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Enzymatic hydrolysis of cuttlefish (*Sepia officinalis*) and sardine (*Sardina pilchardus*) viscera using commercial proteases: Effects on lipid distribution and amino acid composition

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Abstract:

Total lipid and phospholipid recovery as well as amino acid quality and composition from cuttlefish (*Sepia officinalis*) and sardine (*Sardina pilchardus*) were compared. Enzymatic hydrolyses were performed using the three proteases Protamex, Alcalase, and Flavourzyme by the pH-stat method (24 h, pH 8, 50 °C). Three fractions were generated: an insoluble sludge, a soluble aqueous phase, and an oily phase. For each fraction, lipids, phospholipids, and proteins were quantified. Quantitative and qualitative analyses of the raw material and hydrolysates were performed. The degree of hydrolysis (DH) for cuttlefish viscera was 3.2% using Protamex, 6.8% using Flavourzyme, and 7% using Alcalase. DH for sardine viscera was 1.9% (using Flavourzyme), 3.1% (using Protamex) and 3.3% (using Alcalase). Dry matter yields of all hydrolysis reactions increased in the aqueous phases. Protein recovery following hydrolysis ranged from 57.2% to 64.3% for cuttlefish and 57.4% to 61.2% for sardine. Tissue disruption following protease treatment increased lipid extractability, leading to higher total lipid content after hydrolysis. At least 80% of the lipids quantified in the raw material were distributed in the liquid phases for both substrates. The hydrolysed lipids were richer in phospholipids than in the lipids extracted by classical chemical extraction, especially after Flavourzyme hydrolysis for cuttlefish and Alcalase hydrolysis for sardine. The total amino acid content differed according to the substrate and the enzyme used. However, regardless of the raw material or the protease used, hydrolysis increased the level of essential amino acids in the hydrolysates, thereby increasing their potential nutritional value for feed products.

Keywords: By-products; Cuttlefish; Marine lipids; Proteolysis; Viscera; Sardine

1. Introduction

The cuttlefish (*Sepia officinalis)* and the sardine *(Sardina pilchardus)* are among the most exploited marine species in the Mediterranean and Atlantic waters. In the Mediterranean waters, the Gulf of Gabes (southeast of Tunisia) is the main site of cuttlefish and sardine resources and landings occur essentially in the fishing port of Sfax (1). In the Northeast Atlantic waters, the most important commercial cuttlefish fisheries are carried out in the “La Manche” channel, where *S. officinalis* constituted 66% of the total cephalopods that land in Basse-Normandie, which is the leading producer France. The waste material generated by the plants processing marine species, and the concentration of the conditioning activity on the shores, are becoming a major problem in countries where fishing is an important industry. During processing, large quantities of waste material, including viscera, are generated and discarded. Viscera generally represent about 15% to 25% of the original catch, depending on the season. Traditionally, viscera are considered waste material and up-graded only to a minor extent. However, research has developed methods to transform such waste material into useful products (2-6). Among the valuable compounds present in viscera, lipids in general and phospholipids in particular represent a potentially valuable fraction (7-9) as they have several beneficial health effects (10-12).
As previously reported, lipid extraction is enhanced by tissues disruption with the introduction of a pre-hydrolysis step and part of the resulting oil can be recovered after centrifugation of the hydrolysates (13-15). In addition to enhancing oil recovery, proteolysis generates peptidic fractions that are of nutritional and biological interest (16-24).
The aim of this work was to investigate the effect of long-term hydrolysis (24 h) on cuttlefish *S. officinalis* (Cephalopoda) and sardine *S.pilchardus* (Clupeidae) viscera using three wide-spectrum proteases (Protamex, Alcalase and Flavourzyme). Protein content, amino acid profile, and lipids and phospholipids composition are analysed and discussed.

2. Materials and methods

Raw material

Cuttlefish (*S. officinalis*) were provided by Calembo, a seafood processing company (Sfax, Tunisia). Mature individuals were caught during November 2005 by trawling in the Gabes Gulf. Upon capture, the specimens were immediately placed in ice and transported to the laboratory where they were eviscerated. The collected viscera were homogenized for 1 min and then frozen at - 80°C until used. Endogenous enzymes were not inactivated. Viscera included all the organs usually found in the abdomen of mature specimens i.e., the digestive gland, esophagus, stomach, digestive ducts, pyloric caeca, pancreatic diverticula, gonads and accessory nidamental glands, except for only the ink gland that was removed.
Sardines *(S. pilchardus)* were caught in the North-east Atlantic during April and June 2004 and provided by Pêcheries Océanes (Nantes, France). Fish were filleted in less than 12 h after capture and viscera were collected and stored in vacuum at - 80°C. Samples collected in the months of April and June were mixed and homogenized because of the large variations in chemical composition related to the spawning season, sex and age.

Endogenous proteases

Enzymatic extracts of cuttlefish and sardine viscera were obtained by mixing 50 g of the homogenized tissues with 100 mL distilled water for 20 s at 4°C using an Ultra Turrax ® disperser (Bioblock Scientific, Nantes, France). After 10 min, the samples were centrifuged for 30 min (10,000 *g*, 4°C). The supernatant was then filtered and the enzymatic extract was collected.
The activity of endogenous proteolytic enzymes for cuttlefish as well as sardine viscera enzyme extracts was measured at pH 8 and 50°C and expressed as the amount of soluble proteins in the extract, in terms of U (Unit of enzyme activity), where 1 U corresponds to 1 mg of bovine serum albumin cleaved per hour.

External enzymes

The enzymes used for the hydrolysis of sardine and cuttlefish viscera were provided by Novozymes A/S (Bagsvaerd, Denmark). Protamex is a *Bacillus* protease complex; Alcalase 2.4 L is a bacterial serine endopeptidase prepared from a strain of *Bacillus lichenformis.* Flavourzyme 500 MG is a fungal protease/peptidase complex produced by submerged fermentation of a strain of *Aspergillus oryzae*. It exhibits both endoprotease and exoprotease activities.

Hydrolysis

Figure 1 outlines the overall process employed in the production of sardine and cuttlefish viscera hydrolysates. The frozen and minced viscera were stored overnight at 4°C for thawing for thawing and homogenized with Milli-Q-purified water (ratio: 1/1, w/v). For each hydrolysis reaction, 100 g sample of viscera was used. The mixture was directly poured into a 300 mL closed glass vessel with a double jacket to enable thermal exchanges (heating or cooling). All hydrolysis reactions were performed at pH 8 and 50°C for optimal activities of Alcalase, Protamex, and Flavourzyme). Before hydrolysis, mixing was performed for an initial 15 min to adjust the pH (by adding 1M NaOH) and the obtain the desired temperature (using a water bath). Hydrolysis was initiated by adding 0.1% w/w for Alcalase and Protamex (240 and 150 Anson Units, respectively) and 1.5 % w/w Flavourzyme (50,000 Leucine Amino Peptidase Units) according to a previous study (Dumay, J., Ph.D. thesis, Nantes University, Nantes, 2006). The mixtures were continuously stirred at 300 rpm for 24 h. Temperature and pH were controlled by a pH-stat method (TIM 854, Radiometer Analytical, SAS, France) and by the addition of 1 M NaOH. The pH-stat method allowed the estimation of degree of hydrolysis (DH) based on alkali to maintain the pH at the desired level. Thus, DH values were obtained could be obtained according to the following expression (25):

$$DH = \frac{V.M.100}{\alpha.m_p.h_{tot}}$$

where DH is the percent ratio of the number of peptide bonds cleaved and the total number of peptide bonds in the substrate; V is the volume (mL) of 1 M NaOH consumed during hydrolysis; M is the molarity of NaOH; a is the dissociation factor for α-NH_2 groups and is equal to 0.88 at 50°C (25); mp is the mass (g) of protein in the reaction substrate (protein content determined by

the total nitrogen content (% N) × 6.25 (26); and h_{tot} is the total number of peptide bonds in the protein = 8.6 *eq* g kg^{-1} protein in fish samples (27).
Hydrolysis proceeded for 24 h and was stopped by heating at 80°C for 15 min. After cooling at room temperature, the hydrolysates were centrifuged at 20,000 $\times g$ for 30 min. Three fractions were collected: the insoluble sludge , the soluble aqueous phase and the oily fraction. Each fraction was then freeze-dried and stored at - 20°C until further analyses.

Analytical methods

The chemical analyses performed were the same for both sardine and cuttlefish hydrolysates. All methods described below were performed in triplicates.

Dry matter and ash

The percentage of dry matter was estimated gravimetrically. The ash content was calculated by weighing the samples after heating the dried samples overnight at 600°C.

Lipid extraction

Lipids were extracted according to the Folch procedure (28). The total lipid content was determined gravimetrically after solvent extraction and evaporation. Lipid extracts were kept in 10 mL chloroform and stored at - 20°C for phospholipid analysis.

Phospholipid content of the total lipid extract

The phospholipid content was estimated by a colorimetric method based on the formation of a complex between phospholipids and ammonium ferrothiocyanate (29). A 2 mL aliquot of chloroform was added to about 0.35 mg of dry lipid extract. After mixing, 1 mL of a solution prepared from ferric chloride (27 g/L) and ammonium ferrothiocyanate (30 g/L) was added. After vortexing and centrifuging at low speed (1,000 *g*, 15 min), the lower phase was recovered and the absorbance read at 488 nm. A standard curve was plotted with known amounts of a standard phospholipid solution (phosphatidylcholine). Results obtained were expressed as grams of phosphatidylcholine equivalents per gram of dry matter.

Protein content

The total nitrogen content of the non-hydrolyzed raw material and the aqueous phase generated by hydrolysis was dtermined using the Kjeldahl method (26). Crude protein was estimated by multiplying the total nitrogen content (%N) by the factor 6.25.

Amino acid composition

Amino acid composition was determined for the raw (non – hydrolyzed) material and hydrolysates. Ten milligrams of freeze-dried samples was previously digested with 200 µL of 6N HCl under a nitrogen atmosphere at 118°C for 18 h. Then, the samples were completely dried under a nitrogen atmosphere and subsequently diluted by adding 2.5 mL water. The amino acid analysis was performed using the EZ:faast™ procedure (Phenomenex, USA) consisting of a solid phase extraction step followed by derivatization and liquid/liquid extraction. An aliquot from the organic phase was analysed on a GC-FID system (Perkin Elmer Autosystem XL). The

injector temperature was maintained at 250°C while detection occurred at 320°C. Separation was achieved using a Zebron ZB-AAA GC column (10m × 0.25 mm) with the oven temperature linearly increasing from 110 to 320°C at 32°C/min. Helium was employed as the carrier gas at a constant flow rate of 1.5 mL/min. The amino acids were quantified by their response factor relative to the internal standard norvaline added at a concentration of 200 µmol/L.

Statistical analysis

Analyses of variance were performed by XLSTAT 5.0 software.

3. Results and discussion

Proximate chemical composition of the raw material

As indicated into Table 1, the proximate chemical compositions of the raw materials were similar irrespective of the matrix used. Indeed, except for slight differences in the moisture content (77% for cuttlefish viscera and 78% for sardine viscera), both contained 2% ash, less than 5 % lipids, and around 15 % proteins.

Proteolytic activity of endogenous enzymes

The proteolytic activity of cuttlefish viscera extract was estimated at 30558 ± 57 mU with a soluble protein concentration of 8.9 g/L and at 31104 ± 95 mU for sardine viscera with a soluble protein concentration of 8.3 g/L. Due to their digestive functions, viscera usually contain mechanisms with diverse functions, such as pepsin, elastase, trypsin, and chymotrypsin (30-32). Apart from pepsin, most digestive enzymes are active at alkaline pH, in spite of native acidic pH (cuttlefish and sardine viscera pH is about 6.4) over a temperatures range of 35 to 60°C (27). Thus, under the hydrolysis conditions applied in this study, the activities of the proteolytic enzymes of cuttlefish and sardine viscera were maximal. However, it has been proved that in spite of the high amount of endogenous enzymes in viscera, the use of endogenous and exogenous enzymes enhances the solubilization of dry matter (33).

Dry matter distribution

During hydrolysis, both viscera were converted from a viscous mince to a free flowing liquid. Hydrolytic curves Protamex, Flavourzyme, and Alcalase are shown in Figure 2. All the curves exhibited an initial fast reaction corresponding to the rapid consumption of NaOH, followed by a slowdown. The shapes of these curves were similar to those reported for enzymatic hydrolysis of other fish by-product substrates (33-37). After 24 h of hydrolysis, the DH values for cuttlefish viscera were 3.2%, 6.8%, and 7%, respectively, for Protamex, Flavourzyme, and Alcalase. In sardine viscera, Alcalase produced the highest DH (3.3%), followed by Protamex (3.1%) and Flavourzyme (1.9%). Hence, it appears that Alcalase performed the best in terms of DH for both substrates. However, the DH values obtained for cuttlefish were higher than those obtained for sardine, especially for Alcalase and Flavourzyme. This may be due to a difference in protein composition of the tissues and/or to presence of more active endogenous proteases affecting the function of exogenous enzymes.
The dry matter repartitions obtained after centrifugation are shown in the Figure 3. Slight differences can be observed between the substrates and the enzymes. Indeed, without

proteolysis, 45 % of dry matter from cuttlefish viscera was predominantly present in the insoluble phase, while 32 % and 23 % were found in aqueous and lipid phases, respectively. For non hydrolysed sardine viscera, only 24% of the dry matter was found into the sludge while 46% and 30% were found into aqueous and lipidic phases respectively. After 24 h of hydrolysis, the major percentage of dry matter was contained in the supernatant, regardless of the enzyme and substrate. This indicates that proteolysis improved solubilization of dry matter. Similar results were found in recent studies on cod (*Gadus morhua*) viscera (14,30) showing sufficient solubilization of dry matter under similar conditions (24 h hydrolysis at 55°C with Alcalase and Protamex; only the pH and pH control were different). However, it is important to mention that at least 15% of cuttlefish viscera and 9% of sardine viscera remained insoluble (with Alcalase and Protamex, respectively). Moreover, greater differences had been noticed for cuttlefish hydrolysis regarding the enzyme while more homogenous results were obtained for sardine hydrolysis. Indeed, regardless of the enzyme used, nearly 80% of the sardine dry matter was found in the soluble phase (11% in the lipid phase and 9% in the sludge), while for cuttlefish Alcalase treatment produced 75 % of dry matter in the supernatant, while Protamex and Flavourzyme produced 68 % and 53 %, respectively.

Recovery of lipids from the fractions

The recovery and distribution of lipids in different fractions after the enzymatic treatment are presented in Figure 4 As previously noted by some authors, proteolysis of a substrate can lead to an increase of lipid recovery due to better accessibility of lipidic compounds to the organic solvents used for extraction (15,38). In this study, 0.20 and 0.18 g lipid/g dry matter were found in non-hydrolysed cuttlefish and sardine viscera, respectively. However, after a tissue disruption by Flavourzyme, up to 0.31 and 0.28 g lipid/g dry matter was detected in non-hydrolysed cuttlefish and sardine viscera respectively. After calculating the total lipid ratio (lipids in sludge + lipids in oily fraction + lipids in aqueous fraction/lipids in non-hydrolyzed raw material), in cuttlefish, it was found that Protamex and Flavourzyme produced similar values (1.49 and 1.50, respectively), while Alcalase gave the lowest value at 1.24. Flavourzyme also provided the highest ratio value for the sardine substrate (1.52) while Protamex and Alcalase treatment produced a value of 1.4. Consequently, compared to classical extraction, exogenous enzyme disruption of the tissues increased lipid extraction and in some cases released lipids into the aqueous phase.

Regardless of the enzymes used, lipid distribution within the fractions was similar with no significant differences observed according to the statistical analysis. For cuttlefish viscera, the soluble fractions contained the highest amount of lipids (40% - 53%), followed by the oily phases (32% - 36%) and the insoluble fraction (11% - 25%). For sardine viscera, the soluble fractions contained the greatest amount of lipids (50% – 60%) followed by the oily phases (32% – 39%), while the insoluble fraction comprised only 8% to 12% of the total lipids quantified. The results show that almost 90% of the total lipids can be recovered without the use of organic solvents.

Calculations performed using student's *t*-test did not detect any differences between the three enzymes (at the level of 5%), but two clusters were identified; one obtained from classical extraction and the other the enzymatic treatments. This finding indicates that a single classical extraction using an organic solvent (like the Folch procedure) is not complete. Indeed, such yield could result in incomplete lipid extraction and can be enhanced by a second extraction, but this would involve additional time and solvent consumption (39).

Phospholipid content

The distribution of phospholipids among the three fractions in the two viscera substrates is depicted in Fig. 5. The aqueous fraction of cuttlefish hydrolysates contained the highest amount of phospholipids (57% using Flavourzyme, 52% using Alcalase and 51% using Protamex), while the oily fraction contained 25% to 28% and the insoluble fraction usually less than 22%. However, more accentuated distribution differences were observed in was sardine viscera where 74% of the total phospholipids was detected in the soluble phases using Protamex and Alcalase, and 68% using Flavourzyme, whereas the oily and insoluble fractions contained and sludge contained less than 20% each.
As reported above for lipids, tissue disruption also led to better phospholipids extraction with increasing ratios compared to classical organic extraction. However, with regard to the substrate, phospholipid accessibility to the solvent during organic extraction was not the same among enzymes. Indeed, Flavourzyme did not improve the extraction proportion for sardine viscera (1.42) as much as it did with cuttlefish viscera (3.0), while Alcalase extracted 2.82 times more phospholipids from sardine viscera and only 1.5 more from cuttlefish. Because the amount of proteins present in the extract influences greatly the amounts of phospholipids (40), such differences may be explained by the fact that Flavourzyme, an exo-peptidase, removes amino acids from the chain ends resulting in longer peptide chains, which can form complexes with phospholipids. Moreover, in the study of Dauksăs *et al*. (41) on cod viscera, the lipids obtained after treatment with Neutrase had a higher percentage of phospholipids compared to that after Flavourzyme treatment. As it is well known that phospholipids are generally located in cell membranes, the difference in the yield of phospholipids during extraction was probably due to the nature of the organs that constitute fish and cuttlefish viscera.

Protein recovery

Protein recoveries from the soluble fraction after hydrolysis by the three enzymes are given in Table 2. For sardine viscera, they ranged between 57.4% (Flavourzyme) and 61.2% (Protamex) which is in comparable to the recovery levels in previous studies on by-product hydrolysis (42-44). For cuttlefish viscera, Protamex resulted in 57.2% of protein recovery, while with Alcalase, up to 64.3% of proteins were recovered. As expected, these results are in agreement with the aforementioned DH values.

Amino acid composition

The total amino acid compositions before and after a 24 h of hydrolysis with Protamex, Alcalase, and Flavourzyme are shown in Table 3.
From a quantitative point of view, before hydrolysis, the amino acid content was much higher in sardine than in cuttlefish substrates (1.9 and 0.7 mmol/g, respectively). However after 24 h of proteolysis by exogenous enzymes, the total amino acid content in the supernatant was similar in both substrates tested: about 3.0 mmol/g using Protamex and about 1.5 mmol/g using Flavourzyme. The largest differences were observed after Alcalase treatment when 2.7 mmol/g of amino acids were found in sardine viscera and up to 3.5 mmol/g were detected in cuttlefish.
Around 16 to 19 different amino acids (or related compounds) were detected in different fractions. Indeed, it appears that proteolysis led to: - the liberation of additional amino acids (e.g., Protamex hydrolysis of sardine or Alcalase hydrolysis of both sardine and cuttlefish) -and the destruction or complexation of some amino acids (e.g., Flavourzyme on cuttlefish).
The two viscera were quite different in their amino acid compositions. For example, histidine (HIS), sarcosine (SAR), and aspartic acid (ASP) were detected only in the cuttlefish extract,

while α-aminoadipic acid (AAA) was present only in the sardine extract. Before proteolysis, essential amino acids (EAA) accounted for 42% of the cuttlefish supernatant and 37% of the sardine supernatant. It has been reported that proteolysis can increase the amount of EAA in the supernatants (6). In this study, maximum amount of EAA was obtained after Protamex proteolysis of cuttlefish viscera (>46%) and after Flavourzyme proteolysis of sardine viscera (~44%). In terms of concentrations, abundant amino acids in the untreated viscera were Alanine (ALA, 15.0%), glutamic acid (GLU, 14.6%), and glycine (GLY, 11.8%) for sardine, and GLU (11.3%), leucine (LEU, 11.2%),è and ALA (10.6%) for cuttlefish.
Proteolysis of sardine viscera proteolysis did not modify this content, and GLU, GLY, and ALA remained predominant after Protamex and Alcalase treatments. Flavourzyme treatment produced some changes, with the third-most abundant amino acid being LEU and GLU accounting only for 2.1%of the sardine supernatant. By comparison, hydrolysis of cuttlefish viscera produced important modifications to the amino acid content of the supernatant. The most abundant amino acids were in the order ALA > GLY > LEU for cuttlefish viscera treated by Protamex or Flavourzyme and GLY > ALA > GLU for cuttlefish viscera treated by Alcalase. Moreover, two amino acids showed an 8-fold increase (GLY with Protamex or Alcalase, ALA with Protamex) indicating preferential enzymatic actions. Similar results have been reported for squid by-products (6) where GLY and ALA increased by 237% and 173%, respectively following 2 h of hydrolysis using alcalase.
These results indicate that sardine and cuttlefish viscera hydrolysates can be a good source of high - nutritional quality products for feed purposes, notably for aquaculture feeds. For example, GLY can improve food palatability for carnivorous species (45), while GLY and ALA are powerful attractants for cod (46). In addition, a mixture of serine (SER), ASP, GLY, and ALA is reported to be a stimulant for feeding activity in tiger puffer (*Takifugu rubripes*) (47).

This work compares enzymatic hydrolysis of cuttlefish and sardine viscera using the three wide-spectrum proteases Protamex, Alcalase, and Flavourzyme, to enhance lipid extraction and provide a good quality source of amino acids. The results obtained for total lipid and phospholipid extraction were similar in terms of distribution and proportion within the different proteolyzed fractions. Protamex produced the lowest solubilization of dry matter, lipids, and phospholipids in both substrates (cuttlefish and sardine viscera). In the case of cuttlefish viscera, Alcalase allowed the best for lipid recovery from the three fractions (insoluble, oily and aqueous phases), while Flavourzyme allowed the best phospholipids recovery. However, opposite results were obtained using these two enzymes in sardine viscera.
The amount of amino acids in the supernatant, noticeably increased in cuttlefish hydrolysates. Proteolysis can lead to increased amino acid content into the soluble phase and this was most noticeable in cuttlefish hydrolysates after 24 h of hydrolysis, as Alcalase treatment seemed the most efficient in liberating amino acids.
In addition to these quantitative differences, qualitative differences were also observed. Regardless of the enzymes and substrates used, some differences were observed in the amino acid contents that may affect the nutritional value of the hydrolysates. Finally, this study showed a marked increase in total lipids after proteolysis, indicating that classic organic extraction methods may underestimate the lipid levels in tissues. Moreover, as we have previously shown (13), the resultant lipids contained in the proteolyzed liquid fractions can be further purified by solvent-free methods such as filtration.
The high level of essential amino acids in the supernatants may represent an interesting way to upgrade those hydrolysates for feed in aquaculture (6) or for animal nutrition in general, in addition to being a nitrogen source for microbial growth media (48) or to contain bioactive properties (49,50).

References

1. Food and Agriculture Organisation (FAO) : Situation mondiale des pêches et de l'aquaculture. Département des pêches. FAO report, Rome, 13 p (2004).

2. Baca, D.R., Peña-Vera, M.T., and Dìaz-Castañeda, M.: Production of fish protein hydrolysates with bacterial proteases: yield and nutritional value. J. Food Sci*.,* **56**, 309 – 314 (1991).

3. Espe, M., Haaland, H., Njaa, L., and Raa, J.: Growth of young rats on diets based on fish silage with different degrees of hydrolysis. Food Chem*.,* **44,** 195 – 200 (1992).

4. Dufossé, L., De La Broise, D., and Guérard, F.: Evaluation of nitrogenous substrates such as peptones from fish: a new method based on Gompertz modeling of microbial growth. Curr. Microbiol., **42**, 32 – 38 (2001).

5. Coello, N., Montiel, E., Concepción, M., and Christen, P.: Optimization of a culture medium containing fish silage for L-lysine production by *Corynebacterium glutamicum.* Bioresour. Technol., **85,** 207 – 211 (2002).

6. Lian, P.Z., Lee, C.M., and Park, E.: Characterization of squid-processing by-product hydrolysates and its potential as aquaculture feed ingredient. J. Agric. Food Chem., **53,** 5587-5592 (2005).

7. De Koning, A.J.: Phospholipids of marine origin: The octopus *Octopus vulgaris.* J. Sci. Food Agric., **23**, 1471–1475 (1972).

8. Blanchier, B. and Boucaud-Camou, E.: Lipids in the digestive gland and the gonad of immature and mature *Sepia officinalis* (Mollusca: Cephalopoda). Mar. Biol., **80(1)**, 39–43 (1984).

9. Navarro, J.C. and Villanueva, R.: Lipid and fatty acid composition of early stages of cephalopods: an approach to their lipid requirements. Aquaculture, **183,** 161–77 (2000).

10. Dyerberg, J., Bang, H.O., Stoffersen, E., Monkada, S., and Vane, J.R.: Eicosapentaenoic acid and prevention of thrombosis atherosclerosis? Lancet, **2**, 117–119 (1978).

11. Lee, P.G., Turk, P.E., Yang, W.T., and Hanlon, R.T.: Biological characteristics and biomedical applications of the squid *Sepioteuthis lessoniana* cultured through multiple generations. Biol. Bull., **186**, 328–341 (1994).

12. Vanschoonbeek, K., De Maat, M.P.M., and Heemskerk, J.W.M.: Fish oil consumption and reduction of arterial disease. J. Nutr., **133(3)**, 657–660 (2003).

13. Dumay, J., Barthomeuf, C., and Bergé, J.P.: How enzymes may be helpful for up-grading fish by-products: enhancement of fat extraction. J. Aquat. Food Product. Technol., **13(2)**, 69-84 (2004).

14. Šližytė, R., Dauksăs, E., Falch, E., Storrø, I., and Rustad, T.: Yield and composition of different fractions obtained after enzymatic hydrolysis of cod (*Gadus morhua*) by-products. Process Biochem., **40,** 1415–1424 (2005).

15. Dumay, J., Donnay-Moreno, C., Barnathan, G., Jaouen, P., and Bergé, J.P.: Improvement of lipid and phospholipid recoveries from sardine (*Sardina pilchardus*) viscera using industrial proteases. Process Biochem., **41,** 2327-2332 (2006).

16. Tacon, A.G.J.: The nutrition and feeding of farmed fish and shrimp: The essential nutrients. In FAO corporate document repository, 40 p. Brasilia, Brazil (1987).

17. Kolkovski, S., and Tandler, A.: The use of squid protein hydrolysate as a protein source in microdiets for gilthead seabream *Sparus aurata* larvae. Aquacult. Nutr., **6**, 11-15 (2000).

18. Ravallec-Plé, R., Chariot, C., Pires, C., Braga, V., Batista, I., and Van Wormhoudt, A.: The presence of bioactive peptides in hydrolysates prepared from processing waste of sardine (*Sardina pilchardus*). J. Sci. Food Agric., **81**, 1120 – 1125 (2001).

19. Encarnacao, P., De Langea. C., Rodehutscordb, M., Hoehlerc, D., Bureau, W., and Bureau, D.P.: Diet digestible energy content affects lysine utilization, but not dietary lysine

requirements of rainbow trout (*Oncorhynchus mykiss*) for maximum growth. Aquaculture, **235**, 569-586 (2004).
20. Dong, Y., Sheng, G., Fu, J., and Wen, K.: Chemical characterization and anti-anaemia activity of fish protein hydrolysate from *Saurida elongata*. J. Sci. Food Agric., **85(12)**, 2033-2039 (2005).
21. Vercruysse, L., Van Camp, J., and Smagghe, G.: ACE inhibitory peptides derived from enzymatic hydrolysates of animal muscle protein: A review. J. Agric. Food. Chem*.,* **53(21)**, 8106-8115 (2005).
22. Nagai, T., Suzuki, N., and Nagashima, T.: Antioxidative activities and angiotensin I-converting enzyme inhibitory activities of enzymatic hydrolysates from commercial kamaboko type samples. Food Sci. Technol. Int*.,* **12(4)**, 335-346 (2006).
23. Vandanjon, L., Johannsson, R., Derouiniot, M., Bourseau, P., and Jaouen, P.: Concentration and purification of blue whiting peptide hydrolysates by membrane processes. J. Food Eng., **83**, 581 – 589 (2007).
24. Diniz, F.M., and Martin, A.M.: Effects of the extent of enzymatic hydrolysis on functional properties of shark protein hydrolysates. J. Food Sci. Technol., **31**, 419-422 (1996).
25. Adler-Nissen, J.: Enzymic hydrolysis of food proteins. Elsevier (ed.), Applied Science Publishers, New York, p. 110 – 169 (1986).
26. Crooke, W.M., and Simpson, W.E.: Determination of ammonium in Kjeldahl digests of crops by an automated procedure. J. Agric. Food Chem., **27**,1256 – 1262 (1971).
27. Novozymes: Determination of the degree of hydrolysis (DH) based on OPA reaction. Novozymes, Bagsværd, Denmark (2001).
28. Folch, J., Lees, N., and Sloane-Stanley, G.H.: A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem., **226**, 497 – 509 (1957).
29. Stewart, J.C.M.: Colorimetric determination of phospholipids with ammonium ferrothiocyanate. Anal. Biochem., **104**, 10 – 14 (1980).
30. Castillo-Yañez, F.J., Pacheca-Aguilar, R., Garcia-Carreño, F.L., and Navarrete-Del Toro M.: Characterization of acidic proteolytic enzymes from Monterey sardine (*Sardinops sagax caerulea*) viscera. Food Chem., **85**, 343-350 (2004).
31. Kishimura, H., Hayashi, K., Miyashita, Y., and Nonami, Y.: Characteristics of trypsins from the viscera of the true sardine (*Sardinops melanostictus*) and the pyloric caeca of arabesque greenling (*Pleuroprammus azonus*). Food Chem., **97**, 65-70 (2006).
32. Sovik, S.L., and Rustad, T.: Effect of season fishing and ground on the activity of cathepsin B and collagenase in by-products from cod species. LWT, **39**, 43-53 (2006).
33. Aspmo, S.I., Horn, S.J., and Eijsin, V.G.H.: Enzymatic hydrolysis of Atlantic cod (*Gadus morhua* L.) viscera. Process Biochem., **40**, 1957–1966 (2005).
34. Quaglia, G.B., and Orban, E.: Influence of the degree of hydrolysis on the solubility of the protein hydrolysates from sardine (*Sardina pilchardus*). J. Sci. Food Agric., **38**, 271–276 (1987).
35. Shahidi, F., Han, X.Q., and Synowiecki, J.: Production and characteristics of protein hydrolysates from capelin (*Mallotus villosus*). Food Chem., **53**, 285-293 (1995).
36. Guérard, F., Guimas, L., and Binet, A.: Production of tuna waste hydrolysates by a commercial neutral protease preparation. J. Mol. Catal. B: Enzym., **19- 20**, 489 – 498 (2002).
37. Liaset, B., Julshamn, K., and Espe, M.: Chemical composition and theoretical nutritional evaluation of the produced fractions from enzymatic hydrolysis of salmon frames with Protamex. Process Biochem., **38**, 1747-1759 (2003).
38. Roose, P., and Smedes, F.: Evaluation of the results of the QUASIMEME lipid intercomparison: the Bligh & Dyer total lipid extraction method. Mar. Poll. Bull., **32**, 674–680 (1996).
39. Falch, E., Rustad, T., and Aursand, M.: By-products from gadiform species as raw material for production of marine lipids as ingredients in food or feed. Process Biochem., **41(3)**, 666-674 (2006).

40. Chobert, J.M., and Haertle, T.: Protein–lipid and protein–flavor interactions. p. 85-97. In Damodaran, S. and Paraf A. (ed.), Food proteins and their applications. New York: Marcel Dekker Inc. (1997).
41. Daukšas, E., Falch, E., Šližytė, R., and Rustad, T.: Composition of fatty acids and lipid classes in bulk products generated during enzymic hydrolysis of cod (*Gadus morhua*) by-products. Process Biochem., **40(8)**, 2659-2670 (2005).
42. Gildberg, A. and Almas, J.: Utilisation of fish-viscera. p. 383-393. In Le Maguer, M. and Jelens, P. (ed.), Food engineering and process applications-Unit operations, Vol. 2. Elsevier Science Publications, London, UK (1986).
43. Liaset, B., Lied, E., and Espe, M.: Enzymatic hydrolysis of by-products from the fish-filleting industry; chemical characterisation and nutritional evaluation. J. Sci. Food. Agric., **80(5)**, 581-589 (2000).
44. Goddard, S.: Feeding and diet. p27. In Chapman and Hall (ed.), feed management in intensive aquaculture, New York (1996).
45. Pawson, M.G.: Analysis of a natural chemical attractant for whiting *Merlangius merlangus* L. and cod *Gadus morhua* L. using a behavioural bioassay. Comp. Biochem. Physiol., **56A**, 129-135 (1977).
46. Ellingsen, O.F. and Døving, K.B.: Chemical fractionation of shrimp extracts inducing bottom food search behaviour in cod (*Gadus morhua* L). J. Chem. Ecol., **12**, 155-168 (1986).
47. Takaoka, O., Takii, K., Nakamura, M., Kumai, H., and Takeda, M.: Identification of feeding stimulants for tiger puffer. Fish. Sci., **61(5)**, 833-836 (1995).
48. Tamehiro, N., Okamoto-Hosoya, Y., Okamoto, S., Ubukata, M., Hamada, M., and Naganawa, H.: Bacilysocin, a novel phospholipid antibiotic produced by *Bacillus subtilis* 168. Antimicrob. Agents Chemother., **46**, 315 – 320 (2002).
49. Martone, C.B., Pérez Borla, O., and Sànchez, J.J.: Fishery by-product as a nutrient source for bacteria and archaea growth media. Bioresour. Technol., **96**, 383 – 387 (2005).
50. Picot, L., Bordenave, S., Didelot, S., Fruitier-Arnaudin, I., Sannier, F., Thorkelsson, G., Bergé, J.P., Guérard, F., Chabeaud, A., and Piot, J.M.: Antiproliferative activity of fish protein hydrolysates on human breast cancer cell lines. Process. Biochem., **41(5)**, 1217-1222 (2006).

Tables

TABLE 1. Total chemical composition of non-hydrolyzed of cuttlefish and sardine viscera (raw material)

	Moisture	Ash	Total lipids	Proteins
Cuttlefish viscera	77.17 ± 0.10%	2.01 ± 0.01%	4.92 ± 0.70%	15.90 ± 0.25%
Sardine viscera	77.46 ± 0.02%	1.90 ± 0.00%	4.89 ±0.11%	15.76 ± 1.10%

TABLE 2. Protein recoveries (%) from the aqueous phases fraction of centrifuged sardine and cuttlefish hydrolysates

Protein recovery (%)	Protamex	Alcalase	Flavourzyme
Sardine	61.2	60.2	57.4
Cuttlefish	57.2	64.3	60.3

Protein recovery indicates the percentage of total proteins recovered in the soluble phase after hydrolysis compared to that in the raw material.

TABLE 3. Total amino acid concentrations (mmol/mg dry weight) in the soluble fractions of the cuttlefish and sardine hydrolysates produced using Protamex, Alcalase, and Flavourzyme after 24 h of treatment.

Amino acid	Cuttlefish				Sardine			
	Raw	*Prot*	*Alcal*	*Flav*	*Raw*	*Prot*	*Alcal*	*Flav*
AAA	0	0	0	0.003	0.017	0.010	0.019	0.031
ABA	0	0.035	0	0.022	0	0.011	0.011	0.009
ALA	0.078	0.600	0.371	0.344	0.287	0.340	0.349	0.368
ASN	0.010	0	0	0	0.172	0	0	0
ASP	0.050	0.079	0.331	0.063	0	0.309	0.209	0.057
C-C	0	0	0.010	0	0	0	0	0
GLN	0	0	0.047	0	0	0	0	0
GLU	0.083	0.080	0.335	0.092	0.276	0.515	0.346	0.033
GLY	0.044	0.371	0.380	0.218	0.223	0.355	0.365	0.249
HIS	0.002	0	0	0	0	0	0	0
HYP	0	0	0	0	0	0.015	0	0
ILE	0.052	0.302	0.229	0.122	0.106	0.173	0.150	0.112
LEU	0.082	0.357	0.325	0.172	0.186	0.281	0.282	0.193
LYS	0.034	0.057	0.140	0.046	0.067	0.107	0.084	0.028
MET	0.031	0.082	0.099	0.039	0.040	0.073	0.076	0.048
ORN	0.031	0.035	0.024	0.018	0.013	0.019	0.021	0.006
PHE	0.037	0.132	0.164	0.068	0.065	0.099	0.116	0.067
PHP	0	0	0.072	0	0	0	0	0
PRO	0.060	0.256	0.195	0.104	0.12	0.176	0.155	0.096
SAR	0.007	0	0.009	0	0	0	0	0
SER	0.039	0.038	0.228	0	0.065	0.145	0.157	0
THR	0.027	0.119	0.230	0.066	0.096	0.170	0.170	0.071
TYR	0.020	0.058	0.099	0.017	0.018	0.043	0.037	0.023
VAL	0.047	0.293	0.220	0.133	0.144	0.226	0.204	0.158
TOTAL	**0.734**	**2.894**	**3.508**	**1.527**	**1.895**	**3.067**	**2.751**	**1.549**

Enzyme concentrations were 0.1% (w/w) using Protamex *(Prot)* and Alcalase *(Alcal)*, and 1.5% using Flavourzyme *(Flav)*. AAA: α-Aminoadipic acid; ABA: α-Aminobutyric acid; C-C: Cystine; HYP: 4-Hydroxyproline; ORN: Ornithine; PHP: Proline-hydroxyproline (dipeptide); SAR: Sarcosine. TRP is completely lost while ASN and GLN are quantitatively converted to ASP and GLU during acid hydrolysis.

Figures

FIG. 1. Hydrolysis process flow diagram

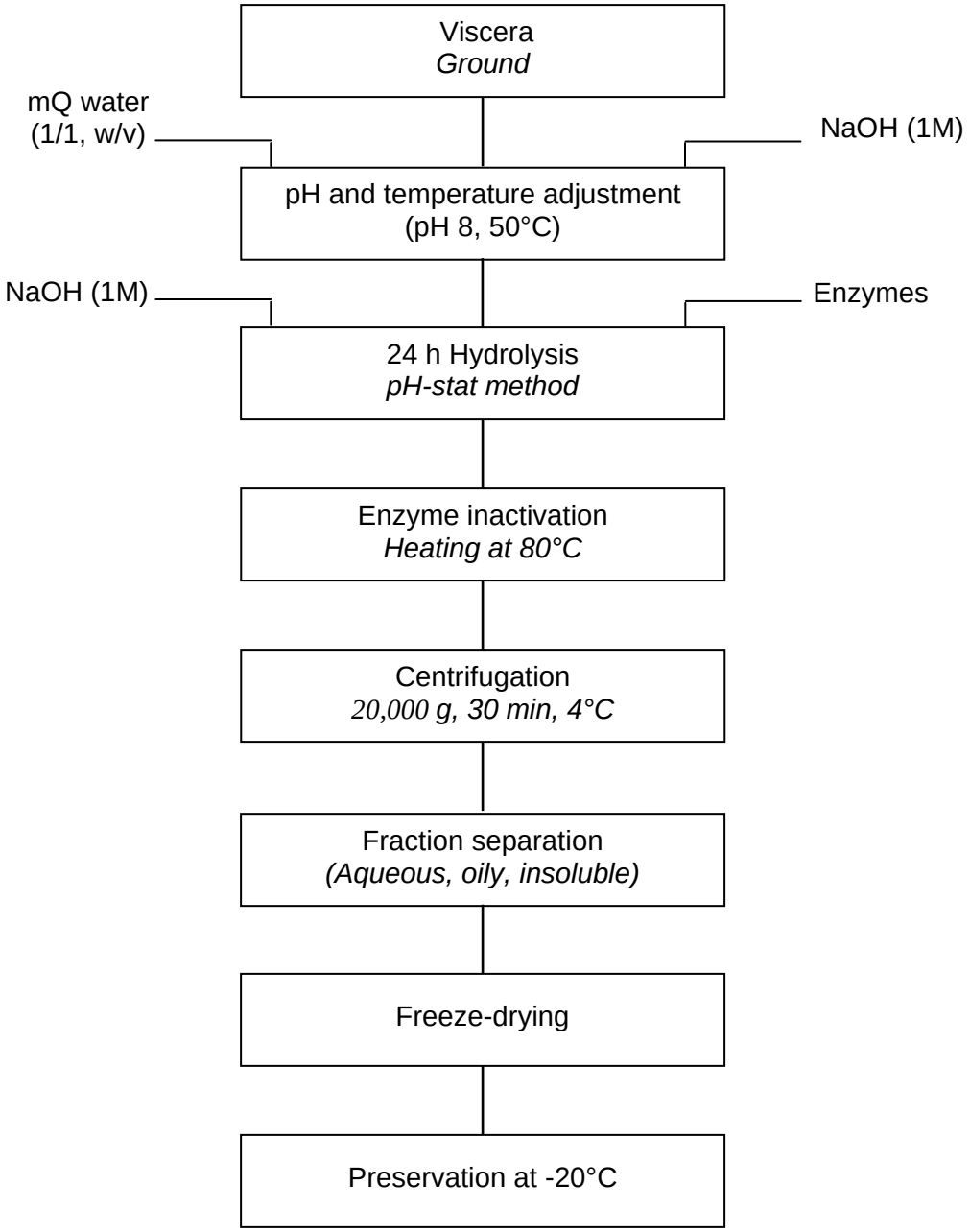

FIG. 1

.**FIG. 2.** Temporal changes in the hydrolysis degree (DH) using Protamex, Alcalase and Flavourzyme: in cuttlefish viscera (A) and sardine viscera (B). Hydrolyses were performed at pH 8, 50°C, with 1 volume of water added to 1 volume of raw material. Enzyme concentrations were 0.1% (w/w) for Protamex and Alcalase, and 1.5% (w/w) for Flavourzyme.

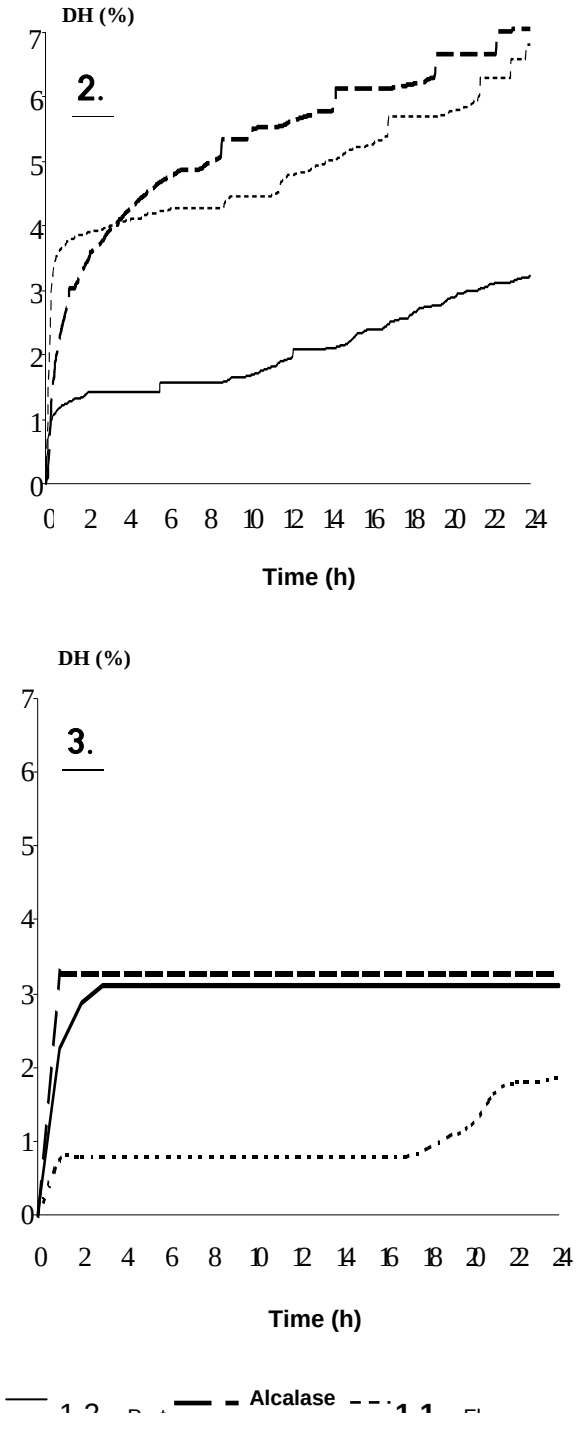

FIG. 2

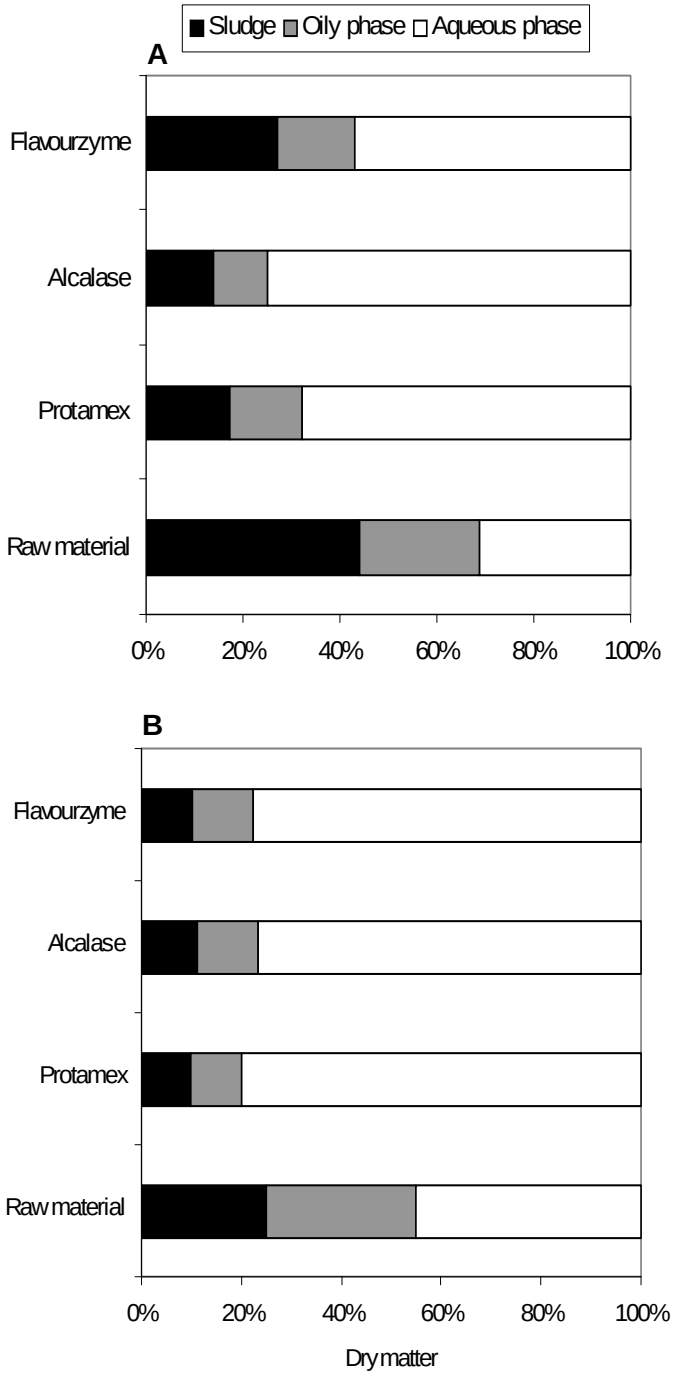
Sludge
Oily phase
Aqueous phase
A
Flavourzyme
Alcalase
Protamex
Raw material
0%
20%
40%
60%
80%
100%
B
Flavourzyme
Alcalase
Protamex
Raw material
0%
20%
40%
60%
80%
100%
Dry matter

FIG. 3

FIG. 3. Dry matter distribution (%) in the three centrifuged fractions of non-hydrolyzed (raw material) and hydrolyzed cuttlefish (A) and sardine viscera (B) with Protamex, Alcalase, and Flavourzyme.(A) cuttlefish viscera. (B) sardine viscera. All hydrolysis reactions were carried out over 24 h, at pH 8 and 50°C, with 1 volume of water added to 1 volume of raw material. Enzyme concentrations were 0.1% (w/w) for Protamex and Alcalase and 1.5% (w/w) for Flavourzyme.

FIG. 4. Lipid distribution in the raw material (non-hydrolyzed viscera) and fractions and in the three fractions generated after hydrolysis of cuttlefish (A) and sardine (B) viscera using different proteolytic enzymes. Hydrolysis reactions were carried out over 24 h, at pH 8 and 50°C with 1 volume of water added 1 volume of raw material. Enzyme concentrations were 0.1% (w/w) for Protamex and Alcalase and 1.5% (w/w) for Flavourzyme. Results are expressed as g of lipid/g of dry matter.

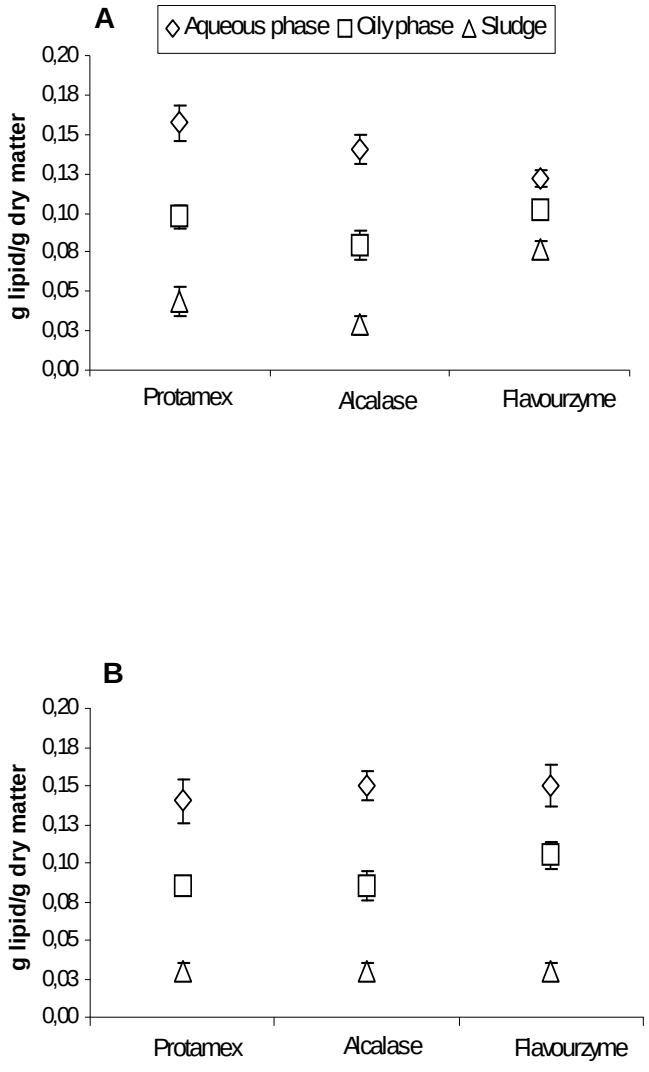

FIG. 4

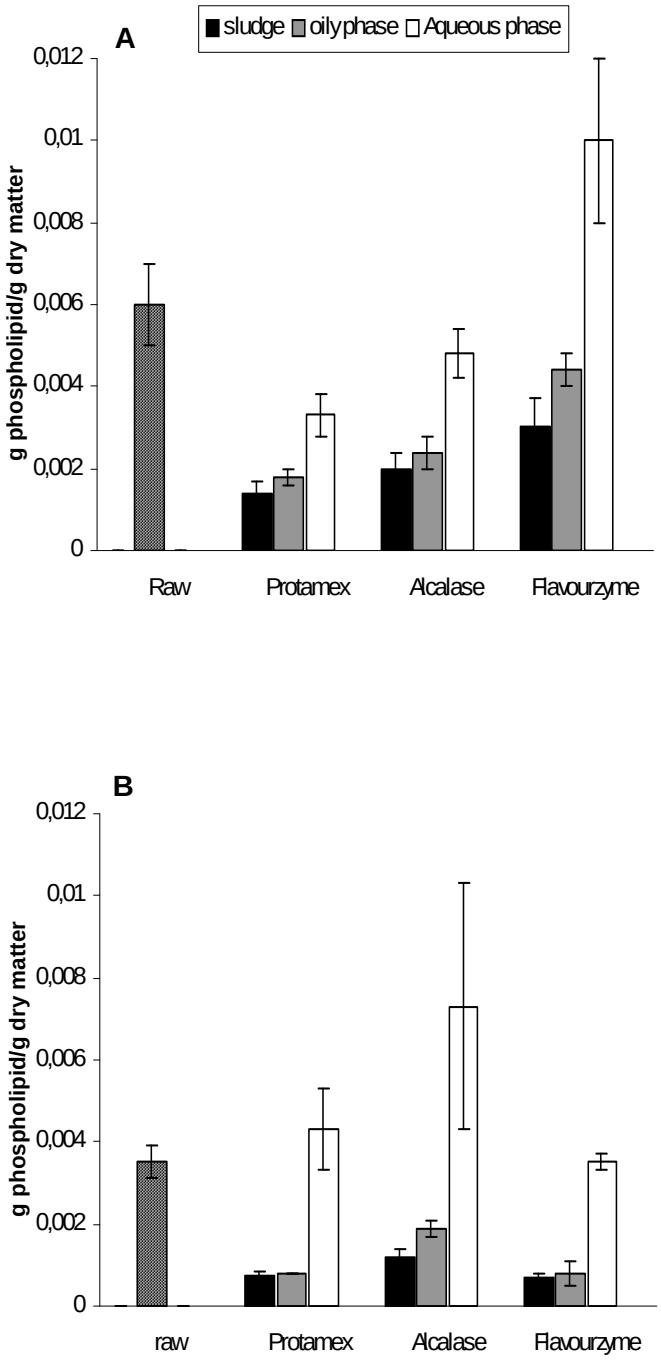
A
sludge
oily phase
Aqueous phase
g phospholipid/g dry matter
0,012
0,01
0,008
0,006
0,004
0,002
0
Raw
Protamex
Alcalase
Flavourzyme
B
g phospholipid/g dry matter
0,012
0,01
0,008
0,006
0,004
0,002
0
raw
Protamex
Alcalase
Flavourzyme

FIG. 5

FIG. 5. Phospholipid distribution in the raw material (non-hydrolyzed viscera) and three centrifuged fractions generated after hydrolysis using different proteolytic enzymes of cuttlefish (A) viscera sardine (B) viscera. Hydrolysis was carried out for 24 h, at pH 8 and 50°C, with 1 volume of water added to 41 volume of raw material. Enzyme concentrations were 0.1% (w/w) for Protamex and Alcalase and 1.5% (w/w) for Flavourzyme. Results are expressed as g of phospholipid/g of dry matter.