CHAPTER 3 MATERIALS AND METHODS

This chapter presents the overall experimental set-up as well as the techniques which are used in the study of the effect of Kraft lignin on rheological properties and functional properties of fish protein-based biomaterial.

1.1 Raw Materials and Equipments

3.1.1 Raw Materials and Chemicals

1. Threadfin bream (*Nemipterus* sp.), Prachauthid 61 market, Bangkok, Thailand as shown in Figure 3.1a.
2. Kraft lignin, Raja engineering. Co. Ltd, Bangkok, Thailand
3. Reagents
   - Acetone, AR Grade, Labscan, Bangkok, Thailand
   - Anhydrous glycerol ($C_3H_5(OH)_3$), AR Grade, Roongsub Chemical Ltd, New South Wales, Australia
   - Magnesium nitrate ($Mg(NO_3)_2$), AR Grade, Ajax Finechem Ltd, Auckland, New Zealand
   - Sodium azide (NaN_3), AR Grade, Merck
   - Phosphorus pentoxide (P_2O_5), AR Grade, Carlo Erba, Italy
   - 4% Boric acid solution, AR Grade, Riedel-de, Seelze, Germany
   - 2-propanol, AR Grade, Carlo Erba, Rodano, Italy
   - 95% Ethyl alcohol, Commercial Grade, Carlo Erba, Rodano, Italy
   - Hexane, AR Grade, J.T.Baker, Phillipsburg, USA
   - Hydrochloric acid, AR Grade, BHD, UK
   - Kjeltabs, AR Grade, Tecator, Höganäs, Sweden
   - Sodium carbonate, AR Grade, Carlo Erba, Rodano, Italy
   - Sodium hydroxide, AR Grade, Carlo Erba, Rodano, Italy
   - Sulfuric acid, AR Grade, BHD, UK
   - Trizma base, AR Grade, Carlo Erba, Rodano, Italy

3.1.2 Apparatus

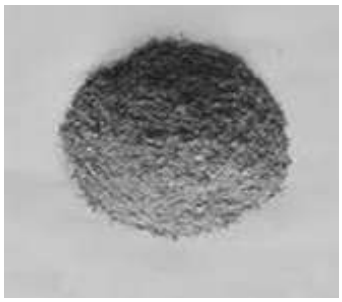
- Centrifuge, Himac, Model CR21, Chiba, Japan
- Chopper, Waring, Model WCG75E, Torrington, LT, USA
- Hot air oven, Memmert, Model ULM 600 II, Germany

- Hot air oven, Termarks, Model TS8000, Bergen, Norway
- Furnance, Ney, Model 2-160, Californea, USA
- Grinder, Waring, Model HSB2WT, Torrington, CT, USA
- Incubator shaker, New Brunswick Scientific, Model G-24, New Jersey, USA
- pH meter, Schott Gerate, Model CG841, Mainz, Germany
- Protein analyzer, Tecator, Model 1002, Hoganas, Sweden
- Fat analyzer, Soxtec System-Texator, Sweden
- Shaking water bath, Heto, Model AT110, Allerod, Denmark
- Sieve shaker, Retsch, Model AS200 basic, Germany
- Stirrer hot plate, Morat-Magnetrüher, Model M21/1, Germany
- Vacuum pack, Mutivac Model A300, Germany
- Weighing machine, Sartorius, Model LA230S, Goettingen, Germany
- Internal mixer, Plasti-corder W50, Brabender, Duisburg, Germany
- Hydraulic Press Machine, 20 T., SMC TOYO METAL Co., Ltd., Thailand
- Thermal analysis instrument, Mettler Toledo TGA/SDTA 851^e, USA
- Differential Scanning Calorimeter, Mettler Toledo DSC 822^e, Mettler Toledo, USA
- Capillary rheometer, RHEO-TESTER 2000, Germany
- Dynamic mechanical thermal analyzer, NETZSCH DMA 242, Piscataway, USA
- Fourier Transform Infrared Spectrometer, Perkin Elmer instruments, Singapore
- Electron Spin Resonance spectroscopy, JES-RE2X, JEOL, Japan.
- Texture Analyzer, Stable Micro System, TA-XT.plus, Surrey, UK

3.2 Preparation of fish protein powder

Threadfin bream (*Nemipterus* sp.) were gutted, headed fish and passed through a meat bone separator. After that, the fish mince were washed twice with tab water, stained in hydraulic machine, chopped in a cutter and dried in hot air oven at 50 ^oC for 5 hours and 40 ^oC for 24 hours or until their weight was constant. Finally, the dried fish protein (FP) was ground less than 425 µm as shown in Figure 3.1b. The fish protein powder was vacuum packed in polyethylene bags.

(a)



(b)

Figure 3.1 Threadfin bream (a), Fish protein powder (b)

3.3 Preparation of plasticized fish protein/ Kraft lignin biomaterial

Materials in this study contain a mixture of fish protein powder: Kraft lignin: glycerol in a weight ratio ranging from 70:0:30 to 0:70:30 as shown in Table 3.1.

Table 3.1 Composition of biomaterial with 0-70% Kraft lignin

Composition (%wt)	**Sample**							
	0% KL	**10% KL**	**20% KL**	**30% KL**	**40% KL**	**50% KL**	**60% KL**	**70% KL**
Fish protein (FP)	70	60	50	40	30	20	10	0
Kraft lignin (KL)	0	10	20	30	40	50	60	70
Glycerol	30	30	30	30	30	30	30	30

3.3.1 Mixing process

Fifty gram of Kraft lignin/fish protein and glycerol were mixed in a mixer (Plasti-corder W50, Brabender, Duisburg, Germany) at temperature 80 °C, 100 rpm for 15 minute. The torque and product temperature were continuously recorded during mixing. The torque rheometer was used to measure the melt flow behavior of biomaterials. Quantitative information for the melt flow of the samples could be obtained by recording the torque and temperature as functions of time during processing. When

sufficiently blended, the solid powders turn into a melt fluid state, and the obtained. Equilibrium torque and temperature were related to the apparent viscosity of the material under process conditions, thus indirectly reflecting the flow behavior.

3.3.2 Compression molding process

Ten or twenty gram of the blend was placed in a squared mould (9 × 9 cm^2) and thermomolded at 100 °C for 15 min. Samples were preheated for 5 min and beginning of the experiments for 10 min in a Hydraulic Press Machine (20 T., SMC TOYO METAL Co., Ltd., Thailand). A pressure of 1 ton was directly applied to the sample in the mold. The thickness of material is approximately about 1 or 2 mm depending on initial weight of blend. Process of fish protein and Kraft lignin specimen preparation is explained below, Figure 3.2.

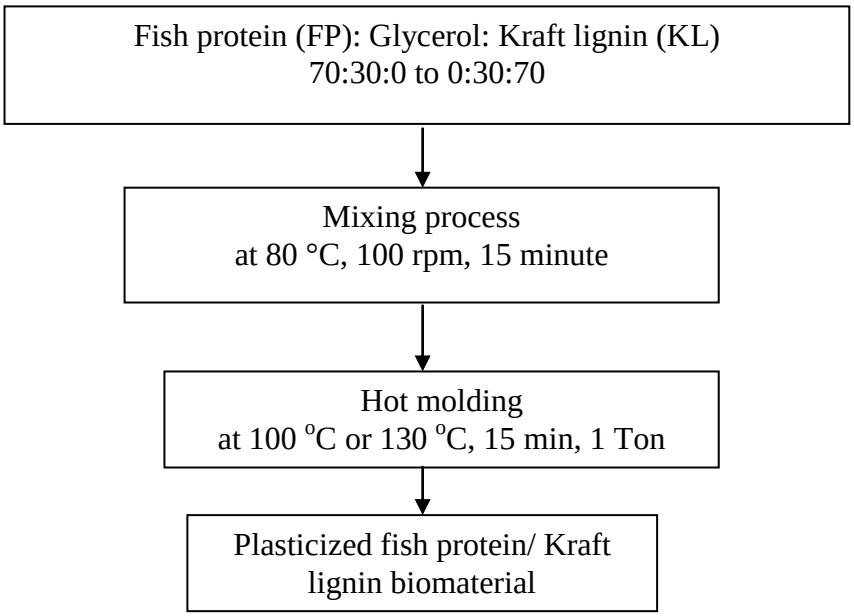


Figure 3.2 Process of plasticized fish protein/ Kraft lignin biomaterial specimen preparation

3.4 Characterization of materials

3.4.1 Proximate composition

The proximate composition was determined according to AOAC methods [47]. Crude protein content was determined using the Kjeldahl method (Kjeltex System-Texator, Sweden). Crude lipid content was determined by the Soxhlet method (Soxtec System-Texator, Sweden). Ash content was determined by burning samples overnight at 550 °C. Moisture content was determined by drying samples overnight at 105 °C until constant weight was achieved. Each sample was analyzed in 3 replicated, standard deviation was calculated and the data was expressed in mean ± SD.

3.4.2 Thermogravimetric analysis (TGA)

Fish protein powders and Kraft lignin were tested by thermal analysis instrument (Mettler Toledo TGA/SDTA 851^{e}, USA). The experiments were carried out in the air. Samples were equilibrated at 0% RH over P_2O_5. The samples were about 5–10 mg, the scope of testing temperature was from the room temperature 30°C to 600 °C and the heating rate was 10 °C/min. Purge the sample chamber with dry nitrogen (or other inert gas) to avoid thermo-oxidative reactions. and record the thermal curve [43]. Select a point on the mass loss thermal curve from before and another on the mass loss plateau immediately after the first mass loss [48]. These temperature points were identified.

3.4.3 Differential Scanning Calorimetry (DSC)

A differential scanning calorimeter was used to measure the glass transition temperature and enthalpy of material melting in the sample. Calorimetric measurements were performed using a Mettler Toledo DSC 822^{e} (Mettler Toledo, USA). An empty stainless steel pan was used in the reference holder. Fish protein powder and Kraft lignin were equilibrated over P_2O_5, 0%RH. The samples were heated first time at 10 ºC/min from 25 to 250 ºC. It was then cooled to 25 ºC at 10 ºC/min and heated a second time at 10 ºC/min to 250 ºC. Finally the samples were cooled to 25 ºC at 10 ºC/min. The glass

transition temperature (T_g), was determined as the temperature midpoint of the heat capacity change observed during the second run [49].

3.5 Characterization of plasticized fish protein/Kraft lignin biomaterial

3.5.1 Rheological Analysis

After mixing, the melt flow behavior of one hundred gram of plasticized fish protein/ Kraft lignin blends were determined by a one barrel capillary rheometer (capillary rheometer RHEO-TESTER 2000, Germany) with a 2-mm capillary die and a length-to-diameter ratio of 15. Measurements were carried out at 140 °C under a shear rate ranging from 10 to 1000 s^{-1}(with step change of 50 s^{-1}).

The power law model was used to determine the rheological behavior that describe relationship between viscosity and shear rate is

$$\tau_w = K \dot{\gamma}_w^n \tag{3.1}$$

where the consistency (K) corresponds to the viscosity value for a shear rate γ of s^{-1} and the power-law index (n) characterizes the deviation from the Newtonian behavior.

The apparent viscosity of samples (η), at constant shear rate, was calculated as follows:

$$\eta = \frac{\tau_w}{\dot{\gamma}_w} = K \dot{\gamma}^{n-1} \tag{3.2}$$

Where: η correspond to apparent viscosity. The values of n are between 0 and 1 for thermoplastic materials, which explains their shear thinning behavior [50-52].

3.5.2 Glass transition temperature (T_g)

Thermomechanical properties were conducted with a dynamic mechanical thermal analyzer (NETZSCH DMA 242, Piscataway, USA). Rectangular samples (15 × 3 × 1 mm^3) were preconditioned at 25 ^oC and 0 % relative humidity over P_2O_5. After that they were analyzed with equipped with a cryogenic system fed with liquid nitrogen. A tensile test was performed with a temperature ramp from –100 to 200°C at a heating rate of

3°C.min^{-1}. A variable sinusoidal mechanical stress was applied to the sample (frequency = 1 Hz) to produce a sinusoidal strain amplitude of 0.05% and the displacement amplitude of 2 µm, which ensure measurements in the linear domain of viscoelasticity [53]. During analysis, the storage modulus (E'), the loss modulus (E") and tan δ (=E"/E') were recorded and plotted against temperature for further evaluation of thermal transition. T_g was identified as the temperature of the tan δ maximum.

3.5.3 FTIR spectra

The dry samples were sprinkled into a matrix of KBr, and ground in an agate mortar (KBr pellet technique). The samples are tested by using a Fourier Transform Infrared Spectrometer (FTIR, Perkin Elmer instruments, Singapore). Investigations have been performed in the transmission mode at the resolution of 4 cm^{-1}. Each sample recording consisted of 64 scans recorded in % Transmittance from 400–4000 cm^{-1} [11, 13]. Prior to recording, the spectra were transformed against a KBr background. Fourier transform infrared spectroscopy (FTIR) was employed to study the structural organization of biomaterials.

3.5.4 Protein solubility in SDS

Protein solubility was determined according to the method of Kunanopparat et al. [13]. Briefly, the fish protein powder (26.67 mg) was stirred for 80 min at 60 ºC in the presence of 20 ml of 0.1 M sodium phosphate buffer (pH 6.9) containing 1% sodium dodecyl sulfate (SDS). SDS is known to be an efficient dissociating detergent able to disrupt hydrophobic, hydrogen and ionic bonds. The addition of strong anionic surfactant like SDS allows the suppression of the intermolecular interactions between proteins and brings them into solution. The SDS-soluble protein extract was recovered by centrifugation (50 min at 15,000g and 20 ºC), and 1000 µl was used to determine protein content. The remaining SDS-insoluble protein fraction can be further extracted in the same SDS buffer, after the cleavage of the disulfide bridges, which are responsible for the protein interchain cross-linking. The pellet was suspended in 5 ml of SDS-phosphate buffer containing 20 mM dithioerythritol (DTE) as reducing agent that is often used to break the disulfide bonds and decrease the polymer size. After it was shaken for 60 min at 60 ºC, the extract was sonicated for 3 min. These treatments

brought insoluble protein from the pellet into the solution. After centrifugation (50 min, 15,000 g, 20 ºC), a part of the supernatant was determined protein content using the Kjeldahl method (Kjeltex System-Texator, Sweden).

3.5.5 SDS-PAGE

SDS-soluble protein fraction and SDS-insoluble protein fraction were solubilized in an SDS–solution containing 10 ml of 10% SDS, 5% 2-mercaptoethanol, 20% glycerol, 0.5 M Tris–HCl (pH 6.8) and Trace bromophenol blue 0.0003 g. The supernatant was used for gel electrophoresis as described by Laemmli [54]. The amount of protein loaded onto the polyacrylamide gel was 30 µl. A total of 100 µl of each protein were mixed with 400 µL of SDS buffer then, used for gel electrophoresis. Stacking gel and separating gel were made of 4% (w/v) and 15% (w/v) polyacrylamide, respectively. The amount of protein loaded onto the polyacrylamide gel was 15 mg. The Precision Plus Protein^{TM} standard (10-250 kDa) as a marker was also injected at the loading of 20 µl in the left lanes of the gel and the electrophoresis was carried out at 200 V for about 60 min. After electrophoresis, gels were stained and detained with silver staining because of the nature of this protein (high stability and hydrophobicity), it is difficult to solubilize and to digest for electrophoretic analysis. In addition, its low protein content difficult ability prevents it from being stained with simple staining procedures, such as the use of Coomassie Blue.

3.5.6 Electron Spin Resonance (ESR) Spectroscopy

The radicals formed on material after treatments were identified according to the method of Ullsten et al. [10] and quantified by electron spin resonance spectroscopy. Firstly, samples were collected immediately after mixing and molding, were put into liquid nitrogen, and were preconditioned at 0 % relative humidity over P_2O_5. In testing, the samples (8-10 mg) were placed into ESR glass containers. These were immediately transferred to a liquid nitrogen container, in order to inhibit further reaction before the spectromagnetic investigation. The ESR spectra were recorded at liquid-nitrogen (77 K) temperature to detect thiyl and di-sulfide radicals that often cannot be detected at room temperature [55]. ESR spectra (first derivatives) were recorded for the frozen samples using a electron spin resonance spectroscopy (JES-RE2X, JEOL, Japan). ESR spectra

using the following instrumental parameters: scan range, 324.0 mT, width 5.0×10 mT at 1 mW microwave power, sweep time 2 min amplitude 1.6×100 and 0.5 mT modulation amplitude. The g values were determined by standardization with α, α'- diphenyl-β-picryl hydrazyl (DPPH). The g value is calculated from the relationship

$$h\nu = g\beta B \tag{3.7}$$

where h is Plank's constant (6.63×10^{-34} J s),

ν is the microwave frequency (9.4 GHz, measured by a frequency counter),

β is the Bohr magneton (9.27×10^{-24}A m^2),

B is the magnetic field (G), Spectra were recorded during runs.

3.5.7 Mechanical properties

Tensile tests were performed on a Texture Analyzer (Stable Micro System, TA-XT.plus, Surrey, UK). Samples were cut into dumb-bell-shaped specimens of 75 mm overall length and 5 mm width for the elongating part and preconditioned at 25 °C, 53% RH over a saturated salt solution of $Mg(NO_3)_2$. Specimen thickness was measured with a caliper. The initial grip separation was 50 mm and elongation speed was 1 mm·s^{-1}.

The tensile strength, elongation at break and Young's modulus was determined. The elastic constants in The Stress values (Pa), Strain values and Young's modulus are calculated according to equations 3.8, 3.9 and 3.10 respectively.

$$\text{Stress (Pa)} = \text{force values (N)} / \text{cross-sectional area (mm}^2\text{)} \tag{3.8}$$

$$\text{Strain (Pa)} = \text{elongation length } (\Delta L) / \text{initial length } (L_0 = 20 \text{ mm}) \tag{3.9}$$

$$\text{Young's modulus (\%)} = \text{Stress} / \text{Strain} \tag{3.10}$$

Tensile properties-tensile strength, elastic modulus and percent elongation and are indicators of mechanical properties in biomaterial [56].

Tensile strength is the maximum stress that a material can withstand while being stretched or pulled before necking, which is when the specimen's cross-section starts to significantly contract. Tensile strength is the opposite of compressive strength and the values can be quite different.

Elongation at break of material is the percentage increase in length that occurs before it breaks under tension.

Young's modulus is a measure of the stiffness of an elastic material and is a quantity used to characterize materials. A high Young's modulus means that the material is rigid. More stress is required to produce a given amount of strain. In polymers, the modulus and compressive modulus can be close or may vary widely.

3.5.8 Water absorption

Samples (20 mm in diameter) were dried in hot air oven at 50 ^{o}C 48 h. or until their weight was constant (W_i). Then, they were immersed in 50 ml distilled water containing 0.05% NaN_3 (to avoid the microbial growth) at 25 ^{o}C. The swollen samples were wiped and weighed (W_w) after 1 week. Then, they were dried in hot air oven at 50 ^{o}C 48 h. or until their weight was constant (W_f). Each sample was analyzed in four replicate. Water absorption was calculated by the following equations (3.6):

$$\text{Water absorption (\%)} = 100 \times (W_w - W_f) / (W_i) \quad (3.11)$$

3.6 Statistical analysis

The experimental data were analyzed and presented as mean values with standard deviations. The one-way ANOVA was used to compare means at 95% confidence ($p \leq 0.05$). The statistical program SPSS (version 11) was used to perform all statistical calculations.