CHAPTER 2

MATERIALS AND METHODS

1. Chemical reagents

Bovine hemoglobin, ethylenediaminetetraacetic acid (EDTA), pepstatin A, soybean trypsin inhibitor, 1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), 2-mercaptoethanol (βME), L-tyrosine, bovine serum albumin, pepsin from porcine stomach mucosa (EC 3.4.23.1; powderized; 750 U mg^{-1} dry matter) and protein maker were purchased from Sigma Chemical Co. (St. Louis, MO, USA.). Trichloroacetic acid, tris (hydroxymethyl) aminomethane, disodium hydrogenphosphate, sodium acetate, Folin-Ciocalteu's phenol reagent, acetic acid and p-dimethylamino-benzaldehyde were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS), Coomassie Blue R-250 and *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA). Type I collagen from calf skin was purchased from Elastin products Co., INC. (Owensville, MO, USA). Food grade gelatin was obtained from Halagel (Thailand) Co., Ltd. (Bangkok, Thailand).

2. Fish stomach and skin preparation

Internal organs and skin of bigeye snapper (*Priacanthus tayanus*), the discards from surimi processing, were obtained from Man A Frozen Foods Co. Ltd., Songkhla, Thailand. Those discards were packed in polyethylene bag, kept in ice with a solid/ice ratio of 1:2 (w/v) and transported to the Department of Food Technology, Prince of Songkhla University, Hat Yai within 1 h. Upon the arrival, pooled internal organs were excised and only stomach was collected and placed in polyethylene bag. For the skin, residual meat was removed manually and cleaned samples were washed with tap water. The skin was then cut into small pieces (0.5 × 0.5 cm.) and placed in polyethylene bag. Both stomach and skin were stored at -20°C until use.

3. Instruments

Instruments	Model	Company/Country
Electrophoresis apparatus	Mini-Protein II	Bio-Rad, USA
pH meter	CG 842	Schott, Germany
Magnetic stirrer	BIG SQUID	IKA labortechnik, Germany
Overhead stirrer	RW 20.n	IKA labortechnik, Germany
Homogenizer	T25 basic	IKA labortechnik, Malaysia
Oil bath	B-490	Buchi, Switzerland
Water bath	W350	Memmert, Germany
Microcentrifuge	MIKRO20	Hettich Zentrifugan, Germany
Refrigerated centrifuge	RC-5B plus	Sorvall, USA
Double-beam Spectrophotometer	UV-16001	Shimadzu, Japan
Freeze dryer	Dura-Top^{TM} µp	FTS system, USA
Colorimeter	ColorFlex	HunterLab Reston, USA
Differential scanning calorimeter	DSC 7	Perkin Elmer, USA
Texture analyzer	TA-XT2	Stable Micro Systems, England
Rotary evaporator	Rotavapor-R	Binkmann, Switzerland
Amino acid analyzer	MLC-730	Atto Co., Japan
Scaning Electron Microscope	JSM-5800LV	JEOL, Japan

4. Preparation and characterization of pepsin from bigeye snapper stomach

4.1 Preparation of stomach extract

Frozen stomach was thawed using running water (26-28°C) until the core temperature reached -2 to 0°C. The sample was cut into pieces with a thickness of 1-1.5 cm. Samples were finely ground in liquid nitrogen using a National Model MX-T2GN blender (Taipei, Taiwan) to a powder form according to the method of Klomklao *et al.* (2004). Stomach powder was suspended in different extracting media including distilled water, 50 mM Sodium

phosphate buffer at pH 7.2 and 50 mM Tris buffer at pH 7.2 at ratio of 1:9 (w/v). The mixtures were stirred continuously at 4°C for 30 min. The suspension was centrifuged for 30 min at 4°C at 7,700×*g* using a Sorvall Model RC-B Plus refrigerated centrifuge (Newtown, CT, USA) to remove the tissue debris. The supernatant was collected and referred to as "stomach extract".

4.2 Enzyme assay

Proteinase activity of stomach extract from bigeye snapper was determined using hemoglobin as a substrate according to the method of Klomkao *et al.* (2004) and An *et al.* (1994) with a slight modification. To initiate the reaction, 200 µl of stomach extract was added into assay mixtures containing 200 µl of 2% hemoglobin, 200 µl of distilled water and 625 µl of reaction buffer. The reaction was conducted at pH 2.5 and 45°C for 20 min. Enzymatic reaction was terminated by adding 200 µl of 50% (w/v) trichloroacetic acid (TCA). Unhydrolyzed protein substrate was allowed to precipitate for 15 min at 4°C, followed by centrifuging at 7,500 rpm for 10 min. The oligopeptide content in the supernatant was determined by the Lowry assay (Lowry *et al.*, 1951) (Appendix 6) using tyrosine as a standard. One unit of activity was defined as that releasing 1 nmole of tyrosine per min (nmol/Tyr/min). A blank was run in the same manner, except that the stomach extract was added into the reaction mixture after addition of 50% TCA (w/v). Protein concentration was measured by the method of Lowry *et al.* (1951) (Appendix 6) using bovine serum albumin as a standard.

4.3 Enzyme fractionation

Stomach extract was adjusted to pH 3 with 1 M HCl and the mixture was allowed to stand at 4°C for 10 min. The suspension was centrifuged for 30 min at 4°C at 5,000×*g* using a refrigerated centrifuge. The supernatant obtained was then fractionated with ammonium sulfate at different ranges of saturation (0-20, 20-40, 40-60 and 60-80% saturation) or acetone at different amounts (0-20, 20-40, 40-60 and 60-80% v/v). The pellet was recovered by centrifuging at 5,000×*g* for 30 min at 4°C. The pellet was then dissolved and dialyzed against 50 mM Na-acetate buffer (pH 3) at a ratio of 1:15 (v/v) at 4°C for 3 times. Proteolytic activity of dialysates was assayed as described in section 4.2. Yield and purification fold were calculated. The fractionation method showing the highest yield and purity was used and the selected fraction was referred to as "bigeye snapper pepsin; BSP".

4.4 Characterization of fish pepsin

4.4.1 pH and temperature profile

To study pH profile, proteolytic activity of BSP was measured at 45°C using hemoglobin as a substrate at different pHs (pH 1.5 using 20 mM Maleate buffer and pHs 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0 and 7.0 using McIlvaine's buffer (0.2 M Na-phosphate and 0.1 M Na-citrate)). For temperature profile, the assay was performed at various temperatures (5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 and 70°C) at pH 2.5.

4.4.2 pH and thermal stability

For pH stability study, BSP was incubated at pH 1 using 20 mM Maleate buffer and pHs 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 using McIlvaine's buffer at room temperature for 30, 60 and 120 min. Residual activity was then determined. For thermal stability, BSP was also incubated at different temperatures (0, 10, 20, 30, 40, 50 and 60°C) for 30, 60 and 120 min before rapid cooling using iced water. The residual activity was then assayed using hemoglobin as a substrate under optimal pH and temperature for 20 min.

4.4.3 Inhibitor study

BSP (200 µl) was mixed with 200 µl of single protease inhibitor to obtain a final designated concentration (0.1 mM E-64, 0.1 mM soybean trypsin inhibitor, 100, 10 and 1 µM pepstatin A and 2 mM EDTA). Mixtures were incubated at room temperature (26-28°C) for 10 min. The residual activity was measured under optimal pH and temperature. Percent inhibition was then calculated.

5. Extraction and characterization of pepsin solubilized collagen from bigeye snapper skin

The collagens were extracted following the method of Jongjareonrak *et al.* (2005b) and Kittiphattanabawon *et al.* (2005) with a slight modification. All preparation procedures were performed at 4°C.

5.1 Preparation of skin for collagen extraction

To remove non-collagenous proteins, the skin was mixed with 0.1 N NaOH at a sample/alkaline solution ratio of 1:10 (w/v). The mixture was stirred for 6 h. The alkaline solution was changed every 2 h. Then, the alkaline treated skins were washed with cold water until neutral

or faintly basic pHs of wash water were obtained. The treated skins were then 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 10 volumes of cold water for three times.

5.2 Effect of pepsin on collagen extraction and composition

5.2.1 Effect of pepsin levels and reaction time on extraction and composition of collagen

Defatted skins were soaked in 0.5 M acetic acid with a solid/solvent ratio of 1:15 (w/v) and subjected to limited hydrolysis with bigeye snapper pepsin (BSP) or porcine pepsin (PP) at levels of 10 and 20 kUnits/g skin. The mixture was stirred at 4°C for 24 and 48 h. At the designated time, the mixture was immediately submerged and held in ice-bath. Prior to determination of hydroxyproline content (Bergman and Loxley, 1963) (Appendix 5) and SDS-PAGE (Leammli, 1970) (Appendix 8), the mixture was centrifuged at 3,500×*g* for 10 min at 4°C. The extraction yield was calculated based on hydroxyproline content in the extract in comparison with that defatted skin.

The concentration of pepsin used for solubilizing the collagen, which was able to extract collagen with the highest yield and retain the major components (β, α1 and α2) of collagen structure, was selected for further step.

5.2.2 Effect of acid swelling process in combination with pepsin on extraction and composition of collagen

Defatted skins were soaked in 0.5 M acetic acid with a solid/solvent ratio of 1:15 (w/v) for 24 or 48 h, followed by the limited hydrolysis with BSP or PP at 20 kUnits/g skin. After addition of pepsins, the mixture was stirred at 4°C for 24 h and 48 h. At the designated time, the mixture was submerged and held in ice-bath to terminate the hydrolysis. The mixtures were centrifuged at 3,500×*g* for 10 min at 4°C and the supernatants were subjected to determination of hydroxyproline content (Bergman and Loxley, 1963) (Appendix 5) and SDS-PAGE (Leammli, 1970) (Appendix 8). The yield was then calculated.

The extraction condition rendering the highest yield and retaining the major components (β, α1 and α2) of collagen structure was chosen for further step.

5.3 Comparative studies on different collagen extracting methods

5.3.1 Acid solubilization process

Defatted skins were soaked in 0.5 M acetic acid with a solid/solvent ratio of 1:15 (w/v) for 48 h. The mixture was filtered with two layers of cheese cloth. The filtrate was collected and subjected to precipitation.

5.3.2 One-step acid/pepsin solubilization process

Defatted skins were soaked in 0.5 M acetic acid containing BSP or PP at 20 kUnits/g skin with a solid/solvent ratio of 1:15 (w/v). The mixture was stirred at 4°C for 48 h. The mixture was then filtered with two layers of cheese cloth. The filtrate was collected for precipitation.

5.3.3 Two-step acid/pepsin solubilization process

Defatted skins were soaked in 0.5 M acetic acid with a solid/solvent ratio of 1:15 (w/v) for 24 h. The mixture was filtered with two layers of cheese cloth. The residue was re-extracted by suspending in 0.5 M acetic acid containing BSP or PP at 20 kUnits/g skin with a solid/solvent ratio of 1:15 (w/v). The mixture was stirred at 4°C for 48 h and filtered with two layers of cheese cloth. Both filtrates were combined and subjected to precipitation.

5.4 Collagen precipitation

The collagen solutions from sections 5.3.1, 5.3.2 and 5.3.3 were precipitated by adding NaCl to a final concentration of 2.6 M in the presence of 0.05 M tris (hydroxylmethyl) aminomethane, pH 7.5. The resultant precipitates were collected by centrifuging at 20,000×*g* for 60 min. The pellets were dissolved in 0.5 M acetic acid, dialyzed against 10 volumes of 0.1 M acetic acid and distilled water for 3 times, respectively, and freeze-dried.

5.5 Characterization of collagen

5.5.1 Hydroxyproline content

Collagens were determined for hydroxyproline content according to method of Bergman and Loxley (1963) (Appendix 5). The conversion factor for calculating the collagen content from hydroxyproline of bigeye snaper skin was 7.7 (Kittiphattanabawon *et al.*, 2005).

5.5.2 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). The mixtures were centrifuged at 8,500×*g* 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 the presence or absence of 10% β-ME. Samples were loaded into polyacrylamide gel made of 7.5% running gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA/gel using a Mini Protein 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. High-molecular-weight markers (Sigma Chemical Co., St. Louis, Mo., USA) were used to estimate the molecular weight of proteins. Type I collagen from calf skin was used as a standard.

5.5.3 Differential scanning calorimetry (DSC)

Prior to analysis, collagen samples were prepared following the methods of Rochdi *et al.* (2000) and Komsa-Penkova *et al.* (1999) with a slight modification. The samples were rehydrated by adding 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). Temperature calibration was run using the Indium thermogram. The collagen suspension or solution (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. An empty pan was used as the reference. Total denaturation enthalpy (△H) was estimated by measuring the area in the DSC thermogram. The maximum transition temperature (T_{max}) was estimated from the thermogram.

5.5.4 Collagen solubility

5.5.4.1 Preparation of collagen solution

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 completely solubilized.

5.5.4.2 Effect of pH on collagen solubility

Collagen solution (8 ml) was added to a 50-ml centrifuge tube (NUNC, Roskilde, Denmark) and the pH was adjusted with either 6 N NaOH or 6 N HCl to obtain the final pHs ranging from 1 to 10. The volume of solutions was made up to 10 ml by distilled water previously adjusted to the same pH as the collagen solution. The solution was centrifuged at 10,000×*g* 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.

5.5.4.3 Effect of NaCl on collagen solubility

Collagen solutions (5 ml) with the concentration of 6 mg/ml were 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 10,000×*g* 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 above.

6. Extraction and characterization of gelatin from bigeye snapper skin using pepsin aided process

The gelatins were prepared by the method of Jongjareonrak *et al.* (2006) and Kittiphattanabawon (2004) with a slight modification.

6.1 Non-collagenous protein removal process

The removal of non-collagenous proteins from the skin was carried out according to the method of Jongjareonrak *et al.* (2006) and Kittiphattanabawon (2004). The skins were mixed with ten volume of 0.025N NaOH. The mixture was stirred for 2 h at room temperature (about 25-28°C). The alkali solution was changed every 1 h. Then, the alkaline-treated skins were washed with tap water until neutral or faintly basic pH of wash water was obtained.

6.2 Effect of bigeye snapper pepsin on gelatin extraction

Alkaline-treated skins were soaked in 0.2 M acetic acid with a solid/solvent ratio of 1:10 (w/v) in the presence of BSP at levels of 0, 5, 10 and 15 kUnits/g skin. The mixture

was stirred at 4°C for 48 h. The pH of mixture was then raised to 7.5 using 10 M NaOH. The mixture was stirred gently for 1 h at 4°C to terminate pepsin activity. To extract the gelatin, pepsin-treated skin mixtures were incubated at 45°C for 12 h with a continuous stirring to extract the gelatin from skin matter. The mixture was then filtered using a Buchner funnel with Whatman No.4 filter paper. The filtrates were subjected to the determination of hydroxyproline content (Bergman and Loxley, 1963) (Appendix 5) and SDS-PAGE (Leammli, 1970) (Appendix 8). The yield of extraction was calculated based on hydroxyproline content in comparison with that of alkaline-treated skin. The concentration of pepsin giving the highest yield was chosen for further study.

6.3 Inactivation of skin endogenous proteases

Due to the presence of heat-activated proteases in bigeye snapper skin (Jongjareonrak *et al.*, 2006; Intarasirisawat *et al.*, 2006), the effective methods were required to prevent the degradation of gelatin molecules.

6.3.1 Use of heat treatment

Pepsin-treated skin mixture (15 kUnits pepsin/g skin) was heated at 70, 80 and 90°C for 5 min. The mixtures were immediately submerged and held into ice-bath for 30 min. Then, the heat-treated mixtures were incubated at 45°C for 12 h with a continuous stirring to extract the gelatin from skin matter. The mixture was then filtered using a Buchner funnel with Whatman No.4 filter paper. SDS-PAGE pattern of resultant filtrate and the mixture before heat treatment were determinated.

6.3.2 Use of protease inhibitor

Pepsin-treated skin mixture (15 kUnits pepsin/g skin) was mixed with pepstatin A (1 and 10 μM) or soybean trypsin inhibitor (0.1, 1 and 10 μM) and the mixtures were allowed to stand for 30 min. Then, the inhibitor-treated mixtures were incubated at 45°C for 12 h with a continuous stirring to extract the gelatin from skin matter. Filtration and determination were made as mentioned in section 6.3.1. Inhibitor at the concentration, which prevented the degradation of the major components (β, α1 and α2) of gelatin structure most effectively, was selected for further step.

6.4 Preparation of fish skin gelatin

Alkaline-treated skins were soaked in 0.2 M acetic acid with a solid/solvent ratio of 1:10 (w/v) in the presence of BSP or PP at a level of 15 kUnits/g skin. The mixture was stirred at 4°C for 48 h, followed by adjusting the pH to 7.5. The mixture was allowed to stand at 4°C for 1 h with gentle stirring to stop pepsin activity. Pepsin-treated skin mixture was added with soybean trypsin inhibitor to obtain the final concentration of 0.1 μM and left for 30 min at room temperature. Then, the inhibitor-treated mixture was incubated at 45°C for 12 h with a continuous stirring to extract the gelatin from skin matter. The mixture was then filtered using a Buchner funnel with Whatman No.4 filter paper. The resultant filtrate was freeze-dried and the dry matter was referred to as "gelatin powder".

6.5 Characterization and functional properties of gelatin

6.5.1 Determination of chemical compositions

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

6.5.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein patterns of gelatin were analyzed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970) (Appendix 8). The gelatin 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. The mixtures were centrifuged at 8,500×*g* for 5 min at room temperature to remove undissolved debris. Solubilized samples were subjected to electrophoresis as described in section 5.5.3.

6.5.3 Gelation of gelatin

6.5.3.1 Gelatin gel preparation

Gelatin gel was prepared by the method of Fernandez-Diaz *et al.* (2001) 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 60°C for 30 min. The solution was stirred until gelatin was solubilized completely, followed by cooling in the refrigerator (4°C, maturation temperature) for 16-18 h.

6.5.3.2 Determination of bloom strength

The bloom strength was determined by the method of Fernandez-Diaz *et al.* (2001) with a slight modification. Bloom strength of gelatin gel 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.

6.5.3.3 Color of gelatin gel

The color of gelatin gels (6.67% (w/v)) were measured by using colorimeter (model ColorFlex, HunterLab Reston, VA, USA) and reported in CIE system color profile of L*, a* and b*.

6.5.3.4 Turbidity of gelatin solution

The turbidity of gelatin solution (6.67% (w/v) in distilled water, 60°C) was determined according to the method of Fernández-Diaz *et al.* (2001). The absorbance was measured at 360 nm using Double-beam spectrophotometer (model UV-16001, Shimadzu, Kyoto, Japan). The values were recorded as the average of three determinations.

6.5.4 Gelatin solubility

The effect of pHs on gelatin solubility was determined by the method of Montero *et al.* (1991) with a 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 was adjusted to different pHs (1-10) with either 6 N NaOH or 6 N HCl. The volume of solution 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×*g* at room temperature for 10 min. Protein content in supernatant was determined by the Biuret method (Robinson and Hodgen, 1940) (Appendix 7) using bovine serum albumin as a standard. Relative solubility was calculated in comparison with that obtained at the pH giving the highest solubility.

6.5.5 Emulsifying properties

Emulsifying activity index (EAI) and emulsifying stability index (ESI) of gelatin solution (0.1% protein) were determined according to the method of Pearch and Kinsella (1978)

and Chobert *et al.* (1988), respectively, with a slight modification. Thirty ml of gelatin solution were homogenized with 10 ml of soybean oil using a homogenizer (POLYTRON® PT-MR 2100, KINEMATICA AG, Switzerland) at 20,000 rpm for 1 min at room temperature. To determine ESI, the emulsions were prepared in the same manner. The emulsions were then heated at 80°C for 30 min in a water bath. The mixtures were left at room temperature until emulsion temperature was equal to the room temperature. The emulsions were diluted to 500-fold with 0.1% SDS containing 0.1 M NaCl (pH 7). Absorbance was read against 0.1% SDS containing 0.1 M NaCl (pH 7) at 500 nm. EAI and ESI were calculated using the following equations:

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

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

where T is turbidity, which is 2.3A/l (A = OD500; l = path length in meters); Φ is oil phase volume (0.25); C refers to the protein content in % (w/v) and EAI_{RT} and $EAI_{80°C}$ are EAI determined before and after being heated at 80°C, respectively.

6.5.6 Foaming properties

Foam formation ability and foam stability were measured by the method of Cho *et al.* (2004) with a slight modification. Gelatin solution (1% protein) was poured into 100 ml cylinders (PYREX®, USA). The foam was prepared by homogenizing the solution at 11,000 rpm for 1 min (model T25 basic, ULTRA TURREX®, IKA LABOTECHNIK, Selangor, Malaysia). The foam formation ability was calculated as the volume ratio of foam liquid. The foam stability was calculates as the ratio of the volume of foam after 30 min to the initial volume of foam.

6.5.7 Scanning electron microscopy

Microstructure of gelatin gels was determined using Scanning Electron Microscopy (SEM). Gelatin gels 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) for 15 min each. 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.

7. 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 a SPSS package (SPSS 10.0 for window, SPSS Inc, Chicago, IL).