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Digestive proteases in juvenile Mexican green abalone, *Haliotis fulgens*

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Abstract

In this study, proteolytic and specific substrate activities were measured in viscera (VIS) and hepatopancreas (HP) extracts from juvenile green abalone, *Haliotis fulgens*. The soluble protein was more abundant in the hepatopancreas than viscera. The HP proteolytic activity was higher than VIS. Proteolytic activity assayed with 1% azocasein and 2% haemoglobin at different pHs showed that juvenile digestive enzymes have activity peaks at acid pH. In alkaline pH, both activities decreased. Regardless of the size of the organism and diet, extracts from hepatopancreas and viscera hydrolysed trypsin, chymotrypsin, and acid phosphatase-specific substrates, but not carboxypeptidase A and B substrates. Specific substrate activities in HP were higher than VIS activity in all groups, except in juveniles fed diatoms in which the VIS trypsin and chymotrypsin activity was higher than the HP activity.

Keywords: Green abalone; Proteolytic activity; Proteinases; Chymotrypsin; Trypsin; Acid phosphate

1. Introduction

The abalone is a marine gastropod. It feeds on microscopic algae during the postlarval phase, changing progressively to macroscopic algae (seaweed), either alive or as detritus. Abalone, like many other herbivores, use enzymes to break down the structural polysaccharides of the algae they consume, and for hydrolysis of ingested proteins. In haliotids, many specific activities of carbohydrases have been measured as well as several other digestive-enzyme activities (Table 1).

Energy metabolism of many gastropods is known to be based on the use of carbohydrates. Available evidence suggests abalone conform to this trend, because their

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Table 1
Studies of digestive enzymes in Haliotids

Activity	Abalone species[a]	Reference
Amylase, emulsin, glycogenase, lactase, lipase, maltase, pepsin, sucrase, urease	not specified	Albrecht (1921)
Amylase, lipase, protease	*H. rufescens*	McLean (1970)
Protease	*H. discus hannai* (110–205 g)	Cho et al. (1983)
Agarase, cellulase, alginase	*H. coccinea canariensis*	Gómez-Pinchetti and García-Reina (1993)
Chymotrypsin, trypsin	*H. rufescens* (150–200 mm)	Groppe and Morse (1993)
Amylase, lipase, protease	*H. midae* (3.2–11.3 mm)	Knauer et al. (1996)
Carboxymethyl cellulase, laminarinase, alginase, carrageenase, agarase	*H. midae* (35 mm)	Erasmus et al. (1997)
Protease, chymotrypsin, trypsin	*H. fulgens* (> 150 mm)	Serviere-Zaragoza et al. (1997)
Protease, chymotrypsin, trypsin, carboxypeptidase	*H. fulgens* (adult)	Hernández-Santoyo et al. (1998)

[a] Weight or shell length.

natural diet is low in fat but rich in storage carbohydrates. Similarly, abalone tissue contains very little fat but rich stores of glycogen (Knauer et al., 1996). Glucoside hydrolases have been described in the abalone *Haliotis coccinea canariensis*, *H. midae*, and *H. rufescens* (Table 1). Additionally, enzymes degrading agarose, carrageenan, laminarin, and alginate from abalone enteric bacteria can hydrolyse the complex polysaccharide components of seaweed that become available for digestion (Erasmus, 1996). Enzymatic systems isolated from the digestive tract of phycophages have been studied as an alternative to degrade both red and brown seaweed cell walls that are not effectively degraded by commercial enzymes (Gómez-Pinchetti and García-Reina, 1993).

Protease activity in the digestive tract is a key determinant of the digestibility and assimilation efficiency of ingested proteins. For mammals, 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). In fish and invertebrates, some mammalian digestive protease-like enzymes has been described (Ikeda et al., 1986; Gildberg, 1988). Proteolytic activity has been detected in the abalone species *H. discus hannai*, *H. midae*, *H. fulgens*, and *Haliotis rufescens* (Table 1). However, knowledge of abalone protease classes is limited. Groppe and Morse (1993) reported the primary structure of a chymotrypsin-like protease from adult red abalone, *H. rufescens*, and found chymotrypsin proteinase expressed in the distal quarter of the intestine. Trypsin activity was detected at its highest concentration in the proximal and distal lumenal, but was localized more specifically to the distal fluid. In adult green abalone, *H. fulgens*, trypsin and chymotrypsin activity were found along the intestine, in rectal fluids (Serviere-Zaragoza et al., 1997), and in the hepatopancreas (Hernández-Santoyo et al., 1998).

Our aim was to use a series of enzymatic techniques to describe proteolytic activity in juvenile green abalone, *H. fulgens*. Knowledge of proteolytic enzyme activity in

juvenile and adult green abalone is instructive and complements a small but growing body of information on carbohydrate and lipid metabolism in abalone. In Mexico, seven abalone species are distributed along the Pacific Coast of Baja California. Two abalone species represent nearly 97% of the total production: green *H. fulgens* and pink abalone *H. corrugata*. The remaining landings include red *H. rufescens*, black *H. cracherodii*, and white *H. sorenseni* abalone (Ramade-Villanueva et al., 1998).

2. Materials and methods

2.1. Abalone sampling

Green abalone *H. fulgens* were obtained from the Laboratory of the ''S.C.P.P. Progreso'' at La Bocana (26°46′30″N lat. and 113°41′35″W long.) on the Pacific Coast of Baja California Sur in October 1996. In La Bocana laboratory, the abalone were held in flow-through, aerated seawater in temperatures ranging from 20 to 25°C and were fed diatoms during the first months, and seaweed later. Digestive systems of the three different groups were studied (Table 2). The number of organisms sampled for each group was related to the quantity required for the assays.

2.2. Sample preparation

At La Bocana laboratory, the digestive systems of juveniles were chilled on ice and dissected while cold. Hepatopancreas (HP) and viscera (VIS) were dissected. Pooled samples of HP and VIS were obtained by mixing samples from 10 to 20 organisms. They were transported on ice to the laboratory and stored at −4°C prior to use. Samples were mixed with an equal weight of distilled water, because extracts were to be assayed with substrates adjusted to different pHs. Samples of HP and VIS were homogenized at 4°C with a hand blender. To eliminate feed residues, solid material, and lipids, the homogenate was centrifuged at $10\,500 \times g$ at 4°C for 15 min in an Eppendorff centrifuge (Model 5403). Aliquots of the supernatant were stored at −30°C.

Table 2
Sample groups of juvenile green abalone, *Haliotis fulgens*. Values are the average ± standard deviation

Group, *n*	Spawning	Shell length (mm)	Weight (g)	Food
I, 152	November 1994	27.95 ± 2.10	2.92 ± 0.70	Diatoms for six months and Seaweed[a] later
II, 75	November 1994	45.00 ± 5.30	10.86 ± 3.06	Diatoms for six months and Seaweed[a] later
III, 155	December 1995	27.61 ± 4.73	2.43 ± 0.71	Diatoms

[a] *Ulva* spp. or *Eisenia arborea*.

2.3. Enzyme assay

The soluble protein content of the extracts was measured according to Bradford (1976) using bovine albumin (1 mg/ml) as standard, in six pooled samples in triplicate. Proteolytic activity of the samples was assayed using 1% azocasein in universal buffer (Stauffer, 1989) according to García-Carreño and Haard (1993), and 2% acidified haemoglobin according to Anson and Mirsky (1932), at different pHs to determine the influence of pH on enzymatic activity and find the optimum condition for evaluation of activity assays. The assays included appropriate blanks and commercial enzymes (1 mg/ml) as internal controls.

Enzyme preparation samples of HP and VIS (0.1 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 Eppendorff tubes for 5 min at $14\,500 \times g$. 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 unit of activity was $\Delta A_{366\ nm}$/min per mg of enzyme protein in the assay.

Digested haemoglobin was estimated by the blue colour it gives with phenol reagent, which reacts with tyrosine, tryptophan, and cysteine groups; tyrosine being used as the standard (Anson and Mirsky, 1932). The substrate (2.2 g) was dissolved in 16 ml of 1 N NaOH, 20 ml of water and 36 g of urea. This mixture was agitated for 1 h at 25°C. Then, 4 g of urea added and the volume made to 100 ml with distilled water. The substrate was adjusted to pH 2, 3, 4, 5, 6, and 7 with 1 M H_3PO_4. To 0.25 ml of substrate incubated for 10 min at 37°C, 50 μl of the enzyme preparation was added. The reaction was stopped 10 min later by adding 0.5 ml of 5% TCA. The reaction mixture was agitated and centrifuged for 5 min at $21\,600 \times g$. The supernatant (0.5 ml) was separated, 1 ml of 1 N NaOH and 0.3 ml of the phenol reagent (1:3) were added, and $A_{650\ nm}$ was recorded, after 30 min. For controls, TCA was added before the substrate. A unit of activity was recorded as μmol tyr/min/mg from a tyrosine standard curve.

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, the enzyme preparation was added (volume adjusted to 100 μg of protein). After 5 min, 0.25 ml of 30% acetic acid was added and $A_{410\ nm}$ was recorded. BAPNA hydrolysis units were evaluated according to Dimes et al. (1994). Amidase activity was expressed in BAPNA units/mg as $(A_{410\ nm}/\text{min} \times 1000 \times \text{vol of the reaction mixture})/(8800 \times \text{mg protein in the assay})$, 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 as substrate (SAPNA). 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. 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$)/(8800 \times$ mg protein in the assay), where 8800 is the extinction coefficient of *p*-nitroaniline (Erlanger et al., 1961).

2.5. Carboxypeptidase A and B activity

Carboxypeptidase A activity was evaluated using 1 mM hippuryl-L-Phe (HLPA) in 25 mM Tris–HCl buffer, pH 7.5, containing 500 mM NaCl (Worthington, 1993). Substrate (1 ml) was mixed with 50 μl of the enzyme preparation in 10% LiCl. The increase of the absorbance at 254 nm caused by the hydrolysis of the substrate was continuously recorded at 25°C during 3 to 4 min.

Carboxypeptidase B activity was evaluated using 1 mM hippuryl-L-Arg (HLA) in 25 mM Tris–HCl buffer, pH 7.65, containing 100 mM NaCl (Worthington, 1993). Substrate (1.45 ml) was mixed with 50 μl of enzyme preparation. The increase of the absorbance at 254_{nm} caused by the hydrolysis of the substrate was continuously recorded at 25°C during 3 to 4 min.

2.6. Acid phosphatase activity

Acid phosphatase activity was evaluated using 2.7 mM *p*-nitrophenol in a ''citrate buffer'', pH 4.8 (according to the Kit of Gerencia General de Biológicos y Reactivos — Secretaria de Salud, Mexico). To 0.250 ml of substrate incubated for 5 min at 37°C, 50 μl of the enzyme preparation was added. The reaction was stopped 30 min later by adding 2.5 ml of 0.02 N NaOH, and $A_{405\ nm}$ was recorded 20 min later. For controls, the enzyme preparation was added at the end. The activity was calculated from a curve of a standard solution of 2.7 mM *p*-nitrophenol in 2 N NaOH with aliquots from each extract.

2.7. SDS-polyacrylamide gel (SDS-PAGE) electrophoresis

Electrophoretic separation of the protein in the enzyme preparations was done according to a modification of Laemmli's method (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 onto 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. 3. A total of 5 μl of molecular weight markers (MWM) was loaded on to each gel for easy comparison of the MW of the protein bands. Electrophoresis was run at a constant current of 15 mA per gel for 120 min at 4°C.

After electrophoresis, gels were 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 were washed with aqueous 40% ethanol–10% acetic acid for two hours. Gels were dried using a Bio-Rad slab gel dryer (Model 583) and photographed.

2.8. Statistical analysis

All assays were done on two pooled samples, each with three replicates. Except where indicated, results are presented as means $\pm$ SD. Statistical analysis was done among extracts of each group and between extracts of group I and II. Group III was not compared because it was from a different spawning and was fed with diatoms only. Differences in protease activity were analyzed by discriminant analysis, followed by Tukey's HSD test. Protein concentration, trypsin, chymotrypsin, and acid phosphatase activity were analyzed by nested analysis of variance (Sokal and Rohlf, 1981). Differences were considered significant at $P < 0.05$. The data were analyzed by using the Statistica software for PCS (StatSoft).

3. Results

Soluble protein of juvenile green abalone was from 8.8 to 12.2 mg/ml for VIS and from 10.0 to 15.2 mg/ml for HP. Protein was more abundant in the hepatopancreas of the three groups. The amount of protein in VIS and HP was similar in juveniles from the same spawning and fed diatoms-seaweed (Group I and II). The concentration of protein in the group fed diatoms was slightly higher (Table 3).

3.1. Effect of pH on proteolytic activity

Proteolytic activity assayed with both 1% azocasein and 2% acidified haemoglobin at different pHs showed that juvenile green abalone digestive enzymes have activity peaks at acid pH. At alkaline pHs, both activities decreased. HP proteolytic activity was higher than VIS activity in all groups. Hydrolysis of azocasein showed that in VIS, the pH of maximum protease activity of group I and II was pH 2 and 3, and of group III was pH 4 (Fig. 1). Nevertheless, there was no significant difference from pH 2 to 4 in the groups. In HP, the pH of maximum protease activity of both group I and III was pH 5, and of group II was 3. The pH range in which the enzyme had at least 70% of the maximum activity was between 2 and 4 in VIS, and 2 and 5 in HP.

Proteolytic activity assayed with 2% haemoglobin at pH 2 to 7 showed that in VIS the pH value of maximum activity of group I and II was pH 3, and of group III was pH 4. In HP, the maximum activity was at pH 4 in the three groups (Fig. 2). The pH range in which the enzyme had at least 70% of the maximum activity was between 3 and 4 in both VIS, and HP. In general, proteolytic activity in juveniles of group I was higher than juveniles of group II between similar extracts.

Table 3
Protein concentration, and protease, acid proteinase, chymotrypsin, and acid phosphatase activity in juvenile green abalone (*H. fulgens*) extracts
The value of maximum activity and pH optimum are reported.
Values are the average of two pooled samples in triplicate ± standard deviations.
Significant differences among extract of each group were found.

Group-extract	Soluble protein (mg/ml)	Protease activity[a] (pH)	Acid proteinase activity[b] (pH)	Trypsin activity[c]	Chymotrypsin activity[d]	Acid phosphatase activity[e]
I Viscera	9.9 ± 0.9	0.168 ± 0.005(2)	1.470 ± 0.055(3)	0.007 ± 0.001	0.006 ± 0.001	0.587 ± 0.032
II Viscera	9.8 ± 1.0	0.124 ± 0.019(3)	0.659 ± 0.052(3)*	0.003 ± 0.001*	0.0031 ± 0.0005*	0.600 ± 0.014
III Viscera	10.5 ± 1.7	0.164 ± 0.024(4)	1.153 ± 0.093(4)	0.0062 ± 0.0003	0.011 ± 0.001	0.457 ± 0.018
I Hepatopancreas	12.1 ± 2.1	0.238 ± 0.009(5)	2.167 ± 0.138(4)	0.011 ± 0.004	0.016 ± 0.004	1.266 ± 0.079
II Hepatopancreas	13.3 ± 1.7	0.168 ± 0.008(3)**	1.447 ± 0.044(4)**	0.005 ± 0.001**	0.015 ± 0.004	1.025 ± 0.121**
III Hepatopancreas	14.3 ± 0.9	0.204 ± 0.009(5)	2.760 ± 0.113(4)	0.0027 ± 0.0003	0.008 ± 0.001	0.700 ± 0.019

[a] Proteolytic activity with azocasein, $Abs_{366\ nm}$/min/mg protein.
[b] Acid proteinase activity with haemoglobin, μmol tyr/min/mg protein.
[c] Specific activity with BAPNA, units/mg protein.
[d] Specific activity with SAPNA, units/mg protein.
[e] Specific activity with *p*-nitrophenol, units/mg protein
* Differences ($P \leq 0.05$) between viscera extract of group I and II.
** Differences ($P \leq 0.05$) hepatopancreas extract of group I and II.

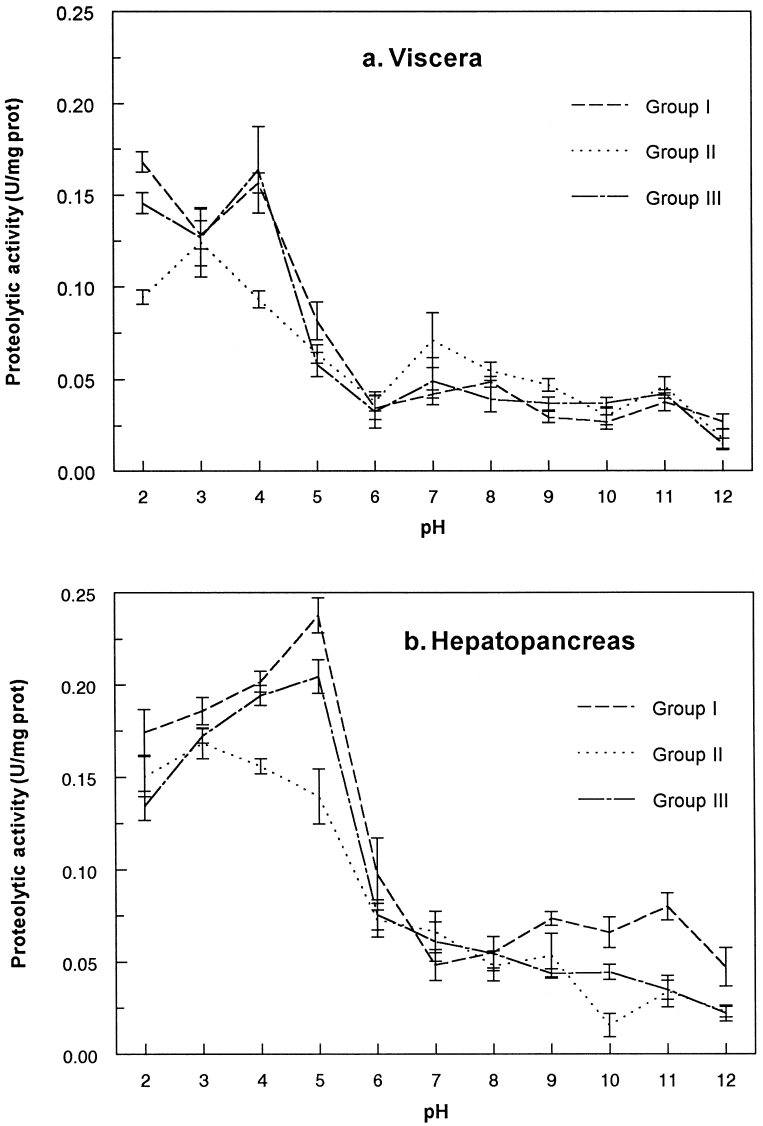

Fig. 1. Effect of pH on the proteolytic activity of viscera (a) and hepatopancreas (b) extracts of juvenile *H. fulgens*. The enzyme preparations were assayed with azocasein following the standard procedure at different pHs.

3.2. Specific substrate activity

Regardless of the size of organisms and diet, extracts from juvenile green abalone *Haliotis fulgens* hepatopancreas and viscera hydrolyzed trypsin, chymotrypsin, and acid phosphatase-specific substrates, but not carboxypeptidase A and B substrates (Table 3).

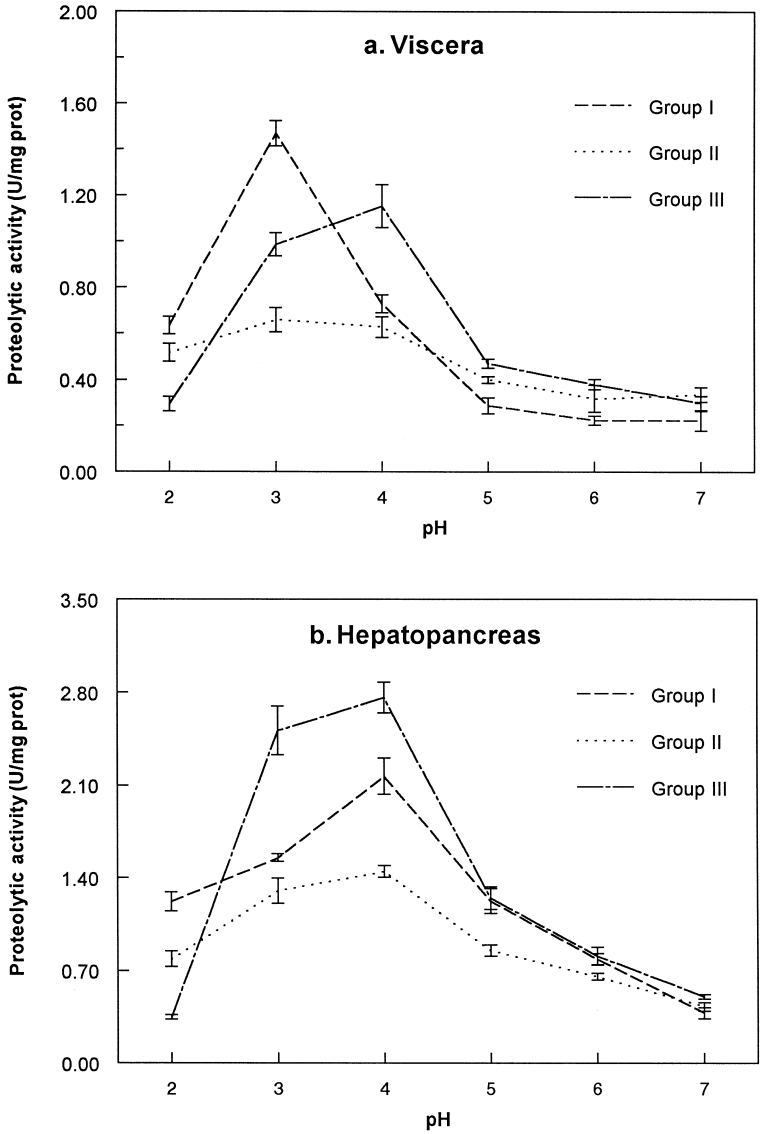

Fig. 2. Effect of pH on the acid proteinase activity of viscera (a) and hepatopancreas (b) extracts of juvenile *H. fulgens*. The enzyme preparations were assayed with haemoglobin following the standard procedure at different pHs.

Specific substrate activities in HP were higher than VIS activity in all groups, except in the juveniles fed diatoms (group III) in which the VIS trypsin and chymotrypsin activity were higher than the HP activity (Table 3). The highest trypsin, chymotrypsin, and acid phosphatase activity was shown by HP from juveniles of group I. There were significant differences in trypsin, and chymotrypsin activity between VIS extracts of group I and II,

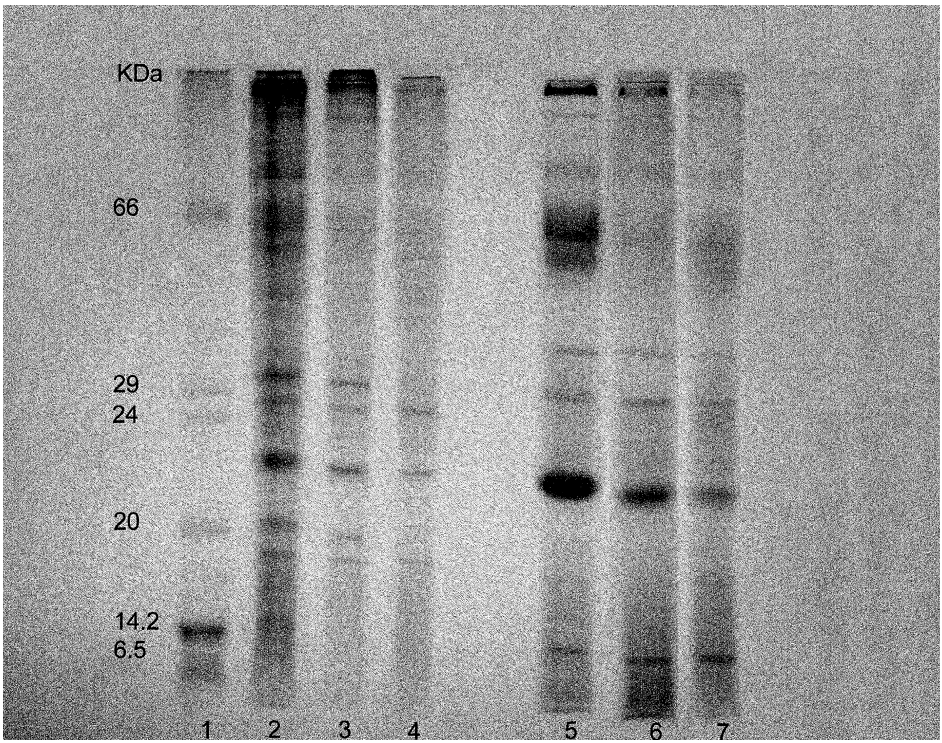

Fig. 3. SDS-PAGE for protein composition in juvenile *H. fulgens*. Column (C) 1, molecular weight marker (MWM); C2, VIS group I; C3, VIS group II; C4, VIS group III; C5, HP group I; C6, HP group II; C7, HP group III. Sample volume CSC activity between s were adjusted to 10 μg for protein content.

and in trypsin, and acid phosphatase activity between HP extracts of these groups. The ratio of chymotrypsin activity to trypsin was 0.9, 1.0, and 1.8 for VIS of group I, II, and III, and for HP 1.4, 3.0, and 3.0.

3.3. Electrophoresis (SDS-PAGE)

The protein composition was similar in group I and II extracts. For VIS extract, protein bands of 29, 27, from 23 to 22, 19, and 17 kDa were detected, and for HP extract of 31, 28, 22, and 11 kDa. Group III showed three protein bands with molecular mass of 27, 23, and 17 kDa for VIS and similar bands of other groups for HP (Fig. 3). It was not possible to show activity in the extracts with substrate SDS-PAGE for protease.

4. Discussion

The larger amount of protein in organisms fed diatoms can be related to the quality of protein in the food. The protein of macroalgae used at La Bocana was 16.82 ± 0.13, mean $\pm$ SD, for *Ulva* spp. and 10.99 ± 0.33 for *Eisenia arborea* (results not shown).

That for diatoms was not studied. The protein reported for diatoms is between 20 and 30% of dry weight (Brown and Jeffrey, 1992). Electrophoregrams of protein composition show differences in number of bands and quantity of protein between group I–II and group III. Britz and Hecht (1997) studied the effect of dietary protein and energy level on body composition of larger juveniles and smaller, postweaned *Haliotis midae* and observed that the protein content of both size-classes increased significantly with increasing dietary protein content, and the protein content of large abalone was greater than that of small abalone. The amount of protein reported in samples of hepatopancreas (HP), crop-stomach content (CSC), intestine (IN), and rectum (RE) along the digestive systems of adult green abalone *H. fulgens* (Serviere-Zaragoza et al., 1997) showed that the protein in HP, 25 mg/ml was higher than HP of juveniles. Other comparisons were not possible because juveniles were too small to obtain samples of different tissues and fluids. HP and VIS were the extracts assayed. Nevertheless, in both juvenile and adults, the larger amount of protein was found in the HP.

Viscera and hepatopancreas digestive enzymes of juveniles of green abalone had at least 70% of the maximum activity at acid pH between 2 and 5, although the value of maximum activity of HP was slightly lower at acid pH than VIS. In adults, Serviere-Zaragoza et al. (1997) reported that the pH range in which the enzyme had at least 50% of the maximum activity was between 4 and 6 in HP, 6 and 8 in the crop-stomach content (CSC), and 7 and 11 in both intestine and rectum. Hernández-Santoyo et al. (1998) reported the maximum activity at pH 8 in HP. Differences between pH of maximum activity along the digestive system have been reported for other species, for example Cho et al. (1983) found, in *Haliotis discus hannai*, that the optimum pH of proteolytic activity of the tissue extracts from the digestive tract was 3.2. McLean (1970) described different protease, amylase, and lipase activity peaks from the crop fluid, midgut, and salivary gland in *H. rufescens*.

Enzyme activities for different stages in abalone are currently lacking. Nevertheless, differences in the optimum pH range for the hydrolysis of azocasein showed that digestive enzymes change from juvenile to adult. In juveniles, HP and VIS digestive enzymes have activity peaks at acid pH between 2 and 5 and at alkaline pHs activities decrease. In adults, HP activity increased between pH 2 and 5 and CSC activity between pH 2 and 7, then decreased with increasing pH. In the intestinal (IN) and rectal (RE) fluids, the activity was detected at alkaline pH between 7 and 11, but it was lower than HP and CSC at acid pH but higher than at alkaline pH (Serviere-Zaragoza et al., 1997). Specific substrate activities support this idea. Trypsin, chymotrypsin, and acid phosphatase activity were found in HP and VIS of juvenile green abalone. Adults show chymotrypsin and trypsin activity in intestinal and rectal fluids, but not in hepatopancreas and crop-stomach content (Serviere-Zaragoza et al., 1997). In juveniles, carboxypeptidase A and B were not found. Hernández-Santoyo et al. (1998) reported a carboxypeptidase-like enzyme in adult *H. fulgens*. In this way, in crustaceans, Fang and Lee (1992) found that in the shrimp *Penaeus monodon* the total protease activity changed dramatically from larva to adult. Rodríguez et al. (1994) described the separate and interactive influences of herbivorous and zooplanktivorous feeding on the biochemical composition of *Penaeus japonicus* larvae, and the adaptative response of digestive enzyme activity to diet.

Extracts of juvenile abalone digested haemoglobin at acid pH suggesting that aspartic proteinases are present in VIS and HP of these organisms. Pepsins have not been detected in invertebrates, and apparently cathepsin D, and other cathepsins, act both as digestive and lysosomal enzymes in many of these animals. Fish with a true stomach have pepsinogen secretion (Gildberg, 1988).

Proteolytic activity was different between abalone groups fed diatoms-macroalgae and diatoms. It was not possible to correlate these observations with the type of diet or size of the organisms, because the group fed diatoms was of a different spawning. Nevertheless, the size of the organisms may have an important effect on proteolytic activity between group I and II, because the organisms were of the same spawning and fed the same diatoms-seaweed, but the size was larger in the second group. Using haemoglobin as substrate, values of maximum activity in VIS of group I were higher than VIS of group II, and HP activity of group I was higher than HP group II. Uki and Watanabe (1992) showed that juvenile *H. discus hannai* (shell length 13 mm) were more efficient in digestion of fish protein that young adults of shell length of 52 mm. Knauer et al. (1996) reported that the enzyme activity in juvenile abalone, fed a pelleted practical diet, was significantly higher than in the group fed diatoms.

It was not possible to detect a band of protease activity from SDS-PAGE electrophoresis according to García-Carreño et al. (1993). Electrophoresis at different pH was done (results not shown) without success. It could be influenced by different problems related to sensitivity of acid proteases to the composition of electrophoresis buffers, and migration to the cathode (Díaz-López et al., 1998). Substrate electrophoresis analysis needs to be done to assess the composition, molecular mass, and classes of acid proteases present in crude extracts from abalone digestive tracts. In adults, specific intestinal proteinases with an alkaline pH optimum were detected with electrophoresesis analysis (Serviere-Zaragoza et al., 1997). Because most studies only focused on intestinal proteases, Díaz-López et al. (1998) evaluated several techniques based upon the use of substrate-polyacrylamide gel electrophoresis to achieve characterization of acid proteases in fish stomachs. The most accurate technique for activity assessment is the neutral substrate-PAGE system, revealing such activity at low pH with haemoglobin as substrate.

These results show the importance of understanding the digestive physiology of a cultured species because this information can be used to formulate a practical diet. A better understanding of the digestive enzymes present in abalone would enable scientists to produce a more digestible diet for abalone. Although growth rates with diets give an indication of the suitability of a diet, the growth rates could be further improved by supplying a diet aimed at the digestive enzymes present.

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