CHAPTER 2

MATERIALS AND METHODS

1. Materials/Chemicals/Enzyme

1.1 Samples

Three different lots of Mungoong were purchased from a local market in Songkhla, Thailand. The samples were stored at 4°C until use and the storage time was not greater than 1 month.

White shrimp (*Litopenaeus vannamei)* cephalothorax were obtained from Chotiwat manufacturing Co., Ltd., Hat Yai, Songkhla. Round scads (*Decapterus maruadsi*) were purchased from the dock in Songkhla, Thailand. The fish, off-loaded approximately 36-38 h after capture, were transported to the Department of Food Technology, Prince of Songkla University in ice with a fish/ice ratio of 1:2 (w/w) within 1 h.

1.2 Chemicals

Methanol was obtained from Merck (Darmstadt, Germany). 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), linoleic acid and L-α-phosphatidylcholine (L-α-lecithin) were purchased from Sigma Chemical (St. Louis, MO, USA). 2,4,6-tripyridyl-S-triazine (TPTZ), ferric chloride hexahydrate, 2-thiobarbituric acid (TBA), β-carotene, butylated hydroxyl toluene (BHT) and potassium persulfate were procured from Fluka Chemical (Buchs, Swizerland).

1.3 Enzyme

Flavourzyme 500MG, declared activity: 500 LAPU/g was provided by Novozymes (Bagsvaerd, Denmark).

2. Instruments

Instruments	Model	Company/Country
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
Fluorescence Spectrophotometer	RF-1501	Shimadzu, Japan
Colorimeter	ColorFlex	HunterLab Reston, VA, USA
Rotary evaporator	Rotavapor-R	Binkmann, Switzerland
Gas Chromatography	GC-14A	Shimadzu, Japan
ICP-OES	4300 DV	Perkin-Elmer, USA
Amino acid analyzer	MLC-703	Atto, Japan

3. Methods

3.1 Proximate analysis and determination of physical properties of Mungoong

3.1.1 Proximate analysis

Moisture, protein, fat and ash were determined according to the method of AOAC (2000) (Appendix) and expressed on wet weight basis.

3.1.2 Salt content

Salt content was determined according to the method of AOAC (2000) (Appendix).

3.1.3 Color measurement

Color of Mungoong was measured by Hunter lab and reported in CIE system. L* a* and b* parameters indicating lightness, redness/greenness and yellowness/blueness, respectively.

3.1.4 Water activity determination

Water activity of Mungoong was measured using water activity analyzer (Thermoconstanter, Novasina, Swizerland).

3.1.5 pH measurement

pH was determined by pH meter CG 842 (Schott, Germany) as described by Benjakul *et al*. (1997) (Appendix).

3.1.6 Fatty acid composition

Lipid in Mungoong was extracted by the Bligh and Dyer method (1959). 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 column was set at 170 $^{\circ}$C and was increased to 225°C with a rate of 1°C /min and then held at 220 $^{\circ}$C for an additional 20 min. The detector temperature was set at 270 $^{\circ}$C, while the temperature at the injection port was maintained at 250°C. Retention time of FAME standards was used to identify chromatographic peaks. Peak area was quantitated and fatty acid content was expressed as g /100 g lipid (AOAC, 1999).

3.1.7 Mineral content

Iron (Fe), copper (Cu), manganese (Mn), cadmium (Cd), nickel (Ni), zinc (Zn), calcium (Ca), magnesium (Mg), phosphorus (P), potassium (K), sodium (Na) and lead (Pb) contents were determined by the inductively coupled plasma optical emission spectrophotometer (ICP-OES) (Perkin-Elmer, Model 4300 DV, Norwalk, CT, USA) according to the AOAC (1999) method at PSU Central Equipment Center, Hat Yai. Mungoong (4 g) was mixed well with 4 ml of nitric acid. The mixture was heated on a hot plate until digestion was complete. The digested sample was transferred to a volumetric flask and the volume was made up to 10 ml with 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, Ni, Zn, Ca, Mg, Na, P, Cd, Pb and K were 238.204, 327.393, 257.610, 231.604, 206.200, 317.933, 285.213, 588.995, 213.617, 228.802, 217.000 and 766.490 nm, respectively.

3.1.8 Amino acid analysis

Mungoong samples 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 the reduced pressure in 4.0 M methanesulfonic acid containing 0.2% (v/v) 3-2 (2-aminoethyl) indole at 115°C 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).

3.2 Study on the extraction of soluble fraction with antioxidative activity from Mungoong

3.2.1 Preparation of soluble fractions from Mungoong

Different extracting media including distilled water, distilled water/ethanol mixture (1:1, 1:2 and 2:1) and ethanol were used to extract the soluble substances from Mungoong. Mungoong (1g) was mixed with extracting medium (100 ml) and the mixture was stirred at room temperature for 30 min. The mixture was then centrifuged at 3000xg for 10 min at room temperature using a Sovall Model RC-5B Plus refrigerated centrifuge (Newtown, CT, USA) to remove undissolved debris. The supernatant was used for further analyses.

3.2.2 Analyses

3.2.2.1 Measurement of absorbance at 280 and 295 nm (A_{280} and A_{295})

A_{280} and A_{295} of soluble fractions from Mungoong were determined according to the method of Ajandouz *et al*. (2001). The formation of Maillard reaction intermediate products was monitored by measuring the absorbance at 280 and 295 nm using a UV-160 spectrophotometer (Shimadzu, Kyoto, Japan).

3.2.2.2 Measurement of browning intensity

The browning intensity of soluble fraction from Mungoong was measured according to the method of Ajandouz *et al*. (2001). Appropriate dilution was made using distilled water and the absorbance was measured at 420 nm using UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan).

3.2.2.3 Measurement of fluorescence intensity

Fluorescent intermediate products from Maillard reaction of soluble fractions from Mungoong were determined as described by Morales and Jimenez-Perez (2001). The fluorescence intensity was measured at an exitation wavelength of 347 nm and emission wavelength of 415 nm using a RF-1501 Fluorescence spectrophotometer (Shimadzu, Kyoto, Japan).

3.2.2.4 Determination of antioxidative activity of soluble fraction from Mungoong

1) Determination of DPPH radical scavenging activity

DPPH radical scavenging activity was determined by DPPH assay as described by Wu *et al.* (2003) with a slight modification. Sample (1.5 ml) was added with 1.5 ml of 0.15 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) in 95% ethanol. The mixture was then mixed vigorously and allowed to stand at room temperature in dark for 30 min. The absorbance of resulting solution was measured at 517 nm using an UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The blank was prepared in the same manner except that the distilled water was used instead of sample. The standard curve was prepared using Trolox in the range of 10 - 60 μM. The activity was expressed as μmol Trolox equivalents (TE)/g Mungoong.

2) Determination of ABTS radical scavenging activity

ABTS radical scavenging activity was determined by ABTS assay as per the method of Arnao *et al.* (2001) with a slight modification. The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulfate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in dark. The solution was then diluted by mixing 1 ml of ABTS solution with 50 ml of methanol to obtain an absorbance of 1.1 $\pm$ 0.02 units at 734 nm using an UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). ABTS solution was prepared freshly for each assay. Sample (150 μl) was mixed with 2850 μl of ABTS solution and the mixture was left at room temperature for 2 h in dark. The absorbance was then measured at 734 nm using the spectrophotometer. The standard curve of Trolox ranging from 50 to 600 μM was prepared. The activity was expressed as μmol Trolox equivalents (TE)/g Mungoong.

3) Determination of ferric reducing antioxidant power

FRAP was assayed according to Benzie and strain (1996). The stock solutions included 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ solution. The working solution was prepared freshly by mixing 25 ml of acetate buffer, 2.5 ml of TPTZ solution and 2.5 ml of $FeCl_3.6H_2O$ solution. The mixed solution was incubated at 37°C for 30 min and was referred to as FRAP solution. Sample (150 μl) was mixed with 2850 μl of FRAP solution and kept for 30 min in dark. Ferrous tripyridyltriazine complex, colored product, was measured by reading the absorbance at 593 nm. The standard curve was prepared using Trolox ranging from 50 to 600 μM. The activity was expressed as μmol Trolox equivalents (TE)/g Mungoong.

The soluble fraction extracted from the extracting media, rendering the hightest antioxidant activity, was selected for further study.

3.3 Effect of the concentration of Mungoong soluble fraction on antioxidative activities and the correlation between antioxidative activities tested by different assays

Selected soluble fraction from Mungoong rendering the highest antioxidative activity (section 3.2) at various concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 mg/ml) was determined for antioxidative activity as mentioned in section 3.2.2.4. The correlations between ABTS and DPPH; DPPH and FRAP; ABTS and FRAP were examined.

3.4 Study on stability of soluble fraction from Mungoong

3.4.1 pH stability

For the pH stability study, selected soluble fraction (3 mg/ml) was adjusted to different pHs (2, 3, 4, 5, 6, 7, 8, 9, 10 and 11) using HCl and NaOH and incubated at room temperature for 30 min. Thereafter, the pHs of sample were then adjusted to pH 7.0. The residual antioxidative activities were determined by DPPH, ABTS and FRAP assays as mentioned in section 3.2.2.4.

3.4.2 Thermal stability

For thermal stability study, selected soluble fraction (pH 7.0) was incubated at 30, 40, 50, 60, 70, 80, 90 and 100°C in the temperature controlled-water bath (Memmert,

Schwabach, Germany) for 30 min. The treated samples were suddenly cooled in iced water. The sample without incubation (25°C) was used as the control. The residual antioxidative activities were determined by DPPH, ABTS and FRAP assays as mentioned in section 3.2.2.4.

The effect of heating times at 100°C on antioxidative activity of selected soluble fraction from Mungoong was also investigated. Soluble fraction (3 mg/ml) was heated at 100 °C for 30, 60, 90, 120 and 150 min. The treated samples were immediately cooled in iced water after time designated. The sample without incubation (25°C) was used as the control. The residual antioxidative activities were measured by DPPH, ABTS and FRAP assays as mentioned in section 3.2.2.4.

3.5 Antioxidative activity and oxidation stability of Mungoong during storage

Mungoong was placed in a polypropylene container and closed tightly. The samples were stored at room temperature (28-30°C) and 4°C for 8 weeks. During storage, the samples were subjected to the following analyses:

3.5.1 Determination of antioxidative activity

Ferric reducing antioxidant power (FRAP), DPPH and ABTS radical scavenging activity of all samples were determined as mentioned in section 3.2.2.4. Water soluble fraction at a concentration of 6 mg/ml was used for all assays.

3.5.2 TBA value

Changes in TBA value of Mungoong stored for various times was determined according to the method of Egan *et al*. (1981). Sample (10 g) was added with 50 ml of distilled water and the mixture was transferred into a distillation flask containing 47.5 ml of distilled water. Thereafter, 2.5 ml of 4 M HCl were added to bring the pH to 1.5. A few glass beads were added into the mixture before heating using an electric mantle (Kando electric, Samutprakarn, Thailand). Distillation was performed until the distillate of 50 ml was obtained. An aliquot (5 ml) of distillate was placed in a glass-stoppered tube, and 5 ml of TBA reagent (0.2883 g / 100 ml of 90 % glacial acetic acid) were added. The mixture was heated in boiling water for 35 min. Distilled water (5 ml) was used as a blank. After heating, the reaction mixture was cooled in

water for 10 min and the absorbance at 532 nm was measured using an UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan).

3.6 Antioxidative activity of soluble fraction from Mungoong in different systems

Soluble fraction from Mungoong at different levels (200, 500 and 1000 ppm) were used in different systems as follows:

3.6.1 Lecithin liposome system

The antioxidative activity of soluble fraction from Mungoong in lecithin liposome system was determined according to the method of Frankel *et al*. (1997) (Appendix).

3.6.2 β-carotene-linoleic system

The antioxidative activity of soluble fraction from Mungoong in β-carotene-linoleic system was tested as described by Taga *et al*. (1984) (Appendix).

3.6.3 Comminuted fish model system

The antioxidative activity of soluble fraction from Mungoong in comminuted fish model system was tested as described by Benjakul *et al*. (2005) (Appendix).

3.7 Study on the use of Flavourzyme on the yield and antioxidative activity of Mungoong

3.7.1 Preparation of Mungoong

Both raw and cooked cephalothorax were used for Mungoong preparation (Figure 4). For typical process, cephalothorax was cooked in boiling water (100°C) with sample/water ratio of 1:2 (w/v) for 15 min. The mixture was then filtered using a screen (1 mm diameter). The cooked cephalothorax was crushed using a blender (National MX-T2GN, Taipei, Taiwan) until the homogeneity was obtained. The crushed sample was mixed with water at ratio of 1:2 and the mixture was filtered using two layers of cheesecloth. Both filtrates were combined and total solid content was measured. The mixture was evaporated using a fire oven with a continuous stirring until the volume was reduced to approximately 30-40%. Salt and sugar at levels of 15 and 30%, respectively, based on total solid content, were then added into the concentrated mixture. The mixture was thereafter concentrated until the heavy paste referred to as ‘Mungoong T’ was obtained.

To prepare Mungoong with the aid of Flavourzyme, the enzyme at two different levels (0.15 and 0.30% (w/w) based on raw materials) was added into a mixture of whole cephalothorax and water (1:2 w/v) preincubated at 50°C for 10 min. The hydrolysis was conducted at 50°C for 90 min and the reaction was terminated by heating the mixture at 100°C for 15 min. The mixture was filtered using a screen as previously described. The remaining cephalothorax was crushed, mixed with water and filtered as mentioned above. The filtrates obtained were combined, concentrated, followed by the addition of salt and sugar. The heavy paste obtained with the aid of Flavourzyme at levels of 0.15 and 0.30% were referred to as 'Mungoong RF15' and 'Mungoong RF30', respectively.

Mungoong was also prepared from cooked cephalothorax as described in typical process. After the extract was filtered, the cooked cephalothorax was mixed with water and the mixture was preincubated at 50°C for 10 min. Flavourzyme was then added into the mixture at two different levels (0.15 and 0.30% (w/w)). The hydrolysis was carried out for 90 min and terminated by boiling the mixture for 15 min. The mixture was then filtered and the remaining cephalothorax was crushed and mixed with water. The mixture was then filtered again. All the filtrates obtained were then combined, concentrated and added with salt and sugar as described above. The heavy paste obtained from the process using 0.15% and 0.30% were referred to as 'Mungoong CF15' and 'Mungoong CF30', respectively.

All Mungoong prepared by different processes were subjected to the analyses.

Figure 4 Scheme for Mungoong production

3.7.2 Analyses

3.7.2.1 Yield

Yield of Mungoong was calculated based on the weight of resulting Mungoong in comparison with the initial weight of cephalothorax and was expressed as the percentage.

3.7.2.2 Proximate analysis and determination of physical properties

1) Proximate analysis

Moisture, protein, fat and ash were determined according to the method of AOAC (2000) (Appendix) and expressed on dry weight basis.

2) pH measurement

pH was determined by pH meter CG 842 (Schott, Germany) as described by Benjakul *et al*. (1997) (Appendix).

3) Salt content

Salt content was determined according to the method of AOAC (2000) (Appendix).

4) Water activity determination

Water activity of Mungoong was measured as mentioned in section 3.1.5.

5) Color measurement

Color of Mungoong was determined as mentioned in section 3.1.3.

6) Solubility

Nitrogen solubility index (NSI) was used to determine the solubility of Mungoong following the procedure of Morr (1985) with a slight modification (Appendix).

3.7.3 Determination of formal nitrogen content

Formal nitrogen content, a convenient index of the degree of protein hydrolysis, was determined by the titration method as described by Thai Industrial Standard (1983) with a slight modification. Mungoong (1 g) was added with 10 ml of distilled water. Then 10 ml of formalin solution (38%, v/v; pH 9) were added and mixed well. The mixture was titrated with 0.1 N NaOH to obtain pH of 9.0. Formal nitrogen content was calculated and expressed as mg formal nitrogen/g sample using the following equation:

$$\text{Formal nitrogen content (mg N/g)} = \frac{\text{ml of NaOH (pH7-pH9)} \times 0.1 \times 14}{\text{Weight of sample (g)}}$$

3.7.4 Determination of ammonia nitrogen content

Ammonia nitrogen content was determined by the titration method as described by Thai Industrial Standard (1983) with a slight modification. Mungoong (1 g) was placed in 400 ml Kjeldahl flask containing 100 ml of distilled water and 3 g of MgO. The mixture was distilled and the distillate was collected in 50 ml of 4% (w/v) boric acid containing the mixed indicator (0.125 g methyl red and 0.082 g bromocresol green in 95% alcohol (100 ml) : 0.1 % methylene blue in distilled water with a ratio of 5:1). The solution was then titrated with 0.05 N H_2SO_4 to reach the end-point. Ammonia nitrogen content was expressed as mg nitrogen/g sample using the following equation:

$$\text{Ammonia nitrogen content (mg N/g)} = \frac{5.6 \times 0.05 \times \text{ml of } H_2SO_4}{\text{Weight of sample (g)}}$$

3.7.5 Determination of amino nitrogen content

Amino nitrogen was calculated based on the difference between formal and ammonia nitrogen contents (TISI, 1983) as follows:

Amino nitrogen content (mg N/g) = Formal nitrogen content - Ammonia nitrogen content

3.7.6 Determination of antioxidative activity of water extract from Mungoong

Mungoong (1 g) was added with 100 ml of distilled water. The mixture was stirred at room temperature for 30 min, followed by centrifugation at 3000×g for 10 min using a Sorvall Model RC-5B Plus refrigerated centrifuge (Newtown, CT, USA) at room temperature to remove undissolved debris. The supernatant was used for determination of antioxidative activity using ferric reducing antioxidant power (FRAP), DPPH and ABTS radical scavenging activity assays as mentioned in section 3.2.2.4.

Mungoong prepared by typical method and that prepared with the aid of Flavourzyme rendering the highest yield and antioxidative activities were subjected to the compositional analysis.

3.7.7 Fatty acid and mineral compositions of Mungoong prepared without and with the aid of Flavourzyme

Lipid in Mungoong was extracted by the Bligh and Dyer method (1959) (Appendix). The fatty acid compositions were determined as mentioned in section 3.1.6.

Iron (Fe), copper (Cu), manganese (Mn), cadmium (Cd), nickel (Ni), zinc (Zn), calcium (Ca), magnesium (Mg), phosphorus (P), potassium (K), sodium (Na) and lead (Pb) contents were determined by the inductively coupled plasma optical emission spectrophotometer (ICP-OES) (Perkin-Elmer, Model 4300 DV, Norwalk, CT, USA) as mentioned in section 3.1.7.

4. Statistical analysis

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