APPENDIX

ANALYTICAL METHODS

1. Measurement of autolytic degradation products (Morrissey *et al*., 1993; Benjakul *et al*., 1997)

Reagent

- 5% Trichloroacetic acid (TCA) (w/v)
- Tyrosine

Method

1. Weigh 3 g of Pacific white shrimp and homogenize in 27 ml of 5% TCA.
2. Keep the homogenate in ice for 1 h, and centrifuge at 5,000 x g for 5 min.
3 Measure tyrosine content in the supernatant using Lowry' s method as and index of autolytic degradation product and express as μmol tyrosine / g Pacific white shrimp gel.

2. Lowry' s procedure for quantitation of protein (Lowry *et al*., 1951)

Reagent

- A: 2% sodium carbonate in 0.1 N NaOH
- B: 0.5% $CuSO_4.5H_2O$ in 1% sodium citrate
- C : 2 N Folin phenol reagent diluted with deionized water (1:1 v/v)
- D: 50 ml reagent A + 1 ml reagent B
- Standard reagent : Bovine serum albumin (BSA) at concentration of 1 mg/ml

Method

1. Add 2 ml of reagent D to 200 μl of the standards and unknown and vortex immediately.
2. Incubate precisely for 10 min at room temperature.
3. Add 0.2 ml of reagent C (previously dilute 1: 1 with distilled water) and vortex immediately.

4. Incubate the mixture for 30 min at room temperature not longer than 60 min.
5. Read the absorbance at 750 nm.(glass cuvate)
6. Plot standard curve and calculate the unknown.

Standard

- Bovine serum albumin (BSA) at concentration of 1 mg / ml (used to determine protein concentration).
- Tyrosine at a concentration of 1 mM (used as index of autocatalyte degradation product).
- Standard volumes (μl) : 0, 20, 40, 60, 100, 140, and 200

Standard curve of Lowry's procedure

Std volumn (μl)	Tyrosine (μl)	Distilled water (μl)
0	0	200
20	20	180
40	40	160
60	60	140
100	100	100
140	140	600
200	200	0

3. Electrophoresis (SDS-PAGE) (Leammli, 1970)

Reagent

- protein molecular weight standards
- 30% Acryamide - 0.8% bis Acrylamide
- Sample buffer: Mix 30 ml of 10% of SDS, 10 ml of glycerol, 5 ml of β-Mercaptoethanol. 12.5 ml 0f 50 mM Tris – HCl, pH 6.8, and 5-10 mg Bromophenol blue (enough to give dark blue color to the solution). Bring the volume to 100 ml with distilled water. Divide into 1 ml aliquot, and store at -20°C
- 2% (w/v) Ammonium persulfate

- 1% (w/v) SDS
- TEMED (*N, N, N′N′*- tetramethylenediamine)
- 0.5 M Tris- HCl, pH 6.8
- 1.5 M Tris- HCl, pH 8.8
- 0.1 M EDTA
- Electrode buffer: Dissolve 3 g of Tris, 14.4 g of glycine and 1 g of SDS in distilled water. Adjust to pH 8.3. Add distilled water to 1.1.
- Staining solution: Disslove 0.04 g of Coomassie blue R-250 in 100 ml of methanol. Add 15 ml of glacoial acetic acid and 85 ml of distilled water.
- Destaining solution I: 50% methanol- 7.5 glacial acetic acid.
- Destaining solution II: 5% methanol- 7.5% glacial acetic acid.

Method

Pouring the separating gel:

1. Assemble the minigel apparatus according to the manufacture's detailed instructions. Make sure that the glass and other components are rigorously clean and dry before assembly.
2. Mix the separating gel solution as shown in Table.
3. Transfer the separating gel solution by using a Pasture pipettes to the center of sandwich, which is about 1.5 to 2 cm from the top of the shorter (front) glass plates.
4. Cover the top of the gel with a layer of isobutyl alcohol by gently squirting the isobutyl alcohol against the edge of one of the spacers. Allow the resolving gel to polymerized fully (usually 30-60 min).

Pouring the stacking gel:

1. Pour off completely the layer of isobutyl alcohol.
2. Prepare a 4% stacking gel solution by adding as defined in Table.
3. Transfer stacking gel solution to tickle into the center of the sandwich along an edge of one of the spacers.

4. Insert comb into the layer of stacking gel solution by placing one corner of the comb into the gel and slowly lowering the other corner in. Allow the stacking gel solution to polymerize 30 to 45 min at room temperature.

Reagents	10%running gel	4% stacking gel
30% Acrylamide-bis	1.167 ml	0.4 ml
1.5 M Tris-HCl buffer, pH 8.8	0.875 ml	-
0.5 M Tris-HCl buffer, pH 6.8	-	1.0 ml
10%SDS	0.35 ml	0.3 ml
Distilled water	0.7585 ml	0.9 ml
0.1M EDTA	-	0.8 ml
2% Ammonium persulfate	0.35 ml	0.4 ml
TEMED	6 µl	5 µl

Sample preparation:

1. Weigh 3 g of sample and homogenize with 5% (w/v) SDS in final volume of 30 ml.
2. Incubate the mixture at 85 °C for 1 h.
3. Centrifuge at 35,000 x g for 5 min at ambient temperature and collect the supernatant.

Loading the gel:

1. Dillute the protein with sample buffer at a ratio of 1:4 (v/v) in microcentrifuge tube and boil for 1 min at 100°C
2. Remove the comb without tearing the edge of polyacrylamide wells.
3. Fill the wells with electrode buffer.
4. Place the upper chamber over the sandwich. Pour electrode buffer into the lower buffer chamber. Place the sandwich attached to the upper buffer chamber into the lower chamber.
5. Fill the upper buffer chamber with electrode buffer so that the sample wells of the stacking gel are filled with buffer.

6. Use a 10-25 μl syring with a flate-tipped needle, load the protein sample into the wells by carefully applying the sample as a thin layer at the bottom of well.
7. Fill the remainder of the upper buffer chamber with additional electrode buffer.

Runing the gel:

1. Connect the power supply to the anode and cathode of the gel apparatus and run at 50 V and 150 V.
2. After the bromophenol blue tracking dye has reached the bottom of the separating gel, disconnect the power supply.

Dissembling the gel:

1. Remove the upper buffer chamber and the attached sandwich.
2. Orient the gel so that the order of the sample well is known, remove the sandwich from the upper buffer chamber, and lay the sandwich on a sheet of absorbent paper or paper towels. Carefully slide the apacers out from the edge of the sandwich along its entire length.
3. Insert a spectula between the glass plates at one corner where the spacer is, and gently pry the two plate apart.
4. Remove the gel from the lower plate. Place the plate with the gel attached into the shallow dish of fixing agent or dye and swishing the plate.

Staining the gel:

1. Place the gel in a small plastic box and cover with the staining solution. Agitate slowly for 1 h or more on a rotary rocker.
2. Pour off the staining solution and cover the gel with a solution of destaining solution I. Agitate slowly for about 15 min.
3. Pour off the destaining solution I and cover the destaining solution II. Discard destaining solution and replace with fresh solution. Repeat unit the gel is clear except for the protein bands.

4. Biuret method quantitation of protein (Copeland *et al*., 1994)

Reagent

- Biuret reagent: combine 1.50 g $CuSO_4.5H_2O$, 6.00 g sodium potassium tartrate, and 500 ml distilled water in a beaker and stir, add while stirring 300 ml of 10 % NaOH (w/v), transfer to plastic bottle for storage.
- Distilled water
- Standard reagent: 10 mg/ml bovine serum albumin (BSA)

Method

1. To each of eight disposible cuvette, add the following reagents according to the table.
2. To tube 6-8, 50 µl of protein sample were added, mix the contents of each tube well.
3. Add 2.0 ml of the biuret reagent to each tube and mix well.
4. Incubate the mixture at room temperature for 30-45 min, then read the absorbance of each tube at 540 nm
5. Using the average absorbance for the three samples of unknown, read the concentration of sample from the plot.

Table Experimental set up for the Biuret's assay.

Tube number	Water (l)	10 mg/ml BSA	Effective BSA concentration (mg/ml)
1	500	0	0
2	400	100	2
3	300	200	4
4	200	300	6
5	100	400	8
6	450	0	unknown
7	450	0	unknown
8	450	0	unknown

5. Solubility (Benjakul *et al.*, 2001)

1. Homogenize the sample (1 g) in 20 ml of solution for 1 min using a homogenizer. Heat homogenate in a boiling water (100°C) for 2 min and stir at room temperature for 4 h.
2. Centrifuge the resulting homogenate at 10,000 x g for 30 min, using a Sorvall model RC-B plus centrifuge (Newtown, CT, USA).
3. Precipitate the protein in the supernatant (10 ml) by the addition of 50% (w/v) cold TCA to a final concentration of 10%.
4. Keep the mixture at 4°C for 18 h and then centrifuge at 10,000 *x* g for 30 min. Wash the precipitate with 10% TCA and solubilize in 0.5 M NaOH.
5. Measure the protein content using the Biuret test (Robinson and Hodgen, 1940). Express the solubility as percent of the total protein.

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List of Publication and Proceeding

Eakpetch, P., Benjakul, S., Visessanguan, W. and Kijroongrojana, K. 2007. Autolytic degradation of Pacific white shrimp (*Litopenaeus vannamei*) meat and its inhibition by protein additives. J. Food Sci. Inpress.

Eakpetch, P., Benjakul, S., Visessanguan, W. and Kijroongrojana, K. 2008. Effect of protein additives on gelling properties of Pacific white shrimp (*Litopenaeus vannamei*) meat. ASEAN Food J. 13(1): 67-74.

Eakpetch, P., Benjakul, S., Visessanguan, W. and Kijroongrojana, K. 2007. Effect of pyrophosphate in combination with magnesium chloride on gelling properties of Pacific white shrimp (*Litopenaeus vannamei*) meat. 7th National Graduate Research Conference. Prince of Songkla University, Suratthani Campus, 4 to 5 April 2007.

Eakpetch, P., Benjakul, S., Visessanguan, W. and Kijroongrojana, K. 2007. Effect of protein additives on gelling properties of Pacific white shrimp (*Litopenaeus vannamei*) meat. 10th ASEAN Food Conference 2007. University Putra Malaysia, 21 to 23 August 2007.