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Trypsin and N-aminopeptidase (APN) activities in the hepatopancreas of an intertidal euryhaline crab: Biochemical characteristics and differential modulation by histamine and salinity ☆

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ABSTRACT

No studies are available about biochemical characteristics and modulation (i.e. by endogenous and/or environmental cues) of trypsin (a key digestive endoprotease) in hepatopancreas of intertidal euryhaline crabs neither on the possible concomitant modulation of key ectoproteases such as aminopeptidase-N (APN) involved in final steps of protein digestion. Furthermore, nothing is still known in decapods crustaceans about the role of histamine as primary chemical messenger for modulation of main components of digestive process (i.e. proteases). We determined biochemical characteristics and investigated the effect of histamine injections; of histamine in vitro and of acclimation of individuals to low and high salinity on trypsin and aminopeptidase-N (APN) activities in the hepatopancreas of the euryhaline crab Cyrtograpsus angulatus (Dana 1851). Trypsin activity was maximal at pH 7.4 and at 45 °C. APN activity increased from pH 6.6 to 7.6–9.0 and was maintained high at 37–45 °C. Both activities exhibited Michaelis-Menten kinetics (apparent Km: trypsin = 0.36 mM; APN = 0.07 mM). The injection of 10^{-4} M histamine decreased trypsin activity (about 40%) in hepatopancreas while did not affect APN activity. Similarly, in vitro 10^{-4} M histamine decreased trypsin activity (about 52%) in hepatopancreas but not APN activity. Trypsin activity in the hepatopancreas was not affected by acclimation of crabs to low (10 psu) or high (40 psu) environmental salinity while APN activity was increased (about 200%) in 10 psu. The results show the differential modulation of trypsin and APN by distinct cues and point to histamine as modulator of intracellular trypsin by direct action on the hepatopancreas.

1. Introduction

Digestive enzymes are a link between ingestion, absorption and assimilation of nutrients thus having a main physiological role in animals (Karasov and del Rio, 2007; Karasov et al., 2011; Karasov and Douglas, 2013; Abumrad et al., 2016). Studies on the mechanisms of regulation of key digestive enzymes (such as different proteases) are therefore of fundamental importance for the integrated knowledge of organisms. However, the information available about chemical messengers involved in the regulation of digestive enzyme activities (i.e. different proteases) is dominated by research on mammals. As pointed out by Karasov and Douglas (2013) the field of comparative digestive physiology at the biochemical level is constrained by the lack of information about most invertebrate groups. Intertidal euryhaline crabs, are a group of major ecophysiological importance in coastal and estuaries ecosystems. However, studies about the occurrence, biochemical characteristics and mechanisms of regulation of key different proteases such as trypsin and aminopeptidase-N (APN) in the hepatopancreas, the main site of digestion and absorption and in which both extra and intracellular digestion is carried out) are still scarce and fragmentary (Michiels et al., 2015a). In fact, chemical messengers involved in the modulation of digestive enzymes in the hepatopancreas of decapod crustaceans in general are far from having been elucidated.

In mammal's digestive tract, various proteases (endo, exo and ectoproteases) catalyze highly specific reactions producing different products from protein digestion (López-Otín and Bond, 2008; Bradley et al., 2013; Trowers and Tischler, 2014). Trypsin (EC 3.4.21.4), an endopeptidase of the serine protease type, is one of the most important proteases in mammalian and non- mammalian vertebrates digestive tract having a central role in protein digestion (Sainz et al., 2004; Muhlia-Almazán et al., 2008; Vandermarliere et al., 2013, Perera et al., 2014, Wang et al., 2014). Aminopeptidase-N (APN) (EC 3.4.11.2) an ectopeptidase attached to the cell membrane with the catalytic site exposed to the extracellular surface, plays a major role in the final stages of dietary proteins digestion in mammal's intestine yielding various products such as peptides which are finally digested by intracellular

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peptidases (Alpers, 1987; Mentlein and Roos, 1996; Goodman, 2010; Fairweather et al., 2012). Mina-Osorio, 2008; Villaseñor-Cardoso et al., 2013; Tang et al., 2016). Thus, APN activity in the digestive tract is used as an indicator of protein digestive capacity of organisms (Ramirez-Otarola et al., 2011). In various decapod crustaceans, trypsin represents the main proteolytic activity in hepatopancreas (Perera et al., 2014; Wang et al., 2014; Perera et al., 2015 while little is known about APN (Michiels et al., 2015a). Circulating biogenic amines play an important role in the regulation of different physiological processes in decapod crustaceans (Fingerman et al., 1994; Cebada and García, 2007; Clark et al., 2008; Christie et al., 2014). Various biogenic amines (i.e. histamine) have been detected in the hepatopancreas which is also an important endocrine organ (Fingerman et al., 1994; Huang et al., 2005). In mammal's digestive tract, histamine is involved in the regulation of digestive and absorptive processes by modulating the activity of key enzymes and being the major regulator of gastric acid secretion (López Mañanes and Vega, 1994; Sander et al., 2006; Breunig et al., 2007; Ramsay and Carr, 2011; Chang and Leung, 2014; Trowers and Tischler, 2014). In some decapod crustaceans, histamine is involved in the control of functions in the digestive tract (Pulver et al., 2003). Works in our lab showed that dopamine is a modulator of distinct digestive enzyme activities in the hepatopancreas of euryhaline crabs (Michiels et al., 2013, 2015a, 2015b) but nothing is known about the role of histamine in any decapod crustacean.

There is growing evidence that the digestive function of the gastrointestinal tract of animals can vary with external factors, one of the levels of this flexibility is biochemical, such as the activity of digestive enzymes (del Valle and López Mañanes, 2011, 2012; Karasov and Douglas, 2013; del Valle et al., 2015). Adaptive variation in digestive enzyme activity can be crucial to the lifestyle of many animals (Karasov and Douglas, 2013). The modulation of specific digestive enzymes activities in the digestive tract can lead to adjustments in the digestive capacity for specific nutrients under changing environmental conditions (Caviedes-Vidal et al., 2000; del Valle and López Mañanes, 2011, 2012; Romano and Zeng, 2012; Karasov and Douglas, 2013). Intertidal zone is an extremely challenging environment in which abiotic factors such as salinity vary abruptly both spatially and temporally. Various studies including several of our lab, pointed out that a differential modulation of specific digestive enzymes activities in the hepatopancreas appears to be one strategy to face environmental salinity in euryhaline crabs (Asaro et al., 2011; Romano and Zeng, 2012; Michiels et al., 2013, 2015a, 2015b; Pinoni et al., 2013, 2015; Wang et al., 2014). In this context, we considered of interest to evaluate whether trypsin and APN activities in the hepatopancreas are under regulation by chemical messengers (histamine) and/or a key external cue (salinity) using as a model the intertidal euryhalinc crab Cyrtograpsus angulatus which is one of the dominant crabs in intertidal areas from Rio de Janeiro (Brazil) to Patagonia (Argentina) on the Atlantic coast and in Peru and Chile on the Pacific coast (Spivak, 1997). Then, we investigated the occurrence and biochemical characteristics of trypsin and APN activity in the hepatopancreas, the effect on histamine injections and the effect in vitro of this biogenic amine on trypsin, APN and total proteolytic activities in the hepatopancreas and the effect of acclimation of individuals to low and high salinity on trypsin and APN activity in the hepatopancreas.

2. Materials and methods

2.1. Chemicals

Azocasein, L-alanine-p-nitroanilide (L-Ala-pNA), N-α-Benzoyl-DL-Arginine-p-Nitroanilide (BAPNA), tris (hydroxymethylamino methane) (Tris), the ethyleneglicol N, N′, N′-tetraacetic acid (EGTA), bovine serum albumi and histamine were from Sigma (St. Louis, MO, USA); Coomassie blue G250 was from Fluka (Germany). All chemicals used were of analytical grade. All solutions were prepared in distilled water.

2.2. Animal collection and maintenance

Crabs were caught from the mudflat area of Mar Chiquita coastal lagoon (Buenos Aires, Province Argentina) (37°32′−37°45′S; 57°19′−57°26′W). Only adult male crabs with a carapace width >2.5 cm were collected. Animals were transported to the laboratory in lagoon water on the day of collection. For all the experiments salinity was measured in practical salinity units (psu). The crabs (20 individuals per aquarium) were maintained in natural seawater (35 psu), dilute sea-water (10 psu) or concentrated seawater (40 psu) for at least 10 days prior to use (Pinoni and López Mañanes, 2004, 2008). Dilute seawater was obtained by dilution of natural seawater with distilled water. Concentrated seawater was obtained by addition of commercial marine salt (Red Sea Salt, Israel) to natural seawater (López Mañanes et al., 2000; Pinoni and López Mañanes, 2004, 2008; Pinoni, 2009). The aquaria contained 36 L of water, continuously aerated and filtered. A regime of 12 h light/12 h dark was applied and the temperature was kept at 22 ± 2 °C. The water was continuously filtered by means of an Atman filter (HF-0400). Aquaria were shielded by black plastic to reduce disturbance. Crabs were fed three times a week with Vita fish commercial food (30% carbohydrates, 44% protein, 12% fat; about 0.07 g individual^{-1}) but they were starved 24–48 h prior to the experiments. No differences in the feeding behavior occurred in the experimental conditions used.

2.3. Assay of trypsin, APN and total proteolytic activity

Trypsin activity was determined using benzoyl- Arg-p-nitroanilide (BAPNA) as substrate with some modifications (dimethyl sulfoxide was not used). The reaction was initiated by addition of substrate (final concentration 1.23 mM) to a reaction mixture that contained an aliquot of the sample in 50 mM Tris buffer pH 7.4. After incubation for 15 min at 45 °C, the reaction was stopped by addition of 250 mL of KOH and absorbance was determined at 410 nm. For characterization studies of trypsin activity, the procedure was the same as described above except that the activity was determined in the presence of various pHs (range 4.5 to 11.0) (50 mM phosphate buffer pH 4. 5 and 50 mM Tris-HCl, pH 6.0–8.0; 50 mM glycine, pH 11.0) and temperatures (4–70 °C). To study the effect of BAPNA concentration on the activity of trypsin, activity was determined at pH 7.4 at 45 °C in the presence of increasing concentrations of BAPNA (from 0.12 to 2.32 mM) in the reaction mixture.

The APN activity was determined using L-alanine-p-nitroanilide (L-Ala pNA) as substrate (Roncari and Zuber, 1969) as described (del Valle and López Mañanes, 2008, 2011; Naya et al., 2011) with some modifications. In the standard assay, the reaction was initiated by adding the substrate (final concentration 0.41 mM) to a reaction mixture containing an adequate aliquot of the corresponding sample (which falls on the linearity zone on an activity vs protein concentration plot) in 50 mM Tris buffer pH 7.6. After incubation for 15 min, the reaction was stopped by the addition of 0.5 mL of cold 2 M acetic acid and absorbance was determined at 384 nm. To study the effect of pH and temperature on APN activity, the procedure was the same as described above except that the activity was determined in the presence of varying pH (range 6.6–9) (50 mM Tris–HCl buffer pH 6.6–9.0) and temperature (4–45 °C) of the reaction mixture. To study the effect of L-Ala-pNA concentration on APN activity, the procedure was the same as described above except that the activity was determined at pH 7.6 at 37 °C in the presence of varying L-Ala-pNA concentrations (0.04–0.6 mM) in the reaction mixture. To study the effect of bestatin on APN activity, the activity was measured as described before but in the absence and/or the presence bestatin (13 mM–55 mM). Bestatin is an inhibitor of APN which is commonly used as a tool to characterize this activity in several tissues (Bauvois and Dauzonne, 2006; Chen et al., 2013).

Total proteolytic activity was assayed by adding an aliquot of the sample (linearity zone on activity vs protein concentration plot) to a

reaction mixture containing 1% w/v azocasein in 0.1 M Tris- HCl buffer (pH 7.5). The assay conditions used were optimal for this activity in the hepatopancreas of *C. angulatus* as described in our previous work (Pinoni, 2009). After incubation at 45 °C for 30 min, the reaction was arrested by adding 0.75 mL of cold trichloroacetic acid (TCA) (10% w/v) (Pinoni, 2009; Pinoni et al., 2011). Absorbance was measured at 440 nm (A440) (Metrolab 330) in the supernatant resulting after centrifuging at 1800 ×*g* for 20 min (IEC-Centra 7R, refrigerated). One unit activity (U) was defined as the amount of enzyme extract that produced an increase of 1 in A440. The proteolytic activity was expressed as U h^{-1} mg $protein^{-1}$.

Individuals acclimated to 35 psu were used in these experiments. The determination of enzyme activity was always performed with samples that had been stored at −20 °C, without any previous thawing. Protein was assayed according to Bradford (1976). Bovine serum albumin was used as standard.

2.4. Hemolymph osmolality

Hemolymph (about 500 μL) was sampled from the infrabranchial sinus of 5–10 individuals by means of a syringe at the base of the cheliped and transferred to an iced centrifuge tube. Serum was separated by centrifugation at 10,000 × *g* (Beckman, Microfuge, B) for 30 s as we described before (Michiels et al., 2013, 2015a). Osmolality was measured with a micro-osmometer (Osmomat 030 D, GONOTEC).

2.5. Effect of the injection of histamine on the activity of digestive enzymes in hepatopancreas

The individuals were anesthetized by cold for 20 min and subsequently injected into the base of the cheliped with 100 μL of saline solution (400 mM NaCl, 10 mM KCl) in the absence (control) or presence of histamine 10^{-4} M. After 30 min of injection, the activities of trypsin and APN in hepatopancreas were determined.

2.6. In vitro effect of histamine on the activity of digestive enzymes in hepatopancreas

Sections of hepatopancreas (100 mg) from crabs maintained in 35 psu were incubated in absence or presence of histamine (10^{-4} M) in 2 mL of a medium containing (mM): 400 NaCl, 13KCl 10 $MgCl_2$ 8.8 H_3BO_3, pH 7.6 at 30 °C (Michiels et al., 2015a,b). After 30 min of incubation, tissue was homogenized in buffer Tris–HCl 50 mM pH 7.4 (4 mL × g $tissue^{-1}$). Total proteolytic, trypsin and aminopeptidase —N activity were assayed (Resch-Sedlmeier and Sedlmeier, 1999; Lwalaba et al., 2010). Trypsin activity was also tested in the medium (indicator of released enzyme activity) (Resch-Sedlmeier and Sedlmeier, 1999; Lwalaba et al., 2010). Trypsin activity was not detected in the medium throughout the experimental period, neither in the absence, nor in the presence of the agents tested.

2.7. Preparation of enzymatic extracts

The crabs were anesthetized by cold by putting them on ice for about 20 min. The hepatopancreas was immediately excised, mixed with homogenizing medium (0.5 M Tris/HCl pH 7.4; 4 mL g^{-1} of hepatopancreas tissue) and homogenized (CAT homogenizer × 120, tool T10) on ice. The homogenate was centrifuged at 10000 ×*g* for 15 min (Sorval, rotor SS34, refrigerated). The hepatopancreas from one individual was used for each preparation of enzyme extract. The supernatant was fractionated into 0.5 mL aliquots and stored at −20 °C until use.

2.8. Statistical analysis

The results of the effect of different substrate concentrations on the enzymatic activities were analyzed by a nonlinear regression analysis (GraphPad Prism4.0 software). The curves that appear are the ones which best fit to the experimental data according to stimation by GraphPad Prism 4.0 software, showing adjustment to the Michaelis-Menten model. Km values (Michaelis–Menten constant) were estimated from this curve (GraphPad Prism 4.0 software). The statistical analysis of the data was realized using the Sigma 3.0 program for Windows, which automatically performs previous test of equality of variances and normality. Analysis of variance (repeated measures ANOVA) or *t*-tests were used to estimate the statistical significance of the differences and $P < 0.05$ was considered significant. A post-hoc (Dunn's) was used to identify differences.

3. Results

3.1. Trypsin and APN activity in hepatopancreas of Cyrtograpsus angulatus: effect of pH, temperature, BAPNA and L-Ala-pNA

Trypsin activity in hepatopancreas increased from pH 4.0 to pH 7.4. At pH 11.0 trypsin activity was approximately 40% lower than the activity at pH 7.4 (Fig. 1A). Trypsin activity was similar at 4 and 37 °C and increased at 45 °C. At 70 °C, the activity decreased about 80% compared to the activity at 45 °C (Fig. 1B). The effect of different concentrations of BAPNA (0.12 to 2.32 mM) on the activity of trypsin is shown in Fig. 1C. Trypsin activity in hepatopancreas of *C. angulatus* exhibited Michaelis-Menten kinetics (apparent Km = 0.36 mM). APN activity increased from pH 6.6 to 7.6. At pH 8.0 APN activity was about 15% lower than the activity at pH 7.6 (Fig. 2A). APN activity increased slightly between 4 °C and 20 °C and the highest value was observed at 37 °C. At

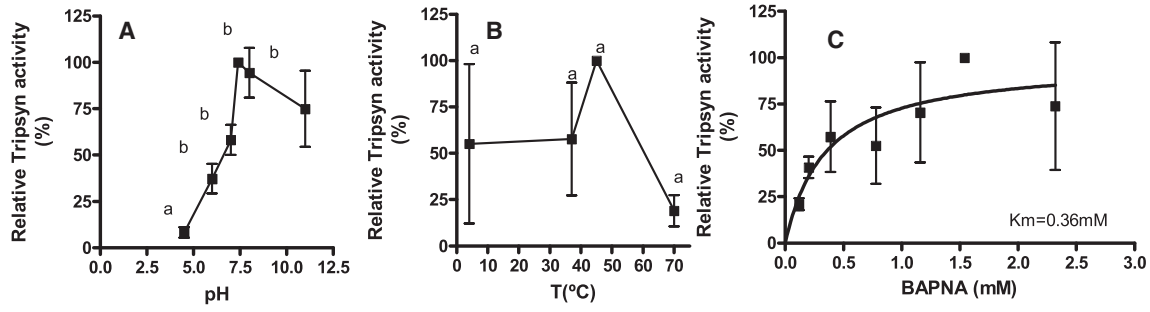

Fig. 1. (A) Effect of pH (4.5 to 11.0) on trypsin activity in hepatopancreas of *C. angulatus*. Activity was measured at 45 °C and in the presence of 1.23 mM of BAPNA. The trypsin activity values are expressed as a relation to the specific activity at pH 7.4 (100%, 3.7 ± 0.2 μmol min^{-1} mg $prot^{-1}$). Data are mean ± 5 individuals S.E. (B) Effect of temperature (4 to 70 °C) on trypsin activity in hepatopancreas of *C. angulatus*. The activity was measured at pH 7.4 and in the presence of 1.23 mM of BAPNA. The trypsin activity values are expressed as a relation to the specific activity at 45 °C (100%, 3.7 ± 2.9 μmol min^{-1} mg $prot^{-1}$). Data are mean ± 5 individuals S.E. (C) Effect of increasing concentrations of BAPNA (0.12 to 2.32 mM) on the activity of trypsin in hepatopancreas of *C. angulatus*. Activity was measured at pH 7.4 at 45 °C. The trypsin activity values are expressed as a relation to the specific activity at 1.60 mM BAPNA (100%, 1.77 ± 0.6 μmol min^{-1} mg $prot^{-1}$). Data are means ± 5 S.E individuals. Different letters indicate significant differences ($p < 0.05$).

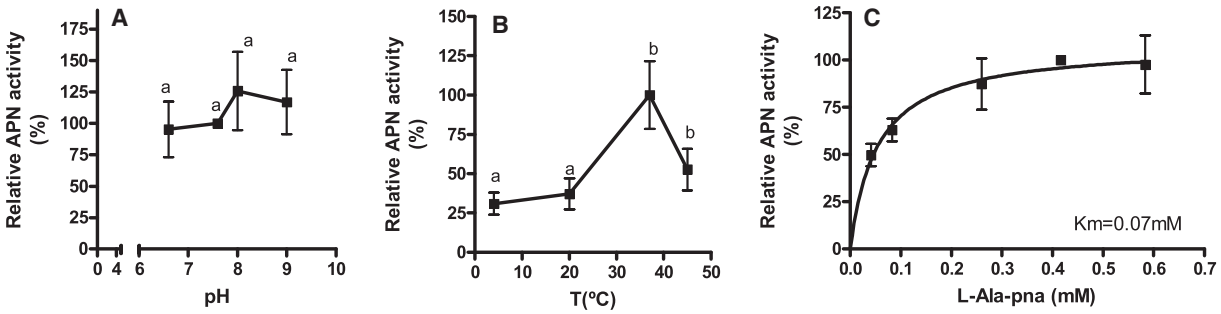

Fig. 2. (A) Effect of pH (6.6 to 9) on the activity of APN in hepatopancreas of *C. angulatus*. Activity was measured at 37 °C and in the presence of 0.41 mM of L-Ala-pNA. The APN activity values are expressed as a relation to the specific activity at pH 7.6 (100%, 3.31 ± 2 μmol min^{-1} mg $prot^{-1}$). Data are mean ± SE 5 individuals. (B) Effect of temperature (4–45 °C) of APN activity in hepatopancreas of *C. angulatus*. Activity was measured at pH 7.6 and in the presence of 0.41 mM of L-Ala-pNA. The APN activity values are expressed as a relation to the specific activity at pH 7.6 (100%, 2.39 ± 1 μmol min^{-1} mg $prot^{-1}$). Data are mean ± S.E 5 individuals. (C) Effect of the concentration of L-Ala pNA (from 0.04 to 0.58 mM) of APN activity in hepatopancreas of *C. angulatus*. Activity was measured at 37 °C and at pH 7.6. The APN activity values are expressed as a relation to the specific activity at pH 7.4 (100%, 2.3 ± 0.8 μmol min^{-1} mg $prot^{-1}$). Data are mean ± S.E 5 individuals. Different letters indicate significant differences ($p < 0.05$).

45 °C, APN activity was approximately 47% lower than the activity recorded at 37 °C Fig. 2B. The effect of different concentrations of L-Ala-pNA (0.04 to 0.58 mM) on APN activity is shown in Fig. 2C. APN activity in hepatopancreas of *C. angulatus* exhibited Michaelis-Menten kinetics (apparent Km = 0.07 mM). APN activity in hepatopancreas was not inhibited by bestatin (range of concentrations tested: 13 mM–55 mM) (data not shown).

3.2. Effect of the injection of histamine on trypsin, APN and total proteolityc activity in hepatopancreas of C. angulatus

The injection of 10^{-4} M histamine produced a decrease of trypsin activity (about a 40%) in hepatopancreas of *C. angulatus* (Fig. 3A). No effect was found on APN activity in hepatopancreas of *C. angulatus* (Fig. 3B). Similar to that found for trypsin activity, total proteolityc activity decreased about 40% by histamine 10^{-4} M (Fig. 3C).

3.3. In vitro effect of histamine on trypsin, APN and total proteolityc activity in hepatopancreas of C. angulatus

In vitro, 10^{-4} M histamine decreased trypsin activity (about 52%) in hepatopancreas of *C. angulatus* (Fig. 4A). APN activity was not affected. (Fig. 4B). A decrease of total proteolytic activity (about 48%) was found in the presence of 10^{-4} M histamine (Fig. 4C).

3.4. Effect of low and high salinity on tripsyn and APN activity in hepatopancreas of C. angulatus

To determine the effect of low and high salinity on trypsin and APN activities in hepatopancreas of *C. angulatus*, individuals were maintained at 35, 10 and 40 psu, salinities in which this crab osmoconforms, hyper and hypo-regulates, respectively (Table 1).

Trypsin activity in hepatopancreas of *C. angulatus* was not affected by low or high salinity (Fig. 5A). In 10 psu APN activity was about

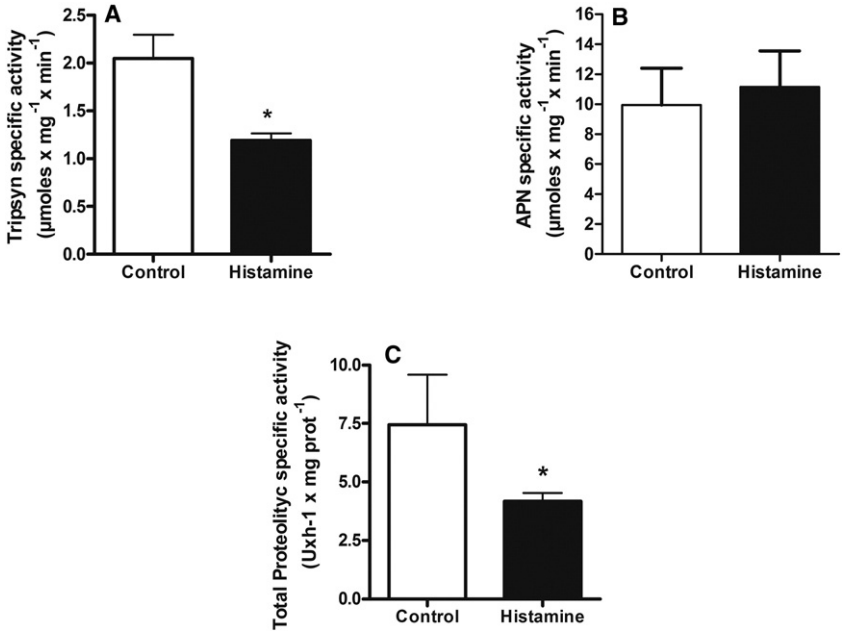

Fig. 3. (A) Effect of the injection of histamine on the activity of trypsin in hepatopancreas of *C. angulatus*. White bars: Control; Black bars: histamine 10^{-4} M.*Indicates significant difference from the corresponding control. A $p < 0.05$ was considered significant. Data are means ± S.E. 5 individuals. (B) Effect of the injection of histamine on the activity of APN activity in hepatopancreas of *C. angulatus*. White bars: Control; Black bars: histamine 10^{-4} M.*Indicates significant difference from the corresponding control. A $p < 0.05$ was considered significant. Data are means ± S.E. 5 individuals. (C) Effect of the injection of histamine on Total proteolytic activity in hepatopancreas of *C. angulatus*. White bars: Control; Black bars: histamine 10^{-4} M. *Indicates significant difference from the corresponding control. A $p < 0.05$ was considered significant. Data are means ± S.E. 5 individuals.

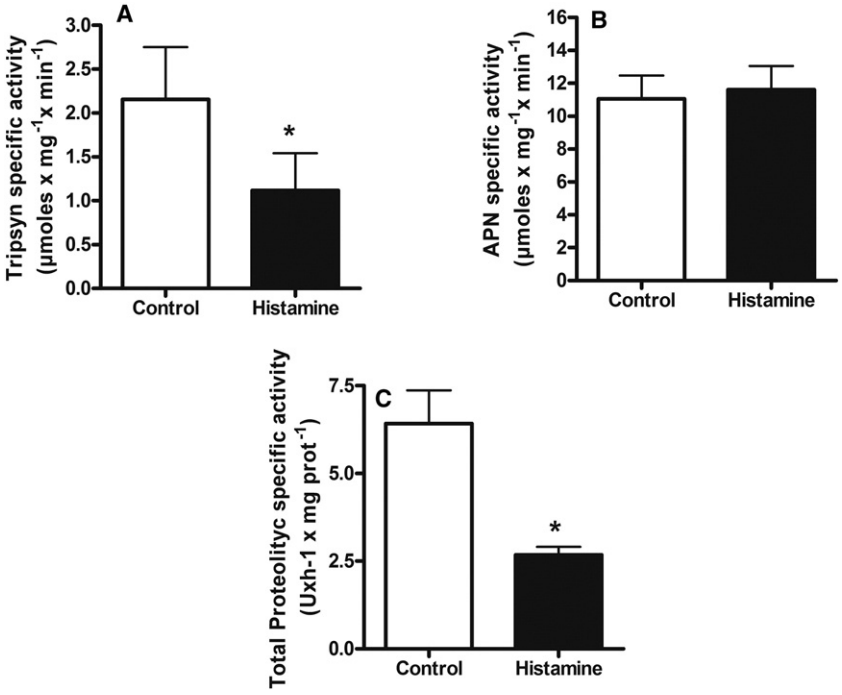

Fig. 4. (A) In vitro effect of histamine on trypsin activity in hepatopancreas of *C. angulatus* acclimated at 35‰ salinity. Slices of hepatopancreas were incubated as described in the Materials and Methods section in the absence (control) and in the presence of histamine 10^{-4} M. After incubation, Trypsin activity was measured as described in the Materials and Methods. White bars: Control; Black bars: histamine 10^{-4} M. *Indicates significant difference from the corresponding control. A $p < 0.05$ was considered significant. Data are means ± S.E. 5 individuals. (B) In vitro effect of histamine on APN activity in hepatopancreas of *C. angulatus* acclimated at 35‰ salinity. Slices of hepatopancreas were incubated as described in the Materials and Methods section in the absence (control) and in the presence of histamine 10^{-4} M. After incubation, APN activity was measured as described in the Materials and Methods. White bars: Control; Black bars: histamine 10^{-4} M. *Indicates significant difference from the corresponding control. A $p < 0.05$ was considered significant. Data are means ± S.E. 5 individuals. (C) In vitro effect of histamine on Total proteolytic activity in hepatopancreas of *C. angulatus* acclimated at 35‰ salinity. Slices of hepatopancreas were incubated as described in the Materials and Methods section in the absence (control) and in the presence of histamine 10^{-4} M. After incubation, Trypsin activity was measured as described in the Materials and Methods. White bars: Control; Black bars: histamine 10^{-4} M. *Indicates significant difference from the corresponding control. A $p < 0.05$ was considered significant. Data are means ± S.E. 5 individuals.

200% higher than in individuals exposed to 35 psu. In 40 psu APN activity was similar to that in 35 psu (Fig. 5B).

4. Discussion

In this work we investigated the occurrence, biochemical characteristics, and modulation by histamine and low and high salinity of key endo (trypsin) and ecto (APN) proteases in the hepatopancreas of the intertidal euryhaline crab Cyrtograpsus angulatus. The occurrence of specific digestive enzyme activities in the hepatopancreas of crustaceans has been linked with the nature of the components of the diet that are potentially used for metabolic processes (Pavasovic et al., 2007; Figueiredo and Anderson, 2009). The hepatopancreas, a multifunction organ, has been pointed out as a site where both extra and intracellular digestion take place (Carter and Mente, 2014). The occurrence of both trypsin and APN activity in the hepatopancreas of *C. angulatus* would indicate an adequate battery to carry out intra and extracellular digestion and then for total digestion of dietary proteins (Mentlein, 2004; Fairweather et al., 2012; Perera et al., 2015). This capacity would be in accordance with the dietary habit is in accordance with the dietary habit of this crab in Mar Chiquita lagoon (Spivak, 1997, Botto et al., 2005). For dietary components such as protein a positive relationship has been predicted between their level in the natural diet and the presence or amount in the digestive tract of enzymes necessary for their breakdown (Karasov and Douglas, 2013). To our knowledge, no reports are available on biochemical characteristics of trypsin activity in the hepatopancreas of intertidal euryhaline crabs. The maintenance of trypsin activity in hepatopancreas of *C. angulatus* at different pHs and high temperatures (45 °C) and the Michaelis–Menten kinetics (Fig. 1) are in agreement with that found in various decapod crustaceans (Murthy and Saxena, 1979; Van Wormhoudt et al., 1980; García-Carreño, 1992a, 1992b; Guizani et al., 1992; Vega-Villasante et al., 1995; Ceccaldi, 1997; Buarque et al., 2009, 2010). A previous work of our lab on the euryhaline crab *Neohelice granulata* was the first to establish the occurrence and biochemical characteristics of APN activity in hepatopancreas of an intertidal euryhaline crab (Michiels et al., 2015a). The highest APN activity in the hepatopancreas of *C. angulatus* at the pH range 7.6–9.0 was similar to that described for this enzyme in vertebrates intestine (Sabat et al., 1998) and to that we found for this activity in hepatopancreas of *N. granulata* as well as the Michaelis-Menten kinetics (Michiels et al., 2015a) while the higher APN activity at 37 °C and the decrease at 45 °C (Fig. 2) differs from *N. granulata* (Michiels,

Table 1
Osmolality (mOsm kg^{-1}) in the external medium and hemolymph of *C. angulatus*.

	35 psu		10 psu		40 psu	
	Medium	Hemolymph	Medium	Hemolymph	Medium	Hemolymph
Osmolality	882 ± 6	872 ± 21	269 ± 15	653 ± 14*	1015 ± 16	878 ± 8*

*Indicates significant difference from corresponding value in the external environment. Data are the mean ± E.S. 5–10 individuals.

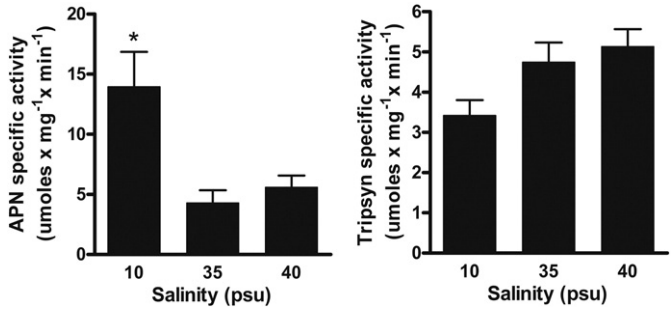

Fig. 5. (A) Effect of acclimation to low (10 psu) and high salinity (40 psu) on Trypsin activity in hepatopancreas of *C. angulatus*. The activity was measured as described in the Materials and Methods section. A $p < 0.05$ was considered significant. Data are mean ± SE for 5–6 individuals (B) Effect of acclimation to low (10 psu) and high salinity (40 psu) on APN activity in hepatopancreas of *C. angulatus*. The activity was measured as described in the Materials and Methods section. A $p < 0.05$ was considered significant. Data are mean ± SE for 5–6 individuals.

2015a). Furthermore, unlike we observed in hepatopancreas of *N. granulata* (Michiels et al., 2015a), bestatin (an inhibitor of APN activities in mammal intestine; Chen et al., 2013) did not affect this activity in hepatopancreas of *C. angulatus* (not shown). Whether the different responses to temperature and bestatin of APN activity in hepatopancreas of both species of euryhaline crabs are due to the occurrence of different forms with distinct features needs further investigation.

Despite its inherent physiological importance the modulation of digestive enzymes by primary chemical messengers in the hepatopancreas of decapod crustaceans has been poorly investigated (Resch-Sedlmeier and Sedlmeier, 1999; Michiels et al., 2013, 2015a, 2015b). In this context, to our knowledge there are no works dealing with the possible modulation by chemical messengers of trypsin activity in the hepatopancreas neither with the possible role of histamine as modulator of digestive enzymes. In mammals, histamine has a main role in regulation of key enzymes and channels involved in digestion and absorption processes (López Mañanes and Vega, 1994; Sander et al., 2006; Breuning et al., 2007; Ramsay and Carr, 2011). In previous works, we showed that dopamine differentially affected distinct digestive enzymes suggesting the role of biogenic amines as primary chemical messengers in mechanisms of regulation of these activities in hepatopancreas of decapod crustaceans (Michiels et al., 2013, 2015a, 2015b). The decrease of trypsin activity as well as of total proteolytic activity in hepatopancreas of *C. angulatus* induced by injection of 10^{-4} M histamine (Fig. 3A.) supports the idea of the role of this chemical messenger and of biogenic amines in the modulation of digestive enzymes. The effect of histamine was specific on trypsin activity because aminopeptidase-N activity was unaltered (Fig. 3B). Furthermore, the concomitant and to the same extent decrease in both trypsin and total proteolytic activity, while no effect occurred on APN activity, suggest that diminished trypsin activity account for the decreased total proteolytic activity induced by the injection of histamine. The differential responses to histamine suggest its specific action on distinct protease activities in hepatopancreas. On the other hand, the similar effect (decrease) induced in vitro by 10^{-4} M histamine on trypsin and total proteolytic activity in the hepatopancreas points out the occurrence of a direct effect of histamine on this organ (Fig. 4A, C). Again, the fact that in vitro 10^{-4} M histamine did not affect APN activity (Fig. 4B) further supports the idea of a distinct modulation of specific proteases by this biogenic amine. Moreover, parallel experiments in our lab show that injection of 10^{-4} M histamine or in vitro 10^{-4} M histamine (using sections of hepatopancreas) induce an opposite effect (increase) on total lipase activity in the hepatopancreas of *C. angulatus* (Michiels, 2015). This suggests that histamine, in fact, differentially modulates specific digestive enzymes in the hepatopancreas. In mammal's digestive tract, the physiological actions of histamine are mediated by specific membrane receptors coupled to distinct signaling pathways such as that of cAMP and Ca++ (Chang and Leung, 2014). The differential effect of histamine in the hepatopancreas of *C. angulatus* (decrease of trypsin/increase of lipase activity), suggests that distinct signaling pathways could be being activated leading, in turn, to a differential modulation (increase/decrease) of specific digestive enzyme activities. Whether the in vitro effect of histamine involves its binding to specific receptors coupled to different signaling pathways in hepatopancreas of *C. angulatus* requires further investigation. It has been proposed that some digestive enzymes activities in the hepatopancreas of decapod crustaceans can be intracellularly regulated (Sánchez-Paz et al., 2006; Michiels et al., 2015b). In this context, we previously showed the stimulation by dopamine of intracellular lipase activity in hepatopancreas of the euryhaline crab *N. granulata* (Michiels et al., 2015b). Since no release of trypsin occurred the results of the present work also suggests the modulation by histamine of intracellular trypsin activity in hepatopancreas of *C. angulatus*.

Differential modulation of specific digestive enzymes activity in response to key environmental cues is an important strategy at the biochemical level in various animals, which could lead to adjustments in digestive capacity and consequently in protein, carbohydrate and/or lipid metabolism (Caviedes-Vidal et al., 2000; del Valle and López Mañanes, 2011, 2012; Romano and Zeng, 2012; Karasov and Douglas, 2013). In some species of euryhaline crabs distinct environmental salinity affects specific digestive enzymes in the hepatopancreas (Asaro et al., 2011; Romano and Zeng, 2012; Wang et al., 2014; Pinoni et al., 2013; Michiels et al., 2015a). Previous work from our laboratory showed that various responses in different tissues occurs in *C. angulatus* upon distinct environmental salinities, which involve a differential modulation of different enzyme activities (López Mañanes et al., 2002; Pinoni and López Mañanes, 2004, 2008; Pinoni, 2009; Michiels et al., 2013). In this context, we have previously shown that an increase of proteolytic activity (about 40%) occurs in hepatopancreas of this crab upon acclimation to low salinity (10 psu) while no change occurs in high salinity (40 psu) (Michiels et al., 2013). In this work, we further investigated the possible components (i.e. specific endo and/or ectoproteases activities) involved in this response. The higher APN activity in hepatopancreas of individuals in 10 psu (Fig. 5B) supports the idea that modulation of APN activity is one of the components accounting for the increased proteolytic activity (by using azocasein as substrate) found in the hepatopancreas in response to low salinity (Michiels et al., 2013). Since trypsin activity was not affected by acclimation to low salinity APN activity appeared to be specifically modulated by environmental salinity. Osmoregulatory adaptation in response to low salinity requires various molecular and biochemical changes such as increased hemolymph amino acids (Romano and Zeng, 2012). The increase of APN activity could lead to an enhanced capacity for final steps of protein digestion and consequently, to a higher availability of amino acids in relation to hyper-regulation (Table 1) as we suggested for *N. granulata* (Michiels et al., 2015a). In mammal's intestine, APN form complexes with amino acid transporters inducing changes in kinetic parameters of the transporters (Fairweather et al., 2012). Upon hypo-osmotic stress gluconeogenesis from amino acids has a role in adjustments of the intracellular level of nitrogenated compounds in hepatopancreas of the euryhaline crab *N. granulata* (Schein et al., 2005; Martins et al., 2011). Nothing is known about the occurrence of these biochemical routes and metabolic pathways or of amino acid transport systems in the hepatopancreas of in *C. angulatus*. We previously found that, contrary to low salinity, total protease activity in the hepatopancreas of *C. angulatus* was not affected by high salinity (40 psu) upon hypo-regulatory conditions (Table 1) (Michiels et al., 2013). The results of this work showing that neither trypsin nor APN activity in hepatopancreas of *C. angulatus* were affected by acclimation to 40 psu further suggests that specific differential adjustments in proteases activities occurs in hepatopancreas in relation to different salinity conditions.

5. Conclusions

The occurrence of trypsin and APN activity in the hepatopancreas of *C. angulatus* are differentially affected by internal (histamine) and environmental (low salinity) cues suggesting that these key endo and ectoproteases are under regulation by different pathways. In this context, the results show the role of histamine as modulator of trypsin activity in the hepatopancreas suggesting its direct action on this tissue to modulate intracellular trypsin activity, while APN activity appears to be one component of responses underlying biochemical adjustments to low salinity.

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