CHAPTER 2

MATERIALS AND METHODS

1. Materials

1.1 Shrimp samples

Black tiger shrimp (*Penaeus monodon*) and white shrimp (*Penaeus vannamei*) with the size of 60 shrimps/kg were obtained from the farms in Songkhla and Suratthani provinces, respectively. The shrimps were placed in ice with an ice/shrimp ratio 2:1 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla within approximately 1 and 4 h, respectively. Upon the arrival, the shrimps were washed with clean water. One lot was used for characterization study. Another lot was used for shelf-life study.

1.2 Chemicals

Chloroform, methanol, sulfuric acid, nitric acid, isopropanol, chloramines T and ρ-dimethylamino-benzaldehyde were purchased from Merck (Darmstadt, Germany). Acrylamide, *N,N,N',N'*-tetramethylethylenediamide (TEMED) and bis-acrylamide were obtained from Fluka (Buchs, Switzerland). Adenosine 5′-triphosphate (ATP), ammonium molybdate, 5-5′-dithio-bis (2-nitrobenzoic acid) (DTNB), ρ-nitrophenyl-α-glucopyranoside, ρ-nitrophenyl-N-acetyl-ß-d-glucose amide,1-anilinonaphthalene-8-sulphonic acid (ANS) and glutaraldehyde were procured from Sigma (St. Louis, MO, USA). Potassium chloride (KCl) was obtained from Ajax Finechem (Wellington, Auckland, New Zealand).

1.3 Microbial media

Peptone water, standard plate count agar (PCA), EC broth, eosin methylene blue agar (EMB), lauryl sulphate tryptose broth (LST), nutrient broth (NB), selenite cysteine broth (SC) and xylose lysine deoxycholate agar (XLD) were purchased from Merck (Darmstadt, Germany).

2. 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, Stanfen, Germany
Homogenizer	T25 basic	IKA labortechnik, Selangor, Malaysia
Oil bath	B-490	Buchi, Flawil, Switzerland
Water bath	W350	Memmert, Schwabach, 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, VA, USA
Rotary evaporator	Rotavapor-R	Binkmann, Switzerland
Scaning Electron Microscope	JSM5800LV	JEOL, Japan

3. Methods

3.1 Characterization of black tiger shrimp and white shrimp meats

Prior to analyses, shells were peeled off. The shrimps were then deveined and the edible portions were ground to obtain the uniformity. The samples were placed in polyethylene bag and kept in ice during the analyses.

3.1.1 Proximate analysis

Shrimp meats of both species were determined for moisture, ash, fat and protein contents according to the method of AOAC (1999). The values were expressed as % (wet weight basis).

3.1.2 Determination of protein and non-protein nitrogenous compounds

Non-protein nitrogenous constituents, sarcoplasmic protein, myofibrillar protein, alkali-soluble protein and stromal proteins in shrimp meats were fractionated according to the

method of Hashimoto *et al.* (1979). Nitrogen content in each fraction was measured by Kjeldahl method (AOAC, 1999). Protein patterns of different fractions were determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% running gel and 4% stacking gel as described by Laemmli (1970).

3.1.3 Determination of collagen

Collagen was isolated into two different fractions including pepsin-soluble (PSC) and insoluble (ISC) collagen fractions according to the method of Sato *et al.* (1988). Hydroxyproline content in each fraction was determined as described by Bergman and Loxley (1963). A factor of 11.42 was used to convert the amount of hydroxyproline to total collagen (Sato *et al.,* 1986). Protein patterns of each fraction were determined according to the method of Laemmli (1970).

3.1.4 Amino acid analysis

Shrimp muscles were analyzed for amino acid composition using an amino acid analyzer at Tokyo University of Marine Science and Technology, Tokyo, Japan. Samples were hydrolyzed under reduced pressure in 4.0 M methanesulfonic acid containing 0.2% (v/v) 3-2 (2-aminoethyl) indole at 115 ºC for 24 h. The hydrolysates were neutralized with 3.5M NaOH and diluted with 0.2 M citrate buffer (pH 2.2). An aliquot (0.4 ml) was applied to an amino acid analyzer (MLC-703; Atto Co., Tokyo, Japan).

3.1.5 Determination of lipid and fatty acid profile

Lipid in the shrimp muscle was extracted using the Bligh and Dyer method (1959). The lipid compositions were determined by thin layer chromatography/flame ionization detection analyzer (TLC-FID). Scanned quartz rods (silica gel powder coated Chromarod S III) were dipped in 3% boric acid solution for 5 min, dried and rescanned with the TLC-FID analyzer. The sample solution (1 µl) was spotted on the rod and the separation was carried out using the mixtures of benzene: chloroform: acetic acid (52: 20: 0.7) for approximately 35 min. Then, the rods were dried in an oven (105ºC) for 5 min before analyzing with the flame ionization detector. The analytical condition was H_2 flow rate of 160 ml/min, air flow rate of 2000 ml/min and scanning speed of 30 sec/scan. Retention time of lipid composition standards was used to identify chromatographic peaks. Peak area was quantitated and expressed as % of total lipid.

Shrimp lipids were analyzed for fatty acid composition using gas chromatography at Thailand Institute of Scientific and Technical Research. The fatty acid compositions were determined as fatty acid methyl esters (FAME) using a gas chromatography, GC-14A (Shimadzu, Kyoto, Japan) equipped with fused silica capillary column Carbowax-30 M (30 m, 0.25 mm ID) and flame ionization detector (FID). Helium was used as the carrier gas at a flow rate of 30 cm/s. The initial temperature of the column was set at 170ºC and increased to 225º C with a rate of 1ºC/min and then held at 220ºC for an additional 20 min. The detector temperature was set at 270ºC, while the temperature at the injection port was maintained at 250º C. Retention time of FAME standards was used to identify chromatographic peaks. Peak area was quantitated and expressed as % of total lipid (AOAC, 1999).

3.1.6 Determination of mineral content

Mineral content of shrimp muscles were determined by the inductively coupled plasma optical emission spectrophotometer (ICP-OES) at PSU Scientific Equipment Center, Hat Yai. Iron (Fe), copper (Cu), manganese (Mn), cadmium (Cd), nickel (Ni), zinc (Zn), cobal (Co) and magnesium (Mg) contents were determined by the inductively coupled plasma optical emission spectrophotometer (ICP-OES) (Perkin Elmer, Model 4300 DV, Norwalk, CT, USA) according to the method of AOAC (1999). Ground shrimp meat (4 g) was mixed well with 4 ml of nitric acid. The mixture was heated on the hot plate until the digestion was completed. The digested samples were transferred to a volumetric flask and the volume was made up to 10 ml with a deionized water. The solution was subjected to (ICP-OES) analysis. Flow rates of argon to plasma, auxiliary and nebulizer were kept at 15, 0.2, and 0.8 L/min, respectively. Sample flow rate was set at 1.5 ml/min. The wavelengths for analysis of Fe, Cu, Mn, Cd, Ni, Zn, Co, Ca and Mg were 238.2, 327.4, 257.6, 228.8, 231.6, 206.2, 228.6, 317.9 and 285.2 nm, respectively. The concentration of mineral was calculated and expressed as mg/kg sample.

3.2 Effect of heat treatment on physicochemical, physical properties and microstructure of black tiger shrimp and white shrimp

3.2.1 Thermal properties of muscle proteins

3.2.1.1 Differential scanning colorimetry (DSC)

Thermal transition of shrimp meat proteins was measured using the differential scanning calorimetry (DSC) (Perkin-Elmer, Model DSCM, Norwalk, CT, USA). The samples (15-20 mg wet weight) were placed in the DSC hermetic pans, assuring a good contact between the sample and the pan bottom. An empty hermetic pan was used as a reference. The samples were scanned at 10ºC /min over the range of 20-100ºC. T_{max} was measured and the denaturation enthalpies (ΔH) were estimated by measuring the area under the DSC transition curve.

3.2.1.2 Thermal stability

Natural actomysin (NAM) was extracted according to the method of Benjakul, Seymour, Morrissey and An (1997). NAM solution (3-5 mg/ml) was incubated at different temperatures (0, 10, 20, 30, 40, 50 and 60ºC). At definite time (0, 5, 10, 30 and 60 min), samples were taken and immediately cooled in iced water. Ca^{2+}-ATPase activity was then measured according to the method of Benjakul *et al.* (1997). The inactivation rate constant (KD) was calculated as described by Arai *et al.* (1973) as follows:

$KD = (\ln C_0 - C_t)/t$

where $C_0 = Ca^{2+}$ -ATPase activity before incubation

$C_t = Ca^{2+}$ -ATPase activity after incubation for time t

t = incubation time (s)

3.2.2 Effect of heat treatment on physical properties of shrimp meats

Both shrimps from three different parts including front, middle and rear were subjected to heating by steaming for different times (0.5, 1.0, 2.0 and 3.0 min). Cooked shrimp meat was immediately cooled in iced water for 30 sec. The shrimps were drained for 5 min at 4 ºC. The samples were then analyzed as follows:

3.2.2.1 Determination of cooking loss

Weight loss was calculated with the following equation:

Cooking loss (%) = (A-B)/A * 100

when : A = weight before steaming

B = weight after steaming

3.2.2.2 Determination of shear force

Shear force of shrimp meats, raw and cooked, were measured using the TA-XT2i texture analyzer equipped (Stable Micro Systems, Surrey, England) with a Warner-Bratzler

shear apparatus (Brauer *et al.,* 2003). The operating parameters consisted of a cross head speed of 10 mm/s and a 25 kg load cell. The shear force, perpendicular to the axis of muscle fibers, was measured in 3 replicates for each treatment of both shrimp meats. The peak of the shear force profile was regarded as the shear force value.

3.2.2.3 Determination of color

The color of shrimps meats, raw and cooked, were measured in the L* a* b* mode of CIE (angle 10°, illuminant D65) using Hunter Lab (ColorFlex, Hunter Associates Laboratory, Verginia USA). L*, a* and b* indicate lightness, redness/greenness and yellowness/blueness, respectively.

3.2.3 Effect of heat treatment on microstructure of shrimp meats

Microstructures of raw and cooked shrimps of three parts (front, middle and tail) were analyzed as described by Jones and Mandigo (1982). Sample was prepared by cutting into a cube (4 x 4 x 4 mm) with a razor blade. The prepared sample was fixed in 2.5% glutaraldehyde in 0.2 M phosphate buffer, pH 7.2 at room temperature for 2 h. All specimens were washed three times with deionized distilled water for 15 min per time and dehydrated with a serial concentration of 50-100% ethanol for 15 min each. All specimens were coated with 100% gold (Sputter coater SPI-Module, PA, USA). The microstructure was visualized using a scanning electron microscopy (JEOL, JSM-5800 LV, Tokyo, Japan).

3.3 Effect of freeze-thawing on physicochemical, physical properties and microstructure of black tiger shrimp and white shrimp meats

Both shrimps were packaged in polyethylene bag and frozen at -20ºC using an air-blast freezer for 48 h. The frozen shrimps were thawed using a running water (27ºC) until the core temperature reached 0-2ºC. The freeze-thawing was performed for 0, 1, 3 and 5 cycles. Total freezing time was approximately 10 days. The shrimps were then subjected to the following analyses.

3.3.1 Determination of exudate loss

The samples (25 g) were chopped into small pieces, followed by centrifuging at 10,000xg for 60 min at 4ºC using a Sorvall RC 26 Plus refrigerated centrifuge (Sorvall, Norwalk, CT, USA). The exudate formed was collected using a Pasteur pipette and the volume obtained

was measured. The exudate was brought to 25 ml with distilled water before enzyme assay. The protein content in exudate was determined by the Lowry method (Lowry *et al.,* 1951).

3.3.2 α -glucosidase (AG) and β-N-acetyl-glucosaminidase (NAG) activity assay

The AG (E.C. 3.2.1.20) and NAG (E.C. 3.2.1.30) activities were assayed according to the method of Benjakul and Bauer (2000) with a slight modification. For AG activity assay, the activity was measured spectrophotometrically using ρ-nitrophenyl-α-glucopyranoside as a substrate. The reaction mixture contained 0.3 ml of 0.1 M Na citrate buffer (pH 4.0), 0.2 ml of 1.0 M NaCl, and 1 ml of diluted shrimp muscle exudate. The reaction mixture was pre-incubated at 37ºC for 10 min. The reaction was initiated by adding 1 ml of 4.2 mM ρ-nitrophenyl-α-glucopyranoside. After 60 min, the reaction was terminated by adding 1 ml of 0.3 M KOH. The absorbance was measured at 405 nm. The blank was performed using distilled water instead of shrimp muscle exudate. The negative control was carried out by adding the stopping reagent prior to the addition of substrate.

NAG activity was determined using ρ-nitrophenyl-N-acetyl- β-D-glucose amide as a substrate. The reaction mixture consisted of 0.3 ml of 0.1 M Na-citrate buffer (pH 4.5), 0.2 ml of 0.6 M KCl and 0.2 ml of diluted shrimp muscle exudate. The reaction was initiated by adding 0.2 ml of ρ-nitrophenyl-N-acetyl- β-D-glucose amide and incubated at 37ºC for 10 min. The reaction was stopped by adding 1 ml of 0.3 M KOH. The blank and negative control was performed as described above. The absorbance was measured at 405 nm. The amount of ρ-nitrophenol released was monitored at 405 nm and calculated using a molar extinction coefficient of 19,500 M^{-1} cm^{-1}. One unit of enzyme was defined as the activity, which released 1 nmol of ρ-nitrophenol per min.

3.3.3 Determination of Ca^{2+}– ATPase activity

ATPase activity was determined according to the method of Benjakul *et al.* (1997). Natural actomysin (NAM) prepared as described by Benjakul et al. (1997) was diluted to 3–5 mg/ml with 0.6 M KCl, pH 7.0. Diluted NAM solution (0.5 ml) was added to 0.3 ml of 0.5 M Tris-maleate, pH 7.0. The mixture was added with 0.5 ml of 10 mM $CaCl_2$ and 3.45 ml of distilled water. To initiate the reaction, 0.25 ml of 20 mM ATP was added. The reaction was conducted for 10 min at 25ºC and terminated by adding 2.5 ml of chilled 15% (w/v) trichloroacetic acid. The reaction mixture was centrifuged at 3500xg for 5 min and the inorganic

phosphate liberated in the supernatant was measured by the method of Fiske and Subbarow (1925). Specific activity was expressed as μmol inorganic phosphate released/mg protein/min. A blank solution was prepared by adding chilled trichloroacetic acid prior to addition of ATP.

3.3.4 Determination of total sulfhydryl content

Total sulfhydryl content was determined using 5, 5′-dithio-bis (2-nitrobenzoic acid) (DTNB) according to the method of Ellman (1959) as modified by Benjakul et al. (1997). To one ml of NAM solution (0.4%), 9 ml of 0.2 M Tris–HCl buffer, pH 6.8, containing 8 M urea, 2% SDS and 10 mM EDTA, were added. To 4 ml of the mixture, 0.4 ml of 0.1% DTNB in 0.2 M Tris–HCl (pH 8.0) was added and incubated at 40ºC for 25 min. The absorbance at 412 nm was measured using a UV-16001 spectrophotometer (Shimadzu, Kyoto, Japan). A blank was conducted by replacing the sample with 0.6 M KCl. Sulfhydryl content was calculated using the extinction coefficient of 13,900 M^{-1} cm^{-1}.

3.3.5 Determination of disulfide bond content

Disulfide bond in NAM was determined by using 2-nitro-5-thiosulfobenzoate (NTSB) assay according to the method of Thannhauser *et al.* (1987). To 0.5 ml of NAM sample (1 mg/ml), 3.0 ml of freshly prepared NTSB assay solution, pH. 9.5 were added. The mixture was incubated in dark at room temperature (25–27ºC) for 25 min. Absorbance at 412 nm was measured. Disulfide bond content was calculated using the extinction coefficient of 13,900 M^{-1} cm^{-1}.

3.3.6 Determination of surface hydrophobicity

Surface hydrophobicity was determined as described by Benjakul *et al.* (1997) using 1-anilinonaphthalene-8-sulphonic acid (ANS) as a probe. NAM dissolved in 10 mM phosphate buffer, pH 6.0 containing 0.6 M NaCl was diluted to 0.1, 0.2, 0.3, and 0.5% (w/v) protein using the same buffer. The diluted protein solution (2 ml) was added with 10 μl of 8 mM ANS in 0.1 M phosphate buffer, pH 7.0. The fluorescence intensity of ANS-conjugates was measured using a FP-750 spectrofluorometer (Shimadzu, Kyoto, Japan) at an excitation wavelength of 374 nm and an emission wavelength of 485 nm. The initial slope of the plot of fluorescence intensity versus NAM concentration was referred to as SoANS.

3.3.7 Determination of protein solubility

Solubility was determined according to the method of Benjakul and Bauer (2000). To 1 g sample, 20 ml of 0.6 M KCl was added and the mixture was homogenized for 1 min at speed of 12,000 rpm using an IKA homogenizer (Salangor, Malaysia). The homogenate was stirred at 4°C for 4 h, followed by centrifuging at 8500*xg* for 30 min at 4ºC. To 10 ml of supernatant, cold 50% (w/v) trichloroacetic acid was added to obtain the final concentration of 10%. The precipitate was washed with 10% trichloroacetic acid and solubilized in 0.5 M NaOH. The sample was also directly solubilized by 0.5 M NaOH and used for total protein determination. Protein content was determined using the Biuret method (Robinson and Hodgen, 1940) and expressed as the percentage of total protein in the sample.

3.3.8 Determination of shear force

Shear force of raw and freeze-thawed shrimp meats (middle part) were measured as described in section 3.2.2.2

3.3.9 Effect of freeze-thawing process on microstructure of shrimp meats

Raw and freeze-thawed shrimp meats (middle part) were subjected to scanning electron microscopy as mentioned in section 3.2.3.

3.4 Effect of ice storage on quality changes of black tiger shrimp and white shrimp meats

Shrimps washed with tap water were separated into two groups; whole and decapitated shrimps. For each group, samples were kept in a styrene foam box containing crushed ice, with a shrimp/ice ratio of 1:2 (w/w). The shrimps were placed and distributed uniformly between the layers of ice. The boxes containing samples and ice were kept at room temperature (28-30°C). To maintain the ice content, different methods were used as follows:

Method A: Molten ice was removed and replaced with an equal amount of ice every 2 days.

Method B: Molten ice was removed and replaced with an equal amount of ice every day.

Both shrimps, whole and decapitated, with different icing methods were taken every 2 days for analyses.

3.4.1 Chemical analysis

3.4.1.1 pH determination (Benjakul *et al.*, 1997)

Shrimps muscle was homogenized with 10 volumes of deionized water (w/v) and the pH was measured using a pH meter (Eutech Instruments Pte Ltd, Singapore).

3.4.1.2 Determination of TVB and TMA (Conway and Byne, 1936).

TVB and TMA contents were determined using the Conway microdiffusion assay according to the method of Conway and Byrne (1936). Sample (2 g) was mixed with 8 ml of 4% tricloroacetic acid (TCA). The mixtures were homogenized at 6,500 rpm using an Ultra Turrax homogenizer (IKA Labortechnik, Selangor, Malasia) for 1 min. The homogenate were filtered using Whatman No. 41 filter paper and the filtrate was used for analysis. To determine the TMA content, formaldehyde was added to the filtrate to fix ammonia present in the sample. TVB and TMA were released after addition of saturated K_2CO_3 and diffused into the boric acid solution. The titration of solution was performed and the amount of TVB or TMA was calculated.

3.4.1.3 Determination of biogenic amines (Eerola *et al*., 1993)

Biogenic amines of shrimp samples were extracted according to the procedure of Koutsoumanis *et al.* (1999) with a slight modification. Sample (10 g) was added with 300 μl of internal standard *(1,7-diaminoheptane ~ 1 mg/ml) and 10 ml of 0.4 N perchloric acid (PCA) solution. The mixture was homogenized at 6,500 rpm using an Ultra Turrax homogeniser (IKA Labortechnik, Selangor, Malasia) for 1 min. The homogenate was made up to 50 ml using of 0.4 N perchloric acid and then centrifuged at 12,000g for 10 min at 4°C using a Sorvall Model RC-5B Plus centrifuge (Newtown, CT, USA). Derivatization of extracted sample was performed according to the method of Senoz *et al.* (2000). The solution was mixed with 100 μl of 2 N NaOH and 150 μl of saturated sodium bicarbonate. One ml of dansyl chloride (10 mg/ml) was added to each sample, mixed very well, and then incubated for 45 min at 40°C. Residual dansyl chloride was removed by adding 50 μl of 25% ammonia and centrifuged at 2500g for 30 min and the supernatant was filtered (0.45 lm); 20 μl of filtrate was then injected into the HPLC (600E Waters, Massachusetts, USA). Quantitative analysis of amines was carried out in a Waters HPLC system (Water Associates, Miliford, MA, USA). Separation was achieved using a column of Hypersil BDS C18 (ThermoHypersil, Hercules, USA). A gradient elution programme was used with the mobile phase of 100% methanol (solvent A) and nanopure distilled

water (solvent B), starting with 55% solvent A and 45% solvent B and finishing with 100% solvent A and 0% solvent B after 45 min. The flow rate was 1.5 ml/min. Data were processed and calculated with a CSW32 programme-based integrator.

The quality index and the biogenic amine index were calculated according to the procedures described by Mieltz and Karmas (1977) and Veciana-Nogues *et al.* (1997), respectively. The equations used were as follows:

$$\text{Quality index (QI)} = \frac{(\text{histamine} + \text{putrescine} + \text{cadaverine})}{(1 + \text{spermidine} + \text{spermine})}$$

Biogenic amine index (BAI) = (histamine + putrescine + cadaverine + tyramine)

3.4.1.4 Determination of glycogen (Rosa and Nunes, 2003).

Shrimp samples (50 mg) were boiled with 400 µl of 33% KOH for 20 min. After cooling, 700 µl of 96% ethanol was added. The mixture was placed in an ice bath to allow the complete precipitation (~ 2 h). Thereafter, the mixture was centrifuged at 7,500 x g for 20 min and the supernatant was discarded. One ml of distilled water was added to the pellet, followed by sonication. Subsequently, 100 µl of sample solution was incubated at 90°C in 2 ml of anthrone reagent (38 ml of concentrated sulphuric acid was added to 15 ml of distilled water and 0.075 g of anthrone) for 20 min in dark and the absorbance was read at 620 nm. Glycogen concentration was calculated from standard curve using glycogen from oyster (II) as standard.

3.4.1.5 Determination of K-value using ion exchange chromatography (Uchiyama and Kakuda, 1984).

Ground sample (5 g) was extracted with 10 ml of 10% PCA. The extracted was run in an ion exchange chromatography with Anion exchange resin-AG (R) 1-X^4, 400 mesh Cl-form.

3.4.1.6 Determination of TBARS (Buege and Aust, 1978).

Ground sample (0.5 g) was dispersed in 10 ml of TBARS (0.375 g of triobituric acid, 15 g of trichloroacetic acid, and 0.875 ml of hydrochloric acid in 100 ml of distilled water). The mixture was heated in boiling water for 10 min, followed by cooling in running tap water. The mixture was centrifuged at 3600xg for 20 min and the absorbance was measured at 532 nm. A standard curve was prepared with malonaldehyde bis (dimethyl acetal)

(MDA) at concentration ranging from 0 to 10 ppm. TBARS was calculated and expressed as mg malondialdehyde/kg.

3.4.1.7 Protein patterns by SDS – PAGE (Laemmli, 1970)

Protein patterns were determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% running gel and 4% stacking gel as described by Laemmli (1970). To prepare the protein sample, 27 ml of 5% (w/v) SDS solution heated to 85°C were added to the sample (3 g). The mixture was then homogenized for 2 min using an IKA homogenizer. The homogenate was incubated at 85 °C for 1 h to dissolve total proteins. The samples were centrifuged at 3500g for 20 min to remove undissolved debris. Protein concentration was determined by the Lowry method (Lowry *et al.,* 1951), using bovine serum albumin as standard. After separation, the proteins were fixed and stained with Coomassie Blue R-250.

3.4.1.8 Determination of TCA soluble peptides (Benjakul *et al.,* 2002).

Ground meat (3 g) was homogenized with 27 ml of 5% (w/v) TCA. The homogenate was kept in ice for 30 min and centrifuged at 5000g for 20 min using a RC-5B plus centrifuge (Sorvall, Norwalk, CT, USA). Soluble peptides in the supernatant were measured according to the Lowry method (Lowry *et al.,* 1951) and expressed as μmol tyrosine/g muscle.

3.4.2 Physical analysis

3.4.2.1 Determination of shear force (Bauer *et al.,* 2003)

Shear force of ice storage shrimp meats (middle part) were measured as described in section 3.2.2.2.

3.4.2.2 Determination of water holding capacity (Lopez-Caballo *et al.,* 2000)

Water holding capacity (WHC) was determined by placing 1.5 g of ground shrimp meat in a centrifugation tube with absorbent paper and centrifuging at 4000 xg at 25°C for 15 min in a refrigerated centrifuge (Sorvall, RC-5B plus, USA). WHC was expressed as the amount of water retained per 100 g of sample.

3.4.2.3 Determination of melanosis (Otwell and Marshall, 1986)

Melanosis or blackening of shrimp samples during iced storage was evaluated by visual inspection described by Otwell and Marshall (1986) using a scale where 0 = absent; 2 = slight, noticeable on some shrimps; 4 = slight, noticeable on most shrimps; 6 =

moderate, noticeable on most shrimps; 8 = heavy, noticeable on most shrimps; and 10 = heavy, totally unacceptable. Twelve trained panelists were used for the assessment.

3.4.2.4 Determination of cooking loss

Cooking loss was determined as the percentage loss of shrimp meat weight after steaming for 5 min, compared with the initial weight.

3.4.2.5 Determination of color

The color of ice storage shrimp was measured by Hunter lab and reported in CIE system. L* a* and b* parameters indicate lightness, redness and yellowness, respectively.

3.4.3 Microbiological analyses

3.4.3.1 Total viable count

Samples without peeling (50 g) were weighed into a stomacher bag containing 450 ml peptone water (2% NaCl). Blending was done in a Stomacher (IUL Instrument, city, Spain) for 2 min. Peptone water was used for diluting the samples. Thereafter, the sample diluted in serial 10-fold steps was used as inoculum for the three-tube MPN procedure and aerobic plate counts (APC). APC was done by the spread plate technique on plate count agar (PCA-Merck). The plates were incubated at 35 °C for 2 days for counting mesophilic bacteria.

3.4.3.2 Determination of coliforms bacteria and *E. coli*

The three-tube MPN procedure for coliforms and *E. coli* was carried out as described by Feng *et al*. (1998). One ml from each diluted tube was transferred into lauryl sulfate tryptose (LST) broth and incubated at 35 °C for 24-48 h. One loopful from tubes with gas formation was transferred to EC broth and incubated at 44.5°C for 24-48 h. The tubes with turbidity and gas formation in the Durham tube of LST indicated the presence of coliforms, whereas the tubes of EC broth indicated the presence of faecal coliforms. Their numbers per 1 g of shrimp were calculated from the MPN table.

A loopful of culture from EC broth was streaked on EMB agar and incubated at 35 °C for 24 h. Colonies with metallic sheen on EMB agar indicated the presence of *E. coli* (Feng *et al*., 1998).

3.4.3.3 Determination of *Salmonella*

Determination of *Salmonella* was carried out following Andrews and Hammack (1998). Shrimp samples (50 g) were weighed into sterile plastic bags and then homogenized with 450 ml NB (Merck). The samples were transferred to a wide-mouth, screw-cap bottle and incubated at 37°C for 24 h. After completion of pre-enrichment process, 10 ml of sample was transferred into the selenite cystine broth (Merck, 107709) and incubated at 35 °C for 24 h. Then, the cultures were streaked onto Xylose Lysine Desoxycholate agar (XLD) (Merck, 105287) and incubated 24 h at 35°C. Suspected *Salmonella* colonies were selected and subjected to standard biochemical tests. (Flowers *et al.,* 1992).

3.4.4 Sensory evaluation

Sensory evaluation was done by twelve panelists according to the method of U.S. Food and Drug Administration (2005). All panelists were trained in evaluation of shrimps. Panelists were screened and those who did not like shrimp or had food allergies were excluded. Training consisted of five sessions until the panelists knew the constituted quality of shrimps and were trained in differentiating odor, appearance and texture of shrimps.

Both raw and cooked shrimps were evaluated for overall appearance, texture, odor and flavor (by olfactory evaluation) using a point structured scale with a value of 1 for excellent (sweet shellfish odor, desirable moist, good color and characteristic firm texture) and 10 for very poor (strong ammoniacal, stale, putrid odor, undesirable discoloration and poor texture). A score more than 5 was taken as score for unacceptable. Two to three shrimps from each treatment were taken and evaluated by each panelist. All samples were identified by a three digit code. Sensory testing was held in a clean, well lighted and well ventilated room.

4. Statistical analysis

All experiments were run with triplicate determination and CRD (Completely Randomized Design) was used. For sensory evaluation, the run with twelve determinations and RCBD (Randomized Completely Block Design) was implemented. Analysis of variance (ANOVA) was performed and mean 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).