Chapter 2

MATERIALS AND METHODS

1. Chemical reagents

All reagents were of analytical grade. Type I collagen from calf skin was purchased from Elastin products Co., INC. (Owensville, MO, USA.). Type II, III and V collagens from porcine cartilage, porcine skin and porcine placenta, respectively, lysyl endopeptidase from *Achromobacter lyticus* were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). V8 protease from *Staphylococcus aureus* was purchased from Sigma Chemical Co. (St. Louis, MO., USA.). Microbial transglutaminase (MTGase) was obtained from Ajinomoto Co., Ltd. (Kawasaki, Japan).

2. Fish skin and bone preparation

The skin and bone of bigeye snapper (*Priacanthus tayenus*) (Figure 15), the waste from surimi processing, were obtained from Man A Frozen Foods Co. Ltd., Songkhla, Thailand. Both skin and bone were placed in ice with a material/ice ratio of 1:2 (w/v) and transported to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, residual meat was removed manually from both skin and bone and cleaned samples were washed with a tap water. Those samples were used for collagen and gelatin extraction.

For collagen and gelatin extraction, the skin was descaled only for collagen extraction, followed by thorough washing. Descaled skin and skin were then cut into

small pieces (1.0 x 1.0 cm.), placed in polyethylene bag and stored at –20 ^{O}C until used. For the bone, it was cut into small pieces (1-2 cm in length) and powderized by blending the samples in liquid nitrogen for 20 seconds using a blender (National, Tokyo, Japan). The prepared samples were kept at –20 °C until used. Bone, descaled skin and skin were subjected to proximate analysis according to AOAC method (1999) (Appendix 1-4). Hydroxyproline content was also determined according to the method of Bergman and Loxley (1963) (Appendix 5).

Figure 15 Bigeye snapper, *Priacanthus tayenus*.

3. Instruments

Instruments	Model	Company/Country
pH meter	CG 842	SCHOTT, Germany
Magnetic stirrer	BIG SQUID	IKA LABORTECHNIK, Stanfen, Germany
Overhead stirrer	RW 20.n	IKA LABORTECHNIK, Stanfen, Germany
Oil bath	B-490	BUCHI, Flawil, Switzerland
Water bath	W350	Memmert, Schwabach, Germany
Vortex mixer	G-560E	NY, USA.
Micorcentrifuge	MIKRO20	Hettich ZENTRIFUGEN, Germany
Refrigerated centrifuge	RC-5B plus	Sorvall, USA
Brookfield Synchorolectic viscometer	DV II+	Brookfield Eng Labs Inc., MA, USA.
Amino acid analyzer	MLC-730	Atto Co., Tokyo, Japan
Compact-PAGE apparatus	PAGEL®-Compact	Atto Co., Tokyo, Japan
Electrophoresis apparatus	Mini-Protean II	Bio-Rad Laboratories, Inc., USA.
Differential scanning calorimeter	DSC 7	Norwalk, USA.
Freeze dryer	Dura-Top™ μp	FTS system, USA
Colorimeter	ColorFlex	HunterLab Reston, VA, USA.
Electron scanning microscope	JSM-5800 LV	JEOL, Tokyo, Japan
Texture analyzer	TA-XT2	Stable Micro Systems, Surrey, England
Double-beam spectrophotometer	UV-16001	SHIMADZU, Australia
Homogenizer	T25 basic	IKA LABORTECHNIK, Selangor, Malaysia

4. Preparation and characterization of acid soluble collagens from skin and bone

4.1 Extraction of collagen

The collagens were prepared by the method of Nagai and Suzuki (2000a) with a slight modification. All the preparation procedures were performed at 4 °C. To remove non-collagenous proteins, the descaled skin and bone were mixed with 0.1 N NaOH at a sample/alkali solution ratio of 1:10 (w/v). The mixture was stirred for 6 h. The alkali solution was changed every 2 h. Then, the deproteinized samples were washed with cold water until neutral or faintly basic pHs of wash water were obtained.

Deproteinized skins were defatted with 10% butyl alcohol with a solid/solvent ratio of 1:10 (w/v) for 18 h and the solvent was changed every 6 h. Defatted skins were washed with cold water, followed by soaking in 0.5 M acetic acid with a solid/solvent ratio of 1:30 (w/v) for 24 h. The mixture was filtered with two layers of cheese cloth. The residue was re-extracted with the same condition. Both filtrates were combined. The collagen was precipitated by adding NaCl to a final concentration of 2.6 M in presence of 0.05 M Tris(hydroxymethyl)aminomethane, pH 7.0. The resultant precipitate was collected by centrifuging at 20,000 *xg* for 60 min. The pellet was dissolved in 0.5 M acetic acid, dialyzed against 0.1 M acetic acid and distilled water, respectively, and then freeze dried.

To extract collagen from bones, deproteinized bones were decalcified with 0.5 M ethylenediaminetetraacetic acid (EDTA) –4 Na (pH 7.4) using a solid/solution ratio of 1:10 (w/v) for 40 h. The solution was changed every 8 h. The residue was washed

thoroughly with cold water (4 ^{O}C). Collagen was further extracted from the bone in the same manner with that used for skin as previously described.

4.2 Characterization of collagen

4.2.1 Proximate analysis

Skin and bone collagens were subjected to proximate analysis including moisture, ash, fat and protein contents according to the method of AOAC (1999) (Appendix 1-4).

4.2.2 Hydroxyproline content

Collagens were determined for hydroxyproline content according to the method of Bergman and Loxley (1963) (Appendix 5).

4.2.3 Amino acid analysis

Skin and bone collagens 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. 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 analyser (MLC-703; Atto Co., Tokyo, Japan).

4.2.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed by the method of Laemmli (1970) (Appendix 8). The collagen samples were dissolved in 0.02 M sodium phosphate containing 1% SDS and 3.5 M urea (pH 7.2) with continuous stirring at room temperature. The mixtures were centrifuged at 8,500 *xg* for 5 min at room temperature to remove undissolved debris. Solubilized samples were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris-HCl, pH 6.8 containing 4% SDS, 20% glycerol) in presence or absence of 10% β-ME. Samples were loaded on the PAGEL®-Compact precast gel

(5% gel) and subjected to electrophoresis at a constant current of 20 mA/gel using a Compact-PAGE apparatus (Atto Co., Tokyo, Japan). 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. High molecular weight markers (Sigma Chemical Co., St. Louis, Mo., U.S.A.) were used to estimate the molecular weight of proteins. Type I, II, III and V collagens were used as standards.

4.2.5 Peptide mapping of collagen from skin and bone

Peptide mappings of collagen samples were performed according to the method of Saito *et al*. (2002) with a slight modification. The freeze-dried samples (0.2 mg) were dissolved in 0.1 ml of 0.1 M sodium phosphate, pH 7.2 containing 0.5% (w/v) SDS. After the addition of 10 μl of the same buffer containing 5 μg of *Staphylococcus aureus* V8 protease (EC 3.4.21.19, Sigma Chemical Co., St. Louis, Mo., U.S.A.) or 0.05 μg of lysyl endopeptidase from *Achromobacter lyticus* (EC 3.4.21.50; 4.5 AU/mg protein; Wako Pure Chemical Industries, Ltd., Tokyo, Japan) to collagen solutions, the reaction mixture was incubated at 37°C for 25 min and 5 min for V8 protease and lysyl endopeptidase, respectively. The reaction was terminated by subjecting the reaction mixture to boiling water for 3 min. Peptides generated by the protease digestion were separated by SDS-PAGE using 7.5% gel. Peptide mapping of calf skin collagen acid-soluble type I was conducted in the same manner and the peptide patterns were compared.

4.2.6 Differential scanning calorimetry (DSC)

The samples were rehydrated by adding deionized distilled water or 0.05 M acetic acid to dried samples at a solid/solution ratio of 1:40 (w/v). The mixture was allowed to stand for 2 days at 4°C.

Differential scanning calorimetry (DSC) was performed using a Model DSC 7 (Norwalk, USA) as described by Rochdi *et al*. (2000) and Komsa-Penkova (1999). Temperature calibration was run using the Indium thermogram. The samples (5-10 mg) were accurately weighed into aluminum pans and sealed. The samples were scanned at 1°C /min over the range of 20-50°C using ice water as the cooling medium. The empty pan was used as the reference. Total denaturation enthalpy (ΔH) was estimated by measuring the area in the DSC thermogram. The maximum transition temperature (T_{max}) was estimated from the thermogram.

4.2.7 Viscosity of collagen solution

A 500 ml of 0.03% collagen in 0.1 M acetic acid was subjected to viscosity measurements using a Brookfield Synchorolectic viscometer (model DV II+, Brookfield Eng Labs INC., Stoughton, MA, USA.) with spindle No. 1 and speed of 100 rpm. Collagen solution was heated from 4 to 50°C with a heating rate of 4°C/min. At designated temperature, solution was held for 30 min prior to viscosity determination. Measurement was carried out in triplicate. The relative viscosity was calculated in comparison with that obtained at 4°C.

4.2.8 Collagen solubility

The collagen solubility was determined by the method of Montero *et al*. (1991) with a slight modification. The collagens were dissolved in 0.5 M acetic acid

to obtain a final concentration of 3 mg/ml and the mixture was stirred at 4°C until collagen was solubilized completely.

4.2.8.1 Effect of pH on collagen solubility

Collagen solution (8 ml) was added to a centrifuge tube and the pH was adjusted with either 6 N NaOH or 6 N HCl to obtain a final pH ranging from 1 to 10. The volume of solutions was made up to 10 ml by distilled water previously adjusted to the same pH of collagen solution. The solution was centrifuged at 20,000 *xg* at 4°C for 30 min. Protein content in the supernatant was determined by the method of Lowry *et al*. (1951) (Appendix 6) using bovine serum albumin as a standard. Relative solubility was calculated in comparison with that obtained at the pH rendering highest solubility.

4.2.8.2 Effect of NaCl on collagen solubility

A 5 ml of collagen (6 mg/ml) in 0.5 M acetic acid was mixed with 5 ml of NaCl in 0.5 M acetic acid at various concentrations (0, 2, 4, 6, 8, 10 and 12 %(w/v)). The mixture was stirred continuously at 4°C for 30 min, followed by centrifuging at 20,000 *xg* at 4°C for 30 min. Protein content in the supernatant was measured by the method of Lowry *et al*. (1951) (Appendix 6) using bovine serum albumin as a standard. Relative solubility was calculated as mentioned before.

5. Extraction and characterization of gelatin from skin and bone of bigeye snapper

The gelatins were prepared by the method of Gomez-Guillen *et al*. (2002) and Grossman and Bergman (1992) with a slight modification as described in the following scheme (Figure 16).

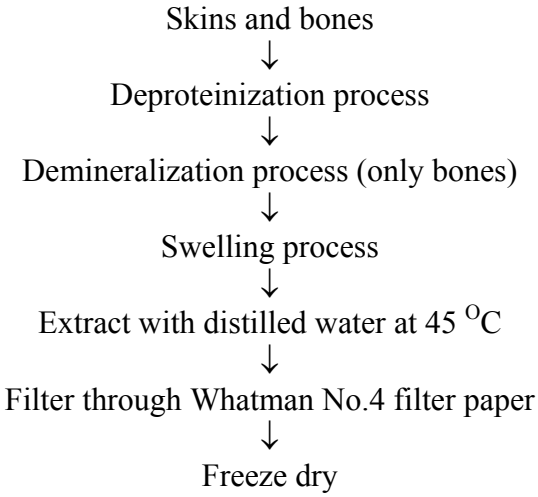

Figure 16 Scheme for extraction of gelatin from skin and bone.

5.1 Deproteinization process for gelatin extraction from skin and bone

To remove non-collagenous proteins, the skin and bone were mixed with ten volume of NaOH (0.025 or 0.050 N). The mixtures were stirred for different times (1, 2 or 3 h) at room temperature (about 25-28 °C). The alkali solution was changed every 1 h. Then, the deproteinized samples were washed with tap water until neutral or faintly basic pH of washed water was obtained. Protein content and hydroxyproline content in deproteinizing solution were determined by the Biuret method (Robison and Hodgen, 1940) (Appendix 7) and the method of Bergman and Loxley (1963) (Appendix 5), respectively.

The deproteinization condition, which was able to remove non-collagenous protein most effectively but showed the lowest hydroxyproline loss, was selected for further step.

5.2 Demineralization process for gelatin extraction from bone

The deproteinized bones were demineralized with either HCl or citric acid at the concentration of 0.6 or 1.2 M with a solid/solution ratio of 1:10 (w/v). The mixture was stirred at room temperature for 5 h. The solution was changed every 1 h. The residue was washed thoroughly with tap water until neutral or faintly acidic pH of washed water was obtained.

The demineralization condition, which reduced the lowest ash content, was chosen for further step.

5.3 Swelling process for gelatin extraction from skin and bone

Deproteinized skins and demineralized bones were swollen with citric acid or acetic acid at various concentrations (0.05, 0.10 or 0.20 M) with a solid/solution ratio of 1:10 (w/v). The mixtures were stirred for 2 h at room temperature. The solvent was changed every 40 min. Swollen skins were washed with tap water until the neutral pH or faintly acidic pH of washed water was obtained and the microstructures of skins, deproteinized skins and swollen skins were determined by SEM. These skins were cut into small pieces (2 x 10 mm.), fixed with 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 5 h. The samples were then rinsed for 45 min in 0.1 M phosphate buffer (pH 7.2) and distilled water, respectively before being dehydrated in a grade ethanol series 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.

After swelling, the skin and bone were subjected to gelatin extraction

5.4 Extraction of gelatin from pretreated skin and bone

5.4.1 Gelatin extraction

The pretreated samples were extracted with distilled water at 45 °C for 12 h. The extract was then filtered using a Buchner funnel with Whatman No.4 filter paper. Finally, the filtrate was collected and freeze-dried.

5.4.2 Gelatin Gel preparation

Gelatin gel was prepared by the method of Fernandez-Diaz *et al.* (2001) with a slight modification. Two g of gelatin was dissolved with 30 ml of distilled water at 60 °C in 50 ml-beaker (PYREX®, USA). The solution was stirred until gelatin was solubilized completely, and cooled in the refrigerator (4 °C, maturation temperature) for 16-18 h.

5.4.3 Determination of bloom strength

The bloom strength was determined by the method of Fernandez-Diaz *et al.* (2001) with a slight modification. The bloom strength at 8-9 °C was determined using a texture analyzer (Stable Micro System, Surrey, UK) with a load cell of 5 kN, cross-head speed 1 mm/s, equipped with a 1.27 cm diameter flat-faced cylindrical Teflon® plunger. The dimensions of the sample were 3.8 cm diameter and 2.7 cm height. The maximum force (in grams), taken when the plunger had penetrated 4 mm into the gelatin gels, was recorded. The measurement was performed in five determinations.

The extracting conditions, which resulted in the highest bloom strength, was chosen for further study.

6 Characterization and functional properties of gelatin from skin of bigeye snapper

6.1 Determination of chemical compositions

Moisture, ash, fat and protein contents of gelatins were determined according to the method of AOAC (1999) (Appendix 1-4).

6.2 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed by the method of Laemmli (1970) (Appendix 8). The gelatin was dissolved in 5% SDS solution. The mixture was then homogenized using homogenizer (model T25 basic, ULTRA TURRAX®, IKA LABORTECNIK, Selangor, Malaysia) for 1 min. The homogenate was centrifuged at 8,500 *xg* for 5 min to remove undissolved debris. Solubilized samples were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris-HCl, pH 6.8 containing 4% SDS, 20% glycerol) in presence or absence of 10% β-ME. Samples were loaded into polyacrylamide gel made of 5% running gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA/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. Type I collagen from calf skin was used as standard.

6.3 Gelation

The 6.67% (w/v) gelatin solutions with different pHs (3, 5, 7 and 9) were prepared. To adjust the pH of gelatin solutions, 6 N NaOH or 6 N HCl were used. The gelatin solutions were incubated at the refrigerated temperature (4°C) for 16-18 h prior to analysis as follows:

6.3.1 Turbidity of gelatin solution

The turbidity of gelatin solution was determined according to the method of Fernandez-Diaz *et al*. (2001). The gelatin solution at different pHs was measured at 360 nm prior to incubation at the refrigerated temperature (4°C) for 16-18 h, using Double-beam spectrophotometer (model UV-16001, SHIMADZU, Australia). The measurement was performed in five determinations.

6.3.2 Bloom strength of gelatin gel

The bloom strength of gelatin gel prepared at different pHs was determined by the method of Fernandez-Diaz *et al*., (2001) with a slight modification as described in section 5.4.3.

6.3.3 Color of Gelatin Gel

The color of gelatin gels prepared at different pHs were determined by a colorimeter (model ColorFlex, HunterLab Reston, VA, USA.). CIE L*, a* and b* values were measured.

6.4 Solubility

The effect of pHs on gelatin solubility was determined by the method of Montero *et al*. (1991) with slight modification. The gelatins were dissolved in distilled water at 60 °C to obtain a final concentration of 2% (w/v) and the mixture was stirred at room temperature until gelatin was completely solubilized. The gelatin solution (8 ml) was added to a centrifuge tube and the pH was adjusted with either 6 N NaOH or 6 N HCl to obtain a final pH ranging from 1 to 10. The volume of solutions was made up to 10 ml by distilled water previously adjusted to the same pH of gelatin solution. The solution was centrifuged at 8,500 *xg* at room temperature for 10 min. Protein content in the supernatant was determined by the Biuret method (Robison and

Hodgen, 1940) (Appendix 7) using bovine serum albumin as a standard. Relative solubility was calculated in comparison with that obtained at the pH rendering highest solubility.

6.5 Emulsifying properties

The emulsifying properties (emulsion activity index, EAI and emulsion stability index, ESI) were determined using turbidimetry method. The gelatin at different concentrations (1, 2 and 3% (w/v)) was used.

The EAI was measured by the method of Pearce and Kinsella (1978) with a slight modification. To prepare the emulsions, soy bean oil (10 ml) was added to the gelatin solution (30 ml). The mixtures were then homogenized using a homogenizer (model T25 basic, ULTRA TURRAX®, IKA LABORTECNIK, Selangor, Malaysia) at 11,000 rpm (no.1) for 1 min at room temperature.

The ESI was measured by the method of Chobert *et al*. (1988) with a slight modification. The emulsions were prepared in the same manner as previously described for EAI measurement. The emulsions were heated at 80 °C for 30 min in a water bath (model B-490, BUCHI, Flawil, Switzerland). The mixtures were left at room temperature until the emulsion temperature equal to the room temperature.

The emulsions were diluted with 0.1% SDS (containing 0.1 M NaCl, pH 7) to obtain a dilution of 1/500. Absorbance was measured against 0.1% SDS (containing 0.1 M NaCl, pH 7) at 500 nm. EAI and ESI were calculated using the following equations:

$$EAI = 2T / \varnothing C$$

$$ESI = 100 \times (EAI_{RT} - EAI_{80\,^{\circ}C}) / EAI_{RT}$$

where T is turbidity as equal 2.3A/l (A = OD_{500}; l = pathlength in meters); ∅ is oil phase volume as equal 0.25; C refers to the protein content in % (w/v) and EAI_{RT} and $EAI_{80\,°C}$ are EAI which determined before and after heated at 80 °C, respectively.

6.6 Foaming properties

The foaming properties (foam expansion, FE and foam stability FS) were determined by the method of Shahidi *et al*. (1995) with slight modification. The gelatin solutions with different concentrations (1, 2, 3, 4 and 5% (w/v)) were added in 100 ml-cylinders (PYREX®, USA). The mixtures were homogenized for 1 min using a homogenizer (model T25 basic, ULTRA TURRAX®, IKA LABORTECNIK, Selangor, Malaysia) at 11,000 rpm (no.1). The mixtures were then incubated with various times (0, 30 and 60 min). FE and FS were calculated using the following equations:

$$FE\ (\%) = (V_T / V_O) \times 100$$

$$FS\ (\%) = (V_t / V_O) \times 100$$

where V_T is total volume; V_O is original volume before whipping and V_t is total volume after leaving at room temperature with different times (30 and 60 min).

7 Improvement of gel properties of gelatin from the skin of bigeye snapper

7.1 Use of $MgSO_4$

The stock solutions of $MgSO_4$ were added into gelatin solution to obtain the final concentrations of 0.1, 0.25, 0.5 and 1.0 M. The gelatin gels were prepared in the same manner as described in section 5.4.2. The gel properties were determined as follows:

7.1.1 Turbidity of gelatin solution

The turbidity of gelatin solution was determined according to the method of Fernandez-Diaz *et al*. (2001) as described in section 6.3.1.

7.1.2 Bloom strength of gelatin gel

The bloom strength was determined by the method of Fernandez-Diaz *et al*., (2001) with a slight modification as described in section 5.4.3.

7.1.3 Color of gelatin gel

The color of gelatin gel was measured by colorimeter (model ColorFlex, HunterLab Reston, VA, USA.). CIE L*, a* and b* values were determined.

7.1.4 Protein patterns of gelatin gel

SDS-PAGE was performed according to the method of Laemmli (1970) as described in section 6.2.

7.1.5 Microstructure of gelatin gel

Microstructure of gelatin gels (2 x 2 mm.) was determined using SEM in the same manner as described in section 5.3.

7.2 Use of microbial transglutaminase (MTGase)

The pH of gelatin solution was adjusted with either 6 N NaOH or 6 N HCl to obtain a final pH of 7. The stock solution of MTGase was then added to that solution to obtain the final concentrations of 0.005, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5 and 1.0% (w/v). The mixture was incubated for 2 h at room temperature followed by the incubation at the refrigerated temperature (4°C) for 16-18 h. Gel properties were then determined as described in section 7.1

To study the effect of incubation time of gelatin solution on gel properties, 6.67% gelatin solutions containing 0.01% (w/v), pH 7, were incubated for different

times (0, 1, 2 and 3 h) at room temperature. The solution was subsequently incubated at the refrigerated temperature (4°C) for 16-18 h prior to analysis of bloom strength and SDS-PAGE.

To study the effect of combination effect of various MTGase concentrations (0.01 and 0.1%, w/v) and different incubation times (0, 20, 40, 60, 120, 180 and 240 min), gelatin solutions added with different MTGase concentrations were incubated for different times. The MTGase were then inactivated in hot water (80 °C) for 1 min. The gels were prepared as described previously in section 6.2.

8 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). Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS for windows: SPSS Inc.).