CHAPTER 2

MATERIALS AND METHODS

1. Materials/Chemicals

Round scad (*Decapterus maruadsi*) were purchased from the dock in Songkhla, Thailand. The fish, off-loaded approximately 18-24 h after capture, were placed in ice with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 2 h. Upon the arrival, fish were filleted and the ordinary muscle was collected and ground. The mince (100 g) was packaged in polyethylene bag and stored under vacuum at -20°C until used.

All chemicals for round scad protein hydrolysate preparation and analyses were of analytical grade. Silica gel plates were procured from Merck (Germany). Alcalase 2.4 L and Flavourzyme 500 L were 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
Purifier	Biologic LP	Biorad, USA
Freeze dryer	Dura-Top^{TM} µp	FTS system, USA
Colorimeter	ColorFlex	HunterLab Reston, VA, USA
Rotary evaporator	Rotavapor-R	Binkmann, Switzerland

3. Methods

3.1 Use of Alcalase and Flavourzyme for the hydrolysis of round scad mince

3.1.1 Effect of hydrolysis time

To prepare the hydrolysates, round scad mince was mixed with water at a ratio of 1:2 (w/v) and homogenized at a speed of 13,000 rpm for 1 min using an IKA labortechnik homogenizer (Selangor, Malaysia). The pH of homogenate was adjusted to 8 for Alcalase and 7 for Flavourzyme reaction (Sathivel *et al*., 2003; Šližyte *et al.,* 2005b). The homogenates were incubated at 50°C for 10 min. The enzymatic hydrolysis was started by adding 0.1 and 0.5% (w/w) of enzymes (Alcalase and Flavourzyme). After hydrolysis time designed (0, 5, 10, 20, 30, 40, 50 and 60 min), 1 ml of sample was taken, mixed with 1 ml of 1% SDS solution (85°C) and placed in a water bath (Model W350, Memmert, Schwabach, Germany) at 85°C for 15 min.

The DH of round scad protein hydrolysate was analyzed at different hydrolysis times according to the method of Benjakul and Morrissey (1997). Diluted protein hydrolysate samples (125 μl) were added with 2.0 ml of 0.2125 M phosphate buffer, pH 8.2 and 1.0 ml of 0.01% TNBS solution. The solution was mixed thoroughly and placed in a temperature-controlled water bath at 50°C for 30 min in dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulfite. The mixtures were cooled at room temperature for 15 min. The absorbance was measured at 420 nm using UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan) and α-amino acid was expressed in terms of L-leucine. The DH was calculated as follows:

$$\text{DH} = [(L_t - L_0)/(L_{max} - L_0)] \times 100$$

where L_t is the amount of α-amino acid released at time *t*. L_0 is the amount of α-amino acid in original scad muscle homogenate. L_{max} is the total α-amino acid in original scad muscle homogenate obtained after acid hydrolysis (6 N HCl at 100°C for 24 h).

3.1.2 Effect of enzyme concentration

Round scad homogenate was prepared as previously described. After pH adjustment and incubating at 50°C for 10 min, different amounts of enzymes (0.05, 0.1, 0.5, 1, 3 and 5% (w/w)) were added and mixed thoroughly. After 1 h of hydrolysis, 1 ml of sample was mixed with 1 ml of 1% SDS solution (85°C) and placed in a water bath at 85°C for 15 min. DH of round scad protein hydrolysate was determined as described by Benjakul and Morrissey (1997).

Log_{10} (enzyme concentration) vs. DH was plotted. From the regression equation, the enzyme concentrations required to hydrolyze round scad mince to obtain the desired DHs (20, 40, 60 %) were calculated.

3.2 Antioxidative activities of round scad protein hydrolysates as affected by proteinase types

3.2.1 Preparation of round scad protein hydrolysates with different DHs

Round scad protein hydrolysates with different DHs (20, 40 and 60%) were prepared as described in section 3.1.2. After 1 h of hydrolysis, the enzymes were inactivated by placing the reaction mixture at 90°C for 15 min in a water bath (Hoyle and Merritt, 1994). The mixture was then centrifuged at 2,000 × g at 4°C for 10 min using a Sorvall Model RC-5B Plus centrifuge (Newtown, CT, USA) and the supernatant was collected and used as protein hydrolysate. Protein hydrolysates obtained were determined for antioxidative activities.

3.2.2 Determination of antioxidative activities of round scad protein hydrolysates

1) DPPH radical scavenging activity

DPPH radical scavenging activity was determined as described by Wu *et al*. (2003) with a slight modification. Diluted sample (1.5 ml) was added with 1.5 ml of 0.15 mM 1,1-diphenyl-2-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. The blank was prepared in the same manner except that the distilled water was used instead of sample. The scavenging effect was calculated as follows:

$$\text{Radical scavenging activity} = [(B-A) / B] \times 100$$

where A is A_{517} of sample and B is A_{517} of the blank.

2) Reducing power

The reducing power was determined according to the method of Wu *et al*. (2003) with a slight modification. Diluted sample (1 ml) was mixed with 1 ml of 0.2 M phosphate buffer (pH 6.6) and 1 ml of 1% potassium ferricyanide. The mixtures were incubated at 50ºC for 20 min, followed by addition of 1 ml of 10% trichloroacetic acid. An aliquot (1 ml) of reaction mixture was added with 1 ml of distilled water and 200 μl of 0.1% $FeCl_3$. The absorbance of resulting solution was measured at 700 nm. Increased absorbance of the reaction mixture indicates the increased reducing power.

3) Chelating activity

The chelating activity on Fe^{2+} was measured using the method of Boyer and McCleary (1987) with a slight modification. Diluted sample (4.7 ml) was mixed with 0.1 ml of 2 mM $FeCl_2$ and 0.2 ml of 5 mM ferrozine. The reaction mixture was allowed to stand for 20 min at room temperature. The absorbance was then measured at 562 nm. The blank was conducted in the same manner but distilled water was used instead of sample. The chelating activity was calculated as follows:

$$\text{Chelating activity} = [(B-A) / B] \times 100$$

where A is A_{562} of sample and B is A_{562} of the blank.

Enzyme rendering the hydrolysate with the higher antioxidative activity was selected for further study.

3.3 Effect of defatting on antioxidative activities of round scad protein hydrolysates

Round scad mince was mixed with ethanol or isopropanol at a ratio of 1:2 (w/v) and heated at 70°C for 30 min. The solvent was removed and the defatted mince was rinsed twice with 5 volumes of water and then centrifuged at 3,000 × g at 4°C for 15 min. The resulting ethanol- and isopropanol-defatted mince was analyzed for moisture (AOAC, 2000) and fat content (Bligh and Dyer, 1959) (Appendix) in comparison with the original mince. The hydrolysis procedure was performed as mentioned in section 3.2.1. The enzyme giving the higher antioxidative activity was used to hydrolyze the round scad mince and defatted mince, in which the moisture contents were adjusted to 75%, to obtain DHs of 20, 40 and 60%. All protein hydrolysates were subjected to the determination of yield and antioxidative activities as mentioned in section 3.2.2. Protein hydrolysate derived from original mince or defatted mince with DH rendering the hightest antioxidant activity was chosen for further study.

3.4 Fractionation of antioxidative peptides from round scad protein hydrolysate

3.4.1 Protein hydrolysate fractionation

3.4.1.1 Gel filtration chromatography

Protein hydrolysate with DH giving the highest antioxidative activity was subjected to fractionation using gel filtration. The hydrolysate was loaded onto a Sephadex G-75 column (2.6 x 70 cm; Amersham Bioscience, Uppsala, Sweden). The elution was carried out with water at a flow rate of 1 ml/min. The 3-ml fractions were collected and the absorbances at 220

and 280 nm were measured. Alcohol dehydrogenase (MW 150,000), bovine serum albumin (MW 66,000), carbonic anhydrase (MW 29,000) and aprotinin (MW 6,500) were used as the molecular weight standard. The fractions obtained were analyzed for yield and antioxidative activities (section 3.2.2).

3.4.1.2 Solvent extraction

Stepwise fractionation was performed using different organic solvents with descending non-polarity in the order of hexane, dichloromethane and ethylacetate, respectively. Protein hydrolysate was mixed with hexane at a ratio of 1:2 (v/v) in separatory funnel and vigorously shaken for 10 min at room temperature. Thereafter, the hexane fraction was collected and hexane was evaporated at 70°C in a rotary evaporator (Model Rotavapor-R, Binkmann, Switzerland). The remaining fraction obtained was further fractionated with dichloromethane and ethylacetate, respectively. The fractions obtained were determined for yield and antioxidative activities (section 3.2.2).

3.4.2 Characterization of antioxidative peptide fraction

Selected peptide fraction with the highest antioxidative activity was separated by thin layer chromatography (TLC). TLC was conducted on analytical silica gel plates (TLC aluminum sheets 20 × 20 cm, Silica gel 60 F_{254}, Merck, Darmstadt, Germany) which had been activated for 30 min at 100°C. TLC plate was developed using chloroform-methanol-water (7:3:0.5, v/v/v) after the samples were spotted. When the solvents had reached the top of the plate, the plate was removed from the developing chamber and dried. Separated peptides were visualized on plates after spraying with a 0.2% ninhydrin solution (Amarowicz and Shahidi, 1997). A second set of plates was dipped in 0.04% DPPH solution (Gocan and Cimpan, 2004) in order to evaluate antioxidative property. The spot with yellow color indicated the peptide with antioxidative activity.

3.4.3 Antioxidative activity of crude protein hydrolysate and peptide fraction in different systems

Crude protein hydrolysate and peptide fraction with the highest antioxidant activity at different levels (100, 500 and 1,000 ppm) were used in different systems in comparison with 25 and 100 ppm BHT.

3.4.3.1 Linoleic oxidation system

The antioxidative activity of crude protein hydrolysate and peptide fraction in linoleic oxidation system was determined according to the method of Sakanaka *et al*. (2004) (Appendix).

3.4.3.2 Lecithin liposome system

The antioxidative activity of crude protein hydrolysate and peptide fraction in lecithin liposome system was tested as described by Frankel *et al*. (1997) and Yi *et al*. (1997) with a slight modification (Appendix).

3.5 Compositions and some properties of round scad protein hydrolysate

Protein hydrolysate which exhibited the hightest antioxidative activity was prepared as mentioned previously. The sample was freeze-dried by Dura-Top^{TM} μp freeze dryer (USA) and used for analyses.

3.5.1 Proximate analysis

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

3.5.2 Amino acid composition

Hydrolysate was analyzed for amino acid composition using reversed phase HPLC at Kasetsart University Research and Development Institute, Bangkok.

3.5.3 Mineral content

Iron (Fe), copper (Cu), manganese (Mn), cadmium (Cd), nickel (Ni), magnesium (Mg), sodium (Na), phosphorous (P), potassium (K), calcium (Ca), and sulfur (S) contents of freeze-dried hydrolysate were determined by the inductively coupled plasma optical emission spectrophotometer (ICP-OES) (Perkin Elmer, Model 4300 DV, USA) at PSU Central Equipment Center, Hat Yai.

3.5.4 Color measurement

The color of freeze-dried hydrolysate was measured by Hunter lab and reported in CIE system. L* a* and b* parameters indicate lightness, redness and yellowness, respectively.

3.5.5 Functional properties

3.5.5.1 Solubility

Nitrogen solubility index (NSI) was used to determine the solubility of protein hydrolysate following the procedure of Morr *et al*. (1985) with a slight modification (Appendix).

3.5.5.2 Emulsifying properties

The emulsifying activity index (EAI) and the emulsion stability index (ESI) were used to measure the emulsifying properties of protein hydrolysate. EAI and ESI were determined according to the method of Pearce and Kinsella (1978) with a slight modification (Appendix).

3.5.5.3 Foaming properties

Foam ability and foam stability of protein hydrolysate was tested according to the method of Shahidi (1995) (Appendix).

3.6 Study on the stability of round scad protein hydrolysate during storage

Freeze-dried hydrolysate prepared as mentioned in section 3.5 (2.5 g) was kept in the amber vial and closed tightly with screw-cap. After storage at 4 and 25^{o}C for 0, 1, 2, 4 and 6 weeks, the samples were taken for analyses.

3.6.1 Antioxidative activities

Changes in DPPH radical scavenging activity, reducing power and chelating activity of all samples were monitored as described in section 3.2.2.

3.6.2 Solubility

All samples were subjected to NSI determination during storage as described in section 3.5.5.1

3.6.3 Color measurement

Samples were dissolved in water to obtain the concentration of 10 mg/ml prior to determination for color during storage and the values were reported as L* a* and b*.

4. Statistical analysis

All experiments were run in triplicate. Analysis of variance (ANOVA) was performed and mean comparisons were performed 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).