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Enzymatic Hydrolysis of Yellowfin Tuna (*Thunnus albacares*) By-Products Using Protamex Protease

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Abstract :

Long-term proteolysis of tuna by-products (head, viscera and tail) by the wide spectrum protease Protamex has been investigated and compared. After hydrolysis, two fractions (soluble aqueous phase and insoluble sludge) were collected. Chemical compositions of each fraction and molecular mass distributions of soluble peptides were determined. Degrees of hydrolysis obtained after 12 h of hydrolysis of head, viscera and tail were 32.3, 16.8 and 22.2 %, respectively. Nitrogen recovery in the soluble fractions was 73.6 % for the head, 82.7 % for the viscera and 85.8 % for the tail. Lipid distribution indicated that the majority of lipids remained in the sludge. Such proteolysis appears useful for the production of very different fractions: one rich in peptides of medium to small molecular mass and poor in lipids, and another one containing the insoluble proteins and the majority of lipids.

Keywords : tuna; by-products; proteolysis; nitrogen recovery; fish protein hydrolysates

1. Introduction

According to the Food and Agriculture Organization of the United Nations, preliminary estimates for 2006 based on reports from some major fishing countries, total world fishery production reached 143.6 million tonnes. Of that, 76.8 % (110.3 million tonnes) was used for direct human consumption, while the remaining 23.2 % (33.3 million tonnes) was determined for non-food products (*1*).

Tuna is one of the most economically important groups of fish species. The principal market tuna species are skipjack, yellowfin, bigeye, albacore and bluefin. Skipjack is the largest species in terms of landed volume while yellowfin is the largest tuna species in terms of international trade. The main forms for international trade are the raw material for canning, the precooked loin for canning or frozen products, tuna for direct consumption (sashimi), canned tuna, smoked tuna and dried tuna.

Seafood processing in general and tuna processing in particular generates a significant amount of wastes. During processing, much is done to maximize the yield of directly edible products but the production of waste or by-products is inevitable. Much of this is generally discarded as waste or as low-value products; this is the case for the tuna head, viscera and tail.

Fish by-products contain valuable protein and lipid fractions as well as vitamins and minerals (*2*), but are also an important source of environmental contamination. Environmental regulations are becoming stricter, requiring new methods for managing those fish by-products. There are many options for seafood waste management that could help to resolve these problems. Among them, the production of fish meal and fish oil is the most used worldwide. However, enzymatic hydrolysis is one of the most efficient methods to recover protein and thus to increase the commercial value of such biomass.

Indeed, a lot of fish by-products have been hydrolyzed such as salmon head (*3*), salmon frame (*4*), sole frame (*5*), sardine viscera and head (*6*). Preferred enzymes are large spectra proteases such as Alcalase (*7*), Neutrase (*8*), Protamex (*9*) and Kojizyme (*10*). Protamex is known to produce bitterless hydrolysates and was therefore chosen for this study.

Among the different biochemical parameters, the degree of hydrolysis (DH) is one of the most important characteristics as it directly influences the peptide length and their nutritional, functional and sensory properties. Moreover, DH is positively correlated to the solubility of the hydrolysates and thereby to the digestibility of the protein. In addition to the DH, protein recovery and molecular mass distribution of the soluble peptides are important as they give interesting information on the possible use of the hydrolysates (*11*).

The objective of this work is to investigate the effect of long-term hydrolysis (12 h) of three tuna by-products (head, tail and viscera) using Protamex. Degree of hydrolysis, protein recovery, molecular mass profile of the soluble peptides and biochemical composition of the resulting fractions are determined and discussed.

2. Materials and Methods

2.1. Raw materials

Yellowfin tuna (*Thunnus albacares*) was provided by Hai Vuong, a seafood processing company in Nha Trang, Vietnam. The tuna were caught in the Pacific Ocean and were filleted at the company. Head, viscera and tail (which also contains a small fraction of the backbone) were collected, frozen and transported to IFREMER (French institute for exploitation of the sea, Nantes, France). After their arrival, they were thawed and ground separately twice. The minced materials were packed in plastic bags (0.5 kg per unit), frozen and stored at -20 °C until their use.

2.2. Enzyme

The enzyme Protamex used for the hydrolysis was provided by Novozymes A/S (Bagsvaerd, Denmark). It is a *Bacillus* protease complex (EC numbers: 3.4.21.62 and 3.4.24.28) developed for the hydrolysis of food proteins (*12*).

2.3. Hydrolysis

Tuna by-products (head, viscera and tail) were thawed at 4 °C overnight and a volume of distilled water was added to an volume of raw material. Enzymatic hydrolysis was performed in a closed glass vessel with a double jacket. The mixture was stirred at 300 rpm until the temperature reached 45 °C, then the enzyme was added at 0.1 % of the masse of raw material. The hydrolysis was carried out for 12 h at 45 °C without any control of the pH (the initial pH of the substrates was 6.3-6.5). Temperature was stabilized with a continuous flow water bath. Aliquots were taken hourly during hydrolysis for the determination of degree of hydrolysis and nitrogen solubilisation. The solution was heated at 95 °C for 15 min in order to inactivate the enzyme. This solution was then centrifuged at 10 000x*g* at 4 °C for 30 min. Two fractions were collected: the soluble aqueous phase and the insoluble sludge. These fractions were then freeze-dried and stored at -20 °C until analyses.

2.4. Chemical analyses

Moisture content in the raw materials and in the produced fractions from hydrolysis was determined after drying at 105 °C. Ash content was measured by incinerating the samples in a furnace at 600 °C. Total nitrogen content was determined by the Kjeldahl method (*13*) and crude protein content was estimated by multiplying total nitrogen content by 6.25. Lipid content was determined after the extraction of lipids from the samples according to the method of Folch et al. (*14*).

2.5. Degree of hydrolysis

The degree of hydrolysis (DH) is defined as the ratio between the number of broken peptide bonds (h) and the total number of peptide bonds per mass unit (h_{tot}):

$$DH = \frac{h}{h\text{tot}} \times 100 \qquad /1/$$

For every hydrolyzed peptide bond, a free α amino group is formed. The degree of hydrolysis was determined by measuring the amount of free α amino groups, based on the reaction

between Sanger's reagent or dinitrofluorobenzene (DNFB) and the amino groups in the amino acids, which results in a yellow complex of dinitrophenyl amino acids (*15*). Absorbance was measured spectrophotometically at 410 nm.

2.6. Nitrogen recovery

Nitrogen recovery (NR) in the soluble fraction was calculated using the following formula (*16*):

$$NR = \frac{\text{Total nitrogen in the soluble fraction}}{\text{Total nitrogen in the substrate}} \times 100 \qquad /2/$$

2.7. Molecular mass distribution

The molecular mass distribution of peptides in the hydrolysates was analysed by gel filtration chromatography. The molecular mass fractions were separated using a high- performance liquid chromatography (HPLC) system equipped with a size exclusion column (Superdex Peptide 10/300 GL, GE Healthcare UK Ltd, Chalfont, UK). The mobile phase consisted of water with trifluoroacetic acid 0.1 % and acetonitrile 0.5 % (70:30) and the flow rate was 0.5 mL/min. Chromatography was monitored by measuring the absorbance at 214 nm. The column was calibrated with standards: ribonuclease A (13 700 Da), aprotinin (6500 Da), renin (1760 Da), vasopressine (1084 Da) and leucine (294 Da). The molecular mass ranges of the different fractions were based on the retention times of the collected fractions and determined from a standard curve.

2.8. Statistical analysis

A statistical program (SPSS, SPSS Inc., Chicago, IL, USA) and Microsoft Excel were used for data processing and statistical analysis. Data were subjected to analysis of variance (ANOVA). Mean values were accepted as significantly different at 95 % level ($p<0.05$).

3. Results and Discussion

Like all raw materials from living beings, tuna by-products possess enzymatic activities and notably proteolytic ones. Indeed, the main proteases found in marine raw material are acid proteases (pepsin), serine proteases (trypsin, chymotrypsin, elastase), cystine proteases (calpain) and metallo protease (collagenase) (*17*). As the raw materials used here had not been heated before the hydrolysis, the endogenous enzymes were still active.

To our knowledge, very few studies have mentioned the proteolytic activity of fish head or fish tail. However, a study on trout heads has revealed that they have a proteolytic activity on a large pH scale (pH=2-11), indicating the presence of several kinds of enzymes, as serine or acid proteases (*18*). On the contrary, viscera, which are well known to possess a high proteolytic activity due to their digestive functions, have been widely studied (*19*). Thus, regarding tuna, the most important digestive enzymes identified are trypsin, chymotrypsin and pepsine (*20-22*). Trypsin and chymotrypsin optimal activities isolated from tuna viscera were found to occur at pH and temperature above 8 and 55 °C respectively (*20,21*). Maximum activity of pepsin was found at pH=2 and 50 °C (*22*).

In the present study, even if the hydrolysis conditions (pH ranging from 6.3 to 6.5 and 45 °C) were not so close to the optima, proteolysis by the endogenous enzymes may have occurred. However, it is well known that a preliminary thermal inactivation of these enzymes changes the protein conformation in the substrate, leading to a great modification of the hydrolysis by the external enzymes. As the aim of this work is to propose to industry a simple (fast and economic) way to upgrade these wastes as useful fractions, it has been decided to keep the digestive enzymes active and to avoid thermal treatment. In addition, it has been proven that in spite of the high amount of endogenous enzymes in viscera, the use of endogenous and exogenous enzymes enhances the solubililsation of the dry matter (*23*).

Without any antimicrobial treatment (of the raw material and during the hydrolysis) bacterial proliferation may have occurred. However, 45 °C is a moderate temperature necessary to perform the proteolysis but adequate to keep the potential microbial growth to a minimum.

Compounds that can be microbially formed during the process may affect the global odour of the products (due to complex mixtures of volatile compounds such as unsaturated carbonyl compounds and alcohols with 6, 8 or 9 carbon atoms). However, non-significant modification of the overall aroma was perceived by a sensory panel even after 12 h of hydrolysis, confirming a reduced bacterial proliferation under such conditions of hydrolysis.

3.1. Proximate chemical composition of tuna by-products

The average proximate chemical compositions (based on triplicates) of tuna by-products are given in Table 1. All the by-products contain a great quantity of water (58-77 %), and medium amount of protein (15-17 %). They differ mostly in their lipid and ash content. Viscera and tails do not contain more than 4 % of lipids, while heads are at least 3 times richer (13 %). Their mineral content varies from 2 % (viscera) to 20 % (tail).

3.2. Degree of hydrolysis

The by-products can be divided in 2 groups: ‘non-solid’ (viscera) and ‘solid’ (head and tail), which contain material more resistant to proteolysis (bones). During the hydrolysis, all the by-products turned into a brownish liquid. Average hydrolytic curves (based on triplicates) of tuna head, viscera and tail are shown in Fig. 1. The degree of hydrolysis measured in the soluble fraction obtained from different materials increased with time. All the curves exhibited an initial fast reaction. Thereafter, the rate of enzymatic hydrolysis decreased and reached a stationary phase. Thus a plateau was obtained after 9 h for the viscera, after 10 h for the tail and 11 h for the head.

Such shapes of hydrolysis curves were similar to those previously published for yellowfin tuna stomach (*24,25*), herring muscle and head (*26,27*), salmon muscle (*28,29*), salmon head (*30,31*) and cod muscle (*32*). Indeed, these kinetics can be divided into two different phases: the first stage where the reaction speed is fast, which corresponds to an easy breakdown of peptide bonds, and the second stage, where the reaction speed decreases, which corresponds to the hydrolysis of more compact proteins. Moreover, it is well known that some inhibition may occur like the one due to lipid oxidation (*33*), or the one due to an increase of peptides. In fact, in the initial period of hydrolysis, a large number of peptide bonds were cleaved, leading to an increase of soluble peptides in the reaction mixture. These soluble peptides may act as effective substrate competitors to the undigested or partially digested proteins. In addition, some partial enzyme inactivation may also have occurred (*25*).

After 12 hours of hydrolysis, the DH values for the 'solid' by-products were 32.3 and 22.2 % (for head and tail, respectively). These values are higher than most of those published previously on marine by-products without hydrolysis optimisation (*8,27,29*). However, most of the authors have conducted short time hydrolysis (less than 4 h). In the case of long time hydrolysis study using Protamex, similar DH was obtained: up to 27 % for fish backbones (*34*). The DH obtained for the viscera was quite low (less than 17 %) even after 12 h. This result was expected as it had been notified by some authors on marine viscera (*23-25*). Such difference in DH among three raw materials may be due to the difference in protein composition of tissues and illustrates that the substrate affects the course of proteolysis.

3.3. Dry matter distribution

Dry matter distributions in soluble and insoluble fractions obtained after centrifugation and freeze-drying are shown in Fig. 2. Large differences can be observed between the hydrolysed and non-hydrolysed substrates. Prior to proteolysis, only 25 % of head dry matter were located in the soluble fraction, while the remaining part was found in the insoluble fraction (75 %). For the non-hydrolysed viscera, 63 % of the dry matter was located in the supernatant, while 37 % was in the sludge. For the non-hydrolysed tail, 61.4 % of dry matter was found in the soluble fraction and 38.6 % in the insoluble fraction.

During hydrolysis, the percentage of dry matter located in the soluble fraction increased. This indicates that the dry matter was solubilised during hydrolysis under the action of Protamex enzyme. After 12 h of proteolysis, 48.2, 83.1 and 77.7 % of dry matter of head, viscera and tail, respectively, were found in the soluble fractions. Similar results were found in recent studies on the proteolysis of fish by-products (*23,35,36*). However, it is important to notice that despite the long time of action, at least 52 % of dry matter of the tuna head, 17 % of the tuna viscera and 22 % of the tuna tail remained insoluble.

3.4. Chemical composition of freeze -dried fractions

Table 2 shows the average chemical composition (based on triplicates) of the freeze-dried soluble and insoluble fractions after 12 h of hydrolysis. Whatever the hydrolysed by-product was, the supernatant appeared rich in proteins (72 to 83 %) and poor in lipids (1 to 3.4 %). Such results illustrate the effectiveness of the protein hydrolysis and its inefficiency on lipid recovery in this fraction (lipids remained in the sludge). These data are in agreement with previous ones where the protein content of the soluble fractions ranged from 62.3 to 91.6 % (*30,37-40*) and the lipid content was 1.6 % for salmon frame (*41*) and 0.4-1.5 % for herring by-products (*27*). However these results are contradictory to some where higher lipid contents (up to 24.5 %) were found in soluble fraction (*31*). This confirms that the composition of substrates influences both the proteolysis process and the composition of resulting fractions (*39*).

In this study, the conditions of proteolysis led to enrichment by factor 2 of the protein content for the tuna head supernatant (36 g of protein per 100 g of dry head and 72 g of protein per 100 g of dry supernatant), while reducing by 22 the content of lipids (33 g of lipid per 100 g of dry head and 1.5 g of lipid per 100 g of dry supernatant) in comparison with the non-hydrolysed head. Similar results were obtained for the tail hydrolysis, where enrichment by 2 of the protein content (42 to 83 %) and a reduction by 8 of the lipid amount (8 to 1 %) were observed. However, this was not the case for the viscera supernatant, where a small variation of the protein content can be observed (from 71 to 83 %), but the great lipid reduction was confirmed (from 16 to 3 %). Regarding the ash content of the soluble fractions, it ranged from 6 (viscera) to 24 % (head). This variability in ash content had previously been observed notably for salmon, herring and pollock (*26,29,42,43*).

As a consequence, the resulting sludges after 12 h of hydrolysis were found to be poorer in proteins and richer in lipids in comparison with the non-hydrolysed materials.

The chemical composition of the initial material strongly influences composition of the resulting fractions. As Protamex is a large spectrum protease, it is the protein content of different fractions (soluble and insoluble) that was mostly affected whatever the raw material. For the lipids, it appears that part of them can be recovered in the supernatants, but great discrepancies were found among the by-products. As previously noticed, it seems that lipid compounds from viscera can be more easily recovered in soluble fraction in comparison with the other by-products ('solid ones'). Further analysis like the lipid characterization (fatty acids, lipid classes, *etc*.) are needed to precise this point.

Thus, if there is a strong relation between the chemical composition of the initial raw materials and that of the fractions obtained from hydrolysis in a quantitative point of view, some qualitative aspects may also interact. Due to their biochemical differences, the resulting fractions may be used for food or feed (*44-48*) or for example as nitrogen sources for microbial growth or fertilizers (*24,49-51*).

3.5. Nitrogen recovery in the soluble fraction

The degree of hydrolysis has been used as an indicator of the cleavage of peptide bond, whereas nitrogen recovery reflects the yield of proteins that can be recovered from the hydrolysis process (*16*).

Fig. 3 shows that the average nitrogen recoveries (based on triplicates) from tuna by-products increased as the hydrolysis progressed. The mass of soluble nitrogen in the tuna head, viscera and tail prior to hydrolysis were 32.8, 64.2 and 51.3 %, respectively. The highest proportion of soluble nitrogen found in the viscera is certainly due to the fact that this raw material is richer in endogenous enzymes, notably digestive proteases. Such results have previously been observed, notably with cuttlefish and sardine viscera (*23*). On the contrary, the 'solid' by-products (head and tail) are richer in hard tissues like bones and cartilage, and poorer in endogenous enzymes, leading to a lower solubilisation of nitrogen without exogenous enzyme action.

Within the first 8 h of hydrolysis, the nitrogen recoveries increased rapidly and reached 72.1 % for the head, 81.8 % for the viscera and 84.6 % for the tail. Afterwards, nitrogen recovery increased slowly or stayed constant until 12 h of hydrolysis. At the end of the proteolysis, nitrogen recovery was high for viscera and tail (82.7 and 85.8 % respectively) and a little bit lower for head (73.6 %). These results are in accordance with those obtained in previous studies on fish by-products (*4,52,53*).

It seems that under such experimental conditions nitrogen recovery with Protamex occurred mainly during the first 8 h. After this period, despite a continuous increase of the DH, Protamex was no longer capable of protein solubilization, but remained active by breaking down peptide bonds of the solubilised proteins, leading to the formation of smaller peptides in the supernatant.

3.6. Molecular mass distribution

As expected, the longer the proteolysis was, the smaller the peptides were (Figs. 4-6). Proteolysis by breaking the peptide bonds enhanced the solubility of proteins (protein recovery), but also shortened these peptides. However, some important differences can be noticed. Before hydrolysis, 45 and 38.3 % of peptides with a molecular mass above 7000 Da

were detected in head and tail, but only 5.8 % in the viscera (84 % were below 2000 Da). During hydrolysis, whatever the tuna by-products, the concentration of all the peptides above 3000 Da diminished. After 12 h of proteolysis, these peptides represented only 10 % of the tuna head hydrolysates, less than 2 % of the viscera supernatant (92.5 % were below 1000 Da), and 27.3 % of the tail hydrolysates.

These results confirm the interest to combine different analyses such as the degree of hydrolysis, protein recovery and molecular mass distribution of the peptides in order to better characterize proteolysis.

3.7. Lipid distribution in the fractions

The distribution of lipids in the different fractions after the enzymatic hydrolysis are presented in Table 3. After 12 h of hydrolysis, regardless of the raw materials used, the majority of lipids were found in the insoluble fractions (98, 80 and 90 % for head, viscera and tail, respectively). A significant modification of the lipid distribution is one of the most noticeable effects of the proteolysis conducted here. These data confirm some results obtained on salmon frames and cod viscera where lipids remained in the sludge after proteolysis (*41,54*). However, they are contradictory to a previous work that had been conducted on the hydrolysis of sardine by-products with the same enzyme, where high amount of lipids was found in the soluble phases (*9,35,36*). Such discrepancies may be explained by a qualitative effect. As previously noticed, it seems that proteolytic enzymes (and notably Protamex) can be 'selective' towards lipids and particularly towards their fatty acid composition as supernatant fraction enriched in unsaturated fatty acids was obtained while the corresponding sludge was found enriched in saturated fatty acids.

Thus, even with a high disruption of tissue by a proteolytic action, the lipid distribution in the soluble fractions appears highly dependent on the substrates and on their lipid and protein compositions.

4. Conclusions

This study compared enzymatic hydrolysis of three tuna by-products with a large spectrum protease. As expected, solubilisation of the raw material occurred, leading to an increase of the DH and nitrogen recovery while the molecular mass of the resulting peptides decreased as the hydrolysis progressed. It appears that under conditions used in the study, 8 h of hydrolysis were enough for the protein solubilisation. However, molecular mass reduction of the soluble peptides was observed. Regarding lipids, very small amounts were recovered in the soluble fraction as they remained in the sludge.

Thus, such proteolysis appears useful for producing fractions of interest: one fraction enriched in proteins (peptides of medium to small molecular size) relatively poor in fat, and another one enriched in lipids and minerals but with significant amounts of proteins (insoluble ones). The relatively high level of proteins found in the supernatants may represent an interesting way to upgrade these by-products for food or feed purposes while the resulting sludge may find applications in feed and notably aquaculture industry.

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Tables

Table 1. Proximate chemical composition of yellowfin tuna by-products

Tuna waste	Moisture/%	Protein/%	Lipid/%	Ash/%
head	59.0±1.1	14.8±0.1	13.5±0.1	11.8±1.1
viscera	77.4±0.1	16.0±0.2	3.7±0.5	1.9±0.2
tail	58.2±2.0	17.4±0.2	3.3±0.4	20.5±0.4

data are given as mean value±S.D. (N=3)

Table 2. Proximate chemical composition (% of dry matter) of the fractions obtained after 12 h of hydrolysis of tuna by-products using Protamex

Fractions	By-products	Protein/%	Lipid/%	Ash/%
Soluble	head	72.1±1.1	1.5±0.2	23.7±0.3
	viscera	82.8±1.0	3.4±0.5	5.8±0.1
	tail	82.7±0.1	1.0±0.1	14.7±0.1
Insoluble	head	23.6±0.8	63.1±0.9	8.6±0.1
	viscera	47.7±0.2	42.7±0.9	7.3±0.2
	tail	46.2±0.2	31.6±0.5	17.2±0.2

data are given as mean value±S.D. (N=3)

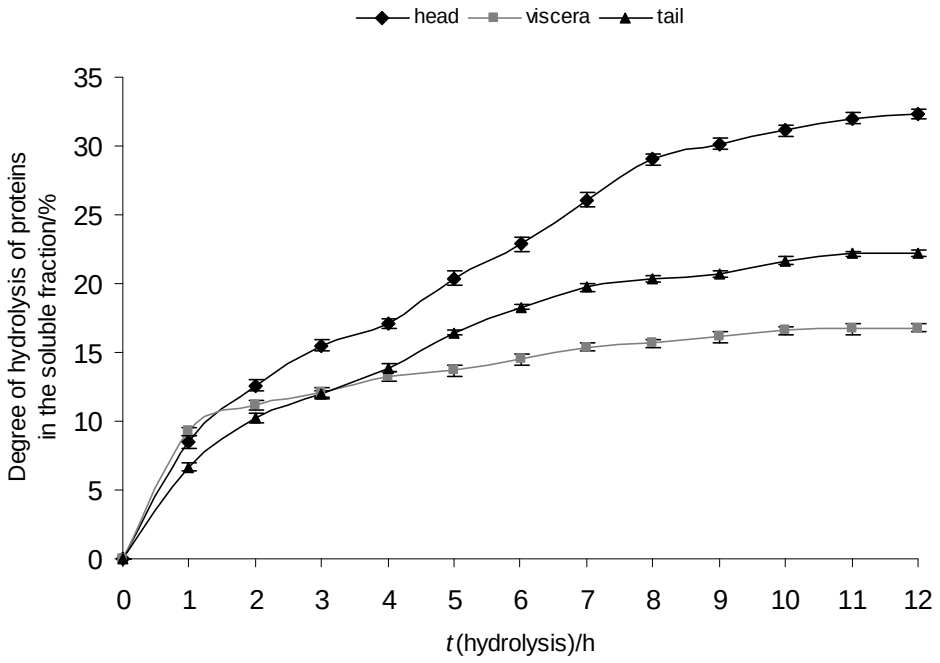
Table 3. Distribution of lipids (% of total lipids) in the fractions obtained after 12 h of hydrolysis of tuna by-products by Protamex

Fractions	Head/%	Viscera/%	Tail/%
soluble	2.1±0.2	20.4±1.6	10.2±0.2
insoluble	97.9±1.4	79.6±1.5	89.8±1.1

data are given as mean value±S.D. (N=3)

Figures

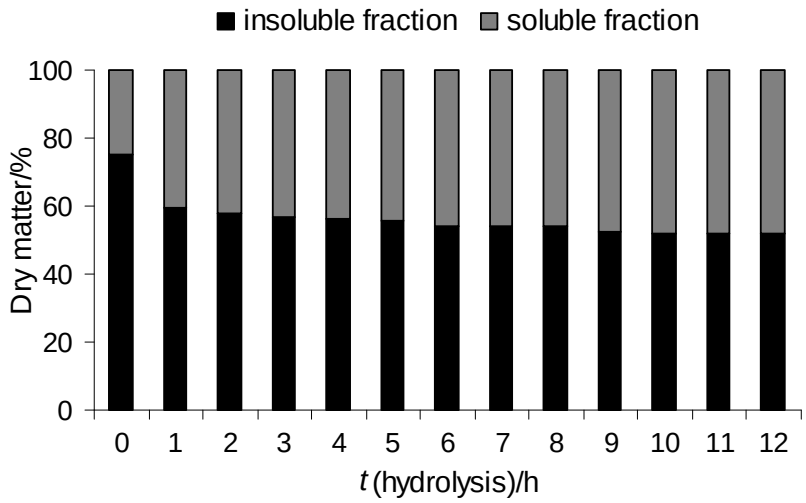
Figure 1. Degree of hydrolysis of soluble fraction at different hydrolysis times



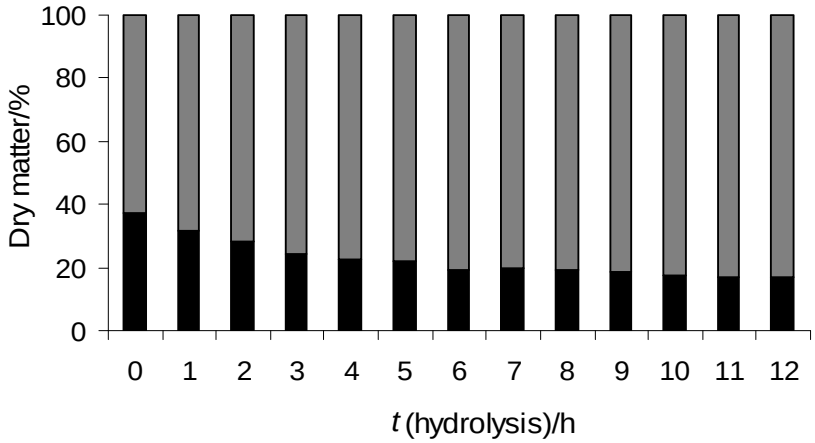
data are given as mean value±S.D. (N=3)

Figure 2. Dry matter distribution in soluble and insoluble fractions of non-hydrolysed and hydrolysed tuna head (a), viscera (b) and tail (c)

(a)



(b)



(c)

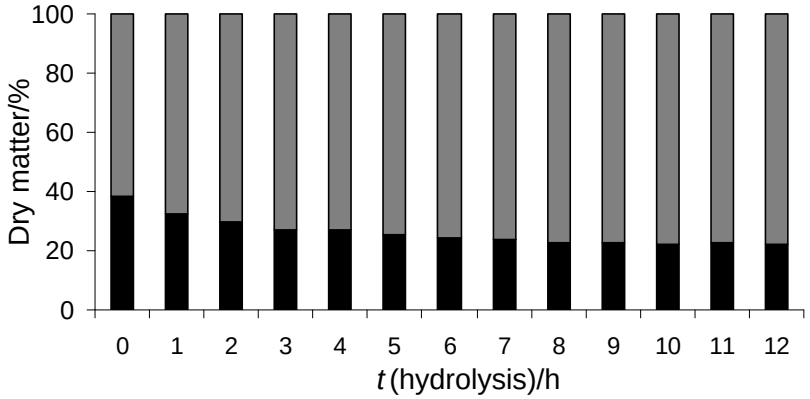
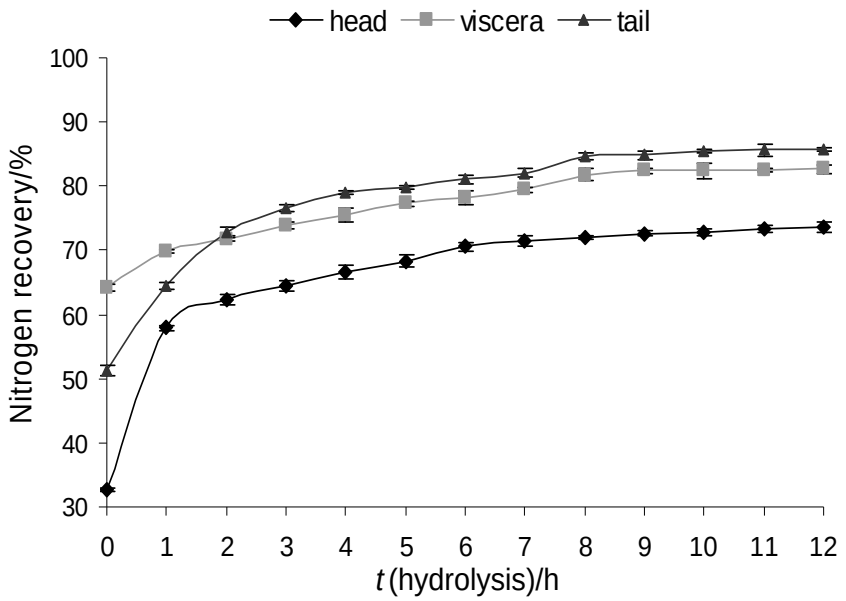


Figure 3. Nitrogen recovery in soluble fraction



data are given as mean value±S.D. (N=3)

Figure 4. Molecular mass distribution of tuna head hydrolysates

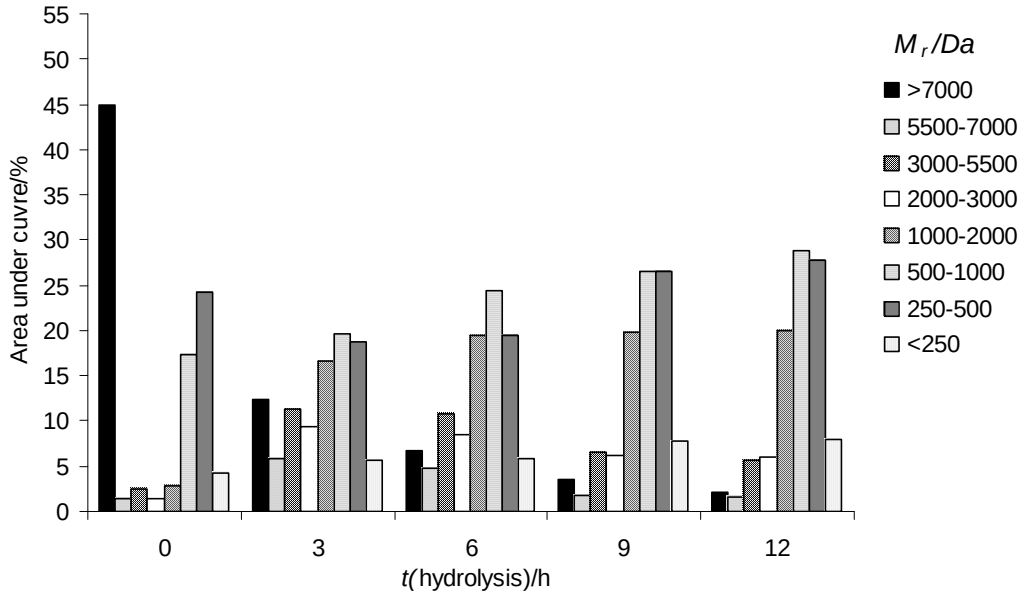


Figure 5. Molecular mass distribution of tuna viscera hydrolysates

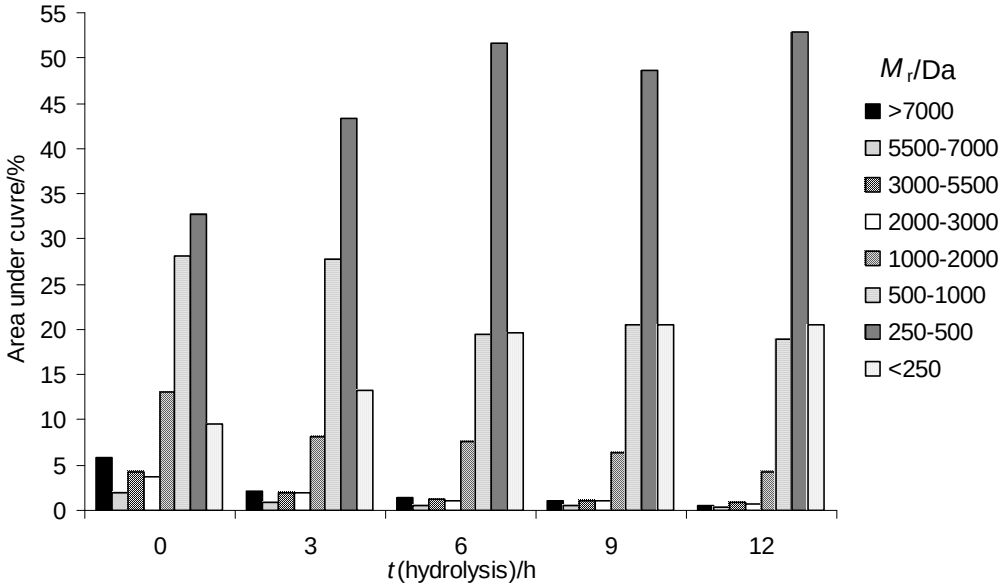


Figure 6. Molecular mass distribution of tuna tail hydrolysates