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Digestive proteinases of *Artemesia longinaris* (Decapoda, Penaeidae) and relationship with molting

A.V. Fernández Gimenez[a], F.L. García-Carreño[b,*], M.A. Navarrete del Toro[b], J.L. Fenucci[a]

[a]Departamento Ciencias Marinas, Facultad Ciencias Exactas y Naturales, CONICET, Universidad Nacional de Mar del Plata, Funes 3350, B7602AYL, Mar del Plata, Argentina

[b]Centro de Investigaciones Biológicas del Noroeste (CIBNOR), A.P. 129, La Paz, B.C.S, 23000, Mexico

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Abstract

Digestive proteinase activities of *Artemesia longinaris* were assayed at different stages of the molting cycle. Total proteolytic activity in the hepatopancreas was highest during postmolt. Trypsin and chymotrypsin activities were highest during intermolt. Specific inhibitors and zymograms of *A. longinaris* hepatopancreas extracts showed four trypsins (14.79, 15.49, 16.60, 17.38 kDa, respectively) and three chymotrypsins (21.38, 22.91, 27.54 kDa, respectively). Our results suggest that proteolytic activity in the hepatopancreas of *A. longinaris* is influenced by the molting cycle. Types and activity of prawn digestive enzymes constitute background information to further study the digestive abilities of these organisms and will lead to understanding their nutritional needs and feeding ecology. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: *Artemesia longinaris*; Chymotrypsin; Hepatopancreas; Inhibitors; Molting cycle; Physiology; Prawn; Serine proteinases; Trypsin

1. Introduction

Proteinases are among the best-studied crustacean digestive enzymes. They have highest activity between pH 5 and 10 (Biesiot and McDowell Capuzzo, 1990; García-Carreño, 1992). Crustaceans digest a variety of food protein materials and possess high concentration of digestive serine proteinases, especially trypsin and chymotrypsin, with the hepatopancreas responsible for synthesis, storage of zymogens, and secretion. Decapod digestive enzymes are synthesized and secreted by the hepatopancreatic F cells (Skinner, 1985). In most cases, the enzyme secretion is holocrine (Ceccaldi, 1997).

Trypsin is a ubiquitous enzyme in crustacean digestive systems. Several trypsins have been isolated and characterized in a number of decapods. Chymotrypsin seems to be less ubiquitous and second in importance, when considering amount of activity (García-Carreño et al., 1994).

Molt is a continuous process leading to acute morphological and physiological changes (Skinner, 1985). Penaeids molt at intervals of a few days or weeks. The pattern of the molting cycle in *A. longinaris* is typically diecdysical. In the laboratory, the duration of the intermolt period of this species is 17 ± 4 days at 19 °C (Petriella, 1986). Usually, ecdysis takes place at night and is achieved in minutes. Prawns stop feeding during ecdysis for 4–6 days (3–4 premolt and 1–2 postmolt) (Petriella, 1986). The hepatopancreas stores nutrients that are used during the periods of

*Corresponding author. Tel.: +52-612-125-3633; fax: +52-612-125-4710.

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

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starvation prior and after molting (Dall et al., 1990).

Because of the importance of digestive enzymes in the assimilation of nutrients, it is essential to identify the main digestive enzymes, and understand how their activity is modified with physiological changes. The understanding of digestive physiology provides clues about nutritional needs and feeding ecology. This study provides preliminary data on the proteolytic enzymes present in the hepatopancreas of *A. longinaris* and the effect of molting stages on the activity of these enzymes for further studies related to the use of food protein for nutrition.

2. Materials and methods

Prawns were captured by a commercial ship from Mar del Plata, Argentina (38°S) and maintained alive on board the ship in circulating seawater. In the lab, the molting stage of each prawn was evaluated by microscopic examination of uropod setae (Díaz and Petriella, 1990). Immediately after, the hepatopancreas of adult individuals in different molting stages were aseptically removed from decapitated specimens. Samples were immediately lyophilized, and stored at −20 °C.

Samples from individuals in the same molting stage were pooled. Freeze-dried hepatopancreas were homogenized in chilled distilled water and centrifuged for 30 min (10 000×*g* at 4 °C). The lipid layer was removed and total soluble protein evaluated in the supernatants (Bradford, 1976), with bovine albumin as standard.

Total proteinase activity was assayed using 1% azocasein in 50 mM Tris–HCl, pH 7.5 (García-Carreño, 1992). Triplicates of 5 μl of enzyme extracts were mixed with 0.5 ml of buffer and 0.5 ml of substrate solution. The reaction mixtures were incubated for 10 min at 25 °C. Proteolysis was stopped by adding 0.5 ml of 20% trichloroacetic acid (TCA), and the mixture was centrifuged in microcentrifuge tubes (5 min at 14 000×*g*). The absorbance of the supernatants at 366 nm for the TCA-soluble peptide-dye was recorded.

The effect of pH on the azocaseinolytic activity was evaluated by using pH 6.5–10 Universal Buffer (Stauffer, 1989) at 25 °C, following the total proteinase activity procedure. The substrate (1% azocasein) was dissolved in each of the pH buffer solutions.

Evaluation of the proteinase class was based on the method of García-Carreño and Haard (1993). Enzyme extracts were incubated with different specific proteinase inhibitors. Phenylmethylsulfonyl fluoride (PMSF) and soybean trypsin inhibitor (SBTI) were used as inhibitors of proteinases belonging to the serine class. Nα-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) was used as the specific inhibitor of trypsin. Solutions (10 μl) of 100 mM PMSF in 2-propanol, 250 μM SBTI in distilled water, or 10 mM TLCK in 1 mM HCl, pH 3, were separately mixed with the enzyme extracts (10 μl) and incubated for 60 min at 25 °C. Then, 0.5 ml of 1% azocasein in 50 mM Tris–HCl, pH 7.5, was added. *N*-Tosyl-L-phenylalanine chloromethyl ketone (TPCK) was used as a specific inhibitor of chymotrypsin. The inhibitor (7.5 μl) was mixed with the enzyme extract (15 μl) and incubated for 60 min at 25 °C before used as the enzyme preparation for activity described above. Assays including distilled water instead of the inhibitor solution were used as controls. The reaction was stopped after 10 min by adding 0.5 ml of 20% TCA and centrifuged for 5 min at 14 000×*g*. The supernatants were read at 366 nm for the released peptide–dye complex. Activity in inhibition assays was reported as a percentage of inhibition. Activity measured in the absence of the inhibitor was considered as 100%. Assays were run in triplicate.

Trypsin activity was measured with Nα-benzoyl-DL-arginine *p*-nitroanilide (BAPNA). BAPNA (1 mM) was dissolved in 1 ml of dimethylsulfoxide (DMSO) and brought to 100 ml with Tris–HCl, pH 7.5, containing 20 mM $CaCl_2$. Triplicates of hepatopancreas samples (5 μl) were added to 0.75 ml of substrate solution at 37 °C. Changes of absorbance at 410 nm were recorded for 10 min (Erlanger et al., 1961). Chymotrypsin activity was evaluated using 0.1 mM Suc–Ala–Ala–Pro–Phe *p*-nitroanilide (SAPNA) in 0.1 M Tris–HCl, pH 7.5, containing 0.01 M $CaCl_2$. Triplicates of hepatopancreas samples (5 μl) were mixed with 0.75 ml of substrate solution. Absorbance at 410 nm was recorded for 5 min (del Mar et al., 1979). Each assay included blanks and commercial enzymes (1 mg/ml) as internal controls. Total proteinase, trypsin, and chymotrypsin units of activity were expressed as change in absorbance/min per mg of protein of the enzyme used in the assays (ΔAbs min^{-1} mg $protein^{-1}$).

Sodium dodecyl sulfate, 12% polyacrylamide

gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). Enzyme preparations, containing 10 mU (U = μmol substrate/min) of each molting stage (diluted 1:1 with sample buffer) were loaded into individual gel wells at 4 °C) in a vertical electrophoresis device. Molecular mass standards (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 of staining, and then destained. In a twin gel, proteinase composition and molecular weight were studied after SDS-PAGE (García-Carreño et al., 1993). Gels were immersed in 3% casein in 50 mM Tris–HCl, 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 the class and type of proteinase and the composition of proteinases in different molting stages, the enzyme extracts were incubated with proteinase inhibitors. Solutions of TLCK, TPCK, PMSF and SBTI were separately added to enzyme extracts containing 10 mU, in a ratio of 1:2 (inhibitor/extract) and incubated at 25 °C for 60 min. Distilled water replaced inhibitors in controls. Samples were mixed in the sample buffer, as described above, and loaded into the gels. After electrophoresis, molecular weight and inhibition lanes were cut apart. They were immediately stained. In a twin gel, control and inhibition lanes were stained for activity, as above.

Bands of enzymes mixed with proteinase inhibitors were compared with controls without inhibitors to identify the inhibitory effects on active bands. Since TLCK and TPCK are specific for trypsin, and chymotrypsin and PMSF and SBTI for serine proteinases, reduction in intensity of the bands on PAGE is attributed to the presence of these enzymes.

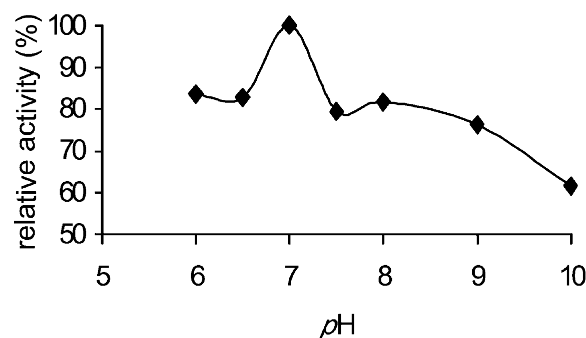

Fig. 1. Effect of pH on the proteolytic activity in hepatopancreas of *Artemesia longinaris*. The enzyme preparations were assayed with azocasein following the standard procedure using Universal buffer at different pH values. Values of activity at pH 7 and 10 are significantly different ($P<0.05$) between them and among other pH values.

Data are expressed as the mean ± S.D. We applied arc sine transformation to percentages. ANOVA and Student's *t*-test were used to find differences among means. In all cases, significance was set as $P<0.05$ (Sokal and Rohlf, 1979). All assays were done three times.

3. Results

The mass of the hepatopancreas of *A. longinaris* did not vary with molting stage; organs were 0.06 ± 0.035, 0.06 ± 0.039 and 0.04 ± 0.016 g as dry matter for postmolt, intermolt, and premolt, respectively, with no significant differences. Also, there were no differences in the protein content of hepatopancreas (Table 1).

The influence of pH on proteinase activity, with azocasein as the substrate, is shown in Fig. 1. Maximum activity occurred at pH 7. Activity was 80% of the maximum at pH 6 and 8. Enzyme activity was therefore assessed at pH 7, including those for substrate-SDS-PAGE, after electrophoresis separation of the fractions.

Table 1
Protein content and specific activity in enzyme extracts from *Artemesia longinaris*

	HP dry matter*	Protein content**	Total proteases ***	Trypsin***	Chymotrypsin***
Stage/substrate			azocasein	BAPNA	SAPNA
Postmolt	0.06	7.1^{a}	1.2^{b}	3.6 ± 0.17^{b}	4.8 ± 0.25^{b}
Intermolt	0.06	6.3^{a}	0.8^{a}	3.9 ± 0.11^{c}	5.4 ± 0.15^{c}
Premolt	0.04	4.7^{a}	0.9^{a}	2.8 ± 0.62^{a}	3.9 ± 0.14^{a}

Means followed by different superscripts are significantly different ($P<0.05$). Specific activity is U/mg of protein in the enzyme extract. *HP = hepatopancreas in g; **mg/ml; ***abs/min per mg protein. Mean of three replicates ± S.D.

Proteinase activity with azocasein as the substrate varied significantly during the molting cycle. Activity was highest in postmolt; a significant decrease was observed in intermolt, while remaining constant in premolt (Table 1). Trypsin was highest during intermolt and lower during premolt. Differences among stages were significant. The specific activity of chymotrypsin mirrored trypsin activity. The highest chymotrypsin activity was found during intermolt, intermediate during postmolt, and lowest during premolt (Table 1). Table 2 shows the percentage inhibition of azocasein hydrolysis by the serine proteinase inhibitors PMSF, SBTI, and TLCK on enzyme extracts at different stages. No significant differences were found.

The percentage inhibition by TLCK (BAPNA as substrate) and TPCK (SAPNA as substrate) is shown in Table 2. Trypsin activity was completely inhibited in all molting stages by TLCK. Proteinase activity was partially inhibited by TPCK in postmolt and intermolt. However, TPCK had almost no effect on the proteinase activity in premolt.

Zymograms analysis of proteinase activity was performed to determine the composition and molecular weight of the enzymes in the prawn hepatopancreas. Hepatopancreas extracts of prawn in different molting stages displayed 16 active zones having caseinolytic activity. The molecular mass of the active zones ranged from 14.79 to 58.88 kDa for all molting stages (Table 3). Specific proteinase activities were identified with synthetic inhibitors. There was no inhibitory effect on proteinase activity when the hepatopancreas extracts were incubated with TPCK. Active bands inhibited with TLCK and SBTI were considered trypsin, while inhibition by PMSF and SBTI indicated chymotrypsin (García-Carreño and Haard, 1993). Several trypsin and chymotrypsin forms were found. Four trypsin forms and three intense bands of chymotrypsin were found in all molting stages (Table 3). One band (19.05 kDa) was partially inhibited by serine proteinase inhibitor PMSF. Two active zones (19.05 and 20.89 kDa) were inhibited by SBTI.

Table 3
Proteinase activity bands in substrate-SDS-PAGE zymograms of *Artemesia longinaris* hepatopancreas extracts in all molting stages

Molecular mass	Inhibition by	Presumptive enzyme
58.88	Nd	ni
46.77	Nd	ni
39.81	Nd	ni
37.15	Nd	ni
33.11	Nd	ni
30.20	Nd	ni
27.54	S, P	Chymotrypsin
23.99	Nd	ni
22.91	S-P	Chymotrypsin
21.38	S, P	Chymotrypsin
20.89	S	serine protease
19.05	S, P	serine protease
17.38	T, S	Trypsin
16.60	T, S	Trypsin
15.49	T, S	Trypsin
14.79	T, S	Trypsin

S, P, and T indicate inhibition by SBTI, PMSF, and TLCK, respectively; nd = no inhibition detected; ni = not identified.

4. Discussion and conclusion

Due to their biological basis, in several species, changes in digestive enzyme activities have been demonstrated during: (1) ontogenetic development

Table 2
Effect of inhibitors on total proteinase, trypsin, and chymotrypsin activity

Stage/inhibitor	Inhibition (%)				
	PMSF*	SBTI*	TLCK*	TLCK**	TPCK***
Postmolt	33.4 ± 4.95^a	51.8 ± 16.85^a	41.2 ± 2.35^a	98.9 ± 0.06^a	34.3 ± 3.84^b
Intermolt	37.2 ± 13.29^a	63.3 ± 6.93^a	42.1 ± 12.88^a	98.6 ± 0.63^a	36.1 ± 5.59^b
Premolt	32.9 ± 18.21^a	59.7 ± 2.58^a	39.1 ± 7.60^a	97.3 ± 2.06^a	14.9 ± 5.17^a

Means followed by different superscripts are significantly different ($P < 0.05$). Substrates: *azocasein; **BAPNA; ***SAPNA. The percentage of inhibition was calculated by using enzyme activity without inhibitor as 100%. Different superscript show statistical differences between groups ($P < 0.05$). PMSF, phenylmethylsulfonyl fluoride; SBTI, soybean trypsin inhibitor; TLCK, Nα-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; BAPNA, *N*-benzoyl-DL-arginine *p*-nitroanilide; SAP-NA, Suc–Ala–Ala–Pro–Phe–*p*-nitroanilide.

Table 4
Proteinase, trypsin, and chymotrypsin activities from adult penaeids and other decapods, in different molting stages

Species	Proteinase			Trypsin			Chymotrypsin			Source
	1	2	3	1	2	3	1	2	3	
Artemesia longinaris	h	l	l	m	H	l	m	h	l	This study
Pleoticus muelleri	h	h	l	h	L	m	h	l	m	Fernández Gimenez et al. (2001)
Penaeus notialis	h	h	l	m	H	l	m	h	h	Fernández et al. (1997)
Penaeus vannamei	l	h	m	l	M	h	l	h	m	Muhlia et al. (personal communication)
Carcinus maenas	h	h	l	na	na	na	na	na	na	Bauchan and Mengeot (1965)

1, 2 and 3 indicate postmolt, intermolt, and premolt, respectively; h = higher activity; l = lower activity; m = middle activity; na = data not available.

(Lovett and Felder, 1990; Biesiot and McDowell Capuzzo, 1990; Fang and Lee, 1992; Lemos et al., 1999); (2) ovarian maturation (Fernández et al., 1997); (3) circadian cycles (Hernández-Cortés et al., 1999); and (4) molting cycle (Fernández Gimenez et al., 2001). In some decapods, the number and organization of genes codifying digestive enzymes is available. But mechanisms of regulation of the enzyme activity in the digestive system in decapods remain unclear. Whether these genes are constitutive or inducible remains unknown.

This work is intended to provide basic information on food protein digestion in *A. longinaris;* what are the number and some operational characteristics of the enzymes responsible for this physiological activity. Characteristics include pH of maximum activity, class, and paralogy (presence of isoenzymes). This work provides a platform for further studies in protein digestion and enzyme regulation.

Proteinases from hepatopancreas of *A. longinaris* had a maximum activity at pH 7. In crustaceans, the optimal pH varies between pH 5.5 and 9 (García-Carreño, 1992; García-Carreño et al., 1994; Ceccaldi, 1997; Fernández Gimenez et al., 2001). Total proteinase activity was highest during postmolt and lowest during intermolt and premolt stages. In crustaceans like *Pleoticus muelleri, Carcinus maenas,* and *Penaeus notiatis*, proteinase activities varied following the same pattern, but differently than that of *A. longinaris* and *Penaeus vannamei* (Table 4).

In this work, the zymogram of *A. longinaris* showed several caseinolytic bands. The hepatopancreas of this species contains several paraloge (homologous proteases from the same species, Southan, 2000) isozymes; four trypsin and three chymotrypsins were detected. The four trypsin isoforms were sensitive to TLCK and SBTI, and their molecular masses ranged from 14.79 to 17.38 kDa. Chymotrypsin forms in *A. longinaris* have molecular masses of 21.38, 22.91 and 27.54 kDa. They were inhibited by PMSF and SBTI.

Reasons for changes in proteolytic activities of digestive enzymes, in relation to the molting cycle, are diverse. Feeding activity declines during early premolt and ceases by late premolt (Dall et al., 1990). The nutritional needs and feeding habits of the mentioned species are diverse (Akiyama et al., 1992). Comparisons between activities of digestive proteinases in different molting stages indicates a species-specific pattern of protein digestion.

The molting process influences crustacean physiology, morphology, behavior, and reproduction (Skinner, 1985). Penaeids molt at intervals of a few weeks, so it is a continuous process, with changes occurring almost daily (Dall et al., 1990). The results of this study suggest that proteolytic activity in the hepatopancreas of *A. longinaris* is influenced by the molting cycle. Information provided by this work strongly suggests that enzyme activities and synthesis regulation is species-specific. It seems that extrapolation is not possible within the taxa and characteristics of species have to be assessed independently. The influence of nutritional condition on the activity of digestive enzymes can shadow the effect of molting, as we demonstrated recently in *Penaeus vannamei* (data to be published elsewhere). It seems that the feeding or absence of feeding during each molting stage is strongly related to the nutritional status of the organism. Recent evidence strongly suggests that mechanisms regulating the presence (paralogy) and abundance (measurable activity) of enzymes responsible for digestion of food protein are more related to feeding habits than to phylogeny. The processing of putative precursors (zymo-

gens) and the secretory mechanisms of proteinases in decapods seem to include features that are species-specific. Studies of these processes provide a basis for detailed understanding of the physiology of digestion of protein to reduce the expense of shrimp aquaculture amount of protein and the cost of ingredients in feed fabrication. To achieve this, it is necessary to describe the enzymes and the characteristics of their operation.

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