Influence of hydrolysis time on protein recovery and amino acid composition of hydrolysates from *Sepia officinalis* viscera

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ABSTRACT

Enzymatic hydrolysis of cuttlefish viscera by Protamex, Pepsin, Delvolase and Multifect Neutral was investigated in a batch reactor during 6 hours with an hourly sampling. The influence of the enzyme used and the hydrolysis conditions (operating pH, temperature) was studied with regards to the extent of proteolytic degradation and to molecular weight distribution of the peptides. After 360 min of hydrolysis, cuttlefish viscera treated with Pepsin yielded significantly ($p < 0.05$) higher protein recoveries compared to the substrate autolysed or treated with other external proteases. However, the amino acid composition showed that this was Protamex hydrolysis which gave the highest percentage of essential amino acids. At least, according to the FPLC patterns, the cuttlefish protein hydrolysates (CPH) were rich in low-molecular-weight peptides, especially with Protamex and Pepsin.

***Keywords**:* Enzymatic hydrolysis, Cuttlefish Protein Hydrolysates (CPH), Cuttlefish By-Products (CBP), Essential Amino Acids (EAA), Protein recovery

INTRODUCTION

Cephalopods are an important economic resource for global fisheries. The cuttlefish (*Sepia officinalis)* is among the most exploited marine species in the Mediterranean and Atlantic waters. In the Mediterranean, the main resource of cuttlefish is located in the Gulf of Gabes (south-east of Tunisia) and the landings occur essentially in the fishing port of Sfax. The most important fisheries of cuttlefish in the northeast Atlantic waters are found in the "La Manche" channel, where *Sepia officinalis* comprised 66% of the total cephalopods landed in Basse-Normandie, which is the foremost producing region in France. During transformation steps in seafood plants, large quantities of waste, including viscera, are generated and discarded. It is estimated that 40% of the total body weight ends up as processing by-product that is not utilized, causing a serious disposal problem. Traditionally, marine viscera have been considered as waste and have been utilized only to a minor extent (Gildberg and Almas, 1986). Nowadays, marine by-product hydrolysates are widely investigated for their biological activities and different purification ways are used for that purpose (Picot *et al.,* 2006; Ravallec-Plé and Van Wormhoudt, 2003; Vandanjon *et al.,* 2007).

Cuttlefish viscera represent an important part of the cuttlefish mass (15 to 25%) depending to the season, and thus their waste represents an important commercial loss. Moreover, the major component in cuttlefish viscera is protein in the range of 72-77% of the solid mass (LeBihan *et al.,* 2007). The cuttlefish protein fraction has shown growth-promoting and attractant properties in shrimp culture at levels low as 1.5% due to the presence of low molecular weight peptides and chemoattractant free amino acids (Takaoka *et al.*, 1995). Lian *et al.*, (2005) have worked on squid-processing by-products and indicated that the level of protein in is high enough for proteolytic hydrolysis for the generation of peptides and free amino acids. It also possesses most of the amino acids essential to the growth and survival of fish (Jobling, 1998). Thus, the conversion of the processing by-products into high-value aquaculture feed ingredients can be a valuable approach to solve the waste disposal problems while simultaneously gaining economic returns. Therefore, one of the approaches for CPB (Cuttlefish Processing By-products) conversion is to hydrolyse the proteins, the primary component,

into smaller and more bioavailable materials, namely, peptides and free amino acids. This makes the product more digestible and could be conveniently formulated into a microdiet to be used as a starter feed. Furthermore, because of their biochemical composition, CPB and specially viscera could be the basis of marine hydrolysates for use as feed in aquaculture diets (Carr, 1982; Goddard, 1996). The changes occurring during the hydrolysis process include protein and dry matter solubility, liberation of free α-amino groups, changes in the molecular weights of the peptides hydrolysed and also in the composition of amino acids (Soufi-Kechaou *et al.,* 2009). Different parameters influence viscera hydrolysis such as endogenous enzymes, which determine the autolysate quality, pH which is an essential parameter to enzymatic activity, and temperature, which influences the degree of autolysis reached after storage and the degree of hydrolysis. Therefore, we essayed four commercial enzymes, acting at different extreme pH, namely Protamex (neutral pH of the substrate), Delvolase (alkaline), Pepsin (acid) and Multifect Neutral (neutral pH of the substrate). Indeed, addition of exogenous enzymes could make a hydrolytic process more controllable and reproducible (Dumay *et al.,* 2006). However, it is also important to point out that addition of exogenous enzymes is the main contributor to the cost of hydrolysis process, meaning that the minimal enzyme consumption is desirable when the goal is to make profitable business from fish hydrolysates (Quaglia and Orban, 1987). However, a comparative study of exogenous enzymes is complicated by the fact that enzymes have different specific activities and optimal working conditions. For that reason, we have screened four enzymes at their optimal hydrolysis conditions, both at the natural and modified pH of the substrate during a 6 hour hydrolysis. No pH control was made to avoid adding more salt to the hydrolysate and to limit the cost of the process. The aim of the present work was to maximise solubilisation of dry matter and proteins in the soluble phase and to shed the light on the biochemical and nutritional (amino acids) composition of the hydrolysates. Solubilisation was primarily monitored by measuring dry matter and nitrogen content of the supernatant and then by the hydrolysis degree (DH). These analyses were carried out after each spent hour of hydrolysis in order to follow the modification of the hydrolysate composition along the time and to conclude about whether it is necessary to conduct a long-time hydrolysis for this type of substrate.

MATERIALS AND METHODS

All analytical methods described below were performed in triplicates.

Material

Fresh cuttlefish (*Sepia officinalis*) were provided by the processing industry "Calembo" (Sfax, Tunisia). Mature individuals (dorsal length 20-25 cm) were caught in the Gabes gulf by trawling during march-october 2008. The viscera were then collected, homogenized for 1 min with a blender and then frozen at - 80°C. Endogenous enzymes were not inactivated. The cuttlefish viscera fraction included all the organs usually found in the abdomen mature specimens, *i.e* the digestive gland, oesophagi, stomach, ink gland, digestive ducts, pyloric caeca, pancreatic diverticula, gonads and accessory nidamental glands.

Enzymes

Enzymes used for the hydrolysis of cuttlefish viscera were provided by Novozymes AS (Denmark) and Genecor (Danisco division, Rochester, NY). Protamex is a *Bacillus* protease complex, Delvolase is a serine protease from a selected GRAS strain of *B. licheniformis*, Pepsin is an exopeptidase and Multifect Neutral, a fungal protease concentrate.

Enzymatic hydrolysis procedure

The frozen and minced viscera were thawed at 4°C during one night and 400 g raw material was homogenized with MilliQ-purified water (ratio 1/1, w/v). Every enzymatic hydrolysis was performed in a thermostatic batch reactor (2L). Before the starting of the hydrolysis reaction, an initial 15 min mixing was done during the adjustment of pH through the addition of NaOH 1M or HCL 2M and the bringing of temperature to the desired value (using a water bath). The operating conditions were 50°C, natural pH of the substrate (6.7) for Protamex, 55°C, pH 10 for Delvolase, 45°C, pH 2 for Pepsin and 50°C, natural substrate pH for Multifect Neutral. The hydrolysis procedure started by adding one of these enzymes with an E/S ratio of 0.1% (w/w). The whole system was continuously stirred with a metallic bar for 6 hours.

The pH was not adjusted during the hydrolysis reaction and samples were withdrawn every hour and incubated at 85°C for 15 min to inactivate the enzymes. After cooling at room temperature, samples were centrifuged at 20,000 *g* during 30 min in a Beckman Coulter Avanti J-25 refrigerated at 4°C. The sludge and the soluble fraction were then separated, freeze-dried, and stored at -20°C for the chemical analyses.

Analytical methods

Dry matter

For the determination of the percentage of dry matter, samples were weighed before and after drying in air at 105°C for at least 16 h.

Total protein content

Total nitrogen content was determined in the raw material and the aqueous phase generated by hydrolysis using the Kjeldahl method (Crooke and Simpson, 1971). Crude protein was estimated by multiplying total nitrogen content (%N) by the factor 6.25.

Molecular weight distribution

Molecular weight distributions of peptides in the supernatants from the original (non- hydrolysed) substrate and the hydrolysates were determined by gel filtration chromatography on a FPLC Superdex Peptide 10/300 GL column (Pharmacia Biotech): exclusion size range of 100–7.000 Da, eluting solvent: 5% acetonitrile/0.1% trifluoroacetic acid/NaCl 50 mM buffer. The flow rate was 0.5 ml/min, and the absorbance 214 nm. Standards injected were Leucin – Tyrosin (294 Da), Vasopressin (1.084 Da) and Ribonuclease (13.700 Da).

Determination of the hydrolysis degree

The DH was determined on the basis of the concentration of α-amino groups using the dinitrofluorobenzene (DNFB) method described by Sanger (Sanger, 1949). The principle is that terminal free amino groups are derivatized with the 2.4 dinitrofluorobenzen forming dinitrophenyl (DNP) derivatives. The sample solution was prepared as follows: the supernatants were diluted by 1/200 and 1 mL dissodium tetraborate (Borax) was added to 1 mL of the diluted sample. The solution was shacked vigorously, then 0.25 mL of a DNFB/ethanol (0.013/1, v/v) mixture was added. The test tubes (6 samples x 2 and the blank) were incubated at 60°C in a water bath for 10 min and then immediately cooled under tap water to stop the reaction and 2 ml HCl 10 N were added to the samples contained in the tubes. The standards were prepared with a solution of glycocol. All spectrophotometer readings were performed at 410 nm with a UNICAM UV4 UV/VIS spectrophotometer.

Amino acid composition

Total amino acid and free amino acid composition of freeze-dried hydrolysates was determined after hydrolysis in 6M HCl at 118°C for 18 h. Then, the samples were completely dried under nitrogen atmosphere and diluted by adding 2.5 mL water. The amino acid analysis was done according to the EZ faast™ procedure byPhenomenex® consisting of a solid phase extraction step followed by derivatization and liquid/liquid extraction. The solid phase extraction is performed via a sorbent packed tip that binds amino acids while allowing interfering compounds to flow through. Amino acids on sorbent are then extracted into the sample and quickly derivatized with reagent at room temperature in aqueous solution. Derivatized amino acids concomitantly migrate to the organic layer for additional separation from interfering compounds. An aliquot from the organic phase is then analysed on a GC-MS system. The mass spectrometer is an Agilent 5973 series network mass selective detector (Agilent, CA, USA). The column is a Zebron ZB-AAA GC column for protein hydrolysates (max. temp. 320/340°C). 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

Data were analyzed using the analysis of variance procedure. Mean difference was determined using the least significant difference (LSD) multiple range test (Statgraphics 6.0, Manugistics Inc., Rockville, MD). Significance of difference was established at $p < 0.05$.

RESULTS AND DISCUSSION

Chemical composition of the raw material

The proximate chemical composition of raw material (non-hydrolyzed cuttlefish viscera) was 79.11 ± 0.1 moisture, 25 ± 0,8 % proteins, 4,53 ± 0,02 % lipids and 1,94 ± 0,04 % ash.

Table 1. Total percentage of the area under the gel filtration chromatograms in the soluble fractions of the cuttlefish 6 hour hydrolysates.

Enzyme	% area							
Protamex	MW>7000D	7000D<MW>5500D	5500D<MW>3000D	3000D<MW>2000D	2000D<MW>1000D	1000D<MW>500D	500D<MW>250D	MW<250D
1 H	4,6	1,0	2,4	2,3	7.1	22.3	38.0	21.8
2 H	3,0	1,0	2,5	2,4	7,3	20,6	48,1	14,4
3 H	3,5	0,8	2,0	2,0	6,5	22,0	44,2	18,4
4 H	3,61	0,84	1,92	1,84	5,96	20,36	43,23	21,65
5 H	3,83	0,89	2,06	1,98	6,55	25,17	41,84	17,18
6 H	4,59	1,01	2,39	2,29	7,13	22,25	38,03	21,76
Pepsin								
1 H	9,2	2,4	4,6	3,6	9,7	25,8	35,0	9,1
2 H	13,2	2,5	5,0	3,5	9,3	15,4	44,4	15,1
3 H	12,7	2,7	5,0	3,8	10,1	21,1	32,7	11,0
4 H	12,19	2,53	4,9	3,85	10,76	23,31	30,03	11,64
5 H	12,42	2,58	5,13	4,12	11,67	21,92	29,43	11,88
6 H	11,77	2,53	4,89	3,85	10,78	23,46	30,22	11,73
Delvolase								
1 H	4.3	1.3	3.4	3.4	12.8	29.4	32.4	12.4
2 H	4,6	1,0	2,8	2,9	11,2	28,0	32,8	16,1
3 H	4,1	0,9	2,6	2,7	10,8	28,9	32,8	16,8
4 H	4,04	0,93	2,65	2,74	11,37	31,57	35,04	11,01
5 H	3,62	1,03	2,58	2,5	10,58	30,2	33,98	15,26
6 H	4,49	1,03	2,34	2,34	10,28	30,82	34,23	13,87
M.Neutral								
1 H	5,5	1,4	3,5	3,3	10,9	26,1	36,9	11,7
2 H	4,7	1,0	2,5	2,4	8,3	22,9	37,5	20,0
3 H	3,7	1,0	2,3	2,3	8,0	24,4	43,4	14,2
4 H	4	0,86	2,01	1,95	6,98	22,36	41,03	20,17
5 H	3,47	0,78	1,86	1,82	6,79	24,23	42,48	15,94
6 H	3,48	0,63	1,53	1,49	5,68	21,06	43,5	21,98

Table 2. Amino acids and related compounds analysed by the EZ :FAAST analysis kit for protein hydrolysates by GC.

Chemical name	**Abbreviation**
α-Aminoadipic acid	AAA
α-Aminobutyric acid	ABA
Alanine	ALA
Asparagine	ASN[a]
Aspartic Acid	ASP
Cystine	C-C
Glutamine	GLN[b]
Glutamic acid	GLU
Glycine	GLY
Histidine	HIS
Hydroxylysine (2 isomers)	HLY
4-Hydroxyproline	HYP
Isoleucine	ILE
Leucine	LEU
Lysine	LYS
Methionine	MET
Ornithine	ORN
Phenylalanine	PHE
Proline-hydroxyproline (dipeptide)	PHP
Proline	PRO
Sarcosine	SAR
Serine	SER
Threonine	THR
Tryptophan	TRP[a]
Tyrosine	TYR
Valine	VAL

[a] TRP is completely lost during acid hydrolysis.
[b] ASN and GLN are quantitatively converted to ASP and GLN during acid hydrolysis.

Enzymatic hydrolysis: *protein and dry matter recoveries and degree of hydrolysis*

The activity conditions of the enzymes experimented was determined from the results for *α*- amino nitrogen value and degree of hydrolysis after 6 hours of digestion at different pH values ranging from 2.0 to 10 and temperatures (45 - 55°C). The initial pH of the substrate was 6.66 at room temperature (25°C). The hydrolysis reaction started by adding one of the enzymes (0.1% w/w, fresh substrate) and without pH control in order to avoid having too much salt in the final substrate and also to observe the behaving of the hydrolysate. The conditions of the reactions were such the pH remained almost stable during all reactions with only a very weak variation that did not exceed 0.2 pH units. During hydrolysis, cuttlefish viscera tissues were converted from a viscous mince to a free flowing hydrolysates within 1 hour. The dark black colour imposed by the ink was conserved but slightly lightened because of the dilution of the substrate. After centrifugation, two fractions were usually obtained: soluble fraction and sludge on the bottom of the centrifugation vessels. The soluble fraction was a light yellowish and the dried powders had a light brown colour and a fishy odour. The sludge was a black pasty layer due to melanin particles of the ink gland. Dried sludge was a very dark black powder, resembling to coal and dying.

Table 3. Total amino acid concentrations (mmol/mg dry weight) in the soluble fractions of the cuttlefish 6 hour hydrolysates with Protamex, Delvolase, Pepsin and Multifect Neutral.

Amino acids	Protamex					
	1 h	2h	3 h	4 h	5 h	6 h
AAA	0,000	0,000	0,000	0,000	0,000	0,000
ABA	0,000	0,015	0,026	0,028	0,034	0,024
ALA	0,340	0,190	0,372	0,395	0,456	0,342
ASP	0,256	0,113	0,056	0,028	0,034	0,057
GLU	0,492	0,013	0,054	0,031	0,029	0,054
GLY	0,356	0,103	0,229	0,208	0,235	0,204
HIS*	0,000	0,000	0,000	0,000	0,000	0,000
ILE*	0,170	0,083	0,142	0,168	0,200	0,158
LEU*	0,252	0,069	0,137	0,111	0,249	0,195
LYS*	0,109	0,016	0,055	0,041	0,029	0,041
MET*	0,059	0,019	0,050	0,038	0,044	0,043
ORN	0,080	0,006	0,020	0,015	0,010	0,023
PHE*	0,116	0,104	0,180	0,071	0,084	0,164
PRO	0,160	0,074	0,138	0,143	0,176	0,141
SER	0,000	0,000	0,000	0,000	0,000	0,000
THR*	0,099	0,027	0,063	0,054	0,065	0,055
TYR	0,033	0,012	0,065	0,024	0,022	0,124
VAL*	0,182	0,082	0,154	0,171	0,186	0,157
Total	**2,702**	**0,926**	**1,740**	**1,526**	**1,852**	**1,881**
Total EAA	**0,985**	**0,400**	**0,780**	**0,669**	**0,856**	**0,811**
% EAA	**36,46%**	**43,19%**	**44,86%**	**43,83%**	**46,23%**	**43,15%**

Amino acid	Pepsin					
	1 h	2 h	3 h	4 h	5 h	6 h
AAA	0,017	0,016	0	0,006	0	0
ABA	0,019	0,033	0,011	0,030	0,051	0,030
ALA	0,305	0,505	0,208	0,454	0,715	0,371
ASP	0,057	0,038	0	0,045	0,063	0,062
GLU	0,029	0,020	0	0,048	0,057	0,054
GLY	0,205	0,289	0,167	0,262	0,391	0,206
HIS*	0	0	0	0	0	0
ILE*	0,104	0,164	0,050	0,103	0,239	0,097
LEU*	0,107	0,276	0,135	0,243	0,382	0,210
LYS*	0,013	0,045	0,026	0,050	0,081	0,077
MET*	0,043	0,156	0,132	0,148	0,265	0,142
ORN	0,009	0,059	0,018	0,022	0,031	0,022
PHE*	0,161	0,191	0,150	0,280	0,223	0,270
PRO	0,116	0,168	0,053	0,154	0,256	0,099
SER	0	0	0	0	0	0
THR*	0,067	0,189	0,037	0,183	0,124	0,035
TYR	0,016	0,131	0,016	0,126	0,045	0,088
VAL*	0,124	0,272	0,062	0,153	0,249	0,106
Total	**1,400**	**2,559**	**0,970**	**2,314**	**3,181**	**1,874**
Total EAA	**0,622**	**1,296**	**0,495**	**1,1632**	**1,5683**	**0,9402**
% EAA	**44,42%**	**50,66%**	**51,04%**	**50,27%**	**49,29%**	**50,10%**

Amino acid	Delvolase					
	1 h	2 h	3 h	4 h	5 h	6 h
AAA	0,022	0	0	0	0	0
ABA	0	0	0	0,028	0	0
ALA	0,389	0,525	0,559	0,525	0,388	0,307
ASP	0,159	0,219	0,097	0,118	0,073	0,065
GLU	0,182	0,2001	0,086	0,092	0,068	0
GLY	0,329	0,406	0,357	0,319	0,214	0,159
HIS*	0	0,011	0	0	0,008	0,004
ILE*	0,156	0,184	0,168	0,158	0,128	0,076
LEU*	0,277	0,356	0,318	0,296	0,210	0,161
LYS*	0,067	0,109	0,056	0,054	0,056	0,038
MET*	0,073	0,086	0,065	0,063	0,044	0,033
ORN	0,032	0,030	0,023	0,020	0,019	0,008
PHE*	0,132	0,148	0,130	0,112	0,084	0,066
PRO	0,168	0,218	0,198	0,183	0,151	0,074
SER	0,109	0,064	0	0	0,081	0
THR*	0,151	0,164	0,100	0,098	0	0,031
TYR	0,045	0,131	0,123	0,043	0,037	0,030
VAL*	0,161	0,191	0,194	0,163	0,137	0,087
Total	**2,4598**	**3,0474**	**2,4803**	**2,2789**	**1,7053**	**1,1442**
Total EAA	**1,0202**	**1,2517**	**1,0342**	**0,9474**	**0,6710**	**0,4999**
% EAA	**41,47%**	**41,07%**	**41,70%**	**41,57%**	**39,35%**	**43,69%**

Acide aminé	Multifect Neutral					
	1 h	2 h	3 h	4 h	5 h	6 h
ALA	0,409	0,380	0,338	0,343	0,359	0,375
ASP	0,489	0,457	0,349	0,441	0,471	0,294
GLU	0,726	0,654	0,429	0,764	0,681	0,367
GLY	0,441	0,419	0,367	0,384	0,380	0,405
HIS*	0,005	0,018	0,032	0,021	0,039	0,031
ILE*	0,289	0,238	0,196	0,220	0,235	0,194
LEU*	0,395	0,342	0,288	0,307	0,317	0,329
LYS*	0,101	0,138	0,120	0,131	0,140	0,114
MET*	0,085	0,075	0,026	0,075	0,070	0,076
ORN	0,070	0,060	0,053	0,065	0,062	0,062
PHE*	0,150	0,134	0,110	0,120	0,120	0,132
PRO	0,248	0,224	0,202	0,213	0,239	0,214
SER	0,105	0,132	0,112	0,145	0,151	0,140
THR*	0,206	0,195	0,188	0,216	0,220	0,209
TYR	0,012	0,040	0,051	0,050	0,068	0,050
VAL*	0,155	0,232	0,199	0,217	0,223	0,189
C - C	0,002	0,017	0,006	0,006	0,016	0,011
Total	**3,894**	**3,744**	**3,067**	**3,718**	**3,780**	**3,188**
Total AAE	**1,38**	**1,374**	**1,162**	**1,310**	**1,367**	**1,278**
% AAE	**35,67%**	**36,71%**	**37,91%**	**35,24%**	**36,18%**	**40,10%**

***** EAA: Essential Amino Acid

Table 4. The amino acid composition of cuttlefish viscera hydrolysates (g/100g) obtained with Protamex, Pepsin, Delvolase and Multifect Neutral in comparison with FAO/WHO and NRC reference protein.

Amino acid	Quantity (g/100g)					
	Protamex	Pepsin	Delvolase	Multifect N.	Reference Protein 1[a]	Reference Protein 2[b]
VAL	2,13	2,24	2,03	2,72	1,3	3,6
LEU	3,31	4,68	3,62	4,49	1,9	3,3
ILE	2,23	2,42	2,15	3,12	1,3	2,5
THR	1,18	1,96	1,07	2,33	0,9	3,9
MET	0,88	1,29	0,85	1,12	1,7	3,1
PHE	1,92	2,45	1,50	2,23	-	6,5
LYS	1,59	1,59	0,67	2,02	1,6	5,7
HIS	0,00	0,18	0,00	0,28	1,6	2,10

[a] Suggested profile of essential amino acid requirements for adults (FAO/WHO, 1990)
[b] Essential amino acid requirements of common carp according to NRC (NRC, 1993)

Table 5. Comparison of the chemical scores between cuttlefish viscera hydrolysates and FAO/WHO and NRC reference proteins.

Amino acid	Chemical score							
	Protamex		Pepsin		Delvolase		Multifect N.	
	RP-1	RP-2	RP-1	RP-2	RP-1	RP-2	RP-1	RP-2
VAL	1,64	0,59	1,72	0,62	1,56	0,56	2,09	0,75
LEU	1,74	1,00	2,46	1,42	1,91	1,10	2,36	1,36
ILE	1,72	0,89	1,86	0,97	1,65	0,86	2,40	1,25
THR	1,31	0,30	2,17	0,50	1,19	0,27	2,59	0,60
MET	0,52	0,28	0,76	0,41	0,50	0,27	0,66	0,36
PHE	-	0,29	-	0,38	-	0,23	-	0,34
LYS	1,00	0,28	1,00	0,28	0,42	0,12	1,26	0,35
HIS	0,00	0,00	0,11	0,08	0,00	0,00	0,18	0,13

RP-1: Chemical score calculated with FAO/WHO reference protein as the base (FAO/WHO, 1990).
RP-2: Chemical score calculated with amino acid requirements of NRC (NRC, 1993).

The shape of the DH curves informs us about the kinetics of the hydrolysis reaction, describing the overall reaction as two first-order processes (Figure 1): one initial fast reaction in which loosely bound polypeptide chains are cleaved from an insoluble protein particle, and second slower reaction in which the more compact core protein is digested. Such a trend can be explained by the fact the rate of hydrolysis may be accounted for by a decrease in the enzyme activity, substrate saturation or product inhibition. The concentration of free α-amino groups in the hydrolysates may be used as an indication of the amount of peptide bonds broken in the reaction after calculation of the DH. Based on these results, Pepsin gave the highest total yield of alpha amino groups (18 %), followed by Delvolase with 9.6 % and finally Protamex and Multifect Neutral (~ 7 %).

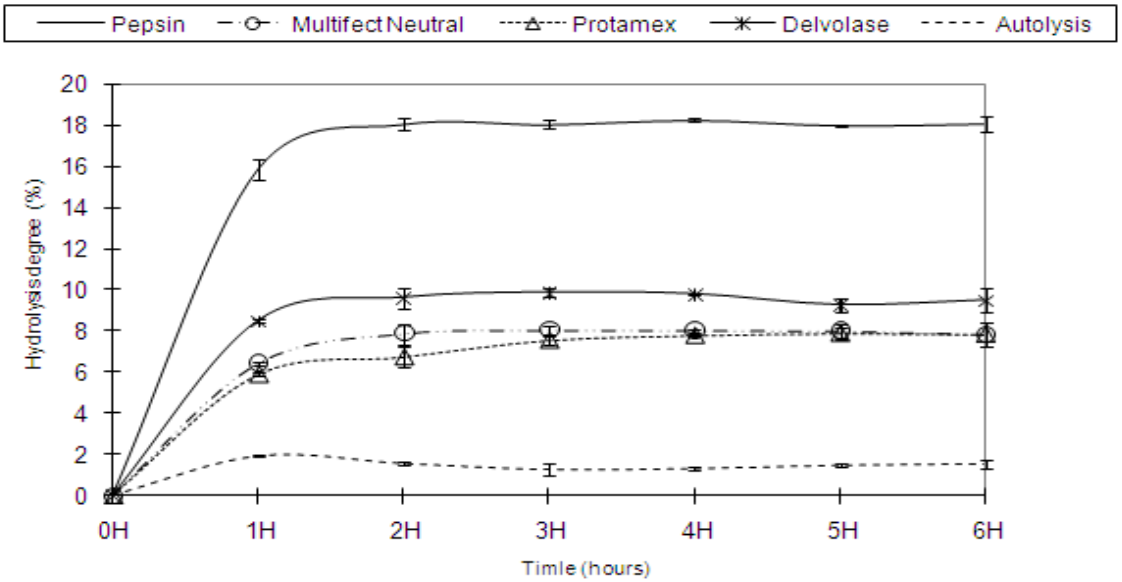

Figure 1

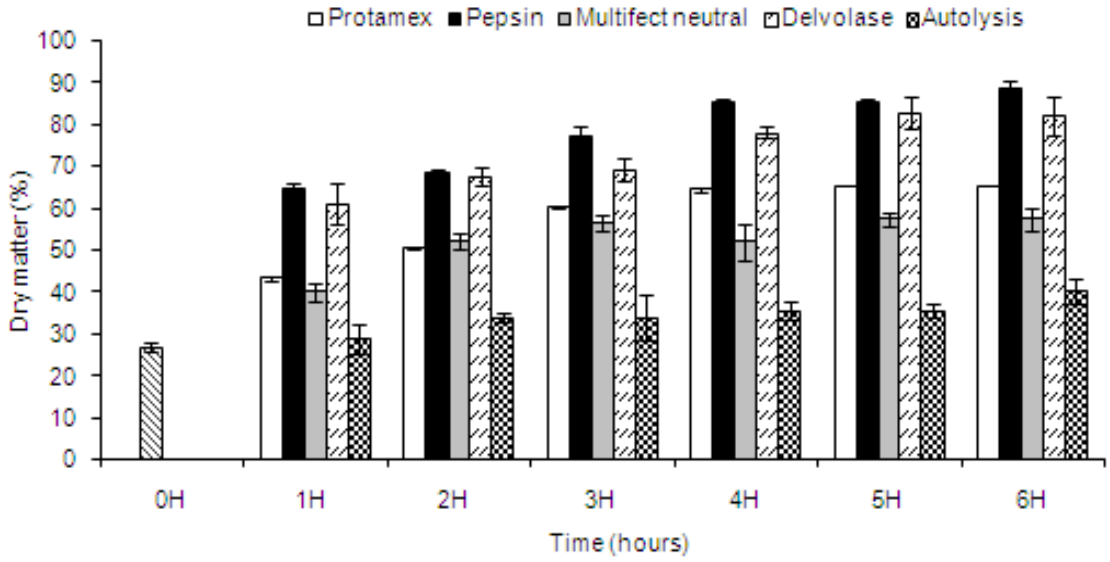

Figure 2

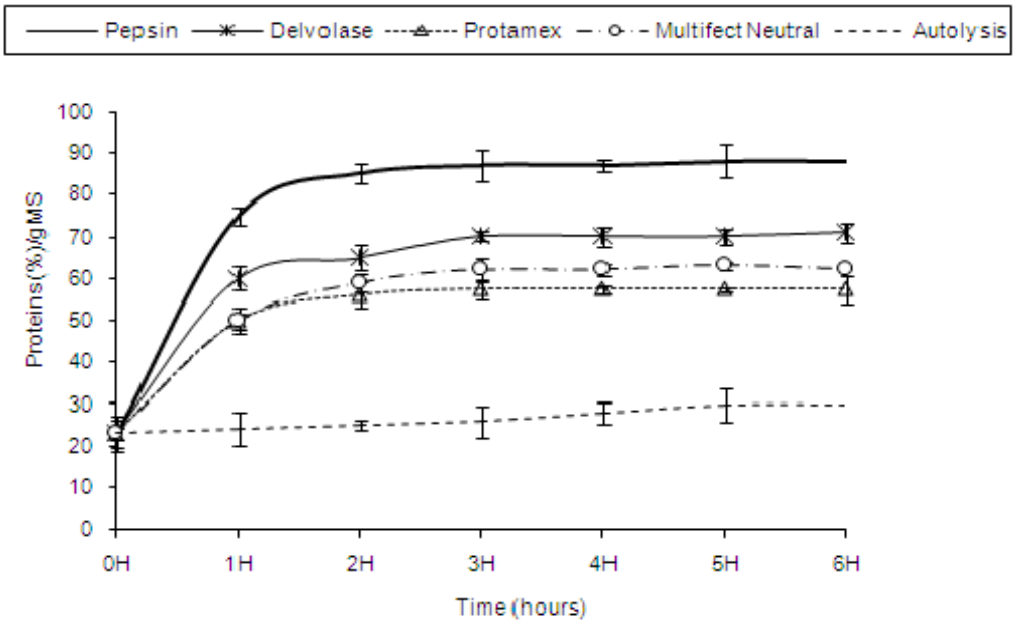

Figure 3

Dry matter distributions in the soluble phases of the 4 hydrolysates show that there was an increase of the amounts compared to the raw (Figure 2). The highest solubilisation of the dry matter occurred when the hydrolysis was achieved by Pepsin. Nitrogen amounts in the soluble fractions obtained after centrifugation and freeze-drying are shown on Figure 3, where important differences can be observed between the hydrolysates obtained with the different enzymes. During hydrolysis, the percentage of nitrogen located in the soluble fraction increased. This indicates that the proteins were solubilized during hydrolysis under the action of the commercial enzymes used and were transferred from the sludge to the soluble phase. As it is shown on the Figure 3**,** the amount of total nitrogen in the soluble fraction increases rapidly during the first two hours and then reaches a steady phase for the rest of hydrolysis reaction while the amount of proteins in the sludge decreases. Indeed, the maximum yields were achieved after approximately 120 min of hydrolysis reaction for almost all of the enzymes tested. In the same context, Studies made on cod frames showed that nitrogen recoveries of 56-64% were obtained after 120 min of enzyme treatment (Liaset *et al.,* 2000). The best nitrogen yields were obtained with pepsin since after hydrolysis, 88 ± 0.2 % of the peptides were located in the soluble phase towards 33.15 ± 0.3% in the soluble phase of the raw material. Studies made on sardine viscera (Soufi-Kechaou *et al.,* 2009) almost gave the same results in terms of hydrolysis degree when the enzyme used was Protamex. However in this study, the enzyme that performed the best is Pepsin. This may in part be due to the hydrolysis temperature. In fact, Mohr (1980) pointed out that during heating to the temperature of hydrolysis, proteins in the sarcoplasmic fraction may denature and precipitate. This is more evident when the raw material is heated before hydrolysis in order to inactivate endogenous enzymes. In our study, the hydrolysis temperature of 45°C of Pepsin is relatively low compared to other temperatures such as 50°C for Protamex and Multifect Neutral and 55°C for Delvolase. Denatured proteins are highly resistant to enzymatic breakdown, and consequently only a minor part of them will be solubilized during enzymatic hydrolysis. In addition, hydrophobic reactions between peptides or self-association between larger peptides probably lead to formation of aggregates which will reduce the susceptibility of proteins towards the enzymatic breakdown, reducing the yield of CPH. However, there may also be concomitant factors such as the acid pH of the substrate and the

cutting sites of Pepsin. The kinetics of total nitrogen formation during Pepsin digestion initially developed a higher rate of evolution than the one obtained with the other enzymes. As digestion proceeds, reaching the stationary phase of the hyperbola after approximately 120 min. This differs from the other enzymes operating in acidic substrates including Papaïn, which reaches this state more slowly (Mohr, 1980; Ritchie and Mackie, 1982).

Molecular weight distributions

The CPH were analysed using size exclusion chromatography. Samples were collected after each hydrolysis hour. The enzymatic treatment by the four enzymes of the cuttlefish viscera produced fractions ranging from 7.000 Da to free amino acids, but the major fractions were comprised betwen 100 and 250 Da.

The molecular weight distributions of the 6-hour hydrolysates are expressed by the percentages of the areas under the chromatograms (Table 1).The results showed a certain correlation between the mass distribution of the soluble peptides and the DH. The more the DH value is high the more the peptides are small-sized. In fact, chromatograms of the supernatants of the hydrolysates obtained with Pepsin showed more material in the low weight area (250-500 Da). Also, Delvolase which ranks just after Pepsin in terms of hydrolysis degree keeps the same order concerning peptide size with 44.4 % material between 250 and 500 Da. However, when we observe the kinetics of formation of small peptides we notice that a long-term proteolysis is not necessary since after 2 hours-hydrolysis we obtain a majority of small peptides. Such a result was noticed for the hydrolysis degree and the nitrogen amounts in the soluble phase where after 2 hours of hydrolysis reaction, almost most of the peptide bonds are broken and we do not observe any modification in the peptide molecular weights. Proteolysis by breaking the peptidic bonds has enhanced the solubility of proteins but has also shortened those peptides. However, some important differences can be noticed. Before hydrolysis 36 % of peptides with a molecular mass above 7000 Da were detected but only 4 to 12 % for the hydrolysed viscera. During hydrolysis whatever the enzyme was, the concentration of all the peptides above 3000 Da has diminished into the supernatant. The majority of the peptides masses were between 250 and 500 Da, which corresponds to free amino acids. This result is important as far as nutritional applications are concerned and confirms the importance of combining those different analyses such as the hydrolysis degree, the nitrogen concentrations in the soluble phase and the molecular mass distribution of the peptides in order to better characterize a proteolysis. Finally we can say that for the four enzymes tested, the percentage of low molecular weight peptides is quite high compared to other marine substrates. The results obtained showed these hydrolysis reactions with different conditions provided in general a high proportion of low molecular weight peptides and free amino acids potentially up-gradable in animal feeding, especially if the essential amino acids represent a high proportion among the total.

Amino acid content

About 18 different amino acids (or related compounds) were detected into the different fractions with 8 EAA. In fact, proteolysis led to the liberation of additional amino acids compared to raw material and to the destruction or complexation of some amino acids. From a quantitative point of view, the hydrolysate where we detected the most important number of amino acids was Pepsin. Also, the maximum amount of EAA was obtained after Pepsin hydrolysis (more than 50 % of the total). For Protamex, the α-aminoadipic acid (AAA) was not detected and the α-aminobutyric acid (ABA) was present with a very low concentration (0.0286 mmol/mg dry matter). Histidine (HIS) also marked a very little presence with the most important amount at 5-hour hydrolysis (0.0081 mmol/mg). In terms of concentrations, the most abundant amino acid for the four enzymes was Alanine (ALA), with the most important amount belonging to Pepsin for the reached after 5 hours of hydrolysis, followed by Glycine (GLY) also regardless to the enzyme used. This indicates that there is no preferential enzymatic cutting in this case. Similar results have been previously noticed (Lian *et al.,* 2005) on squid by-products where Glycine and Alanine increased by 237% and 173% respectively upon 2 hour-hydrolysis.

The concentrations and compositions of amino acids in the soluble part of the 6-hour hydrolysates with Protamex, Pepsin, Delvolase and Multifect Neutral are shown on Table 3. All amino acids that can be quantified by the kit EZ:faast™ are listed on Table 2. In a quantitative point of view, it seems that the amino acid content in Pepsin hydrolysates was higher than the ones of the other enzymes, especially during the 2 first hours of hydrolysis. In fact for the 4 enzymes, a peak was reached during the second hour of hydrolysis. In enzymatic hydrolysis reaction, the ability of the protease to cut peptide bonds is dependent on physical interactions between the substrate and the enzyme in the aqueous environment within hydrophobic regions of the peptide chain in the biological materials (Liaset and Espe, 2008). It is likely that the access for the protease of these hydrophobic regions might be limited (Chotia, 1975). The most plausible would therefore be that the insoluble peptide fractions had higher proportions of hydrophobic EAA. Another contributing factor to the lower proportion of EAA is the reduced levels of Tryptophan (TRP). The reason is that TRP is completely degraded during acid hydrolysis (Lee and Rogers, 1988) and that oxidation of TRP could possibly be accelerated by factors such as oxygen (Cuq *et al.*, 1983), and light (Holt *et al.*, 1977). During the enzymatic hydrolysis process, taking place in a reactor with water, enzymes and raw material, adding some form of stirring introducing oxygen into the water, and with a heating step to inhibit the protease action at the end of the hydrolysis, the proteolysis procedure might have caused partial decomposition of the TRP in the protein fraction which could have contributed to the findings in the present study. It is worth mentioning that let alone the hour of hydrolysis, the amount of EAA makes up a considerable part of the total amino acids present in the hydrolysates. This amount varies between 44.42 % and 51.04 % for Pepsin, 39.46 % and 46.23 % for Protamex, 39.35 % and 43.69 % for Delvolase and finally 35.24 % and 40.10 % for Multifect Neutral.

The comparison of the amino acid composition between CPH and reference proteins and their chemical scores are given in the Table 4 and Table 5. Chemical score provides an estimation of the nutritive value of a protein. This parameter compares levels of EAA between the test and standard proteins. In this study, chemical scores computed are based on the reference protein of the FAO/WHO (1990) for adults, and amino acid requirements of juvenile common carp, as listed by NRC (1993). The amino acid composition in this study in comparison with reference proteins revealed that the amino acid profiles of the cuttlefish hydrolysates were generally higher in EAA compared to the suggested pattern of requirements by FAO/WHO for adult humans regardless to the enzyme used. These results also revealed that Histidine and methionine are the most limiting amino acids since our hydrolysates are very poor or do not contain this amino acid. The results of the common carp chemical score showed that the composition in EAA of cuttlefish viscera hydrolysates is usually lower than the amount required by juvenile carp except for leucine where chemical score are quite high and sometimes for lysine (with Protamex, Pepsin and Multifect Neutral). Consequently, based on the cuttlefish viscera hydrolysate amino acid composition, and the FAO/WHO and NRC standards, the hydrolysates fulfil human requirements and partly common carp needs. Based on the results, in spite of minor deficiencies in certain essential amino acids, the protein hydrolysate does no lose its nutritional value, so it can be considered as an ingredient in balanced fish diets. These results comfort the hypothesis that cuttlefish viscera can be a quite good source of high nutritional quality products for feeding purposes notably aqua cultural feeds. Indeed Glycine an Alanine for example is powerful feeding attractants for some carnivorous fish species (Pawson, 1977).

The results of the comparative part of this study show that all the externally added enzymes increased the yield of the hydrolysis process in terms of solubilized dry matter, protein recovery and hydrolysis degree. The results also showed that there is an important variation between the performance of commercial enzymes and that endogenous enzyme. In fact, adding external enzymes contribute a lot to dry matter solubilisation and protein recovery, but with noticeable differences between the different enzymes. In general, when fish viscera are stored for further processing, the material is usually frozen or boiled to prevent microbial spoilage.

Such treatments have significant denaturing effects on proteins and this should be taken into account when comparing hydrolytic processes. For example, one of the effects which are observed is lipid peroxydation, which in return causes lipid hydroperoxyde activity reactivity with proteins and amino acids (Gardner, 1979). Reducing the time between tissue grinding and initiation of hydrolysis should therefore lower the initial lipid oxidation and also lower the inhibitory effects the peroxydations have on proteolysis (Zamora and Hidalgo, 2001). Reducing the oxidative environmental parameters during hydrolysis should also lower the inhibitory effect peroxydations may have on binding enzymes as well as substrate. In other words, stirring during hydrolysis should probably only be performed under a nitrogen atmosphere. These carbonyl-amino reactions may explain the observed decline of α-amino group concentrations in most of the samples after 4 hours of hydrolysis. An additional plausible explanation may be that α-amino groups react with carbohydrate substances released over time, through Maillard reactions that occur during the thermal denaturation step performed when stopping the hydrolysis reactions. Reactions between amino groups and reducing carbohydrates may be measured by the OPA-assay. Then, the visually darker appearance of the boiled 24-hour-hydrolysates may support these explanations. When the temperature reaches 40°C, the majority of the intestinal proteolytic enzymes of fish become thermally denatured. However, some enzymes in the liver fraction thrive at temperatures up to 60°C. It is therefore likely that the proteolytic activity at 55°C is largely attributable to externally added enzymes, but some contribution to from endogenous enzymes cannot be excluded. It should be noted however that most of the solubilisation is due to a subtile interplay between endogenous and exogenous enzymes which are both under the influence of temperature and which probably interfere in terms of performance. It is quite acceptable that conducting the hydrolytic reactions at ambient temperatures, without adding exogenous enzymes can be an effective and financially interesting alternative for protein recovery and dry matter solubilisation. Somehow, this strategy is precluded by microbial spoilage at theses low temperatures. Also, the endogenous viscera activity probably varies with viscera quality and the addition of exogenous enzymes may be needed to achieve a reproducible process.

The results show in all cases a hyperbolic tendency towards total nitrogen solubilisation, α-amino nitrogen formation and degree of hydrolysis with digestion time. The hydrolytic curves allowed a comparison of the different enzyme activities on the same substrate. The kinetics shows that for the four enzymes, significant differences were observed *($p < 0.05$)* with regard to *α*-amino nitrogen value and degree of hydrolysis. In their study on the rayfish, Pastoriza *et al.,* (2004) showed that the degrees of hydrolysis achieved after 1 hour of digestion were 25.26% for multienzyme preparation, 17.85% for endogenous enzymes and 15.88% for Pepsin. The raw material for hydrolysis was a homogenate of rayfish residues. Therefore, in the experiments using commercial enzymes, the medium also contained the endogenous enzymes of the species. Contrarily to what was expected, the activity of these digestions was lower than that for samples containing the rayfish enzymes only. This implies that in these cases the reaction conditions, which were characteristic for hydrolysis with such commercial enzymes, were not the most adequate for solubilising rayfish residue proteins, but were, whenever the hydrolysis took place under the conditions used for endogenous enzymes, both when digested by autolysis or by addition of the multienzyme isolate. In addition, the characteristics of activity, pH and temperature of native enzymes are typical of alkaline proteases. Accordingly, the greater degree of hydrolysis of these enzymes indicates that their proteolytic activity is greater the one of neutral and acidic enzymes such as Papain and Pepsin. Other studies have shown the effectiveness of Alcalase for preparing protein hydrolysates from cod muscle or Pacific whiting solid wastes (Benjakul and Morrissey, 1997). This finding confirms the high effectiveness of these enzymes for the preparation of fish protein hydrolysates.

CONCLUSIONS

This study revealed that cuttlefish viscera hydrolysates through the action of Protamex, Delvolase, Pepsin and Multifect Neutral provided a high proportion of peptides from 1000 Da to di-

peptides and free amino acids, with a considerably important proportion of essential amino acids. This is an interesting result since the nutritional composition, especially the composition in essential amino acids is a determining factor in human and animal feeding. Starting from the results of this study, protein hydrolysates from cuttlefish viscera may potentially serve as a good source of desirable peptides and amino acids. The concentration of available hydrolysable bonds was one of the main factors controlling the hydrolysis rate. The total nitrogen concentrations using Pepsin increased during the first two hours of the hydrolysis process. Pepsin also yielded higher protein and dry matter recoveries than Protamex Delvolase and Multifect Neutral. Future work will consist in the fractionation of peptide hydrolysates according to their molecular weight with UF membranes of intermediate MWCO (approximately 4000–5000 Da), since the molecular weight of the peptides is related to the antimicrobial activity and this later will be investigated. If the results are encouraging, the process of concentration and fractionation will be optimised from a technical-economic point of view.

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