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Post-mortem changes in viscera of cuttlefish *Sepia officinalis* L. during storage at two different temperatures

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Abstract

We observed the post-mortem changes in viscera of cuttlefish *Sepia officinalis* in order to apply the data to autolysate production. Cuttlefish viscera were stored at 4 or 25 °C and sampled regularly over 4 months. Our results showed that total acid proteases and cathepsins were rapidly released to the extracellular medium due to the breakdown of lysosomes. Total alkaline protease activity increased 2 h after death due to the breakdown of zymogene vesicles. The same patterns were found with trypsin, chymotrypsin and α-amylase activities. After 10 days of incubation, no endogenous enzymatic activity was found. After 50 days of storage, the TCA soluble protein levels decreased rapidly to approximately 30% due to protein degradation and aggregation. After 10 days, the pH of viscera stored at 25 °C was alkaline, whereas in the viscera stored at 4 °C the pH increased more slowly. As significant reduction in the protein molecular weight due to autolysis, was also observed.
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Keywords: Digestive enzymes; Molecular weight; pH; Post-mortem; *Sepia officinalis* L. (Mollusca Cephalopoda); TCA soluble proteins; Viscera

1. Introduction

The cuttlefish, *Sepia officinalis*, is known to have a short life cycle and is characterized by a high growth rate (Clarke, Rodhouse, Holmes, & Pascoe, 1989; Le Goff & Daguzan, 1991; Mangold & Boletsky, 1973; Mangold, 1983). Such rapid growth must involve a high rate of body protein turnover, and thus, intense activity of the proteolytic enzymes. Previous studies have examined the occurrence of digestive enzymes along the digestive tract of the cuttlefish *S. officinalis* (Boucaud-Camou, 1968, 1973, 1974). In recent studies, Perrin, Le Bihan, and Koueta (2004) measured digestive enzymes such as trypsin, acid phosphatases, total acid proteases and chymotrypsin in the juvenile cuttlefish. Moreover, Le Bihan, Perrin, and Koueta (2004b) have characterized cuttlefish cathepsins. The lysosomal system (Carajaville, Robledo, Etxeberria, & Marigomez, 1995; Likholat, Anan'eva, Antoniuk, & Lutova, 2000; Pihan, 2001; Sindermann, 2000; Villalba, Mourelle, Lopez, Carballal, & Azedo, 1993a, Villalba, Mourelle, Carballal, & Lopez, 1993b), which contains cathepsins has been implicated in numerous mechanisms of intracellular digestion. Lysosomes are cytoplasmic granules that contain several acid hydrolases within the lysosomal membrane (De Duve, Pressman, Gianetto, Wattiaux, & Applemans, 1955). These hydrolases, such as catheptic enzymes, are considered to be the main cause of post-mortem tissue softening (Eino & Stanley, 1973; Ono, 1971; Penny, 1980).

The cephalopods are an important economic resource for global fisheries. Basse-Normandie is the foremost Cephalopod-producing region in France. Cuttlefish are mainly exported in frozen form to the Mediterranean countries and to Japan; France is the second most

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important exporter of frozen cuttlefish in the European Union. The valorisation can permit to increase the value of viscera. Traditionally, viscera have been considered as waste and utilized only to a minor extent (Gildberg & Almas, 1986). Nevertheless, the cuttlefish viscera represent an important part of the cuttlefish mass (15–25%). So, their waste represents an important commercial loss. Moreover, the digestive gland, which correspond to 10–15% of viscera, contains about 10% of lipids rich in polyunsaturated fatty acids (Boucaud-Camou, 1973). So, because of their biochemical composition, viscera could be the basis of marine autolysates for use as feed in aquaculture diets. In order to reveal mechanisms of autolysis, it is essential to study the post-mortem process in relation to the activities of hydrolytic enzymes in viscera. Different parameters influence the viscera autolysis such as endogenous enzymes, which determine the autolysate quality; pH, which is an essential parameter to enzymatic activity; temperature, which influences both the degree of autolysis reached after storage and the degree of hydrolysis of the amide groups (Haaland & Njaa, 1889). So, we assayed enzymatic activity, pH, the TCA (trichloroacetic acid) soluble protein level, because it reflects the degree of hydrolysis of peptide bonding and the molecular weight of proteins and peptides.

2. Materials and methods

2.1. Biological material

Cuttlefish *S. officinalis* were caught in the English Channel during Spring 2004. Individuals (mature animals; dorsal length 20–25 cm) were placed in tanks for 72 h at 15 °C, in the marine station in Luc-sur-Mer (France), to ensure that the cuttlefish used were not stressed. The animals were fed with live crab (*Carninus maenas*) during one week. The viscera were removed from anaesthetized live cuttlefish and immediately disrupted using a liquidizer. The viscera (40 l) were placed into two treatments in acid resistant plastic containers: one was stored at 4 °C and the second group at 25 °C, for 4 months. Tissue samples were taken at regular intervals (0, 1 h, 2 h, 3 h, 4 h, 5 h, 8 h, 24 h, 48 h,...4 months), frozen in liquid nitrogen and stored at −80 °C until analyzed.

2.2. Enzyme extraction

For acid protease and cathepsin assays, the frozen viscera were homogenized in 2.5 volume of the extraction buffer (1% KCl containing 1 mM of EDTA) (Le Bihan, Perrin, & Koueta, 2004a). The homogenate was centrifuged for 60 min at 10,000*g* at 4 °C. The supernatant liquid was used for the assays. For the alkaline proteases, trypsin, chymotrypsin, lipases and amylase assays, the frozen viscera were extracted in buffer (1 ml of buffer per 60 mg of sample) containing: 0.09 M TRIS-base, 0.08 M boric acid, 3 mM EDTA, 0.5 mM mercapto-ethanol, and glycerol 10%, pH 8.3 (Koueta, 1983). The crude extract was centrifuged for 30 min at 10,000*g* at 4 °C, and the supernatant used for assays.

2.3. Enzymatic assays

Total acid and alkaline protease activities were measured according to Charney and Tomarelli (1947) and Van Wormhoudt and Sellos (1980). The substrate was casein Yellow 0.005% in a phosphate buffer (0.096 M, KH2P04, 0.004 M, NaH2PO4), pH 2 for the acid proteases and pH 10 for alkaline proteases. 0.5 ml was used for each 0.1 ml of supernatant. The incubation was carried out for 1 h at 37 °C, and the intensity of the yellow coloration was estimated at 442 nm. Enzyme activity was expressed as specific activity (U mg^{-1} protein), where one unit is the variation of one unit of O.D. min^{-1}.

Cathepsin activity was measured according to Bonete, Manjon, Llorca, and Iborra (1984). The assay mixture was composed of 0.1 ml of sample (containing catheptic activity), 0.05 ml of 0.4 M acetate buffer (pH 4.0), and 0.05 ml of 2% (w/v) haemoglobin solution. The mixture was incubated at 37 °C for 60 min. The reaction was stopped by the addition of 1 ml of 3% (w/v) trichloroacetic acid. After 10 min, the assay was centrifuged at 4000 rpm for 10 min. An aliquot was used for the estimation of the released proteolytic end-product. Appropriate blanks were used, and the products were evaluated by using the Folin–Lowry reaction, according to Barrett (1977), employing tyrosine as standard. The activity was expressed as specific activity (U mg^{-1} protein) where one enzymatic unit corresponds to 1 μmol of tyrosine mg^{-1} protein.

Trypsin activity was measured according to Tsunematsu, Nishimura, and Mizusaki (1985) using 1 mM Nα-benzoyl-Arg-*p*-nitroanilide as substrate in a 0.1 M Tris buffer, pH 9, and chymotrypsin according to Delmar, Largman, Brodrick, and Geokas (1979) using 1.142 mM succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (SAAPNA) as substrate in a buffer of 0.1 M Tris–base, 0.01 M CaCl2, pH 11. Then, for both assays, 0.1 ml of supernatant was added to 0.5 ml of substrate. Time incubation was 30 min at 37 °C for chymotrypsin and 1 h at 25 °C for trypsin. The final absorbance was recorded at 410 nm. Enzyme activity was expressed as specific activity (U mg^{-1} protein) where one enzymatic unit corresponds to 10 μmol l^{-1} of pNa min^{-1}.

Lipase activity was dosed according to a modified method of Versaw, Cuppet, Winters, and Williams (1989) using β-naphtyl caprilate as substrate. The assay mixture contained: 50 μl of 200 mM sodium taurocholate; 450 μl of BES 50 mM, pH 7.2, 100 μl of extract

and 5 μl of substrate at 200 mM (dissolved in DMSO). The mixture was incubated for 30 min at 40 °C and 5 μl of fast Blue BB 100 mM (dissolved in DMSO) was added. The second incubation was 5 min at 40 °C. The reaction was stopped by addition of 50 μl of trichloroacetic acid 0.72 N and 675 μl of 95% ethanol/ethylacetylate (V/V) solution. The colored product was read at 540 nm. The lipase activity was expressed as specific activity (U mg^{-1} protein).

Amylase activity assayed was described by Sigma–Aldrich, using starch as substrate. The substrate buffer contained starch at 1% in a monobasic phosphate buffer at 20 and 6.7 mM of sodium chloride, pH 6.9. We add 100 μl of substrate buffer at 100 μl of extract. The incubation was 3 min at 20 °C, after, 100 μl of revelator was added and incubated for 15 min in ebullition water. The revelator corresponds to tartrate sodium potassium 0.94 M, NaOH 0.4 M and 3-5-dinitrosalicylic acid 48 mM. The absorbance was recorded at 640 nm. Enzyme activity was expressed as specific activity (U mg^{-1} protein).

2.4. pH

One gramme of fresh tissue was added to 8 ml of twice distilled water. After vortexing, the pH was read using a pH-meter.

2.5. TCA soluble protein and protein content

The TCA soluble protein was assayed according to Benjakul, Visessanguan, and Tueksuban (2003). Twenty-seven milliliter of TCA 5% (w/v) was added to 3 g of fresh tissue. After homogenization, the mixture was stored for 1 h at 4 °C, and then centrifuged at 5000*g* during 5 min at 4 °C. Protein content was estimated using the Lowry method (1951).

The concentration of protein in each extract (in extraction buffer) was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951) with bovine serum albumin as the standard.

2.6. Molecular weight of proteins and peptides

Fresh tissue was added to Tris buffer containing 10 mM Tris–HCl and 150 mM NaCl, pH 8 (0.1 g to 10 ml). The mixture was stored at 4 °C for 1 h, then centrifuged for 10 min at 4 °C at 10,000*g*. The supernatant contained the Tris soluble proteins. The protein content was assayed according to the Lowry method (1951) using BSA as standard. The protein concentration in the supernatant was then adjusted at 10 mg/ml. The molecular weight of the extract obtained was estimated using a Sephadex G25 column (Pharmacia G25M PD 10 Sephadex), which had been calibrated with the following molecular weight standards: β-amylase (200,000), alcohol deshydrogenase (150,000), albumine (66,000), carbonic anhydrase (29,000), vitamin B12 (1350). The amount of protein in the different fractions (500 μl) was appreciated by the absorbance at 280 nm.

2.7. Statistical analysis

The results are given as means ± standard deviation ($n = 6$ for each treatment). Data between treatments were compared with an ANOVA followed by a Tukey's test when significant differences ($p < 0.05$) were found (Sokal & Rohlf, 1981).

3. Results and discussion

3.1. Intracellular enzyme specific activity change

Fig. 1 presents cathepsin specific activity in crude extracts of cuttlefish digestive viscera during the first 24 h of storage at two different temperatures. Significant increase ($p < 0.05$) of cathepsin activity was observed 2 h after the animal's death (90–100% of maximal activity), activity that remained stable for 24 h when stored at 4 °C, but which decreased over 24 h when stored at 25 °C. As described by Aoki and Ueno (1997), we also observed cathepsin liberation from the lysosomes during post-mortem storage. This suggests that the increase of activity 2 h after death was due to disruption of lysosomes. Aoki and Ueno (1997) considered that most post-mortem change with regard to muscle degradation was due to the liberation of cathepsins from lysosomes. Whiting, Montgomery, and Anglemier (1975) prepared a lysosomal fraction from rainbow trout white muscle and demonstrated that catheptic activity (possibly cathepin D) was released from the lysosomes in aging muscle at 4 °C. Moreover, activity of cathepsins B and L gradually decreased in the lysosomal fraction and increased in the supernatant fraction as rainbow trout muscle aged at 0 °C. It appears that cathepsins are quickly liberated from the lysosomes during ageing (Aoki & Ueno, 1997). Beyond 24 h after death, the cathepsin specific activity significantly decreased ($p < 0.05$) to 30% of maximal activity after 96 h (Fig. 2). After storage for 4 months, cathepsins activity viscera was 10% of maximal activity. The lysosome breaking leads to the liberation of the cathepsins in an environment that possesses a pH at 6.3 (Table 1). Le Bihan et al. (2004b), who have characterized the digestive gland cathepsins in the cuttlefish *S. officinalis*, indicated that cathepsin stability was weak above pH 5. The low cathepsin activity detected in viscera after several days may, therefore, be due to too high a pH.

Fig. 3 describes total acid protease specific activity in the crude extracts of cuttlefish digestive viscera during the first 24 h of storage at the two different temperatures.

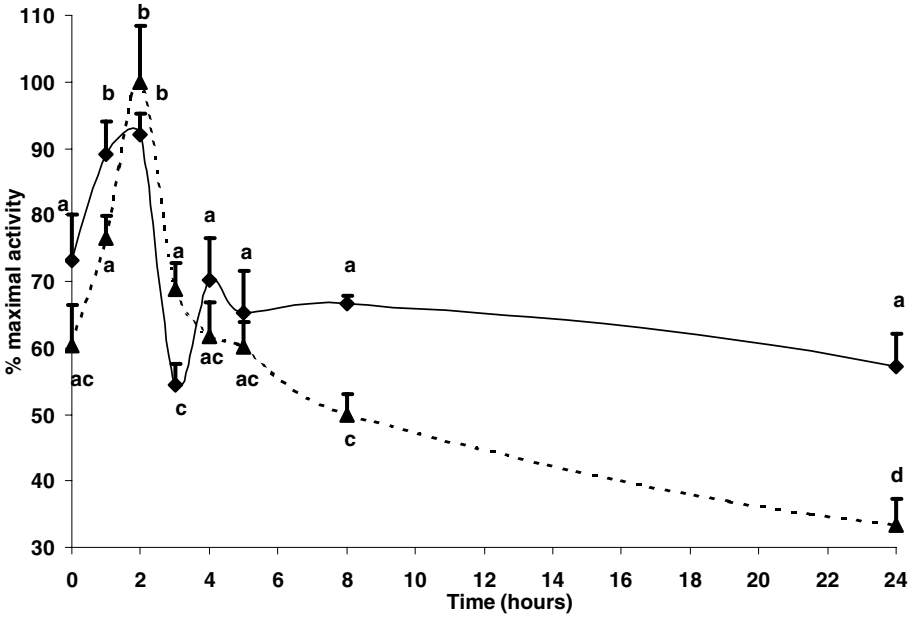

Fig. 1. Cathepsins specific activity in crude extracts of cuttlefish digestive viscera during the first 24 h of storage (% of maximal activity) at two different temperatures (−4 or −25 °C). Points not bearing the same superscript letter are significantly different ($p < 0.05$).

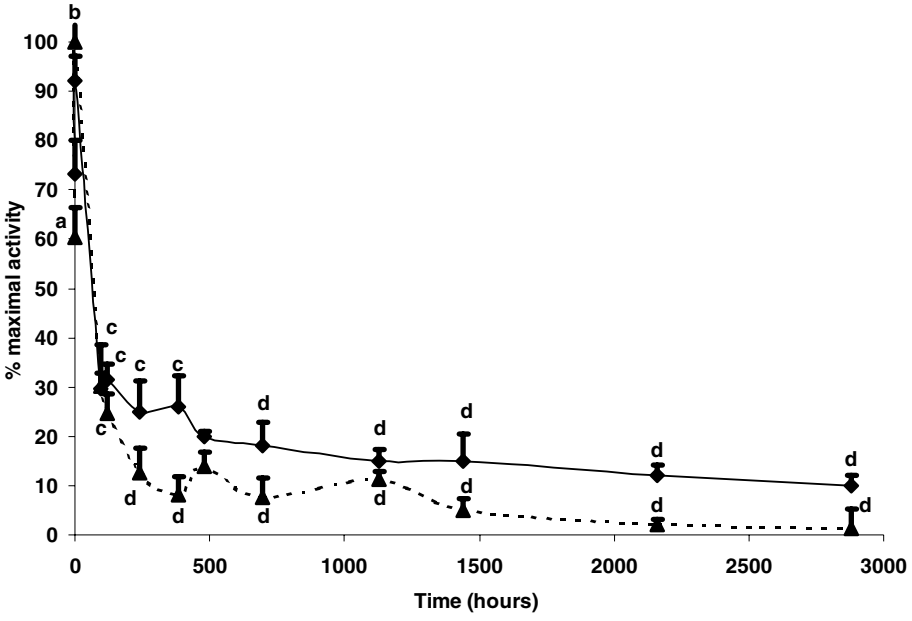

Fig. 2. Cathepsins specific activity in crude extracts of cuttlefish digestive viscera during 4 month of storage (% of maximal activity) at two different temperatures (−4 or −25 °C). Points not bearing the same superscript letter are significantly different ($p < 0.05$).

We can observe a significant increase ($p < 0.05$) of acid protease activity 2 h after the animal death (60% of maximal activity), next, this activity stay stable to 24 h when the storage was made at 4 °C, or increase to 24 h when the storage was made at 25 °C. We observed that as with cathepsin activity, the total acid protease activity increased rapidly after the animal's death. In this way, it appears that in invertebrates, only lysosomes contain an acidic pH below 5. Then, acid proteases where always localized on lysosomes in invertebrates.

Beyond 1 day, total acid protease specific activity decreased significantly ($p < 0.05$) to approximately 20% of maximal activity at four month after the cuttlefish death (Fig. 4). As observed for the cathepsins activity, which contributes to total acid protease activity, have weak stability above pH 5. So, enzymes that mainly partici-

Table 1
Effect of storage temperature on pH, TCA soluble proteins and molecular weight of peptides and proteins

	pH	TCA soluble proteins (mg/ml)	Molecular weight (%)	
			<20 kDa	>6.5 kDa
Storage time at 4 °C				
0 h	6.42 ± 0.011a	54.6 ± 2.57a	80	20
2 h	6.285 ± 0.005b	25 ± 0.1b	80	20
8 h	6.315 ± 0.015b	61.6 ± 2.24c	70	30
24 h	6.31 ± 0.001b	59.8 ± 0.52c	60	40
10 Days	6.485 ± 0.015c	68 ± 0.1e	55	45
50 Days	6.69 ± 0.03d	70.8 ± 5e	40	60
4 Months	6.815 ± 0.015d	28.7 ± 3.9g	30	70
Storage time at 25 °C				
0 h	6.42 ± 0.011a	54.6 ± 2.57a	80	20
2 h	6.285 ± 0.015b	25 ± 0.9b	80	20
8 h	6.47 ± 0.02a	71.5 ± 2.3d	65	35
24 h	6.65 ± 0.03e	71.5 ± 2.17d	50	50
10 Days	6.975 ± 0.015f	76.2 ± 0.5e	40	60
50 Days	7.51 ± 0.01g	84.8 ± 2.9f	25	75
4 Months	7.19 ± 0.01g	29.9 ± 2.2g	16	84

Points not bearing the same superscript letter are significantly different ($p < 0.05$).

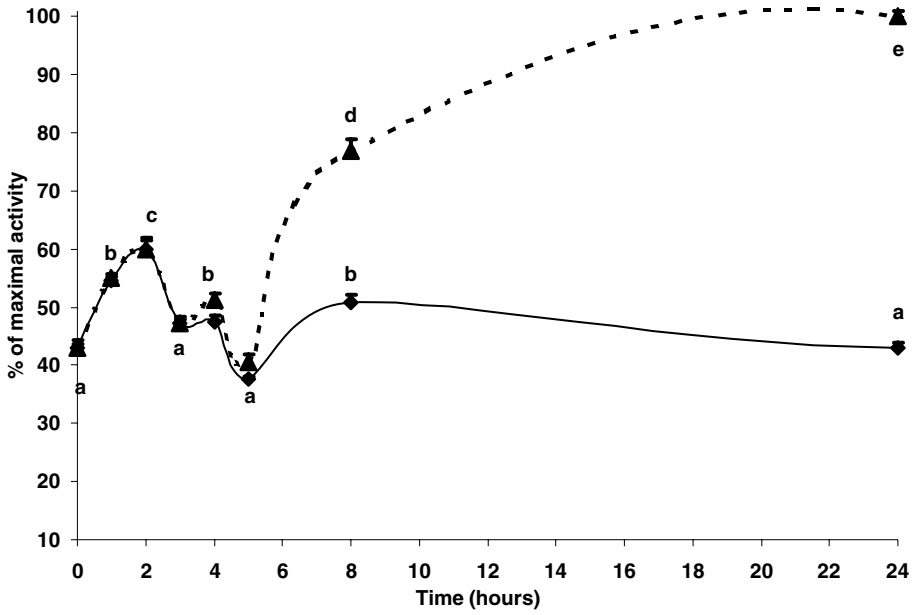

Fig. 3. Total proteolytic acids specific activity in crude extracts of cuttlefish digestive viscera during the first 24 h of storage (% of maximal activity) at two different temperatures (−4 or −25 °C). Points not bearing the same superscript letter are significantly different ($p < 0.05$).

pate in the total acid protease activity were cathepsins, as observed by Le Bihan et al. (2004b).

3.2. Extracellular enzymes specific activity change

Fig. 5 presents total alkaline protease specific activity in the crude extracts of cuttlefish digestive viscera during the first 24 h of storage at two different temperatures. A significant increase ($p < 0.05$) of alkaline protease activity was observed 2 h after the animal's death (90–100% of maximal activity), followed by a decrease (70–80%) after 24 h. The animal's death results in major changes in the biochemical features of the tissue, leading to its disintegration (Delbarre-Ladrat, Verrez-Bagnis, Noël, & Fleurence, 2004). This suggest that zymogene vesicles present in cells, containing secreted proteolytic alkaline enzymes, break down a few hours after the death of the cuttlefish inducing the increased alkaline protease activity observed 2 h after death.

Beyond 96 h after death, the total alkaline protease specific activity decreased significantly ($p < 0.05$) to 25% of maximal activity after four months (Fig. 6).

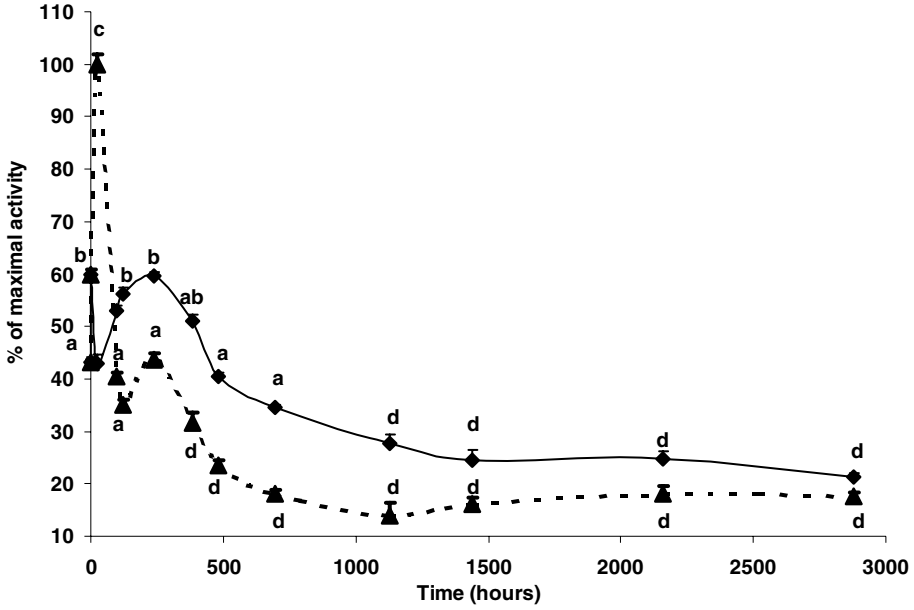

Fig. 4. Total proteolytic acids specific activity in crude extracts of cuttlefish digestive viscera during 4 month of storage (% of maximal activity) at two different temperatures (−4 or −25 °C). Points not bearing the same superscript letter are significantly different ($p < 0.05$).

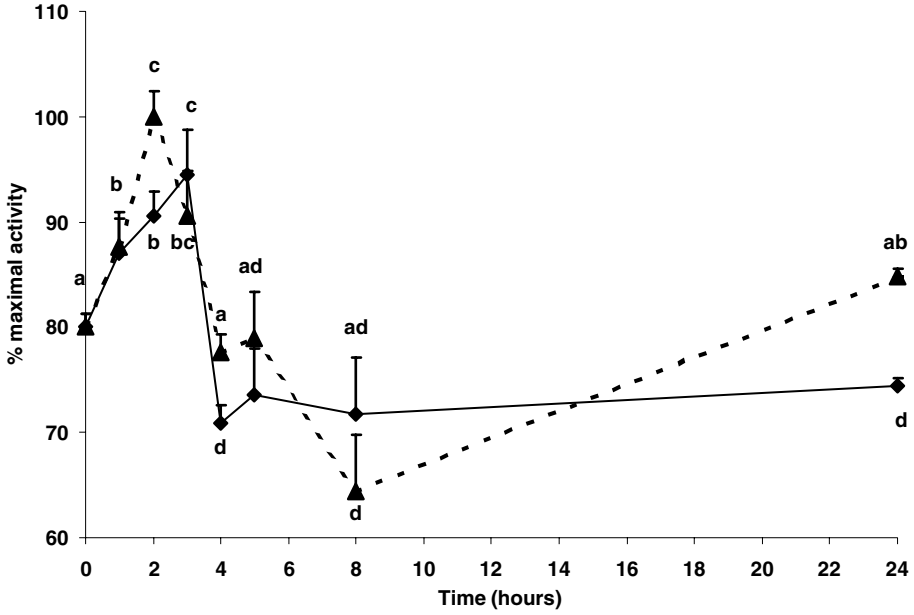

Fig. 5. Total proteolytic alkaline specific activity in crude extracts of cuttlefish digestive viscera during the first 24 h of storage (% of maximal activity) at two different temperatures (−4 °C or −25 °C). Points not bearing the same superscript letter are significantly different ($p < 0.05$).

So, total alkaline protease activity declines a few days after the animal's death under our storage conditions.

Fig. 7 presents the trypsin specific activity in the crude extracts of the digestive viscera cuttlefish during the first 24 h of storage at the two different temperatures. A significant increase ($p < 0.05$) of trypsin activity was observed 2 h after the animal's death (30% of maximal activity), after, which activity decreased to 20% at 4 h. Between 4 and 24 h, the activity remained stable when stored at 4 °C, but increased when stored at 25 °C. So, as for the alkaline proteases, zymogene vesicles may be breaking down a few hours after death, inducing the trypsin activity increases observed 2 h after death. In the case of storage at 25 °C, trypsin activity increased to a maximum at 12 days, after which activity decreased over 4 months to 20% of maximal activity. In contrast, when tissues were stored at 4 °C trypsin activity remained stable up to 20 days after death at 30% of max-

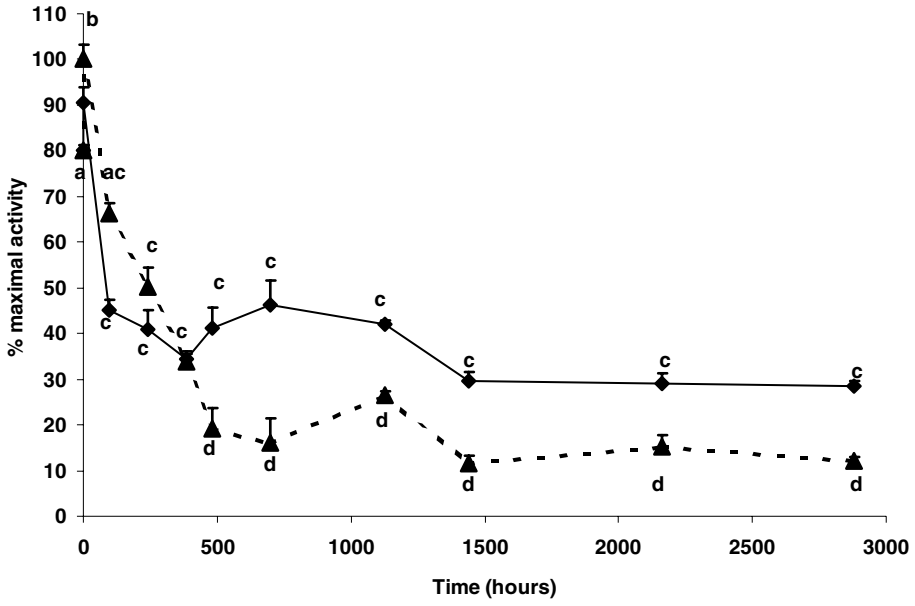

Fig. 6. Total proteolytic alkaline specific activity in crude extracts of cuttlefish digestive viscera during 4 month of storage (% of maximal activity) at two different temperatures (−4 or −25 °C). Points not bearing the same superscript letter are significantly different ($p < 0.05$).

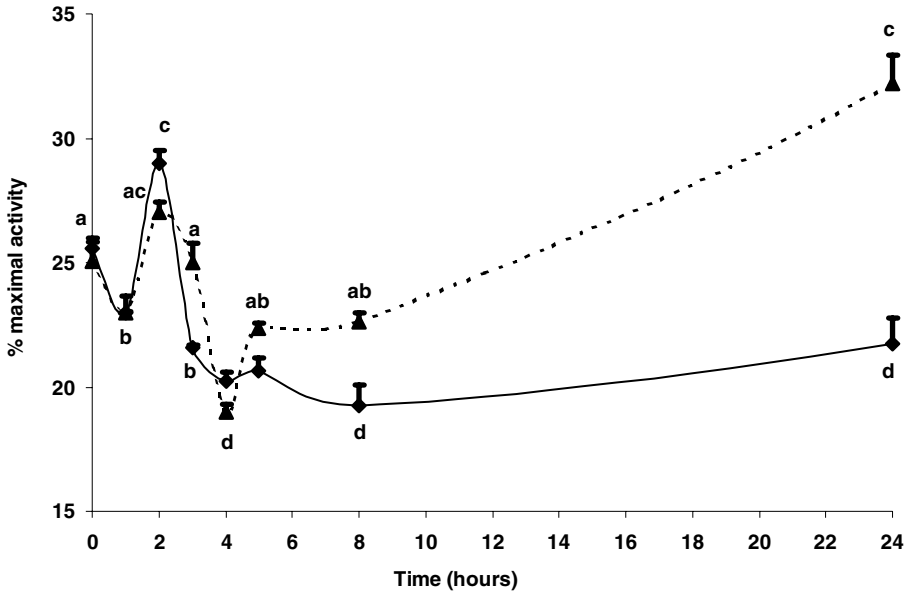

Fig. 7. Trypsin specific activity in crude extracts of cuttlefish digestive viscera during the first 24 h of storage (% of maximal activity) at two different temperatures (−4 or −25 °C). Points not bearing the same superscript letter are significantly different ($p < 0.05$).

imal activity, followed by a significant increase ($p < 0.05$) at 30 days (60% of maximal activity). After 30 days, trypsin activity decreased significantly to 20% of maximal activity four months after the death of the cuttlefish (Fig. 8). Trypsin stability and activity therefore appears to be dependent on storage temperature. Nevertheless, trypsin activity remains high for at least 12 days.

There is a significant increase ($p < 0.05$) of chymotrypsin activity in crude extracts of cuttlefish viscera 2 h after the animal's death (90–100% of maximal activity). This activity decreases after 4 h (77%) in viscera stored at 4 °C, and 8 h (60%) in viscera stored at 25 °C (Fig. 9). This activity decreases after 4 h (77%) in viscera stored at 4 °C, and 8 h (60%) in viscera stored at 25 °C. After decreasing, chymotrypsin activity stays stable up to 24 h. It would seem, therefore, that zymogene vesicles were breaking few hours after the cuttlefish death releasing alkaline proteases and trypsin and inducing the chymotrypsin activity increases 2 h after the death.

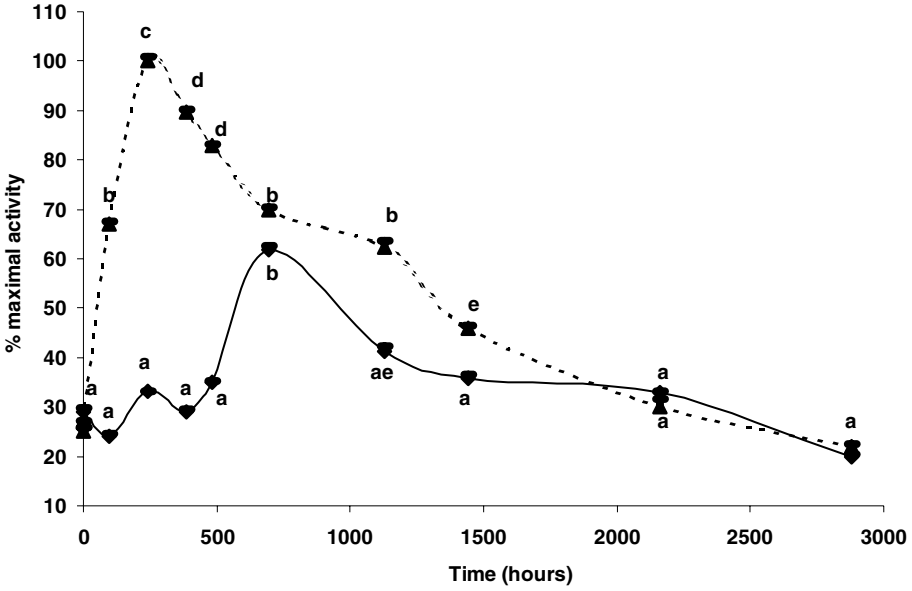

Fig. 8. Tryspin specific activity in crude extracts of cuttlefish digestive viscera during 4 month of storage (% of maximal activity) at two different temperatures (−4 or −25 °C). Points not bearing the same superscript letter are significantly different ($p < 0.05$).

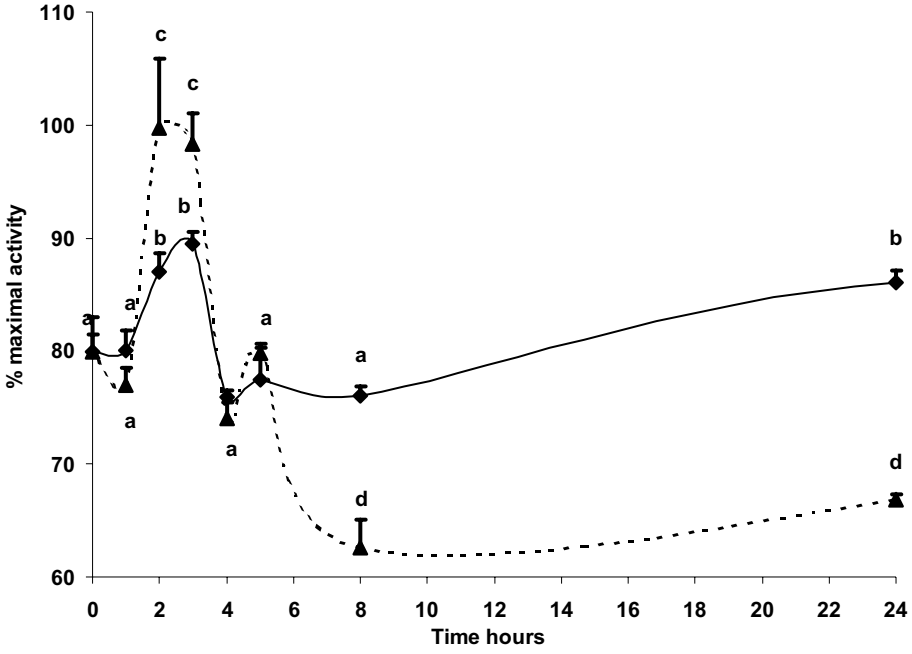

Fig. 9. Chymotrypsin specific activity in crude extracts of cuttlefish digestive viscera during the first 24 h of storage (% of maximal activity) at two different temperatures (−4 or −25 °C). Points not bearing the same superscript letter are significantly different ($p < 0.05$).

Chymotrypsin activity decreased significantly ($p < 0.05$) after 50 days to be at 30% of maximal activity four months after the death of the cuttlefish (Fig. 10). Chymotrypsin was very stable on storage. It is a serine protease, which acts at alkaline pH and is stable in poor acidic pH conditions. Chymotrypsin and trypsin are quantitatively important in the digestive system of carnivorous cuttlefish due to their high proteolytic activities (Heu, Kim, & Pyeun, 1995). They actively participate in the viscera autolysis.

Fig. 11 shows that lipase specific activity in the crude extracts of cuttlefish digestive viscera does not change during the first 24 h of storage at the two different temperatures. Lipases appear to be stable under the storage conditions at 4 or 25 °C.

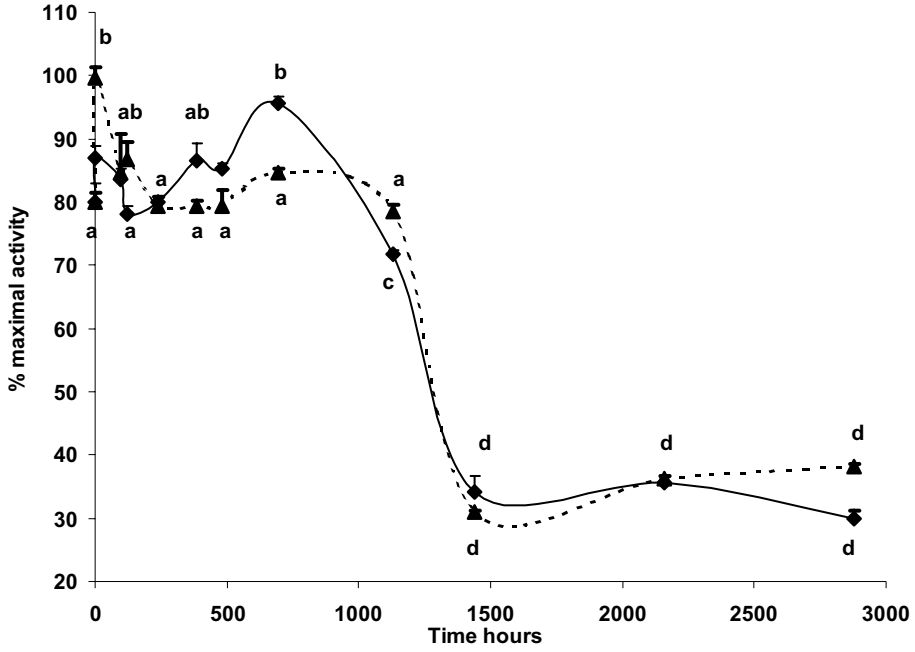

Fig. 10. Chymotrypsin specific activity in crude extracts of cuttlefish digestive viscera during 4 month of storage (% of maximal activity) at two different temperatures (−4 or −25 °C). Points not bearing the same superscript letter are significantly different ($p < 0.05$).

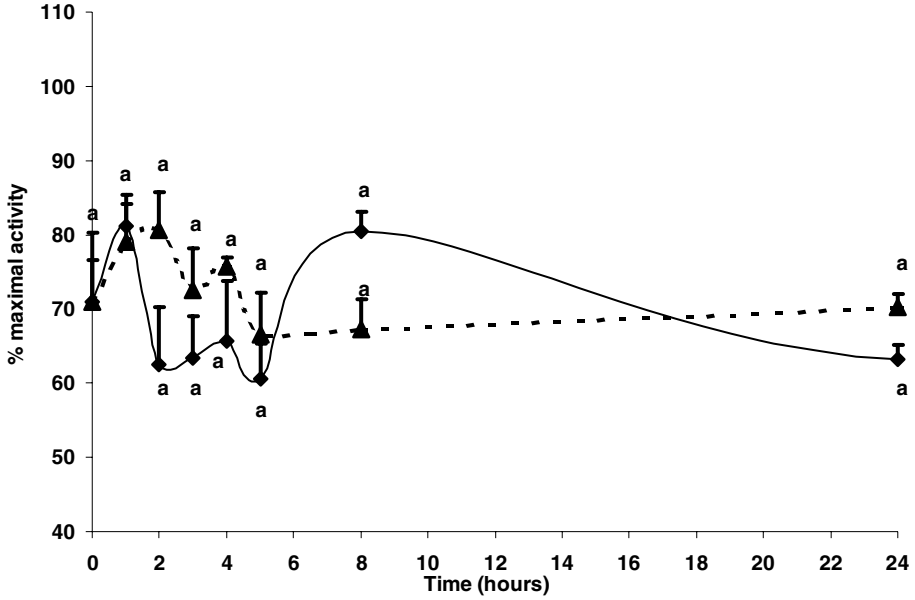

Fig. 11. Lipases specific activity in crude extracts of cuttlefish digestive viscera during the first 24 h of storage (% of maximal activity) at two different temperatures (−4 or −25 °C). Points not bearing the same superscript letter are significantly different ($p < 0.05$).

Lipase activity stays significantly stable up to 50 days (90% of maximal activity). After 50 days, lipase activity decreased significantly ($p < 0.05$) to be at 10% of maximal activity four months after the death of the cuttlefish (Fig. 12) and after this to remain stable. In this way, Abbas, Hiol, Deyris, and Comeau (2002) showed that lipases have optimal stability at pH 5–7. Lipases are essential in autolysis because they determine the lipid quality in the final product. Their high stability in time can result in the accumulation of polyunsaturated fatty acids in high quantity in the viscera autolysate.

Fig. 13 describes the amylase specific activity in the crude extracts of cuttlefish digestive viscera during the first 24 h of storage at the two different temperatures. We observed a significant increase ($p < 0.05$) of amylase activity 2 h after the animal's death (90–100% of maximal

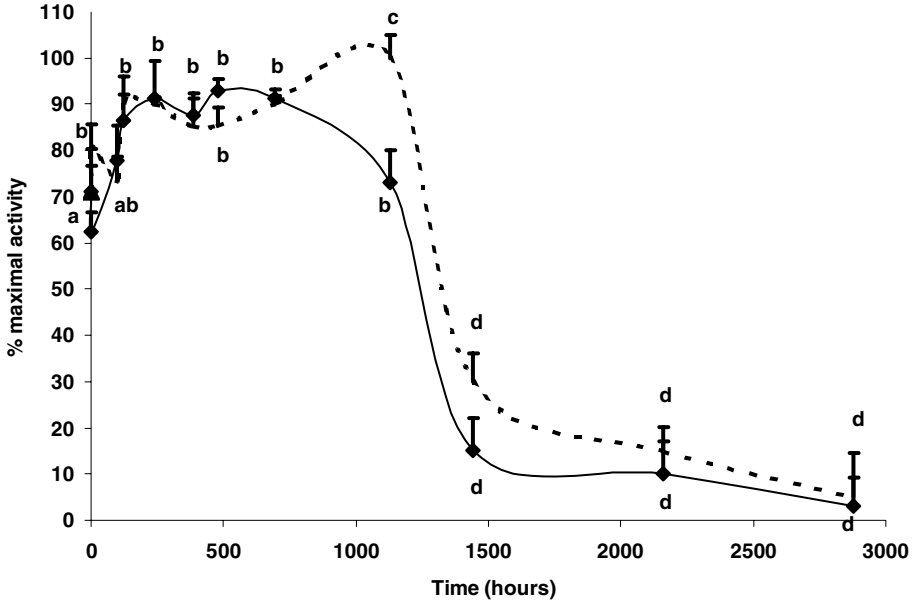

Fig. 12. Lipases specific activity in crude extracts of cuttlefish digestive viscera during 4 month of storage (% of maximal activity) at two different temperatures (−4 or −25 °C). Points not bearing the same superscript letter are significantly different ($p < 0.05$).

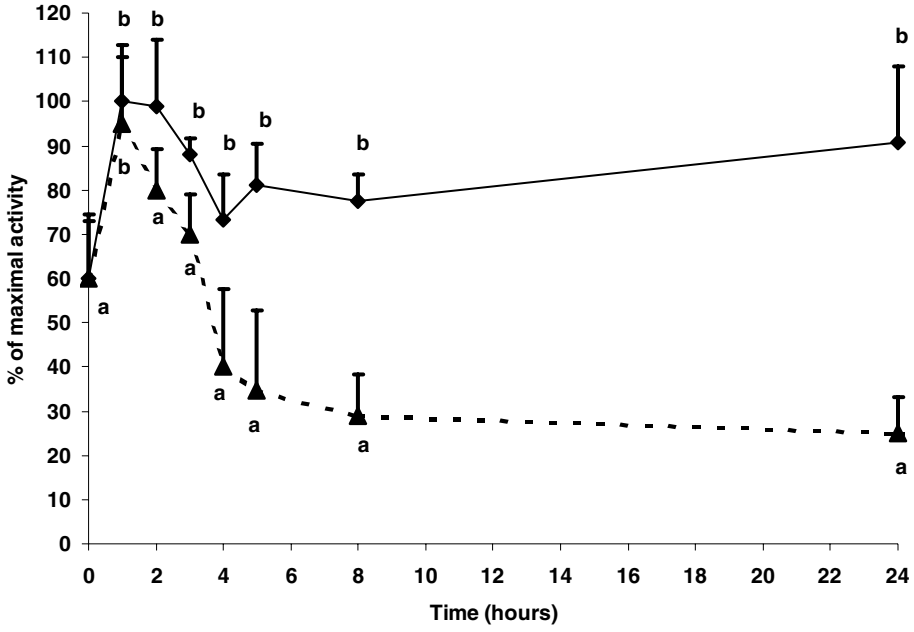

Fig. 13. Amylase specific activity in crude extracts of cuttlefish digestive viscera during the first 24 h of storage (% of maximal activity) at two different temperatures (−4 or −25 °C). Points not bearing the same superscript letter are significantly different ($p < 0.05$).

activity), followed by a decrease after 4 h (30%) in the viscera stored at 25 °C, which was not seen in the viscera stored at 4 °C. After these variations, amylase activity stays stable up to 24 h.

Amylase activity decreased significantly ($p < 0.05$) after 10 days in the viscera stored at 25 °C and after 30 days in the viscera stored at 4 °C to be at 10% of maximal activity four month after the death of the cuttlefish (Fig. 14). Like carnivores (Hidalgo, Urea, & Sanz, 1999), cuttlefish have low amylase activity. Nevertheless, amylase was relatively stable under our storage conditions, even if this stability varied according to the temperature of storage.

3.3. pH changes

The pH at the animal's death was 6.42 (Table 1). We observed a significant pH decrease ($p < 0.05$) 2 h after

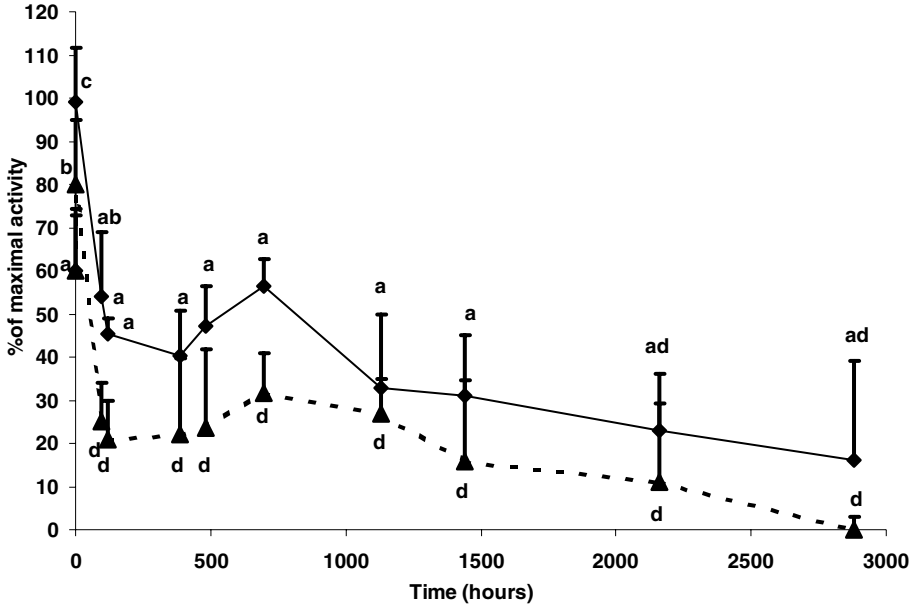

Fig. 14. Amylase specific activity in crude extracts of cuttlefish digestive viscera during 4 month of storage (% of maximal activity) at two different temperatures (−4 or −25 °C). Points not bearing the same superscript letter are significantly different ($p < 0.05$).

the death of the cuttlefish. In the viscera stored at 4 °C, the pH stayed significantly stable up ($p < 0.05$) to 24 h. After 24 h, in the viscera stored at 4 °C the pH increased slowly to be at 6.81 after 4 months. On the other hand, in viscera stored at 25 °C there was a significant increase in pH ($p < 0.05$) after 4 months (7.19). The death of an animal initiates major changes in the biochemical features of tissue, leading to its disintegration (Delbarre-Ladrat et al., 2004). After death, there is a decrease in pH and a reduction of cell membrane integrity (Church, 1998; Cottin & Ducastaing, 1996). These changes interact with other biochemical processes occurring after death, especially proteolysis (Delbarre-Ladrat et al., 2004).

3.4. TCA soluble protein changes

The TCA soluble protein level at the animal's death was 54.6 mg/ml (Table 1). We observed a significant decrease in the TCA soluble protein level ($p < 0.05$) 2 h after the death of the cuttlefish due to a decrease in pH. In the viscera, the TCA soluble protein level significantly increased ($p < 0.05$) over 50 days. After 50 days, the TCA soluble protein level significantly decreased ($p < 0.05$) to be at 29 mg/ml after 4 months. Haaland and Njaa (1889) have observed a stabilization of the TCA soluble protein level after 1–3 months storage time at 4 °C. Benjakul et al. (2003) have shown an increasing of TCA-soluble peptides and amino acids in

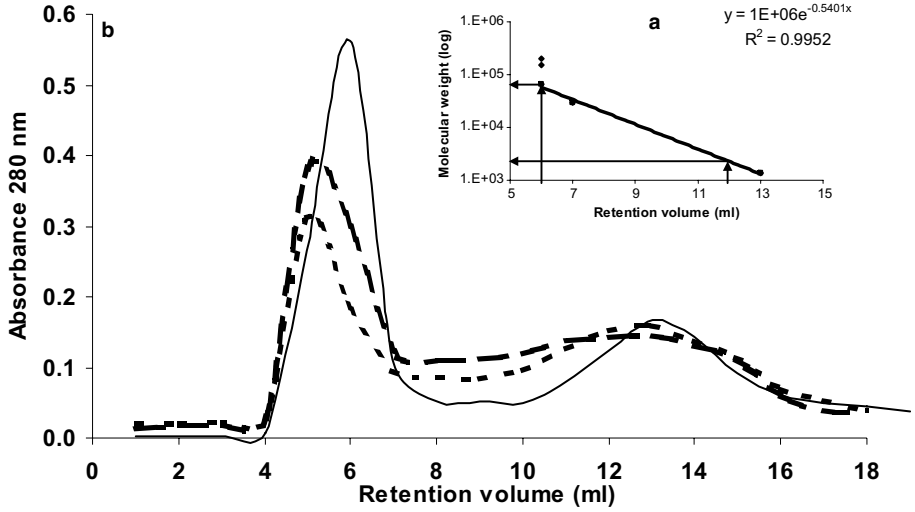

Fig. 15. (a) Calibration graph of molecular weight for Pharmacia G25M PD 10 Sephadex. (b) Effect of storage temperature on molecular weight of peptides and proteins between the cuttlefish death to 24 h.

the lizardfish throughout 15 days of iced storage, suggesting the autolytic degradation of protein. Moreover, they observed that, generally, whole fish had more TCA-soluble peptides and amino acids than heated/eviscerated fish, especially when the storage time increased. In this way, viscera contain a variety of digestive proteases, which play a role in the softening of abdominal tissues during post-mortem storage (Haard, 1994). The protein solubility increased during the initial months of storage, and then fell, as is characteristic of protein aggregation (Ruiz-Capillas, Moral, Morales, & Montero, 2002). After 50 days of storage of the viscera, there was denaturation and aggregation of proteins.

3.5. Molecular weight of proteins and peptides changes

The molecular weight of protein at the animal's death was greater than at 20 kDa for 80% of protein (Table 1 and Fig. 15). The molecular weight of protein decreases after the animal's death. After 4 months, the molecular weight of protein was lower than 6.5 kDa for 70% of proteins in the viscera stored at 4 °C and for 84% of proteins in the viscera stored at 25 °C. This implies that there is a significant autolysis ($p < 0.05$) reflected by a high decrease of the molecular weight of proteins contained in stored viscera. In this way, after 4 months of storage the main protein components have a molecular weight lower than 6500 (composed of about 65 amino acids).

4. Conclusion

Properties of viscera enzymes are important in the understanding degradation of proteins and lipids, and may provide information useful in the production of cuttlefish viscera silage. Autolysate is used as a feed for farmed fish and other animals (Haaland & Njaa, 1889). The nutritional quality of the silage may depend on the degree of autolysis and the products formed. Haaland and Njaa (1889) showed that temperature influences both the degree of autolysis reached after storage and the degree of hydrolysis of the amide groups. Moreover, the undissolved fraction, which always remains in the autolysate, was smaller in the autolysate stored at high temperatures than in the autolysate at 2 °C (Haaland & Njaa, 1889). The liquefaction of silages is markedly favored at acid pH values and at temperatures above room temperature (Raa & Gildberg, 1976). Despite the weak stability of viscera enzymes in our experiments after 96 h, we observed a high degree of autolysis in the first few days. Our results allow us to conclude that there are two different phenomena in the post-mortem evolution of cuttlefish viscera. There are fast phenomena, occurring a few hours after the animal's death, which induce irreversible and uncontrollable mechanisms. Slow phenomena occur several days after the animal's death and imply autolysis over which we may have control by controlling temperature, pH, etc. Autolysis may be restricted to less than 50 days because beyond that time, native proteins are denatured and aggregated. Acid needs to be added to obtain the optimum pH for enzyme activities, autolysis acceleration and to stop bacteria development (Gildberg & Almas, 1986). Finally, concerning the temperature, our results are insufficient to decide between storage at 4 or 25 °C. In future experiments, autolysate fabrication will be made at 4 and 25 °C. Autolysate quality (protein and lipid concentrations as well as toxicity tests heavy metals, hydrocarbon and pesticides concentrations) will be examined. Finally, the study of the dietary value of viscera autolysate must be observed in vivo, via its use as protein complement in cuttlefish rearing and in vitro on the viability of the cell isolated from the cuttlefish digestive gland and digestive physiology.

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