Chapter 2

Materials and methods

1. Materials

Chemical reagents

Sodium pyrophosphate (PP), sodium tripolyphosphate (TPP), sodium hexametaphosphate (HMP), sodium dedocyl sulfate (SDS), calcium chloride, β-mercaptoethanol (βME), adenosine 5′-triphosphate (ATP) and sodium dedocyl sulfate (SDS) were purchased from Sigma (St Louis, MO, USA). Microbial transglutaminase was obtained from Ajinomoto Co., Ltd. (Kawasaki, Japan) with activity of 45.8 unit/g powder. *N,N,N′,N′*-tetramethylethylenediamine (TEMED), acrylamide, bis-acrylamide, and urea were procured from Fluka (Buchs, Switzerland).

Raw materials

Bigeye snapper (*Priacanthus tayenus*) and threadfin bream (*Nemipterus bleekeri*), caught off the Trang Coast along Andaman sea of Thailand, off-loaded approximately 24–36 h after catching were kept in ice using the fish:ice ratio of 1:2 (w/w). Fish were transported to the Department of Food Technology, Prince of Songkla University. Upon arrival, the fish were washed and used for surimi preparation.

Surimi preparation

Fish were washed and filleted. The flesh was minced using a mincer

with a hole of 2-3 mm. The mince was subjected to washing with cold water (5°C) using a ratio of 1:3 (w/v) for two times. Washed mince was centrifuged using a model CE 21 K basket centrifuge (Grandiumpiant, Belluno, Italy) at 700 x g for 10 min. The scheme of surimi preparation is shown in Figure 6.

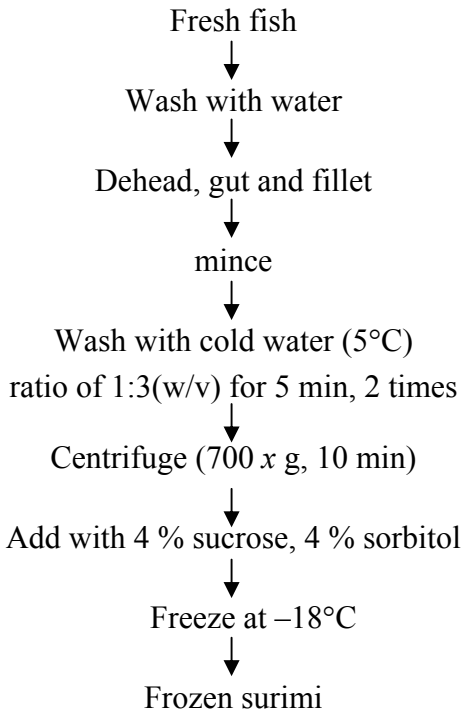

Figure 6. Scheme for surimi preparation

2. Instruments

Instruments	MODEL	Company
- Electrophoresis apparatus	Mini-Protein II	Bio-Rad, USA
- Spectrofluorometer	RF-1501	SHIMADZU, Japan
- Texture analyzer	TA-XT2	Stable Micro Systems, England
- Homogenizer	T25B	Ultra turrax, Malaysia
- Refrigerated centrifuge	RC-5B plus	Sorvall, USA
- Magnetic stirrer	RO 10 power	KIKAL labortechnik, Gernamy
- Basket centrifuge	CE 21K	Grandimpianti, Italy
- Mixer	MK-K77	National, Japan
- pH meter	Denver 15	Scientific, USA
- Scanning Electron Microscope	JSM 5800LV	JEOL, Japan

3. Study on the effect of phosphate compounds on setting and gel forming ability of surimi

To study the effect of various phosphates on gel properties of surimi, phosphates at different levels were added into the surimi paste and gels were prepared as depicted in Figure 7. Phosphate compounds with different types at different levels used were shown as follows:

- PP (0, 0.025, 0.05, 0.1 and 0.3 %)
- TPP (0, 0.025, 0.05, 0.1 and 0.3 %)
- HMP (0, 0.025, 0.05, 0.1 and 0.3 %)

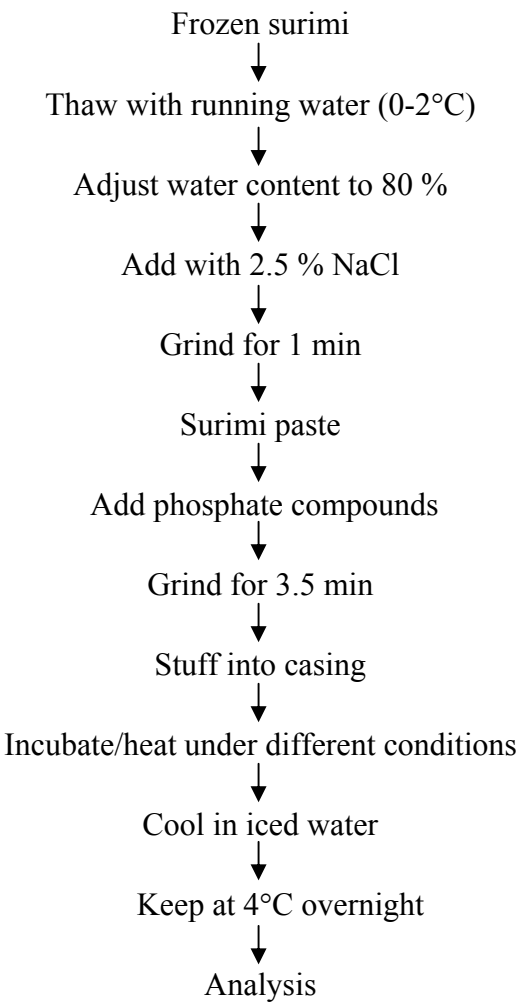

Figure 7. Scheme for surimi gel preparation

Surimi paste containing phosphates with different types and levels was then stuffed into polyvinylidine casing with a diameter of 3.6 cm. Both ends of the casing were sealed tightly. Surimi mixtures were subjected to heating under different conditions to obtain the different gels as follows:

- Directly heated gels were prepared by heating the mixture at 90°C for 20 min.

- Kamaboko gels were prepared by incubating the mixture at 40°C for 30 min, followed by heating at 90°C for 20 min.

- Suwari gels were prepared by incubating the mixture at 40°C for 30 min.

The gels were cooled in iced water and stored over night at 4°C prior to analysis. Gels were then determined as follows:

1. Breaking force and deformation

Textural properties of gel were determined by measuring breaking force (gel strength) and deformation (elasticity/deformability) using a punch test according to the method of Benjakul *et al*. (2001)

2. Whiteness

Whiteness of gel was determined as described by Park (1994) using the following equation.

$$\text{Whiteness} = 100-[(100-L^*)^2 + a^{*2} + b^{*2}]^{1/2}$$

where: L* = lightness

a* = redness/greenness

b* = yellowness/blueness

3. Protein pattern

Protein patterns of surimi gels were determined under reducing and non-reducing conditions using SDS-PAGE according to method of Laemmli (1970) with 4 % stacking gel and 10 % separating gel.

4. Expressible moisture

Expressible moisture was measured according to the method of Ng (1987). Cylindrical gel samples were cut into a thickness of 5 mm, weighed (X) and placed between two pieces of Whatman paper No.1 at the bottom and one piece on the top of the sample. The standard weight (5 kg) was placed at the top and hold for 2 min. The sample was then removed from the papers and weighed again (Y). Expressible moisture was calculated with the following equation:

$$\text{Expressible moisture (\%)} = 100 \times \{(X-Y)/X\}.$$

5. Solubility

Solubility of surimi gel was determined in solvent mixtures (20 mM Tris-HCl containing 1% sodium dodecyl sulfate, 8 M urea and 2% β-mercaptoethanol, pH 8) according to the procedure of Roussel and Cheftel (1990)

Phosphate type rendering the highest breaking force and deformation for both surimi from bigeye snapper and threadfin bream was chosen for further study.

4. Study on the effect of phosphate compounds and calcium chloride or microbial transglutaminase on setting and gel forming ability of surimi

Selected phosphate which showed the highest breaking force of surimi gel from bigeye snapper and threadfin bream in section 3 was added into surimi paste

in combination with calcium chloride ($CaCl_2$) or microbial transglutaminase (MTGase) at different levels as follows:

- $CaCl_2$ (0, 25 and 50 mmole/kg)
- MTGase (0 and 0.05%)

The kamaboko gels were prepared from the surimi mixtures as mentioned in section 3 and gel properties were determined as described in section 3. Surimi mixtures containing $CaCl_2$ at a level of 50 mmole/kg or MTGase at a level of 0.05% were subjected to setting at 40°C for 0, 30, 60, 90, 120 and 150 min, followed by heating at 90°C for 20 min. Gels obtained under different conditions were determined as described in section 3.1. Additionally, microstructure was examined.

To determine the microstructure of surimi gels, the gel samples (0.25x0.25x0.25 cm) were fixed with 2.5 % glutaraldehyde in 0.2 M phosphate buffer, pH 7.2 for 2 h at room temperature. Fixed specimens were dehydrated in graded ethanol solution with serial concentrations of 50, 70, 80, 90 and 100 % and critical point dried (Balzers mod. CPD 030, Balzers Process Systems, Liechtenstein) using CO_2 as transition fluid. The prepared samples were mounted on copper specimen holders, sputter-coated with gold (Balzer mod. SCD 004) and examined on a JSM 5200 scanning electron microscope (JEOL, Ltd., Akishima, Japan)

5. Study on the effect of phosphate on transglutaminase activity from fish muscle

5.1 Preparation of transglutaminase crude extract

Transglutaminase (TGase) crude extract was prepared according to the method of Tsukawasa *et al*. (2002) with a slight modification. Fish meat was homogenized with a blender at 10000 r.p.m. for 2 min with five volumes of 50 mM

Tris-HCl buffer (pH 7.5) containing 5 mM EDTA, 10 mM NaCl and 3 mM dithiothreitol (buffer A) at 4°C. Homogenate was centrifuged at 1600 x *g* for 30 min, and the precipitate was collected after addition of saturated ammonium sulfate (30-60%). The precipitate dissolved in a small amount of buffer A was dialyzed against buffer A overnight with three changes. The resulting crude TGase extract was assayed for TGase activity.

5.2 Determination of TGase activity

TGase activity was measured by the hydroxamate method (Folk, 1970). Freshly prepared substrate mixture containing 350 μl of 0.1 M Tris-acetate, pH 6.0, 25 μl of 2.0 M hydroxylamine, 75 μl of 0.1 M N-ε-CBZ-L-glutamidylglycine and 25 μl of deionized water was used. To initiate the reaction, 25 μl of crude extract were added and reaction was performed for 10 min at 37°C. The reaction was terminated by adding 500 μl of 15% TCA containing 5% $FeCl_3$. The resulting suspension was centrifuged at 9000 x g for 5 min and the absorbance was measured at 525 nm using a spectrophotometer. The calibration was performed using L-glutamic acid-γ-monohydroxamic acid as standard. One unit of MTGase was defined as the amount of enzyme required to catalyze the formation of 1 μmole hydroxamic acid min^{-1} at pH 6.0 and 37°C (Ho *et al*., 2000). To study the effect of phosphate in the absence or presence of $CaCl_2$, 25 μl of crude extract were mixed with phosphate and/or $CaCl_2$ to obtain the different final concentrations. The mixture was allowed to stand at room temperature for 10 min prior to starting reaction assay.

6. Study on the effect of phosphate on fish muscle protein and gel forming ability of surimi

6.1 Effect of phosphate and Ethylene glycol-bis (2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA) on gel forming ability

PP or TPP (12% (w/v); neutralized) were added into surimi paste to obtain different final levels (0, 0.025, 0.05, 0.1 and 0.3%) in combination with 20 mmole EGTA/kg. Kamaboko gel and directly heated gels were then prepared as mentioned in section 3 and gels properties were determined as described in section 3.

6.2 Effect of phosphate compounds on fish muscle protein

6.2.1 Preparation of natural actomyosin (NAM)

Natural actomyosin (NAM) was prepared according to the method of Benjakul *et al*. (1997) with a slight modification. Fish muscle (10 g) was homogenized in 100 ml of chilled 0.6 M KCl, pH 7.0 for 4 min using a homogenizer. The container with sample was placed in ice. Each 20 sec of homogenization was followed by a 20 sec rest interval to avoid overheating during extraction. The homogenate was centrifuged at 5,000 x g for 30 min at 4°C. Three volumes of chilled water (0-2°C) was added to precipitate NAM. The NAM was collected by centrifuging at 5,000 x g for 20 min at 4°C. The pellet was then dissolved by stirring in an equal volume of chilled 0.6 M KCl, pH 7.0 for 30 min at 4°C.

6.2.2 Effect of phosphate on thermal stability of NAM

One ml of NAM solution (3-5 mg/ml) without and with 0.1% neutralized pyrophosphate was incubated at different temperatures (0, 10, 20, 30, 40,

50, 60 and 70°C). At definite time (0, 5, 10, 20, 30 and 60 min), a sample solution was immediately cooled in ice water. The sample was then equilibrated at 25°C prior to the assay for Ca^{2+}-ATPase and Mg^{2+}-ATPase activities. The inactivation rate constant (K_D) of actomyosin ATPase was calculated according to Arai *et al*. (1973) as follows:

$$K_D = (\ln C_0 - \ln C_t)/t$$

where C_0 = Ca^{2+}-ATPase or Mg^{2+}-ATPase activity before treatment, C_t = Ca^{2+}-ATPase or Mg^{2+}-ATPase activity after treatment for time t; and t = treatment time (s).

6.2.3 Effect of various phosphates on ATPase activities

NAM containing different phosphates (PP, TPP, HMP) at different final concentrations (0.025, 0.05, 0.1 and 0.3%) were allowed to stand at 4°C for 10 min. The solutions were then dialyzed using 10 volumes of 20 mM Tris-HCl containing 0.6 M KCl, pH 7.0 with 6 times changes at 4°C. The dialyzates were determined for ATPase activities.

6.2.4 ATPase activity determination

ATPase activity was determined according to the method of Benjakul *et al*. (1997). NAM was diluted to 0.5-3.0 mg/ml with 0.6 M KCl, pH 7.0. Diluted NAM solution (0.5 ml) was added with one of the following solutions for each ATPase activity assay to a total volume of 9.5 ml: 10 mM $CaCl_2$ for Ca^{2+}-ATPase; 2 mM $MgCl_2$ for Mg^{2+}-ATPase; 0.1 mM EGTA for Mg^{2+}-EGTA-ATPase. To each assay solution, 0.5 ml of 20 mM ATP was added to initiate the reaction. The reaction was conducted for 10 min at 25°C and terminated by adding 5 ml of chilled 15 %

(w/v) trichloroacetic acid. The reaction mixture was centrifuged at 3,500*xg* for 5 min and the inorganic phosphate liberated in the supernatant was measured by the method of Fiske and Subbarow (1925). Specific activity was expressed as μmoles inorganic phosphate released/mg protein/min. A blank solution was prepared by adding chilled trichoroacetic acid prior to addition of ATP.

7. Statistical analysis

Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's Multiple Range Test (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS 10.0 for windows: SPSS Inc., Chicago, IL).