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Research Article

Partial characterization of digestive proteases in adults of bigclaw river shrimp *Macrobrachium carcinus*

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ABSTRACT. The present research was focused to characterize the digestive proteases in *Macrobrachium carcinus* adults using biochemical and electrophoretic techniques. Our results showed that the alkaline proteolytic activity from males and females did not show significant differences ($P > 0.05$) between them, the optimum pH for digestive proteases is 8, and is very stable to changes in alkaline pH (8 and 10). The optimum temperature for alkaline proteases is 45ºC and is stable from 25 to 45ºC. The activity was totally inhibited with phenylmethylsulfonyl fluoride (PMSF), additionally the inhibition with trypsin soybean inhibitor 1 (SBT1) and tosyl-lysine-methyl ketone (TLCK) indicate high effect over serine proteases. Eight active bands were found using sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) zymogram (range 17.8-94.0 kDa), which were partially inhibited with ovalbumin (Ovo), SBT1, phenanthroline (Phen), tosyl-phenylalanine-methyl ketone (TPCK), TLCK and ethylenediaminetetraacetic acid (EDTA), indicating an omnivorous digestive capacity, which remarks that the mainly alkaline protease in *M. carcinus* hepatopancreas are trypsin like enzymes.

Keywords: *Macrobrachium carcinus*, enzymes, nutrition, proteases, inhibitors.

INTRODUCTION

Nutrition is an essential factor than influence many physiological process in all animal species, in their different stages of life (Abrunhosa & Melo, 2008). In the case of native prawns, such as bigclaw river shrimp *Macrobrachium carcinus*, there is little information about their nutrition, which supports their growth, survival, reproduction, etc. under growing conditions. In this sense, commercial feed for fattening was started in the mid-1980s, mainly for penaeid shrimp and Malaysian shrimp (*Macrobrachium rosenbergii*), to date there are no diets available that are suitable for the metabolism of adult shrimp (Sudaryono *et al*., 1999); therefore, it is essential to study the digestive physiology of these organisms with a view to developing formulations suitable for its culture (Sainz *et al.*, 2004; Abrunhosa & Melo, 2008).

In crustaceans the chemical digestion of proteins begins in the cardiac cavity and continues in the tubules of the hepatopancreas; it is at the level of this gland that the digestion becomes more active, with the participation of enzymes produced by specialized cells producing a greater enzymatic activity, this suggests the importance of this organ in the synthesis and secretion of digestive enzymes (Guillaume & Ceccaldi, 2001), which depend of the amount of enzymes available, number of isoforms, type of digestive proteases (endo and exopeptidases), environmental condition, feeding habit, among others that improve the hydrolysis of nutrients (Carrillo-Farnés *et al*., 2007).

A key determinant of the hydrolysis and efficiency of assimilation of ingested proteins is the presence of the protease enzymes in the digestive tract (Divakaran *et al.*, 2004). Thus, nutrition is focused on the characterization of digestive enzymes as one of the most studied areas in the feeding of crustaceans (Fang & Lee, 1992; García-Carreño *et al*., 1994). For example, in some lobster species (*Panulirus* spp.), studies have focused on proteolytic digestive enzymes such as trypsin, chymotrypsin, carboxypeptidases,

leucine aminopeptidases, elastases, and collagenases (Galgani & Nagayama, 1987; Glass & Stark, 1994, 1995). Studies on white shrimp (*Penaeus vannamei*) show that the enzymatic activity of chymotrypsin increases in relation to weight gain (Gambóa-Delgado *et al*., 2003). Similar activities have also been found for trypsin, carboxypeptidases A and B (Galgani *et al*., 1985), aminopeptidase and dipeptidases, which gives them a high capacity to hydrolyze various foods (Lee *et al*., 1980; Muramatsu & Morita, 1981).

In the case of prawns of the genus *Macrobrachium*, the characterization of the activity of digestive enzymes in the digestive tract indicates the presence of digestive enzymes such as trypsin, aminopeptidases, amylases, chitinases, cellulases, esterases and lipases (Lee *et al*., 1980), where the chemical breakdown of the food by the digestive enzymes is what determines the types of nutrients that will be available for assimilation (Lee *et al*., 1984), where it has been possible to determine the requirement of protein as a function of digestive capacity, particularly in giant river prawn, *M. rosenbergii* (Sagar *et al*., 2009). However, for *M. carcinus*, the information generated in this line of research is null. In this way, the present research work will contribute to the characterization of digestive enzymes in this species, which will serve to the understanding of its digestive physiology.

MATERIALS AND METHODS

Prawn selection and protein extraction

Five adults females and five adult males (153 ± 18 g average weight) were collected in the Usumacinta River in the municipality of Tenosique, Tabasco, Mexico, which were transported to the Tropical Aquaculture Laboratory of the UJAT-DACBIOL. The organisms were placed in two circular plastic tanks of 2000 L each and were fasted for 24 h to empty the gastro-intestinal tract. After that period, the organisms were weighed, measured and sacrificed by cephalic dissection. The hepatopancreas were extracted, weighed, stored in Eppendorf tubes and kept at -80°C for processing. Extraction of the digestive enzymes of the hepato-pancreas was performed individually by disrupting and homogenizing each organ with distilled water (300 mg of hepatopancreas in 1 mL H_2Od), using a tissue disruptor and its subsequent centrifugation at 16,000 g at 4°C for 30 min (Eppendorf 5810R, Rotor F45F45-30-11). The soluble fraction was recovered and the pH was adjusted to 8 with sodium hydroxide (0.1 M) to activate the digestive proteases. Each fraction was dispensed in 1 mL aliquots and stored at -80°C until use.

Alkaline protease activities

Previous to the determination of the protease activities, soluble protein was determined in each hepatopancreatic extract according to Bradford (1976) at 595 nm. All measurements were performed in triplicate using a standard curve of bovine albumin (1 mg mL^{-1}). The determination of alkaline digestive proteases was performed by the method of Kunitz (1947) modified by Walter (1984) using 0.5% casein as a substrate in buffer (50 mmol L^{-1} Tris-HCl, 10 mmol L^{-1} $CaCl_2$ at pH 9). The enzymatic extract was incubated at 37°C for 60 min; the reaction was stopped by the addition of 0.5 mL of trichloroacetic acid (20% TCA). After standing the reaction mixture at 4°C (30 min), it was centrifuged at 16,000 g for 10 min. In the supernatant, a UV/visible spectrophotometer measured the amount of released tyrosine (280 nm). One unit of activity is defined as the amount of enzyme that catalyzes the formation of 1 mg of tyrosine per min. To determine the molar extinction coefficient of tyrosine, a standard straight line with different concentrations of tyrosine (0 to 300 mg mL^{-1}) was performed. All assays were performed in triplicate.

Determination of trypsin activity was performed by the method of Erlanger *et al*. (1961), using 100 mM BAPNA (Na-Benzoyl-DL-Arginine-P-NitroAnilide) solution as substrate, dissolved in dimethylsulfoxide (DMSO) and suspended in 50 mmol L^{-1} Tris-HCl and 10 mmol L^{-1} $CaCl_2$ at pH 8.2 as buffer solution. To initiate the reaction, 990 mL of substrate (37°C) was mixed with 10 mL of the enzymatic extract. After 2 h of linear incubation, the reaction was stopped with the addition of 0.25 mL of 30% acetic acid. The absorbance was then measured at 410 nm using a blank as a control. One unit of activity is 1 µmol of p-nitroanilide released per min using 8800 cm^{-1} M^{-1} molar extinction coefficient (MEC). All assays were performed in triplicate.

Chymotrypsin-like activity was determined at 256 nm by hydrolysis of BTEE (Nbenzoyl-L-tyrosine ethyl ester) at 37°C, according to the methodology described by Ásgeirsson & Bjarnason (1991). The standard method for 1 mL reaction was as follows: to 0.1 mL BTEE and 5 mmol L^{-1} in DMSO was added 0.89 mL of buffer (44.4 mmol L^{-1} Tris-HCl, 55.5 mmol L^{-1} $CaCl_2$, pH 7.8). The reaction was started with the addition of 10 µL enzymatic extract; the reaction was stopped after 4 min. One unit of enzyme activity was defined as 1 µmol of hydrolyzed BTEE per min using as the molar extinction coefficient 964 cm^{-1} M^{-1}. All assays were performed in triplicate.

Leucine-aminopeptidase activity was determined by the method of Maraux *et al*. (1973) using leucine p-nitroanilide (0.1 mmol L^{-1}) as substrate. The substrate was dissolved in 200 µL of dimethyl sulfoxide (DMSO) and loaded with 10 mL of buffer (sodium phosphate 50 mmol L^{-1}, pH 7.2). The reaction was started with l0 µL of test extract in 0.980 mL of substrate, and incubated for 10 min at 37°C. The reaction was stopped with 0.250 mL of 30% acetic acid and read at 410 nm. One

unit of activity is 1 μM p-nitroanilide released per min using as the molar extinction coefficient 8200 cm^{-1} M^{-1}. All assays were performed in triplicate.

Carboxypeptidase A activity was determined by the method of Folk & Schirmer (1963) using as substrate Hippuryl-L-phenylalanine (1 mmol L^{-1}). The substrate was dissolved in 10 mL buffer (25 mmol L^{-1} Tris-HCl, 0.5 mol L^{-1} NaCl, pH 7.5). The reaction was started with the addition of 10 μL of enzymatic extract, stopped after 6 min and activity was read at 254 nm. One unit of enzyme activity was defined as 1 μM of hydrolyzed hippuryl-L-phenylalanine per min, using as the molar extinction coefficient 19.4 cm^{-1} M^{-1}. All assays were performed in triplicate.

Units of enzyme activity (U) were plotted as mU mg $protein^{-1}$, mU mg $hepatopancreas^{-1}$ and mU $organism^{-1}$.

Effect of pH on the activity and stability of alkaline proteases

The optimum pH of alkaline protease was determined using buffered casein (1%) as substrate in the universal buffer (Stauffer, 1989) from 2 to 12 pH values. All these assays were performed in triplicate. The effect of pH on the stability of alkaline protease activity was determined by pre-incubating the extracts at different pH (2, 4, 6, 8, 10 and 12) for 30, 60 and 90 min. At 25°C in its substrate and then its activity was measured at pH 9. Residual activity was quantified at regular intervals without pre-incubating control. All assays were performed in triplicate.

Effect of temperature on the activity and stability of alkaline proteases

To determine the influence of temperature on the activity of the alkaline proteases, the enzymatic extracts were incubated in casein (1%), stabilizing them for 10 min at increasing temperatures (25, 35, 45, 55 and 65°C). The reaction was started by the addition of the extract. On the other hand, the influence of temperature on the stability of the alkaline protease activity was determined by pre-incubating the extracts at increasing temperatures (25, 35, 45, 55 and 65°C) for 0, 30, 60 and 90 min. After pre-incubation the activity was determined at regular time intervals, taking samples that were rapidly cooled in an ice bath at 4°C for 5 min. Residual activity was then determined without pre-incubating control. All assays were performed in triplicate.

In vitro enzyme inhibition

The inhibition of digestive enzymes in *M. carcinus* was carried out according to the method described by Dunn (1989). For the characterization of the alkaline protease activity, the extracts were incubated with the inhibitors phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor 1 (SBT1), tosyl-phenylalanine-methyl ketone (TPCK), Tosyl-lysine-methyl ketone (TLCK), phenanthroline (Phen), and ovalbumin (Ovo) and the chelating inactivator ethylenediaminetetraacetic acid (EDTA), for which 500 μL buffer (Tris-HCl 10 mmol L^{-1}, 20 mmol L^{-1} $CaCl_2$, pH 9.0), 20 μL enzyme extract with 20 μL inhibitor. The mixture was preincubated for 60 min at 37°C, then 0.5 mL of casein (1%, pH 9.0) was added to the reaction mixture and incubated for 3 h at 37°C and reaction was stopped adding 0.5 mL of trichloroacetic acid (TCA), the mixture was centrifuged at 16,000 g for 15 min at 4°C and the absorbance read at 280 nm. Activity was expressed as the percent inhibition relative to an activity control without inhibitors (100% activity). All assays were performed in triplicate.

Inhibition by electrophoretic techniques for digestive proteases

The study of proteases was complemented by zymogram using SDS-PAGE electrophoresis and the use of the same inhibitors. The activity of alkaline proteases was studied in order to quantify the number of caseinolytic bands incubated in casein (2%), as well as their molecular mass, in addition to establishing the types of proteases that compose the extracts. For this purpose, the enzymatic extracts of *M. carcinus* (20 μL) were incubated with 5 μL of each of the inhibitors described above for 60 min and then 25 μL of the mixture were taken to perform SDS-PAGE zymogram. The electrophoresis was performed according to Laemmli (1970), and adapted by García-Carreño *et al*. (1993). The electrophoresis was carried out in the Mini PROTEAN® II (Bio-Rad) cell with 2 vertical gel plates of 8×10×0.075 cm. A low-range marker (LRMWM, SIGMA, M-3913) was used to calculate the molecular weights in addition to the Quality One program v. 4.6.5 (Hercules, CA, USA).

Statistical analysis

In order to compare the specific activities of digestive proteases and the effect of the inhibitors, by not complying with the normality (Kolmogorov-Smirnov test) and homoscedasticity (Levine test) postulates, data were analyzed by means of U Mann-Whitney test comparing between sexes. The analysis were performed using a significance value of 0.05 by means of the statistical package Statistica v.7.0.

RESULTS

Characterization of specific activities

The activities of digestive proteases for both females and males did not show significant differences ($P \geq$

0.05) between sex for each of the digestive enzymes studied (Table 1).

Effect of pH on the activity and stability of digestive proteases

The optimum pH level of the alkaline proteases showed a peak at pH 8 with an activity level of 13.65 U mg protein^{-1}, after which it decreased as the pH value increased (Fig. 1a). To test the stability of alkaline digestive proteases, the extracts were subjected to different pre-incubation times at different pH and then their residual activity was evaluated under standard conditions. Alkaline proteases showed a high activity at pHs 8 and 10 where their residual activity is maintained between 75 and 95%, the residual activity of the digestive proteases 2 and 6 showed a decrease after 30 min of pre-incubation, reaching values between 20 and 28%; the digestive enzymes show a drastic reduction from 30 min of pre-incubation to pH 12, decreasing to 22% their residual activity (Fig. 1b).

Effect of temperature on the activity and stability of digestive proteases

The optimal temperature for the activity of the digestive proteases was detected at 45°C reaching its maximum activity with 14.67 U mg protein^{-1} (Fig. 1c). On the other hand, the residual activity of the digestive proteases is highly stable at 45°C, maintaining a value of 92%; in the same sense, at 55ºC, digestive proteases gradually decrease their stability until reaching 66%, while for the rest of the temperatures, the residual activity falls from the 30 min of pre-incubation, maintaining near values to 28% at 90 min of pre-incubation (Fig. 1d).

In vitro enzyme inhibition

Inhibition of alkaline proteases in enzymatic extracts of *M. carcinus* in females and males did not show significant differences between the sexes ($P \geq 0.05$). Trypsin activity was inhibited by 63% for females and 75% for males, using TLCK as an inhibitor; the serine protease activity was inhibited by 91% for females and 87% for males using PMSF, and 85% for females and 89% for males using SBT1; chymotrypsin activity was inhibited by 56% for females and 61% for males when using TPCK; while metal protease activity was inhibited by 85% for females and 95% for males using EDTA, and 75% for females and 83% for males when using phenanthroline; finally, protease activity was inhibited in 62% for females and 75% for males using ovalbumin (Fig. 2).

Inhibition by electrophoretic techniques for digestive proteases

Inhibition of digestive proteases by electrophoretic techniques showed eight bands of proteolytic activity in the uninhibited multienzymatic extract (line 1) showed four bands with a high molecular weight (94.0, 87.0, 83.7 and 81.0 kDa), two of them with medium molecular weight (45.8 and 28.5 kDa respectively) and finally two with low molecular weight (21.5 and 17.8 kDa respectively). With the use of OVO (line 2), only one band (28.3 kDa) was inhibited, using SBT1 and Phen (lines 3 and 6 respectively) the same two bands (45.8 and 28.5 kDa) were inhibited, while using the TPCK (line 5), bands of 45.8 and 94.0 kDa were inhibited; on the other hand, with EDTA and TLCK (lines 6 and 7 respectively) only one band was inhibited (28.5 kDa); finally, the PMSF (line 4) inhibited the total of the bands with proteolytic activity (Fig. 3).

DISCUSSION

To our knowledge, the present work is the first approaching in digestive enzyme characterization in *Macrobrachium carcinus*, an economically valuable species in southern of Mexico, and could provide bases for future studies in formulation for practical diets to

Table 1. Specific digestive proteases activity (average ± SE) in females (♀) and males (♂) adults of *Macrobrachium carcinus.*

Enzyme	Sex	Enzymatic activity units (mU mg protein^{-1})	Enzymatic activity units (mU g hepatopancreas^{-1})	Enzymatic activity units (mU ind^{-1})
Alkaline protease	♀	3.62 ± 0.59	0.059 ± 0.001	0.018 ± 0.000
	♂	4.30 ± 0.21	0.079 ± 0.004	0.023 ± 0.001
Trypsin	♀	$1.33\times10^{-5} \pm 6.86\times10^{-7}$	$2.17\times10^{-7} \pm 1.13\times10^{-8}$	$6.63\times10^{-8} \pm 3.43\times10^{-9}$
	♂	$1.23\times10^{-5} \pm 1.18\times10^{-6}$	$2.24\times10^{-7} \pm 2.17\times10^{-8}$	$6.13\times10^{-8} \pm 5.92\times10^{-9}$
Quimotrypsin	♀	$4.78\times10^{-3} \pm 1.47\times10^{-4}$	$7.85\times10^{-5} \pm 2.41\times10^{-6}$	$2.39\times10^{-5} \pm 7.35\times10^{-7}$
	♂	$7.82\times10^{-3} \pm 3.58\times10^{-4}$	$1.28\times10^{-4} \pm 5.87\times10^{-6}$	$3.91\times10^{-5} \pm 1.79\times10^{-6}$
Carboxypeptidase A	♀	0.97 ± 0.13	$0.016 \pm 2.11\times10^{-3}$	$0.005 \pm 6.43\times10^{-4}$
	♂	0.42 ± 0.05	$0.007 \pm 7.97\times10^{-4}$	$0.002 \pm 2.43\times10^{-4}$
Leucine aminopeptidase	♀	$1.03\times10^{-5} \pm 1.38\times10^{-6}$	$1.69\times10^{-7} \pm 2.26\times10^{-8}$	$5.06\times10^{-8} \pm 6.87\times10^{-9}$
	♂	$1.63\times10^{-5} \pm 4.90\times10^{-7}$	$2.67\times10^{-7} \pm 8.04\times10^{-9}$	$8.13\times10^{-8} \pm 2.45\times10^{-9}$

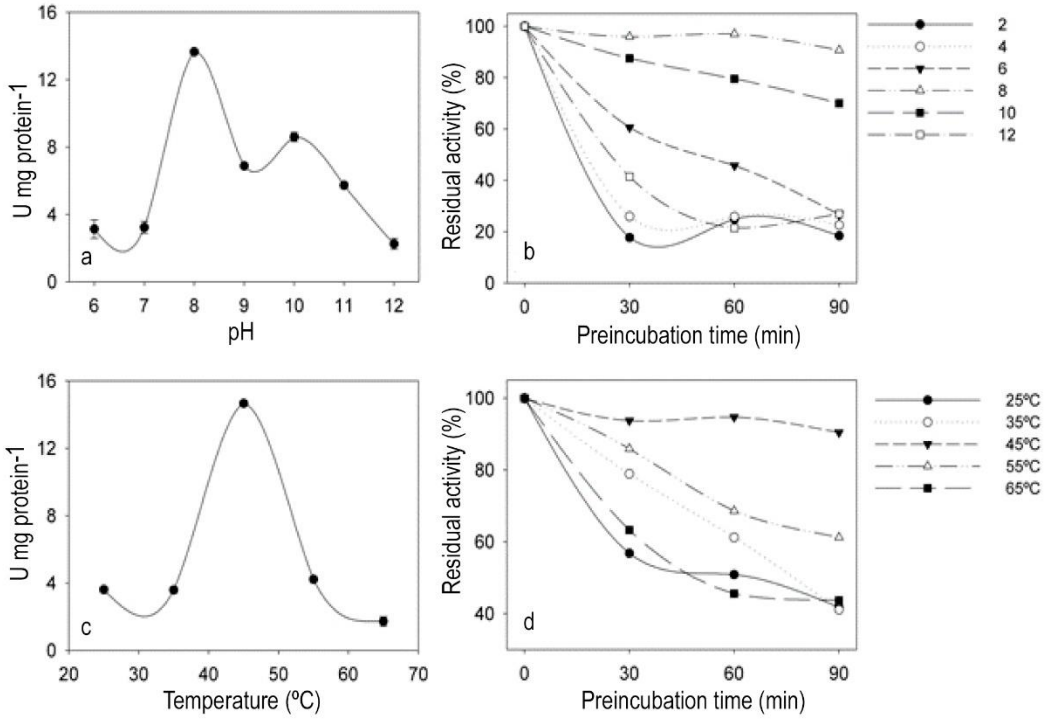

Figure 1. a) pH optimum, b) stability to pH changes, c) optimum temperature, and d) stability to temperature changes on the activity of alkaline proteases in enzymatic extracts of *Macrobrachium carcinus* adults (mean ± SD; n = 5).

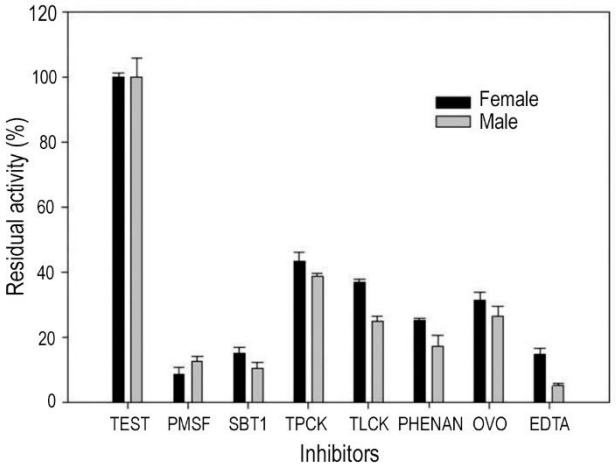

Figure 2. *In vitro* inhibition of digestive proteases in enzymatic extracts in males and females of *Macrobrachium carcinus*.

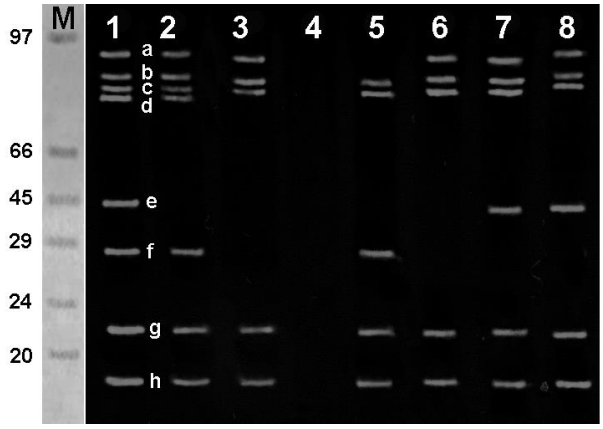

Figure 3. Inhibition zymogram of digestive proteases in the enzymatic extracts of the *Macrobrachium carcinus* adults. Line M) low molecular weight marker: 97 kDa phosphoryl b, 66 kDa serum bovine albumin, 45 kDa egg albumin, 29 kDa carbonic anhydrase, 24 kDa trypsinogen, and 20 kDa triphosphate soybean inhibitor; line 1) enzymatic extract without inhibitor (control); line 2) ovalbumin; line 3) SBT1; line 4) PMSF; line 5) TPCK; line 6) phenanthroline; line 7) EDTA; line 8) TLCK. The letters represent the calculated molecular weights of the isoforms: a) 94.0, b) 87.0, c) 83.6, d) 81.0, e) 45.8, f) 28.5, g) 21.5, h) 17.8 kDa.

sustainable aquaculture development for this species. Our results show that the optimal pH of the digestive proteases of *M. carcinus* is 8 and remains highly stable at alkaline pH. Generally crustaceans proteases show maximum activity at pH ranges 5.5 to 9 (García-Carreño, 1992; García-Carreño *et al*., 1994; Ceccaldi, 1997), where digestive enzymes have been studied for several years due to their importance as auxiliaries in the transformation of food into decapod crustaceans (García-Carreño & Haard, 1993; García-Carreño *et al*., 1997; Hernández-Cortés *et al*., 1997; Lemos *et al*.,

1999; Albuquerque *et al*., 2002; Pongsetkul *et al*., 2016). However, Hernández-Cortés *et al*. (1997) showed that chymotrypsin of *P. vannamei* can be activated in a pH range of 5 to 11. Following the same idea, lepidopteran and coleopteran digestive proteases show their maximum activity in pH ranges of 10 to 11 and 6 to 7 respectively (Purcell *et al*., 1992; Ortego *et al*., 1996). The digestive proteases of the American lobster (*Homarus americanus*) and the Japanese spiny lobster (*Panulirus japonicus*), presents an optimal pH range of 7 to 8 (Brockerhoff *et al*., 1970; Galgani & Nagayama, 1987), while the maximum digestive enzymatic activity of the multienzymatic extracts of *M. carcinus* was obtained in pH ranges from 8 to 10, indicating that proteases of this organism works optimally at alkaline pH's similar to that reported for other species of crustaceans and differs compared to reports in the lobster *Homarus gammarus* and the spiny lobster *Panulirus interruptus* (Glass & Stark, 1994; Celis-Guerrero *et al.*, 2004). It is important to emphasize that in the case of *M. rosenbergii*, the characterization of the digestive proteases varies when compared to *M. carcinus*, since the presence of peaks with acidic proteolytic activity are present (pH 3 and 6), coupled with alkaline digestive proteases, which works at pH 9, and confers to this species a greater ability to hydrolyze the peptide bonds of proteins (Hasan-Chisty *et al*., 2009). Another species of the same genus *M. amazonicus* and *M. lanchesteri* shows a high similarity to *M. carcinus*, where it was determined that the optimum pH was between 8 and 8.5 (Silva-Santos *et al*., 2014; Pongsetkul *et al*., 2016).

On the other hand, the optimal temperature of the activity in the adult digestive proteases of *M. carcinus* was 45°C, which is similar to that observed in crab species such as *C. affinis* and *Chionoecetes* sp. (Galgani & Nagayama, 1987) and spiny lobster *Panulirus interruptus* (Celis-Guerrero *et al*., 2004), however differs with species of the same genus *M. amazonicus* and *M. lanchesteri*, showing optimal temperature between 60 to 65°C (Silva-Santos *et al*., 2014; Pongsetkul *et al*., 2016). The highest stability of *M. carcinus* proteases occurs at 45°C at all preincubation times, which is similar to that reported in two species of crabs, *Callinectes bellicosus* and *C. arcuatus* (Díaz-Tenorio *et al*., 2006), crab *Pacifastacus astacus* (García-Carreño & Haard, 1993) with 75% residual activity between 30°C and 50°C for 30 min pre-incubation, which strongly agrees with the way in which crustaceans tend to feed by remaining a long time grinding and digesting the food, a way that allows them to maximize the ability to hydrolyze and assimilate nutrients within the hepatopancreas (Ceccaldi, 1997; Vega-Villasante *et al.*, 1999). Likewise, *M. carcinus* compared with the studies in *M. amazonicus* and *M. lanchesteri* (Silva-Santos *et al*., 2014; Pongsetkul *et al*., 2016) where the optimal temperature differs as previous mentioned, it is probable that the capacity of protein hydrolysis in the present species is more limited, particularly because of the lower resistance to temperature changes.

The number and type of caseinolytic bands in hepatopancreas with proteolytic activity for *M. carcinus* in the present work is higher (eight bands) than reported in whole body of *M. rosenbergii* and *M. lanchesteri*, where six and seven bands with caseinolytic activity are reported respectively, while for *M. amazonicus*, a number between 14 to 15 bands are reported in whole body and seven bands are reported in hepatopancreas (Hasan-Chisty *et al*., 2009; Silva-Santos *et al*., 2014; Pongsetkul *et al*., 2016). On the other hand, the large number of bands with proteolytic activity detected in *M. carcinus*, presents a wide molecular weight ranges (17.8 to 94.0 kDa), which coincides with studies in the crab *Pacifastacus astacus*, where bands with activity are in the molecular weight ranges between 14 and 81 kDa, while *M. rosenbergii* presents bands with activity in the molecular weight ranges between 13.48 and 136.1 kDa. In both cases the wide type of bands correspond to serine proteases (trypsin and chymotrypsin-like enzymes) and metallo-proteases (García-Carreño *et al*., 1993; Hasan-Chisty *et al*., 2009).

The study of inhibitors both *in vitro* and through the zymogram in adults of *M. carcinus* showed a residual inhibition of more than 80% by PMSF and SBT1, indicating the presence of serine proteases (García-Carreño & Haard, 1993); While SBT1 inhibited two bands (45.8 and 28.5 kDa) and PMSF inhibited all bands of proteolytic activity. Recent studies with *Artemisa longinaris* (Fernández-Giménez *et al*., 2002) showed that trypsins have a molecular weight ranging from 14.79 to 17.32 kDa and chymotrypsins can vary from 21.38 to 27.54 kDa as previous reports in various species of decapods such as *P. monodon, P. japonicus, Marsopenaeus japonicus, P. penicillatus, Fenneropenaeus penicillatus, P. vannamei, Metapenaeus conoceros* and *M. rosenbergii* (Tsai *et al.*, 1986). The results in zymograms and inhibition with the use of inhibitors such as phenanthroline and EDTA, denotes the presence of metalloproteases in the specie, which represents a higher digestive capacity for the species (Figs. 2-3). In this sense, inhibition with PMSF, SBT1 and TLCK indicates that a large part of the proteolytic activity in the extracts of *M. carcinus* is due to the presence of trypsin-like enzymes as demonstrated in other studies with the lobster *Pleuroncodes planipes* (García-Carreño & Haard, 1993). On the other hand,

ovalbumin inhibited up to 75% residual activity of *M. carcinus* proteases, which shows a similar response with *P. vannamei* postlarvae (Alarcón *et al*., 2007), which is already explained by the dietary habits of peneids and palemonids in their natural environment, presenting mainly omnivorous-detritivorous nutritional behaviors, nevertheless some species shows carnivore tendency (Guerao, 1994; Soares-Pontes *et al*., 2008; Sethi *et al*., 2013; Lima *et al.*, 2014), Therefore, the use of general proteolytic inhibitors, such as SBT1 (from soybean) and PMSF, show that the use of plant ingredients within the diet of this type of organism could have a direct effects on digestive enzymatic activity, fattening process under culture process or for the breeding process, which could presents an effect of the quality of the seed produced. Thus, the digestive proteases of *M. carcinus* are tolerant to alkaline pHs, whereas the high percentages of inhibition by the presence of PMSF and SBT1 indicate the presence of serine proteases, therefore a large part of the proteolytic activity is given by action of trypsin like enzymes, which has been detected in other crustaceans. Hence, it is possible to conclude that this species presents an adequate enzymatic package, which could indicate an omnivorous digestive capacity with a possible tendency to carnivorous adaptation.

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