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Digestive enzymes present in crustacean feces as a tool for biochemical, physiological, and ecological studies

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Abstract

The effect of food composition on the digestive system of *Penaeus vannamei* shrimp was used to determine the suitability of feces for analysis of class, type, composition of digestive proteinases, and whether alterations in the digestive gland are mirrored in feces composition. Enzymes recovered from feces and the midgut gland of white shrimp *P. vannamei* were used for comparison purposes. Three groups of shrimp were assembled: two groups fed two different brands of commercial feeds (PI and SC) with different content of protein, and the last group fed 50% PI feed and 50% thawed giant squid. Composition of proteinases in the midgut gland and feces were identical, and trypsin and chymotrypsin paralogues were identified in both samples by substrate-electrophoresis. Total proteolytic, trypsin, and chymotrypsin enzyme activities were higher in both samples from organisms fed SC, than in the other two groups. In the hepatopancreas, trypsin activity was ~ 30% higher in SC fed group. Final average weights of shrimp were close in three groups, but hepatopancreas weight was 20% higher in the SC group. The degree of protein hydrolysis (DH) in vitro for the SC and PI was evaluated by the pH-stat method, using enzymes from feces and hepatopancreas of each group. The DH of food was no different, but it was affected by enzyme source, hepatopancreas extract (HPE) or feces extract (FE). DH was always higher when FE was the enzyme source than when HPE was the source. The proposed methods for recovery of enzymes from shrimp feces can be applied to other crustaceans. Measurements were sufficiently sensitive to allow quantifying the effects of feed on digestion physiology and other ecological and physiological applications, without the necessity of killing specimens.

Keywords: Crustaceans; Digestive enzymes; Feces; Proteases; Shrimp

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1. Introduction

Animals relate to the environment through several stimuli, food being one of the most important. Heterotrophs obtain the necessary carbon and energy sources to synthesize autologous molecules needed to grow, survive, and conduct actions such as movement, reproduction, and defense. To get carbon and energy sources from food, food is subjected to the action of enzymes, which contribute to breakdown into compounds that can be absorbed by the cells in the gut. The gut lumen is a space where material is concentrated and converted to molecules that can pass through the gut wall to reach body cells and be metabolized. (Dall, 1992; Wotton and Malmqvist, 2001). Unassimilated material is passed out as feces, together with animal tissues and materials, such as enzymes excreted into the gut lumen.

Digestion of food to obtain nutrients for growth, maintenance, motion, and reproduction is one of the most important functions in organism physiology. Intake of food, breakdown of the materials, and assimilation of nutrients comprise the process known as nutrition. Digestive enzymes accomplish digestive breakdown. Protein is one of the most important components of food because, after digestion, it supplies amino acids needed to construct the organism's proteins. Construction of proteins is requisite for maintenance of tissues and growth. Crustaceans also use amino acids as a source of energy (Fox et al., 1994; Shiau, 1998).

Enzymes collectively called proteases accomplish digestion of protein in food by protein hydrolysis. The study of protein digestion usually starts with identification of class, type, and composition of enzymes responsible for food protein hydrolysis. In large organisms like mammals, it is possible to use a canula to sample body fluids. In small crustaceans, this is not possible. For study of decapod crustaceans, anatomizing and homogenizing digestive organs yields digestive enzyme samples (Hernández-Cortés et al., 1997; Ezquerra et al., 1997; Córdova-Murueta and García-Carreño, 2002), but this procedure does not allow subsequent observation of the same organism. These drawbacks call for alternative methods.

In sciences like zoology, and veterinary and human medicine, information about physiological and other characteristics are obtained from body fluids and wastes. Saliva, feces, or urine have been used and validated (Desaulniers, 1989; Garner et al., 2000; von Borell, 2000). Our group is looking for alternative methods of collecting information about digestive system of decapod crustaceans, mainly for experiments where consecutive samplings of a single organism following a treatment are needed. There is no information available about this kind of methodology in crustaceans. We wanted to answer these questions: are digestive enzymes from crustaceans present and active in feces? Can feces serve as material for analysis of digestive system status, including class, type, composition, and kinetic characteristics of proteinases synthesized in the digestive gland? Another objective was to evaluate procedures for measuring changes in the midgut gland. To address these questions, we sampled feces and evaluated proteinase activities in white shrimp *Penaeus vannamei* during a challenge with various compositions of food, and compared enzyme characteristics in feces and digestive gland extracts. The technique described here makes it possible to avoid killing study specimens, and allowed consecutive sampling. This will assist in understanding digestive system function.

Additionally, we compared in vitro digestibility of protein by enzymes from shrimp midgut and feces.

2. Materials and methods

2.1. Feeding experiment

From a commercial shrimp farm in La Paz, Baja California Sur, Mexico, 54 shrimp, weighting an average 16.3 g were purchased and stocked in 70-l rectangular plastic tanks at the CIBNOR facilities, each tank provided with six sections of 1.27 cm mesh plastic-coated screens. One organism was placed in each tank section of 23 × 19 cm. Aeration was provided through air stones, and temperature and salinity were maintained at 28 °C and 37‰, respectively.

Prior to the experiment, shrimp were acclimated to experimental conditions for 1 week. The specimens were assigned to one of three experimental groups in triplicate. To observe changes in the digestive system, all groups were fed ad libitum, twice a day for 30 days. Three different feeds were tested: SC (commercial feed, 45% protein), PI (commercial feed, 35% protein), and SQ (thawed giant squid *Dosidicus gigas*). Because feces from the SQ group were scarce and barely solid, PI feed was substituted for squid in the afternoons from day 14 until the end of the experiment. The weight and molt stage of shrimp were recorded every 7 days. Exoskeletons from molted shrimp were collected daily. At the end of the experiment, the digestive glands from all specimens were extracted, weighed, and stored in individual 1.5-ml test tubes at − 20 °C until used. Proximate analysis of food was conducted using standard methods of A.O.A.C. (1990).

2.2. Feces collection and enzymatic extracts

Feces were collected 2 h after feeding every other day, by siphoning the bottoms of the tanks, starting on day 8. Feces were gently rinsed with distilled water to eliminate excess salt, and placed individually in 1.5-ml test tubes. Tubes were chilled on ice during feces collection, and maintained at 4 °C until used.

Enzymatic extracts (EE) were prepared by adding to each tube 300 μl distilled water, or 200 μl when feces volume was less than half the tube. These samples were homogenized and centrifuged for 10 min at 10,000 × *g* at 4 °C. Supernatant was decanted and stored at 4 °C.

2.3. Enzymatic activity measurements

Soluble protein in the EE was evaluated by the Bradford (1976) method, adapted to micro-assays, using bovine serum albumin as standard. For evaluation, 10 μl EE, 10 μl distilled water, and 200 μl Bradford reagent were placed in a 96-well microplate in triplicate. Absorbance was measured at 595 nm in a microplate reader (BIO RAD 550).

Total proteolytic activity of extracts was evaluated using the method of García-Carreño and Haard (1993) with 1% azocasein as substrate in 50-mM TRIS–HCl buffer at pH 7.5 at 25 °C for 10 min. The reaction was stopped by adding 200 μl 20% TCA. The amount of

peptides released in 50 μl samples of reaction mixture in 96-well microplates was evaluated by reading absorbance at 340 nm in a microplate reader (Labsystems Mulitskan Ascent 354).

Specific activity of trypsin (EC 3.4.21.4) was assayed in 96-well microplates. Samples were prepared with 15 μl EE and 200 μl substrate, consisting of. 0.1-mM benzoyl-Arg-*p*-nitroanilide (BAPNA, SIGMA B4875) in 50-mM TRIS–HCl at pH 7.5 and 20-mM $CaCl_2$. Progress of the reaction at 405 nm was recorded at 3-min intervals at 37 °C. Activity was evaluated using the ratio (Abs 405 × ml reaction volume)/(8800 × mg protein) as described by García-Carreño et al. (1994). Chymotrypsin (EC. 3.4.21.2) activity was measured at 25 °C using 0.1 mM succinyl-(Ala)2-Pro-Phe-*p*-nitroanilide (SAPNA, SIGMA S7388) in 50 mM TRIS–HCl at pH 7.5 and 20 mM $CaCl_2$ as substrate. The method and formula used for trypsin activity was applied.

Individual shrimp midgut glands (hepatopancreas, HP) were homogenized with enough distilled water to comprise 1.2 ml total volume. Homogenates were centrifuged for 30 min at 4 °C and prepared in the same manner as EE. HP extracts (HPE) were assayed for soluble protein, total proteolytic activity, and specific activities of trypsin and chymotrypsin. Assays of 1:40 dilutions of HPE were performed, following the same procedure as described for EE.

Electrophoresis for composition of protein and proteinases in feces and hepatopancreas was performed following the technique of García-Carreño et al. (1993). In brief, sodium dodecyl sulfate, 12% polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli (1970). Enzyme preparations, containing 6 mU enzyme and diluted 1:1 with sample buffer, were loaded into individual gel wells at 4 °C in a vertical electrophoresis device. Molecular mass standards of 4 μl were loaded on each gel. After electrophoresis, gels were stained with 0.05% Coomassie brilliant blue R-250 for at least 24 h, and then destained. Proteinase composition and molecular weight in a twin gel were studied after SDS–PAGE. Gels were immersed in 3% casein in 50-mM TRIS–HCl at pH 7.5 for 30 min at 5 °C to allow the substrate to diffuse into the gel. The temperature was then raised to 25 °C for 90 min. Gels were washed in water and immediately fixed and stained with Coomassie brilliant blue.

To characterize class, type, and composition of proteinases in different samples, enzyme extracts were incubated with proteinase inhibitors. Solutions of TLCK, PMSF, and EDTA were added separately to enzyme extracts containing 10 mU, in the ratio 1:2 inhibitor to extract, and incubated at 25 °C for 60 min. Distilled water replaced inhibitors in controls. Samples were mixed in the sample buffer described above and loaded into the gels. After electrophoresis, molecular weight and inhibition lanes were cut apart, and stained immediately. In a twin gel, control and inhibition lanes were stained for activity as described above. Bands of enzymes mixed with proteinase inhibitors were compared with those of controls without inhibitors to identify active bands. Since TLCK is specific for trypsin, PMSF for serine proteinases, and EDTA for metalloproteases, reduction in intensity of the bands on PAGE is attributed to the presence of these enzymes.

Individual EE data were analyzed daily to compare enzymatic activities among groups. HPE activities were also compared among groups. Final weights of shrimp were evaluated for differences between treatments. The effect of molt on proteolytic activity was analyzed using the data observed at the end of the experiment for HP and the data obtained during the entire experiment for EE. Statistical differences among groups were determined using

Table 1
Approximate composition in experimental foods

Item	Moisture (%)	Protein[a]	Lipids[a] (%)	Ash[a] (%)	Crude fiber[a] (%)	Energy (kJ g^{-1})
SC feed	6.5 ± 0.06	46.6 ± 0.1	7.19 ± 0.07	11.5 ± 0.02	1.9 ± 0.03	18.8 ± 0.04
PI feed	6.9 ± 0.06	36.8 ± 0.2	4.9 ± 0.04	7.4 ± 0.02	1.9 ± 0.09	19.4 ± 0.05
Thawed squid	80.4 ± 0.4	79.3 ± 0.2	1.3 ± 0.03	6.4 ± 0.12	0.0	21.4 ± 0.01

[a] Expressed as dry basis. Nitrogen free extracts are considered the difference from 100%.

two-way ANOVA and HSD multiple-comparison test (Tukey's truly significant differences, Statgrphics Plus v. 6.1) when differences were detected.

2.4. In vitro digestibility

The degree of protein hydrolysis (DH) of the SC and PI food sources was evaluated by the pH-stat method using enzymes from EE and HPE from their respective shrimp group. The SQ group enzymes were not assayed because insufficient EE was obtained from this group. Additionally, in vitro digestibility of casein was measured using HPE and EE of the two shrimp groups. The pH-stat method for in vitro digestibility was applied, as described in Ezquerra et al. (1997) using ion analyzer (718 Stat Titrino, Metrohom Ion Analysis, Switzerland) with a computer interface program (Metrodata Menu 718 STAT TitrinoPC). Powdered feed containing 0.08 g protein was weighed out. Feed was stirred with the required enzyme and enough distilled water in the hydrolysis vessel to yield 10 g substrate mixture. The pH was adjusted to 7.9 using a solution of 1 M l^{-1} NaOH and stirred for 1 h to dissolve the protein completely and stabilize the pH. Prior to starting the reaction, the

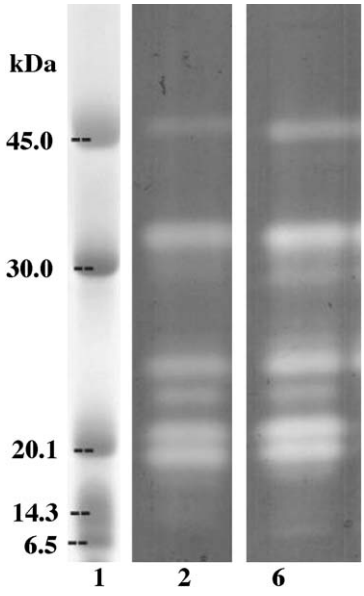

Fig. 1. Zymograms of feces (lane 2) and hepatopancreas (lane 6) extracts. Lane 1 is the molecular weight marker.

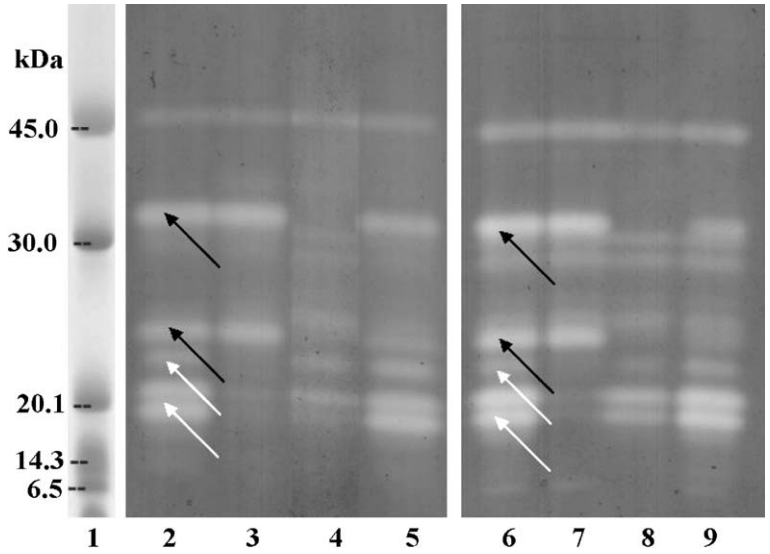

Fig. 2. Zymogram of feces enzymes (lane 2) and hepatopancreas enzymes (lane 6), and the same preparation incubated with specific inhibitors. Lanes 3, 4, and, 5 show feces enzymes incubated with TLCK, PMSF, and EDTA, respectively and lanes 7, 8, and, 9 show hepatopancreas enzymes incubated with TLCK, PMSF, and EDTA, respectively. White arrows indicate trypsin; black arrows indicate chymotrypsin.

pH was automatically raised to 8.0 by the pH-stat, which added 0.1 M l^{-1} NaOH. To start the reaction, volume equivalent to 0.15 units enzyme activity (pH adjusted to 8.0) was added. All assays were done in triplicate. The reaction mixture was maintained at 28 °C using a jacketed reaction vessel and a circulating water bath. DH was calculated using the formula presented by Navarrete-del-Toro (1999):

$$\mathrm{DH}\% = \left(B N_{\mathrm{B}} 1.4 \left[\frac{(S\%/100)}{8} \right] \right) 100,$$

where B is the volume in ml of the 0.1 M l^{-1} NaOH solution consumed to maintain the reaction mixture at pH 8.0; N_{B} is the normality of the NaOH solution; S% is the percentage protein in the reaction mixture.

Table 2
Enzyme activities of feces extracts in U

Group[1]	Total[2]	Trypsin[3]	Chymotrypsin[3]
SC	0.126[c]	0.028[b]	0.087[b]
PI	0.060[a]	0.017[a]	0.043[a]
SQ	0.081[b]	0.020[a]	0.034[a]

Different letters in the same column indicate significant difference ($P < 0.05$).

[1] PI = shrimp fed PI, SQ = shrimp fed thawed giant squid plus PI, SC = shrimp fed SC.

[2] One unit of activity = absorbance at 340 nm min^{-1} mg $protein^{-1}$.

[3] One unit of activity for synthetic substrate is the amount of enzyme needed to hydrolyze 1 μmol of substrate in 1 min.

Table 3
Enzyme activities of hepatopancreas extracts in U, and final shrimp weight

Group*	Total activity[1]	Trypsin activity[2]	Chymotrypsin activity[2]	Hepatopancreas weight (g)	Final shrimp weight (g)
SC	0.300b	0.121b	0.104a	0.469c	16.71a
PI	0.274b	0.067a	0.089a	0.396a	16.67a
SQ	0.169a	0.071a	0.070a	0.378a	16.47a

*PI = shrimp fed PI, SQ = shrimp fed thawed giant squid plus PI, SC = shrimp fed SC.
Different letters in the same column indicate significant difference ($P < 0.05$).
[1] One unit of activity = absorbance at 340 min^{-1} mg $protein^{-1}$.
[2] One unit of activity for synthetic substrate = the amount of enzyme needed to hydrolyze 1 μmol of substrate in 1 min.

DH data of feeds were analyzed using two-way ANOVA and HSD Tukey test for the effect of PI and SC groups and HPE and EE enzymes sources on DH. One analysis was performed for DH of feeds and another for DH of casein.

3. Results

The proximate composition of food is given in Table 1. In previous work (Córdova-Murueta and García-Carreño, 2002; Muhlia-Almazán and García-Carreño, 2002), effects of varying food composition on digestive proteinase activity of the shrimp were differentiable. Protein percentages evaluated as nitrogen by the Kjeldahl method were 79%, 47%, and 37% for SQ, SC, and PI, respectively. Lipids and energy were also different among the three feeds, which allows an expectation that the effects of feed treatments on organisms are differentiable.

The zymogram in Fig. 1 shows composition of proteinases in digestive gland and feces extracts (FE). Compositions in the two preparations are comparable. The zymogram of enzyme preparations incubated with specific inhibitors (Fig. 2) shows that some active bands are turned off as a consequence of inhibitor action. With this technique, called substrate SDS–PAGE (García-Carreño et al., 1993), it is possible to identify classes of proteinase, such as serine, cysteine, metallo, or aspartic, and type, such as trypsin or

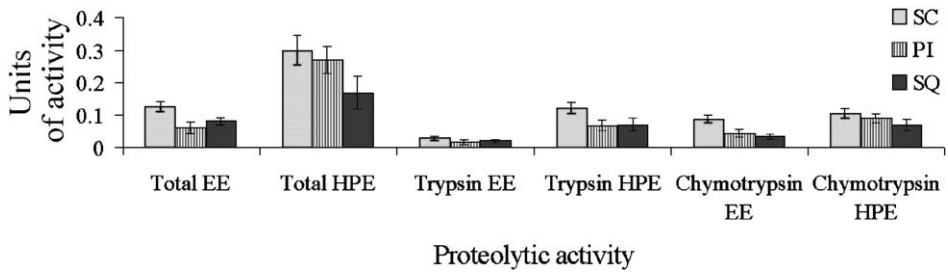

Fig. 3. Proteolytic activity (in U) measured in feces (EE) and hepatopancreas extracts (HPE) of shrimp fed PI = commercial feed (35% protein), SC = commercial feed (45% protein), or SQ = thawed giant squid plus PI.

chymotrypsin. Also, the presence of paralogue enzymes can be detected. The presence of at least two paralogue chymotrypsins and one trypsin in both hepatopancreas and feces extracts are shown in Fig. 2.

Averages of total and specific activities of feces digestive proteinases are shown in Table 2. Total proteolytic, trypsin, and chymotrypsin enzyme activities were 30%, 40%, and 100% higher ($P < 0.05$) in specimens fed with the SC feed than in specimens fed with the other two feeds.

Total and specific activities of hepatopancreas digestive proteinases are shown in Table 3. Trypsin activity was ~ 30% higher ($P < 0.05$) in SC group organisms. Table 3 also shows hepatopancreas and whole body weights at the end of the experiment. Whole body

Table 4
Total and specific proteolytic activities in shrimp feces shown by day sampled and by group from day 7 to 29 (in U)

Sample Day	Group*	Total	Trypsin	Chymotrypsin
7	SC	0.187^{b}	0.056^{a}	0.056^{a}
	PI	0.082^{a}	0.027^{a}	0.027^{a}
	SQ	0.178^{b}	0.029^{a}	0.026^{a}
8	SC	0.127^{b}	ND	ND
	PI	0.054^{a}	ND	ND
	SQ	0.042^{a}	ND	ND
10	SC	0.081^{b}	0.032^{b}	0.095^{c}
	PI	0.025^{a}	0.011^{a}	0.059^{b}
	SQ	0.034^{a}	0.024^{b}	0.015^{a}
12	SC	0.097^{b}	0.042^{b}	0.142^{b}
	PI	0.035^{a}	0.011^{a}	0.044^{a}
	SQ	0.042^{a}	0.019^{a}	0.032^{a}
15	SC	0.137^{b}	0.017^{a}	0.072^{b}
	PI	0.052^{a}	0.016^{a}	0.032^{a}
	SQ	0.121^{b}	0.014^{a}	0.061^{ab}
17	SC	0.149^{b}	0.032^{a}	0.110^{b}
	PI	0.078^{a}	0.040^{a}	0.061^{a}
	SQ	0.090^{a}	0.020^{a}	0.048^{a}
20	SC	0.197^{b}	0.028^{b}	0.126^{b}
	PI	0.083^{a}	0.011^{a}	0.040^{a}
	SQ	0.083^{a}	0.020^{a}	0.034^{a}
22	SC	0.091^{b}	0.006^{a}	0.067^{b}
	PI	0.054^{a}	0.007^{a}	0.031^{a}
	SQ	0.061^{a}	0.004^{a}	0.023^{a}
24	SC	0.152^{a}	0.023^{b}	0.092^{b}
	PI	0.110^{a}	0.021^{ab}	0.046^{a}
	SQ	0.129^{a}	0.010^{a}	0.031^{a}
26	SC	0.083^{b}	0.013^{ab}	0.059^{a}
	PI	0.043^{a}	0.009^{a}	0.039^{a}
	SQ	0.058^{ab}	0.018^{b}	0.048^{a}
29	SC	0.083^{a}	0.017^{a}	0.056^{a}
	PI	0.052^{a}	0.014^{a}	0.040^{a}
	SQ	0.059^{a}	0.010^{a}	0.032^{a}

*PI = shrimp fed PI, SQ = shrimp fed thawed giant squid plus PI, SC = shrimp fed SC.
Different letters in the same day sampled and column indicate significant differences ($P < 0.05$).

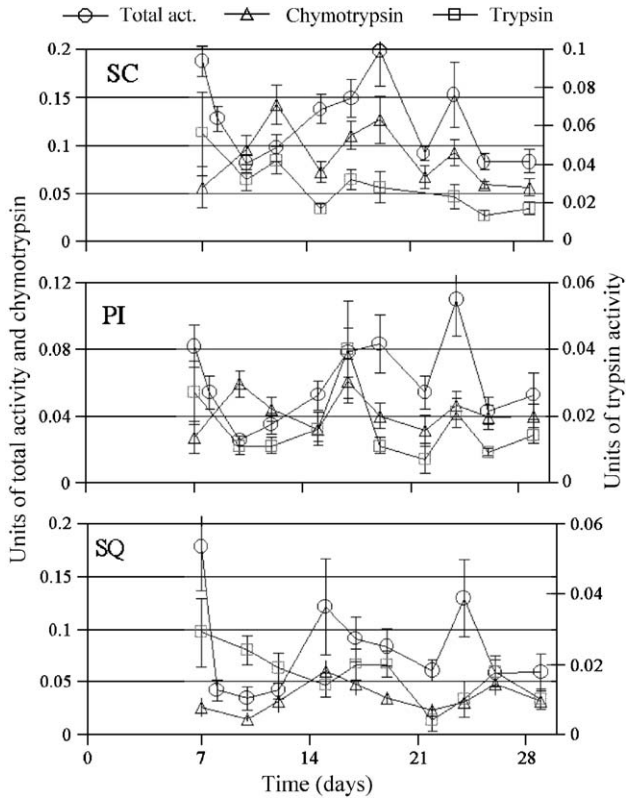

Fig. 4. Proteolytic activities against time in shrimp feces extracts of groups fed SC = commercial feed 45% protein PI = commercial fed 35% protein, or SQ = thawed giant squid plus PI.

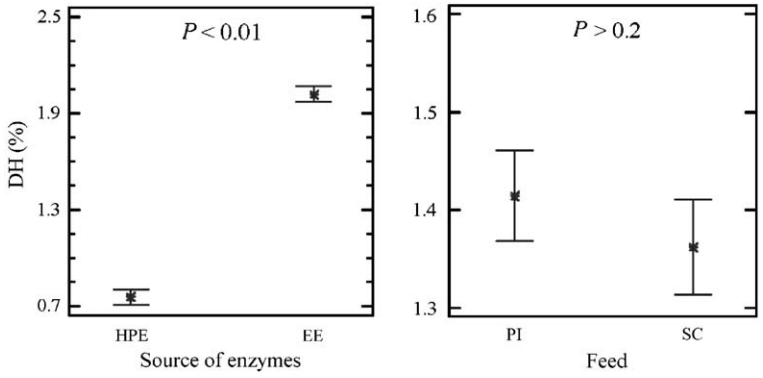

Fig. 5. Average degree of hydrolysis (DH (%)) of feed and Tukey intervals for source of enzymes and shrimp feed group, using two-way ANOVA.

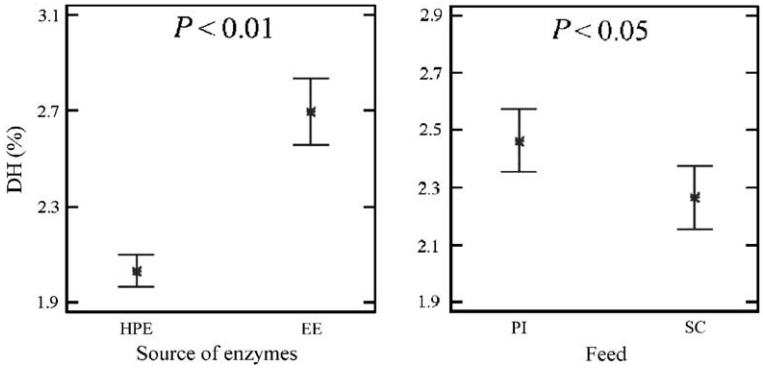

Fig. 6. Average percentage casein hydrolysis (DH (%)) and Tukey intervals for source of enzyme and shrimp feed group, using two-way ANOVA.

weights were close in the three groups. Hepatopancreas weight was 20% higher in the SC group. Fig. 3 shows that the SC group's HPE and EE activities were the highest and that the SC group's chymotrypsin EE and HPE activities were comparable.

Total proteinase, trypsin, and chymotrypsin activities in feces sampled periodically are shown in Table 4. High activity variability over time was found (Fig. 4). Of particular interest in this figure are the low activities for days 10, 15, and 22, which followed the day that shrimp were manipulated for weight measurements and to establish molt stage.

Significant differences were detected in total proteolytic activity ($P < 0.05$; Table 4) among groups on each day sampled, except for the 24th and 29th days (no difference was found, $P > 0.05$). EE total proteolytic activity of the SC group was highest on most days sampled, followed by the SQ and PI groups. Trypsin activity was significantly higher ($P < 0.05$) for the SC group in 4 of the 11 samples, and chymotrypsin activity was significantly higher in 6 samples. The average of combined enzyme activities was higher, at 0.1 U, in SC specimens, compared to 0.06 and 0.08 U for PI and SQ specimens, respectively (Fig. 4).

The DH of food sources was no different ($P > 0.2$), but it was affected by enzyme source, HPE or EE ($P < 0.01$, Fig. 5). No interaction of the two sources of variation was detected ($P > 0.1$). Also, DH for casein was no different for the two shrimp groups ($P > 0.05$), but it was affected by enzyme source ($P < 0.01$, Fig. 6), and was always higher with EE as the enzyme source than HPE.

4. Discussion

Study of biochemical physiology in small organisms is demanding, and killing a small animal to obtain organ or tissue samples is unavoidable. Such a strategy not only eliminates the organism, but also precludes subsequent monitoring a phenomenon in the same organism over time. In these studies, a large number of organisms are necessary to obtain successive samples. We have been studying protein digestion in crustacean decapods for several years (García-Carreño and Haard, 1993; García-Carreño et al.,

1997; Hernández-Cortés et al., 1997; Lemos et al., 1999; Albuquerque et al., 2002, among others), mainly enzymes responsible for digestion of food protein. Usually, we have to anatomize the digestive gland to extract enzymes. While this procedure provides information about class and type of enzymes present in the gland, it is not possible to distinguish between enzymes secreted into the digestive cavity and those stored in intracellular granules. Therefore, the exact amount of enzymes secreted and used to digest food protein is unknown.

Alternative methods for sampling are needed in the pursuit of such information. Because penaeid shrimp are too small to tolerate a canula, we conceived of looking at feces composition. The idea is consistent with studies of fish in which pepsin activity was recovered from intestine contents, even after chyme was mixed with the pancreas juice and pH was raised to alkaline levels (Díaz-López et al., 1998). Also, we wanted to investigate possible reabsorption of digestive enzymes in the distal part of the penaeid intestine, as what happens in mammals (Diamond, 1978). According to our results, there is no evidence of reabsorption, or at least not extensive activity because we recovered significant quantities of digestive enzymes in feces to perform all the measurements, including in vitro digestibility.

Shrimp build a peritrophic membrane, which is a thin tubular sheet secreted in the midgut, extending from the lumen to the outer wall of the intestine (Martin and Chiu, 2001). It functions in the midgut as a lubricant to prevent abrasion of the gut wall by food particles (Wotton and Malmqvist, 2001). The peritrophic membrane is chitinous in nature, helping the organism to resist attack from pathogens present in food particles that may penetrate intestine tissue (Martin and Chiu, 2001). In fecal pellets this membrane might act as a protection barrier to retard leakage of material, including digestive enzymes; this feature facilitates recovery of the digestive enzymes. Observations of fecal pellets with microscopy show that this membrane constitutes a complete wrapping over the fecal material that remains intact for several hours, as demonstrated in the amphipod *Gammarus tlacustris*, lasting up to 7 h (Lautenschlager et al., 1978).

The secretion of this membrane in shrimp could be constant because unfed shrimp can eject the empty membrane (Córdova-Murueta, unpublished data). The rate of secretion of the peritrophic membrane is as fast as the ingested material is expelled out of the organism (1–2 h after ingestion).

Proteinolytic enzymes are present and active in shrimp feces, and their composition matches that of the enzymes in the hepatopancreas (Fig. 1), supporting the conclusion that the source of feces enzymes is the midgut gland. Also, we found that *P. vannamei* proteinases are stable for more than 72 h at 25 °C (unpublished data). Proteinases of *P. vannamei* are optimum at alkaline pH, but Hernández-Cortés et al. (1997) demonstrated that chymotrypsin from *P. vannamei* can be active at pH 5 to 11. This explains the stability of digestive enzymes in *P. vannamei* FE when released in seawater. Both trypsin and chymotrypsin were found in feces extracts, as demonstrated by inhibition substrate SDS–PAGE (Fig. 2). In the experiment, we intended to discover if changes in proteolytic activity in the midgut gland are mirrored in feces. We used food composition as a stimulant for the digestive gland, as in other experiments (Córdova-Murueta and García-Carreño, 2002).

Groups fed different foods had different final hepatopancreas weights, and because final body weights among groups were statistically identical, the hepatosomatic indices

were also different. The SC group had the highest index, meaning that food composition profoundly affects digestive gland condition. Food composition also affected total proteolytic and trypsin activity in hepatopancreas extract. In this study, chymotrypsin activity was not affected (Table 3). Variables affected in the midgut gland were mirrored in feces (Fig. 3).

Total proteolytic, trypsin, and chymotrypsin activities in feces varied during the study (Fig. 4), and significant differences were found. Food composition significantly affected enzyme activities. Total, trypsin, and chymotrypsin activities were highest in feces of the SC group on most sampling days, followed by the SQ group. Fig. 4 indicates that feces of SC organisms had highest enzyme activities, demonstrating that changes in the midgut gland can be traced in feces composition.

We do not know the ultimate cause of these variations over time, but several possibilities exist, including lixiviation or at least some effect on enzymes during the period between feces excretion and collection. If molt stage had no significant effect on feces or midgut gland proteolytic activities ($P>0.05$) in the experiment, we could not discard physiological stress was at least partially responsible for the huge variations in enzyme activity, as suggested by low activities in samples taken the day after shrimp were manipulated for weighing and observation of molt stage. Also, individual variations within the species must be considered. To obtain some clues on this variability, we are conducting further experiments.

Feces are suitable for evaluating midgut gland condition, mainly by analyzing activity, class, type, and composition of digestive enzymes. The use of feces for evaluation of biochemical and physiological processes is already applied in several settings, as in human and veterinary medicine (Kita et al., 1989), forensic medicine, and identification of organisms present in the wild. However, there is no report of using this analytical procedure on invertebrates, such as crustaceans, including decapods and penaeids, or on other aquatic organisms. The procedure could be useful in studies of ecology, biochemistry, and physiology, and in helping researchers in these fields with aquaculture applications.

Little attention has been paid to the role of feces in ecosystems, yet fecal pellets are often abundant, represent a repackaging of available organic matter, and are a source of nutrients for many organisms (Wotton and Malmqvist, 2001). Some organisms could be fed proteases from feces as an aid to protein digestion. For example, when comparing guinea pigs fed its feces with a group prevented from coprophagy, the last group showed lower digestibility of nitrogen and fiber components, and lower nitrogen retention (Yutaka and Sakaguchi, 2002). Shrimp usually eat their own feces, and coprophagy is considered to be a suitable strategy for further use of material for which one gut passage is too short and/or inefficient (Heinz, 1997), and proteases in feces probably play an important role in protein digestion.

In ecology studies, analysis of proteases in feces will assist in understanding the influence of prey on group performance. In physiological biochemistry, information obtained from feces will help to learn about composition and activity of enzymes. Sampling digestive proteinases of feces can help determine ontogeny of particular species. Composition of feces will be especially enlightening when studying organisms with short digestion periods, where very active enzymes and low-efficiency digestion are expected, as in decapods. Regarding in vitro digestibility studies, the results showed that it is

possible to use the enzymes in feces for digestibility studies as a substitute of enzymes from the digestive gland.

In feces, we expect to find only those enzymes physiologically secreted into the gut lumen. This enzymatic cocktail should contain the exact amount and composition of proteases needed to break down protein in the ingested food. Such enzymes are excreted with feces, which are protected by the peritrophic membrane that prevents leakage. In contrast, when homogenizing the whole digestive gland to obtain an enzyme extract, the cocktail may contain enzymes, zymogens, enzyme inhibitors (Albuquerque et al., 2002) and other compounds that could affect protease efficiency during in vitro digestibility assay. This effect could account for the higher DH of substrates assayed with shrimp feces enzymes than with HPE. Further investigation is needed to explore this hypothesis. We analyzed only proteolytic enzymes, but we believe that other digestive enzymes and metabolites might be present in feces.

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