of gelatin at pH 3 was lower than control (pH~5). Choi and Regenstein (2000) reported that the bloom strength of gelatin from tilapia skin and porcine skin and bone markedly decreased when pH was below 4.

From the result, it was suggested that pI of gelatin from bigeye snapper skin might be in the alkali pH range. As a result, the aggregation of gelatin polymer was maximized at pH 9. Thus, further study on pI of gelatin from bigeye snapper skin needs to be conducted.

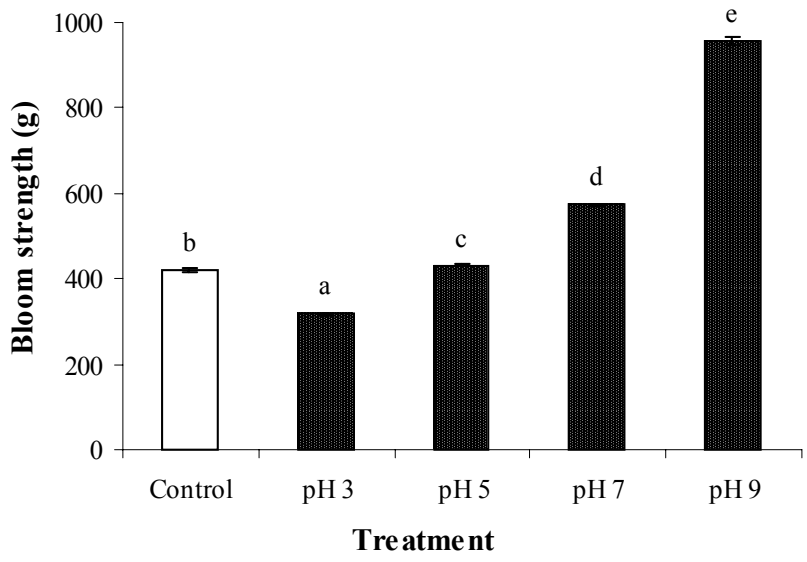

Figure 29 Bloom strength of bigeye snapper skin gelatin at different pHs. Bars represent the standard deviation from five determinations.

Different letters on the bars denote the significant differences ($p<0.05$).

3.3.3 Color of gelatin gel

The color (L*, a* and b*-value) of gelatin gel at different pHs is shown in Table 10. L*-value increased when pH of gelatin gel increased ($p<0.05$) and was different from that of the control ($p<0.05$). Decrease in a*-value was observed at pH 9. Increase in L*-value and decrease in a*-value of gelatin gel at pH 9 might be associated with the loss in solubility at this pH, possibly close to its pI. However, at pH of 3, L*-value was considerably low. At this pH, gelatin-water interactions increased due to the repulsive forces predominated (Matsumura and Mori, 1996). This phenomenon resulted in completely solubilized gelatin with negligible aggregate. Thus, L*-value of gelatin gel at this pH was low.

Table 10 Color of gelatin gel from bigeye snapper skin at different pHs.

Treatments	Color**		
	L*	a*	b*
Control	30.39 ± 0.73^{b}	-0.80 ± 0.11^{bc}	-1.76 ± 0.35^{ab}
pH 3	8.54 ± 0.28^{a}	-0.71 ± 0.15^{c}	-1.54 ± 0.22^{b}
pH 5	30.03 ± 0.20^{b}	-0.68 ± 0.11^{c}	-1.76 ± 0.07^{ab}
pH 7	35.21 ± 0.15^{c}	-0.97 ± 0.08^{ab}	-1.89 ± 0.26^{a}
pH 9	34.86 ± 0.37^{c}	-1.03 ± 0.19^{a}	-2.01 ± 0.19^{a}

Different superscripts in the same column indicate the significant difference ($p<0.05$).
** Average $\pm$ SD from five determinations.

3.4 Solubility of gelatin

The effect of pH on solubility of gelatin from bigeye snapper skin in comparison with egg white is depicted in Figure 30. The minimum solubility of bigeye snapper skin gelatin and egg white was found at pH 8 and 5, respectively. The pI of gelatin has been reported to be in the range of 7-9 (Zayes, 1997), while pI of egg white is 4.5-6 (Powrie and Nakai, 1986). At pH = pI, large dipoles of protein molecules attract themselves through the counterchanged domain and there is no electrostatic repulsion between neighboring molecules. As a consequence, protein molecules tend to precipitate (Milewski, 2001). The difference in solubility between gelatin from bigeye snapper skin and egg white suggested the differences in amino acid compositions and sequence, molecular weight and conformation and the content of polar and non-polar groups in amino acids (Zayes, 1997).

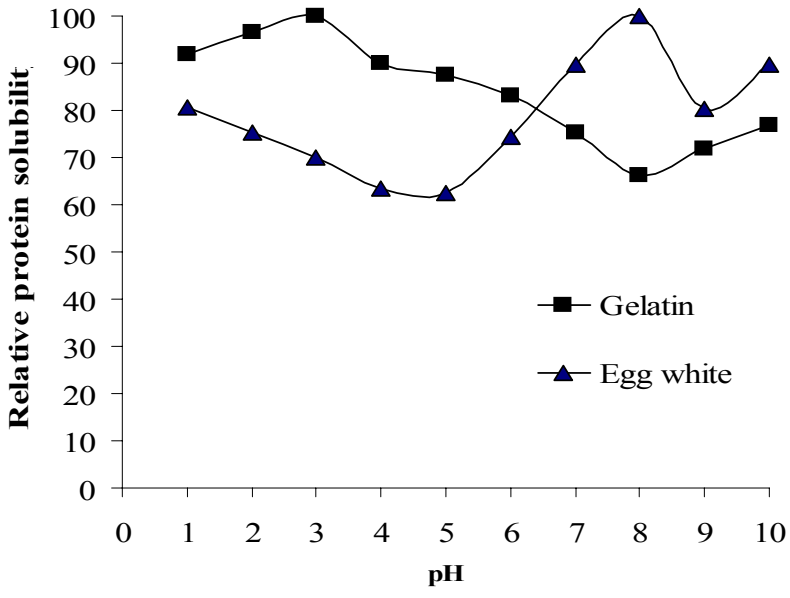

Figure 30 Relative solubility of gelatin from bigeye snapper skin and egg white at different pHs.

3.5 Emulsifying properties

Emulsion activity index (EAI) and emulsion stability index (ESI) of gelatin from bigeye snapper skin and egg white at concentrations of 1, 2 and 3%(w/v) are shown in Table 11. EAI and emulsion stability of both proteins increased with increasing concentrations. The result suggested that protein at high concentration facilitated more protein adsorption at interface (Yamauchi *et al*., 1980). When comparing the emulsifying properties between skin gelatin and egg white, EAI of egg white was higher than that of gelatin, while emulsion stability was opposite. From the result, it indicated that the difference in emulsifying properties of both proteins was the result of the difference in the intrinsic properties of proteins, compositions and conformations of proteins (Kinsella and Srinivasan, 1981). High solubility of the protein in the dispersing phase increases the emulsifying efficiency, because the protein molecules should be able to migrate to the surface of the fat droplets (Sikorski, 2001). However, insoluble protein particles incorporated during emulsification could increase stability of emulsion (Paulson *et al*., 1984). Conformation of egg white was globular shape (Lehninger, 1975), while that of gelatin was rod shape (Gaman and Sherrington, 1990). Moreover, Feeney and Osuga (1976) reported that ovalbumin which was the major proteins in egg white denatured easily. Thus, egg white might be more flexible than gelatin. Hill (1996) reported that the flexibility of the backbone of protein molecules was the one of major important for emulsifying properties. Zayes (1997) reported that ovalbumin in egg white had a great surface hydrophobicity which influenced emulsifying activity. However, if the percent fraction of hydrophobic patches on the surface of protein exceeds a critical level, it might impair the solubility of the protein and thus affect its surface activity.

Therefore, an optimum ratio of hydrophilic to hydrophobic patches on the surface of a protein seemed to be critical to ensure maximum surface activity (Damondaran, 1996). Hill (1996) reported that gelatin in bulk solution will form gels which become stronger with increasing time. This behavior is also shown in emulsions where gelatin is adsorbed at the interface and in the additional reversible adsorbed protein layers. When the protein level was low, it was possible that the oil droplets approached each other easily, leading to flocculation (Hill, 1996). Thus, increasing in protein concentration resulted in the increased emulsion stability, as evidenced by the lowered ESI.

Table 11 Emulsifying properties of gelatin from bigeye snapper skin and egg white at different concentrations.

Protein types	Concentration (%)	Emulsifying activity index (EAI)*	Emulsion stability index (ESI)*
Egg white	1	48.63 ± 4.44^{a}	55.86 ± 1.36^{c}
	2	60.72 ± 2.68^{b}	45.15 ± 0.68^{b}
	3	83.54 ± 4.97^{c}	19.38 ± 1.97^{a}
Gelatin	1	10.43 ± 0.43^{a}	27.25 ± 4.02^{b}
	2	20.79 ± 0.50^{b}	8.85 ± 2.42^{a}
	3	41.46 ± 5.61^{c}	4.71 ± 2.63^{a}

Different superscripts in the same column under the same protein indicate the significant difference ($p<0.05$).
* Average $\pm$ SD from triplicate determinations.

3.6 Foaming properties

The foaming properties (foam expansion, FE and foam stability, FS) of gelatin from bigeye snapper skin at concentrations of 1, 2, 3, 4 and 5%(w/v) were compared with egg white at the same concentrations as shown in Table 12. FE increased with increasing protein concentrations ($p<0.05$). Foams with higher concentration of proteins were more dense and more stable because of an increase in the thickness of interfacial films (Zayes, 1997). Moreover, FS also increased as protein concentrations increased ($p<0.05$). FS is directly affected by protein concentration, which influences the thickness, mechanical strength and cohesiveness of the film (Zayes, 1997). Foam stability rapidly increased with protein concentration up to 0.1% depending on the protein surface properties (Halling, 1981). Bolnedi and Zayes (1993) reported that high viscosity at higher concentrations was effective in preventing gravity deformation of the film in protein foams. The bulk viscosity of protein solutions affected the foam stability which in turn extends the lifetime of the film and hence the foams were stable (Townsend and Nakai, 1983; Kumagai *et al*., 1991). Thicker and rigid interfacial films at higher protein concentration might have reduced drainage within the lamella structure (Bolnedi and Zayes, 1993). From the result, FS decreased when incubation time increased ($p<0.05$). The result suggested that during foam aging, gravitational forces will cause water to drain, and air cells will come closer together (Zayes, 1997). When comparing foaming properties between skin gelatin and egg white, gelatin exhibited slightly better foaming properties. Foaming properties of proteins might be influenced by the source of the protein, intrinsic properties of protein: the compositions and conformations of the proteins in solution and at the air/water interface (Zayes, 1997; Wilde and Clark, 1996; Phillips *et al*., 1989).

Table 12 Foaming properties of bigeye snapper skin gelatin and egg white at different concentrations.

Protein types	Concentration (%)	Foam expansion, FE (%)*	Foam stability, FS (%)*	
			30 min	60 min
Egg white	1	120.94 ± 1.33^{Ba}	119.38 ± 0.88^{ABa}	116.88 ± 0.88^{Aa}
	2	131.25 ± 1.77^{Ab}	130.31 ± 1.33^{Ab}	128.13 ± 0.88^{Ab}
	3	135.00 ± 0.00^{Cc}	133.44 ± 0.44^{Bc}	132.19 ± 0.44^{Ac}
	4	140.94 ± 1.33^{Cd}	137.81 ± 0.44^{Bd}	135.00 ± 0.00^{Ad}
	5	145.00 ± 0.00^{Ce}	142.81 ± 0.44^{Be}	137.50 ± 0.00^{Ae}
Gelatin	1	128.13 ± 0.88^{Ba}	126.25 ± 1.77^{ABa}	122.19 ± 1.33^{Aa}
	2	131.56 ± 1.33^{Ba}	129.69 ± 0.44^{Aab}	124.38 ± 0.88^{Aa}
	3	138.75 ± 1.77^{Cb}	133.44 ± 0.44^{Bb}	128.44 ± 1.33^{Ab}
	4	145.00 ± 3.54^{Bb}	141.56 ± 2.21^{ABc}	135.94 ± 1.33^{Ac}
	5	155.00 ± 3.54^{Bc}	148.75 ± 1.77^{ABd}	145.94 ± 2.21^{Ad}

Different superscripts in the same column under the same protein indicate the significant difference ($p<0.05$).
Different capital superscripts in the same row indicate the significant difference ($p<0.05$).
* Average $\pm$ SD from duplicate determinations.

4. Improvement of gel properties of gelatin from skin of bigeye snapper

4.1 Turbidity of gelatin solution

The turbidity of gelatin solution added with magnesium sulphate ($MgSO_4$) or MTGase at various concentrations are shown in Figure 31. The turbidity of gelatin solution added with $MgSO_4$ decreased with increasing concentration of $MgSO_4$ ($p < 0.05$). At 1.0 M $MgSO_4$, the solution became coagulated, possibly caused by "salting out" effect. The result indicated that low salt concentration resulted in the "salting in", leading to the increased solubility of gelatin. Ions of neutral salts decrease the electrostatic attraction between the countercharged surface domains of neighboring protein molecules (Milewski, 2001). This phenomenon resulted in the decrease in turbidity of gelatin solution. At the high concentration of salt (1.0 M $MgSO_4$), the solubility of gelatin decreased and lost its solubility. Milewski (2001) reported that at sufficiently high ionic strength, a protein can be completely precipitated from solution. Water molecules are strongly bound to the salt and there is competition between the salt ions and the protein molecules for the water molecules (Zayes, 1997). With the addition of MTGase, no changes in turbidity of gelatin solution were observed at all levels added. Thus, the use of MTGase is a potential approach for increasing bloom strength without causing the changes in turbidity. Fernandez-Diaz *et al*. (2001) found that the addition of 0.1 or 0.5 M $MgSO_4$, 15% glycerol or 10 or 15 mg TGase/ml resulted in the changes in turbidity of gelatin from cod (*Galus marhua*) and hake (*Merluccius merluccius*) skin.

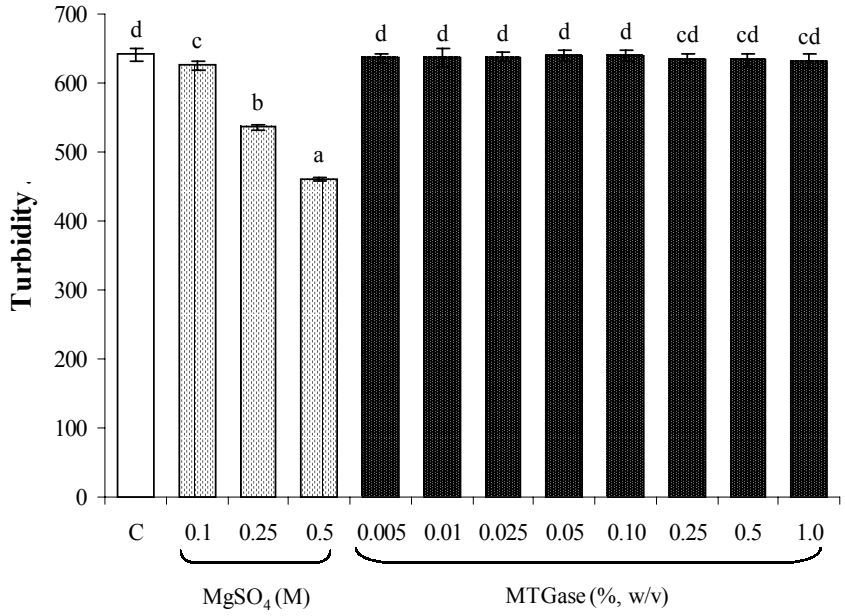

Figure 31 Turbidity of gelatin solution from bigeye snapper skin added with $MgSO_4$ or MTGase at different concentrations. C denote the control gelatin without adding $MgSO_4$ or MTGase. Bars represent the standard deviation from five determinations.

Different letters on the bars denote the significant differences ($p<0.05$).

4.2 Bloom strength of skin gelatin gels

The bloom strength of skin gelatin gel added with $MgSO_4$ or MTGase at various concentrations is shown in Figure 32. The bloom strength of skin gelatin added with $MgSO_4$ decreased with increasing concentrations and was lower than that of the control (without addition of $MgSO_4$ or MTGase). Salts destabilize gelatin structure, probably due to a direct competition for water to hydrate (Slade and Levine, 1987; Elysee-Collen and Lencki, 1996). Additionally, promotion of helix formation by screening of ionic interactions by salts does not necessarily result in stronger gels (Stainsby, 1987). NaCl decreased the bloom strength of several commercial gelatins from different sources (Choi and Regenstein, 2000). NaCl is capable of breaking both

hydrophobic and hydrogen bonds, which prevents stabilization of gel junction zones, either directly by preventing hydrogen-bond formation and/or by modifying the structure of the liquid water in vicinity of these sites (Finch *et al*., 1974). The lowest bloom strength was found with the addition of 1.0M $MgSO_4$. This result suggested that at the high ionic strength, protein aggregated and formed a coagulum. This interfered with the formation of a gel network and resulted in formation of soft and turbid gel (Pomeranz, 1991). The decrease in bloom strength of gelatin gel added with $MgSO_4$, especially at high concentration, was possibly caused by the disruption of hydrophobic and hydrogen bonds, which are the important bonds stabilizing gel network.

The addition of MTGase at the concentration up to 0.01% (w/v) resulted in the marked increase in bloom strength. The result suggested that non-disulfide covalent bond, particularly ε-(γ-glutamine) lysine bonds were formed by MTGase. A lower bloom strength was observed with the samples added with MTGase above 0.25% (w/v). Those gels were not only soft but also fragile. The result was in accordance with Kolodzjejska *et al*. (2003) who reported that modification of 2% fish gelatin with transglutaminase at a concentration of 0.25 mg/ml resulted in a decrease in deformation of gel. The highest bloom strength was found at the concentration of 0.01% (w/v) which was about 2 times higher than the control. In general, the bloom strength of skin gelatin increased with increasing of MTGase concentration. Sakamoto *et al*. (1994) reported that TGase with the concentration of 30 units/g protein produced maximum values of breaking strength of gelatin gels. The increased formation of TGase-mediated covalent bonds is detrimental to gel strength, probably because it flavors intramolecular covalent bonds which hinder the aggregation of the

poly-L-proline II helix into a collagen fold triple helix and thus do not contribute to the gel net work formation (Ledward, 1986). Fernandez-Diaz *et al*. (2001) found that MTGase at levels higher 0.015% (w/v) increased the melting and gelling points and produced a very elastic gel with lower gel strength, whereas lower quantities of enzyme resulted in an increase in gel strength. Therefore, MTGase at the concentration of 0.01 % (w/v) was adequate to improve bloom strength of the gelatin from bigeye snapper skin. With addition of MTGase at this level, bloom strength increased by 98.61%, compared with that of the control.

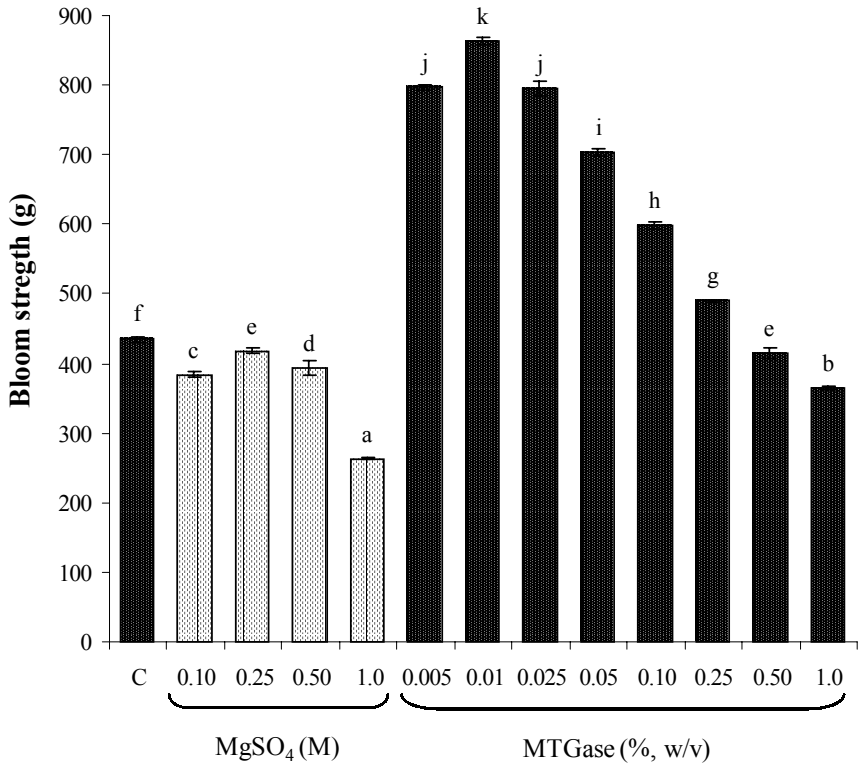

Figure 32 Bloom strength of gelatin gel from bigeye snapper skin added with $MgSO_4$ and MTGase at different concentrations. C denote the control gelatin without adding $MgSO_4$ or MTGase. Bars represent the standard deviation from five determinations.

Different letters on the bars denote the significant differences ($p<0.05$).

4.3 Color of gelatin gel

The addition of $MgSO_4$ resulted in the decrease in L*-value, but caused no changes in a*-value (Table 13). This was postulated to be due to "salting in" effect at low salt concentration. No gel was formed with addition of 1.0 M $MgSO_4$ since the coagulum was produced and gel network could not be formed. For the gel added with MTGase, a slight increase in L*-value was observed at the concentration increased but no differences in a*-value were found.

Table 13 Color of gelatin gel from bigeye snapper skin added with $MgSO_4$ or MTGase at different concentrations.

Treatments	Color**		
	L*	a*	b*
Control	30.39 ± 0.73^{def}	-0.80 ± 0.11^{a}	-1.56 ± 0.31^{abcde}
0.10 M $MgSO_4$	28.49 ± 0.34^{c}	-0.86 ± 0.07^{a}	-1.93 ± 0.22^{ab}
0.25 M $MgSO_4$	23.77 ± 0.25^{b}	-0.67 ± 0.18^{a}	-1.36 ± 0.04^{cde}
0.50 M $MgSO_4$	21.53 ± 0.17^{a}	-0.84 ± 0.29^{a}	-1.73 ± 0.17^{abcd}
0.005 % MTGase	29.63 ± 0.68^{d}	-0.84 ± 0.28^{a}	-1.71 ± 0.26^{abcd}
0.01 % MTGase	29.80 ± 0.62^{d}	-0.76 ± 0.17^{a}	-1.19 ± 0.24^{e}
0.025 % MTGase	29.86 ± 0.87^{d}	-0.79 ± 0.15^{a}	-1.31 ± 0.30^{de}
0.05 % MTGase	29.70 ± 0.40^{d}	-0.81 ± 0.18^{a}	-1.48 ± 0.28^{bcde}
0.10 % MTGase	29.84 ± 1.09^{d}	-0.80 ± 0.17^{a}	-1.79 ± 0.30^{abc}
0.25 % MTGase	30.13 ± 0.58^{de}	-0.74 ± 0.11^{a}	-1.83 ± 0.16^{ab}
0.5 % MTGase	30.88 ± 0.56^{ef}	-0.72 ± 0.20^{a}	-1.18 ± 0.10^{e}
1.0 % MTGase	31.04 ± 0.47^{f}	-0.89 ± 0.12^{a}	-1.96 ± 0.11^{a}

Different superscripts in the same column indicate the significant difference ($p<0.05$)
** Average $\pm$ SD from five determination

4.4 SDS- polyacrylamide gel electrophoretic (SDS-PAGE) patterns of gelatin gels

The electrophoretic patterns of skin gelatin gel added with magnesium sulphate ($MgSO_4$) or MTGase at various concentrations are shown in Figure 33 and 34, respectively. For the addition of $MgSO_4$ (Figure 33), similar protein patterns were observed for the gel without and with the addition of $MgSO_4$ at various concentrations Therefore, addition of $MgSO_4$ did not cause the formation of non-disulfide covalent bond in the gelatin gel. Moreover, it might destroy the hydrogen or hydrophobic bond between gelatin polymers (Figure 33).

For the addition of MTGase (Figure 34), α- and β-components continuously decreased as the higher concentrations of MTGase were used and disappeared at the MTGase concentration above 0.1% (w/v). For the γ-component, the marked decrease was observed with addition of MTGase above 0.01% (w/v). From the result, the decrease in protein bands was most likely due to the cross-linking via non-disulfide covalent bond formation. The virtual absence of bands corresponding to the α-, β- and γ-components in the sample indicated that these fractions were polymerized and were retained in the precipitate (Gomez-Guillen *et al*., 2001). From the result, it indicated that γ-component underwent cross-linking to much higher extent, compared to β- and α-components, respectively. This was possibly because γ-component contained more acyl donor and receptor aligned in the appropriate position for reaction induced by MTGase than β- and α-components, respectively.

From the results, although cross-linking of α-, β-and γ-components of gelatin gel was higher when MTGase concentration increased, it could not improve bloom

strength of gelatin gel. This was probably because excessive formation of ε-(γ-glutamyl) lysine crosslinks would inhibit uniform development of the protein network during incubation (Sakamoto *et al*., 1995). Sakamoto *et al*. (1994) reported that the decrease in breaking strength of caseinate, gelatin and egg yolk gels was observed when MTGase concentration was above 15, 30 and 30 units/g protein, respectively. Those gels with high enzyme concentration were not only soft but also fragile (Sakamoto *et al*., 1994).

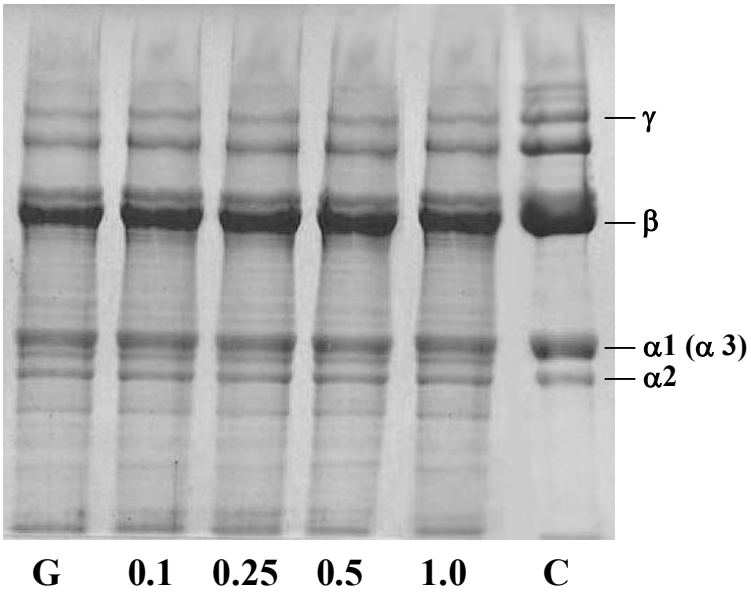

Figure 33 SDS-PAGE pattern of gelatin gel from bigeye snapper skin added with $MgSO_4$ at different concentrations. G and C denote gelatin and collagen from bigeye snapper skin, respectively. Numbers denote $MgSO_4$ concentration (M) added in gelatin gel.

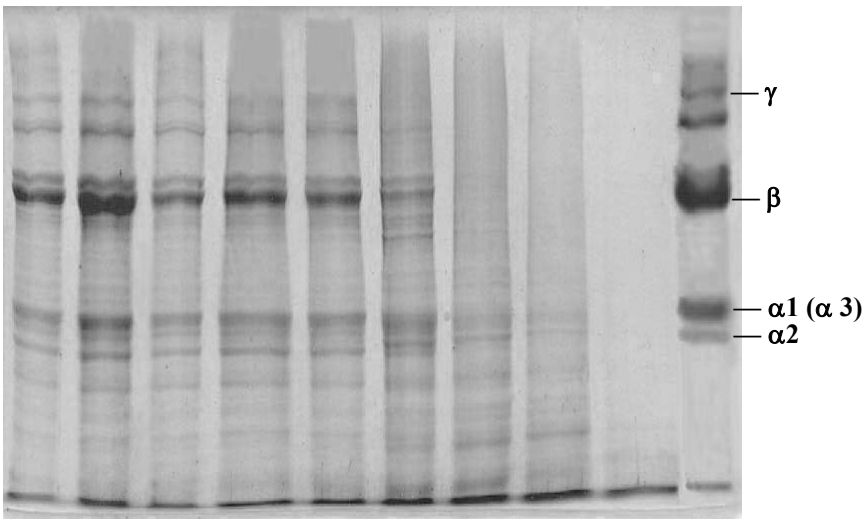

Figure 34 SDS-PAGE pattern of gelatin gel from bigeye snapper skin with MTGase at different concentrations. G and C denote gelatin and collagen from bigeye snapper skin, respectively. Numbers denote MTGase concentration (%, w/v) added in gelatin gel.

4.5 Scanning Electron Microscopy of Gelatin gels

The microstructures of gelatin gel added with different ingredients including 0.25 and 1.0 M $MgSO_4$ or 0.01, 0.1 and 1.0%(w/v) MTGase are shown in Figure 35, 36 and 37. Gelatin gel add with $MgSO_4$ showed an aggregate structure, especially at the high concentration (1.0M $MgSO_4$), which might be caused by "salting-out" effect. For the microstructure of gelatin gels added with MTGase, it was found that the bead type structure was observed, particularly with the higher MTGase concentrations. MTGase at high level might cause the excessive cross-link, leading to the large coagulum as observed in the gel. From the result, gel added with MTGase higher than 0.01%(w/v) had less uniformity, compared with that of the control. The result

suggested that this network might be formed through inter-molecular ε-(γ-glutamyl) lysine linkage induced by added MTGase.

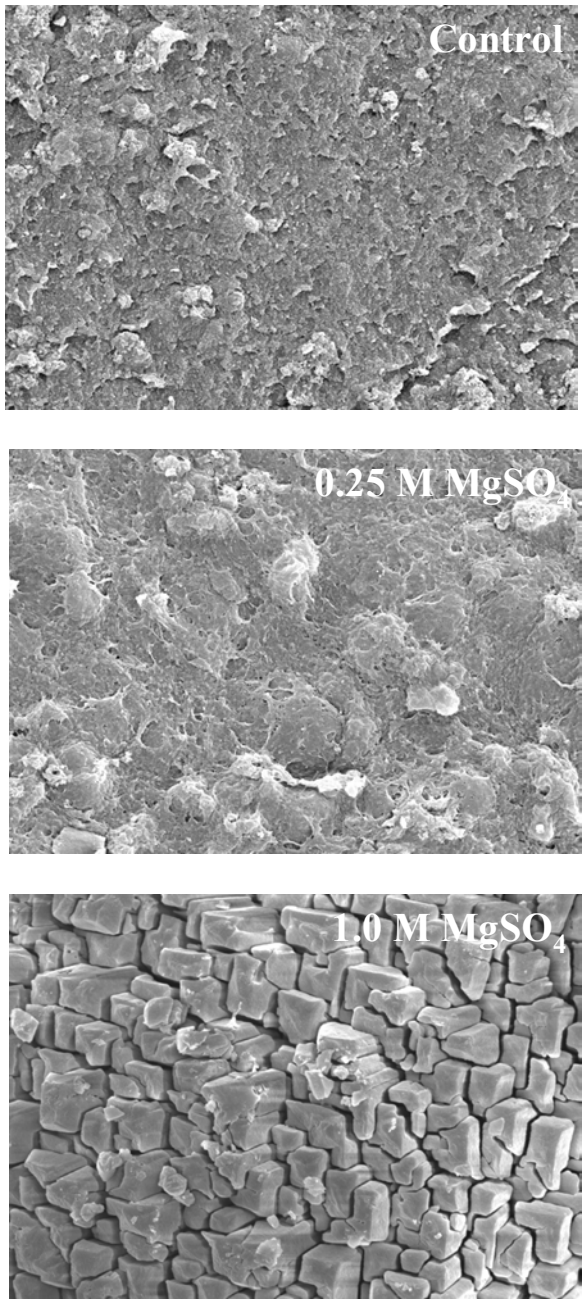

Figure 35 Microstructure of gelatin gel from bigeye snapper skin with and without addition of $MgSO_4$ at different concentrations (Magnification: 3,000X).

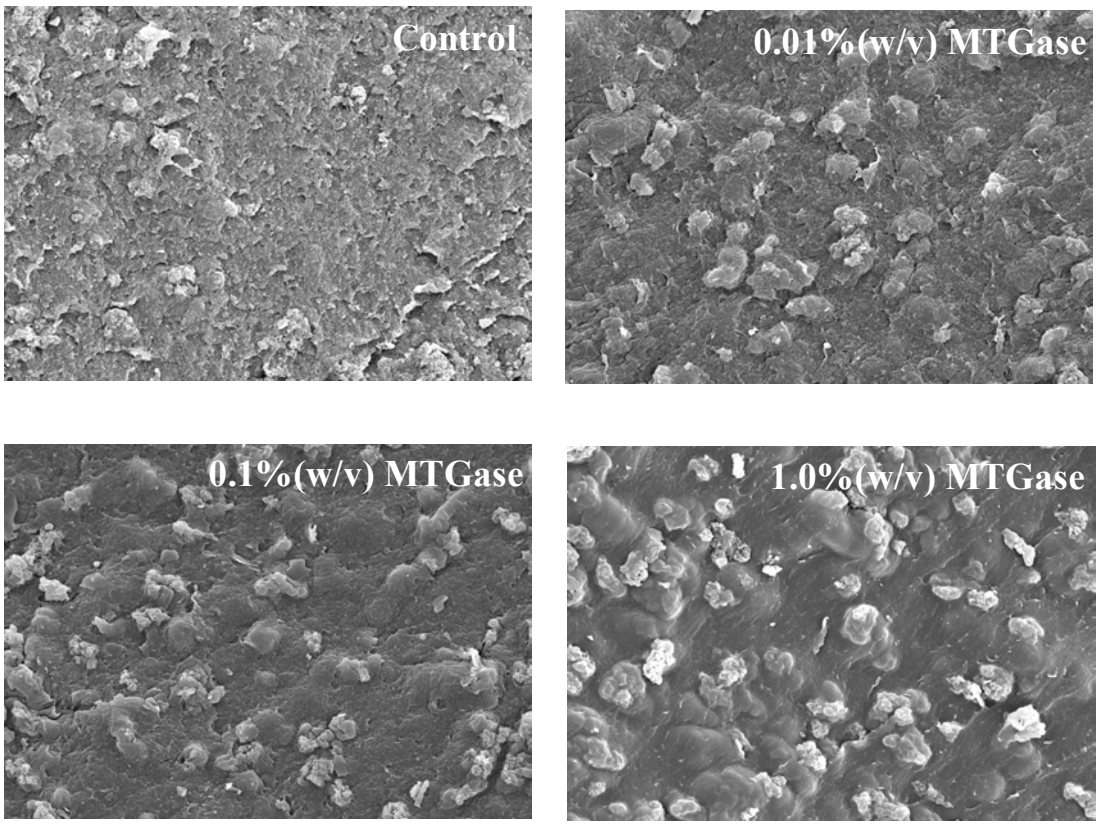

Figure 36 Microstructure of gelatin gel from bigeye snapper skin with and without addition of MTGase at different concentrations (Magnification: 3,000X).

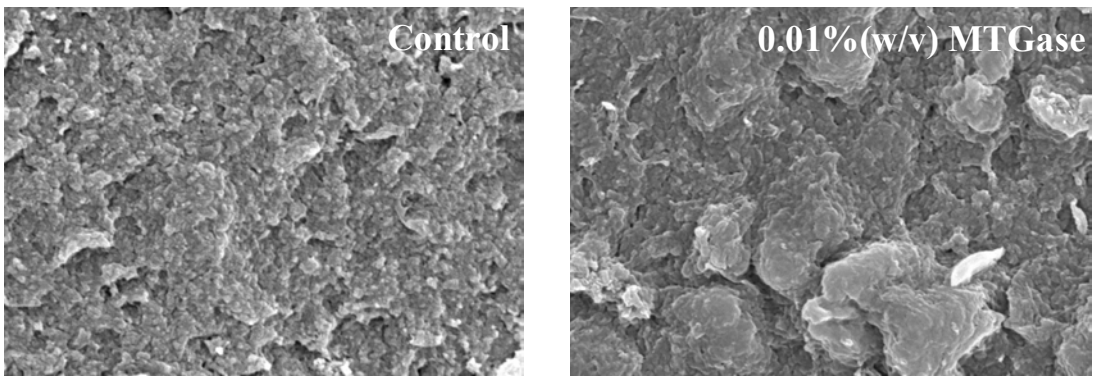

Figure 37 Microstructure of gelatin gel from bigeye snapper skin with and without adding MTGase at the concentration of 0.01 %(w/v) (Magnification: 10,000X).

From the Figure 37, the gelatin gel without MTGase showed a continuous network structure, while the gelatin gel added with MTGase showed some cross-linked structure, stabilized by non-disulfide covalent bond. Additionally, some cross-link between those particulates might be formed, leading to the enhanced bloom strength.

The bloom strength of skin gelatin gels added with 0.01%(w/v) MTGase and incubated at room temperature for different times is shown in Figure 38. The bloom strength increased with increasing incubation times ($p<0.05$). However, it decreased when incubation time was 3 h ($p<0.05$). Thus, incubation time of 2 h was the optimum time for strengthening skin gelatin gel added with MTGase, which could increase the bloom strength by 97.18%, compared with that of control. This suggested that the inter and intra linkages of gelatin gel were gradually constructed and accordingly the bloom strength was increased. The decrease in bloom strength at incubation time of 3 h suggested that the inter and intra linkages of gelatin gel might be formed excessively by MTGase.

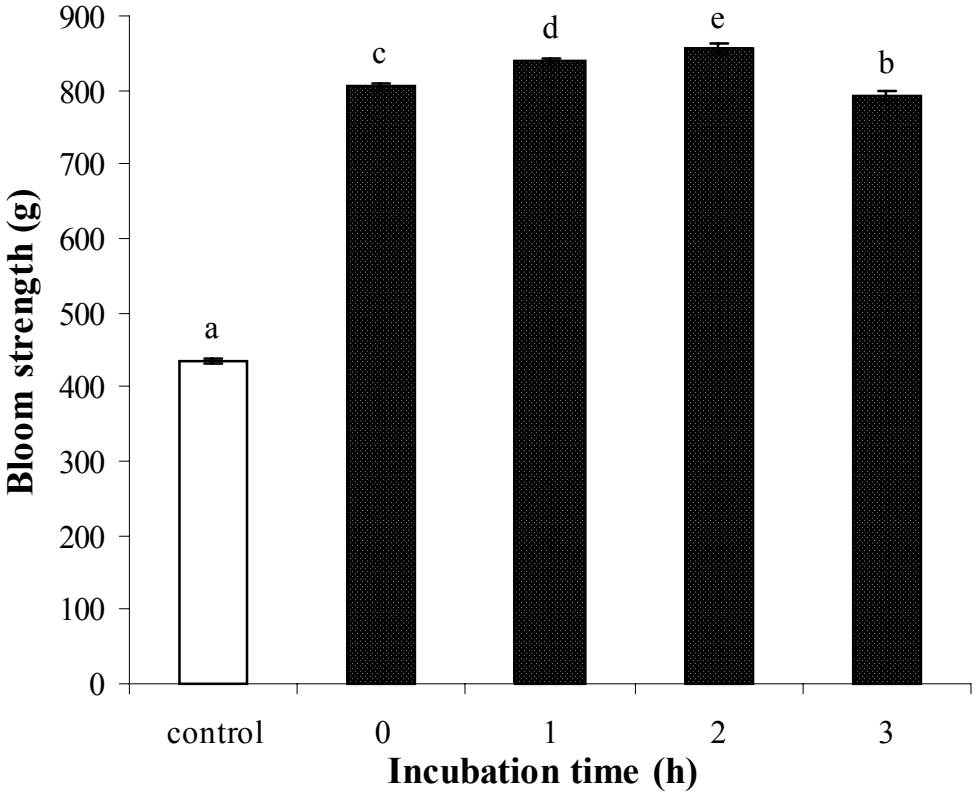

Figure 38 Bloom strength of gelatin gel from bigeye snapper skin containing 0.01 %(w/v) MTGase incubated for different times. Bars represent the standard deviation from five determinations.

Different letters on the bars denote the significant differences ($p<0.05$).

From electrophoretic study, protein patterns of skin gelatin gel were not different when incubation time at room temperature increased (Figure 39). However, difference in bloom strength was observed ($p<0.05$) (Figure 38). From the result, it was suggested that the cross-linking of proteins induced by MTGase under those conditions might be formed to the extent, which could not be clearly observed in the SDS-PAGE but favored the formation of strong gel network.

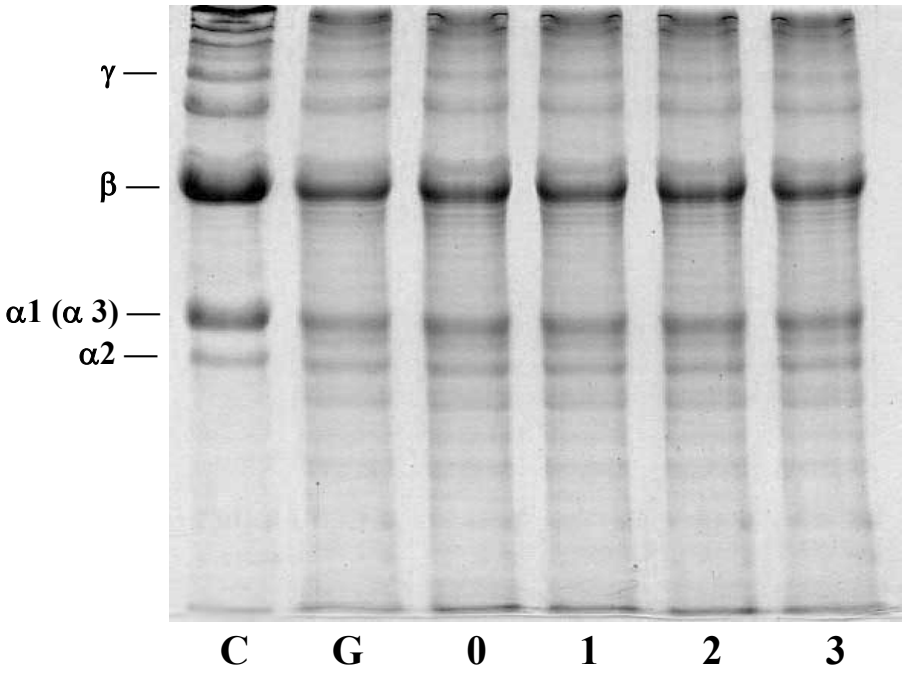

Figure 39 SDS-PAGE pattern of gelatin gel from bigeye snapper skin containing 0.01 %(w/v) MTGase incubated for different times. C and G denote collagen and gelatin from bigeye snapper skin, respectively. Numbers denote the incubation time at room temperature (h.).

To verify if MTGase at higher concentration and longer incubation time resulted in the changes of gelatin proteins, the electrophoretic patterns of skin gelatin gel added with MTGase at the concentration of 0.1 and 1.0%(w/v) with different incubation time (0, 20, 40, 60, 120, 180 and 240 min) was determinied (Figure 40). At the level of 0.1%MTGase, γ-component decreased with increasing incubation time and disappeared at incubation time of 180 min, while α- and β-components decreased to some extent and no changes were observed with increasing incubation time (Figure 40A).

At a level of 1.0%MTGase, α-, β- and γ-components continuously decreased with increasing incubation time and disappeared after incubation for 240, 180 and 120 min, respectively (Figure 40B).

The result suggested that γ-component was more polymerized by MTGase than β- and α-components, respectively. TGase is a transferase which catalyzes the acyl transfer reaction between γ-carboxyamine groups of glutamine residues and primary amines (Nio *et al*., 1986; Nonaka *et al*., 1989; Folk and Chung, 1973; Folk and Finlayson, 1977; Haard, 2001). When the ε-amino groups of lysine residues act as acyl receptor, it results in polymerization and intra- and intermolecular crosslinking of proteins via formation of ε-(γ-glutamyl) lysine linkages (Ashie and Lanier, 2000; Totosaus *et al*., 2002). Thus, γ-component might be the preferable substrate for MTGase rather than β- and α-components, respectively.

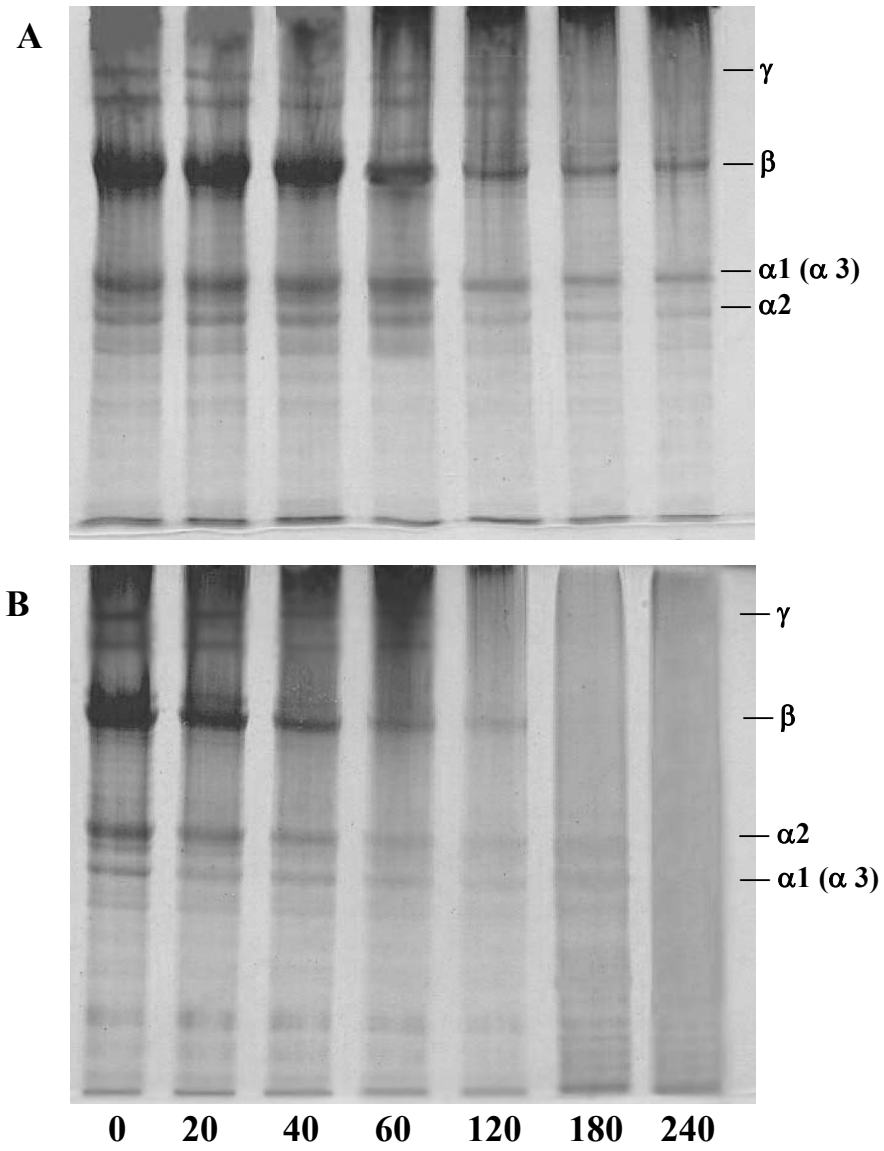

Figure 40 SDS-PAGE pattern of gelatin gel from bigeye snapper skin added with 0.1 (A) or 1.0 (B) %(w/v) MTGase and incubated for different times. Numbers represent incubation time (min).