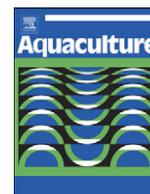




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## Comparison of digestive proteinases in three penaeids

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### ABSTRACT

Recently, several groups of researchers reported that besides serine proteinases, other classes of proteinases may be involved in crustacean's food protein digestion, including cysteine and aspartic proteinases. In this paper, a comparative study of the class and type of digestive proteinases of whiteleg shrimp *Penaeus vannamei*, blue shrimp *Penaeus stylirostris*, and yellowleg shrimp *Penaeus californiensis* is addressed, along with some operational characteristics. The substrate-SDS-PAGE zymogram of the three species showed varieties of different proteinases that were species-specific and proteinase composition that may be used for species identification or population studies. In the three species, trypsin and chymotrypsin were present as isoenzymes. Some active bands were active at acid pH and were partially inhibited by pepstatin A and based on bibliography information they are digestive enzymes. The involvement of these enzymes in food protein digestion is discussed and compared with digestive enzymes present in other decapod species. This and additional information expand the knowledge about enzyme food protein digestion in crustaceans, making clear that digestion process is more complex than previously alleged and if common patrons exist, there are some individualities. This information opens a line of research trying to fully understand the mechanism of protein digestion.

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### 1. Introduction

With the advent of genomic era, nutrition and food sciences are moving to individualize or personalize food and nutrition, and ultimately health by tailoring the food to the individual or population genotype, this merged science is called Nutrigenomics. Into doing so, it is necessary to identify what genome derived molecules are involved in processing food. In this study we describe proteinases of digestive system of three commercial shrimp species, including operational parameters. This information, with others to come, will help to understand if proteinases, as it happens for *Penaeus vannamei*, are present as isoenzymes, enzymes with same specificity for the substrate and reaction but coming from different genes, and can be used as population markers for feeding trials while aquafarming. The research is supported by the knowledge that shrimp nutritional, functional and organoleptic properties are influenced by what is fed and how food or feed is processed by the digestive system. Other important potential contribution is that the precise knowledge of enzymes present in the digestive gland of penaeids may help to design strategies to extend the shelf life of the seafood product.

Hydrolyzing of exogenous protein by the digestive system and endogenous protein for metabolism regulation and reuse of amino acids in the whole organism is one of the most ancient metabolic processes

evolved (Neurath, 1984). A typical genome contains 2–4% of genes encoding for proteinolytic enzymes, from these, serine-proteinases emerged during evolution as the most abundant functionally diverse group. Among these enzymes, only the trypsin family is responsible for a variety of functions, besides digestion, trypsin is involved in blood coagulation, fibrinolysis, development, fertilization, apoptosis and immunity (Di Cera, 2009).

For exogenous protein digestion, decapod crustaceans synthesize zymogens in the digestive gland. Because the digestive gland in crustaceans is holocrine (Ceccaldi, 1997), all the content of B-cells is poured into the lumen of the gland tubules. Digestive secretion flows anteriorly along the lateral grooves of the foregut into the heart chamber, also known as the stomach, where it is mixed in the anterior chamber with food, yielding chyme and facilitating the first step in peptide bond hydrolysis for food protein (Dall et al., 1990).

The most common proteinases in the digestive system of decapods so far characterized are the serine proteinases trypsin and chymotrypsin (García-Carreño et al., 1994; Sainz et al., 2005; Titani et al., 1983; Tsai et al., 1991). These enzymes have their highest activities under neutral or slightly alkaline conditions, ca pH 8.0. Synthesis of non-serine proteinases, as prevailing in vertebrates, has proved contradictory in invertebrates such as crustaceans (Gildberg, 1988) and was eventually neglected. Contrary to expectations, we found proteinases with activity at acid pH appearing in the gastric fluid of several decapods (Celis Guerrero, 2003; Navarrete del Toro et al., 2006) and in mollusks (García-Carreño et al., 2003). Such enzymes have their highest activity around pH 3. These activities were inhibited, in varying degrees, by pepstatin A, which

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indicates a high share of aspartic proteinases, mostly cathepsin (Navarrete del Toro et al., 2006; Rojo et al., 2010). Recently, we demonstrated, by means of mass mapping, N-terminal, and full-length cDNA sequencing, that proteinases with maximum activity at acid pH are cathepsin Ds in lobsters *Homarus americanus* and *Homarus gammarus* (Rojo et al., 2010). Zymogram at pH 3.0 of European lobster, *H. gammarus* showed a band of activity, with the apparent molecular weight of 32 kDa. Such band of activity was heavily inhibited by pepstatin A (Navarrete del Toro et al., 2006). Recently, Chisty et al. (2009) also reported acid and alkaline activity in *Macrobrachium rosenbergii* postlarvae. They found 3 peaks of activity at pH 3, 6, and 9, but they identified only serine- and metallo-class proteinases using specific inhibitors in S-SDS-PAGE at pH 9.0. They did not identify the proteinases working at acid pH.

Serine proteinases show their highest activity at neutral or mildly alkaline pH, while other proteinases, such as aspartic or cysteine proteinases are active under slightly acidic conditions, pH 4.7–6.0, that prevail in the cardiac chamber of the stomach in most crustaceans (Vonk and Western, 1984). The reason for pH of the gastric fluid is uncertain. However, early researchers suggested that it is caused by the presence of acid salts, such as mono-disodium phosphate, rather than the release of free acids, like in a mammal stomach. The pH of the cardiac chamber, along with the amount of proteinase activity found in several crustaceans including crabs, lobsters and shrimps (Navarrete del Toro et al., 2006), lead us to propose that acid proteinases need to be active at the conditions in the cardiac chamber if they are involved in extra-cellular digestion of food.

In this study, we analyze the presence of alkaline and acid proteinases and compared them among three ecologically and economically important penaeids along the Pacific coast of Mexico. By using non-reduced electrophoresis (substrate-SDS-PAGE), we made simultaneous comparisons of crude extracts and determined the molecular masses of the enzymes. Also, the effects of inhibitors and pH were evaluated by using this technique to assign the class or type of proteinase. The research was launched by the knowledge that shrimp nutritional, functional and organoleptic properties are influenced by what is fed and how feed is processed by the digestive system and the fact that current knowledge about the classes of proteinases involved in crustaceans is rapidly evolving.

## 2. Materials and methods

### 2.1. Reagents

Bovine serum albumin (BSA), bovine hemoglobin, casein from bovine milk, glycine, trizma base, pepstatin A, phenylmethylsulfonyl fluoride (PMSF), tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E-64), trichloroacetic acid (TCA), succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (SAPNA), N-benzoyl-D-L-arginine p-nitroanilide hydrochloride (BAPNA), acrylamide, Coomassie brilliant blue G-250, and R-250, ammonium persulfate (APS) were purchased from Sigma-Aldrich. Low molecular weight standards were purchased from Amersham Biosciences.

### 2.2. Specimens

Yellowleg shrimp *Penaeus californiensis* Holmes, 1900 (Syn: *Farfantepenaeus californiensis*), and whiteleg shrimp *Penaeus vannamei* Boone, 1931 (Syn: *Litopenaeus vannamei*) were provided by CIBNOR aquafarming facilities. Blue shrimp *Penaeus stylirostris* Stimpson, 1874 (Syn: *Litopenaeus stylirostris*) was provided by the commercial farm "Acuacultores de la Peninsula" in La Paz, B.C.S., Mexico. All adult animals weighing 20 g average were collected directly from the pond. Specimens of the three species were acclimatized in an aquarium for two days in the absence of food to get them in basal condition before anatomizing the digestive gland.

### 2.3. Enzyme preparation

The digestive gland of 200 specimens of each species were excised, pooled, and stored in a beaker in an ice bath, weighed, and homogenized twice with cold, distilled water (1:3 w/v) for 20 s each time, using a kitchen blender operating at low speed. The homogenate was centrifuged for 30 min at 4 °C and 10,000 g to eliminate tissue debris and lipids. The supernatant containing the digestive enzyme proteins, was evaluated (Bradford, 1976), using BSA as the protein standard. Enzyme activity was evaluated according to García-Carreño and Haard (1993). The supernatant was freeze-dried and the powder was maintained at –20 °C for further analysis.

### 2.4. Enzyme activity assays

Acid enzyme activity was measured, as described by Celis-Guerrero et al. (2004), using 0.5% (w/v) bovine hemoglobin in 0.2 M glycine·HCl at pH 4.0 as the substrate. For alkaline activity, 0.5% w/v casein in 50 mM TRIS·HCl at pH 8.0 was used as the substrate. An enzyme preparation of 10 µl was mixed with 1 ml of substrate solution in a 1.7-ml micro-centrifuge tube and incubated for 30 min at 25 °C. In acid and alkaline assays, the reaction was stopped by adding 500 µl 20% (w/v) trichloroacetic acid (TCA) and cooling on ice for 10 min. The undigested substrate precipitate was separated by centrifugation for 5 min at 10,000 g. The absorbance of the supernatant was measured at 280 nm against distilled water. For blanks, TCA was added before the substrate. Enzyme activity was expressed as change of absorbance per min per mg protein (activity units =  $\Delta \text{Abs}_{280} \text{ m}^{-1} \text{ mg}^{-1} \text{ protein}$ ). Assays were run in triplicate. Trypsin and chymotrypsin activities were measured as described in García-Carreño et al. (1994), using synthetic substrates. For trypsin activity 5 µl of enzyme solution was assayed with 1 mM BAPNA as substrate in 50 mM Tris-HCl containing 20 mM CaCl<sub>2</sub> at 30 °C, the reaction was monitored at 410 nm for 3 min within intervals of 30 s. For chymotrypsin activity 0.1 SAPNA as substrate in 50 mM Tris-HCl containing 20 mM CaCl<sub>2</sub>, the reaction was monitored at 410 nm for 3 min within intervals of 30 s at 30 °C. Specific activity for trypsin and chymotrypsin was calculated with the algorithm: activity units  $\text{mg}^{-1} = ((\text{Abs } 410 \text{ nm}/\text{min}) \cdot 1000 \cdot \text{vol. of reaction mixture})/8800 \times \text{mg of enzyme}$ , where 8800 is the extinction coefficient of paranitroaniline. One unit of enzyme activity was expressed as 1 µmol of p-nitroaniline released/min.

### 2.5. The effect of pH and temperature on proteinase activity

To assess the effect of pH on enzyme activity, an assay was done using different pH, as described by García-Carreño and Haard (1994), using universal buffer (Stauffer, 1989). For the pH range 2–5, 0.5% w/v bovine hemoglobin was used as the substrate; for the pH range 6–10, 0.5% w/v casein were used as the substrate.

To determine the effect of temperature on enzyme activity, an assay was performed at different temperatures, according to García-Carreño and Haard (1994). Triplicate aliquots of each enzyme preparation from each species were incubated at selected temperatures at intervals between 10 and 70 °C.

### 2.6. Identification of proteinases by specific inhibition

The percentage of inhibition or the remaining activity after treating the enzyme preparations with class and type-specific inhibitors was measured (García-Carreño, 1996). A 10 µl enzyme preparation was mixed with 5 µl different inhibitors and 500 µl glycine·HCl at pH 4.0 or 50 mM TRIS·HCl at pH 8.0. For aspartic proteinases, including pepsin, cathepsin E, and cathepsin D, 29 mM pepstatin A in DMSO was used. For cysteine proteinases, including cathepsin B and cathepsin H, 5.8 mM E-64 in 1:1 ethanol:water was used. For trypsin and chymotrypsin, 10 mM TLCK in 1 mM HCl or 100 mM PMSF in ethanol were used as

inhibitors. After one hour incubation at 25 °C, 500 µl 1% w/v bovine hemoglobin in glycine·HCl at pH 4.0 or 1% bovine casein in 50 mM Tris·HCl at pH 8.0 were used as the substrates. Reaction mixtures were treated the same way as the enzyme activity assays. The percentage of inhibition was reported with the enzyme activity, in the absence of the inhibitor, as 100%. Blanks contained the inhibitor solvent with no inhibitor. Assays were run in triplicate.

### 2.7. Electrophoresis

Proteins present in the enzyme preparation were separated by electrophoresis on 12% polyacrylamide gels (Laemmli, 1970). When needed, samples were not treated with β-mercaptoethanol nor boiled to keep the enzyme activity. Aliquots of enzyme preparations containing 20 µg of protein were diluted in loading buffer 1:1 v/v; and 3 µl low molecular mass protein standards were loaded into individual wells of the gel. The electrophoresis was run at 15 mA per gel in an electrophoresis unit (model SE-260, Hoefer, San Francisco) in a refrigerated, circulating bath at 4 °C. Once the front dye reached the bottom of the gel, electrophoresis was stopped. Gel was stained with an aqueous solution containing 0.5% w/v Coomassie blue R-250 in 40% v/v methanol, 7% v/v acetic acid. The excess dye in the gel was washed with aqueous solution containing 40% v/v methanol and 7% acetic acid.

### 2.8. Substrate-SDS-electrophoresis

Evaluation of proteins with proteinolytic activity was performed by substrate-SDS-electrophoresis (García-Carreño et al., 1993). Aliquots of enzyme preparations containing 10 µg protein for alkaline activity and 20 µg protein for acid activity were treated as described in the previous subsection. Once electrophoresis was stopped, the gel was processed as follows: for activity, each independent gel was transferred to a tray and washed with distilled water, the pH of the washing solution was continuously monitored with a pH meter and 100 mM HCl or NaOH solution was added until the desired pH was reached. Then gels were soaked in 60 ml of 3% w/v casein in universal buffer at pH from 6.0 to 9.0 as the substrate and maintained in an ice bath under slow circular agitation to allow substrate to diffuse into the gel. After 30 min, the temperature was raised to 25 ± 1 °C and maintained for 90 min under slow circular agitation. Then, each gel was washed thoroughly with distilled water to eliminate excess substrate and immediately stained and washed, as described in Section 2.7. Clear bands over blue background show proteinolytic activity. For acid activity, once electrophoresis was stopped, the gel was transferred to a tray and washed several times with distilled water. The pH of the washing solution was continuously monitored with a pH meter and 100 mM HCl solution was added until the desired pH was reached, which takes ~25 min. Then, 60 ml substrate, consisting of 0.25% w/v hemoglobin in universal buffer at the appropriate pH from 2.0 to 5.0, was poured into the tray and incubated, stained, and destained, as described above.

### 2.9. Substrate-SDS-electrophoresis with specific inhibitors

For class or type characterization of proteinases present in the enzyme preparation, each enzyme aliquot was individually mixed with specific inhibitors. After incubation for 1 h, the enzyme and inhibitor mixture was diluted with loading buffer (1:1 v/v) and placed into the gel. For controls, enzyme samples were incubated with distilled water instead of inhibiting solution. Previously, we demonstrated that the solvent of the inhibitors does not interfere with enzyme activity (García-Carreño and Haard, 1993).

### 2.10. Digestibility in vitro

The digestibility in vitro (hydrolysis) of casein (SIGMA C7078, used as a reference protein), commercial fishmeal (64% protein) and shrimp commercial-feed (38% protein) was performed using enzymatic extracts from *P. vannamei* and *P. californiensis* in a 718 Stat Titrimeter (Metrohm Ion Analysis, Herisau, Switzerland). The degree of hydrolysis (DH%) was assayed as described in Lemos et al. (2004). Briefly: triplicate samples containing 0.08 g protein were weighed and transferred to a jacketed hydrolysis vessel and stirred with the appropriate volume of distilled water to reach 9.6 g total weight. The mixture was continuously adjusted to pH 8.0 at 28 °C using a 4 g l<sup>-1</sup> NaOH solution to facilitate complete solubilization of protein. Once the reaction mixture was stable at pH 8.0 hydrolysis was started by adding the equivalent to 4 units of enzyme activity plus enough distilled water to reach 10 g of total reaction mix. The DH% was calculated as follows  $DH\% = (B \times N_B \times 1.5 \times 12.5 \times 0.125) \times 100$  where *B* is the volume of NaOH used to maintain the pH at pH 8.0, *N<sub>B</sub>* is the normality of the base, 1.5 is 1/α (pK for amino groups at 25 °C), 12.5 is 1/Mp (mass of protein in the reaction) and 0.125 is 1/htot {content of peptide bonds in meq/g (N × f<sub>N</sub>)}.

### 2.11. Statistics

ANOVA was used to compare trypsin, chymotrypsin, and total proteolytic activity among species after testing of normality and homogeneity of variances of the data. Significant differences were considered at *P* ≤ 0.05. When significant differences were found, a Tukey multiple range test was performed to locate these differences (Statgraphics Plus, v. 5.1).

## 3. Results

### 3.1. Samples

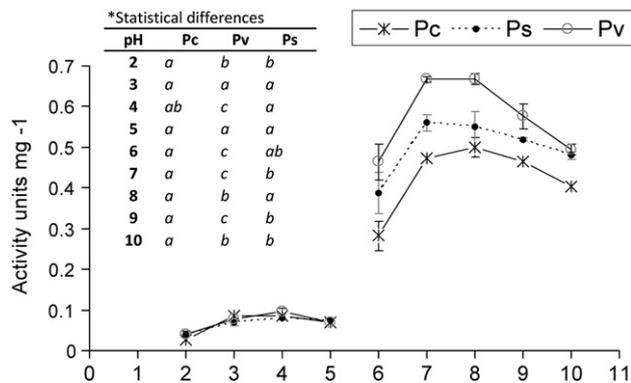
The digestive gland preparations of the three species had similar protein content in mg ml<sup>-1</sup>, *P. vannamei* 5.1 ± 0.241, *P. californiensis* 5.3 ± 0.139, and *P. stylirostris* 5.2 ± 0.276. Freeze-dried samples showed somewhat lower activity than fresh ones, but were not significant enough to overcome the advantage of storage for long periods.

### 3.2. The effect of pH on proteinase activity

Paramount for this study, the three species showed proteinase activity at both alkaline and acid pHs; the three species had maximum activity at pH 8.0 and 4.0. Acid and alkaline conditions are not strictly comparable because alkaline activity was evaluated using casein as the substrate and acid activity was evaluated with hemoglobin. Activity was far lower at acid pH, but not negligible (Fig. 1). It is noteworthy that *P. vannamei* had the highest activity at alkaline pH, followed by *P. stylirostris*, then *P. californiensis*. For acid proteinolytic activity, there were no differences among the species.

Fig. 2 shows the composition of proteins and proteinases with activity at pH 8.0 in the three digestive gland preparations from the three species of shrimp. The figure shows that the composition of proteins and proteinases is species-specific. For *P. vannamei* (Lane 5), the three bands around 20 kDa apparent molecular mass match to isotrypsins C, B, and A, according to the nomenclature of Sainz et al. (2004a). All the species showed several bands of proteinase activity using substrate SDS-PAGE.

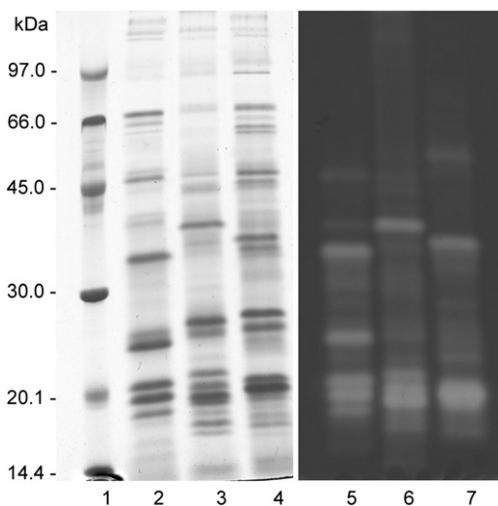
Fig. 3 shows total, trypsin, and chymotrypsin activity in the midgut gland of the three shrimp species. Total activity was assessed with casein as the substrate at pH 8.0 and reported as total caseinolytic activity (activity units mg<sup>-1</sup>). Trypsin and chymotrypsin activities were assessed by using specific substrates bearing an amide scissile bond formed by the specific amino acid residue in the carboxylic side;



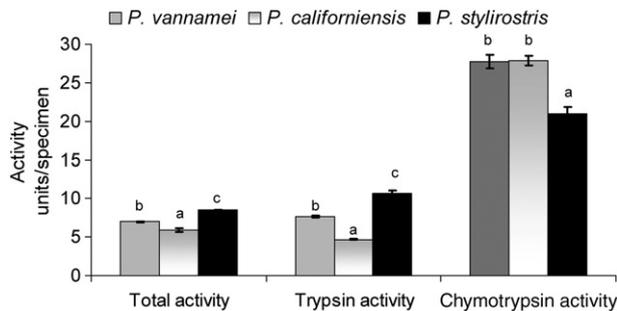
**Fig. 1.** The effect of pH on the enzyme activity of the three penaeid species. (Left) The activity at acid pHs using hemoglobin as the substrate, (right) the activity at alkaline pHs using casein as the substrate. Hemoglobin for pH 2–5 and casein for pH 6–10 were used as substrates. Assays were performed in triplicate at 25 °C.

the activity was reported as amidase activity for BAPNA and SAPNA. *P. californiensis* had the lowest caseinolytic and trypsin activities, while, *P. stylirostris* had the lowest chymotrypsin activity. These data support each other with what is shown in Fig. 1.

To assess the class or type of proteinase catalyzing at alkaline pH in the enzyme preparations, we incubated the preparations with TLCK and PMSF, inhibitors that reduce trypsin and serine proteinase activities and then separated proteins by SDS-PAGE and tested activity at pH 8.0. Fig. 4 shows that, for the three specimens, TLCK reduced the intensity of those bands for paralogous enzymes: isotrypsins; MW of 19.9, 18.4 and 17.2 kDa in *P. vannamei* (Lanes 2–4), of 21.2, 19.6 and 18.6 kDa in *P. californiensis*, and of 19.3, 18.6 and 15.9 kDa in *P. stylirostris*. PMSF also reduced the intensity of some bands present in the three specimens, demonstrating the presence of paralogous isochymotrypsins. At least two isochymotrypsins were found in each species: *P. vannamei* 35.6 and 24.1 kDa, *P. californiensis* 41.5 and 22.6 kDa, and *P. stylirostris* 38.4 and 28.1 kDa. The three species synthesize proteinases following a common general pattern with some individualities. Differences were enough to distinguish among them and confirming they are paralogous enzymes that diverged by gene duplication and are related by speciation.



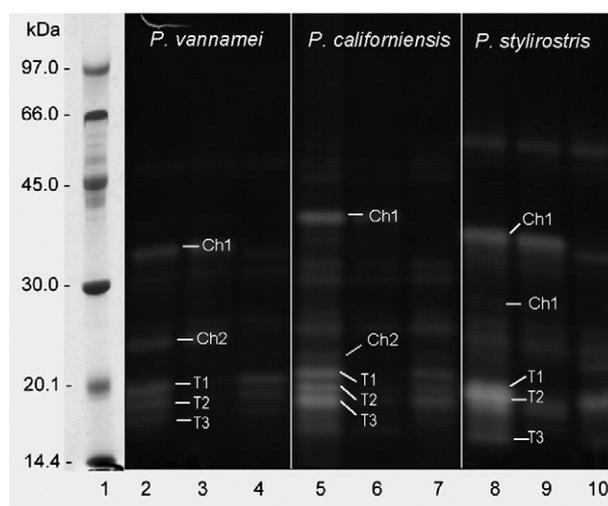
**Fig. 2.** Composition of proteins in the three enzyme preparations after separation by electrophoresis and proteinase activity of the same enzyme preparations using casein as the substrate at pH 8.0. (1) MWM; (2) protein from *P. vannamei*; (3) protein from *P. californiensis*; (4) protein from *P. stylirostris*; (5) zymogram of *P. vannamei*; (6) zymogram of *P. californiensis*; (7) zymogram of *P. stylirostris*. 3% casein was used as substrate.



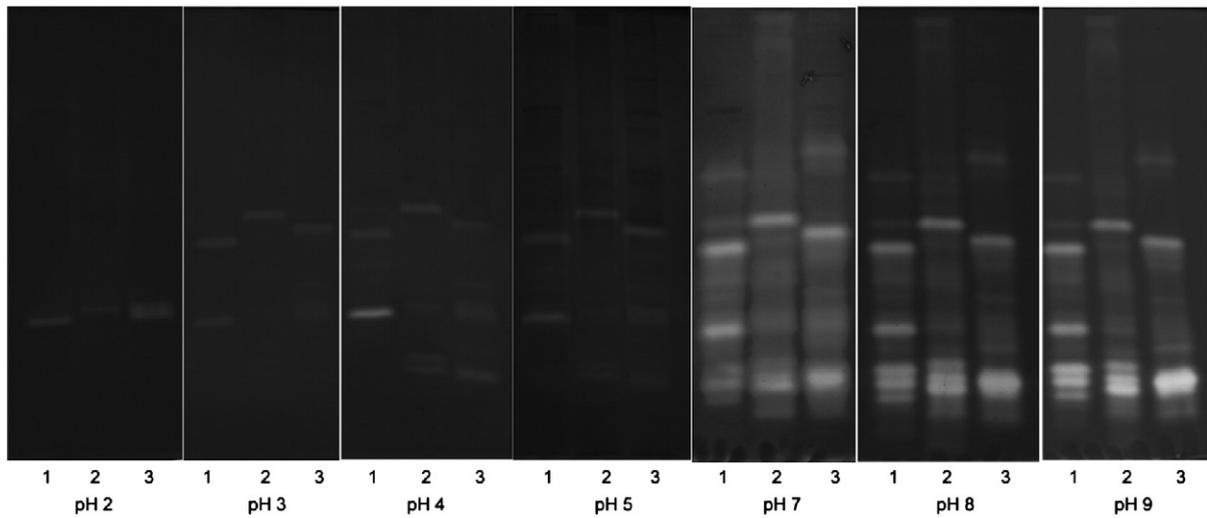
**Fig. 3.** Activity of total proteinases in the three enzyme preparations using casein as the substrate, trypsin using BAPNA as the substrate, and chymotrypsin using SAPNA as the substrate at pH 8.0. Different letters in the same enzyme indicate significant differences ( $P < 0.05$ ). Trypsin and chymotrypsin are expressed as  $U\ mg^{-1}$  of amidase activity. Total activity is expressed as  $U\ mg^{-1}$  of caseinolytic activity.

In addition to the advantages already shown previously for the substrate SDS-PAGE technique (García-Carreño et al., 1993), it reveals bands of activity at any desired pH. Fig. 5 shows the composition of proteinases found by substrate-SDS-PAGE in the three species as revealed at pH ranging from 2.0 to 9.0. We are familiar with zymograms at alkaline pH and can identify isotrypsins and isochymotrypsins. What is original is the presence of a few bands of activity at acid pH. Most of the bands at acid pH coincide with those at pH 7, 8, or 9. These results agree with those in Fig. 1.

Fig. 6 shows the in vitro residual activity of the enzyme preparations from the three species as affected by the reduction in activity by specific inhibitors, pepstatin A and E-64, and pH from 3.0 to 5.0. Porcine pepsin was used as positive control to confirm the effect of pepstatin A (data not shown). As expected at pH 3.0, pepstatin A inhibited all mammalian enzyme activity, validating the assay. The effect of inhibitors on the total proteinase activity was affected by the pH and was species-specific. At pH 4.0, the enzyme preparation from *P. vannamei* was affected by pepstatin A and E-64, with almost no effect on the two other species. At pH 5.0, E-64 inhibited the preparation from *P. vannamei* and produced only a negligible effect on the two other species.

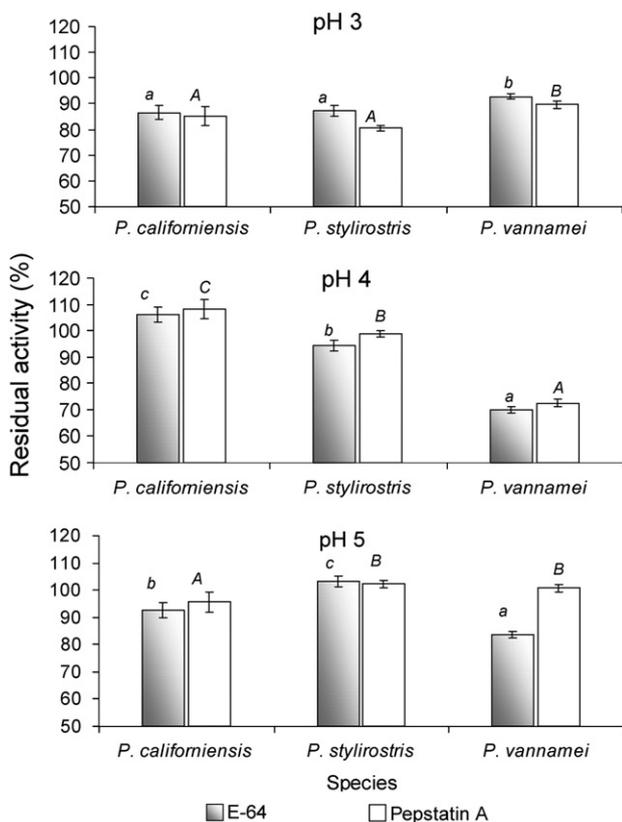


**Fig. 4.** Identification of proteinase with activity at alkaline pHs by specific inhibition on substrate SDS-PAGE and activity revealed at pH 8.0 using 3% of casein as the substrate. Gel incubated with 3% casein in 50 mM Tris-HCl at pH 8.0 for 30 min at 4 °C and 90 min at 26 °C. (1) MW; (2) *P. vannamei* control; (3) *P. vannamei* – TLCK; (4) *P. vannamei* – PMSF; (5) *P. californiensis* control; (6) *P. californiensis* – TLCK; (7) *P. californiensis* – PMSF; (8) *P. stylirostris* control; (9) *P. stylirostris* – TLCK; (10) *P. stylirostris* – PMSF. T = trypsin and Ch = chymotrypsin.

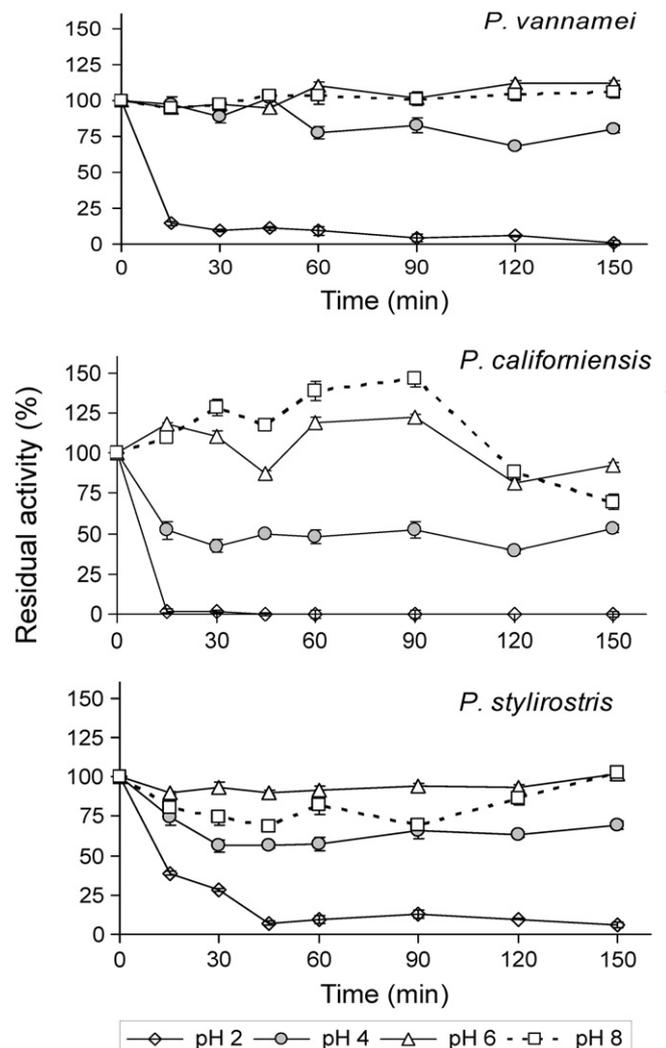


**Fig. 5.** The effect of pH on the composition of proteinases, as detected by substrate SDS-PAGE. Line 1 = *P. vannamei*; 2 = *P. californiensis*; 3 = *P. stylirostris*. Identifying abbreviations as in Fig. 2. For pH 2 to 5 0.25% hemoglobin was used as substrate and for pH 6 to 9, 3% casein was used as substrate.

Fig. 7 shows the effect of pH on the proteinase activity of the three species. For *P. vannamei*, enzyme activity was quite stable at pH 6.0 and 8.0. At pH 4.0, total proteinolytic activity was reduced 30% at 60 min. At pH 2.0, enzyme activity was reduced by 90% at 15 min. In *P. californiensis*, functioning is more dramatic at pH 4.0, for which diminished activity started at 30 min with a 50% loss; at pH 2, the enzyme showed no activity after 15 min incubation. For *P. stylirostris*, the effect is similar. The study of stability of proteinases in the three



**Fig. 6.** Residual activity from enzymes exposed to protease inhibitors E-64 and pepstatin. The effect of inhibitors on shrimp enzymes is compared at each pH value (3.0, 4.0 and 5.0). Different lowercase letters (E-64) at each pH means significant differences at  $P < 0.05$ . Different capital letters (pepstatin) at each pH means significant differences at  $P < 0.05$ .



**Fig. 7.** The effect of incubation time on proteinase activity at different pH values. Enzyme preparations were incubated at the indicated pH and samples were taken in intervals and then activity assessed.

species at varying pHs shows that in spite of peak activity at acid pH occurring at 4.0, this was not the best pH for keeping the preparations. Enzyme activity was severely reduced in *P. stylirostris*, more than in the other two species, with *P. vannamei* the less affected. It didn't escape our attention that an increase in activity after 60 min at alkaline pHs for *P. californiensis* enzyme extract. Because we are assessing crude extracts, no hypothesis can be advanced to explain the phenomena; further research on pure enzymes is required.

Fig. 8 shows the effect of temperature on the proteinase activity at pH 4. A similar pattern was obtained for the three species. Proteinase activity at pH 4.0 is negligible at up to 20 °C. Some activity is shown at 30 °C. The highest activity, namely the optimum activity, at the conditions of the assay was 60 °C. At 70 °C all the enzyme preparations diminished about 50% of the activity at 60 °C. What is notable is that the three species had the same optimum temperature and after 60 °C, the three enzyme activities greatly declined. Again highest activity was found in the *P. vannamei* preparation.

The in vitro hydrolysis of casein was higher when *P. vannamei* enzymes were used, but when a commercial feed was hydrolyzed, the highest DH% was observed using *P. californiensis* enzymes. Both enzymes performed equal hydrolyzing commercial fishmeal (Table 1).

#### 4. Discussion

This research was aimed at knowing the composition of proteinases in the digestive gland of three penaeids in their baseline physiology, mostly to know and compare the full set of proteinases regardless of the activity assessed in vitro using synthetic specific substrates. Anyhow, an unexpected result of the comparison of the proteinase activity of the three species is that *P. vannamei* had the highest one, with chymotrypsin activity as the most conspicuous. We don't know the reason for this. We biologists never expect an effect being the result of only one cause, like food composition or so; biological phenomenon have multivariate causes, however, coincidentally the result agrees with the highest DH% for casein (Table 1), and with the fastest growth of the organism and the reason is that it is the choice species for aquafarming in northwest México, and Latin-American and Asian countries. Here we only give empiric results and provided there is no conceptual basis to elaborate further, additional research is needed to assess the topic and give credit to the amount of proteinase activity found in the species studies.

The three species synthesize proteinases following a common general pattern with some individualities. Differences were enough to distinguish among them and confirming they are paralogous enzymes that diverged by gene duplication and are related by speciation. Besides serine-proteinases, cysteine- and aspartic-proteinases seem to be involved in food protein digestion in decapod crustaceans (Navarrete del Toro et al., 2006; Teschke and Saborowski, 2005). For example, in the gastric juice of the American lobster *H. americanus*, cysteine-proteinase accounts for 80% of the proteolytic activity (Laycock et al., 1989, 1991),

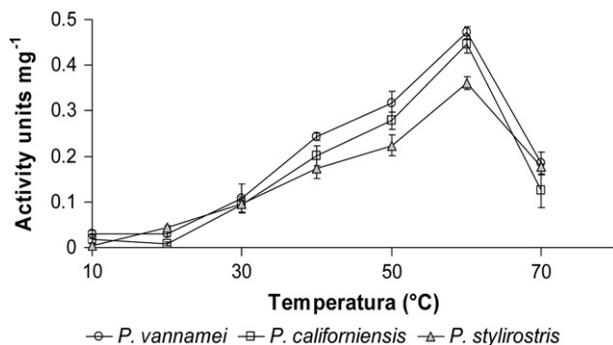


Fig. 8. The effect of temperature on proteinase activity at pH 4.0. Enzyme preparations were incubated at the indicated temperature and samples were taken in intervals and then activity assessed. Thermoresistance.

Table 1

Degree of hydrolysis of different substrates using enzymes from *Penaeus vannamei* and *Penaeus californiensis*.

Source of enzymes	Casein DH%	Commercial feed DH%	Commercial fishmeal DH%
<i>P. californiensis</i>	15.97 ± 0.28 <sup>a</sup>	5.81 ± 0.31 <sup>b</sup>	3.56 ± 0.12 <sup>a</sup>
<i>P. vannamei</i>	17.30 ± 0.51 <sup>b</sup>	5.20 ± 0.29 <sup>a</sup>	3.51 ± 0.26 <sup>a</sup>

<sup>a</sup> Different letters in the same column means significant differences at  $P < 0.05$ .

<sup>b</sup> Data correspond to the average value of four assays ± SD.

and in North Sea shrimp *Crangon* sp. for 70% (Teschke and Saborowski, 2005). Recently, in clawed lobsters *H. americanus* and *H. gammarus*, our group found active proteins and identified them as aspartic proteinase by specific inhibition with pepstatin A and confirmed been cathepsin D by mass mapping, N-terminal, and full-length cDNA sequencing. Both lobster species transcribed two cathepsin D mRNAs: cathepsin D1 and cathepsin D2. Cathepsin D1 mRNA was detected only in the midgut gland, suggesting its function as a digestive enzyme (Rojo et al., 2010). Finally confirming that proteinases different from serine proteinases are involved in digestion of food protein. But science is not about findings, but explaining mechanisms about how it happens. We are in that quest.

In the three species in this study, we found that some digestive proteinases have activity at both acid and alkaline pH. The pH range 4.7–6.0 prevails in the cardiac chamber of the stomach in most crustaceans (Vonk and Western, 1984), making possible that digestive enzymes with different catalytic properties can contribute to the digestion of food components, and most probably more efficient, mainly because the pH of the gastric chamber may change as a result of the hydrolysis of the food protein, as reported by Vonk and Western (1984) by ionization of carboxylic moiety of amino acid residues freed during hydrolysis. The pH of gastric juice of several crustaceans is: *Panulirus interruptus* 6.0, *H. gammarus* 4.7, *Cancer pagurus* 5.8, and *Callinectes arcuatus* 6.1 (Navarrete del Toro et al., 2006). The pH of the digestive gland of the three species in this study, as measured with a contact pH probe, is  $\sim 5.7 \pm 0.1$ . So, a set of proteinases having activity at pH from 4.0 to 8.0 may be advantageous for an assortment of food and the conditions in the digestive system. Using a buffer at pH 7.5–8.0 for the in vitro assessment of activity is more operational than physiological. In our study, it is evident that proteinases are not working at the optimal pH during physiological conditions. This is not surprising because the same pattern happens at optimal temperature of the enzymes that were studied.

Regarding the class and type of proteinases found in the digestive gland of the three species, trypsin and chymotrypsin are present and have some combination of substrate specificities; possessing such combination of proteinases an organism may hydrolyze food proteins in a way that allows most of the essential amino acids to be released. In nature all organisms have specific niches in which food source may be different in quality and quantity. That is why different species have enzymatic packages in their digestive system to take advantage of all the food items that they can eat (Johnston and Freeman, 2005). The existence of different proteinases and isoenzymes with different catalytic properties, in the same and in related species, may constitute an adaptive strategy to the success of the species in their different habitats (Baani and Liesack, 2008). Food sources can be different along the year and from one place to another, even in their life cycles. The fact that inhibitors of cysteine- and aspartic proteinases like E-64 and pepstatin A reduce the measured activity of the enzyme preparations supports the presence of enzymes belonging to those classes. It has not escaped to our attention that the pH in which enzymes and inhibitors collide affects the degree of reduction of activity, vg. At pH 4 *P. vannamei* enzyme preparation is more affected than at more acidic and more alkaline pHs. We speculate this depends on the structure of the enzymes and remains to be addressed.

This is an important issue regarding feed qualities while aquafarmed shrimp species have species-specific enzyme sets and their

digestion capabilities are dependent on it, then the feeds should be species specific. Also different responses to protease inhibitors on the proteases from several shrimp species had been demonstrated (De Albuquerque-Cavalcanti et al., 2002) including *P. vannamei*, *P. stylirostris* and *P. californiensis*. Also in this work we demonstrated that shrimp enzymes from *P. vannamei* and *P. stylirostris* yield different DH% for protein substrates like casein, a reference protein, and commercial ingredient and feed. Casein protein is not a natural feed item for shrimp, but the different degree of hydrolysis on this substrate demonstrates different enzyme capabilities. Regarding the commercial feed, it is widely documented that different ingredients are included in commercial feeds; some of them are plant in origin and may contain protease inhibitors (Lemos et al., 2004). Different DH% on the same substrate denotes different performance of enzymes. These results support the idea that better feeds could be designed attending the different digestion capabilities of the digestive enzymes of the different aqua-farmed shrimp species.

Here, we demonstrated by using specific inhibitions and substrates, that isotrypsins as well as isochymotrypsins are present in the three species. The presence of serine proteinases, such as trypsin and chymotrypsin in decapods, is well-documented (García-Carreño et al., 1994; Hernández-Cortés et al., 1997; Sainz et al., 2004a; Tsai et al., 1991). Previously, it was demonstrated that *P. vannamei* specimens fed with different feeds synthesized the same enzymatic set regardless of the type of food ingested (Sainz et al., 2005). In *P. vannamei*, three isotrypsins A, B, and C are synthesized in the midgut gland (Sainz et al., 2004b) yielding three phenotypes, 1, 2, and 3, derived from four genotypes: AA, AB, BB, and CC (Sainz et al., 2005). Isotrypsin A, B, and C genes rest on two loci: isotrypsins C lies on the homozygous locus  $\beta$ , while isotrypsins A and B lie on the heterozygous locus  $\alpha$ . From offspring from 20 families analyzed, we know that isotrypsins A and B, from locus  $\alpha$ , are segregated observing Mendelian rules yielding three phenotypes in *P. vannamei* and that external stimuli do not affect the phenotype; for further reading see Sainz et al. (2005). For the other two shrimp species, such research is yet to be addressed; we hypothesize that they will have similar characteristics in spite of their different molecular masses.

Question arises concerning why the three species show activity at acid pH. What proteins are those with activity at pH 4.0? And more important for our research topic, are those proteinases contributing to digestion of food protein? Results are not clear, but what we do know is that crustaceans can have proteinases belonging to the cysteine and aspartic classes (Navarrete del Toro et al., 2006; Teschke and Saborowski, 2005). Our paper dealt only with showing the fact of their presence. Ongoing research should expand on this subject.

We hypothesize that bands of activity at acid pH not belonging to the aspartic proteinase class may be serine proteinases that happen to be at some extent affected by pepstatin A and have some activity at acid pH, as reported for chymotrypsin in *P. vannamei* (Hernández-Cortés et al., 1997). Using SAPNA as the substrate at pH as low as 5.0, pure chymotrypsin showed activity. Also, the enzyme remained active at pH 5 for 40 min. For *P. californiensis*, we already know that at least two chymotrypsins are active at pH as low as 4, indicating, that some crustaceans possess digestive proteinases different from class serino; like *H. gammarus* while others have serine proteinases that are active at a wide rank of pH.

This paper demonstrated that the shrimp species have proteinase activity at basic and acid pH and are inhibited by inhibitors specific for class and type. Identifying these enzymes and more importantly, what their function remains to be resolved. A paramount result of this research is that the zymograms and the SDS-PAGE of proteins from enzymatic extracts of the three species are species-specific and may be used for identification of species as done using different proteins from different species (Etienne et al., 2000; Hasnain et al., 2005; Rehbein, 1990) or, by identification of isoenzymes for population studies as done for *P. vannamei* (Sainz et al., 2005). For these shrimp species, enzymes

have activity at the pH of the gastric fluid and the digestive gland. Digestive proteinases keep activity higher than 60% at pH 6–8 for 120 min, longer than the residence period of chyme in the digestive system and good enough for digesting food protein. Regardless of the pH of the gastric fluid and digestive gland, pH is heavily influenced by food. Shrimp seem to thrive under such conditions and possessing enzymes working at varied pH is an advantageous feature. Regarding temperature, proteinases work far from optimum, usually at 60 °C in test tube assays. Besides, digestion is never 100% efficient and much food protein and enzymes remain in the feces (Córdova-Murueta et al., 2004). This seems to have importance in ecology and pond management: protein and enzymes from feces are in the medium and could be ingested by other animals as a protein source and as digestive enzyme supplement.

With this work we are showing that digestion of food protein in penaeids can involve proteinase classes besides serine proteinases, namely aspartic proteinases and opening the study of such enzymes, their catalytic characteristics and eventually the mechanisms for participating in food protein digestion. We are aware that we are far from knowing all about these topics, further research is in course to expand knowledge about the digestion mechanisms involved in crustaceans that in future could give the support for better understanding food protein digestion and all its technological consequences.

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