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Abstract

The physiology of protein digestion by adult abalone was investigated. The female and male digestive systems of blue abalone were studied by using enzymatic techniques, including proteinaceous-substrate hydrolysis, specific synthetic-substrate hydrolysis, specific inhibitors in proteinaceous-substrate hydrolysis, and proteinase composition by substrate SDS–PAGE. The highest proteolytic activity was found in the intestinal and rectal fluids at alkaline pH, followed by hepatopancreas and crop–stomach content at acid pH. Trypsin and chymotrypsin activity were found along the intestine and rectum. Chymotrypsin activity was 10 times higher than trypsin activity. Several unknown proteinase activities were found in the hepatopancreas, crop–stomach content, intestinal fluid, and rectal fluid. © 1997 Elsevier Science B.V.

Keywords: Blue abalone; Proteolytic activity; Proteinases; Chymotrypsin; Trypsin

1. Introduction

In Japan, China, Korea, Thailand, Australia, New Zealand, South Africa, Canada, México and the USA, feeding of juvenile abalone has shifted from seaweeds to nutritionally suitable and cost-effective artificial diets (Wee et al., 1992; Viana et al., 1993; Britz, 1996b; Fleming et al., 1996). Artificial diets using different sources of protein have been evaluated or developed in countries with a history of abalone culture

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(Britz, 1996a,b; Fleming et al., 1996; Knauer et al., 1996). In México, attempts have been made to formulate artificial diets from different protein sources, preferably at low cost and with locally available ingredients (Viana et al., 1993, 1994, 1996; López and Viana, 1995; Rivero and Viana, 1996). It is still necessary to investigate natural diets, digestive processes, and food digestibility of Mexican species to develop suitable formulated feeds for all abalone life stages.

Abalone, like many other animals, cannot synthesize 10 of the 20 L-amino acids required to assemble proteins, and their growth rate is thus dependent on the quantity and proportion of these amino acids in their diet. Protease activity in the digestive tract is a key determinant of the digestibility and assimilation efficiency of ingested proteins.

In general, pepsin in the stomach, and trypsin, chymotrypsin, and carboxy- and aminopeptidases in the intestine, are responsible for the hydrolysis of ingested proteins (Whitaker, 1994). Activity of the digestive enzymes varies significantly among animal groups, reflecting, in a complex way, the influences of evolutionary adaptation to diet.

In Haliotids, studies on enzyme activity are meager, with most focusing on enzymes that break down structural polysaccharides and proteins. Lipases, proteases, and glucoside hydrolases, e.g., cellulase, laminarinase, alginase, carrageenanase, and agarase, have been described in abalone such as *Haliotis coccinea canariensis*, *H. midae*, and *H. rufescens* (Albrecht, 1921; McLean, 1970; Gómez-Pinchetti and García-Reina, 1993; Erasmus, 1996; Fleming et al., 1996; Knauer et al., 1996). The presence of some of these enzymes has been related to the diet of the abalone. Additionally, enzymes from abalone enteric bacteria can hydrolyze the seaweed polysacccharides agarose, carrageenan, laminarin, and alginate (Erasmus, 1996). However, only limited studies on proteases and their zymogens have been reported (Groppe and Morse, 1993).

This present work is based on the hypothesis that a better knowledge of the digestive system of *H. fulgens* will promote the development of formulated diets for the commercial cultivation of abalone. The aim of this paper is to use a series of enzymatic techniques to describe proteolytic activity in the hepatopancreas, crop–stomach content, and intestinal and rectal fluids of both male and female blue abalone.

2. Materials and methods

2.1. Abalone sampling

Blue abalone *H. fulgens* were obtained by 'Sociedad Cooperativa Leyes de Reforma' at Bahía Asunción (27°15′N lat, and 114°15′W long) on the Pacific Coast of Baja California Sur in March 1996 during commercial harvest. The digestive systems of 100 adults (> 150 mm) were donated for the study. Adult female ($n = 50$) and male ($n = 50$) samples were analyzed separately.

2.2. Sample preparation

The digestive systems of both adult females (♀) and males (♂) were chilled on ice and dissected while cold. Hepatopancreas, crop–stomach content, intestine, and rectum

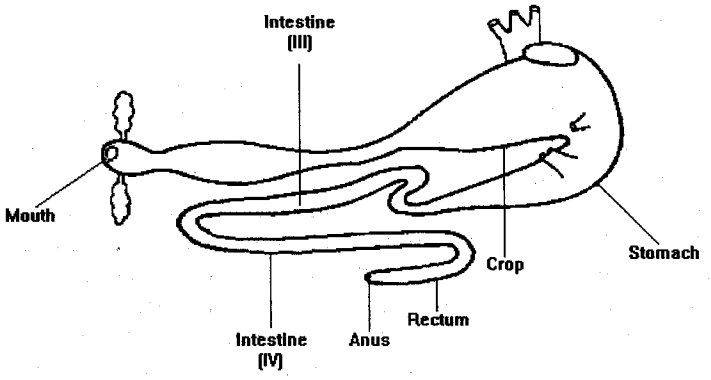

Fig. 1. Schematic diagram of digestive tract of abalone. (III) anteriorly-directed limb of intestine, (IV) posteriorly-directed limb of intestine McLean (1970).

were dissected using the illustrations of McLean (1970) as a guide (Fig. 1). Pooled samples of hepatopancreas (HP) and crop–stomach content (CSC) extracts were obtained by mixing samples from 20 organisms, and pooled samples of intestinal (posteriorly-directed limb of intestine) (IN) and rectal (RE) fluid extracts by mixing samples from 50 organisms. Pooled samples were mixed with one volume (by weight) of 50 mM Tris · HCL, pH 8.0 (Groppe and Morse, 1993). They were transported on ice to the laboratory and stored at −4°C prior to use. Samples of HP, CSC, IN, and RE were homogenized at 4°C with a hand blender. To eliminate feed residues, solid material and lipids, the homogenate was centrifuged at 10500 *g* at 4°C for 10 min in a Beckman centrifuge. Aliquots of the supernatants were stored at −30°C.

2.3. Enzyme assay

The soluble protein content of the extracts was measured in triplicate according to Bradford (1976) using bovine albumin (1 mg/ml) as the standard. Proteolytic activity of the samples was assayed using 1% azocasein in Universal Buffer (Stauffer, 1989) at different pH values according to García-Carreño and Haard (1993) to: (1) determine the influence of pH on enzyme activity and find the optimal condition for evaluation of activity assays, (2) to predict variations in activity by exogenous factors, such as food composition, and (3) to determine the correlation with endogenous pH. Enzyme preparation samples of IN♀, IN♂, RE♀, and RE♂ (0.04 mg protein), HP♀ and CSC♀ (0.4 mg protein), and HP♂ and CSC♂ (0.08 mg protein) were mixed with 0.5 ml of Universal Buffer at 25°C and with pH varying from 2.0 to 12.0. The reaction was initiated by the addition of 0.5 ml of 1% azocasein and stopped 60 min later by adding 0.50 ml of 20% trichloroacetic acid (TCA). The reaction mixture was centrifuged in Eppendorf tubes for 5 min at 14500 *g*. The supernatant was separated from the undigested substrate and the $A_{366\ nm}$ of the released dye was recorded. For controls, TCA was added before the substrate. The activity unit was $\Delta A_{366\ nm/min}$ per mg of enzyme protein in the assay.

2.4. Trypsin and chymotrypsin activity

Trypsin amidase activity was assayed in test tubes using benzoyl-Arg-*p*-nitroanilide (BAPNA) as substrate (Erlanger et al., 1961; García-Carreño and Haard, 1993). BAPNA was dissolved in 1 ml of DMSO to make a 1 mM solution of the substrate and then made to 100 ml with 50 mM Tris · HCl buffer, 20 mM $CaCl_2$, pH 7.5, at 37°C, to allow substrate solubilization. The reaction mixture was maintained at 25°C during the enzyme assay period. To 1.25 ml of fresh substrate solution, 25 μl of the enzyme preparation was added. After 10 min, 0.25 ml of 30% acetic acid was added and $A_{410\ nm}$ was recorded against a water blank. BAPNA hydrolysis units were evaluated according to Dimes et al. (1994). Amidase activity was expressed in BAPNA units/mg as: ($A_{410\ nm}/^{min} \times 1000 \times$ vol of the reaction mixture)/(8800 × mg protein in the assay), where 8800 is the extinction coefficient of *p*-nitroaniline (Erlanger et al., 1961).

Chymotrypsin activity was assayed using succinyl-$(Ala)_2$-Pro-Phe-*p*-nitroanilide (SAPNA) as substrate. The assays were run at 25°C. Hydrolysis of SAPNA was continuously recorded as the increase in $A_{410\ nm}$. Ten μl of enzyme preparation was mixed with 0.590 ml of 0.1 mM SAPNA solution in 50 mM Tris · HCl buffer, 20 mM $CaCl_2$, pH 7.5, 8.0, 9.0, or 10.0. The absorbance was recorded for 5 min. Chymotrypsin activity was expressed in SAPNA units/mg as: ($A_{410\ nm}/^{min} \times 1000 \times$ vol of the reaction mixture)/(8800 × mg protein in the assay), where 8800 is the extinction coefficient of *p*-nitroaniline (Erlanger et al., 1961).

2.5. SDS–polyacrylamide gel (SDS–PAGE) electrophoresis

Electrophoretic separation of the protein in the enzyme preparations was done according to a modification of the method of Laemmli (1970) using 12.5% acrylamide gels. The enzyme extracts were diluted (1:4) in sample buffer containing SDS, but with no reducing agents. The diluted samples were not boiled before loading into the gels. Samples of 2.5 to 12.0 μl were applied to the gel in each well. The amount of protein loaded for each sample is indicated in the legend of Fig. 2. A total of 5 μl of molecular weight markers (MWM) was loaded on each gel for easy comparison of the MW of the protein bands and activity zones.

2.6. Substrate SDS–PAGE

Zymograms of protease activities of the fractions separated by electrophoresis were made according to García-Carreño et al. (1993). Electrophoresis was run at a constant current of 15 mA per gel for 90 min at 5°C.

The characterization of enzymes responsible for protein hydrolysis in the abalone extracts was done in zymograms using proteinase-specific inhibitors (García-Carreño and Haard, 1993). Three volumes of the enzyme preparation were mixed with one volume of one of the following serine proteinase inhibitors: 100 mM phenylmethanesulfonyl fluoride (PMSF) in 2-propanol, 10 mM tosyl-Lys-chloromethyl ketone (TLCK) in 1 mM HCl, or 5 mM tosyl-Phe-chloromethyl ketone (TPCK) in MeOH (García-Carreño, 1992). Mixtures were incubated for 1 h at 25°C. Aliquots of the mixture were

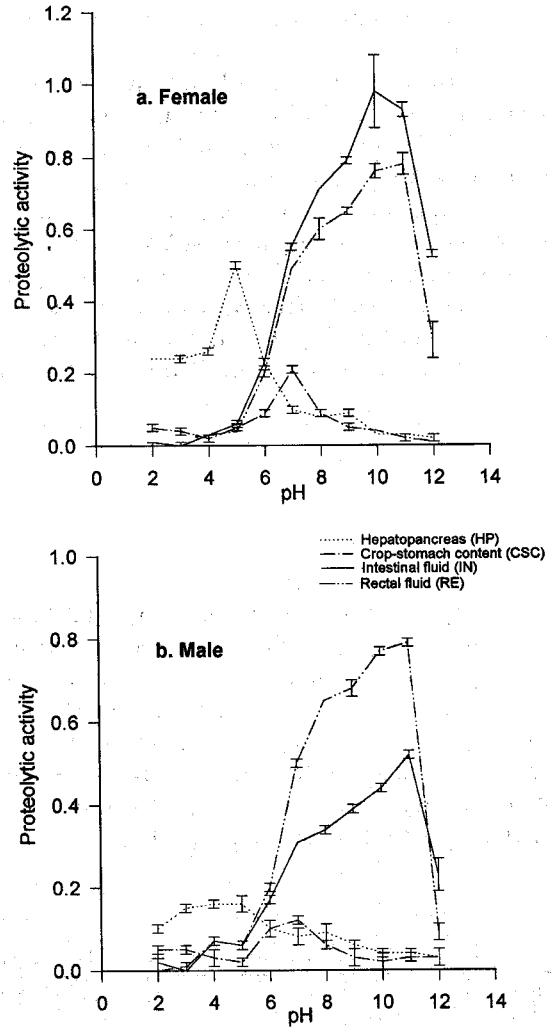

Fig. 2. Effect of pH on the proteolytic activity of female (a) and male (b) extracts of *H. fulgens*. The enzyme preparations were assayed with azocasein following the standard procedure using Universal buffer at different pHs.

diluted 1:4 in sample SDS–PAGE buffer and 2.5 μl of the mixture was loaded into SDS–PAGE gels and developed for zymograms. Samples of enzyme preparations without inhibitor and corresponding controls treated with inhibitor solvent were included in the assay. MWMs were incorporated into the same gel to determine the MW of the proteinases.

After electrophoresis, gels were immersed in 50 ml of 3% casein, 50 mM Tris–HCl buffer, pH 7.5, or Universal buffer (Stauffer, 1989), for 30 min at 5°C. The low temperature allowed the substrate to diffuse into the gel at reduced enzyme activity.

Then the temperature was raised to 25°C and the gels were incubated for 90 min for the digestion of the protein substrate by the active fractions.

After substrate hydrolysis, gels were washed with distilled water and immediately fixed and stained in a one-step process by immersing them in a filtered staining solution containing 40% ethanol, 10% acetic acid, and 0.05% Coomassie brilliant blue R-250. Gels used for detection of MWM and proteins other than proteinases were stained as well. The staining period was 2 h. Clear zones on a blue background, indicating proteinase activities, were observed at this stage. MWM bands and proteins other than proteinases had a higher intensity of blue color than the background caused by the staining of the undigested casein. Two hours of washing with aqueous 40% ethanol–10% acetic acid was enough to destain and improve the contrast of the clear zones indicating proteinase activity. Gels were dried using a Bio-Rad slab gel dryer (Model 583) and photographed.

3. Results

3.1. Proteolytic activity

The following characteristics were considered for enzyme activity description: (1) the protein content, (2) protease activity using the proteaceous substrate, azocasein, and (3) enzyme composition using specific substrates, substrate gel electrophoresis, and specific inhibition in substrate gel electrophoresis from both female and male extracts.

The amount of soluble protein in hepatopancreas, crop–stomach content, and intestinal and rectal fluids was similar in females and males (Table 1), although the concentration of protein in female extracts was slightly lower. The largest amount of protein was found in HP, followed by CSC, IN, and RE.

Table 1
Protein concentration, and protease, trypsin, and chymotrypsin activity in blue abalone (*H. fulgens*) extracts

Extracts	Soluble protein (mg/ml)	Protease activity[a] (pH)	Trypsin activity[b]	Chymotrypsin activity[c]
♂ Hepatopancreas	25.19	0.50 (5)	0.0	0.0
♀ Hepatopancreas	25.63	0.16 (5)	0.0	0.0
♀ Crop–stomach	18.43	0.21 (7)	0.0	0.0
♂ Crop–stomach	21.14	0.12 (7)	0.0	0.0
♀ Intestine	0.97	0.78 (11)	0.05	3.38
♂ Intestine	1.05	0.79 (11)	0.02	3.05
♀ Rectum	0.62	0.98 (10)	0.18	1.85
♂ Rectum	0.77	0.52 (11)	0.11	5.67

[a]Proteolytic activity with azocasein, $\Delta Abs_{366\,nm}/^{min}$/mg protein. The value of maximum activity and pH optimum are reported.
[b]Specific activity with BAPNA, units/mg protein.
[c]Specific activity with SAPNA, units/mg protein.
Values are the average of pooled sample triplicates.
The coefficient of variation was less than 12%.

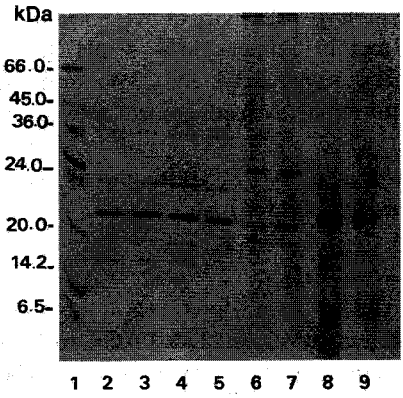

Fig. 3. SDS–PAGE for protein composition in *H. fulgens*. Column (C) 1, molecular weight marker (MWM); C2, IN ♀; C3, IN ♂; C4, RE ♀; C5, RE ♂; C6, CSC ♀; C7, CSC ♂; C8, HP ♀; C9, HP ♂. Sample volumes were adjusted to 5.0 μg of protein in intestinal fluids and 10.0 μg in CSC and HP.

The protease activity in female extracts was higher than in male extracts. In both, the highest activity was found in the intestine and rectum, followed by HP and CSC (Table 1). A gradient of decreasing protein concentration down the length of the intestine was found. In females, the protein gradient was inversely proportional to a gradient of protease activity, but not in males.

3.2. Effect of pH on protease activity

The pH of frozen-thawed digestive system extracts was 5.7 for HP and CSC, 6.0 for IN, and 6.4 for RE. Similar trends in protease activity as a function of pH were observed in female and males (Fig. 3a,b). In both sexes, HP activity increased between pH 2 and 5 and then decreased with increasing pH. Maximum activity was observed at a lower pH than the physiological pH found in the extract (5.7). In both sexes, the pH of maximum activity of CSC was pH 7, one unit higher than in the extract. The pH of maximum proteolytic activity in IN and in RE of both males and females was about 10 (Fig. 2a,b).

3.3. Trypsin and chymotrypsin activity

Trypsin activity was found in both intestine and rectum, but not in HP and CSC. In both sexes, an increase in trypsin activity down the intestine was found. However, a higher trypsin activity was detected in females than in males (Table 1).

Extracts of both intestinal and rectal fluids possessed chymotrypsin activity. In contrast, in HP and CSC, chymotrypsin activity was not found. Regardless of sex, chymotrypsin activity was similar in the intestine. However, in females intestinal chymotrypsin activity was higher than in the rectum, whereas in males the activity was higher in the rectum (Table 1).

Chymotrypsin activity using SAPNA as substrate was measured at increasing pH. In all extracts, similar to proteolytic activity, an increase in chymotrypsin activity with pH

was found. In females, chymotrypsin activity at pH 7.5 in the intestinal extract increased up to 238%, and in the rectum extract 211% at pH 9.0. In males, the increase in chymotrypsin activity at pH 7.5 was 263% and 339% in intestinal and rectal extracts at pH 9.0. Enzymes in the intestinal extract seemed to work better in a highly alkaline environment.

The ratio of chymotrypsin activity to trypsin activity was IN = 68 and RE = 10 for females, and IN = 150 and RE = 52 for males.

3.4. Electrophoresis (SDS–PAGE)

SDS–PAGE for protein and substrate SDS–PAGE for protease were used to determine the composition of sections of the digestive system. The protein composition was similar in female and male extracts, except for the HP extract (Fig. 3). Proteins of 22 kDa and 14 kDa were more abundant in females than in males. The differences observed in HP may be related to the stage of maturation of gonads because a mobilization of tissue components between hepatopancreas and gonads is found. Protein bands in the intestine and rectum were no more than ten. Most of these proteins were lower than 22 kDa. Three of about ten bands of protein with MW of 37,000, 26,000, and 22,000 were identified as proteinases.

Zymograms revealed several bands of protease activity in intestinal and rectal fluids (Fig. 4). The protease composition of IN and RE was similar when comparing sections of intestine and sex. The active zones in both had molecular weights of 22,000 and 26,000, and a series of high-molecular-weight enzymes. The rectum had the more evident and complex high molecular weight active zones.

Enzyme activity of abalone hepatopancreas and crop–stomach content was negligible in alkaline solutions, and active bands in zymograms were not detected. They have maximum activity at acid pH.

A reduction in active zones of abalone intestinal and rectal fluids was found in

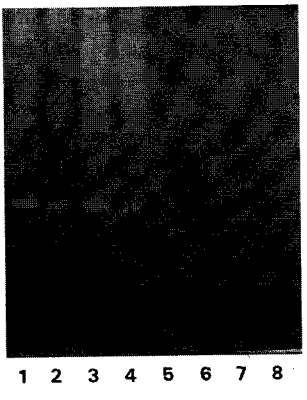

Fig. 4. Substrate SDS–PAGE for composition of proteases in *H. fulgens*. Column (C) 1, IN♀; C2, IN♂; C3, RE♀; C4, RE♂; C5, CSC♀; C6, CSC♂; C7, HP♀; C8, HP♂. Sample volumes were adjusted to 3.0 μg for protein content in intestinal and rectal fluids and 10 μg in CSC and HP.

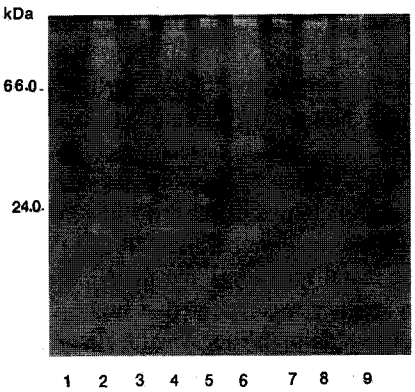

Fig. 5. Substrate SDS–PAGE for proteinase inhibitors in female intestinal fluids of *H. fulgens*. Column (C) 1, 5.0 μg MWM; C2, IN control; C3, IN+PMSF; C4, IN+TLCK; C5, IN+TPCK; C6, RE control; C7, RE+PMSF; C8, RE+TLCK; C9, RE+TPCK. Sample volumes were adjusted to 3.0 μg for protein content.

zymograms treated with inhibitors for serine proteinases (Fig. 5). The inhibition by PMSF suggests that at least one blue abalone enzyme is a serine protease. TLCK and TPCK are known inhibitors for chymotrypsin and trypsin (García-Carreño, 1992). The active zone between 25 and 29 kDa was inhibited by TLCK in both fluids, and the zone over 66 kDa was slightly inhibited by TPCK in IN.

4. Discussion

The amount of soluble protein in all pooled samples was similar in females and males. The largest amount of protein was found in hepatopancreas, followed by crop–stomach content, and intestinal and rectal fluid. Protein in crop–stomach content, intestine, and rectal extracts is derived from food and tissue debris and secretions, whereas protein in the hepatopancreas comes from the tissue itself.

Proteolytic activity in blue abalone adults was detected in hepatopancreas, crop–stomach content, and intestinal and rectal fluids. These results agree with the work of Albrecht (1921) and McLean (1970) who reported protease, amylase, and lipase (among others) in the digestive fluid of *H. rufescens*. In female extracts, proteolytic activity was higher than in male extracts. Comparable results on enzyme activities regarding sex of other species are currently lacking. These possibilities remain to be assessed, because of the importance of this finding for abalone physiology and culture.

The pH of maximum activity was different between extracts. In both sexes, the pH range in which the enzyme activity was 50% or more of the maximum activity was between 4 and 6 in HP, 6 and 8 in CSC, and 7 and 11 in IN and RE. This catalytical characteristic of enzymes in abalone extracts guarantees that about 50% of the enzyme activity is available to break down food protein at physiological conditions. In the same way, McLean (1970) described, in *H. rufescens*, that the optimum pH of proteolytic, amylolytic, and lipase activities was different between crop fluid and homogenates of various tissues (midgut gland, salivary gland, and intestine).

Proteolytic activity has been described in other abalone species, but the knowledge of enzyme classes is limited. Trypsin and chymotrypsin activity were demonstrated in the intestine and rectum by using specific-synthetic substrates and inhibitors in kinetic and electrophoresis assays. The results show chymotrypsin activity was at least ten times greater than trypsin activity. The physiological meaning of ratio activities in abalone remains unknown. Regardless of sex, trypsin activity was higher in the rectum. Chymotrypsin activity was higher in the intestine in females, but not in males. These results are consistent with those of Groppe and Morse (1993) who found chymotrypsin proteinase expressed in the distal quarter of the intestine of *H. rufescens*. Additionally, they described that trypsin activity was detected at highest concentration in the proximal and distal lumenal fluids of two animals, but was localized more specifically to the distal fluid of a third animal. Therefore, it may be secreted by the epithelial cells of the proximal and distal intestine, or may be secreted by the hepatopancreas and transported down the gastrointestinal tract into the lumen of the intestine (Groppe and Morse, 1993).

Blue abalone enzyme classes from the hepatopancreas and stomach remained unknown. They have maximum activity at acid pH. It is necessary to use specific synthetic substrates and inhibitors in kinetic and electrophoresis assays of different acid enzymes to identify specific enzymes in abalone HP and CSC extracts. In bivalve stomachs and digestive diverticula, acid enzymes such as cathepsins, carboxypeptidases and acid phosphatases have been reported (Reid, 1981). In the squid *Illex argentinus*, the main source of acid proteases in the viscera was the hepatopancreas, and the proteolytic activity was almost all aspartic proteases (Kolodziejska and Mazurek, 1995).

Electrophoresis analysis of proteinases is a powerful tool helping confirm participation of both trypsin and chymotrypsin activities in both intestine and rectum in blue abalone. The active zone between 25 and 29 kDa was inhibited by the chymotrypsin inhibitor TLCK in intestinal and rectal fluids, and the zone > 66 kDa was slightly inhibited by the trypsin inhibitor TPCK in IN. Therefore, the molecular weight of the *H. fulgens* chymotrypsin protease was close to 25,000. In accordance with our results, the molecular weight of the *H. rufescens* chymotrypsin protease calculated from cDNA-encoded amino acid sequence is 24,900 (Groppe and Morse, 1993), essentially equal to the molecular weight estimated of mammal, crustacean, and insect chymotrypsin (Le Chevalier et al., 1995). Other activities could be involved, however, specificities have to be studied on pure enzymes.

These results show the importance of continuing the characterization of digestive enzyme in abalone to understand how these herbivorous organisms use the protein content in macroalgae.

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