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Amylase in the hepatopancreas of a euryhaline burrowing crab: characteristics and modulation

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Abstract: In spite of its inherent physiological importance, studies on the occurrence, characteristics, and modulation of amylase in euryhaline crabs are lacking. We investigated the occurrence of amylase forms and the effect of acclimation to different salinities on their number and made a partial purification and characterization of the major form present in the hepatopancreas of *Neohelice granulata*. Zymogram analysis revealed 5 amylase forms in crabs acclimated to 35 psu (seawater) and 37 psu, and an additional band at 10 psu, but with a major form (29 kDa) in all cases, which was partially purified and characterized. Amylolytic activity was maximal between 30 and 40 °C; maintained at high NaCl concentrations (up to 4 M); increased by 5 mM K^+, Li^+, Co^{2+}, and Mg^{2+} (36%–45%); inhibited by Cu^{2+}, Zn^{2+}, Cd^{2+}, Fe^{2+}, and Mn^{2+} (92.4% and 23.7%); not affected by Ni^{2+} or Ba^{2+}; and enhanced almost 100% by Ca^{2+}. Amylase exhibited Michaelis–Menten kinetics (starch: K_m = 1.24 mg mL^{-1}; glycogen: K_m = 16.19 mg mL^{-1}). The potential physiological significance and relationship to habitat conditions of the extra form in low salinity and the biochemical characteristics of the partially purified amylolytic activity (halotolerant, differential sensitivity to ions, capability to hydrolyze starch and glycogen) are discussed.

Key words: Phenotypic flexibility, amylolytic activity, digestive tract, environment, euryhaline crabs

1. Introduction

In animals, α-amylases (α-1,4 glucan-4-gluconohydrolase, EC 3.2.1.1) have a central role in the initial steps of the digestion of key glycogenic substrates such as dietary starch and dietary and/or storage glycogen (Singh and Kayastha, 2014; Date et al., 2015). However, in spite of their inherent physiological importance, the current knowledge about forms, modulation by environmental factors, and biochemical features of amylase in the hepatopancreas (the main site of digestive enzyme synthesis and of digestion and absorption of nutrients and storage of energy substrates) of groups of ecological importance such as intertidal euryhaline crabs is still scarce and fragmentary (Blandamer and Beechey, 1966; Van Wormhoudt et al., 1995; Asaro et al., 2011; Zeng et al., 2010).

In decapod crustaceans, the maintenance of suitable glucose levels in the hemolymph is essential to support the regular functions of various organs and in responses to environmental stress (Verri et al., 2001; Lorenzon et al., 2005; Dutra et al., 2008). The digestion of glycogenic substrates and absorption of glucose via the hepatopancreas are the main sources of hemolymphatic glucose (Verri et al., 2001; Obi et al., 2011). Thus, the level and modulation of amylase activity in the hepatopancreas will determine the ability to digest and/or use glycogenic carbohydrates. Several amylase forms have been found in the hepatopancreas of various decapod crustaceans, although with a high degree of interspecific variability. Moreover, the number of amylase forms present in the hepatopancreas can be influenced by internal and external factors in some species (Van Wormhoudt and Sellos, 2003; Perera et al., 2008a, 2008b; Coccia et al., 2011; Le Moullec et al., 2011; Aragón-Axomulco et al., 2012; Rodríguez-Viera et al., 2016). To our knowledge, the work by Van Wormhoudt et al. (1995) in *Carcinus maenas* represents the only available study on the occurrence of amylase forms in the hepatopancreas of a euryhaline crab. Furthermore, studies on the effect of low and high salinity on amylase forms are lacking.

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Euryhaline crabs successfully occupying intertidal areas of estuaries and coastal lagoons have to cope with broad changes in environmental salinity (McNamara and Faria, 2012; Romano and Zeng, 2012). Phenotypic flexibility involves reversible within-individual variations in phenotypic traits (from molecular to organism), which can increase the chances of survival for animals exposed to frequent changes in environmental conditions (Piersma and Drent, 2003; Pfenning et al., 2010; Kelly et al., 2012). Various animals that inhabit heterogeneous habitats exhibit digestive flexibility at the biochemical and molecular level (i.e. modulation of key digestive enzymes) in response to variations in environmental factors (del Valle et al., 2004, 2006; Karasov et al., 2011; Karasov and Douglas, 2013). In some species of intertidal euryhaline crabs, biochemical adaptation to low and high salinity involves adjustments in various tissues and organs such as the hepatopancreas, although the mechanisms are still not fully understood from a metabolic perspective (Pinoni et al., 2005, 2008; Pinoni and López Mañanes, 2009; Asaro et al., 2011; Romano and Zeng, 2012; Romano et al., 2014; Michiels et al., 2013, 2015a; Pinoni et al., 2013, 2015). The modulation of digestive enzyme activities in the hepatopancreas (e.g., amylase) in response to different environmental salinity could lead to adjustments in the digestive capacity for specific substrates (starch/glycogen) and therefore in the availability of key metabolites (i.e. glucose) (Romano and Zeng, 2012; Michiels et al., 2013, 2015a; Pinoni et al., 2015). However, studies at the molecular level, as we pointed out above, are lacking.

Neohelice granulata is considered an animal model for biochemical, physiological, and ecological research (Spivak, 2010). *N. granulata* inhabits intertidal areas of the southwestern Atlantic from southern Brazil to northern Argentinean Patagonia (Spivak, 1997; Luppi et al., 2013). In the Mar Chiquita coastal lagoon (Argentina), this crab successfully occupies the whole intertidal area, where it is exposed to a broad range of environmental salinities (Spivak et al., 1994; Luppi et al., 2013). Previous work of our laboratory showed that complex and integrative responses occur upon acclimation to low and high salinity (López Mañanes et al., 2000; Schleich et al., 2001; Pinoni et al., 2005, 2013, 2015; Pinoni and López Mañanes, 2009; Asaro et al., 2011; Michiels et al., 2015a). The aims of this work were to determine the occurrence of amylase forms in the hepatopancreas of the intertidal euryhaline crab *N. granulata*, to examine the effect of acclimation of crabs to low and high salinity on the pattern of amylase forms, and to determine biochemical characteristics of partially purified amylase. We hypothesized that modulation and characteristics of amylase forms present in the hepatopancreas allow *N. granulata* to sustain digestive capacity for key glycogenic substrates in different environmental conditions with the following predictions: individuals of *N. granulata* acclimated to low and high salinity should exhibit a major number of amylase forms in the hepatopancreas with a concomitant increase in total amylase activity, and individuals of *N. granulata* should exhibit halotolerant and ion-tolerant amylase activity in the hepatopancreas, with the ability to digest both starch and glycogen.

2. Materials and methods

2.1. Chemicals

3,5-Dinitrosalicylic acid, Tris-(hydroxymethylamino-methane) (Tris), hydrochloridic acid, dibasic sodium phosphate, monobasic sodium phosphate, sodium hydroxide, and bovine serum albumin with membrane dialysis were from Sigma (St. Louis, MO, USA); sodium potassium tartrate tetrahydrate and Coomassie Blue G250 were from Fluka (Germany). All solutions were prepared in glass-distilled water.

2.2. Animal collection and maintenance

The crabs were caught from the mudflat area of the Mar Chiquita coastal lagoon (Buenos Aires Province, Argentina) (37°32′–37°45′S, 57°19′–57°26′W). In this area, *N. granulata* is exposed to a broad range of varying salinities (Spivak, 1997; Luppi et al., 2013; Pinoni et al., 2013). For all of the experiments, salinity was measured in practical salinity units (psu). Only adult male crabs with a carapace width greater than 2.5 cm were collected. Animals were transported to the laboratory in lagoon water on the day of collection. The crabs were maintained in natural seawater (35 psu), dilute seawater (10 psu), or concentrated seawater (37 psu) for at least 10 days prior to use (Michiels et al., 2015a). 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 (Michiels et al., 2015a). 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. The crabs were fed 3 times a week, with a ratio of 0.07 g/individual, using commercial food (55% carbohydrates, 31% protein, 5% fat, 2% fiber, Tetra Pond, Koi Vibrance, Germany), and were starved for 120 h prior to the experiments. Studies in our lab showed that in male individuals of *N. granulata* from the Mar Chiquita coastal lagoon, digestive and metabolic adjustments related to ingestion occur up to 120 h after feeding (Asaro et al., 2014; Pinoni et al., 2015). No differences in the feeding behavior occurred in the experimental conditions used. No mortality of individuals occurred throughout the experimental period. This

research project was performed following the regulations and statements of Ethics Committee CICUAL (OCA 1499/12), FCEyN Universidad Nacional de Mar del Plata.

2.3. Preparation of hepatopancreas enzyme extract

The crabs were cryoanesthetized by putting them on ice for about 20 min. A sample of hemolymph was withdrawn for assaying osmolality as described below. The hepatopancreas was immediately excised, mixed with 50 mM Tris/HCl of pH 8.0 (4 mL g^{-1} of hepatopancreas tissue), and homogenized (CAT homogenizer × 120, tool T10) on ice (Asaro et al., 2011; Michiels et al., 2015a). The homogenate was centrifuged at 10,000 × g for 15 min at 4 °C. Zymogram analysis of amylolytic activity of crabs acclimated to different salinities (10, 35, and 37 psu) was performed from hepatopancreas extracts obtained from 5 individuals for each condition.

2.4. Amylase partial purification

Enzyme extracts obtained from the hepatopancreas of 13 crabs acclimated to 35 psu were used. Hepatopancreas enzyme extract was initially treated with 10% w/v $(NH_4)_2SO_4$, allowed to stand for 4 h at 4 °C, and centrifuged at 10,000 × g for 30 min at 4 °C. Ammonium sulfate (60% w/v) was added to the supernatant and allowed to stand overnight. The precipitate was recovered by centrifugation at 10,000 × g for 30 min at 4 °C, dissolved in 50 mM Tris/HCl buffer (pH 8.0), dialyzed overnight against the same buffer, and used as enzyme solution. The enzyme solution was loaded onto an FPLC (fast protein liquid chromatography) system (Superose 12 10/300 GL, GE Healthcare) equilibrated with the same buffer. Fractions of 0.25 mL were collected at the rate of 0.25 mL min^{-1} and assayed for amylase activity. The protein concentration in the fractions collected was measured by continuous monitoring of the absorbance at 280 nm.

2.5. Zymogram analysis of amylase activity

Zymogram analysis (substrate-SDS-PAGE) for amylolytic activity was performed in the corresponding samples according to Perera et al. (2008a) with minor modifications. The samples were neither boiled nor treated with mercaptoethanol before electrophoresis on 12% polyacrylamide gels. After electrophoresis, the gels were immersed in starch solution (1%, w/v) at pH 6.0 for 90 min and then stained with iodine/KI solution (10 mM). Molecular weight (MW) markers (12–250 kDa, Amersham Full-Range Rainbow) were used for apparent MW determination (Perera et al., 2008a).

2.6. Biochemical assays

Amylolytic activity was measured as previously described (Asaro et al., 2011). In the standard assay, amylolytic activity was determined in a reaction mix containing 15 mg mL^{-1} starch in 50 mM phosphate buffer (pH 5.2) at 30 °C. The reaction was initiated by the addition of an aliquot of the sample (15 µL). Following incubation for 15 min, dinitrosalicylic acid reagent (DNS) was added and the reaction mix was boiled for 10 min and immediately cooled on ice. The amount of released maltose was determined by reading the absorbance at 540 nm (A_{540}), and the amylase activity was expressed as mg maltose min^{-1} mg $protein^{-1}$. To study the effect of temperature on amylolytic activity, the procedure was the same as described above, except that the enzyme activity was determined at varying temperatures (4–70 °C). To examine the effect of NaCl, the activity was determined in the absence and presence of increasing concentrations of NaCl (0.5–4 M) in the reaction mix. The effect of several ions was determined by measuring amylase activity in the absence and presence of 5 mM of K^+, Li^+, Mn^{2+}, Co^{2+}, Mg^{2+}, Ni^{2+}, Ba^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, and Fe^{2+} (as chloride or sulfate salts) in the reaction mix. The effect of Ca^{2+} was analyzed by measuring amylolytic activity in the absence and presence of 5 mM Ca^{2+} (as chloride salt), 5 mM EDTA, or 5 mM Ca^{2+} plus 5 mM EDTA in the reaction mix. To study the effect of starch, the procedure was the same as described above except that the activity was determined in the presence of varying starch concentrations (0.15–15 mg mL^{-1}) in the reaction mix. To determine the glycogen hydrolytic capacity of the samples, the enzyme activity was assayed as described above in the presence of varying glycogen concentrations (0.15–15 mg mL^{-1}) in the reaction mix. K_m (Michaelis–Menten constant) and Vmax values were estimated by analysis of data using a Lineweaver–Burk plot (GraphPad Prism 5.01 software).

Protein concentration was determined by the method of Bradford (1976), and bovine serum albumin was used as the standard.

2.7. Hemolymph osmolality

Hemolymph (about 500 µL) was sampled from the infrabranchial sinus of 5–19 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 for 30 s as described before (Michiels et al., 2013). Osmolality (mOsm kg^{-1}) was measured in an aliquot of 50 µL of hemolymph and medium with a cryoscopic osmometer (Osmomat 030, Gonotec).

2.8. Statistical analysis

The statistical analysis of the data was realized using the Sigma-Stat 3.0 program for Windows, which automatically performed the previous test of equality of variances and normality. A t-test was used to estimate the statistical significance of the differences between hemolymph osmolality and those of the external medium. Analysis of variance (one-way ANOVA) was used to estimate the statistical significance of the differences in total amylase activity at different salinities. $P < 0.05$ was considered significant. An a posteriori test to ANOVA (Holm–Sidak method) was used to identify differences.

3. Results

To determine the possible occurrence of amylase forms, a zymogram analysis of amylolytic activity in the hepatopancreas of individuals acclimated to 35 psu was carried out. At 35 psu, the hemolymph osmolality of the crabs was similar to that of the external medium, indicating osmoconformation (Table). Zymogram analysis shows that in the hepatopancreas of crabs acclimated to 35 psu, at least 5 bands with amylolytic activity were detected (Figure 1a). These bands can be divided into 2 groups: a group of 2 slower migrating bands with electrophoretic mobility around 36 kDa, and a second one with electrophoretic mobility in the range of 26–29 kDa. The band of 29 kDa appeared to exhibit the highest activity (Figure 1a).

To evaluate the effect of low and high environmental salinity on amylolytic activity, hepatopancreas extracts from crabs acclimated to low (10 psu) or high (37 psu) salinity were analyzed by zymography. Hemolymph osmolality was significantly higher or lower than that of the external medium at 10 and 37 psu, indicating hyper- and hyporegulation responses, respectively (Table). The hepatopancreas of crabs acclimated to 10 psu showed an additional band with amylolytic activity of about 30 kDa when compared with crabs at 35 psu. The hepatopancreas of crabs acclimated to 37 psu exhibited a pattern of amylolytic activities similar to that at 35 psu (Figure 1b). Similarly to 35 psu, in the hepatopancreas from crabs acclimated to low and high salinity, the 29 kDa amylolytic band appeared to exhibit the highest activity (Figure 1). Biochemical analysis of total amylase activity (sample used in zymogram analysis) showed that enzyme activity was higher at 10 psu (2.77 ± 0.49 mg maltose mg protein $^{-1}$ min^{-1}) compared to 35 psu (1.35 ± 0.22 mg maltose mg protein $^{-1}$ min^{-1}), while the activity at 37 psu was similar (1.68 ± 0.079 mg maltose mg protein $^{-1}$ min^{-1}).

Hepatopancreas extracts of individuals acclimated to 35 psu were used to obtain an enriched fraction of the amylase species that showed the highest activity on zymograms for further biochemical characterization.

Table. Osmolality (mOsm kg^{-1}) in external medium and *N. granulata* hemolymph.

Salinity (psu)	Medium	Hemolymph
10	168 ± 9	684.4 ± 30 *
35	820 ± 12	827.53 ± 27
37	984 ± 23	852.6 ± 27*

* Significantly different from the corresponding concentration of the external medium (t-test, P < 0.05). Data are the mean ± SEM, n = 5–19.

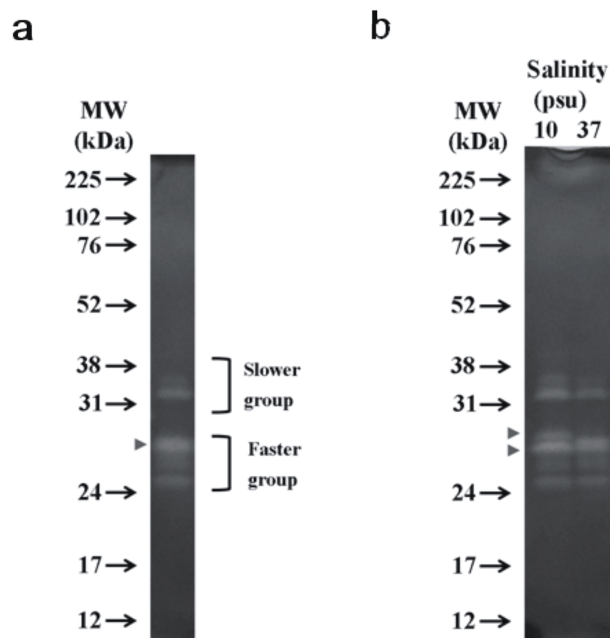

Figure 1. (a) Zymogram of amylolytic activity from hepatopancreas extract of *N. granulata* acclimated to 35 psu. After electrophoresis, the gel was immersed in a starch solution (1%) at pH 6.0 for 90 min and then stained with an iodine/KI solution (10 mM). The gray arrow indicates the major active band (29 kDa). (b) Zymogram of amylolytic activity from the hepatopancreas extract of *N. granulata* acclimated to low (10 psu) and high (37 psu) salinity. After electrophoresis, the gel was immersed in a starch solution (1%) at pH 6.0 for 90 min and then stained with an iodine/KI solution (10 mM). The gray arrows indicate the bands of 29 and 30 kDa.

Initially, amylolytic activity was precipitated with 10%–60% saturated ammonium sulfate (data not shown). This fraction was dialyzed against 50 mM Tris/HCl buffer (pH 8.0) and then separated by FPLC (Figure 2a). Protein (A_{280nm}) and amylase activity profiles in all fractions collected from the column are shown in Figure 2a. The fractions with the lowest (41–42) and the highest (46–48) activity peaks were pooled (pools A and B, respectively) and used for zymogram analysis (Figure 2b). Pool B was enriched in the 29 kDa form and the amylolytic activity was purified about 2-fold. Based on this result, pool B was used as the enzyme source (partially purified amylase) for further characterization (Figures 3a–3d and 4).

Figure 3a shows the effect of temperature (4–70 °C) on partially purified amylase. The activity increased from 20 °C to 30 °C, being maximal in the range of 30–40 °C. At 50 °C and 70 °C, the activity decreased to 90% and 33% of the activity attained at 30 °C, respectively. The effect of varying NaCl concentrations (0.5 to 4 M) is shown in Figure 3b. Amylolytic activity was highest from 0 to 1.5 M NaCl.

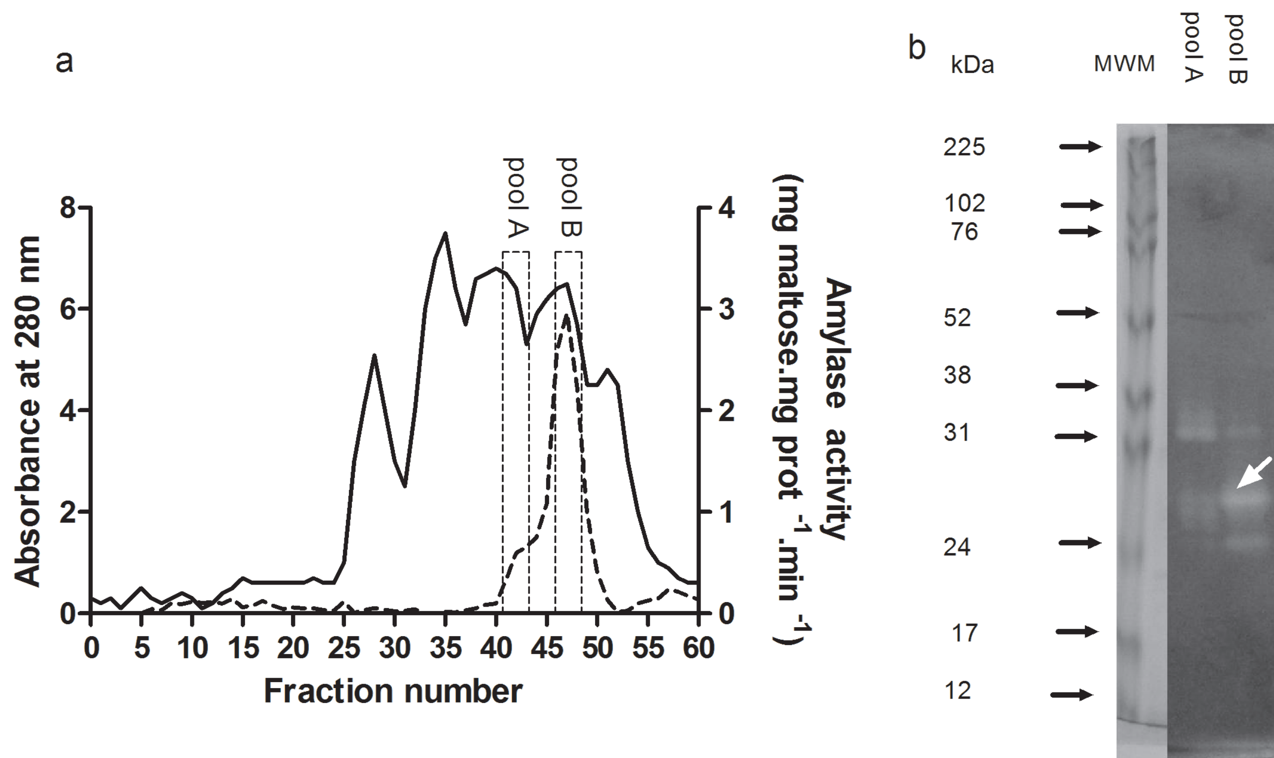

Figure 2. (a) FPLC elution profile of amylolytic activity. Hepatopancreas extract of crabs acclimated to 35 psu were precipitated with ammonium sulfate (10%–60%); the supernatant was dialyzed and chromatographed by FLPC as indicated (M & M). (-) A_{280}; (----) amylolytic activity. Pool A and pool B indicate fractions with amylolytic activity. The values of amylolytic activity are the average of triplicate assays. (b) Zymogram of amylolytic activity of pools A and B. The white arrow indicates the major active band (29 kDa).

At higher concentrations the activity decreased, being in the presence of 4 M NaCl about 30% of the activity in the absence of NaCl (Figure 3b). The effect of several ions on amylolytic activity was tested at a concentration of 5 mM (Figure 3c). Cations such as K^{+}, Li^{+}, Co^{2+}, and Mg^{2+} increased the enzyme activity 36%–45%; on the contrary, Cu^{2+} (92.4%) Zn^{2+}, Cd^{2+}, and Fe^{2+} (around 55.53%) as well as Mn^{2+} (23.7%) decreased amylolytic activity. Ni^{2+} and Ba^{2+} had no effect. The amylolytic activity was enhanced almost 100% in the presence of Ca^{2+}. On the other hand, it was not affected by the presence of Ca^{2+} plus EDTA or in the presence of EDTA alone (Figure 3d). Amylolytic activity exhibited Michaelis–Menten kinetics (K_m = 1.24 mg mL^{-1}, Vmax = 3.86 mg maltose mg $protein^{-1}$ min^{-1}) in response to varying starch concentrations (Figure 4). This partially purified amylase-hydrolyzed glycogen exhibited Michaelis–Menten kinetics (K_m = 16.19 mg mL^{-1}, Vmax = 3.71 mg maltose mg $protein^{-1}$ min^{-1}) (Figure 4).

4. Discussion

The use of zymograms with starch as a substrate has allowed the identification of several amylase forms (1 to 10 active bands) in the hepatopancreas of different decapod crustaceans (Van Wormhoudt et al., 1995; Perera et al., 2008a, 2008b; Coccia et al., 2011; Castro et al., 2012; Rodríguez-Viera et al., 2016). Using this technique, in this work we have detected 5 amylolytic species with an apparent molecular mass within the range of 26–37 kDa in the hepatopancreas of the euryhaline crab *N. granulata* upon osmoconforming conditions (35 psu, Table) (Figure 1a). These bands were grouped as "slower" and "faster" migrating amylase forms according to their electrophoretic migration, with one major amylolytic 29 kDa species (Figure 1). Our results differ from those shown in the hepatopancreas of the euryhaline crab *Carcinus maenas*, in which 2 amylase forms of about 30 kDa were reported by zymogram analysis (Van Wormhoudt et al., 1995).

The differences in the number of amylase forms present in the hepatopancreas of crustaceans have been associated with variations in environmental and/or physiological factors (Borowsky, 1984; Le Moullac et al., 1997; Perera et al., 2008a, 2008b; Castro et al., 2012). *N. granulata* behaves as a hyper/hyporegulator since it exhibits hemolymph osmolarity values higher and lower than those of the corresponding external medium upon acclimation to 6–10 psu and 37–60 psu, respectively, while it osmoconforms at 35 psu (Asaro et al., 2011; Pinoni et al., 2013; Michiels et al., 2015a; Pinoni et al., 2015; this work,

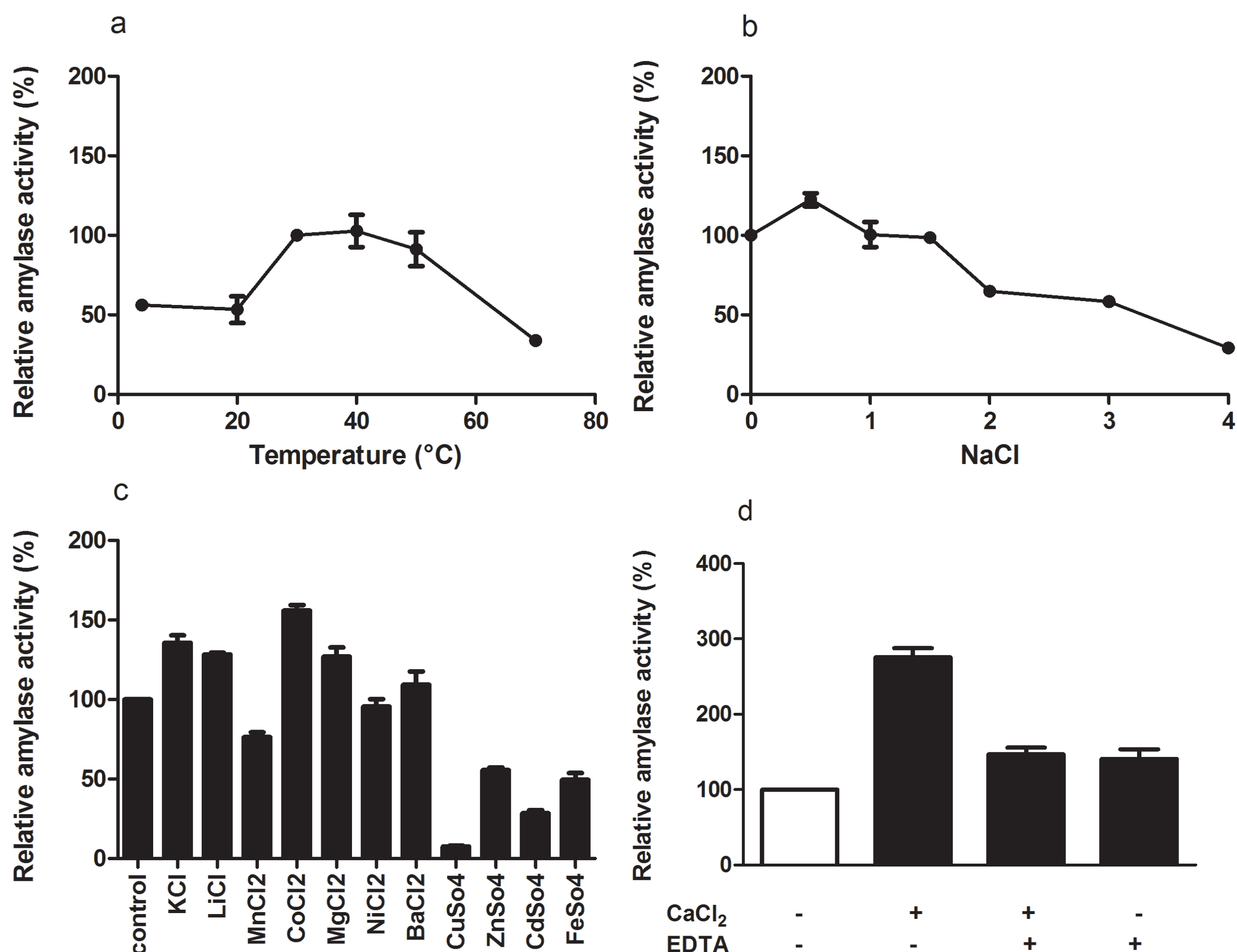

Figure 3. Effect of temperature, NaCl concentrations, various ions, and Ca^{2+} on amylolytic activity of partially purified amylase from hepatopancreas of *N. granulata*. (a) Effect of temperature (4–70 °C). The activity was measured at pH 5.2 in the presence of 15 mg mL^{-1} of starch. The activity values are expressed in relation to the activity at 30 °C (100% = 4.3 mg maltose mg^{-1} protein min^{-1}). (b) Effect of NaCl concentrations (0–4 M). The activity was measured at pH 5.2 and 30 °C in the presence of 15 mg mL^{-1} of starch. The activity values are expressed in relation to the activity in the absence of NaCl (100% = 6.16 mg maltose mg^{-1} protein min^{-1}). (c) Effect of ions. The activity was measured in the presence of 15 mg mL^{-1} of starch at pH 5.2, 30 °C, in the absence (control) or the presence of the indicated ions (5 mM) (chloride or sulfate salts). The activity values are expressed in relation to the control (100% = 7.5 mg maltose mg^{-1} protein min^{-1}). (d) Effect of Ca^{2+}. The activity was determined in the presence of 15 mg mL^{-1} of starch at pH 5.2, 30 °C, in the absence or presence of 5 mM $CaCl_2$, 5 mM EDTA, or both. The activity values are expressed in relation to the activity in the absence of $CaCl_2$ or EDTA (100% = 3.3 mg maltose mg^{-1} protein min^{-1}). The values are the average of triplicate assays.

Table). The additional 30 kDa species found by zymogram analysis in the hepatopancreas from crabs acclimated to low salinity (10 psu) (hyperregulation conditions) (Figure 1b) suggests that mechanisms of modulation at the molecular level occur, which could lead to the higher total amylase activity detected by biochemical analysis. Whether a differential expression/synthesis of amylase and/or posttranslational modifications are occurring upon acclimation to low salinity, as well as hormonal regulation, remains to be investigated. In mammals, amylase synthesis in the pancreas is under hormonal control (Mössner et al., 1985, 1989; Tsai et al., 1994). The mechanisms of regulation by chemical messengers of digestive enzymes in the hepatopancreas of crustaceans have not been elucidated yet. Recently, we showed that dopamine and glucagon modulate the activity of key digestive enzymes in the hepatopancreas of *N. granulata* (Michiels et al., 2015a, 2015b). An ecdysteroid-responsive amylase gene was identified in the hepatopancreas of the red crayfish *Procambarus clarkii* (Peng et al., 2015). The additional

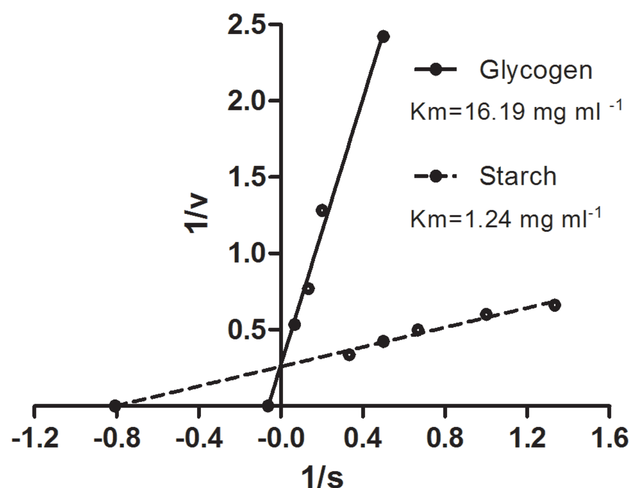

Figure 4. Lineweaver–Burk plot of activity of partially purified amylase from the hepatopancreas of *N. granulata* using starch or glycogen as substrates. The corresponding activities were measured at pH 5.2 and 30 °C. The values are the average of triplicate assays.

amylase form in the hepatopancreas of *N. granulata* at low salinity could be associated with a differential digestive capacity for glycogenic substrates, which would lead to modifications in glucose availability. In vitro experiments with *N. granulata* from other geographical areas showed the de novo synthesis of glycogen in posterior gills via uptake of glucose and suggested that adjustments in this uptake could be a strategy to support varying environmental conditions (Kucharski et al., 2002; Valle et al., 2009). Hyper- and hypoosmoregulation in crabs appear to require different mechanisms (McNamara and Faria, 2012; Romano and Zeng, 2012). The results of this work suggest that this appears to also be the case for amylase forms in the hepatopancreas of *N. granulata* since, unlike at low salinity, no differences were found at high salinity under hyporegulatory conditions (Figure 1; Table). Whether differential mechanisms are operating upon osmotic adaptation to low and high salinity leading to a differential use of digestive and metabolic pathways has yet to be elucidated.

To further characterize amylolytic activities present in the hepatopancreas of *N. granulata*, we carried out a partial purification from the hepatopancreas of crabs acclimated to 35 psu, which included ammonium sulfate precipitation as an initial step and FLPC chromatography of the active fractions (Figure 2). Zymogram analysis of pool B with the highest amylolytic activity obtained from the FPLC column revealed the presence of the 29 kDa form (Figure 2b). Therefore, this pool was used as an enzyme source (partially purified amylase) from the hepatopancreas of *N. granulata* and further characterized (Figures 3 and 4).

The maximal activity within the range of 30–40 °C for amylolytic activity (Figure 3a) is similar to that found for partially purified amylase from the copepods *Acartia clausi* and *Heliodiaptomus viduus* (Mayzaud, 1985; Dutta et al., 2006). Amylolytic activity was maintained at low (4 °C) and high (70 °C) temperatures (56% and about 34% of activity at 30 °C, respectively) (Figure 3a). Since in the Mar Chiquita coastal lagoon, *N. granulata* is exposed to a wide range of temperatures (Spivak, 1997; Luppi et al., 2013; personal observations), an extreme temperature-tolerant amylolytic activity in the hepatopancreas may play a role in thermal acclimation, as we suggested for lipase and aminopeptidase-N activities (Michiels et al., 2015 a, 2015b).

The effect of varying NaCl concentrations and of several ions is commonly used for characterization of amylases from crustaceans since they can differentially regulate the activity, although the effects (activation/inhibition, no effect) appear to be species-specific (Mayzaud, 1985; Dutta et al., 2006; Figueiredo and Anderson, 2009; Castro et al., 2012; Muralisankar et al., 2014; Wu et al., 2014). The presence of specific ions along with food content can inhibit or enhance amylase activity and therefore differentially affect the rate of carbohydrate digestion (Dutta et al., 2006; Coccia et al., 2011; Muralisankar et al., 2014; Wu et al., 2014). In most marine and brackish waters, the main dissolved inorganic ions come from NaCl; thus, salinity constitutes a measure of the water content of this dissolved salt (Romano and Zeng, 2012; Kültz, 2015). In the mudflats of the Mar Chiquita coastal lagoon, adult males of *N. granulata* prefer to feed during submersion periods but can also can eat sediments during emersion periods while the soil remains wet (Bas et al., 2014). Furthermore, they can tolerate high concentrations of zinc, copper, and manganese, which accumulate in the hepatopancreas (Beltrame et al., 2010, 2011). Taking into account these facts, it was of interest to evaluate the effect of varying concentrations of NaCl and of several ions on partial purified amylolytic activity, This activity was not affected by high NaCl concentrations within 0.5–1.5 M, and it was maintained at values of about 50% and 30% of the maximal activity even in the presence of 2–3 M and 4 M NaCl, respectively. This outcome indicates that this amylolytic activity is halotolerant. This differs from results shown for partially purified amylase from the copepod *H. viduus*, which exhibits maximum activity in the absence of NaCl, which was attributed to conditions experienced in its freshwater habitat (Dutta et al., 2006). Partially purified amylolytic activity from the hepatopancreas of *N. granulata* was higher in the presence of K^+, Li^+, Co^{2+}, and Mg^{2+}, while Ni^{2+} and Ba^{2+} had no effect (Figure 3c). Partially purified amylase activity from the copepod *H.*

viduus was reported to be increased by K^+, Co^+, Ni^{2+}, and Ba^{2+} (Dutta et al., 2006). These results support the idea that the effect of ions on amylolytic activity is species-specific (Castro et al., 2012). On the other hand, enzyme activity of *N. granulata* was lower in the presence of Mn^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, and Fe^{2+} (Figure 3c). Whether the diminished activity could represent a potential impact on carbohydrate digestion and/or absorption remains to be addressed. Ca^{2+} is a potent activator of amylase from the hepatopancreas of *C. maenas* (Bladamer et al., 1966). The fact that the amylolytic activity from *N. granulata* was enhanced by Ca^{2+}, but not in the presence of the EDTA, suggests the specific effect of the cation. A Ca^{2+} binding site has been suggested to be a common structural feature of all amylases to stabilize the active site (Boel et al., 1990; D'Amico et al., 2000; Peng et al., 2015).

The Michaelis–Menten kinetics of partially purified amylase from the hepatopancreas of *N. granulata* in response to varying starch concentrations (Figure 4) is in agreement with purified amylase from the copepods *A. clausi* and *H. viduus* (Mayzaud, 1985; Dutta et al., 2006), and to what we previously described for total amylase activity in the hepatopancreas of this crab (Asaro et al., 2011). The results in Figure 4 show that partially purified amylase from the hepatopancreas of *N. granulata* also exhibited ability to hydrolyze glycogen, similarly to partially purified amylase from the copepod *H. viduus* (Dutta et al., 2006). The ability to hydrolyze starch and glycogen suggests the capacity of *N. granulata* to utilize glycogenic carbohydrates from vegetal and/or animal sources, which is in agreement with its dietary habits in the mudflat of the Mar Chiquita lagoon (Iribarne et al., 1997; Pinoni et al., 2011; Bas et al., 2014). The ability to hydrolyze glycogen could also be linked to the capacity for using this reserve in the hepatopancreas, which is a site of glycogen storage and exhibits a high content of free glucose (Pinoni et al., 2011). In decapod crustaceans, it has been proposed that when the internal reserves of cells must be mobilized, digestive enzymes could be activated intracellularly and finely regulated (Sanchez-Paz et al., 2006). Recently, we showed the modulation of intracellular lipase in parallel with the regulation of triglyceride reserves in the hepatopancreas of *N. granulata* (Michiels et al., 2015b).

In summary, our results show the occurrence of several forms with amylolytic activity in the hepatopancreas of the intertidal crab *N. granulata*, with a major form of 29 KDa. The extra band in individuals acclimated to 10 psu indicated that a number of forms can be affected by external factors and furthermore suggests the possible occurrence of mechanisms of modulation at the molecular level of amylase as part of the complex process of biochemical adaptation to environmental salinity. In addition, the partial purification of the major amylase form present and its further characterization showed biochemical characteristics (halotolerant, differential sensitivity to different ions, and capability to degrade glycogen besides starch) that could be related to environmental conditions and diet experienced by this crab in its natural environment. Future studies should be focused on elucidating the exact physiological consequence of the occurrence, modulation, and characteristics of amylase forms (i.e. differential key carbohydrates' digestive capacity) in the hepatopancreas of *N. granulata* in particular and in intertidal euryhaline crabs in general.

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