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Amylase, maltase and sucrase activities in hepatopancreas of the euryhaline crab *Neohelice granulata* (Decapoda: Brachyura: Varunidae): partial characterization and response to low environmental salinity

ANTONELA ASARO [1,2], JUANA CRISTINA DEL VALLE [1] and ALEJANDRA ANTONIA LÓPEZ MAÑANES [1,3]

[1] Departamento de Biología, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, Mar del Plata, Argentina. E-mail: mananes@mdp.edu.ar
[2] CIC, Buenos Aires, Argentina.
[3] CONICET, Argentina.

SUMMARY: Studies on digestive adjustments at the biochemical level in relation to salinity in euryhaline crabs are lacking. Moreover, knowledge of biochemical digestive characteristics of euryhaline crabs (i.e. occurrence and characteristics of key digestive enzyme activities) is still scarce and fragmentary. We studied the occurrence, characteristics and response to low salinity of amylase, maltase and sucrase activities in the hepatopancreas of the euryhaline crab *Neohelice* (*Chasmagnathus*) *granulata*. Maximal amylase and maltase activities were found at pH 5.2. Sucrase activity was maximal within the pH range 3.6-5.2. Amylase, maltase and sucrase activities showed a Michaelis-Menten kinetics (km = 0.41±0.10 mg ml^{-1}; 1.37±1.03 mM and 0.55±0.45 mM, respectively). In crabs acclimated to low salinity (10 psu; hyperregulating conditions), amylase activity (7263±980 μg maltose min^{-1} mg $prot^{-1}$) was higher than in 35 psu (osmoconforming conditions) (3605±340 μg maltose min^{-1} mg $prot^{-1}$). Maltase and sucrase activities (497±98 and 64±16 μg glucose min^{-1} mg $prot^{-1}$, respectively) were similar in both salinities. The response of amylase activity to low salinity suggests a role in digestive adjustments upon hyperregulation. This study contributes to a better understanding of the complexity of the biochemical adaptations to low salinity in euryhaline crabs.

Keywords: euryhaline crabs, amylase, maltase, sucrase, hepatopancreas, hyperregulation.

RESUMEN: Actividades de amilasa, maltasa y sacarasa en hepatopáncreas del cangrejo eurihalino *Neohelice granulata* (Decapoda: Brachyura: Varunidae): caracterización parcial y respuesta a baja salinidad ambiental. – El conocimiento sobre las características digestivas a nivel bioquímico en cangrejos eurihalinos es aún escaso y fragmentario. Así, faltan estudios sobre identificación y caracterización de actividad de enzimas digestivas clave en el hepatopáncreas y sobre el posible efecto de la salinidad sobre las mismas. Se estudió la existencia, características bioquímicas y respuesta a baja salinidad de amilasa, maltasa y sacarasa en el hepatopáncreas del cangrejo eurihalino *Neohelice (Chasmagnathus) granulata*. La mayor actividad de amilasa y maltasa se encontró a pH 5.2. La actividad de sacarasa fue máxima dentro de un rango de pH de 3.6-5.2. Las actividades de amilasa, maltasa y sacarasa mostraron una cinética michaeliana (km=0.41±0.10 mg ml^{-1}; 1.37±1.03 mM y 0.55±0.45 mM, respectivamente). En cangrejos aclimatados a baja salinidad (10 psu, condición de hiperregulación), la actividad de amilasa fue mayor (7263±980 μg maltosa min^{-1} mg $prot^{-1}$), que en individuos aclimatados a 35 psu (condición de osmoconformación) (3605±340 μg maltosa min^{-1} mg $prot^{-1}$). Las actividades de maltasa y sacarasa (497±98 y 64±16 μg glucosa min^{-1} mg $prot^{-1}$, respectivamente) fueron similares en ambas salinidades. La respuesta de la actividad de amilasa a baja salinidad permite sugerir un rol en mecanismos de ajustes bioquímicos secundarios a la hiperregulación. Los resultados constituyen un aporte relevante al conocimiento sobre las complejas adaptaciones a nivel bioquímico en respuesta a baja salinidad en cangrejos eurihalinos.

Palabras clave: cangrejos eurihalinos, amilasa, maltasa, sacarasa, hepatopáncreas, hiperregulación.

INTRODUCTION

Euryhaline crabs inhabiting coastal waters, tide areas or estuaries are exposed to frequent and abrupt changes in the environmental salinity that require biochemical, physiological, morphological and/or behavioural strategies for controlling movements of water and ions between the individuals and their medium (Anger, 2001). In low salinities, hyperregulating crabs maintain the hemolymph osmotic concentration above that of the external medium by absorbing both sodium and chloride from the environment mainly via the posterior gills. Hyperregulation is a costly physiological process requiring a metabolic reorganization to support the energy demands necessary for activation of the osmoregulatory machinery (Freire *et al.*, 2008). In spite of the extensive work that has been done on different aspects of the adaptive responses of hyperregulating crabs to low salinity, studies on the possible digestive adjustments at the biochemical level (i.e. modulation of the activity of key digestive enzymes) are lacking. Moreover, it is striking that in comparison with the broad knowledge of other decapod crustaceans, information on the occurrence and biochemical characteristics of key digestive enzyme activities in the hepatopancreas of euryhaline crabs is still scarce and fragmentary. The effect of extreme environmental salinity on digestive enzymes activities (i.e. total amylase activity) has been shown to occur in the hepatopancreas of the euryhaline pacific white shrimp *Litopenaeus vannamei*, suggesting the possibility of deriving extra energy upon osmoregulation (Li *et al*., 2008).

In decapod crustaceans, the maintenance of suitable levels of glucose in the hemolymph is essential for supporting several key functions and in the response to various environmental stress (Lorenzon, 2005). The digestion of glycogenic carbohydrates (i.e. complex polysaccharides such as starch and disaccharides) is one of the main sources of hemolymphatic glucose (Verri *et al*., 2001). The ability to digest starch depends on the existence and level of activity in the hepatopancreas (the main site of digestive enzyme production) of the key enzymes intervening in its degradation (i.e. amylase and maltase). Though amylase activity has been detected in the hepatopancreas of *Carcinus maenas* (Johnston and Freeman, 2005), *Uca minax*, *U. pugnax* and *U. pugilator* (Azzalina and Trainer 1985), information on the biochemical characteristics and response to environmental salinity of euryhaline crabs is lacking. A suitable level of maltase and other disaccharidases (i.e sucrase) in the hepatopancreas would further allow the potential utilization of dietary specific glycogenic disaccharides (i.e. maltose and sucrose) as glucose sources. The information about maltase and sucrase activities in the hepatopancreas of crabs is scarce. To our knowledge, so far the study of McClintock *et al*. (1991), which showed the occurrence of maltase and sucrase activity in hepatopancreas of *Callinectes sapidus*, is the only one done in a euryhaline crab.

Neohelice (*Chasmagnathus*) *granulata* Dana, 1851 is a semi-terrestrial euryhaline crab that is found from southern Brazil to Patagonia (Argentina) (Spivak, 1997). In Mar Chiquita coastal lagoon (Buenos Aires Province, Argentina) it is one of the dominant crabs in the outer parts, where it is exposed to highly variable environmental salinity ranging from 4 to 35 psu. The differential responses upon acclimation to low salinity of various key energy-demanding enzyme activities in individual gills and in chela muscle of *N. granulata* from the mudflat area of Mar Chiquita lagoon show the occurrence of complex and integrative responses at the biochemical level upon hyperregulation (López Mañanes *et al*., 2000; Schleich *et al*., 2001; Pinoni *et al*., 2005; Pinoni, 2009; Pinoni and López Mañanes, 2009). Thus, *N. granulata* is an excellent model for studying the integrative responses at the biochemical level to environmental salinity. Studies on different aspects of the biochemical digestive physiology increase our understanding of the biochemical-physiological strategies underlying hyperregulation. However, studies on the modulation of digestive enzymes, particularly in relation to environmental factors (i.e. salinity) are lacking. Moreover, although field studies demonstrate that *N. granulata* living in the mudflat area of Mar Chiquita lagoon shows an omnivorous-detritivorous behaviour (Iribarne *et al.*, 1997), nothing is known about the digestive battery at the biochemical level (i.e. occurrence and level of carbohydrases) underlying this feeding behaviour. To increase knowledge of different aspects of the biology of *N. granulata* as part of our integrative studies on the identification of enzyme activities involved in biochemical adaptations to salinity in euryhaline crabs, the aims of this work were to determine the occurrence, characteristics and response to low salinity of amylase, maltase and sucrase activities in hepatopancreas of *N. granulata* from Mar Chiquita coastal lagoon. We hypothesized that the modulation of key digestive enzymes in the hepatopancreas constitutes a digestive adjustment at the biochemical level in *N. granulata*, allowing a positive energy balance under hyperregulation conditions. In this context, we predict that amylase, sucrase and maltase activities in the hepatopancreas should be enhanced upon acclimation of *N. granulata* to low salinity. To our knowledge this is the first attempt to determine the occurrence of amylase and disaccharidase in hepatopancreas of this crab and the first work to evaluate the possible effect of environmental salinity on these activities in a euryhaline crab. This work will increase the scarce knowledge of digestive biochemical characteristics and responses in euryhaline crabs.

MATERIALS AND METHODS

Chemicals

Malic acid, 3,5-Dinitrosalicylic acid, Tris-(hydroxymethylamino-methane) (Tris), hydrochloridic

acid, dibasic sodium phosphate, monobasic sodium phosphate, sodium potassium tartrate tetrahydrate, sodium hydroxide and bovine serum albumin were from Sigma (St. Louis, MO, USA); sucrose was from Merck (Darmstadt, Germany); maltose was from ICN (Ohio, USA); Coomassie Blue G250 was from Fluka (Germany). All solutions were prepared in glass-distilled water.

Animal collection and maintenance

The crabs were caught from the mudflat area of Mar Chiquita coastal lagoon (Buenos Aires, Province Argentina) (37º32' 37º45'S; 57°19' 57°26'W), which exhibited variations in salinity ranging from 4 to 35 psu. For all 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) or dilute seawater (10 psu) for at least 10 days prior to use. 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. Aquaria were shielded by black plastic to reduce disturbance. Crabs were fed 3 times a week with commercial food (Cichlind T.E.N., Wardley, USA) (48% carbohydrates, 40% protein, 3% fat, 4% fibre; about 0.07 g individual^{-1}) but they were starved 48 h prior to the experiments. Our laboratory results show that no differences in the feeding behaviour occur in *N. granulata* in the experimental conditions used in this work and that behavioural and osmoregulatory responses are not altered by short-term starvation (Pinoni and López Mañanes, unpublished results; Asaro *et al*, unpublished results).

Preparation of enzyme hepatopancreas extract

The crabs were cryoanesthetized by putting them on ice for about 20 min. The hepatopancreas was immediately excised, mixed with homogenizing medium (0.1 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 x g for 15 min. The hepatopancreas from 1 individual was used for each preparation of enzyme extract. The supernatant was fractionated into 500 ul aliquots and stored at –20°C until use. Glycerol (1.3% v/v) was added to samples before freezing.

Assay of amylase activity

Amylase activity was determined using the method described by Biesiot and Capuzzo (1990), with some modifications. In the standard assay, amylase activity was determined in a reaction medium 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 corresponding sample (linearity zone on activity vs protein concentration plot). Following incubation for 15 min, dinitrosalicylic acid reagent (DNS) was added and the reaction medium was boiled for 10 min. Assay tubes were immediately cooled in ice and the amount of released maltose was determined by reading the absorbance at 540 nm. To study the effect of starch concentration and pH on amylase activity, the procedure was the same as that described above except that the activity was determined in the presence of varying starch concentrations or at different pH levels of the reaction mixture, respectively. Five 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 previous thawing.

Assay of disaccharidase activities

In the standard assay, maltase and sucrase activities were assayed by measuring the glucose released from the hydrolysis of the corresponding substrate (maltose and sucrose, respectively). The reaction was initiated by adding an aliquot of the corresponding sample (linearity zone on activity vs protein concentration plot) to a reaction mixture containing 28.0 mM of the corresponding substrate in 0.1 M maleate/NaOH buffer (pH 5.2) at 37°C. After incubation for 10 min, the reaction was stopped by addition of 1.5 mL of the combined enzyme colour glucose reagent solution (glucose oxidaseate gluc 10 kU l^{-1}, 1 kU peroxidase, 0.5 mmol l^{-1} l, 4-aminophenazone, 100 mmol l^{-1} phosphates pH 7.0, 12 mmol l^{-1} hydroxybenzoate) (Wiener Lab Kit cod. 1400101). After 5 min at 37°C, the amount of glucose released was determined by reading the absorbance at 505 nm of the coloured quinone complex (Beckman UV-visible spectrophotometer). To study the effect of maltose or sucrose concentration and pH on the corresponding activity, the procedure was the same as that described above except that the activities were determined in the presence of varying substrate concentrations or at different pH levels of the reaction mixture, respectively. Five individuals acclimated to 35 salinity were used in these experiments. The determination of enzyme activities was always performed with samples that had been stored at –20°C, without previous thawing.

Hemolymph osmolality and ionic concentration

Hemolymph (about 500 µl) was sampled from the infrabranchial sinus of 4-9 individuals by mean of a syringe at the base of the cheliped and transferred to an iced centrifuge tube. Serum was separated by centrifugation at 10000 × g (Beckman, Microfuge, B) for 30 s. Osmolality was measured with a micro-osmometer (Radiometer, Copenhagen, 3MO). Sodium ion was determined by flame photometry. Chloride ion was determined by a colorimetric method (Randox Commercial

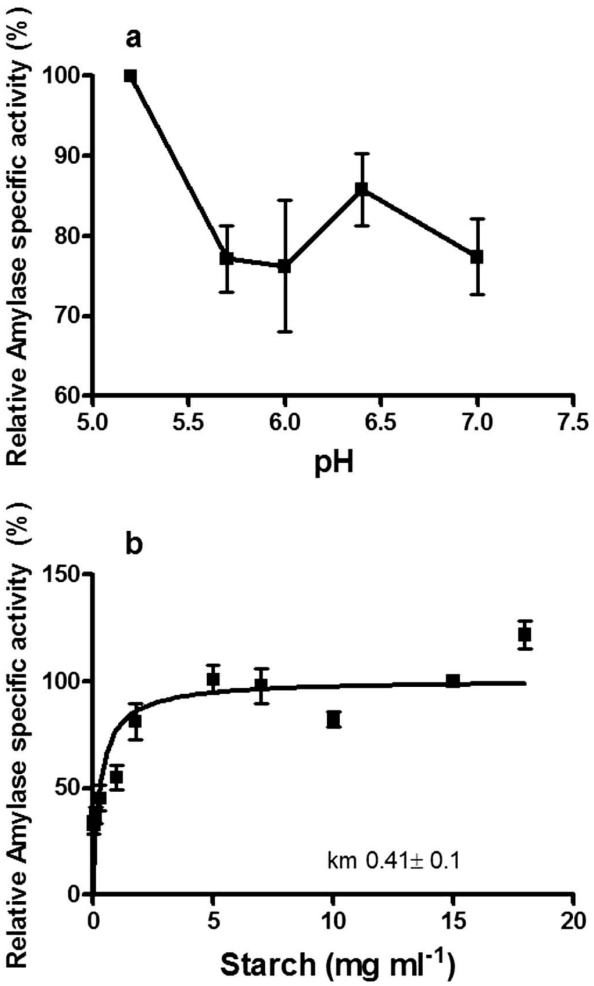

FIG. 1. – (a) Effect of pH (5.2-7.0) on amylase activity in hepatopancreas of *N. granulata*. The amylase activity values are expressed as relation to the specific activity at pH 5.2 (100%). Data are the mean ±SE for 5 individuals. (b) Effect of starch concentration on amylase activity in hepatopancreas of *N. granulata*. The curves are the ones which best fit the experimental data (GraphPad Prism 2.01). The activity values are expressed as relation to the corresponding activity in the presence of 15 mg ml^{-1} starch (100%). In some cases, error bars were smaller than the symbols used. Data are the mean ±SE for 5 individuals.

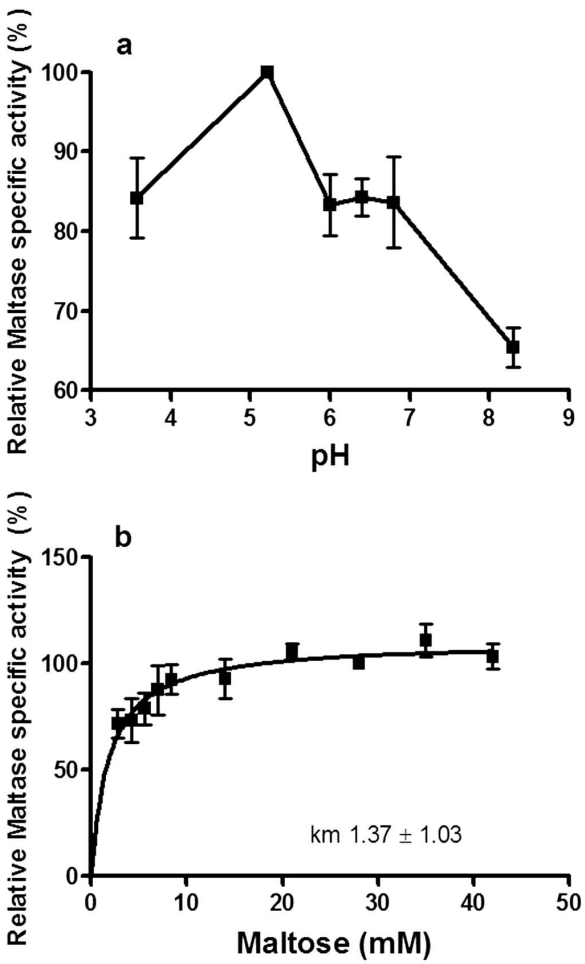

FIG. 2. – a) Effect of pH (3.5-8.3) on maltase activity in hepatopancreas of *N. granulata*. The maltase activity values are expressed as relation to the specific activity at pH 5.2 (100%). Data are the mean ±SE for 5 individuals. (b) Effect of maltose concentrations on maltase activity in hepatopancreas of *N. granulata*. The curves are the ones which best fit the experimental data (GraphPad Prism 2.01). The activity values are expressed as relation to the corresponding activity in the presence of 28 mM maltose (100%). In some cases, error bars were smaller than the symbols used. Data are the mean ±SE for 5 individuals.

Kit) based on the formation of a blue Fe-2,4,6-tri-(2-pyridyl)-1.3.5-triazine–ferrous sulphate complex.

Protein determination

Protein was assayed according to the method of Bradford (1976). Bovine serum albumin was used as standard.

Statistical analysis

Statistical analyses were performed using the Sigma Stat statistical package for Windows operating system, which automatically performs a previous test for equal variance and normality. A parametric (one-way ANOVA) analysis of variance was used. An a posteriori ANOVA test (Holm–Sidak) was used to identify differences and $P<0.05$ was considered significant. Results of effect of varying concentrations of amylase, maltose and sucrose were analyzed by means of non-linear regression analysis (GraphPad Prism 2.01 software). The corresponding curves shown are those which best fit the experimental data. Michaelis-Menten constant (km) values were estimated by analysis of these curves (GraphPad Prism 2.01 software).

RESULTS

Amylase activity was determined within the range of pH 5.2-7.0. Maximal activity was found at pH 5.2. The activity was similar within the range of 5.7-7.0, being about 20% lower than the activity at pH 5.2 (Fig. 1a).

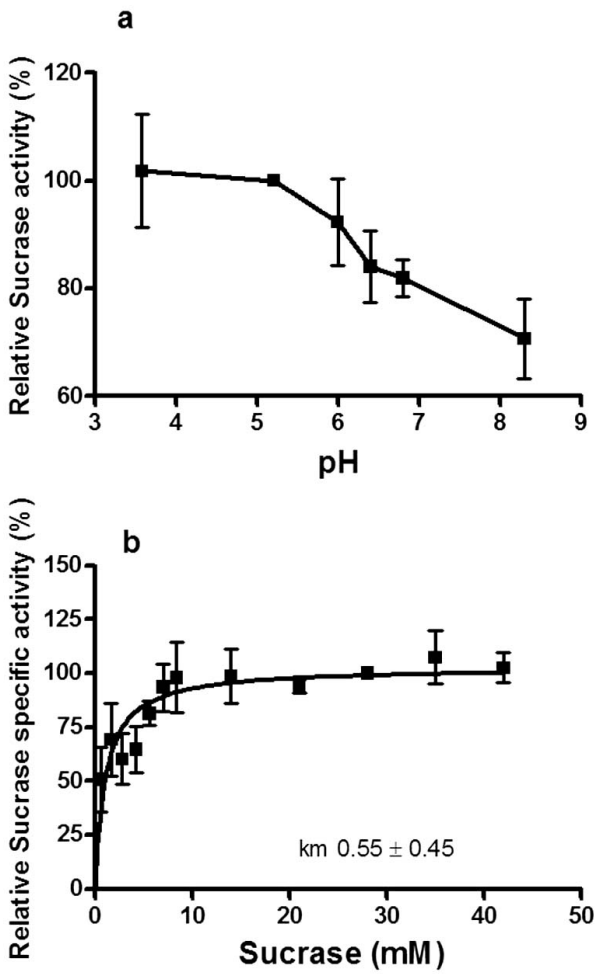

FIG. 3. – (a) Effect of pH (3.5-8.3) on sucrase activity in hepatopancreas of *N. granulata*. The sucrase activity values are expressed as relation to the specific activity at pH 5.2 (100%). Data are the mean ±SE for 5 individuals. (b) Effect of sucrose concentrations on sucrase activity in hepatopancreas of *N. granulata*. The curves are the ones which best fit the experimental data (GraphPad Prism 2.01). The activity values are expressed as relation to the corresponding activity in the presence of 28 mM sucrose (100%). In some cases, error bars were smaller than the symbols used. Data are the mean ±SE for 5 individuals.

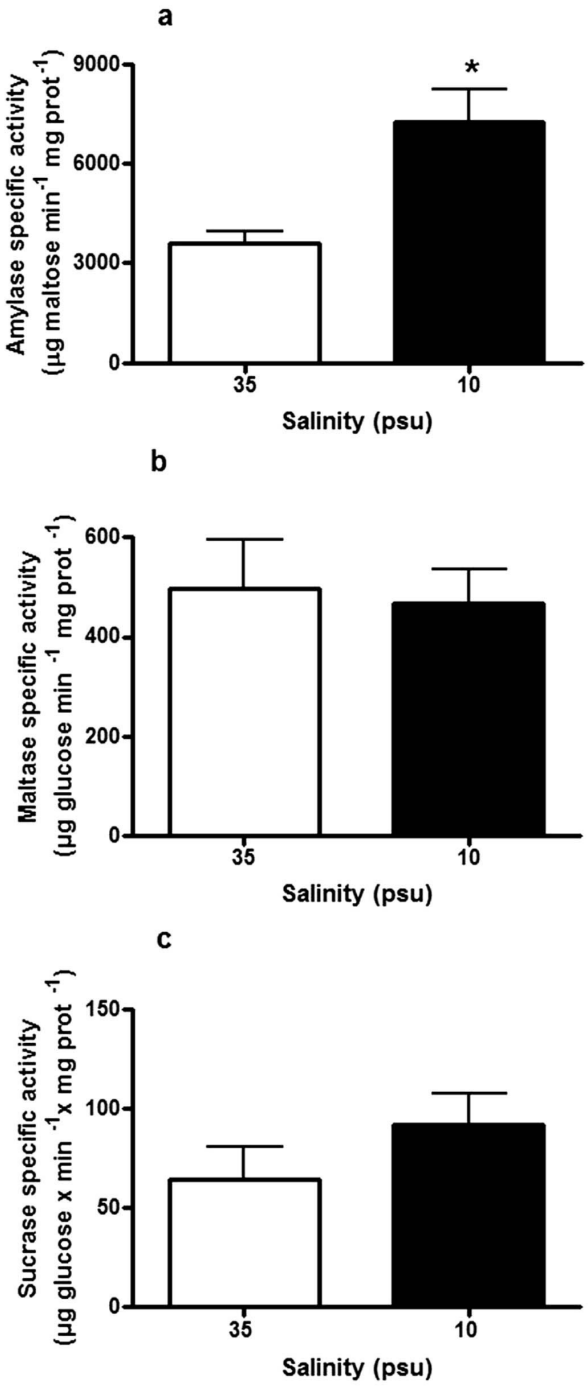

FIG. 4. – Effect of acclimation to low salinity on amylase (a), maltase (b) and sucrase (c) specific activities in hepatopancreas of *N. granulata*. *Significantly different from the corresponding activity of individuals acclimated to 35 psu (P<0.05). Data are the mean ±SE for 5 individuals.

The effect of starch concentrations on amylase activity is shown in Figure 1b. Amylase activity in hepatopancreas of *N. granulata* exhibited Michaelis–Menten kinetics (apparent km=0.41±0.10 mg ml^{-1}).

Maltase activity in hepatopancreas was determined within the range of pH 3.5-8.3. Maximal maltase activity was found at pH 5.2 (Fig. 2a). At pH 6.0-6.8 maltase activity decreased but to values similar to those found at pH 3.5. At pH 8.3 the activity was about 65% of the activity at pH 5.2. The effect of maltose concentrations on maltase activity of hepatopancreas of *N. granulata* is shown in Figure 2b. Maltase activity showed Michaelis-Menten kinetics (apparent km=1.37±1.03 mM).

Sucrase activity in hepatopancreas was determined within the range of pH 3.5-8.3. Highest sucrase activity was found at pH 3.5-5.2. At pH 6.0-6.8 activity decreased slightly, being about 80-90% of the activity at pH 5.2. At pH 8.3 sucrase activity further decreased, being about 70% of the activity at pH 5.2 (Fig. 3a).

The effect of sucrose concentrations on sucrase activity of hepatopancreas of *N. granulata* is shown in

TABLE 1. – Concentration of ions (mEq l^{-1}) and osmolality (mOsm kg^{-1}) in external medium and in *N. granulata* hemolymph

	35 psu		10 psu	
	Medium	Hemolymph	Medium	Hemolymph
Na^+	427.9±19.9	419±14.9	148.2±8.1	341±13.1*
K^+	10.1±0.5	11.3±0.5	3.4±0.1	9.2±0.4*
Cl^-	492±13.5	427±26.5	167±7.7	384,7±30,2*
Osmolality	902.9±52.2	868.8±14.0	311±5.6	689.7±48.3*

*Significantly different from the corresponding concentration of the external medium (ANOVA, $P < 0.05$). Data are the mean ±SE of 4-9 individuals

Figure 3b. Sucrase activity showed Michaelis-Menten kinetics (apparent km = 0.55 ± 0.45 mM).

To determine the effect of low environmental salinity on amylase, maltase and sucrase activities in hepatopancreas of *N. granulata*, specimens were acclimated to salinities 35 and 10, at which this crab osmoconforms and hyperegulates, respectively (Table 1).

In crabs acclimated to 35 psu, hepatopancreas exhibited a high amylase activity (3605±340 μg maltose min^{-1} mg prot $^{-1}$). However, in individuals acclimated to low salinity (10 psu) amylase activity (7263±980 μg maltose min^{-1} mg $prot^{-1}$) was about 100% higher than the activity at 35 psu (ANOVA, F=0.066, P<0.05) (Fig. 4).

Crabs acclimated to 35 psu also exhibited a high maltase activity (497 ± 98 μg glucose min^{-1} mg $prot^{-1}$). This activity was not affected by acclimation of crabs to 10 psu (467±67 μg glucose min^{-1} mg prot $^{-1}$) (ANOVA, F=0.066, P>0.05) (Fig. 4).

In crabs acclimated to 35 psu, sucrase activity was 64 ± 16 μg glucose min^{-1} mg prot $^{-1}$. No differences were found in this activity in 10 psu (91±15 μg glucose min^{-1} mg $prot^{-1}$) (ANOVA; F=1.49; P>0.05) (Fig. 4).

DISCUSSION

Our results show the occurrence of amylase, maltase and sucrase activities in hepatopancreas of *N. granulata* from Mar Chiquita coastal lagoon and the response of amylase activity to low environmental salinity. The high amylase and maltase activity in hepatopancreas of *N. granulata* from Mar Chiquita lagoon suggest the ability of this crab to perform complete starch degradation and, furthermore, the potential use of dietary maltose as a nutrient source. The occurrence of high sucrase activity also suggests this crab's capacity for digestion and potential use of sucrose as a nutrient sourceb. The pH for value maximal amylase activity in hepatopancreas of *N. granulata* (Fig. 1a) is quite different to those described for this activity in the hepatopancreas of the marine crab *Thalamita crenata* (Van Weel, 1960) and the mud crab *Scylla serrata* (Pavasovic *et al*., 2004) but is in agreement with optimum values at acid pH found for amylase activity in hepatopancreas of various decapod crustaceans (Johnston, 2003; Figueiredo and Anderson, 2009). The Michaelis–Menten kinetics of amylase activity of hepatopancreas of *N. granulata* in response to varying starch concentrations (Fig.1b) is in agreement with that previously described for amylase activity of hepatopancreas of the crayfish *Procambarus clarki* (Hammer *et al*., 2003). Information about the occurrence and biochemical characteristics of maltase and sucrase activity in hepatopancreas of decapod crustaceans, and particularly in crabs, is quite scarce. As in amylase activity, the pH value for maximal maltase activity found in hepatopancreas of *N. granulata* (Fig. 2a) appears to be different to that found in the marine crab *T. crenata* (Van Weel, 1960), but it is within the range of optimal acid pH values reported for maltase activity in hepatopancreas of other decapod crustaceans (Figueiredo and Anderson, 2009). Maltase activity in hepatopancreas of *N. granulata* exhibited a Michaelis–Menten kinetics (Fig. 2b) similar to that described for this activity in mammal intestine (del Valle and López Mañanes, 2008) and insect midgut (Fonseca *et al*., 2010). To our knowledge no report is available on the kinetic behaviour of maltase activity in hepatopancreas of decapod crustaceans. pH values for maximal activity and the Michaelis–Menten kinetics of sucrase activity in hepatopancreas of *N. granulata* (Fig. 3) are in agreement with those found by Saxena and Murthy (1982) for this activity in hepatopancreas of the shrimp *Macrobrachium lamarrei*. As in the amylase and maltase activities, pH values for maximal sucrase activity (Fig. 3) differ from those reported for the marine crab *T. crenata* (Van Weel, 1960). In crustaceans, digestive enzymes synthesized in the hepatopancreas appear to be released into the gastric chamber during the digestive cycle (Muhlia-Almazán and Garcia Carreño, 2003). Since the pH of gastric juice of *N. granulata* has been found to be between 4.7 and 6.6 (Buckup *et al*., 1991), the high activity at acidic pH and maintenance of about 70-90% of maximal activity over a wide range of amylase, maltase and sucrase activities in *N. granulata* could suggest a high hydrolytic capacity after secretion from the hepatopancreas. The ability of hyperregulating crabs to adapt to varying environmental salinities may involve responses from the molecular to the organism level. In low salinity, hyperregulating crabs absorb both sodium and chloride from the external medium mainly via the posterior gills, thus regulating their concentrations in the hemolymph and compensating for the salt losses (Freire *et al*., 2008). Although hyperregulation appears to involve a metabolic reorganization, little is known about the possible digestive adjustments at the biochemical level (i.e. activity of key digestive enzymes) that could improve the digestive capacity for essential energy substrates (i.e. carbohydrates). The higher amylase activity in hepatopancreas of *N. granulata* acclimated to 10 psu (Fig. 4), a salinity at which this crab exhibits a strong hyperregulatory capacity (Table 1), suggests a role of this enzyme in adjustments at the biochemical level secondary to hyperregulation. The higher amylase activity in hepatopancreas in low salinity could be associated with an enhanced digestive capacity for starch degradation which, in turn, could lead to an increase

in the glucose available to be used in organs or tissues involved in biochemical adaptation to low salinity in this crab (i.e. gills, chela muscle and hepatopancreas) (López Mañanes *et al.*, 2000; Schleich *et al*., 2001; Pinoni. 2009; Pinoni and López Mañanes, 2009). Previous work in our lab showed that, in *N. granulata* from the mudflats area of Mar Chiquita lagoon acclimated to low salinity, an increase in glycogen content occurred in posterior gills a long time after feeding, concomitant with a decrease in glucose hemolymph (Pinoni, 2009). In vitro experiments carried out with *N. granulata* from other geographical areas show the de novo synthesis of glycogen in posterior gills via uptake of glucose from the incubation medium (Kucharski *et al*., 2002) and suggest that adjustments in the uptake of glucose by posterior gills could be a strategy to support the varying environmental conditions in its natural habitat (Valle *et al*., 2009). The fact that maltase activity was similar in the hepatopancreas of crabs acclimated to 35 and 10 psu (contrary to our prediction) could indicate that the level of this activity is high enough to support a complete hydrolysis of starch. However, we cannot rule out the possibility that adjustments of the activity of other enzymes involved in the final steps of starch degradation occur in low salinity. Since nothing is known about the occurrence of these activities in *N. granulata*, further experiments are needed to test this hypothesis.

A sodium-dependent transport activity for fructose has recently been identified in the hepatopancreas of the lobster *Homarus americanus* (Sterling *et al*., 2009). The occurrence of a high sucrase activity in hepatopancreas of *N. granulata* suggests the capacity for digestion and potential use of sucrose not only as a glycogenic substrate but also as a fructose source. Since the sucrase activity in the hepatopancreas was not affected by acclimation to low salinity, it can be speculated that an enhanced capability for the degradation of sucrose does not constitute one of the biochemical adjustments underlying hyperregulation. However, since studies about the fructose metabolic and transporting pathways in *N. granulata* are lacking, further studies are needed to elucidate the biochemical-physiological role of sucrase activity (and other digestive enzyme activities yielding fructose) in hepatopancreas of this crab

The mechanisms of regulation (i.e. chemical messengers) or factors involved in the modulation of the activity and/or secretion of digestive enzymes in the hepatopancreas of crustaceans are far from having been elucidated**.** The fact that amylase activity in the hepatopancreas of *N. granulata* was affected by acclimation of crabs to low salinity, while maltase and sucrase activities were not (Fig. 4), suggests the occurrence of a differential and specific regulation of the activity of digestive enzymes upon hyperregulation. A differential modulation of the activity and secretion of specific digestive enzymes by various regulatory factors has been shown to occur in insects (Lwalaba *et al*., 2010). In the American crayfish *Orconectes limosus* in vitro experiments showed a differential release of digestive enzymes activities (i.e. amylase) from the hepatopancreas stimulated by several vertebrate gastrointestinal hormones (Resch-Sedlmeir and Sedlmeir, 1999). Whether this is the case for carbohydrase activities in hepatopancreas of *N. granulata* and, furthermore, whether the enhanced amylase activity in hepatopancreas in low salinity is due to modulation of pre-existent enzyme or by synthesis/degradation processes require further investigation.

In conclusion, the results of this study show the occurrence of high amylase, maltase and sucrase activities in hepatopancreas of *N. granulata*. The response of amylase activity to low salinity under hyperregulating conditions suggests the participation of this enzyme in responses to varying environmental salinity at the biochemical level. Whether amylase activity is involved in biochemical-physiological processes secondary to hyperregulation (i.e. digestive adjustments leading to differential capacity for digestion of key dietary items and for absorption of metabolites) remains to be investigated. Future studies should focus on establishing the exact physiological role of amylase activity in hepatopancreas and the regulation mechanisms involved, in order to provide a better understanding of the integrative responses of *N. granulata* to varying salinity and of the complex mechanisms underlying biochemical-physiological adaptations to environmental conditions in euryhaline crabs.

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