CHAPTER 4

SKIN GELATIN FROM BIGEYE SNAPPER AND BROWNSTRIPE RED SNAPPER: CHEMICAL COMPOSITIONS AND EFFECT OF MICROBIAL TRANSGLUTAMINASE ON GEL PROPERTIES

4.1 Abstract

Fish skin gelatin was extracted from the skin of bigeye snapper (*Priacanthus macracanthus*) and brownstripe red snapper (*Lutjanus vitta*) with yields of 6.5% and 9.4% on the basis of wet weight, respectively. Both skin gelatins having high protein but low fat content contained high hydroxyproline content (75.0 and 71.5 mg/ g gelatin powder). The bloom strength of gelatin gel from brownstripe red snapper skin gelatin (218.6 g) was greater than that of bigeye snapper skin gelatin (105.7 g) ($P < 0.05$). The addition of microbial transglutaminase (MTGase) at concentrations up to 0.005% and 0.01% (w/v) increased the bloom strength of gelatin gel from bigeye snapper and brownstripe red snapper, respectively ($P<0.05$). However, the bloom strength of skin gelatin gel from both fish species decreased with further increase in MTGase concentration. SDS-PAGE of gelatin gel added with MTGase showed the decrease in band intensity of protein components, especially, β- and γ- components, suggesting the cross-linking of these components induced by MTGase. Microstructure studies revealed that denser and finer structure was observed with the addition of MTGase.

4.2 Introduction

Gelatin and collagen, its native form, have been widely applied in the food industry as ingredients to improve the elasticity, consistency and stability of foods. It can be used for encapsulation, production of hard and soft capsules, wound dressing, adsorbent pads and edible film formation, making it of interest to the pharmaceutical, biomaterial-based packaging, and photographic industries (Digenis *et al.*, 1994). Gelatin is normally manufactured on a large scale from the waste generated during animal slaughter and processing, such as skin and bone (Patil *et al.*, 2000). In general, there are two methods to

obtain gelatin from skins and bones, an acid process (gelatin A with isoelectric point at pH 6-9) and an alkaline process (gelatin B with isoelectric points at pH 5) (Stainsby, 1987). Chemicals used for pretreatment as well as extraction condition such as temperature and time can influence the length of polypeptide chains and the functional properties of the gelatin (Kolodziejska *et al*., 2004). The gelling properties of gelatin are also influenced by the source of raw material, which vary in proline and hydroxyproline contents. Collagen with different compositions from varying species is associated with the temperature of animals living habitat. The melting temperature of gelatin prepared from the skins of warm-blooded animals and warm-water fish is generally higher than that of gelatin from the skin of fish living in cold-water, owing to the greater imino acid content and increased proline hydroxylation degree (Piez and Gross, 1960; Yamaguchi *et al*., 1976; Norland, 1990; Gilsenan and Ross-Murphy, 2000). To improve the gel property of gelatin, some chemicals such as $MgSO_4$, and glycerol were used (Fernandez-Diaz *et al*., 2001). Moreover, microbial transglutaminase has been reported to increase the bloom strength of Baltic cod skin gelatin (Kolodziejska *et al*., 2004).

So far, the main sources of gelatin are limited to those of land animal origin such as bovine or porcine skin and bone. However, the outbreak of bovine spongiform encephalopathy (BSE) and the foot-and-mouth disease (FMD) crisis have resulted in anxiety among users of collagen and gelatin products from land-based animals (Helcke, 2000). In addition, gelatin produced from porcine skins or bones cannot be used for some foods due to esthetic and religious objections (Sadowska *et al*., 2003). Furthermore, the increases in consumer need for kosher and halal foods have gained a demand for fish gelatin. Therefore, alternative sources, especially fish processing wastes including skin, bone or scale, have been paid increasing attention for gelatin extraction. Fish gelatin has been produced from cod, hake skin (Fernandez-Diaz *et al*., 2001), megrim skin (Sarabia *et al*., 2000), tilapia (*Oreochromis* spp.) skin (Grossman and Bergman, 1992; Jamilah and Harvinder, 2002), Nile perch skin and bone (Muyonga *et al*., 2004b). However, little information regarding the characteristics of gelatin from tropical fish, especially from commercially important species involving those used for surimi production, such as bigeye snapper and brownstripe red snapper, has been reported. Therefore, the aims of this investigation were to prepare and characterize skin gelatins from bigeye snapper and brownstripe red snapper and to study the effect of microbial transglutaminase on their gelling properties.

4.3 Materials and Methods

Chemicals

Microbial transglutaminase (MTGase) powder (Activa™ TG) was obtained from Ajinomoto Co (Kawasaki, Japan). MTGase powder had 625 units TGase/g powder as assayed by hydroxamate method (Folk, 1970) All chemicals for electrophoresis were purchased from Sigma Chemical Co. (St. Louis, Mo., USA).

Fish skin preparation

Bigeye snapper (*Priacanthus macracanthus*) and brownstripe red snapper (*Lutjanus vitta*) with an average total length of 22-25 cm were caught from Songkhla coast along the Gulf of Thailand, stored in ice and off-loaded after 24-36 h of capture. Upon arrival to the dock in Songkhla, fish were stored in ice with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai. Fish were washed with a tap water. Skins were then removed, descaled and cut into small pieces (0.5x0.5 cm). Prepared skins were kept on ice prior to gelatin extraction.

Extraction of fish skin gelatin

Gelatin was extracted from fish skin according to the method of Sarabia *et al.* (2000) with a slight modification. Skins were soaked in 0.2 M NaOH with a skin/solution ratio of 1:10 (w/v) at 4°C with a gentle stirring. The solution was changed every 30 min for 3 times to remove noncollagenous proteins and pigments. Alkaline treated skins were then washed with tap water until neutral or faintly basic pHs of wash water were obtained. The skins were then soaked in 0.05 M acetic acid with a skin/solution ratio of 1:10 (w/v) for 3 h at room temperature (25°C) with a gentle stirring to swell the collagenous material in fish skin matrix. Acid treated skins were washed as previously described. The swollen fish skins were soaked in distilled water with a skin/water ratio of 1:10 (w/v) at 45°C for 12 h with a continuous stirring to extract the gelatin from skin

matter. The mixture was then filtered using two layers of cheesecloth. The resultant filtrate was freeze-dried and the dry matter was referred to as "gelatin powder".

Proximate analysis of gelatins

The moisture, ash and fat contents of the gelatin powder were determined according to the AOAC methods (AOAC, 1999). Protein content was measured by Kjeldahl method (AOAC, 1999) and a nitrogen conversion factor of 5.4 was used for calculation of protein content (Eastoe and Eastoe, 1952). Hydroxyproline content was determined according to the method of Bergman and Loxley (1963). The conversion factor for calculating the collagen content from hydroxyproline of bigeye snaper and brownstripe red snapper skin were 11.49 and 12.34, respectively (Jongjareonrak *et al*., 2005a; 2005b).

Determination of amino acid composition

Gelatin samples were hydrolyzed under reduced pressure in 4.0 M methanesulfonic acid containing 0.2% (v/v) 3-2(2-aminoethyl)indole at 115°C for 24 h, and the hydrolysates were neutralized with 3.5 M NaOH and diluted with 0.2 M citrate buffer (pH 2.2). An aliquot of 0.4 ml was applied to an amino acid analyzer (MLC-703; Atto Co., Tokyo, Japan).

Gelatin gel preparation

Gelatin gel was prepared according to the British Standard 757:1975 method (BSI, 1975) with a slight modification. Gelatin (2.0 g) was mixed with 30 ml of distilled water in a 50 ml-beaker (PYREX®, USA) to obtain a final concentration of 6.67% (w/v). The mixture was stirred thoroughly and left at room temperature for 30 min to allow the gelatin to absorb water and swell. The mixture was then incubated at 42°C for 30 min in a temperature-controlled water bath with occasional stirring. The beakers were then kept in a temperature-controlled chamber at 10°C and allowed to stand for 16-18 h before determination of bloom gel strength.

To study the effect of MTGase on gel properties of fish skin gelatin, MTGase at various concentrations (0.001, 0.0025, 0.005, 0.01, and 0.05% (w/v)) was added after the total dissolution. The mixture was then incubated at 40°C for 30 min, followed by incubating at 10°C for 16-18 h. The resultant gels were subjected to analysis.

Determination of bloom gel strength

Bloom gel strength was determined according to the British Standard 757: 1975 method (BSI, 1975) with a slight modification. Bloom gel strength at 10°C was determined by a Model TA-XT2 Texture analyzer (Stable Micro System, Surrey, UK) using a load cell of 5 kN equipped with a 1.27 cm diameter flat-faced cylindrical Teflon® plunger. The dimensions of the sample were 3.8 cm in diameter and 2.7 cm in height. The maximum force (in grams) taken, when the penetration distance of 4 mm was obtained, was recorded. The speed of the plunger was 0.5 mm/s. The measurement was performed in triplicate.

Electrophoretic analysis

Protein patterns of gelatin and gelatin gel samples were determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). The samples (1 g) were dissolved in 10 ml of 5% (w/v) SDS solution. The mixture was heated at 85°C for 1 h in a water bath to dissolve total proteins. Supernatants were collected after centrifuging at 3000 x ***g*** for 3 min. The supernatants were then mixed with sample buffer (0.5 M Tris-HCl, pH 6.8 containing 4% (w/v) SDS, 20% (v/v) glycerol and 10% (v/v) βME at the ratio of 1:1 (v/v). Samples (20 μg protein) were loaded into the polyacrylamide gel made of 7.5% running gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini Protean II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After electrophoresis, gel was stained with 0.05% (w/v) Coomassie blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid and destained with 30% (v/v) methanol and 10% (v/v) acetic acid. Calf skin acid-soluble type I collagen (Sigma Chemical Co., St. Louis, Mo., USA) was used as a standard collagen.

Scanning electron microscopy

Microstructure of gelatin gels was determined using Scanning Electron Microscopy (SEM). Gelatin gels from bigeye snapper and brownstripe red snapper skin with and without MTGase having a thickness of 2-3 mm were fixed with 2.5% (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 7.2). The samples were then rinsed with distilled water for 1 h and dehydrated in ethanol with a serial concentration of 50, 70, 80, 90 and 100% (v/v). Dried samples were mounted on a bronze stub and sputter-coated with gold (Sputter coater SPI-Module, PA, USA). The specimens were observed with a scanning electron microscope (JEOL JSM-5800 LV, Tokyo, Japan) at an acceleration voltage of 10 kV.

Statistical analysis

Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test (Steel and Torrie, 1980). Analysis was performed using a SPSS package (SPSS 8.0 for window, SPSS Inc, Chicago, IL).

4.4 Results and discussion

Composition of fish skin gelatin

Gelatins were extracted from bigeye snapper and brownstripe red snapper skin with yields of 6.5% and 9.4%, respectively, on the basis of wet weight. The yield of gelatin extracted from bigeye snapper skin was lower than that of brownstripe red snapper skin. The gelatin yields have been reported to vary among the fish species, mainly due to the differences in collagen content, the compositions of skin as well as the skin matrix. The different yields of skin gelatin have been reported for sole (8.3%), megrim (7.4%), cod (7.2%), hake (6.5%) (Gomez-Guillen *et al.*, 2002), red and black tilapia (7.8% and 5.4%, respectively) (Jamilah and Harvinder, 2002), young and adult Nile perch (12.5% and 16%, respectively) (Muyonga *et al.*, 2004b). From the results, the lower yield of gelatin from bigeye snapper skin was in accordance with the lower contents of collagen (6.4% ASC and 1.1% PSC), compared with those of brownstripe red snapper skin (9.0%

ASC and 4.7% PSC) (Jongjareonrak *et al.*, 2005a; 2005b). Furthermore, the high degree of cross-linking via covalent bonds caused the decrease in solubility of collagen and might lead to the lower content of extractable gelatin (Foegeding *et al.*, 1996).

Table 6. Proximate composition of gelatins from bigeye snapper and brownstripe red snapper skins

Compositions	Source of skin gelatin	
	Bigeye snapper	**Brownstripe red snapper**
Protein*	87.9±0.8[#]	88.6±0.7
Moisture*	8.2±0.7	7.6±0.2
Ash*	3.2±0.2	1.9±0.1
Fat*	0.6±0.0	0.8±0.1
Hydroxyproline content**	75.0±0.4	71.5±0.5
Collagen content**	862.0±4.6	882.0±5.5

* % wet wt. ** mg/ 1 g gelatin powder. [#] Values are given as mean ± SD from triplicate determinations.

The proximate compositions of bigeye snapper and brownstripe red snapper skin gelatins are shown in Table 6. Generally, skin gelatins of both species contained high protein content but low moisture and fat contents, suggesting the efficient removal of water and fat from skin. However, gelatin from bigeye snapper skin was higher in ash content (3.2%), compared with that from brownstripe red snapper (1.9%). Also the ash content obtained was higher than the recommended maximum of 2.6% (Jones, 1997). The different mineral contents between skins of both species might be associated with the varying ash contents obtained. From the results, high hydroxyproline and total collagen contents were observed in the extracted gelatins. Collagens constituted approximately 86% and 89% of the dry gelatin from bigeye snapper and brownstripe red snapper skins, respectively, indicating a high purity with negligible contaminating constituents.

Table 7. Amino acid compositions of gelatins from bigeye snapper and browstripe red snapper skin (residues per 1000 total amino acid residues)

Amino acids	Source of skin gelatin	
	Bigeye snapper	**Brownstripe red snapper**
Hydroxyproline	91	84
Aspartic acid	61	56
Threonine	32	31
Serine	38	39
Glutamic acid	103	105
Proline	134	141
Glycine	193	204
Alanine	103	108
Valine	21	17
Methionine	17	15
Isoleucine	10	9
Leucine	27	25
Tyrosine	6	5
Phenylalanine	21	20
Lysine	38	38
Histidine	12	9
Arginine	92	94
Total	1000	1000

The amino acid composition of both skin gelatins expressed as residues per 1,000 total amino acid residues is shown in Table 7. Slight differences in the amino acid profiles between skin gelatins of two species were observed. In general, both skin gelatins had the amino acid composition similar to that of mother collagens reported by Jongjareonrak *et al.* (2005a; 2005b). Bigeye snapper and brownstripe red snapper skin gelatins were rich in glycine (19.3% and 20.4%), alanine (10.3% and 10.8%), and proline (13.4% and 14.1%). For hydroxyproline, it accounted about 61 and 56 residues per 1,000 residues for bigeye snapper and brownstripe red snapper skin gelatins, respectively. The numbers of imino acids, proline and hydroxyproline, in bigeye snapper

and brownstripe red snapper skin gelatins were 195 and 197 residues per 1,000 residues, respectively. The imino acid content in skin gelatin from both species were higher than that reported in sole, megrim, cod and hake (174, 175, 156, 173 residues per 1,000 residues, respectively) (Gomez-Guillen *et al.*, 2002) but lower than that found in porcine skin gelatin (222 residues per 1000 residues) (Cho *et al.*, 2004). The stability of triple helical structure in renatured gelatins was associated with the total content of pyrrolidine imino acids (Ledward, 1986). Hydroxyproline plays an essential role in the stabilization of the triple-helix strands of collagen via its hydrogen bonding ability through its -OH group (Burjandze, 1979; Ledward, 1986). Gelatin with higher content of hydroxyproline is believed to have higher viscoelastic properties and its ability to develop triple helix structures, which are important for stabilizing the gelatin gel structure (Gomez-Guillen *et al.*, 2002).

Effect of MTGase on bloom strength of skin gelatin gels

Bloom strength of gelatin gels with and without MTGase was shown in Figure 17. Bigeye snapper skin gelatin gels exhibited the lower bloom strength (105.7 g) than that of brownstripe red snapper skin gelatin (218.6 g), suggesting the difference in gel forming ability of gelatin between two species. The bloom strength of fish skin gelatin gel was reported to be varied with species. Fish skin gelatins with different bloom strength were reported for young and adult Nile perch (217 g and 240 g, respectively) (Muyonga *et al.*, 2004b), tilapia (263 g) (Grossman and Bergman, 1992), sole, megrim, hake, and cod (~ 350, 340, 100, and 70 g, respectively) (Gomez-Guillen *et al.*, 2002). The difference in bloom strength between species was possibly due to the different composition, particularly in terms of amino acid composition and size of protein chains (Muyonga *et al.*, 2004b). Protein degradation during gelatin extraction was found in bigeye snapper (Jongjareonrak *et al.*, 2006), while less degradation was noticeable in brownstripe red snapper skin gelatin. Protein degradation fragments, if present, may reduce the ability of α-chains to anneal correctly by hindering the growth of the existing nucleation sites (Ledward, 1986; Normand *et al.*, 2000).

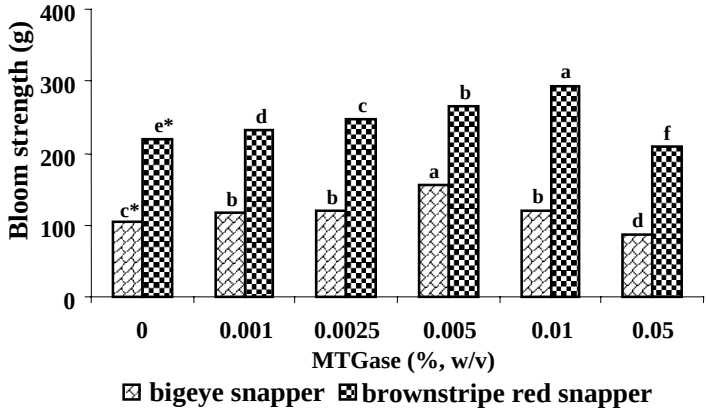

Figure 17. Bloom strength of gelatin gels from bigeye snapper and browstripe red snapper skins without and with MTGase added at different concentrations. * Values are given as mean ± SD from triplicate determinations. Different letters on the bars under the same species indicate the significant differences ($P<0.05$).

From the results, the addition of MTGase generally increased the bloom strength of gelatin gels of both species ($P<0.05$). The marked increase in bloom strength of gelatin gel from bigeye snapper and brownstripe red snapper skin gelatin was observed with the addition of MTGase up to 0.005% and 0.01%, respectively. The results suggested that MTGase might catalyze the formation of ε-(γ-glutamyl) lysine linkages between protein chains. However, bloom strength decreased with further increase in MTGase concentration. This result was in agreement with Gomez-Guillen *et al.* (2001) who reported that the increases in MTGase concentration from 0.015% to 0.075% lowered the bloom strength of megrim skin gelatin gel. From the result, the addition of MTGase at the appropriate concentration increased bloom strength of skin gelatin of both species effectively. The excessive formation of MTGase-catalyzed covalent bonds was detrimental to gel strength due to formation of intramolecular covalent bonds. As a consequence, intermolecular aggregation might be impeded, leading to reduced gel network formation. Therefore, MTGase at the concentrations of 0.005% and 0.01% (w/v) were appropriate to improve the bloom strength of gelatin gels from bigeye snapper and brownstripe red snapper skins, respectively.

Effect of MTGase on SDS-PAGE patterns of gelatin gels

The protein patterns of gels from bigeye snapper and brownstripe red snapper skin gelatins under reducing condition (in the presence of βME) are shown in Figure 18. For gelatin gels without MTGase, α_1-chains was found as the major component for both samples. However, α_2-chains were not found in gelatin gels from brownstripe red snapper skin. Protein patterns of gelatin gels were generally relevant to those of the starting gelatin powder, especially for brownstripe red snapper gelatin. However, proteins with molecular weights of 71, 65 and 57 kDa were not found in gel sample. No β-component was noticeable in the gel sample, however protein with molecular weight of 143 kDa was found in bigeye snapper gelatin sample. This might be owing to the degradation of these components during gel preparation, particularly during heat treatment. This result was in accordance with Muyonga *et al.* (2004b) who observed that Nile perch skin gelatins contained low-molecular-weight peptides, especially, gelatins extracted with higher temperature. Heat-induce cleavage of protein chains occurred during the extraction process of Nile perch skin gelatin and was enhanced with higher temperature extraction (Muyonga *et al.*, 2004b). From the results, a greater amount of peptides with molecular weight lower than α-chains were observed in bigeye snapper skin gelatin than that of brownstripe red snapper skin gelatin. This suggested that collagen of bigeye snapper skin was more degraded during the extraction and some proteinase might be involved in the degradation of gelatin (Jongjareonrak *et al.*, 2006). The formation of degradation fragments is associated with the low viscosity, low melting point, low setting point, high setting time, as well as decreased bloom strength of gelatin (Ledward, 1986; Tavernier, 1989; Normand *et al.*, 2000; Muyonga *et al.*, 2004b). From the results, greater bloom strength was found in brownstripe red snapper skin gelatin, compared with that from bigeye snapper skin (Figure 17).

When MTGase was added, band intensity of all components in gelatin gels from both species decreased continuously with increasing MTGase concentration. When MTGase concentration increased, band intensity of β- and γ-components as well as α-chains decreased with the concomitant increase in high-molecular-weight aggregate. At the highest concentration used (0.05%), all protein bands almost disappeared. The decrease in protein band intensity was most likely due to the cross-linking via non-disulfide covalent bond formation induced by MTGase (Folk, 1983). The results indicated that all

components underwent cross-linking induced by MTGase. This was possibly because most of protein components contained both acyl donor and receptors aligned in the appropriate position for reaction induced by MTGase. Lysine was found in skin gelatin (Table 7) and might act as the acyl receptor of gelatin from both species. Glutamine, reported as glutamic acid, probably functioned as the acyl donor of gelatin. Generally, glutamine undergoes deamidation to glutamic acid under the harsh acid hydrolysis conditions used for analysis (Pickering and Newton, 1990) (Table 7). The decrease in protein band intensity (Figure 18) was coincidental with the increase in bloom strength (Figure 17). The aggregation of protein components via the covalent bond directly resulted in the development of a strong gel network, particularly with the appropriate range of MTGase levels. However, excessive cross-linking caused the adverse effect on gel network formation. Thus, the alignment or aggregation pattern of gelatin protein components also played an important role in gel formation as well as the strength of gelatin gel obtained.

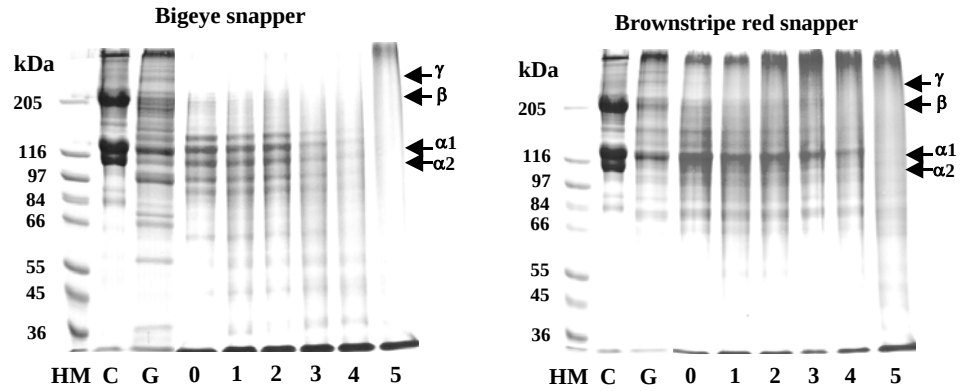

Figure 18. Protein pattern of gelatin gel from bigeye snapper and brownstripe red snapper skins added with MTGase at different concentrations. HM, C, and G, denote high molecular weight markers, standard collagen type I, and gelatin powder, respectively. Numbers, 0, 1, 2, 3, 4, and 5 indicate MTGase concentrations of 0, 0.001, 0.0025, 0.005, 0.01, and 0.05% (w/v), respectively.

Microstructure of gelatin gels

The microstructure of gelatin gels from bigeye snapper and brownstripe red snapper skins without and with MTGase were visualized by SEM as shown in Figure 19. In general, bigeye snapper skin gelatin gel showed the finer and denser strands compared with those of brownstripe red snapper skin gelatin (Figure 19A and 19C). This was possibly due to the different characteristics of gelation between species in terms of aggregation and alignment of protein molecules. With addition of MTGase, the denser aggregates with negligible voids in the gel network were observed (Figure 19B and 19D). It was suggested that gelatin underwent cross-linking via non-disulfide covalent bonding, resulting in greater intermolecular aggregation between adjacent molecules. The result was coincidental with the lower α- and β- components as shown in Figure 18. These cross-links might be associated with the higher bloom strength of gelatin gel when MTGase was added (Figure 17).

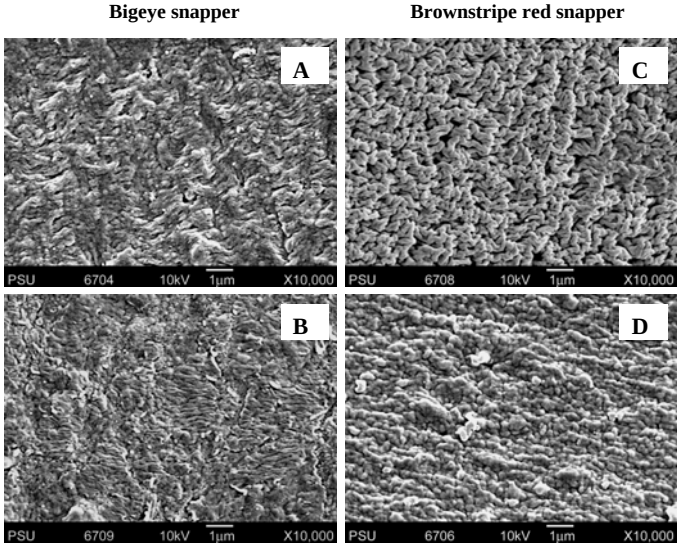

Figure 19. Microstructure of gelatin gels (magnification: 10,000 X). A and B: gelatin gels of bigeye snapper skin without and with 0.005% (w/v) MTGase, respectively. C and D: gelatin gels of brownstripe red snapper skin without and with 0.01% (w/v) MTGase, respectively.

4.5 Conclusion

Gelatin was extracted from the skin of bigeye snapper and brownstripe red snapper. Both gelatins contained slightly different amino acid composition. The bloom strength of gelatin gel from bigeye snapper skin was lower than that of brownstripe red snapper skin. The addition of MTGase induced the aggregation of proteins, which was associated with the increase in bloom strength of fish skin gelatin from both species. However, bloom strength decreased with the excessive MTGase amount.