CHAPTER 2

MATERIALS AND METHODS

1. Materials

Raw materials

Frozen surimi (grade A), produced from bigeye snapper (*Priacanthus tayenus*), threadfin bream (*Nemipterus bleekeri*) and goat fish (*Parupeneus indicus*) were purchased from Man A Frozen Foods Co., Ltd., Muang, Songkla, Thailand. Surimi was stored at –20ºC until used. Fresh sardine (*Sardinella gibbosa*) with an average weight of 55-60 g were obtained from the dock in Songkhla. The fish were transported in ice with the fish/ ice ratio of 1: 2 (w/w) to the Department of Food Technology, Prince of Songkla University within 30-45 min. Palm oil (Emeral), butter (Orchid) and shortening (Olympic kream) were obtained from the market in Hat Yai, Songkhla.

Chemical regents

Microbial transglutaminase (MTGase, protein-glutamine γ-glutamyltransferase, EC 2.3.3.13) was obtained from Ajinomoto., Ltd. (Kawasaki, Japan). Sodium dodecyl sulfate (SDS), β-mercaptoethanol (βME), glutaraldehyde (25% solution), glycerol and formaldehyde (37% solution) were purchased from Sigma (St. Louise MO, USA). Glyoxal (40% solution) was procured from Fluka (St. Quentin Fallavier, France).

2. Instruments

Instruments	Model	Company
-Homogenizer	T25	Ultra turrax, Malaysia
-Spectrophotometer	UV-16001	SHIMADZU, Australia
-Electrophoresis	Mini-Protean II	Bio-Rad, USA
-Magnetic stirrer	RO 15 power	IKA labortechnik, Germany
-pH meter	CG 842	SCHOTT, Germany
-Universal testing machine	LR 30K	LLOYD, England
-Environmental chamber	KBF 115	WTB BINDER, Germany
-CIE colorimeter	Color Flex	HUNTER, USA
-Differential scanning calorimeter	DSC 7	PERKIN ELMER, USA
-Thermogravimetric analyzer	TGA 7	PERKIN ELMER, USA
- Scanning Electron Microscopy	JSM-5800 LV	JEOL, Japan

3. Chemical analyses of surimi

Moisture, protein, ash, fat and carbohydrate contents of surimi were determined according to the methods of AOAC (1999). Trichloroacetic acid soluble peptide content was measured as described by Morrissey et al. (1993). Protein patterns were determined by SDS-PAGE using 4% stacking gel and 10% running gel according to the method of Laemmli (1970).

4. Preparation of surimi film

Frozen surimi was thawed using a running water (26-27ºC) until the core temperature reached 0ºC. The film-forming solution was prepared as described by Shiku et al. (2003) with a slight modification. Thawed surimi was added with the distilled water to obtain the final protein concentrations of 1 and 2% (w/v). The mixture was homogenized at 13,000 rpm for 1 min using a homogenizer (IKA Labortechnik, Selangor, Malaysia). Glycerol was then added at 50% (w/w) of protein content. The mixture was stirred gently for 30 min at room temperature. The pH of the mixture was adjusted to 3 or 11 using 1 N HCl and 1 N NaOH, respectively. The film-forming solution obtained was filtered through a layer of nylon sheet. The filtrate was used for film casting. The film-forming solution (4 g) was cast onto a rimmed silicone resin plate (50x50 mm) and air blown for 12 h at room temperature prior to further drying at 25ºC and 50% relative humidity (RH) for 24 h in an environmental chamber (WTB Binder, Tuttlingen, Germany). The resulting films were manually peeled off and used for analyses.

5. Determination of film properties

5.1 Film thickness

The thickness of film was measured using a micrometer (Gotech, Model GT-313-A, Gotech testing machines Inc, Tawai). Five random positions of each film of ten films were used for thickness determination.

5.2 Mechanical properties

The films were conditioned for 48 h at 25ºC and 50%RH prior to testing. Tensile strength (TS) and elongation at break (EAB) were determined as described by Iwata et al. (2000) with a slight modification using the Universal Testing Machine (Lloyd Instruments, Hampshire, UK). Eight samples (2x5 cm) with initial grip length of 3 cm were used for testing. Cross-head speed was 0.5 mm/s.

5.3 Water vapor permeability (WVP)

WVP of films was determined using a modified ASTM method (American Society for Testing & Materials, 1989) as described by Shiku et al. (2003). The film was sealed on a glass permeation cup containing silica gel (0%RH) with silicone vacuum grease and a rubber band. The cups were placed at 30ºC in a desiccator containing the distilled water. The cups were weighed at 1 h intervals over a 7 h period. WVP of the film was calculated as follows (McHugh et al., 1993):

$$\text{WVP (g m}^{-1}\text{ s}^{-1}\text{ Pa}^{-1}) = wxA^{-1}t^{-1}(P_2\text{-}P_1)^{-1}$$

where w is the weight gain of the cup (g), x is the film thickness (m), A is the area of exposed film (m^2), t is the time of gain (s) and (P_2-P_1) is the vapor pressure difference across the film (Pa). Five films were used for WVP testing and the measurement was run in duplicate.

5.4 Color and film transparency

Color of the film was determined as *L**, *a** and *b** using CIE colorimeter (Hunter associates laboratory, Inc., VA, USA) (Paschoalick et al., 2003). The films were subjected to the measurement at 600 nm using the UV-16001 spectrophotometer (Shimadzu, Kyoto, Japan) as described by Han and Floros (1997). The transparency of the films was calculated by the following equation:

$$\text{Transparency} = -\log T_{600}/x$$

where T_{600} is the transmittance at 600 nm and x is the film thickness (mm).

5.5 Film solubility and protein solubility

Film solubility was determined according to the method of Gennadios et al. (1998). The conditioned film samples (2x2 cm) were weighed and placed in 50-ml centrifuge tubes containing 10 ml of distilled water with 0.1% (w/v) sodium azide, and then stored at 30ºC for 24 h with continuous gentle stirring. Undissolved dry matter was determined by centrifugation at 3000xg for 20 min and drying them at 105ºC for 24 h. The weight of solubilized dry matter was calculated by subtracting the weight of unsolubilized dry matter from the initial weight of dry matter and expressed as the percentage of total weight. To determine the protein solubility, the samples were prepared in the same manner with film solubility test. Solubilized protein was determined by the Lowry method (Lowry et al., 1951). Protein solubility was expressed as percentage of total protein in the film which was solubilized with 0.5 M NaOH at 30ºC for 24 h.

5.6 Protein pattern

Protein patterns were determined by SDS-PAGE using 4% stacking gel and 10% running gel according to the method of Laemmli (1970).

Film prepared under condition rendering the highest mechanical property and lowest WVP was chosen and used for further study.

6. Effect of pH on the compositional changes of film-forming solution

The surimi was solubilized using acid and alkaline at pH 3 and 11, respectively, as described previously. The solution was allowed to stand at room temperature and taken for analysis at 0, 2, 5, 7 and 10 h. At the time designated, the solution was neutralized using either 1 N NaOH or 1 N HCl. Then, the neutralized solution was mixed with 5%SDS at a ratio of 1:1 (v/v). The mixture was incubated at 85ºC for 15 min and the undissolved debris was removed by centrifuging at 3,500xg for 5 min. The supernatants were subjected to SDS-PAGE analysis using 4% stacking gel and 10% running gel according to the method of Laemmli (1970). The reducing sugar was determined according to the method of Nelson-Simogyi as modified by Chaplin and Kennedy (1994).

7. Effect of lipids on the film properties

Thawed surimi was added with the distilled water and homogenized for 1 min at 13,000 rpm using a homogenizer. The homogenate with 2% protein content was adjusted to pH 3 using 1 N HCl. The film-forming solution was filtered

through a layer of nylon sheet. The lipids were added as a substitute of glycerol at 0, 25, 50, 75 and 100% (w/w). To stabilize the emulsion, Tween-20 at 10% of lipids added was used. The mixture containing lipids and/or glycerol as well as Tween-20 was then homogenized at 13,000 rpm for 4 min. Subsequently, the film-forming solution was used for film casting. The film-forming solution was cast onto a rimmed silicone resin plate and dried overnight at 25ºC and 50% relative humidity (RH). All films were determined as described in section 5. Moisture sorption isotherms of the film samples were determined at 4ºC and room temperature as described by Kim and Ustunal (2001). Eight different humidity conditions (18$\pm$0.5, 23$\pm$0.5, 34$\pm$0.5, 46$\pm$0.5, 54$\pm$0.5, 64$\pm$0.05, 73$\pm$0.05, 90$\pm$0.5%) were obtained by using saturated salt solutions of $LiCl•H_2O$, $KC_2H_3O_2$, $CaCl_2•2H_2O$, $K_2CO_3•2H_2O$, $Mg(NO_3)_2•6H_2O$, $NaNO_2$, NaCl and KCl, respectively.

Film samples which had the highest mechanical properties and lowest WVP were chosen for further study.

8. Effect of crosslinker on surimi film properties

8.1 The use of aldehydes

Aldehydes (formaldehyde, glutaraldehyde and glyoxal) were added to film-forming solutions (section 7) to obtain the final concentrations of 0, 0.5, 1 and 5 mM. The mixture was cast onto a rimmed silicone resin plate and dried overnight at 25ºC and 50% relative humidity (RH). All films were subjected to testing as described in section 5.

8.2 The use of microbial TGase

Microbial TGase was added to film-forming solutions with the pH of 5.0 at various levels (0, 5, 10, 20, 40 and 80 units/ g protein). The mixture was cast onto a rimmed silicone resin plate and incubated at 37ºC for 2 h before drying overnight at 25ºC and 50% relative humidity (RH). All films were analyzed as described in section 5. Moisture sorption isotherms of the film samples were also determined as described in section 7.

Film samples obtained from section 8 which had the highest mechanical properties and lowest WVP was chosen and used for further study.

9. Characterization of surimi film

The film samples obtained from section 4, 7 and 8 with the highest mechanical property and lowest WVP were subjected to the following analysis:

9.1 Microstructure

Microstructure of the film samples was determined using Scanning Electron Microscopy (SEM). The film was coated by gold (Sputter coater SPI-Module, PA, USA). The surface was observed at an acceleration voltage of 10 kV.

9.2 Hydrolysis by protease

Ground film sample (50 mg) was suspended in 50 ml of α-chymotrypsin solution (40 μg/ml in 40 mM Tris-HCl buffer, pH 7.6). The suspension was then incubated at 37ºC for 2 h. To 2.5 ml aliquot, an equal volume of 20% TCA

was added to terminate the reaction. After standing for 30 min at room temperature, the precipitate was removed by centrifugation (1800xg for 15 min). The peptide and amino acid content of supernatant was determined by the Lowry method. The degree of hydrolysis was calculated as described by Yildirim and Hettiarachchy (1998).

9.3 Protein solubility

Protein solubility of surimi film in various solvents were determined as described by Chawla et al. (1996). The solvents used included:

1) 20 mM Tris-HCl (pH 8.0) containing 1% (w/v) SDS.

2) 20 mM Tris-HCl (pH 8.0) containing 1% (w/v) SDS and 8.0 M Urea.

3) 20 mM Tris-HCl (pH 8.0) containing 1% (w/v) SDS and 8.0 M Urea and 2% βME.

Samples were also solubilized in 0.5 M NaOH. Protein content in 0.5 M NaOH extract was used as the reference value, i.e., 100%.

9.4 Thermal properties

Glass transition temperature (T_g) of surimi film was determined by differential scanning calorimeter (DSC) as described by Sobral et al. (2005). The instrument was calibrated with Indium as a standard. Films were conditioned over siliga gel at 23ºC for 3 weeks before testing. Dry samples (10 mg) were placed in a hermetically sealed aluminium pan and heated at 5ºC/min between 20-150ºC.

Samples were also determined for thermal degradation temperature (T_d) using thermogravimetric analyzer (TGA). The heating rate of 10ºC/min was used between 30 and 400ºC.

10. Changes of films during storage

Film samples were stored in desicators with varying RH using different saturated salt solutions including $MgCl_2$, NaBr, NaCl or KCl as described by Cuq et al. (1996b). The desicators were placed in room temperature and 4ºC. Film samples stored in air at both temperatures were also used for study.

Film samples were taken for analysis of color and transparency (section 5.4) every week for totally eight weeks.

Film samples were also taken every two weeks for the following analyses:

- Film solubility (as described in section 5.5)

- Mechanical properties (as described in section 5.2)

- Water vapor permeability (as described in section 5.3)

11. Uses of surimi film to extend the shelf-life of dried fish powder

Dried sardine muscle powder was prepared. The fish were washed and filleted. The fillets were subjected to drying using hot-air oven with the air velocity of

1.5 m/s at 60ºC for 5 h. The dried fish were powderized using the blender until the uniformity was obtained.

To study the effect of surimi film on shelf-life extension of dried fish powder, fish powder (15g) were placed in a cylindrical bottle. The selected films from section 7, 8 and polyethylene film were placed on the bottle containing fish powder and sealed tightly with O-ring. The samples stored at room temperature (30-32ºC) were taken for analysis every 3 days for 15 days as follows:

- TBARS (Thiobarbitaric acid reactive substances) were determined according to the method of Buege and Aust (1978).

- Color was measured using a colorimeter (Hunter Lab, Model ColorFlex) and reported in CIE system color profile of L*, a*, and b*.

- Moisture content was determined according to the method of AOAC (1999).

12. Statistical analysis

Analysis of variance (ANOVA) was performed and mean comparisons were carried out by Duncan's multiple range test (Steel & Torrie, 1980). Analysis was performed using the SPSS package (SPSS 11.0 for Windows, SPSS Inc, Chicago, IL).