Chapter 2

MATERIALS AND METHODS

1. Materials

Porcine blood was collected from a slaughter house in Hat Yai, Thailand. The blood was mixed with 3.8% trisodium citrate at a ratio of 1:9 (v/v) to prevent blood coagulation and transported in ice to the Department of Food Technology, Prince of Songkla University.

Sardine (*Sardinella gibbosa*) was purchased from the dock in Songkhla. The fish, off-loaded approximately 36-48 h after capture, were transported to Department of Food Technology, Prince of Songkla University in ice with a fish/ice ratio of 1:2 (w/w) within 1 h.

Frozen surimi produced from sardine (*S. gibbosa*) were given by Man A Frozen Foods Co, Ltd., Maung, Songkhla.

All chemicals for MRPs preparation and analysis were of analytical grade. Glucose was purchased from Merck (Damstadt, Germany). Fructose and galactose were obtained from Fluka (Messerchmittstr, Switzerland). Alcalase 2.4 L was obtained from Novozymes (Bagsvaerd, Denmark).

2. Instruments

Instrument	Model	Company/Country
pH meter	CG 842	Schott, Germany
Magnetic stirrer	BIG SQUID	Ikalabortechnik, Stanfen, Germany
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
Freeze dryer	Dura-Top^{TM}µp	FTS system, USA
Colorimeter	ColorFlex	HunterLab Reston, VA, USA
Double-beam Spectrophotometer	UV-16001	Shimadzu, Japan
Fluorescence Spectrophotometer	RF-1501	Shimadzu, Japan
Homogenizer	T25 basic	Ikalabortechnik, Selangor, Malaysia
Rotary evaporator	Rotavapor-R	Binkmann, Switzerland

3. Methods

3.1 Preparation of porcine plasma protein

Porcine plasma protein was prepared by the method of Benjakul *et al*. (2001a). Porcine blood mixed with trisodium citrate was centrifuged at 1,000 xg for 30 min at 4ºC using a Sorvall Model RC-5B Plus centrifuge (Newtown, CT, USA) to remove red blood cells. The resulting supernatant was dialyzed with 10 volumes of distilled

water at 4ºC for 3 times. Dialyzed plasma protein was freeze-dried and kept at 4ºC until used. The dry powder was referred to as porcine plasma protein (PPP).

3.2 Effect of sugar types and heating time on the characteristics and antioxidant activity of Maillard reaction products (MRPs) from PPP-sugar model system

3.2.1 Preparation of MRPs

PPP (2 g) was mixed with different reducing sugars (glucose, fructose and galactose) at two different levels (1 and 2 g). Distilled water was added to dissolve the mixture and the volume was adjusted to 100 ml. The mixture was then transferred to screw-sealed tubes, tightly capped and heated in oil bath (Buchi labortechnik AG, Switzerland) at 100ºC. The samples were taken after heating for 0, 1, 2, 3, 4 and 5 h. The heated samples were cooled immediately in iced water. MPRs samples obtained were kept at 4ºC until analyzed.

3.2.2 Analysis

3.2.2.1 Measurement of pH

pH of MRP samples was measured using a pH meter (CG 842, Schott, Germany)

3.2.2.2 Measurement of absorbance at 294 nm (A_{294})

The A_{294} of MRP samples was determined 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 UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan).

3.2.2.3 Measurement of fluorescence intensity

Fluorescence of MRP samples with an appropriate dilution 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 RF-1501 Fluorescence spectrophotometer (Shimadzu, Kyoto, Japan).

3.2.2.4 Measurement of browning intensity

The browning intensity of MRP 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 420 nm using UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan).

3.2.2.5 Determination of free amino group content

Free amino group content was determined according to the method of Benjakul and Morrissey (1997). MRP samples with an appropriate dilution (125 μL) was 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 mixtures were mixed thoroughly and placed in a temperature-controlled water bath (Memmert, Bavaria, Germany) at 50ºC for 30 min in the 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. Free amino group content was expressed in term of L-leucine.

3.2.2.6 Determination of antioxidative activity of MRPs

1) Determination of reducing power

The reducing power of MRP samples was determined according to the method of Oyaizu (1986) with a slight modification. One ml of MRPs sample with an appropriate 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 mixtures were incubated in a temperature controlled water bath at 50ºC for 20 min, followed by addition of 1 ml of 10% trichloroacetic acid. The mixtures were then centrifuged at 750 xg using Mikro 20 centrifuge (Hettick zentrifugen, Germany) for 10 min at 25ºC. The supernatant obtained (1 ml) was added with 1 ml of distilled water and 200 µl of 0.1% $FeCl_3$. The absorbance of the reaction mixture was measured at 700 nm. An increase in absorbance was used as the measure of reducing power.

2) Determination of DPPH radical scavenging activity

DPPH radical scavenging activity was determined according to the method of Yen and Hsieh (1995) with a slight modification. An aliquot of MRPs sample (80 µl) with an appropriate dilution was added with 320 µl of distilled water and 2 ml of 0.12 mM DPPH in methanol was added. The mixture was then mixed vigorously and allowed to stand at room temperature in the dark for 30 min. The absorbance of mixture was measured at 517 nm using UV-1601 spectrophotometer. Blank was conducted in the same fashion but deionized water was used instead of DPPH solution. DPPH radical scavenging activity was calculated as follows (Singh and Rajini, 2004):

$$\text{Radical scavenging activity (\%)} = (1-(A_{sample\ (517\ nm)} / A_{control\ (517\ nm)})) \times 100$$

where $A_{sample\ (517\ nm)}$ is the absorbance of sample after blank substraction and $A_{control\ (517\ nm)}$ is the absorbance of the control.

The PPP-sugar MRPs, derived from the sugar types and sugar/plasma ratio, rendering the hightest antioxidant activity, was selected for further study.

3.3 Effect of pHs on the characteristics and antioxidant activity of MRPs from PPP-glucose model system

3.3.1 Preparation of MRPs

PPP (2 g) and glucose (2 g) were dissolved in 90 ml of different buffers (0.05 M sodium phosphate buffer pH 8 and 9; 0.05 M Sodium hydrogen carbonate buffer pH 10, 11 and 12). The pH of solution was readjusted and the volume was brought to 100 ml using the same buffer. The solutions were then transferred to screw-sealed tubes, tightly capped and heated in an oil bath at 100ºC. The samples were taken after heating for 0, 2, 4, 6 and 8 h. The heated samples were cooled immediately in iced water. MRP samples obtained were kept at 4ºC until analyzed.

3.3.2 Analysis

3.3.2.1 Measurement of pH, A_{294}, fluorescence intensity, browning intensity and free amino group content was conducted as described in section 3.2.2.

3.3.2.2 Determination of reducing sugar content

Reducing sugar content was determined according to the method of Chaplin (1994) (Appendix).

3.3.2.3 Determination of antioxidative activity

Reducing power and DPPH radical scavenging activity were determined as described in section 3.2.2.6.

The PPP-glucose MRPs, derived from the initial pH and heating time, which possessed the hightest antioxidant activity, was chosen for further study.

3.4 Characteristics and antioxidative activity of MRPs from PPP hydrolysate-glucose model system

3.4.1 Preparation of PPP hydrolysate

3.4.1.1 Preparation of PPP enzymatic hydrolysate

PPP enzymatic hydrolysate was prepared according to the method of Benjakul and Morrissey (1997). PPP (1 g) was dissolved in 0.2 M borate buffer, pH 8.5. The pH of the mixture was rechecked and adjusted with 6N NaOH or 6N HCl. The volume was brought to 25 ml and transfered to the flask. The mixture was preincubated and well-shaken in a shaking water bath at 60°C for 10 min. The reaction was initiated by adding alcalase at different levels (0, 3.01, 16.84 and 93.41 AU/L PPP solution). After 4 h, reaction was stopped by heating at 90°C for 5 min. The degree of hydrolysis (DH) of PPP enzymatic hydrolysate was determined according to the method of Benjakul and Morrissey (1997) as follows:

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

where L_t corresponded to the amount of α-amino group released at time t. L_0 was the amount of α-amino group released in original PPP. L_{max} was the maximum amount of α-amino group in PPP obtained after acid hydrolysis (6 N HCl at 100°C for 24 h.

3.4.1.2 Preparation of PPP acid hydrolysate

PPP acid hydrolysate was prepared according to the method of Alflawaz *et al.* (1994) with a slight modification. PPP (1.5 g) was suspended in 4 N HCl and the final volume was adjusted to 25 ml. The mixture was then refluxed for 7, 20 and 62 min. The reaction was stopped by rapid cooling and the mixture was neutralized to pH 7. The mixture was diluted to obtain the final concentration of 4% PPP and filtered using a Whatman® filter paper No.4. The degree of hydrolysis of PPP acid hydrolysate was determined as described previously.

3.4.2 Preparation of MRPs

Either PPP enzymatic hydrolysate or PPP acid hydrolysate with DH of 0, 20, 40 or 60% (2%) were mixed with 2% glucose in 0.05 M borate buffer, pH 10 and 12. The reaction mixtures were then transferred to screw-sealed tubes, tightly capped and heated in an oil bath at 100ºC. The samples were taken after heating for 2 h. The heated samples were cooled immediately in iced water. MRP samples obtained were kept at 4ºC until analyzed.

3.4.3 Analysis

3.4.3.1 pH, A_{294}, fluorescence intensity, browning intensity, free amino group content and reducing sugar content were determined as described in section 3.3.2.

3.4.3.2 Reducing power and DPPH radical scavenging activity were determined as mentioned in section 3.2.2.6

3.4.3.3 Metal chelating activity was determined according to the method of Tang *et al*. (2002). One ml of MRP samples with an appropriate dilution was reacted with 0.1 ml of 2mM $FeCl_2$ and 0.2 ml of 5mM 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine) for 20 min. The absorbance was read at 562 nm. One ml of distilled water, instead of MRPs, was used as a control. Blank was conducted in the same fashion but deionized water was used instead of ferrozine solution. Chelating activity (%) was calculated as follows:

$$\text{Chelating activity (\%)} = [1-(A_{562} \text{ of sample}/ A_{562} \text{ of control})] \times 100$$

PPP hydrolysate-glucose MRPs, derived from hydrolysate with DH rendering the hightest antioxidant activity was chosen for further study.

3.5 Fractionation of PPP acid hydrolysate-glucose MRPs

3.5.1 MRPs preparation

The MRPs with highest antioxidative activity were prepared as mentioned in section 3.4.2. Stepwise fractionation of MRPs was performed using different organic solvents in the order of hexane, dichloromethane and ethylacetate. To fractionate MRPs, hexane (the lowest polarity organic solvent) was mixed with MRPs at a ratio of 1:1 (v/v) in separatory funnel and vigorously shaken for 10 min at room temperature. Thereafter, the hexane fraction was collected and hexane was evaporated in a rotary evaporator. The remaining fraction obtained was then fractionated with dichloromethane and ethylacetate, respectively. The five fractions obtained (crude MRPs, hexane, dichloromethane, ethyl acetate and residual fractions) were lyophilized and subjected to analysis.

3.5.2 Analysis

3.5.2.1 The browning intensity of PPP acid hydrolysate-glucose MRP fractions were determined as mentioned in section 3.2.2.4.

3.5.2.2 Antioxidative activity of MRP fractions was measured as described in section 3.4.3. MRP fractions (0.6 g) were dissolved in deionized water and the final volume was adjusted to 5 ml. Reducing power, DPPH radical scavenging activity and metal chelating activity of MRP fractions were determined.

3.6 Decolorization of PPP acid hydrolysate-glucose MRPs

3.6.1 Decolorization by activated carbon

The MRPs with highest antioxidant activity were prepared as mentioned in section 3.4.2. Decolorization of MRPs by activated carbon was performed according to the method of Synowiecki and Al-Khateeb (2000) with a slight modification. Activated carbon at different levels (1, 2 and 5%) was added in MRPs and shaken at

120 rpm for 30 min at room temperature. Then, the mixtures were filtered using Whatman® filter paper No.42. The filtrate was centrifuged at 10000 xg for 20 min to remove the activated carbon particles. The decolorized MRPs were then lyophilized.

3.6.2 Decolorization by Sep-Pak Cartridge C18

The MRPs with highest antioxidative activity were prepared as mentioned in section 3.4.2. Decolorization of MRPs using Sep-Pak Cartridge C18 (Vac 3cc, 500 mg) was performed according to the method of Lee (1992). A 10 ml of PPP acid hydrolysate-glucose MRPs was transferred into a syringe and passed through a Sep-Pak Cartridge C18 which had been prewetted with 3 ml of methanol, followed by 5 ml of water. The decolorized MRPs obtained were subjected to decolorization for another 1 and 2 times. MRPs decolorized by a Sep-Pak Cartridge C18 for different times (1, 2 and 3) were liophilized and used for analysis.

3.6.3 Analysis

3.6.3.1 Color measurement

The color of MRPs and decolorized MRPs powder samples were measured by Hunter lab and reported in CIE system. L* a* and b* parameters indicate lightness, redness and yellowness, respectively.

3.6.3.2 Measurement of browning intensity

MRPs and decolorized MRPs (0.6 g) were dissolved in deionized water and the final volume was adjusted to 5 ml. The browning intensity were determined as mentioned in section 3.2.2.4.

3.6.3.3 Determination of antioxidative activity

Reducing power, DPPH radical scavenging activity and metal chelating activity of all samples were determined as mentioned in section 3.4.3.

PPP hydrolysate-glucose MRPs with decolorization treatment, which resulted in the lowest color and hightest antioxidant activity, was prepared, liophilized and used for further study.

3.7 Study on antioxidant activity of MRPs and decolorized MRPs

Antioxidative activity of MRPs and decolorized MRPs from PPP-acid hydrolysate-glucose model system was investigated.

3.7.1 Effect of MRPs and decolorized MRPs amount on antioxidative activity

MRPs and decolorized MRPs at the concentrations of 2, 4, 8 and 12% were prepared. Reducing power, DPPH radical scavenging activity and metal chelating activity of MRPs and decolorized MRPs were determined as mentioned in section 3.4.3. Furthermore, MRPs and decolorized MRPs were subjected to the following analysis:

1) Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of MRPs and decolorized MRPs was determined according to the method of Halliwell *et al*. (1987) (Appendix).

2) Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity of MRPs and decolorized MRPs was determined according to the method of Ruch *et al*. (1984) (Appendix).

3.7.2 Antioxidative activity of MRPs and decolorized MRPs in different systems

MRPs and decolorized MRPs with different levels (100, 200 and 500 ppm) were used in different systems.

3.7.2.1 Lecithin liposome system

The antioxidative activity of MRPs and decolorized MRPs in lecithin liposome system was determined according to the method of Frankel *et al*. (1997) (Appendix).

3.7.2.2 β-carotene-linoleic system

The antioxidative activity of MRPs and decolorized MRPs in β-carotene-linoleic system was tested as described by Taga *et al*. (1984) (Appendix).

3.8 Study on the antioxidant activity of MRPs and decolorized MRPs during storage

The MRPs and decolorized MRPs were prepared as mentioned previously. Both MRPs samples were liophilized and each sample (0.6 g) was kept in a microtube. After storage at 4 and 25°C for 0, 1, 2, 4, 6 and 8 weeks, the samples were taken for analysis. Samples were dissolved in deionized water and the volume was brought to 5 ml. Reducing power, DPPH radical scavenging activity and metal chelating activity of all MRPs were determined as described in section 3.4.3.

3.9 Uses of MRPs in sardine mince and sardine emulsion sausage

3.9.1 Uses MRPs and decolorized MRPs in sardine mince

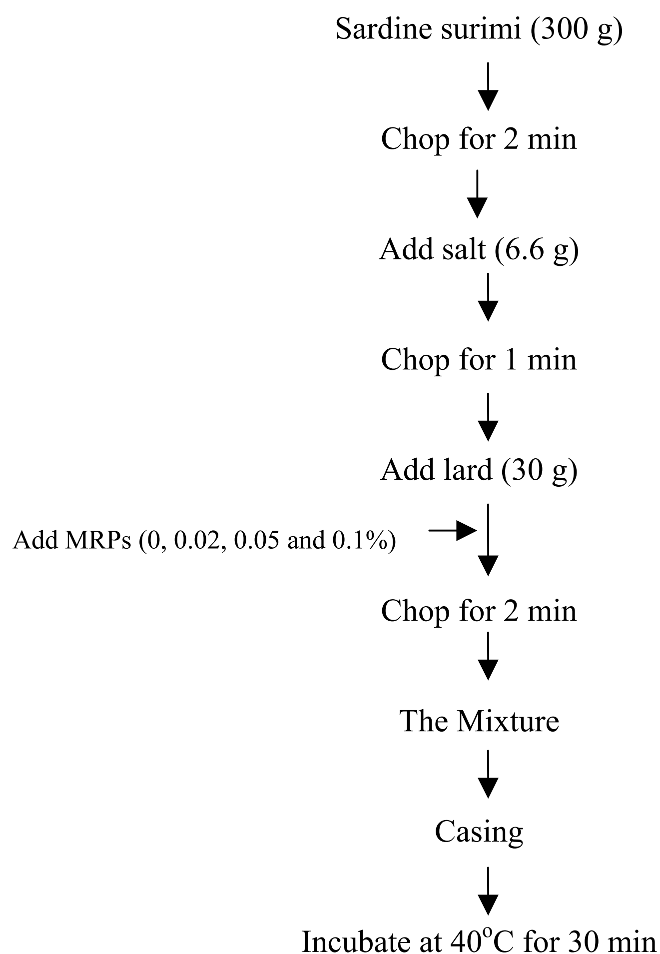
Sardines (*S. gibbosa*) were washed, filleted and minced. The mince was mixed well with MRPs and decolorized MRPs at levels of 0, 100, 200, and 500 ppm. All samples were kept in polyethylene bags and stored in ice for 15 days. The molten ice was replaced with the same amount of ice every 2 days. After 0, 3, 6, 9, 12 and 15

days of storage, the samples were subjected to TBARS determination as described by Buege and Aust (1978) (Appendix).

3.9.2 Uses of MRPs in sardine emulsion sausage

3.9.2.1 Preparation of sardine emulsion sausage

Sardine surimi was used to prepare sardine emulsion sausage as shown in the following scheme.



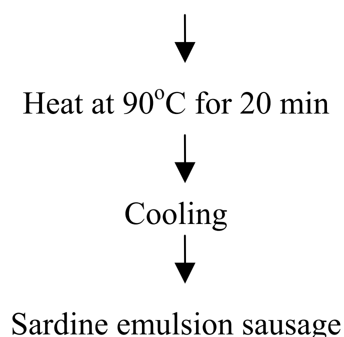


Figure 4 Scheme for sardine emulsion sausage production

3.9.2.2 Changes in lipid oxidation of sardine emulsion sausage

Sardine emulsion sausages were stored at 4°C. The samples were taken after 0, 3, 6, 9, 12 and 15 days of storage for the analysis. TBARS, peroxide value (PV) and conjugated diene were determined as described by Buege and Aust (1978), IUPAC (1979) and Frankel and Huang (1996), respectively (Appendix). Prior to PV and conjugated diene analysis, lipids in the samples were extracted by Bligh and Dyer method (1959) (Appendix).

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 8.0 for windows, SPSS Inc, Chicago, IL).