2. MATERIALS AND METHODS

The first samples were taken at Songkhla Canning, Songkhla, November 1997, with subsequent approximately monthly sampling until March 1999. The workplan has been divided into 3 parts

A. Seasonal variation of tuna viscera, roe and milt content

The seasonal variations is monitored monthly at Songkhla Canning, by weighing tuna (10 fish), viscera , roe and milt (if available). If possible all species produced should be assayed; skipjack, tonggo, bonito etc. The purpose of this study is to get an "guesstimate" of roe, viscera and milt content.

The fish were taken at random from the conveyor belt prior to evisceration of the fish.The fish, viscera (with roe) , roe and milt were individually weighed, using
a) Balance 0-10 kg, +/- 100g,
b) Balance (kitchen model) 0-3 kg, +/- 100 g and
c) Balance (laboratory model) 0-1kg +/- 1 g.

The samples were analyzed for chemical composition

- dry matter (DM),protein and ash (AOAC, 1984)
- lipid content (Bligh and Dyer, 1959)

Information regarding the amount of raw material processed will be collected from the factory after approximately one year of monitoring.

B. Extraction and characterization of lipids from viscera, roe and milt of tuna

The lipids from all raw materials are extracted using 2 different organic solvent methods (chloroform-methanol, Bligh&Dyer,1959 and hexane-isopropanol , Hara&Radin, 1979)
The extracted lipid were determined for

- Total lipid content (Bligh and Dyer, 1959 ; Hara and Radin, 1979)
- Total phospholipid content (Bartlett, 1959 ; Christie, 1982)
- Free fatty acid (FFA) content (IUPAC, 1979)
- Iodine value (IUPAC, 1979)
- Saponification value (IUPAC, 1979)
- Fatty acid acid profile is determined by GC after the preparation of methylesters (acetyl-methanol in benzene method).

Methyl-esters were prepared by the acetyl chlorid method (Loewaas,1998) at a concentration of 10 mg/ml. 1 µl was injected on a fused silica capillary (Machery-Nagel: Permabond FFAP-DF-0.25, 25 m x 0.25 mm.) with Helium at 25 Psi as carrier gas and column temperature at 60oC for 1 min, rate 20°C/min to 200°C , 8 min at 200°C, rate 10°C/min to 220°C and 12 min at this temperature. The GC was a Perkin Elmer GC with Turbochrom program for peak calculations.

C. Protein hydrolysate and oil separation from tuna viscera, roe and testies by enzyme hydrolysis

Materials and Reagents

By-products (roe, testies, and viscera) from Skipjack tuna (*Katsuwonus pelamis*) which caught in the Pacific Ocean in October 2002 were obtained from tuna canning company (Songkhla Canning Public Co., Ltd., Songkhla, Thailand).

2,4,6-Trinitrobenzenesulfonic acid (TNBS),, and L-leucine were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium dodecyl sulfate (SDS) and sodium sulfite were purchased from Fluka Chemie (Switzerland). Alcalase 2.4 L (a declared activity of 2.4 AU/g and density of 1.18 g/ml) was provided by Novo Nordisk (Denmark). All reagents used were of analytical grade.

Composition analysis

Chemical composition of samples including roe, testies, and viscera were determined as described below. Moisture content was measured by oven drying at 105°C until a constant weight (AOAC, 1990, method 985.14). Protein and ash were determined by according to method 928.08 (Kjeldahl method) and 920.153, respectively (AOAC, 1990). Lipid was assayed using solvent extraction (Bligh and Dyer, 1959). Iodine value and saponification number of oil was determined by method 920.159 and 920.160, respectively.

Enzymic hydrolysis of tuna by-products

Ground tuna by-products (15 grams) was added with 0.2 M Tris-HCl buffer (pH 8.0) at the ratio of 1 : 2 (w/v) and pH of mixture was rechecked and adjusted with 6 N NaOH or 6 N HCl. The mixtures were incubated and well-shaken at 50°C for 10 min. Alcalase was added to the reaction mixtures at enzyme/substrate concentration 0.2% (w/w). The reaction mixtures were periodically sampled at 30, 60, 90, 120, 150, and 180 min. The reaction was stopped by heating at 85°C for 15 min, the supernatant was obtained by centrifuging at 8000 rpm for 15 min at 4°C. The hydrolysate obtained from different times was determined for α-amino acid, DH, NR (Benjakul and

Morrissey, 1997), oil content (Bligh and Dyer, 1959), and polar lipid content (Kaitaranta and Ackman, 1981).

Determination of α-amino acid and degree of hydrolysis (DH)

Properly diluted sample (500 μL) were mixed with 2.0 mL of 1% hot SDS solution (85°C) and place in a water bath at 85°C for 15 min. Then 125 μL of mixtures were mixed thoroughly with 2.0 mL of 0.2125 M phosphate buffer, pH 8.2, followed by the addition of 1.0 mL of 0.01% TNBS solution. The mixtures were then placed in a water bath at 50°C 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 down at ambient temperature for 15 min. The absorbance was measured at 420 nm and α-amino acid wasexpressed in terms of L-leucine. The DH was determined and defined as follows:

$$DH = \{(Lt - Lo) / (Lmax - Lo)\} \times 100$$

where Lo was the amount of α-amino acid in original tuna by-products, Lt corresponded to the amount of α-amino acid released at time t, Lmax was the maximum amount of α-amino acid in tuna by-products obtained after acid hydrolysis (Benjakul and Morrissey, 1997). The suspension of tuna by-products (500 μL) was mixed with 4.5 mL of 6 N HCl. The tube with sample mixtures was flashed with nitrogen gas and sealed tightly with screw-cap. The hydrolysis was run at 100°C for 24 h (Beak and Cadwallader, 1995). The acid-hydrolyzed sample was filtered through Whatman paper No. 1 to remove the unhydrolyzed materials. The filtrate was neutralized with 6 N NaOH before determined for α-amino acid.

Determination of nitrogen recovery (NR)

After the hydrolysis reaction, the supernatant was obtained by centrifuging at 8000 rpm for 15 min. The dense lipid layer was skimmed using two-layers of cheese cloth. The total nitrogen in supernatant was determined using Kjeldahl method (AOAC, 1990). NR was calculated using the following equation: NR (%) = [total nitrogen in supernatant (mg) / total nitrogen in substrate (mg)] x 100 (Benjakul and Morrissey, 1997).

Extraction of oil from hydrolysate

Modified method of Bligh and Dyer (1959) was used to determine oil content. The hydrosate (20 g) was mixed with 80 mL of MeOH, 40 mL of chloroform, and 20 mL of water, then homogenized at 11000 rpm for 2 min. The mixture was added with 40 mL of chloroform and homogenized at 11000 rpm for 1 min. After adding of 40 mL of water, the mixture was homogenized at 11000 rpm for 0.5 min. The mixture was centrifuged at 10000 rpm for 20

at 11000 rpm for 0.5 min. The mixture was centrifuged at 10000 rpm for 20 min at 4°C. Oil in chloroform phase was separated and the chloroform was evaporated under vacuum. Oil content was measured by weighting and the oil was determined for iodine value, saponification number (AOAC, 1999), and polar lipid content (Kaitaranta and Ackman, 1981).

Determination of polar lipid content

The polar lipid content was determined using thin-layer chromatography (TLC) according to method of Kaitaranta and Ackman (1981) with slightly modification. The TLC separation of polar lipid for the Iatroscan TH-10 analyzer (Iatron Laboratories Inc., Japan) was accomplished on Chromarod S (silica gel) rods. These were stored in 3% boric acid, dried in hot air oven at 105°C for 5 min, and activated by passing them through the flame of the detector of the analyzer under conditions of 30 sec/scan for scan rate, H2 flow rate at 160 mL / min, and air flow rate at 2000 mL / min. After spoted the sample, the Chromarods were developed in paper-lined glass tanks using the solvent mixtures petroleum ether:benzene: formic acid, 92:17:1 (by vol), and petroleum ether:diethyl ether:formic acid, 97:4:1 (by vol). After development for 17.5 min in the former solvent system, the rods were dried for about 5 min at 105°C before the second development in the latter solvent system (17.5 min). After drying the polar lipid was quantitated on the Iatroscan analyzer. The peak areas were changed to weight percent using conversion factors established with standard compounds.