CHAPTER 2

MATERIALS AND METHODS

1. Chemicals and microbial media

Bovine haemoglobin, ethylenediaminetetraacetic acid (EDTA), pepstatin A, soybean trypsin inhibitor, 4-hexylresorcinol, sodium benzoate, citric acid, ascorbic acid, 1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), L-β-(3,4 dihydroxylphenyl) alanine (L-DOPA), Brij-35, L-tyrosine, ninhydrin, bovine tendon collagen type I, bovine serum albumin, malonaldehyde bis (dimethyl acetal) (MDA) and thiobarbituric acid (TBA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA.). Trichloroacetic acid (TCA), ammonium sulfate, Folin-Ciocalteu's phenol, sodium pyrophosphate, peptone water, standard plate count agar (PCA), EC broth, eosin methylene blue agar (EMB), lauryl sulphate tryptose broth (LST), baird parker medium (BP), nutrient broth (NB), selenite cysteine broth (SC), glucose salt teepol broth (GSTB), thiosulfate citrate bile salts sucrose agar (TCBS), xylose lysine deoxycholate agar (XLD) and Man rogaso sharpe broth (MRS broth) were procured from Merck (Darmstadt, Germany).

2. Instruments

Instruments	Model	Company/Country
pH meter	PB10	Eutech Instruments Pte Ltd, Singapore
Magnetic stirrer	BIG SQUID	IKA labortechnik, Germany
Homogenizer	T25 basic	IKA labortechnik, Malaysia
Water bath	W350	Memmert, Germany
Microcentrifuge	MIKRO20	Hettich Zentrifugan, Germany
Refrigerated centrifuge	RC-5B plus	Sorvall, USA
Stomacher	M400	SEWARD,UK
Texture analyzer	TA-XT2	Stable Micro Systems, England
Double-beam Spectrophotometer	UV-16001	Shimadzu, Japan

3. Shrimp samples

Black tiger shrimp (*Penaeus monodon*) and white shrimp (*Litopenaeus vannamei*) with the size of 60 shrimps/kg were purchased from the farms in Suratthani and Songkhla provinces, respectively. The shrimps were transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla in ice with a shrimp/ice ratio of 1:2 (w/w) within 1-3 h. Upon arrival, the shrimps were washed with clean water. The shrimps were kept in ice until used.

Cephalothorax was removed and the muscle was collected. Both portions were powderized in the presence of liquid nitrogen and ground into a fine powder using a National Model MX-T2GN blender (National, Taipei, Taiwan) at high speed. The powder was placed in the polyethylene bags and kept at -20°C until used.

4. Characterization of shrimp enzymes associated with the quality changes

4.1 Preparation of shrimp enzyme extracts

Cephalothorax or muscle powder (50g) was mixed with 2 volumes of 0.01 M sodium phosphate buffer (pH 7.6) (Brauer *et al*., 2003). The mixture was homogenized for 5 min using an IKA labortechnik homogenizer (Selangor, Malaysia) at a speed of 11,000 rpm. The homogenate was stirred for 30 min at 4°C, followed by centrifugation at 10,000x*g* for 30 min at 4°C using a Sovall Model RC-B Plus refrigerated centrifuge (Newtown, CT, USA). The supernatant was referred to as 'crude protease extract' and used for the assays of protease and collagenase activities.

For PPO activity assay, cephalothorax powder (10g) was added with 3 volumes of 0.05 M sodium phosphate buffer, pH 7.2 containing 1 M NaCl and 0.2% Brij 35 as described by Chen *et al*. (1997). The extract was homogenized at a speed of 11,000 rpm for 2 min. The homogenate was stirred for 30 min at 4°C and the mixture was then centrifuged at 8,000x*g* for 30 min at 4°C. The supernatant was subjected to ammonium sulfate fractionation at 0-40% saturation (Montero *et al*., 2001a). The pellet obtained was dissolved in a minimum volume of 0.05 M phosphate buffer, pH 6.5 and dialyzed against 15 volumes of the same buffer at 4°C with three changes of dialysis buffer. The dialysate was used as 'crude PPO extract'.

4.2 Enzyme assays

4.2.1 Protease activity

Proteolytic activity of crude protease extract from shrimp muscle was determined using haemoglobin (pHs 2.0-6.0) and casein (pHs 6.0-11.0) as substrates according to the method of An *et al*. (1994). To the preincubated reaction mixture containing 2 mg of casein or haemoglobin, 200 µl of distilled water and 625 µl of reaction buffer, the crude extract (200 µl) was added to initiate reaction. The mixture was incubated at pH and temperature specified for 30 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 and centrifuged at 8,500xg for 10 min. The oligopeptide content in the supernatant was determined by the Lowry method (Lowry *et al*., 1951) using tyrosine as a standard. One unit of activity was defined as that releasing 1 mmole of tyrosine per min (mmol/Tyr/min). A blank was run in the same manner, except the enzyme was added after addition of 50 % TCA (w/v).

4.2.2 Collagenase activity

The collagenolytic activity of crude extract from cephalothorax and muscle was determined using bovine tendon collagen (collagen type I) as substrate according to the method of Brauer *et al*. (2003). Reaction mixture comprised 6 mg of bovine tendon collagen type I, and 1.25 ml of 0.1 M buffer (pH 7.5) containing 0.35 mM $CaCl_2$. To initiate the reaction, 25 µl of crude extract were added into the reaction mixture. The reaction was incubated at pH and temperature specified for 5 h. Thereafter, the reaction mixture was centrifuged at 4,000xg for 5 min. The supernatant obtained (0.2 ml) was transferred into test tube containing 0.5 ml of 1.5% ninhydrin. The mixture was incubated at 100°C for 15 min and cooled to room temperature; 2.5 ml of 50% ethanol was added to the mixture. The absorbance at 600 nm was recorded. The concentration of hydrolyzed amino acid was determined using L-leucine as a standand. One unit of collagenolytic activity was expressed as micromoles of leucine released/ 5 h. For blanks, substrate or enzyme extract were left out from the reaction mixture. Reaction was performed for 5 h and the activity was determined and calculated as previously described.

4.2.3 PPO activity

PPO activity of crude PPO extract from cephalothorax was assayed using *L*-DOPA as a substrate according to the method of Chen *et al*. (1997) with a slight

modification. The assay system consisted of 150 µl of the enzyme solution, 150 µl of distilled water, 150 µl of 0.1 M phosphate buffer, pH 6.0 and 750 µl of *L*-DOPA. PPO activity was determined for 3 min at 45°C by monitoring the formation of dopachrome at 475 nm. One unit of PPO was defined as the change in 0.001 unit of A_{475}/min. Blank was prepared by excluding the substrate from the reaction mixture.

4.3 Characterization of shrimp enzymes

4.3.1 pH and temperature profiles

The activities of protease, collagenase and PPO were measured at pH 7.0 (using 0.2 M McIlvaine's buffer) at various temperatures (25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80°C). The pH profile was studied under optimal temperature over the pH range of 2.0-11.0 (0.2 M McIlvain's buffer for pHs 2.0-7.5 and 0.1 M glycine-NaOH for pHs 8.0-11.0). The optimum pH and temperature were used for further studies.

4.3.2 Thermal and pH stability

Thermal stability of protease, collagenase and PPO was determined by incubating 200 µl of crude extracts at different temperatures (25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80°C) for 30 min. The sample was then cooled rapidly in the iced water before assaying for the residual activity under the optimal condition. For pH-stability study, crude extract were mixed with 200 µl of sodium-citrate buffer (0.1 M) or phosphate buffer (0.1M) at various pHs and incubated at 25°C for 30 min. The residual activity was measured under the optimal condition.

4.3.3 Inhibitor study

For the protease and collagenase, various inhibitors at different concentrations were added into the crude extract at the ratio of 1:1 (v/v). The inhibitors and final concentrations used included pepstatin A (1 µM, 10 µM), EDTA (2 mM, 20 mM, 50 mM, 100 mM), soybean trypsin inhibitor (1 µM, 10 µM, 50 µM, 100 µM) and E-64 (1 µM, 10 µM, 50 µM, 100 µM). The mixtures were allowed to stand at room temperature for 15 min. The residual activity was measured under the optimum condition. Percent inhibition was calculated.

Different inhibitors at different concentrations were used to test for their inhibitory activity towards PPO. The inhibitors and final concentrations used involved

sodium benzoate (0.1 g/l, 1.0 g/l), 4-hexylresorcinol (1.0 g/l, 10.0 g/l), citric acid (0.5 g/l, 5.0 g/l), ascorbic acid (0.5 g/l, 5.0 g/l) and EDTA (0.25 g/l, 2.5 g/l). The inhibitors were mixed with crude extract at a ratio 1:1 (v/v) and the mixtures were kept at room temperature for 15 min. The residual activities of the crude extracts were determined under the optimal condition. Percent inhibition was then calculated.

5. Effect of different MAP conditions on melanosis and quality of black tiger shrimp and white shrimp

5.1 Sample preparation

Whole black tiger shrimps and white shrimp (head-on) were divided into two groups, without and with ascorbic acid treatment. To treat the shrimps with ascorbic acid, the samples were socked in five volumes of ascorbic acid solution (0.5%) for 15 min at 4°C, followed by draining for 10 min at 4°C. Ten shrimps for each sample were placed orderly on polystyrene trays. A tray containing shrimp was inserted in a vacuum bag (polyethylene bag) (7 inch x 11 inch) with gas permeability (O_2 transmission rate of 0.66 g $m^{-2}day^{-1}$at 23°C, 1 atm pressure) and was packaged with a shrimps/gas ratio of 1:3 (w/v) using a Henkovac type 1000 (Tecnovac, Italy). Gas mixtures used included 45% CO_2, 10% O_2, 45% N_2 (MAP1); 60% CO_2, 10% O_2, 30% N_2 (MAP2), and 80% CO_2, 10% O_2, 10% N_2 (MAP3). Shrimps kept in air were used as the control. All samples packaged under the different atmospheres with and without ascorbic acid treatment were stored at 4°C. The samples were removed for chemical, physical and microbiological analyses every 3 days up to 12 days. Samples were also determined for enzyme activity.

5.2 Chemical analyses

5.2.1 pH determination

Shrimps meat (2 g) was homogenized thoroughly with 10 volumes of distilled water (w/v) and the homogenate was used for pH determination (Benjakul *et al.*, 1997). The pH was measured using a pH meter (Eutech Instruments Pte Ltd, Singapore).

5.2.2 Determination of TVB and TMA contents

Total volatile base (TVB) and trimethylamine (TMA) contents in shrimp meat were determined using the Conway microdiffusion method as described by Conway (1950). The 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 through Whatman No. 41 filter paper and the filtrate was used for analyses. Sample extract (1 ml) was placed in the outer ring. The inner ring solution (1% boric acid containing the Conway indicator) was then pipetted into the inner ring. To initiate the reaction, K_2CO_3 (1 ml) was mixed with sample extract. The Conway unit was closed and incubated at 37°C for 60 min. The inner ring solution was then titrated with 0.02 N HCl until the green color turned to pink. TMA content was determined with the same procedure as TVB but 10%, formaldehyde was added to the filtrate to fix ammonia present in the sample. TVB and TMA were released after addition of saturated and diffused into the boric acid solution. The titration of solution was performed and the amounts of TVB or TMA were calculated.

5.2.3 Determination of thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) were determined according to the method of Buege and Aust (1978). Ground sample (0.5 g) was dispersed in 10 ml of TBARS solution (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 boiled water for 10 min, followed by cooling in running water. The mixture was centrifuged at 3,600xg for 20 min (MIKRO20, Hettich Zentrifugan, Germany) and the absorbance was measured at 532 nm using UV 1601 spectrophotometer (Shimadzu, Kyoto, Japan). A standard curve was prepared with malonaldehyde bis (dimethyl acetal) (MDA) at concentrations ranging from 0 to 10 ppm. TBARS was calculated and expressed as mg malondialdehyde/ kg shrimp meat.

5.2.4 Determination of formaldehyde content

Formaldehyde was determined with the acetylacetone reagent as described by Nash (1953). The sample (5 g) was homogenized with 20 ml of 5% trichloroacetic acid at a speed of 11,000 rpm. The homogenate was then filtered with Whatman No 41 paper. The residue was homogenized with 10 ml of 5% trichloroacetic acid at the same speed and time and

filtered. The combined filtrate was neutralized to pH 6.0-6.5 using 1 or 0.1 M KOH. Then the neutralized filtrate was made up to 50 ml with distilled water. The filtrate (3 ml) was mixed thoroughly with 3 ml of acetylacetone reagent. The mixture was heated at 60°C for 15 min to develop color and the absorbance was measured spectrophotometrically at 412 nm using a UV 1601 spectrophometer (Shimadzu, Kyoto, Japan). Formaldehyde content was expressed as µg/ g shrimp meat as determined from a standard curve.

5.2.5 Determination of TCA soluble peptide content

TCA soluble peptides were determined as per the method of Benjakul *et al*. (2002). Shrimp meat (3 g) was homogenized with 27 ml of 5% (w/v) TCA at a speed of 1,100 rpm for 2 min. The homogenate was kept in ice for 30 min and centrifuged at 5,000xg 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 shrimp meat.

5.3 Physical analyses

5.3.1 Determination of shear force

Shear force of shrimp were measured at the middle part using the TA-XT2i texture analyzer (Stable Micro Systems, Surrey, England) equipped with a Warner-Bratzler shear apparatus. 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 shrimp meats. The peak of the shear force profile was regarded as the shear force value (Brauer *et al.,* 2003).

5.3.2 Determination of water holding capacity

Water holding capacity (WHC) was determined by placing 1.5 g of ground shrimp meat in a centrifugation tube containing absorbent paper and centrifuging at 4,000xg at 25°C for 15 min in a centrifuge (Sorvall, RC-5B plus, USA). WHC was expressed as the amount of water retained per 100 g of sample. Water holding capacity was calculated following Lopez-Caballo *et al.* (2000).

5.3.3 Determination of melanosis

Melanosis or blackening of shrimp samples during storage was evaluated by visual inspection as 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.

5.4 Microbiological analyses

5.4.1 Total viable count

Whole samples (without peeling) (50 g) were weighed into a stomacher bag containing 450 ml peptone water (2% NaCl). Blending was done in a Stomacher (M400, SEWARD, UK) for 2 min. Thereafter, the sample was diluted using peptone water in serial 10-fold steps and 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). The plates were incubated at 35°C for 2 days according to the method of Speck (1976). Microbial counts were expressed as CFU/g.

5.4.2 Lactic acid bacteria count

Whole samples were blended as previously described (section 5.4.1). A series of ten-fold dilutions was made using the saline solution for microbiological analyses. Lactic acid bacteria were counted in double-layer in man rogaso sharpe (MRS) agar and incubated at 35°C for 3 days according to the method of Ordonez *et al*. (1991). Microbial counts were expressed as CFU/g.

5.5 Enzyme activities

Protease, collagenase and PPO activity were measured as described in section 4.2.

MAP condition rendering the shrimps with the lowest melanosis with the high quality during the extended storage was chosen for further study. Only white shrimps were used for further study due to the unavailaility of black tiger shrimp.

6. Combination effect of melanosis inhibitors and MAP on the quality changes of white shrimp

6.1 Effect of phosphate and ascorbic acid pretreatment on quality of white shrimp kept under MAP

Whole white shrimp (head-on) were soaked in five volumes of 2% (w/v) sodium pyrophosphate solution for 2 h at 4°C and drained for 10 min at 4°C. After being drained, shrimps were separated into two groups; with and without ascorbic acid socking. Ascorbic acid was shown to exhibit the most effectiveness in inhibitory PPO extract (section 4.3.3). For shrimps with ascorbic acid pretreatments, the shrimps were socked in five volumes of ascorbic acid solution (5 g/l) for 15 min at 4°C and drained for 10 min at 4°C. For individual treatment, ten shrimps were placed on polystyrene trays. A tray containing shrimp was inserted in a vacuum bag (7 inch x 11 inch) with a shrimps/gas ratio of 1:3 (w/v). Gas mixtures containing 80% CO_2, 10% O_2, 10% N_2 was used. Shrimps with different treatments packed under MAP were stored at 4°C. The control samples were soaked in distilled water, drained and packed in air. All samples were taken for chemical, physical and microbiological analyses as well as the determination of enzyme activity every 3 days for up to 12 days as described in sections 5.2, 5.3, 5.4 and 5.5, respectively.

6.2 Effect of phosphate and 4-hexylresorcinol pretreatment on quality of white shrimp

Whole white shrimp (head-on) were soaked in five volumes of 2% (w/v) sodium pyrophosphate solution for 2 h at 4°C and drained for 10 min at 4°C. The shrimps were then separated into two groups: without and with PPO inhibitor treatment. Pretreatment of 4-hexylresorcinol was carried out by socking the shrimp in five volumes of 4-hexylresorcinol solution (0.25%) for 15 min at 4°C and drained for 10 min at 4°C. Prior to MAP, shrimps were separated into two groups: whole and decapitated shrimps. For individual treatment, ten shrimps were placed on polystyrene trays and packed under MAP (80% CO_2, 10% O_2 and 10% CO_2) for the sample treated with PP and 4-hexylresorcinol, two different atmospheres (80% CO_2, 10% O_2, 10% N_2; 80% CO_2, 20% N_2) was used. The control samples were soaked in distilled water, drained and packed in air. All samples were stored at 4°C and taken for chemical, physical

analyses and the determination of enzyme activity every 3 days for up to 12 days as described in section 5.2, 5.3 and 5.5. Microbiological analyses of all samples were performed as follows:

6.2.1 Total viable count

Fish sample (25 g) was collected aseptically in a stomacher bag and 10 volumes of sterile saline solution (0.85%) were added. After homogenizing in a stomacher (M400, SEWARD, UK) a series of ten-fold dilutions was made using the saline solution for microbiological analyses. Mesophilic and psychrophilic bacterial count were determined by plate count agar (PCA) with the incubation at 35°C for 2 days (Speck, 1976) and 7°C for 7 days (Cousin *et al*., 1992), respectively.

6.2.2 Lactic acid bacteria count

Lactic acid bacteria count of shrimp samples were measured as described in section 5.4.2

6.2.3 Determination of coliforms bacteria and *E. coli*

The three-tube MPN procedure for coliforms and *E. coli* was carried out as described by Geissler *et al*. (2000). 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 indicate 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* (Geissler *et al*., 2000).

6.2.4 Determination of *Salmonella*

Salmonella was determined following the method of Hammack (1999). Shrimp samples (50 g) were weighed into sterile plastic bags and then homogenized with 450 ml nutrient broth using a stomacher (M400, SEWARD, UK). 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 and incubated at 35°C for 24 h. Then, the cultures were streaked on Xylose Lysine Desoxycholate (XLD) agar

and incubated for 24 h at 35°C. Suspected *Salmonella* colonies were selected and subjected to standard biochemical tests. Pink colonies with or without black centers on XLD agar indicate the presence of *Salmonella*. Many cultures of *Salmonella* may produce colonies with large, glossy black centers or may appear as almost completely black colonies.

6.2.5 Determination of *Staphylocoous aureus*

Determination of *Staphylocoous aureus* was carried out according to the method of Bennett *et al*, (1986). Whole samples were blended as previously described (section 5.4.1). A series of ten-fold dilutions was made using the saline solution for microbiological analyses. The 0.1 ml of samples was spreaded onto baird parker medium and incubated for 48 h at 35°C. Colonies of *Staphylocoous aureus* are circular, smooth, convex, moist, 2-3 mm in diameter on uncrowded plates, gray to jet-black, frequently with light-colored (off-white) margin, surrounded by opaque zone and frequently with an outer clear zone; colonies have buttery to gummy consistency when touched with inoculating needle.

6.2.6 Determination of *Vibrio parahaemolyticus*

Vibrio parahaemolyticus was determined by the method of Bradshaw (1974). Whole samples were blended as previously described (section 5.4.1). A series of ten-fold dilutions was made using the saline solution for microbiological analyses. One ml of samples were transferred into the glucose salt teepol broth (GSTB). Then, the inoculated tubes were incubated at 37°C for 24 h and used as inoculum for the three-tube MPN procedure. Every tube showing turbidity was checked for the presence of *Vibrio parahaemolyticus* by streaking on thiosulphate citrate bile salts sucrose agar (TCBS) and incubated 24 h at 35°C. *Vibrio parahaemolyticus* appear as round, opaque, green or bluish colonies, 2 to 3 mm in diameter on TCBS agar.

7. Statistical analyses

All experiments were run with 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).