

Chapter 2

Materials and methods

1. Chemicals

Ammonium molybdate, 5-5'-dithio-bis (2-nitrobenzoic acid) (DTNB), adenosine 5'- triphosphate (ATP), sodium dedocyl sulfate (SDS), *L*-ascorbic acid, calcium chloride and β -mercaptoethanol (β -ME) were purchased from Sigma (St. Louise, MO, USA). Microbial transglutaminase (MTGase) was obtained from Ajinomoto Co., Ltd. (Kawasaki, Japan). *N,N,N,N'*-tetramethylethylenediamine (TEMED), acrylamide, bis-acrylamide, and urea were procured form Fluka (Buchs, Switzerland). Sodium hypochlorite was purchased from Ajax Finechem (Auckland, New Zealand). Hydrogen peroxide was obtained from Merck (Hohenbrunn, Germany).

2. Instruments

Instruments	Model	Company
- Electrophoresis apparatus	Mini-Protein II	Bio-Rad, USA
- Spectrofluorometer	RF-1501	SHIMADZU, Japan
- Double-beam spectrophotometer	UV-16001	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, Germany
- Basket centrifuge	CE 21K	Grandimpianti, Italy
- Mixer	MK-K77	National, Japan
- pH meter	Denver 15	Scientific, USA
- Scanning Electron Microscope	JSM5800LV	JEOL, Japan

3. Fish preparation

Bigeye snapper (*Priacanthus tayenus*), threadfin bream (*Nemipterus hexodon*) and starry triggerfish (*Abalistes stellaris*) were caught from Songkla coast along the Gulf of Thailand, stored in ice and off-loaded after 72 h of capture. Upon the arrival to the dock in Songkhla, fish were kept 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 1 h. The fish were immediately washed and drained. Fish were kept in the insulated box containing ice with a fish/ice ratio of 1:2. The box was placed at room temperature (25-30°C). The molten ice was replaced by an equal amount every two days. The fish were taken for analysis and surimi preparation at week 0, 1 and 2.

4. Study on the effect of oxidizing agent in wash water on washed mince

4.1. Chemical composition and gel-forming ability

Fish stored in ice for 0, 1 and 2 weeks were washed, filleted and minced. The mince was subjected to washing with different washing media as follows:

1. water
2. 10 ppm H₂O₂
3. 20 ppm H₂O₂
4. 40 ppm H₂O₂
5. 10 ppm NaOCl
6. 20 ppm NaOCl
7. 40 ppm NaOCl

Mince washed with different washing media was centrifuged using a model CE 21 K basket centrifuge (Grandiumpian, Belluno, Italy) at 700xg for 10 min as described in Figure 5. Washed mince and unwashed mince were subjected to the following analysis.

- pH (Benjakul *et al.*, 1997).

- TCA-soluble peptide (Morrissey *et al.*, 1993).
- Thiobarbituric acid reactive substances (TBARS) (Buege and Aust, 1978).
- Total volatile base (TVB) and trimethylamine (TMA) (Conway and Byme, 1936).
- Gel forming ability (Benjakul *et al.*, 2001).

Surimi gel was prepared as described in Figure 6. The prepared surimi was added with 2.5 % salt, and the moisture content was adjusted to 80 %. The mixture was chopped for 3.5 min at 4°C to obtain a homogeneous sol. The sol was then stuffed into polyvinylidene casing with a diameter of 3.6 cm, and both ends of the casing were sealed tightly. Surimi gels were prepared by incubating the sol at 40°C for 30 min, followed by heating at 90°C for 20 min. The gels were cooled in iced water and stored over night at 4°C prior to analysis. Gel was 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 punch test according to the method of Benjakul *et al.* (2001)

2. Whiteness

Whiteness of gel was also determined as described by Benjakul *et al.* (2001) 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.

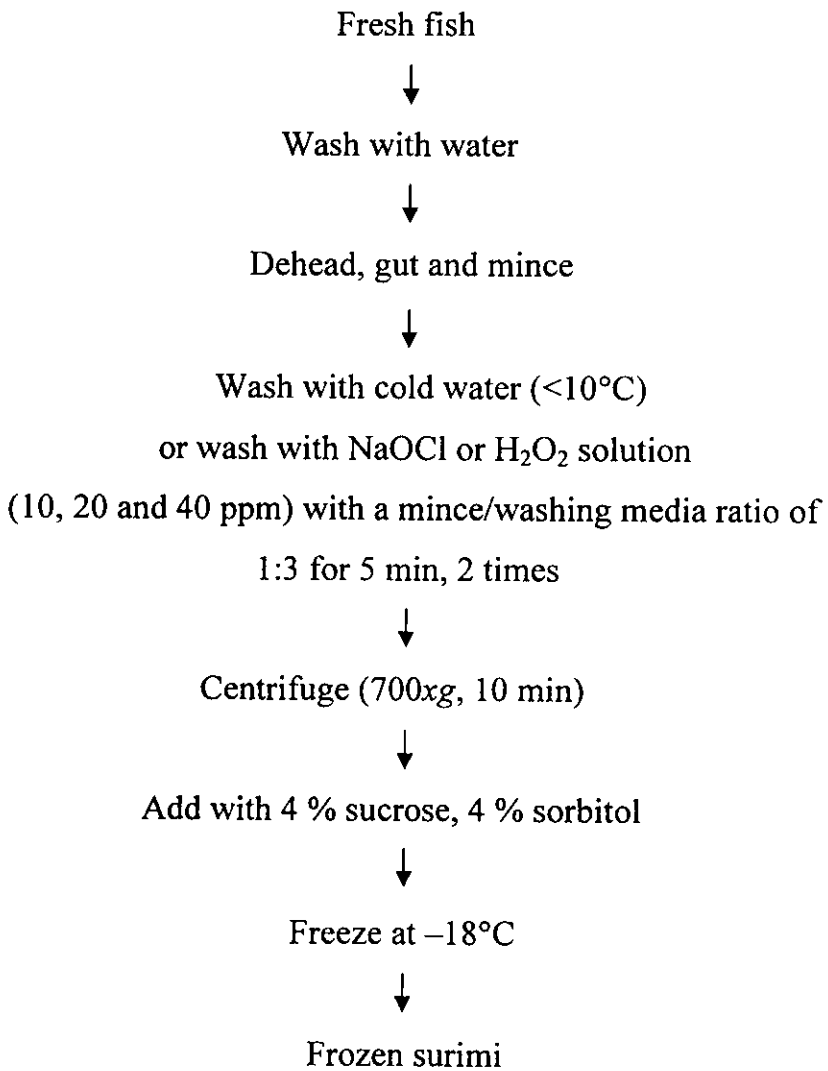


Figure 5 Scheme for surimi preparation

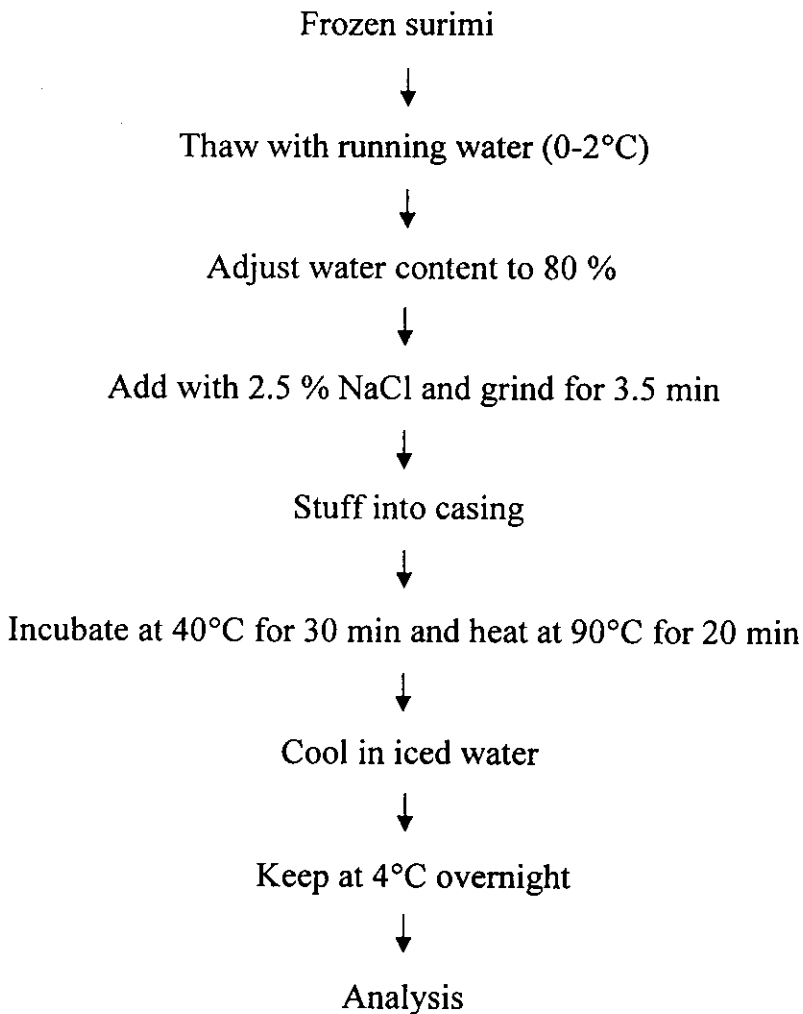


Figure 6 Scheme for surimi gel preparation

4.2. Physicochemical properties of muscle proteins

Washing medium rendering the highest breaking force and deformation was chosen. The mince washed with the appropriate washing medium was subjected to the following analysis in comparison with unwashed mince.

Determination of ATPase activity

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 5,000xg for 30 min at 4°C. Three volume of chilled water (0-2°C) was added to precipitate NAM. The NAM was collected by centrifuging at 5,000xg 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.

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 CaCl₂ for Ca²⁺-ATPase; 2 mM MgCl₂ for Mg²⁺-ATPase; 0.1 mM EGTA for Mg²⁺-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 3,500xg 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 trichloroacetic acid prior to addition of ATP.

Determination of total sulfhydryl content

Total sulfhydryl content was determined using 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB) according to the method of Ellman (1959) as modified by Benjakul *et al.* (1997). To 1.0 ml of NAM solution (4.0 mg/ml), 9 ml of 0.2 M Tris-HCl buffer, pH 6.8, containing 8 M urea, 2 % SDS and 10 mM EDTA, were added. To 4 ml of the mixture, 0.4 ml of 0.1 % DTNB was added and incubated at 40°C for 25 min. Absorbance at 412 nm was then measured. A blank was conducted by replacing the sample with 0.6 M KCl. Sulfhydryl content was calculated using the extinction coefficient of 13,600 M⁻¹cm⁻¹.

Determination of disulfide bond content

Disulfide bond content in NAM was determined by using 2-nitro-5-thiosulfobenzoate (NTSB) assay according to the method of Thannhauser *et al.* (1987). To 0.5 ml of NAM sample (4.0 mg/ml), 3.0 ml of freshly prepared NTSB assay solution were added. The mixture was incubated in the dark at room temperature (25-30°C) for 25 min. Absorbance at 412 nm was measured. Disulfide bond content was calculated using the extinction coefficient of $13,900 \text{ M}^{-1}\text{cm}^{-1}$.

Determination of surface hydrophobicity

Surface hydrophobicity was determined as described by Benjakul *et al.* (1997) using 1-anililonaphthalene-8-sulphonic acid (ANS) as a probe. NAM dissolved in 10 mM phosphate buffer, pH 6.0 containing 0.6 M NaCl was diluted to 0.1, 0.2, 0.3, and 0.5 % (w/v) protein using the same buffer. The diluted protein solution (2 ml) was added with 20 μl of 8 mM ANS in 0.1 M phosphate buffer, pH 7.0. The fluorescence intensity of ANS-conjugates was measured at an excitation wavelength of 374 nm and an emission wavelength of 485 nm. The initial slope of the plot of fluorescence intensity versus NAM concentration was referred to as SoANS.

4.3. Protein pattern (SDS-PAGE)

Protein patterns of sample 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.

5. Study on the effect of washing time on gelling properties and physicochemical properties of mince washed with oxidizing agent

Mince was washed with washing medium showing the greatest gel strengthening effect for different times (5, 10 and 15 min). Washed mince was subjected to analysis as described in section 4. Surimi gel was prepared and gel properties were determined as previously mentioned in section 4.

6. Study on the effect of some chemicals and MTGase on gel properties of surimi prepared by washing with oxidizing agent

Bigeye snapper, threadfin bream and starry triggerfish stored in ice for 14 days were used for surimi preparation using the washing medium rendering the greatest breaking force and deformation. Different chemicals or MTGase at different levels were added into surimi sol as follows:

- CaCl₂ (20, 50 and 100 mmole/kg)
- *L*-ascorbic acid (0.1, 0.2 and 0.3 %)
- MTGase (0.1, 0.2 and 0.3 %)

The surimi gels were prepared as mentioned in section 4 and gel properties were determined as described in section 4. Bigeye snapper, threadfin bream and starry triggerfish stored in ice for 14 days were used for surimi preparation. MTGase or chemicals with the optimal level rendering the maximal gel strength was added into surimi gel. Gel was then prepared and determined as described in section 4.1. Additionally, microstructure was examined.

To determine the microstructure of surimi gel, 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 CO₂ 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)

7. Study on the effect of washing with oxidizing agent on setting of surimi

Fresh bigeye snapper, threadfin bream and starry triggerfish, were used for surimi preparation by either water or 20 ppm NaOCl washing. Both surimi was added with 100 mmole CaCl_2/kg or 10 mmole EGTA/kg. The surimi gel was prepared by setting at 40°C for 30 min, followed by heating at 90°C for 20 min. The gel properties were determined as follows:

- Breaking force and deformation as described in section 4.
- Protein patterns under reducing and non-reducing conditions according to the method of Laemmli (1970)
- Protein solubility

To 3 g sample, 30 ml of 1 % SDS, 8 M urea and 2 % β ME was added and the mixture was homogenized for 1 min. The homogenate was stirred at room temperature (25-30°C) for 4 h, followed by centrifuging at 10,000xg for 30 min at 4°C. To 10 ml of supernatant, cold 50 % (w/v) trichloroacetic acid was added to obtain the final concentration of 10 %. The precipitate was washed with 10 % thrichloroacetic acid and solubilized in 0.5 M NaOH. Protein content was determined using Biuret method (Robinson and Hodgen, 1940).

8. 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 for windows: SPSS Inc.)