ELSEVIER

Comparative Biochemistry and Physiology Part B 134 (2003) 143–150

CBP

www.elsevier.com/locate/cbpb

Digestive enzymes in juvenile green abalone, *Haliotis fulgens*, fed natural food

F.L. García-Carreño*, M.A. Navarrete del Toro, E. Serviere-Zaragoza

Centro de Investigaciones Biológicas del Noroeste (CIBNOR), P.O. Box 128, La Paz, BCS 23000, Mexico

Received 26 July 2002; received in revised form 17 September 2002; accepted 9 October 2002

Abstract

Enzymes responsible for the digestion of food protein by juvenile green abalone (*Haliotis fulgens*) were studied when fed algae or a sea grass (*Phyllospadix torreyi*) naturally occurring in the habitat. The effect of food on the composition and activity of the enzymes was also evaluated. Acid, serine proteinases and aminopeptidases, as confirmed by pH profile of activity, specific inhibition and synthetic substrate hydrolysis were found in the digestive organs of juvenile green abalone. Algae and sea grass differentially affected the digestive system in abalone.

Keywords: Aminopeptidases; Chymotrypsin; Digestion; Green abalone; *Haliotis fulgens*; Proteinases; Proteolytic activity; Trypsin

1. Introduction

Abalone, coastal marine herbivorous mollusks, feed chiefly on macroalgae after they have reached a size >10 mm (Cox, 1962; Kawamura et al., 1998). Their digestive system is anatomically and biochemically suited to feed on such food. Their digestive system uses enzymes to break down structural polysaccharides of algae and to hydrolyze algal proteins. To increase the understanding of digestion, enzymes with proteolytic activity have been studied and some of their properties described in several abalone species including *Haliotis rufescens* (McLean, 1970; Groppe and Morse, 1993), *H. discus hannai* (Cho et al., 1983), *H. midae* (Knauer et al., 1996), *H. fulgens* (Serviere-Zaragoza et al., 1997; Hernández-Santoyo et al., 1998; Picos-García et al., 2000) and *H. rubra* (Edwards and Condom, 2001). Using in vitro and electrophoretic (SDS-PAGE) techniques, Serviere-Zaragoza et al. (1997) studied some proteases related to protein digestion in Mexican populations of adult green abalone, *H. fulgens*. Trypsin and chymotrypsin were found in the intestine, with chymotrypsin the predominant proteinase. Extracts from juvenile green abalone hydrolyzed trypsin, chymotrypsin and acid phosphatase-specific substrates (Picos-García et al., 2000).

Research on green abalone nourished with seaweed species has shown that good growth rates in juveniles and young adults was achieved when fed alariacean brown algae *Egregia menziesii* (Leighton and Peterson, 1998). *E. menziesii* is part of algae populations along the northern and central Pacific coasts of the Peninsula of Baja California, but is not present in the habitats of abalone species along the southern third part of the peninsula, where the best growth has been observed when fed *Macrocystis pyrifera* (Serviere-Zaragoza et al., 2001).

The objective of this study was to compare the effects of macroalgae and sea grass diets on acid

*Corresponding author. Tel.: +52-612-1253633; fax: +52-612-1254710.

E-mail address: fgarcia@cibnor.mx (F.L. García-Carreño).

1096-4959/03/$ - see front matter © 2002 Elsevier Science Inc. All rights reserved.
PII: S1096-4959(02)00221-X

proteinases, trypsin, chymotrypsin and aminopeptidase activities, using crude enzyme extracts of juvenile, *H. fulgens*. Particularly, our goal was to deepen the understanding of protein digestion, and to know how enzyme activities are affected by exogenous factors, such as food, and to get some clues about the regulation of digestive enzyme activity. Data about the growth of abalone juveniles used to analyze enzyme activities are included.

2. Materials and methods

2.1. Feeding trials

The experiment to compare digestive enzymes in abalone fed natural diets was conducted at the Northwest Biological Research Center laboratory (CIBNOR) on Bahia de La Paz, Baja California Sur, Mexico (24 °N, 110 °W). Eight-month-old green abalone (*H. fulgens*) were supplied by a commercial hatchery near Ensenada, Baja California, México and transferred to the CIBNOR laboratory. Specimens were acclimatized in tanks supplied with running, aerated seawater and fed with brown algae (*Eisenia arborea*).

After 6 months on this regime, animals were transferred to 16-l fiberglass rearing containers with a concave bottom ($50\times30\times35$ cm, $L\times W\times H$). Each rearing container (experimental unit, EU) was stocked with 40 abalone juveniles, shell length 17.3 ± 2.2 mm (S.D.) and body mass 0.44 ± 0.2 g (S.D.). Animals were marked with plastic tags glued to the shell. Triplicates of the four food groups were included. EUs were supplied with temperature-controlled (20 ± 1 °C), filtered (10-μm filter) seawater at a flow rate of 73 ml/min. The water was vigorously aerated. During the following experimental period, water quality was monitored every 8 days. Analyses showed the following averages ($\pm$ s): pH 8.03 (±0.06), oxygen 6.52 (±0.74) mg/l, salinity 40 (±1.29) ppm, nitrites 0.0052 (±0.0004) μmol/l, nitrates 0.1253 (±0.0029) μmol/l, ammonium 0.0226 (±0.00001) mg/l and phosphate 0.00552 (±0.000017) μmol/l.

The feeding trials ran for 226 days. Food was supplied ad libitum at intervals of 2 days in the afternoon. Every second morning, uneaten food and feces were removed. Micro algae growing on the inner walls of the EUs were removed twice a week with a soft brush. Shell length was measured with calipers, and body mass measured with an electronic balance (to 0.001 g) at 0, 34, 65, 106, 135, 191 and 226 days. Dead animals were removed and replaced to maintain the standard density.

Organisms were fed with one of four foods, the macroalgae *Macrocystis pyrifera*, *Eisenia arborea*, *Gelidium robustum* and the sea grass *Phyllospadix torreyi*. The kelp *M. pyrifera* is the dominant species in southern part of the Pacific shore of California (Dawson et al., 1960). Other species are thought to be important in abalone communities and have potential as food along the Pacific coast of Baja California (Guzmán del Próo et al., 1972, 1991; Serviere-Zaragoza et al., 1998). Macroalgae and sea grass used in the feeding experiment were harvested near abalone communities of Baja California (27 °N) the same day to reduce chemical composition variation due to seasonal changes. The material was immediately air-dried, due to the difficulty of maintaining fresh material in the laboratory. Foods were stored in cardboard boxes. To detect changes in the chemical composition from extended storage, proximate analyses (crude protein, ether extractables, crude fiber and ash) were run at the beginning and end of the feeding experiment with the methods described by the Association of Official Agricultural Chemists (1995).

2.2. Enzyme analysis

At the completion of the feeding trials (226 days), 10 abalone from each replicate (30 per diet) were randomly sampled and shell length and body mass recorded. The digestive organs, comprising the esophagus, stomach, hepatopancreas, intestine and rectum were removed, weighed and kept in an ice bath until all animals were sampled. Pooled viscera were stored at −30 °C. Enzymatic extracts were obtained by adding cold, double-distilled (dd) water (1:3 w/v) to the thawed viscera, which were then homogenized in a glass Potter homogenizer in an ice bath. The homogenates were centrifuged for 30 min and $10\,000\times g$ at 4 °C in a refrigerated microcentrifuge. Supernatants containing the enzyme extracts were stored at −30 °C. Soluble protein content in the enzymatic extracts was evaluated by the method described by Bradford (1976) using BSA as a standard.

2.3. Acid proteinase activity

Assays to find the pH optimum of enzymes active at acid pH and to evaluate the activity of

such enzymes were made following the method of Díaz-López et al. (1998). Ten microlitre of each enzyme preparation was mixed with 1 ml of 0.5% hemoglobin in 0.1 M Gly–HCl buffer at pH 2.0, 3.0 and 4.0 and incubated for 10 min at 25 °C. The reaction was stopped by adding 500 μl of 20% trichloroacetic acid (TCA), left for 10 min at 0 °C to precipitate undigested substrate, then centrifuged for 5 min and 10 000×*g*. Absorbance of the supernatant was recorded at 280 nm against dd water as the blank. Negative controls received TCA solution before the substrate. Activity units were calculated by the equation:

$$\text{Activity units} = [(\text{Abs}_{280}/\text{min}) \times \text{ml of reaction mixture})]/0.051 \times \text{min of reaction} \times \text{mg of protein}$$

where 0.051 is the extinction coefficient of 1 mg/ml of tyrosine and min is the duration of the reaction.

For the inhibition of acid proteinases, 10 μl of enzyme preparation were incubated for 60 min with 10 μl of 100 μg/ml of pepstatin A in DMSO, as described by García-Carreño (1992). The remaining enzymatic activity was assayed with hemoglobin as noted above, including a solvent control. The remaining activity was evaluated as percentage of the activity of the enzyme preparation without the inhibitor. DMSO did not affect the enzyme activity. The linearity of the enzyme extracts with time was confirmed.

2.4. Trypsin and chymotrypsin activity

Trypsin and chymotrypsin activity was evaluated as described by García-Carreño et al. (1994), using synthetic substrates. For trypsin activity, 10 μl of enzyme preparation was mixed with 750 μl of 0.1 mM BAPNA in 50 mM TRIS–HCl at pH 7.5 and 20 mM $CaCl_2$ buffer. The reaction was monitored for 10 min at 30 °C at 410 nm. For chymotrypsin activity, 10 μl of enzyme preparation was mixed with 750 μl of 0.1 mM SAPNA in TRIS–HCl at pH 7.5 and 20 mM $CaCl_2$ buffer. The reaction was monitored for 3 min at 30 °C at 410 nm. Trypsin and chymotrypsin amidase activity units were calculated by the equation:

$$\text{Activity units} = [(\text{Abs}_{410}/\text{min}) \times 1000 \times \text{ml of reaction mixture}]/8800 \times \text{mg}$$

where 8800 is the molar extinction coefficient of *para*-nitroaniline liberated from chromgens BAPNA and SAPNA and mg is the protein content in the reaction mixture.

2.5. Aminopeptidase activity

Aminopeptidase activity was evaluated by the method described by Ezquerra et al. (1999), assaying the quantity of *p*-nitroaniline produced from each of nine substrates (Lys-*p*-NA, Val-*p*-NA, Ala-*p*-NA, Leu-*p*-NA, Pro-p-NA, Gly-*p*-NA, Glu-*p*-NA, Arg-*p*-NA and Met-*p*-NA). Each amino acid derivative substrate at 1 mM was dissolved in 1 ml DMSO, then in the appropriate volume of 50 mM phosphate buffer at pH 7.2. In a disposable spectrophotometer cuvette, 10 μl of enzyme preparation and 750 μl of substrate were mixed. The reaction was immediately monitored for 10 min at 30 °C at 410 nm. All assays were done in triplicate. Distilled water was used as the blank. The activity units for aminopeptidases were calculated using the same equation for amidase activity noted above.

2.6. Electrophoresis

Electrophoretic separation of proteins was performed according to Laemmli (1970). Bands with alkaline enzymatic activity and their molecular masses were evaluated according to García-Carreño et al. (1993). Acid proteinase activity was evaluated following Díaz-López et al. (1998).

2.7. Statistical analysis

Data on growth, acid, trypsin, chymotrypsin and aminopeptidase activities were analyzed by ANOVA. Differences between the means were analyzed by HSD Tukey test (Sokal and Rohlf, 1995). Differences were considered significant at $P < 0.05$. Statistical analysis was performed with the STATISTICA software program (Tulsa, OK).

3. Results

3.1. Viscera analysis

Because of the small size of the specimens, the viscera included all the digestive organs. The visceral wet mass was approximately 0.25 g for all groups, except for the group fed on alga *M. pyrifera*, where it was 0.7 g. The protein content

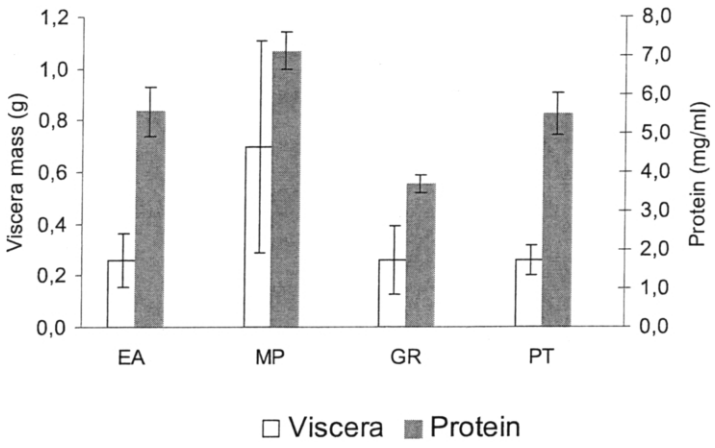

Fig. 1. Soluble protein content in crude enzymatic extract and viscera mass of juvenile green abalone (±S.D.).

of the viscera varied between 3.7±0.2 (S.D.) in organisms fed on *G. robustum* and 7.1±0.5 (S.D.) mg/ml in organisms fed on *M. pyrifera* (Fig. 1). In spite of the differences in average viscera mass, there were no significant differences among groups. In contrast, the protein content of viscera was significantly different with the highest found in organisms fed *M. pyrifera*, and the lowest those fed *G. robustum*.

3.2. Acid proteinase activity

In all groups, the pH for maximum acid proteinases activity was between 2 and 3, with <50% of the maximum activity at pH 4. At pH 1, the activity in all groups was negligible (Fig. 2). Therefore, pH 2 was chosen for further studies. Differences in the activity of acid proteinases among the groups were found ($P<0.05$). The proteinase acid activity was the lowest in organisms fed on *M. pyrifera*. Higher activity was found in organisms fed on *E. arborea*, *P. torreyi* and *G. robustum* (Table 1). In all samples assayed with the inhibitor pepstatin A, the acid enzyme activity was fully eliminated (data not shown).

3.3. Trypsin and chymotrypsin activity

Trypsin activity showed significant differences ($P<0.05$) among the groups. It was lowest in specimens fed on *P. torreyi* and *Eisenia arborea*, and highest in specimens fed on *G. robustum* and

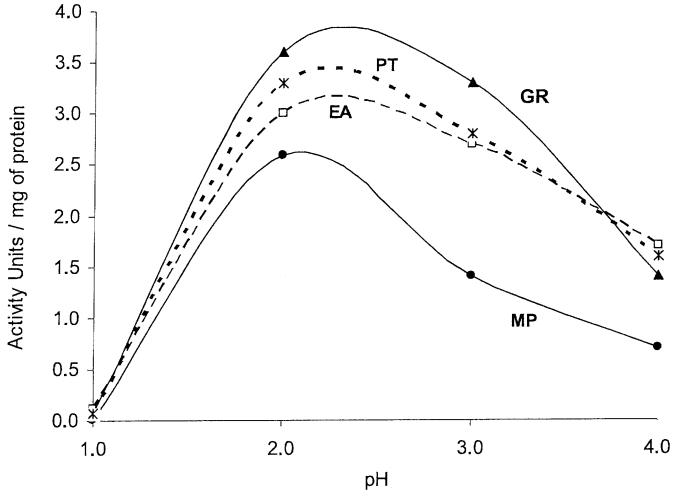

Fig. 2. The effect of pH on acid activity of juvenile green abalone.

Table 1
Acid proteinase, trypsin and chymotrypsin activity in juvenile green abalone fed seaweeds

Diet	Acid proteinase[1]	Trypsin[2]	Chymotrypsin[3]
E. arborea	$3.0 \pm 0.4^{a,c}$	3.5 ± 0.6^{a}	3.2 ± 0.4^{a}
M. pyrifera	2.7 ± 0.3^{c}	5.2 ± 1.7^{b}	1.1 ± 0.3^{b}
G. robustum	3.6 ± 0.5^{b}	5.8 ± 1.3^{b}	6.8 ± 1.4^{c}
P. torreyi	$3.3 \pm 0.2^{a,b}$	3.4 ± 0.6^{a}	1.5 ± 0.2^{b}

[1] Substrate hemoglobin at pH 2, μmol tyr min^{-1} mg^{-1} protein.
[2] Specific activity with BAPNA, units/mg protein.
[3] Specific activity with SAPNA, units/mg protein.
Values are the average of three pooled samples in triplicate ±S.D. Values with different letters in each column are statistically different ($P < 0.05$).

M. pyrifera. Chymotrypsin activity was statistically different among juveniles fed different seaweeds ($P < 0.05$). Specimens fed on *M. pyrifera* showed the lowest activity, but not statistically different to *P. torreyi*. The activity was higher in specimens fed *E. arborea*, and the highest activity was found in specimens fed *G. robustum* (Table 1).

3.4. Aminopeptidase activity

Activities of lysine-, alanine-, leucine-, glycine, arginine- and methionine-aminopeptidase were detected in the four groups. Proline aminopeptidase activity was negligible in three groups. In *M. pyrifera*, it was not evaluated. Valine- and glutamate-NA aminopeptidase activity was not detected. There were significant differences in aminopeptidase activities among the abalone fed with the four diets. The highest values were in specimens fed with *G. robustum*, except to glycine aminopeptidase activity. Lower values of lysine-, alanine-, leucine- and proline aminopeptidase were detected in specimens fed *E. arborea* and *M. pyrifera*. Specimens fed *M. pyrifera* showed the lowest values of glycine, arginine and methionine activity (Table 2).

3.5. Protein composition

Electrophoregrams of enzyme preparations of the abalone digestive system showed differences in the composition of proteins present. Although the samples came from the same species, the molecular mass and amount of protein in the enzyme preparations were distinctive for each group, depending on diet. In the substrate SDS-PAGE, several bands of proteinase activity between 14.2 and 36 kDa were observed. Acid proteinolytic activity was revealed by substrate-neutral-PAGE. All groups showed the same composition of enzymes, with *P. torreyi* showing the highest intensity (data not shown). *E. arborea*, *P. torreyi* and *M. pyrifera* enzyme preparations shared three similar bands, whereas *G. robustum* had an intense band of activity near the anode.

3.6. Growth

Animals sampled for enzyme analysis showed the highest growth rate when fed on *M. pyrifera*. After 226 days, both mean shell length (data not shown) and mean body wet mass 2.12 ± 0.20 g (S.E.) were statistically higher from the means of juveniles fed *E. arborea*, 1.08 ± 0.08 g (S.E.); *G. robustum*, 1.12 ± 0.11 g (S.E.); and *P. torreyi*, 1.21 ± 0.06 g (S.E.) ($P < 0.05$). There were no statistical differences among juveniles fed the latter three foods (Fig. 3). Table 3 shows the chemical composition of the natural foods. Significant

Table 2
Aminopeptidase activity in juvenile green abalone fed seaweeds[1]

Substrate	*E. arborea*	*M. pyrifera*	*G. robustum*	*P. torreyi*
Lys-*p*-NA	0.139 ± 0.020^{a}	0.169 ± 0.002^{a}	0.325 ± 0.011^{c}	0.254 ± 0.018^{b}
Ala-*p*-NA	0.081 ± 0.004^{a}	0.097 ± 0.005^{a}	0.158 ± 0.021^{b}	0.142 ± 0.004^{b}
Leu-*p*-NA	0.042 ± 0.002^{a}	0.045 ± 0.002^{a}	0.124 ± 0.010^{c}	0.101 ± 0.004^{b}
Pro-*p*-NA	0.006 ± 0.001^{a}	[2]	0.136 ± 0.002^{b}	0.005 ± 0.001^{a}
Gly-*p*-NA	0.080 ± 0.001^{a}	0.040 ± 0.001^{c}	0.114 ± 0.006^{b}	0.122 ± 0.006^{b}
Arg-*p*-NA	0.340 ± 0.013^{a}	0.245 ± 0.045^{d}	0.774 ± 0.025^{c}	0.464 ± 0.011^{b}
Met-*p*-NA	0.178 ± 0.010^{a}	0.145 ± 0.013^{d}	0.266 ± 0.000^{c}	0.235 ± 0.007^{b}

[1] Specific activity, units/mg protein.
[2] Not evaluated.
Values are the average of three pooled samples in triplicate ±S.D. Values with different letters in each row are statistically different ($P < 0.05$).

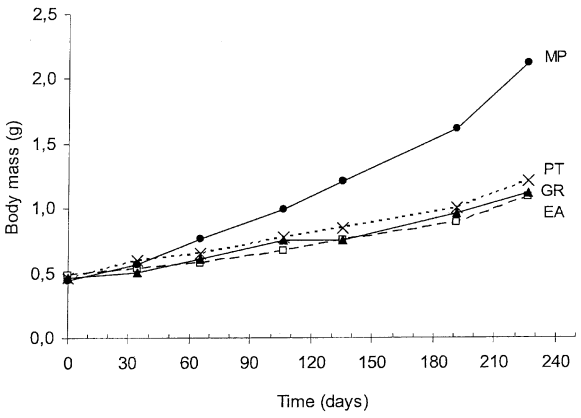

Fig. 3. Effect of macroalgae and sea grass food on growth of green abalone.

changes in chemical composition because of storage of seaweeds were not detected.

4. Discussion

According to this study and previous ones (Serviere-Zaragoza et al., 1997; Picos-García et al., 2000), proteases in the digestive system of green abalone seem to belong to two main proteinase classes; aspartic proteinases (EC 3.4.23.x) and serine proteinases (EC 3.4.21.x). In this study, aspartic proteinase was identified according to the pH of maximum activity and inhibition with highly specific pepstatin A. Because the K_i of pepstatin A for pepsin is 45 pM, this suggests the presence of pepsin-like activity in the abalone. The fact that some acid proteinases are present in the digestive system of abalone is not exceptional. Serviere-Zaragoza et al. (1997) found enzyme activity at pHs as low as 2 in adult of green abalone, as Edwards and Condom (2001) in blacklip abalone. The precise identification of the enzyme responsible for the proteinase activity at acid pHs is to be addressed. Evidence so far indicates that some pepsin-like enzyme is present in the green abalone. Serine proteases were identified as trypsin and chymotrypsin. Both enzymes are found along the intestine and rectum in several abalone species: green abalone *H. fulgens* (Serviere-Zaragoza et al., 1997; Hernández-Santoyo et al., 1998); red abalone *H. rufescens* (Groppe and Morse, 1993); and blacklip abalone *H. rubra* (Edwards and Condom, 2001). In a previous work, Picos-García et al. (2000) did not find A or B carboxypeptidase activity. Now, aminopeptidase activities were detected in the abalone digestive system.

Acid proteinases, serine proteinases (trypsin and chymotrypsin) and aminopeptidases gave varied responses to the feeding regime. Organisms fed *G. robustum*, the seaweed with the highest content of protein, showed the highest activities in both acid and serine protinases and six of the nine aminopeptidases assayed. There was a significant reduction of acid proteinase and chymotrypsin activity in specimens fed on *M. pyrifera*, the ones that showed the highest growth. These results suggest that synthesis and secretion of digestive enzymes is somehow regulated, and indicates some kind of rapid adaptation to the feeding regime, like that observed in fish, as a response to poor protein quality or the presence of inhibitors of digestive enzymes in feed (Olli et al., 1994). In contrast to the results presented in this work, Edwards and Condom (2001) did not find variations in acid- or serine-proteinase activity by effect of the composition of the food or feed supplied.

We noticed important differences in aminopeptidase activities in abalone fed with different food sources. Proline-, leucine-, alanine-, lysine-, methionine-, arginine- and glycine-aminopeptidases were lower in specimens fed *E. arborea* and *M. pyrifera*, and higher in organisms fed *G. robustum* and *P. torreyi*. The biological function of aminopeptidases in haliotids remains unknown. The intestinal alanine aminopeptidase in the rat participates in proteolytic reactions essential for cell growth (Constam et al., 1995). The leucine aminopeptidase of *Salmonella typhimurium* hydrolyzes peptides to promote protein turnover during starvation (Yen et al., 1980). The highest aminopeptidase activities in hepatopancreas of white shrimp (*Penaeus vannamei*) were found in specimens fed with fishmeal protein of poor nutritional quality (Ezquerra et al., 1999). In *P. vannamei*, there

Table 3
Analysis of macroalgae and sea grass used as food for juvenile green abalone

Diet	Crude protein	Ash	Crude fiber	Ether extract	N-free extract
E. arborea	7.6	27.1	6.4	1.1	57.7
M. pyrifera	12.0	41.3	7.0	1.3	38.4
G. robustum	17.6	21.3	10.2	1.1	49.9
P. torreyi	15.9	31.9	13.5	1.3	37.4

% Dry weight.

seems to be a pattern indicating that the better the food, the lower the amount of enzymes needed to digest the food. Food quality depends on several factors, such as the quantity of components, but also, and more conspicuously, on the availability of the components. Why *M. pyrifera* provides the highest growth in abalone remains to be discovered. Mai et al. (1994) found that different feeding strategies will significantly affect the free amino acid profiles of two abalone species, *Haliotis tuberculata* and *H. discus hannai*. They mentioned that abalone, like other marine gastropods, have high concentrations of free amino acids (FAA) in tissues, especially taurine, alanine, glycine and arginine. It could be possible to obtain better growth of abalone by selecting well-balanced, mixed algae species in which some algae are richer in arginine, methionine and threonine.

Rehydrated seaweeds and sea grass used in this experiment supported acceptable abalone growth. After 226 days of feeding, the average growth rate of organisms fed re-hydrated algae *M. pyrifera* (43 μm day^{-1}, data not shown) was satisfactory, in comparison to those reported by other authors (Viana et al., 1993, 1996). These authors reported averages of 12 μm day^{-1} and 16 μm day^{-1} for green abalone juveniles fed fresh kelp, *M. pyrifera*. Data for other seaweed species are not available.

The group fed *M. pyrifera* reached a significant 14–18% larger shell size and 43–49% higher body mass than groups fed on other seaweed taken from abalone habitat along the western coast of Baja California. In the wild, different growth rates between abalone populations may be related to both differences in the locally available food (nutrient composition, bioavailability and palatability) and digestive capabilities. In Baja California, México, flora of the benthic environments inhabited by abalone change from north to south. Stands of the kelp *M. pyrifera* were found around Punta San Roque, over 15 km northwest of Bahía Asunción (27°N) (Serviere-Zaragoza et al., 1998). *Macrocystis* is not present in abalone banks from Punta San Roque to Bahia Magdalena (24°N), the southern limit of the commercial abalone range, although as drift, *M. pyrifera* occurs as far south as Punta Abreojos (26°N).

Because chemical assays for composition of foodstuffs are usually harsher than those found in biological systems, it is difficult to correlate results from laboratory assays with assays in vivo. Further studies are needed to determine if protein composition or availability of specific food is responsible for the induction of enzyme synthesis in the digestive system. Also, improved laboratory techniques are needed to precisely evaluate the nutrient ingredients of feed or food. This research is now underway for shrimp (Ezquerra et al., 1997).

Acknowledgments

We would like to thank Ms Alejandra Mazariegos for technical support related to the feeding assays, and BC Abalone (Ensenada, B.C., Mexico) for supplying juvenile abalone. Financial support was provided by project CONACYT-4107P and project SIMAC990107002. Mr Ira Fogel at CIBNOR edited the English language text.

References

Association of Official Agricultural Chemists, 1995. Official Methods of Analysis. Association of Official Agricultural Chemists, Washington, DC, pp. 1141.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle dye binding. Anal. Biochem. 72, 248–254.

Constam, D.B., Tobler, A.R., Rensing, E., Ehl, A., Kemler, I., Hersh, L.B., et al., 1995. Puromicyn sensitive aminopeptidase-sequence analysis, expression and functional characterization. J. Biol. Chem. 270, 26291–26939.

Cox, K.W., 1962. California abalones, family Haliotidae. Calif. Fish Game Fish. Bull. 118, 130.

Cho, D.M., Pyeun, J.H., Byun, D.S., 1983. Proteolytic activity of the crude enzyme extracted from the digestive tract of marine gastropods. Bull. Korean Fish. Soc. 16, 216–224.

Dawson, E.Y., Neushul, M., Wildman, R.D., 1960. Seaweeds associated with kelp beds along southern California and northwestern Mexico. Pacific Nat. 1, 3–61.

Díaz-López, M., Alarcón-López, F.J., García-Carreño, F.L., Navarrete del Toro, M.A., Moyano-López, F.J., 1998. Substrate-gel electrophoresis for the characterization of high pH aspartic proteinases. Comp. Biochem. Physiol. 121B, 369–377.

Edwards, S., Condom, C., 2001. Digestive protease characterization, localization and adaptation to blacklip abalone (*Haliotis rubra* Leach). Aquaculture Res. 32, 95–102.

Ezquerra, J.M., García-Carreño, F.L., Civera, R., Haard, N., 1997. pHstat Method to predict protein digestibility in white shrimp (*Penaeus vannamei*). Aquaculture 157, 249–260.

Ezquerra, J.M., García-Carreño, F.L., Arteaga, G., Haard, N., 1999. Effect of feed diet on aminopeptidase activities from hepatopancreas of white shrimp (*Penaeus vannamei*). J. Food Biochem. 23, 59–74.

García-Carreño, F.L., 1992. Protease inhibition in theory and practice. Biotechnol. Educ. 3, 145–150.

García-Carreño, F.L., Dimes, N., Haard, N., 1993. Substrate-gel electrophoresis for composition and molecular weight of proteinases or proteinaceous proteinase inhibitors. Anal. Biochem. 214, 65–69.

García-Carreño, F.L., Hernández-Cortés, M.P., Haard, N.F., 1994. Enzyme with peptidase and proteinase activity from the digestive systems of a freshwater and marine decapod. J. Agricul. Food Chem. 42, 1456–1461.

Groppe, J.C., Morse, D.E., 1993. Molluscan chymotrypsin-like protease: structure, localization, and substrate specificity. Arch. Biochem. Biophys. 305, 159–169.

Guzmán del Próo, S.A., de la Campa, S., Pineda, B.J., 1972. Flora macroscópica asociada a los bancos de abulón (*Haliotis* spp.) en algunas áreas de la costa occidental de Baja California. Memorias IV Congreso Nacional de Oceanografía, pp. 257–263.

Guzmán del Próo, S.A., Mille, P.S.R., Guadarra, G.R., de la Campa, G.S., Carrillo, L.J., Pereira, C.A., et al., 1991. La comunidad bentónica de los bancos de abulón (*Haliotis* spp. Molusca: gastropoda) en Bahia Tortugas, Baja California Sur, México. An. Esc. Cien. Biol. Mex. 36, 27–59 The benthic community from abalone banks (*Haliotis* spp. Molusca: gastropoda) at Bahia Tortugas, Baja California Sur, México.

Hernández-Santoyo, A., Hernández-Arana, A., Arreguín-Espinoza, R., Rodríguez-Romero, A., 1998. Purification and characterization of several digestive proteases from the blue abalone, *Haliotis fulgens*. Aquaculture 159, 203–206.

Kawamura, T., Roberts, R.D., Takami, H., 1998. A review of the feeding and growth of postlarval abalone. J. Shellfish Res. 17, 615–625.

Knauer, J., Britz, P.J., Hecht, T., 1996. Comparative growth performance and digestive enzyme activity of juvenile South African abalone, *Haliotis midae*, fed on diatoms and a practical diet. Aquaculture 140, 75–85.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T. Nature 227, 608–685.

Leighton, D.L., Peterson, D.L., 1998. The superiority of Egregia as a food in cultivation of the green abalone (*Haliotis fulgens*). World Aquaculture Society and the National Shellfisheries, p. 331, Abstract.

Mai, K., Mercer, J.P., Donlon, J., 1994. Comparative studies on the nutrition of two species of abalone, *Haliotis tuberculata* L. and *Haliotis discus* hannai Ino. II. Aminoacid composition of abalone and six species of macroalgae with an assessment of their nutritional value. Aquaculture 128, 15–30.

McLean, N., 1970. Digestion in *Haliotis rufescens* Swainson (Gastropoda: Prosobranchia). J. Exp. Zool. 172, 303–318.

Olli, J., Hjelmeland, K., Krogdahl, A., 1994. Soybean trypsin inhibitors in diets for Atlantic salmon (*Salmo salar* L.): effects on nutrient digestibilities and trypsin in pyloric caeca homogenate and intestinal content. Comp. Biochem. Physiol. 109A, 923–928.

Picos-García, C., García-Carreño, F.L., Serviere-Zaragoza, E., 2000. Digestive proteases in juvenile Mexican green abalone, *Haliotis fulgens*. Aquaculture 181, 157–170.

Serviere-Zaragoza, E., Navarrete del Toro, M.A., García-Carreño, F.L., 1997. Protein-hydrolyzing enzymes in the digestive systems of the adult Mexican blue abalone, *Haliotis fulgens* (Gastropoda). Aquaculture 157, 325–336.

Serviere-Zaragoza, E., Gómez-López, D., Ponce-Díaz, G., 1998. The natural diet of the green abalone (*Haliotis fulgens* Philippi) in the southern part of its range, Baja California Sur, México, assessed by an analysis of gut contents. J. Shellfish Res. 17, 777–782.

Serviere-Zaragoza, E., Mazariegos-Villareal, A., Ponce-Díaz, G., Montes-Magallón, S., 2001. Growth of juvenile abalone, *Haliotis fulgens* Philippi, fed different diets. J. Shellfish Res. 20, 689–693.

Sokal, R.R., Rohlf, J.F., 1995. Biometry. The Principles and Practice of Statistics in Biological Research. W.H. Freeman and Company, New York, pp. 887.

Viana, M.T., López, L.M., Salas, A., 1993. Diet development for juvenile abalone *Haliotis fulgens*. Evaluation of two artificial diets and macroalgae. Aquaculture 117, 49–156.

Viana, M.T., López, L.M., García-Esquivel, Z., Mendez, E., 1996. The use of silage made from fish and abalone viscera as an ingredient in abalone feed. Aquaculture 140, 87–98.

Yen, C., Green, L., Miller, C.G., 1980. Degradation of intracellular protein in *Salmonella typhimurium* peptidase mutants. J. Mol. Biol. 143, 21–33.