

CHAPTER 2

MATERIALS AND METHODS

1. Materials

1.1 Pacific white shrimp samples

Pacific white shrimps (*Litopenaeus vannamei*) with an average size of 55-60 shrimp/kg were purchased from a farm in Songkhla province. Samples were kept in ice using the shrimp/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. Upon the arrival, shrimps were washed with clean water, immediately deheaded, peeled and deveined. The meat was minced using a mincer with a hole diameter of 5 mm. The mince was placed in polyethylene bag and kept on ice during preparation (Thammatinna, *et al.*, 2007).

1.1.1 Chemicals

All chemicals for analyses were of analytical grade. Sodium chloride, trichloroacetic acid, sodium pyrophosphate, magnesium chloride, Folin-Ciocalteu's phenol reagent and Coomassie blue R-250 were obtained from Merck (Darmstadt, Germany). Calcium chloride, β -mercaptoethanol (β -ME), ethylenediaminetetraacetic acid (EDTA), 1,10-phenanthroline monohydrate, ethylene-bis (oxyethylenitrilo) tetraacetic acid (EGTA), trans-epoxysuccinyl-L-leucyl-amido (4-guanidino) butane (E-64), pepstatin A, soybean trypsin inhibitor (SBTI) and L-tyrosine were procured from Sigma Chemical Co. (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS) and *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Bovine plasma protein (BPP) was obtained from Merrick's, Inc (Middleton, WI, USA). Whey protein concentrate (WPC) was obtained from Hilmar Ingredients (Hilmar, CA, USA) and egg white (EW) were purchased from Food EQ Co., Ltd (Bangkok, Thailand). Microbial transglutaminase (MTGase) was obtained from Ajinomoto Co., Ltd. (Kawasaki, Japan). Modified starch, hydroxypropylated distarch

phosphate (GelPro HC30) was obtained from General starch (Bangkok, Thailand). *I*-Carrageenan was purchased from Union Chemical 1986. Co., LTD (Bangkok, Thailand).

2. Instruments

Instruments	Model	Company/City/Country
- pH meter	CG 842	Schott, Mainz, Germany
- Magnetic stirrer	BIG SQUID	IKA labortechnik, Stanfen, Germany
- Homogenizer	T25 basic	IKA labortechnik, Selangor, Malaysia
- Water bath	W350	Memmert, Schwabach, Germany
- Microcentrifuge	MIKR 020	Zentrifugan, Hettich, Germany
- Refrigerated centrifuge	RC-5B plus	Sorvall, Norwalk CT, USA
- Colorimeter	ColorFlex	HunterLab Reston, VA, USA
- Electrophoresis apparatus	Mini-Protein II	Bio-Rad, CA, USA
- Double-beam Spectrophotometer	RF-1501	Shimadzu, Kyoto, Japan
- Texture analyzer	TA-XT2	Stable Micro Systems, Surrey, UK
- Mixer	MK-K77	National, Tokyo, Japan
- Scanning Electron Microscope	JSM 5800LV	JEOL, Akishima, Japan

3. Methods

3.1 Study on the gel properties of Pacific white shrimp meat.

3.1.1 Effect of sodium chloride concentrations on gel properties of Pacific white shrimp meat

Pacific white shrimp meat was mixed with NaCl at different levels (2.0, 2.5, 3.0, 3.5 and 4.0% (w/w)). The mixture was adjusted to obtain the moisture content of 80% and mixed thoroughly using a mixer (National Model MKK77, Tokyo, Japan) for 4 min. The sol obtained was then stuffed into polyvinylidene casing with a diameter of 2.5 cm and both ends of casing were sealed tightly. The sol was heated at 90°C for 20 min, followed by rapid cooling in iced water.

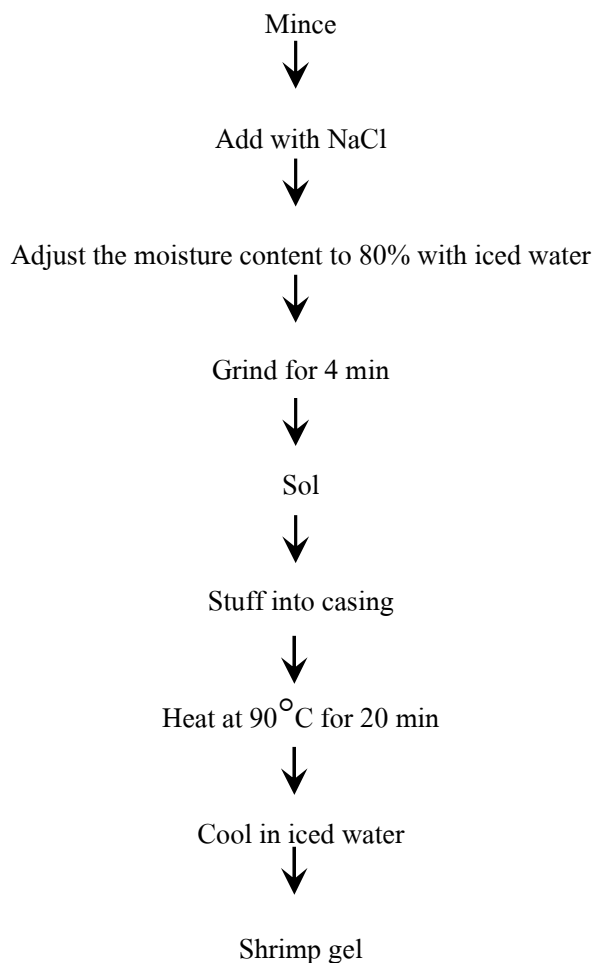


Figure 15 Scheme for shrimp gel preparation

The gel samples were stored for 12 h at 4°C prior to analyses. Gels were subjected to analyses as follows:

1. Breaking force and deformation

Breaking force (strength) and deformation (cohesiveness/elasticity) of shrimp gels were determined using a Model TA-XT2 texture analyzer (Stable Micro System, UK). Gels were equilibrated at room temperature (28-30°C) before analyses. Five cylindrical samples (2.5 cm in diameter) were cut into the length of 2.5 cm (Benjakul *et al.*, 2003). A spherical probe with a diameter of 5 mm was pressed into the cut surface of a gel specimen perpendicularly at a constant depression speed (60 mm/min) until the puncture occurred. The force to puncture into

the gel (breaking force) and the distance at which the probe punctured into the gel (deformation) were both recorded.

2. Expressible moisture content

Expressible moisture content was measured according to the method of Ng (1978). Gel samples were cut into a thickness of 0.5 cm, weighed (X) and placed between two pieces of Whatman filter paper No. 1 at the top and two pieces of the same filter paper at the bottom. The standard weight (5 kg) was placed on the top of the sample and maintained for 2 min. The sample was then removed from the papers and weighed again (Y). Expressible moisture content was calculated and expressed as the percentage of sample weight with the following equation:

$$\text{Expressible moisture content (\%)} = 100 \times [(X-Y)/X]$$

3. Color

The color of gel sample was measured in the $L^* a^* b^*$ mode of CIE using a colorimeter (ColorFlex, Hunter Associates Laboratory, Reston, VA, USA). L^* , a^* , and b^* indicate lightness, redness/greenness, and yellowness/blueness, respectively.

4. TCA-soluble peptide content

TCA-soluble peptide content of gel sample was determined according to the method of Morrissey *et al* (1993). The sample (3 g) was homogenized with 27 ml of 5%TCA for 1 min at a speed of 11,000 rpm using a homogenizer (Model T25 basic, IKA, LABORTECHNIK, Selangor, Malaysia). The homogenate was kept in ice for 1 h and centrifuged at $5,000 \times g$ for 5 min using a microcentrifuge (Model MIKRO20, Hettich ZENTRIFUGEN, Germany). The soluble peptide content in the supernatant was measured by the method of Lowry *et al* (1951) and expressed as $\mu\text{mole Tyrosine/g sample}$.

5. Protein patterns

Protein patterns of gels were determined using SDS-PAGE according to method of Laemmli (1970) with 10% running gel and 4% stacking gel. To prepare the protein sample, 27 ml of 5% (w/v) SDS solution heated to 85°C were added into the sample (3 g). The mixture was then homogenized for 2 min at a speed of 11,000 rpm using a homogenizer. The homogenate was incubated at 85°C for 1 h to dissolve total proteins. The samples were centrifuged at $8500 \times g$ for 20 min to remove undissolved debris. Protein concentration was

determined by the Biuret method (Robinson and Hodgen, 1940) using bovine serum albumin as a standard. Sample with the protein content of 20 μg was loaded onto the gel. Electrophoresis was conducted at 15 mA/plate. After separation, proteins were fixed and stained for 5 h in 0.125% Coomassie Brilliant Blue R-250 in 40% methanol and 10% glacial acetic acid. Gels were destained with destaining solution I (50% methanol and 7.5% glacial acetic acid) for 15 min and with the destaining solution II (5% methanol and 7.5 % glacial acetic acid) for 3 h.

6. Microstructure

Gel samples (0.25x0.25x0.25 cm) were fixed with 2.5% glutaraldehyde in 0.2 M phosphate buffer, pH 7.2 for 2 h at room temperature (Hayat, 1981). Fixed specimens were dehydrated in graded ethanol solution with serial concentrations of 50, 70, 80, 90 and 100% and critical point dried (Balzers mod. CPD 030, Balzers Process Systems, Liechtenstein) using CO_2 as transition fluid. The prepared samples were mounted on copper specimen holders, sputter-coated with gold (Balzers mod. SCD 004) and examined on a JSM 5200 scanning electron microscope (JEOL, Ltd., Akishima, Japan).

3.1.2 Effect of pyrophosphate in combination with magnesium chloride and/or calcium chloride on gel properties of Pacific white shrimp meat

Pacific white shrimp meat was added with NaCl at a level rendering the highest breaking force and deformation of Pacific white shrimp gel (section 3.1.1). Thereafter, PP at different levels (5 and 10 mmol/kg) in combination with MgCl_2 (0, 5 and 10 mmol/kg) was added into the mixture with or without CaCl_2 (150 mmol/kg). The different treatments are shown as follows:

- 150 mmole CaCl_2/kg
- 5 mmole PP/kg + 150 mmole CaCl_2/kg
- 5 mmole PP/kg + 5 mmole MgCl_2/kg + 150 mmole CaCl_2/kg
- 5 mmole PP/kg + 10 mmole MgCl_2/kg + 150 mmole CaCl_2/kg
- 10 mmole PP/kg + 150 mmole CaCl_2/kg
- 10 mmole PP/kg + 5 mmole MgCl_2/kg + 150 mmole CaCl_2/kg
- 10 mmole PP/kg + 10 mmole MgCl_2/kg + 150 mmole CaCl_2/kg

The sols were then stuffed into polyvinylidene casing with a diameter of 2.5 cm and both ends of casing were sealed tightly, followed by heating at 90°C for 20 min. The gels were cooled rapidly in iced water and stored for 12 h at 4°C prior to analyses as described in 3.1.1.

The treatment resulting the highest breaking force and deformation was used for further study.

3.2 The effect of some protein additives on gel properties of Pacific white shrimp meat

Pacific white shrimp meat was added with the selected chemicals rendering the highest breaking force and deformation (section 3.1). Protein additives including bovine plasma protein (BPP), egg white (EW) and whey protein concentrate (WPC) were added at levels of 0, 0.5, 1.0, 2.0 and 3.0% (w/w) into the mixture. The mixture was chopped for 4 min to obtain the homogenous sol. The sol was then stuffed into polyvinylidene casing with a diameter of 2.5 cm and both ends of casing were sealed tightly. Sols were subject to heating under different conditions to obtain different gels as follows:

-One-step heating (90°C for 20 min)

-Two-step heating (40°C for 30 min and 90°C for 20 min)

The gels were cooled rapidly in iced water and stored for 12 h at 4°C prior to analyses as described in 3.1.1.

3.3 Study on autolysis of Pacific white shrimp meat

3.3.1 Effect of temperature on autolysis of Pacific white shrimp meat

Pacific white shrimp mince (2 g) was incubated at different temperatures (30, 35, 40, 45, 50, 55, 60, 65 and 70°C) in the absence and in the presence of 2.5% NaCl in a temperature-controlled water bath (Mettert, Schwabach, Germany) for 30 min and 60 min. The autolytic reaction was terminated by addition of 18 ml of cold 7.5% trichloroacetic acid. The mixture was homogenized at the speed of 11,000 rpm using a homogenizer (Model T25 basic, IKA, LABORTECHNIK, Selangor, Malaysia) for 2 min. The homogenate was subjected to centrifugation at 7,500 \times g for 10 min using a microcentrifuge (Model MIKRO20, Hettich ZENTRIFUGEN, Germany). TCA-soluble peptide content in the supernatant obtained was

determined by the Lowry method (Lowry *et al.*, 1951) using L-tyrosine as a standard and expressed as μmol Tyrosine/g sample. To determine the autolytic protein pattern, the autolytic reaction was conducted in the same manner, but 18 ml of 7.5% SDS (85°C) were added to terminate the reaction and homogenized at a speed of 11,000 rpm for 2 min. The homogenate was then incubated at 85°C for 1 h. To remove undissolved debris, the mixture was centrifuged at $8,500 \times g$ for 15 min. The supernatant was subjected to SDS-PAGE analysis.

3.3.2 Effect of pH on autolysis of Pacific white shrimp meat

Pacific white shrimp mince (2 g) was mixed with 6 ml of different buffers having various pH values. Those included 0.2 M McIlvaines' buffer (0.2 M Na-phosphate and 0.1 M Na-citrate) for pH values of 2.0 - 7.0 and 0.1 M Na_2HPO_4 - 0.05 M $\text{Na}_2\text{B}_4\text{O}_7$ for pH values of 8.0-10.0. The mixture was homogenized at a speed of 11,000 rpm for 2 min. The homogenate was incubated at the optimum temperature in the absence and in the presence of 2.5% NaCl. Autolysis was terminated after 60 min of incubation by addition of 12 ml of cold 7.5% TCA or 7.5% SDS solution (85°C) as previously described. TCA-soluble peptide content and SDS-PAGE protein patterns were determined as mentioned above.

3.3.3 Effect of various proteinase inhibitors on autolysis of Pacific white shrimp meat

The effect of various inhibitors on autolysis was determined by mixing 1 ml of shrimp mince homogenate having the pH values of 3, 7 and 9 with 1 ml of proteinase inhibitor solution to obtain the final concentration designated (2 μM pepstatin A, 0.1 mM E-64, 0.1 mM soybean trypsin inhibitor, 20 mM EDTA and 10 mM EGTA). The mixture was mixed thoroughly and then incubated in a temperature controlled water bath at either 35°C or 40°C . Autolysis was terminated by either addition of 1 ml of 15% TCA or 15% SDS solution (85°C). TCA-soluble peptide content was measured using the Lowry method (Lowry *et al.*, 1951) and autolytic protein patterns were determined by SDS-PAGE.

3.3.4 Effect of various protein additives on autolysis of Pacific white shrimp meat

Pacific white shrimp mince (2 g) was mixed with 2.5% NaCl. Various protein additives including egg white (EW), whey protein concentrate (WPC) and bovine plasma protein

(BPP) at different levels (0, 0.5, 1.0, 2.0 and 3.0% (w/w)) were then added and mixed thoroughly. The mixture was incubated at 40°C for 60 min. Autolysis was terminated by addition of 18 ml of cold 7.5% TCA or 7.5% SDS solution (85°C) as previously described. TCA-soluble peptide content and SDS-PAGE protein patterns were determined as mentioned before.

3.4 Study on the effect of setting condition on gel properties of Pacific white shrimp meat

3.4.1 Characterization of endogenous TGase activity

3.4.1.1 Preparation of crude extract

Transglutaminase (TGase) crude extract was prepared according to the method of Tsukawasa *et al* (2002) with a slight modification. Pacific white shrimp meat was homogenized with five volumes of 50 mM Tris-HCl buffer (pH 7.5) containing 5 mM EDTA, 10 mM NaCl and 3 mM dithiothreitol (buffer A) at a speed of 10,000 rpm for 2 min at 4°C. After centrifugation at 1,600 x g for 30 min, the supernatant was subjected to ammonium sulfate precipitation using 30-60% saturation. The precipitate was dissolved in small amount of buffer A and dialyzed against buffer A overnight with three changes. The resulting crude TGase extract was assayed for TGase activity.

3.4.1.2 Effect of temperature on TGase activity

TGase activity was determined by the hydroxamate method (Folk, 1970). Freshly prepared substrate mixture containing 350 µl of 0.1 M Tris-acetate, pH 6.0, 25 µl of 2.0 M hydroxylamine, 75 µl of 0.1 M N-ε-CBZ-L-glutamidylglycine and 25 µl of deionized water was used. To initiate the reaction, 25 µl of crude extract were added and reaction was performed for 10 min at different temperatures (25, 30, 35, 40, 45, 50, 55 and 60°C). The reaction was terminated by adding 500 µl of 15% TCA containing 5% FeCl₃. The resulting suspension was centrifuged at 9,000 x g for 5 min and the absorbance was measured at 525 nm using a spectrophotometer. The calibration was performed using L-glutamic acid-ε-monohydroxamic acid as the standard. One unit of TGase was defined as the amount of enzyme required to catalyze the formation of 1 µmole hydroxamic acid/min at pH 6.0 and 37°C (Ho *et al.*, 2000).

3.4.1.3 Effect of CaCl₂ on TGase activity

To study the effect of CaCl₂, 25 μ l of crude extract were mixed with 25 μ l CaCl₂ to obtain the different final concentrations (0, 10, 20, 50, 100 mM). The mixture was allowed to stand at room temperature for 10 min prior to starting reaction assay at the optimum temperature of crude TGase activity (section 3.4.1.2).

3.4.2 Effect of setting condition on gel properties

To study the effect of setting condition on gel property, shrimp mince was added with NaCl in the presence and in the absence of PP in combination with MgCl₂ at the levels yielding the highest breaking force and deformation (section 3.1.2). The mixture was then added with 150 mmoleCaCl₂/kg. The mixture was chopped for 4 min to obtain the homogenous sols and the moisture content was adjusted to 80%. The sols were then stuffed into polyvinylidene casing with a diameter of 2.5 cm and both ends of casing were sealed tightly. Sols were subjected to setting at the optimum temperature (section 3.4.1.2) for 0, 0.5, 1, 2 and 3 h, followed by heating at 90°C for 20 min. The gels were cooled rapidly in iced water and stored for 12 h at 4°C prior to analyses as described in section 3.1.

3.4.3 Effect of CaCl₂ on gel properties

Pacific white shrimp mince was added with all additives giving the resulting gel with the highest breaking force and deformation (section 3.4.2). CaCl₂ at different levels (0, 10, 20, 50, 100 and 150 mmol/kg) was then added. The mixture was chopped for 4 min to obtain the homogenous sols and the moisture content was adjusted to 80%. The sols were then stuffed into polyvinylidene casing with a diameter of 2.5 cm and both ends of casing were sealed tightly. Sols were set at the optimum temperature (section 3.4.1.2) and time (section 3.4.2) followed by heating at 90°C for 20 min. The gels were cooled rapidly in iced water and stored for 12 h at 4°C prior to analyses as described in section 3.1.

3.5 The effect of MTGase on gel properties of Pacific white shrimp meat

Pacific white shrimp was mixed with all additives, yielding the gel with the highest breaking force and deformation (section 3.4.3). MTGase at different levels (0, 0.2, 0.3,

0.4 and 0.5% (w/w)) was then added. The mixture was chopped for 4 min to obtain the homogenous sols and the moisture content was adjusted to 80%. The sols were then stuffed into polyvinylidene casing with a diameter of 2.5 cm and both ends of casing were sealed tightly. Sols were set at selected temperature (section 3.4.1.2) and time (section 3.4.2), followed by heating at 90°C for 20 min. The gels were cooled rapidly in iced water and stored for 12 h at 4°C prior to analyses as described in section 3.1.

3.6 The effect of hydrocolloids on freeze-thaw stability of Pacific white shrimp gel

Gel with the highest breaking force and deformation was prepared as described in section 3.4.3. Modified starch or *I*-carrageenan at different levels (0, 2 and 4% (w/w)) were added. The mixture was chopped for 4 min to obtain the homogenous sol and the moisture content was adjusted to 80% with iced water. The sols were then stuffed into polyvinylidene casing with a diameter of 2.5 cm and both ends of casing were sealed tightly. Sols were set at the optimum temperature (section 3.4.1.2) and time (section 3.4.2), followed by heating at 90°C for 20 min. The gels were cooled rapidly in iced water and stored for 12 h at 4°C prior to analyses and freezing. To freeze the gel samples, gels were placed in an air-blast freezer at -18°C for 24 h. The frozen samples were thawed using running water (26-28°C) until the core temperature reached 0-2°C. The freeze-thawing was performed for 0, 3 and 5 cycles. Pacific white shrimp gel without either modified starch or *I*-carrageenan was used as the control. Gels obtained under different conditions were determined as follows:

1. Breaking force and deformation as described in section 3.1.1.
2. Expressible moisture content as described in section 3.1.1.
3. Microstructure

Microstructures of the gels were determined using scanning electron microscopy as described in section 3.1.1. Shrimp gels added without and with hydrocolloid at the level yielding lowest changes in breaking force and deformation during multiple freeze thawing were examined for microstructure.

4. Statistical analysis

All experiments were run in triplicate and CRD (Completely Randomized Design) was used. Analysis of variance (ANOVA) was performed and means comparisons were carried out by Duncan's multiple range tests (Steel and Torrie, 1980). Analyses were conducted using a SPSS package (SPSS 11.0 for windows, SPSS Inc, Chicago, IL).