

Chapter 2

Materials and Methods

1. Materials

The cephalothorax of black tiger prawn (*Penaeus monodon*) was obtained from Piti Seafood Co.Ltd, Songkhla, Thailand. The cephalothorax was kept in ice with a sample/ice ratio of 1: 2 (w/w) and transported to the Department of Food Technology, Prince of Songkhla University, Thailand within 1 h.

Black tiger prawns with the size of 40-50 shrimp/kg were purchased from the dock in Pattani province. The prawns off-loaded approximately 6-12 h after capture, were transported to Department of Food Technology, Prince of Songkhla University in ice with a prawn/ice ratio of 1:2 (w/w) within 2 h.

Chemicals

2,4,6-trinitrobenzenesulfonic acid (TNBS), L-leucine, L- β -(3,4 dihydroxylphenyl) alanine (L-DOPA), Brij-35 and potassium ferricyanide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trichloroacetic acid was obtained from Riedel-deHaen (Seelze, Germany). Ferric chloride, ammonium sulfate and sodium sulfite were purchased from Merck (Damstadt, Germany). Fructose and glycine were obtained from Fluka (Messerchmittstr, Switzerland).

2. Instruments

Instruments	Model	Company/Country
pH meter	CG 842	Schott, Germany
Magnetic stirrer	BIG SQUID	IKA labortechnik, Stanfen, Germany
Oil bath	B-490	Buchi, Flawil, Switzerland
Water bath	W350	Memmert, Schwabach, Germany
Refrigerated centrifuge	RC-5B plus	Sorvall, USA
D o u b l e - b e a m Spectrophotometer	UV-16001	Shimadzu, Japan
Spectrofluorophotometer	RF-1501	Shimadzu, Japan
Homogenizer	T25 basic	IKA labortechnik, Selangor, Malaysia
Colorimeter	ColorFlex	HunterLab Reston, VA, USA

3. Methods

3.1. Extraction of black tiger prawn PO

The cephalothorax were powdered by grinding in the presence of liquid nitrogen with a blender. The powder was placed in the polyethylene bag and kept at -20°C until used. To extract the PO, cephalothorax powder was homogenized with 0.05 M phosphate buffer containing 1 M NaCl, pH 7.2 at a ratio of 1:3 (w/v) using 11,000 rpm an IKA Labortechnik (Selangor, Malaysia) for 2 min. The

homogenate was then stirred for 30 min at 4°C. After centrifugation at 8,000×g for 30 min at 4°C, the supernatant was fractionated with solid ammonium sulfate (0-40% saturation). The precipitate formed was collected by centrifugation at 12,000×g for 30 min at 4°C. The pellet was dissolved in a minimum volume of 0.05 M phosphate buffer, pH 6.5. The crude extract was dialyzed against 15 volumes of cold 0.05 M phosphate buffer, pH 6.5 at 4°C with three changes of dialysis buffer. The dialysate was used as 'PO solution'.

3.2. Characterization of black tiger prawn PO

3.2.1. PO activity assay

PO activity was assayed using *L*-DOPA as a substrate as described by Simpson *et al.* (1987) with a slight modification. PO solution (150 µl) was mixed with 150 µl of distilled water and 150 µl of 0.1 M phosphate buffer, pH 6.0. The assay mixture was added with 750 µl of *L*-DOPA preincubated at 45 °C to initiate the reaction. The reaction was run for 3 min and A_{475} was measured. One unit was defined as the change in 0.001 unit of A_{475}/min .

3.2.2. pH profiles

The pH profiles of PO were studied over the pH range of 4.5 to 8.5 (4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8 and 8.5) at 25°C. Sodium-citrate buffer (0.1 M) was used for pH ranges of 4.5-5.5 and phosphate buffer (0.1 M) was used for pH ranges of 6-8.5. The activity was measured at 475 nm after 3 min.

3.2.3. Temperature profiles

For the temperature profile study, the activity of PO was assayed at different temperatures (30, 35, 40, 45, 50, 55, 60, 65°C) at pH 6.0. The activity assay was carried out using *L*-DOPA as substrate as described in section 3.2.1.

3.2.4. pH stability

To determine the influence of pH on enzyme stability, PO solution was incubated in various buffers with different pHs (pH 5.0-8.5) for 10 and 30 min. The residual activity was determined under optimum condition

3.2.5. Thermal stability

PO solution was preincubated at different temperatures (30-55°C) for 10 and 30 min. After cooling in iced water, the residual activity was determined under optimum condition.

3.2.6. Inhibitor study

PO solution (150 µl) was mixed with various inhibitors (150 µl) to obtain the final concentration designated (1 and 2 mM L-cysteine, thiourea or glutathione; 0.005 and 0.01% SDS; 15, 25 and 45% methanol). The mixture was allowed to stand at room temperature (25-27°C) for 10 min. The remaining PO activity was determined under optimum pH and temperature.

3.3. Effect of cysteine concentration on the PO activity

PO solution (150 µl) was mixed with cysteine (150 µl) at various concentrations (0.25, 0.5, 0.75, 1 and 1.5 mM). The mixture was allowed to stand at room temperature for 10 min prior to assay as described in section 3.2.1. The cysteine concentration rendering the inhibitory activity of 20% and 40% were chosen for further study.

3.4. Effect of types of amino acid and sugar in model system on inhibition of PO

3.4.1. Preparation of MRPs

Amino acids (cysteine, glycine) and different reducing sugars (glucose, fructose and galactose) were mixed in equimolar of 0.5 and 0.75 mM in 0.1 M borate buffer, pH 8. The mixture was then transferred to screw-sealed tubes, tightly capped and heated in an oil bath (Buchi labortechnik AG, Switzerland) at 100°C. The samples were taken after heating for 8 h. The heated samples were cooled immediately in iced water. MRPs samples obtained were kept at 4°C until analyzed.

3.4.2. Analysis

3.4.2.1. Measurement of pH

The pH of MRPs samples was measured using a pH meter (CG 842, Schott, Germany).

3.4.2.2. Measurement of absorbance at 294 nm (A_{294})

A_{294} of MRPs samples was measured according to the method of Ajandouz *et al.* (2001). Appropriate dilution was made using distilled water and the absorbance was measured at 294 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan).

3.4.2.3. Measurement of fluorescence intensity

Fluorescence of MRPs samples was determined as described by Morales and Jimenez-Perez (2001) with a slight modification. The fluorescence intensity was measured at an excitation wavelength of 347 nm and emission wavelength of 415 nm using a RF-1501 Fluorescence Spectrophotometer (Shimadzu, Kyoto, Japan).

3.4.2.4. Measurement of browning intensity

The browning intensity of MRPs samples was measured according to the method of Ajandouz *et al.* (2001). Appropriate dilution was made using distilled water and the absorbance was measured at 294 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan).

3.4.2.5. Determination of free amino group content

Free amino group content was determined according to the method of Benjakul and Morrissey (1997). MRPs samples (500 μ l) were mixed with 2.0 ml of 0.2125 M phosphate buffer, pH 8.2 and 1.0 ml of 0.01% TNBS solution was added. The solution was mixed thoroughly and placed in a temperature-controlled water bath (Memmert, Bavaria, Germany) at 50 °C for 30 min in the dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulfite. The mixture was cooled at room temperature for 15 min. The absorbance was measured at 420 nm. Free amino group content was expressed in term of L-leucine.

3.4.2.6. Determination of reducing sugar content

The reducing sugar content was determined according to the method of Simogyi (1951). One ml of MRPs samples was mixed with 1 ml of reagent C. The reaction mixture was heated at 100 °C for 15 min and cooled rapidly to room temperature. The reaction mixture was added with 1 ml of reagent D, followed by addition of 3.0 ml of distilled water. The absorbance at 520 nm was measured. Reducing sugar content was read from the standard curve. The loss of reducing sugar was calculated based on the differences between initial reducing sugar content and that obtained after reaction.

3.4.2.7. Determination of reducing power

The reducing power of MRPs samples was determined according to the method of Oyaizu (1986) with a slight modification. One ml of MRPs samples (5-fold dilution) was mixed with 1 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 1 ml of 1% potassium ferricyanide ($K_3Fe(CN)_6$). The reaction mixture was incubated in a temperature-controlled water bath at 50 °C for 20 min, followed by addition of 1 ml of 10 % trichloroacetic acid. The mixtures were added with 1 ml of distilled water and 200 μ l of 0.1% $FeCl_3$. The absorbance at 700 nm was determined and used as the measure of reducing power.

3.4.2.8 Determination of copper chelating

The copper binding capacity of MRPs samples was determined according to the method of Wijewickreme *et al.* (1997) with a slight modification. The MRPs samples (20-fold dilution) (500 μ l) were mixed with 10 mM hexamine buffer (pH 5) containing 10 mM KCl (1.5 ml). The mixture was then added with 500 μ l of 0.1 mM $CuSO_4$ and incubated for 10 min at room temperature. The reaction mixture was mixed with 100 μ l of 1 mM TMM in hexamine buffer. The amount of free copper in the solutions was obtained from a standard curve, where the absorbance ratio A_{460}/A_{530} , in a solution of 500 μ l of $CuSO_4$ (0.02-0.1 mM), 2.0 ml of hexamine buffer and 100 μ l of TMM was plotted against the amount of $CuSO_4$ (0.02-0.1 mM). The amount of copper bound to MRPs mixtures was calculated as the difference between the amount of copper added and free copper present in the solution.

3.4.2.9. Determination of PO inhibition

To study the effect of MRPs on PO activity, 150 μ l of MRPs was added instead of distilled water. The mixture was allowed to stand at room temperature for 10 min prior to the assay as described in section 3.2.1. The residual activity was measured and % inhibition was calculated.

The amino acid/sugar MRPs, derived from the system rendering the highest PO inhibition, was selected for further study.

3.5. Effect of heating time of fructose/glycine model system on inhibition of PO

3.5.1. Preparation of MRPs

Fructose and glycine were used to prepared equimolar fructose/glycine (0.75 mM) model system in 0.1 M borate buffer, pH 8. Aliquots of mixture were placed in screw-sealed tubes. The samples were then heated in an oil bath at 100°C. The samples were taken after heating for 0, 2, 4, 6, 8, 10, 12 h. The heated samples were cooled immediately in iced water. MRPs samples obtained were kept at 4°C until analyzed.

3.5.2. Analysis

All analyses were performed as mentioned in section 3.4.2.

The fructose/glycine system with heating time exhibiting the highest inhibitory activity towards PO was chosen for further study.

3.6. Effect of temperature on the PO inhibitory activity of MRPs from fructose/glycine model system

3.6.1. Preparation of MRPs

An equimolar fructose/glycine (0.75 mM) model system in 0.1 M borate buffer, pH 8.0, was prepared. The mixture was transferred to screw-sealed tubes and then heated in an oil bath at different temperatures (80, 90, 100 and 110°C) for 12 h. The samples were taken after heating for 12 h and cooled immediately in iced water. MRPs samples obtained were kept at 4°C until analyzed.

3.6.2. Analysis

All analyses were performed as mentioned in section 3.4.2.

The fructose/glycine system heated at the temperature showing the highest inhibitory activity against was chosen for further study.

3.7. Effect of reactant concentration of fructose/glycine model system on inhibition of PO

3.7.1. Preparation of MRPs

MRPs were prepared by heating the equimolar of fructose and glycine in 0.1 M borate buffer, pH 8 at 100 °C. Different reactant concentrations (0.75, 1.5, 4.5, 7.5, 15 and 30 mM) were used. To produce MRPs, the mixtures were transferred to screw-sealed tubes, tightly capped and heated in an oil bath at 100 °C for 12 h. The heated samples were cooled immediately in iced water. MRPs obtained were kept at 4 °C until analysed.

3.7.2. Analysis

MRPs sample were subjected to analysis as described in section 3.4.2.

The model system derived from fructose/glycine with the concentration exhibiting the highest inhibitory activity towards PO was chosen for further study.

3.8. Effect of pHs of fructose/glycine model system on inhibition of PO

3.8.1. Preparation of MRPs

MRPs were prepared by heating the equimolar of fructose and glycine in different buffer with various pHs (0.1 M tris-buffer for pH 7; 0.1 M borate buffer for pH 8, 9 and 10; 0.1 M sodium hydrogen carbonate buffer for pHs 11 and 12). The mixtures were then transferred to screw-sealed tubes, tightly capped and heated in an oil bath at 100 °C for 12 h. The heated samples were cooled immediately in iced water. MRPs obtained were kept at 4 °C until analysed.

3.8.2. Analysis

All analyses were carried out as described in section 3.4.2.

The fructose/glycine model system with initial pHs rendering the highest inhibitory activity towards PO was chosen for further study.

3.9. Decolorization of fructose/glycine MRPs

3.9.1. Decolorization by activated carbon

The MRPs with highest PO inhibitory activity were prepared as mentioned in section 3.4.-3.8. Decolorization of MRPs by activated carbon was performed according to the method of Synowiecki *et al.* (2000) with a slight modification. Activated carbon at different levels (1, 2 and 5%) was added in MRPs and shaken at 80 rpm for 30 min at room temperature. Then, the mixtures were

filtered using Whatman filter paper No. 54. The decolorized MRPs were then analyzed.

3.9.2. Decolorization by Sep-Pak Cartridge C18

The MRPs with highest PO inhibitory activity were prepared as mentioned in section 3.4.-3.8. Decolorization of MRPs using Sep-Pak Cartridge C18 (Vac 300, 500 mg) was performed according to the method of Lee (1992). A 5 ml of MRPs was transferred into a syringe and passed through a Sep-Pak Cartridge C18 which had been prewetted with 3 ml methanol, followed by 5 ml of water. MRPs decolorized by Sep-Pak Cartridge C18 were used for analysis.

3.10.3. Analysis

All analyses were performed as mentioned in section 3.4.2.

Additionally, the color of MRPs and decolorized MRPs sample were measured by Hunter lab Colorimeter and reported in CIE system. L*, a* and b* parameter indicate lightness, redness greenness and yellowness blueness, respectively.

3.10. Effect of MRPs on changes in chemical and sensory properties of black tiger prawn during iced storage

3.10.1. Preparation of prawn

Black tiger prawns were soaked in an equimolar fructose/glycine MRPs model system (30 mM) heated at 100°C, pH 11 (0.1 M Na-carbonate buffer) for 12 h with a with a soaking time of 10 min. Prawns soaked in 1.25% sodium metabisulphite for 1 min were also prepared. The treated prawns were drained on the screen for 3 min. Prawns without inhibitors were used as the control. All sample placed in polyethylene bags were stored in ice. Twelve prawns were taken for each

treatment and evaluated every 2 day up to 10 days for chemical analysis and sensory evaluation as follows:

3.10.2. Chemical measurement

3.10.2.1. Total volatile base (TVB) and trimethylamine contents (TMA) were determined using Conway's method according to the method of Conway and Byrne (1939).

3.10.2.2. pH measurement was performed by homogenizing the flesh with water at a ratio of 1:5 (w/w) for 1 min. The homogenate was subjected to pH measurement using a pH meter.

3.10.3. Sensory evaluation

Melanosis assessment was performed by 15 trained panelists. Melanosis as manifested by black spots on the prawns was determined by the visual scale using 10-point scoring test (Montero *et al.*, 2001; Marshall *et al.*, 2000) as follows: 0, absent; 2, slight (up to 20% of prawns' surface affected); 4, moderate (20 to 40% of prawns' surface affected); 6, notable (40 to 60% of prawns' surface affected); 8, severe (60 to 80% of prawns' surface affected); 10, extremely heavy (80 to 100% of prawns' surface affected) (Appendix 2). Sensory evaluation for texture, color, flavor and overall acceptability were evaluated by 9-point Hedonic scale as follows: 1, dislike extremely; 2, dislike very much; 3, dislike moderately; 4, dislike slightly; 5, neither like nor dislike; 6, like slightly; 7, like moderately; 8, like very much; 9, like extremely.

4. Statistical analysis

All analyses were run in triplicate. Analysis of variance (ANOVA) was performed and mean comparisons were done by Duncan's multiple range test (Steel and Torrie, 1980). Analysis was performed using a SPSS package (SPSS 11.0 for windows, SPSS Inc, Chicago, IK).