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Chitobiase of planktonic crustaceans from South Atlantic coast (Southern Brazil): Characterization and influence of abiotic parameters on enzyme activity

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ABSTRACT

Chitobiase is one of the enzymes involved in chitin degradation in nature. It is produced and released by a variety of organisms from bacteria to fish. In crustaceans, it is associated with digestive function and acts on the epidermis during the molting process. In the present study, the influence of water pH, temperature and salinity on maximum chitobiase activity (MCA), as well as the enzyme affinity (*Km*) for a substrate, the methylumbelliferyl N-acetyl-ß-D-glucosaminide (MUFNAG) was evaluated in the copepod *Acartia tonsa*. *Km* values for chitobiases of other crustaceans from the Patos Lagoon estuary and Cassino Beach (Southern Brazil) were also determined. For *A. tonsa*, MCA was observed at pH 5–6 and 30–35 °C. The range of pH was quite similar to that reported for other aquatic organisms. However, the range of temperature was lower than that previously reported. For salinity, no previous studies have considered the influence of this parameter on MCA. For *A. tonsa*, MCA was observed in freshwater, showing a significant linear decrease with increasing salinity. Considering that maximum copepod survival and growth rates are observed between 15 and 25 ppt, these findings suggest that the observed enzyme activity in this range of salinity (68 to 47% of that measured in freshwater) is not a limiting factor for *A. tonsa* growth. However, the extremely decreased enzyme activity observed in salinity 30 ppt (33% of that measured in freshwater) suggests that chitobiase activity might be one of the limiting factor for copepod growth at 30 ppt salinity or higher. *Km* values (μM) determined for organisms evaluated in the present study (copepod *A. tonsa* = 20.77; mysid *Metamysidopsis elongata atlantica* = 14.67; nauplii barnacle *Balanus improvisus* = 18.19; decapod zoea = 14.30; decapod megalopa = 24.77) were lower than those reported for other crustaceans from Northern Hemisphere. Also, they were much lower than those of organisms from different taxonomic groups like bacteria and fungi, but much higher than in protozoans and dinoflagelates. These findings suggest that chitobiase might be differentially evolved in each specific group of organism, and even within different ontogenetic stages of the same species, for a better adaptation to cope with its respective environmental needs.

1. Introduction

Chitin is one of the more abundant polysaccharides in nature. It is part of the exoskeleton of invertebrates like insects and crustaceans. Therefore, enzymes degrading chitin are produced by a variety of organisms for digestion like in bacteria (Vrba et al., 1993), gastropod mollusks (Brendelberger, 1997), fish (Lindsay, 2006), and some omnivore crustaceans (Saborowski and Buchholz, 1999). In insects and crustaceans, these enzymes are also involved in molting, thus being necessary for animal growth (Chang, 1993).

Two enzymes play a key role in chitin digestion during crustacean molting. Chitinase degrades chitin in oligosaccharides, including dimers and trimers of N-acetyl-ß-D-glucosaminide. In turn, chitobiase (N-acetyl-ß-D-glucosaminidase) degrades these oligosaccharides into monomers (Muzzarelli, 1977), which will be re-absorbed into the new cuticle (Buchholz, 1989).

Due to its importance in the life cycle of crustaceans, chitobiase has been employed in aquatic pollution studies (Richards et al., 2008; Zou and Fingerman, 1999a,b) and as index of planktonic secondary production (Oosterhuis et al., 2000; Sastri and Dower, 2006). Some studies consider the enzyme from tissues of the studied animal, while others are based on the analysis of the free chitobiase in the water (Oosterhuis et al., 2000; Sastri and Dower, 2006). In this compartment, different sources of enzyme might contribute for the observed concentration. Therefore, it is important to characterize the kinetic properties to test the activity of the desired enzyme.

Chitobiases from organisms of different taxonomic groups have been characterized (O'Connell et al., 2008; Oosterhuis et al., 2000; Zou and Fingerman, 1999c). However, chitobiases from zooplanktonic crustaceans are still not well studied. Furthermore, most studies are performed on species from the Northern Hemisphere adapted to

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temperatures typical from temperate/sub-polar environments (Oosterhuis et al., 2000; Sastri and Dower, 2006).

Zooplanktonic organisms are recognized for their importance as the secondary trophic level in food chains. In general, crustaceans dominate assemblies in coastal environments (Sabatini and Martos, 2002). They can be part of the holoplankton, such as copepods and mysids (Wooldridge, 1983), or the meroplankton, such as larvae of Cirripedia and Decapoda (Sekiguchi, 1979).

Among Calanoida copepods, *Acartia tonsa* has a cosmopolitan distribution, contributing for more than 80% of the secondary production in coastal waters in subtropical and temperate zones (Mauchline, 1998). It is also important to mention the mysid *Metamysidopsis elongata atlantica*, which can be also found at high densities in these environments because of its patched distribution (Mclachlan, 1990). Larval stages of the barnacle *Balanus improvisus* and of decapods are also very representative during their reproduction period.

In light of this background, the main goal of the present study was to characterize the chitobiase of the holoplanktonic copepod *A. tonsa* in relation to the influence of water temperature, pH and salinity on maximum enzyme activity. The enzyme affinity for its substrate (*Km*) was also determined and compared for *A. tonsa*, *M. elongata atlantica*, nauplii barnacle *B. improvisus*, and zoea and megalopa of decapods.

2. Material and methods

2.1. Animal collection and acclimation

Animals were collected in the Patos Lagoon estuary (32° 05′05.16″ S; 052°13′04.69″W; Southern Brazil) and at the surf zone in the Cassino Beach (32°12′14.69″S; 052° 10′37.71″W, Southern Brazil), using a 200-μm mesh plankton net fitted with a non-filtering cod end. Collections were performed from July to December 2008. Samples were diluted in water from the sampling site and brought to the laboratory. Copepods *A. tonsa* and mysids *M. elongata atlantica* at different stages from nauplii to adults, as well as nauplii of the barnacle *B. improvisus* and larval stages of decapods were sorted out using a stereoscopic microscope and transferred to 5-L glass jars containing filtered (1 μm pore size) seawater at 30 ppt.

Animals were kept under laboratory conditions at fixed temperature (20 °C) and photoperiod (12L: 12D) and fed *ad libitum* with a mixture of the diatoms *Thalassiosira weissfloggi* and *Isocrysis galbana* obtained from cultures.

2.2. Enzyme source

Chitobiase from crustaceans was obtained using different numbers of organisms, depending on their biomass. For *A. tonsa* (nauplii, copepodites, and adults) and *B. improvisus* (nauplii), 100 individuals were homogenized in 500 μl of diluent, *i.e.*, autoclaved and filtered (0.2-μm mesh polycarbonate filters) seawater. For *M. elongata atlantica* (from nauplii to adults) and Decapoda larvae, 4–10 individuals were used. Animals were homogenized using a Potter-Elvehjem tissue grinder with Teflon pestle. Homogenates were centrifuged (10 min; 10,000×*g*) and the supernatant was filtered (0.2-μm mesh polycarbonate filter). The filtrate was used as enzyme source.

2.3. Calibration of the enzyme assay

Chitobiase reaction with its substrate methylumbelliferyl N-acetyl-ß-D-glucosaminide (MUFNAG) generates the fluorescent methylumbelliferone (MUF). Therefore, the enzyme activity was determined by transforming the fluorescence units in nM MUF using a calibration curve built from a series of MUF (Sigma-Aldrich, USA) standards diluted in dimethyl sulfoxide (DMSO) and added to the diluent to achieve the desired final MUF concentrations (6.25, 12.5, 25, 50, 100 and 200 nM). The same concentration of MUF can generate a different fluorescence value according to the assay medium conditions. Therefore, tests were performed to determine the influence of medium pH and salinity on MUF fluorescence adding MUF (50 nM final concentration) dissolved in the diluent at different pH (4.0–7.7) and salinities (0–30 ppt). All calibration tests were performed at 25 °C.

2.4. Enzyme assay

Chitobiase activity was measured using the method described by Oosterhuis et al. (2000) and Sastri and Dower (2006) with some modifications. The substrate MUFNAG was used from a 9-mM stock solution prepared in DMSO. For tests, the stock solution was diluted to give a final concentration of 250 μM MUFNAG. Test tubes were filled with diluent, enzyme source (sample homogenate) and enzyme substrate (MUFNAG). Control tubes were also performed without addition of the enzyme source and/or enzyme substrate. Reaction mixture was transferred to a 96-well microplate and the fluorescence was read (excitation: 360 nm, emission: 450 nm) at 25 °C in a spectrofluorometer (Victor 2, Perkin Elmer, USA). Fluorescence was measured every 5 min for up to 1 h. Increase in fluorescence over the reaction period corresponded to the free chitobiase activity, which was expressed as nM MUF·h^{-1}.

2.5. Enzyme characterization

Assays to determine the optimum pH and temperature for maximum enzyme activity were performed with chitobiase from *A. tonsa*. A pH range (4.0–7.7) was tested adjusting the pH of the diluent with HCl. These tests were performed at 25 °C and salinity 30 ppt. Fluorescence readings were corrected considering the effect of reaction mixture pH on MUF fluorescence (see Results section). A temperature range (20–45 °C) was also tested using a water bath. These tests were run at salinity 30 ppt and pH 7.7, characteristic of the estuarine water where copepods were collected. Considering that MUFNAG decomposes spontaneously at temperatures above 30 °C, a previous assay was run without chitobiase to check the influence of temperature on the rate of spontaneous MUFNAG degradation. Data obtained were used to correct results from the final assays. As *A. tonsa* is a euryhaline copepod, maximum enzyme activity was also measured in a range of water salinity (0–30 ppt) diluting the diluent with distilled water. These tests were performed at 25 °C and pH 6.0. Considering that pH varies according to salinity, a pH condition should be fixed to evaluate the isolated effect of salinity on enzyme activity. Water pH 6.0 was selected because of a maximum enzyme activity was observed in the range of pH 5.0–6.0 (see Results section), making this condition "ideal" to perform the enzyme assays.

Chitobiase affinity values for the substrate (*Km*) were determined for *A. tonsa*, *M. elongata atlantica*, *B. improvisus* nauplii, and Decapoda larvae. Homogenates for each group of organism tested were individually prepared and spiked into a series of substrate concentrations (15, 31, 62, 125, 250 and 500 μM of MUFNAG). These assays were run at 25 °C, pH 7.7 and salinity 30 ppt. *Km* values were estimated using non-linear regression analysis (hyperbolic function).

2.6. Data analysis

Data were expressed as mean ± standard error (n = 4). Mean data comparisons were performed through analysis of variance followed by the Tukey test using the software Statistica 7 (StatSoft, USA). Linear or non-linear regression analysis was applied for data modeling using the software SigmaPlot 2001 for Windows version 7.0 (SPSS, USA).

3. Results

3.1. Enzyme assay calibration

MUF fluorescence was influenced by pH and salinity of the reaction mixture. It augmented with increasing pH (Fig. 1A) and salinity (Fig. 1B) following a sigmoid- and hyperbolic-shape curve, respectively. The calibration curve obtained was adjusted to a linear-shape regression ($r^2 = 0.99$) and was applied to transform fluorescence units in nM MUF in all *Km* assays.

3.2. Acartia tonsa chitobiase

A. tonsa chitobiase showed a maximum activity at a pH range of 5.0–6.0, decreasing linearly its activity between pH 6.0 and 7.7 (Fig. 2A). The enzyme activity also showed a negative linear relationship as a function of salinity (Fig. 2B). Regarding temperature, maximum chitobiase activity was achieved at 30–35 °C, being reduced at higher temperatures (40 and 45 °C) (Fig. 2C). The *Km* value was 20.77 μM MUFNAG with significant inhibition at 500 μM MUFNAG (Fig. 2D).

3.3. Crustacean chitobiases

Km values changed according to the animal group considered. These values were 14.67, 18.19, 14.30, and 24.77 μM MUFNAG for the mysid *M. elongata atlantica* (Fig. 3A), nauplii barnacle *B. improvisus* (Fig. 3B), decapod zoea (Fig. 3C), and decapod megalopa (Fig. 3D), respectively. In all cases, enzyme activity was significantly inhibited at 500 μM MUFNAG.

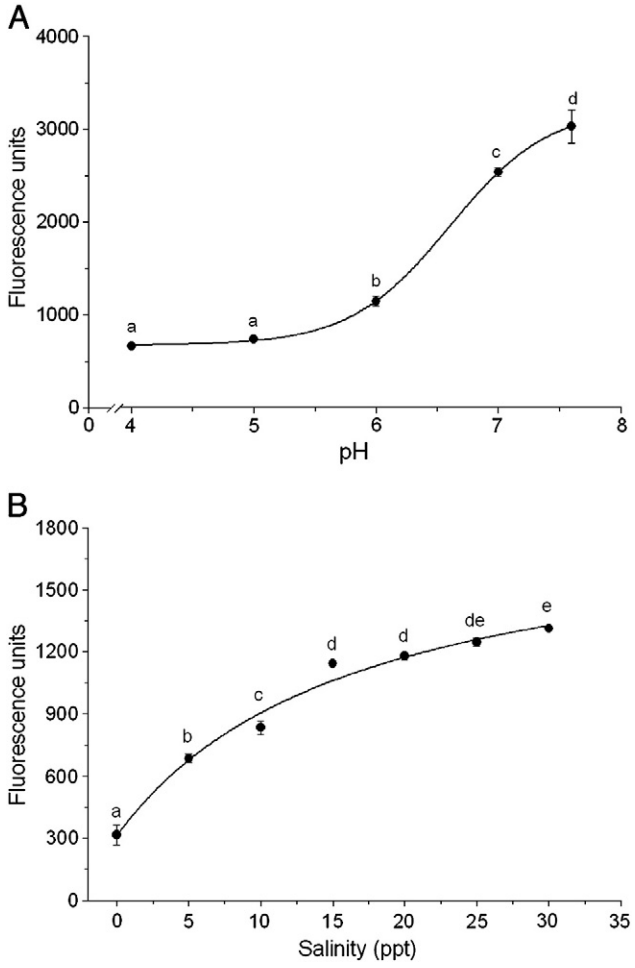

Fig. 1. Fluorescence of methylumbelliferone (MUF; 50 nM) as a function of (A) pH [$y = 672.44 + 2560.07/(1 + \exp(-(x\text{-}6.60)/0.41))$; $R^2 = 0.99$] and (B) salinity [$y = 311.25 + 1573.10x/(16.47 + x)$; $R^2 = 0.98$]. Data are expressed as mean ± standard error ($n = 4$). Different letters indicate significantly different mean values ($p < 0.05$).

4. Discussion

Results from the present study showed that MUF fluorescence resulting from the enzymatic reaction between chitobiase and its substrate (MUFNAG) is dependent on water pH and salinity. Therefore, results from tests performed at different reaction conditions need to be corrected for the influence of those parameters (pH and salinity) before comparisons could be made. Calibration curves showed in the present study can be employed for this purpose in the case of studies using the same methodology employed here. However, these studies report the pH of the reaction media but not salinity, complicating such corrections and comparisons.

In the present study, the whole body chitobiase activity was evaluated in the copepod *A. tonsa*. Studies performed with Euphausiacea and Decapoda crustaceans have reported the presence of chitobiase activity in both epidermis and digestive tract (Saborowski and Buchholz, 1999; Zou and Fingerman, 1999c). Considering that copepods show omnivore and carnivore food habits, it is suggested that these animals also present chitobiase not only in the epidermis for molting, but also in the digestive tract for food digestion. Despite the enzyme likely has a different function in these tissues they were never evaluated separately.

Chitobiase from epidermis would be associated with the degradation of the chitin present in the crustacean cuticle. It is important to note that data reported in the present study were obtained using whole copepod homogenates. Considering that copepods at different developmental stages (nauplii, copepodites, and adults) were used, maximum enzyme activity can thus be also attributed to chitobiase from epidermis. Considering the direct contact of the epidermis with the environmental medium, enzyme activity could be influenced by water physicochemical parameters such as pH, salinity and temperature.

Chitobiases from different sources showed a similar response to water pH. In the copepod *A. tonsa,* maximum enzyme activity was observed between pH 5 and 6 (Fig. 2A), as reported for other organisms (Oosterhuis et al., 2000; Vrba and Machácek, 1994), including the copepod *Neocalanus plumchrus* (Sastri and Dower, 2006), different tissues of decapods (Zou and Fingerman, 1999a) and the fungi *Talaromyces emersonii* (Table 1). Other enzymes like amylase in the copepod *Heliodiaptomus viduus* (Dutta et al., 2006) and chitinase of *Artemia* (Funke and Spindler, 1989) also showed maximum activity at pH close to those reported for chitobiase (pH 6.0 and 5.8, respectively).

Despite the excellent pH buffering capacity of seawater, *A. tonsa* can be subjected to pH changes when exposed to the low salinities occurring in estuaries. One could expect that a decrease in water pH to values close to that for maximum activity of both chitobiase and chitinase would favor copepod growth. However, previous studies showed that an increase in CO_2 concentration and a decrease in water pH did not affect survival, body size or growth rate in the copepod *A. tsuensis* (Kurihara and Ishimatsu, 2008). Therefore, pH does influence maximum enzyme activity indeed but the process of degrading the old chitin remains unchanged, the time being triggered by other mechanisms (usually environmental, biotic). In fact, changes in growth rate as a function of environmental pH would be associated with a shift in energy allocation from growth to other metabolic processes related to animal survival such as respiration, osmoregulation and excretion. A lower ability to take up and process food at low water pH also cannot be ruled out. In fact, a raise in water pH up to 8.5 was shown to increase the filtration rate and grazing in the copepod *Schmackeria dubia* (Changling et al., 2008). Therefore, less food would be obtained at pH 6 than at pH 8.5, *i.e.*, a lower amount of carbon is available for copepod growth at pH 6. Taken all together, these findings indicate that

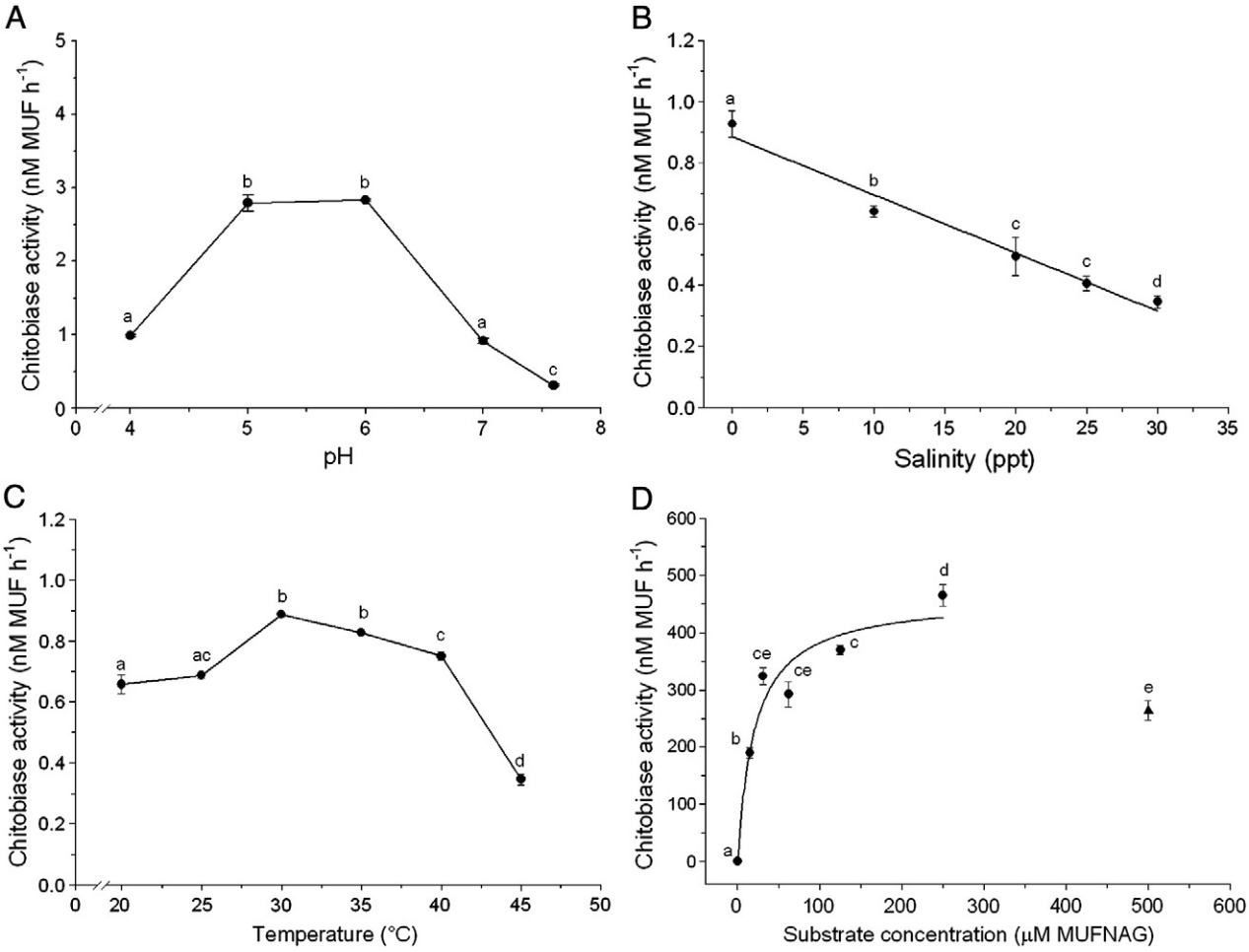

Fig. 2. Chitobiase activity of the copepod *Acartia tonsa* as a function of (A) pH, (B) salinity [($y = 0.8866 - 0.01902x$ ($R^2 = 0.97$))], (C) temperature, and (D) substrate concentration [$Km = 20.77$ μM MUFNAG; $y = 461.8x/20.77 + x$ ($R^2 = 0.94$)]. ▲: inhibitory concentration. Data are expressed as mean ± standard error ($n = 4$). Different letters indicate significantly different mean values ($p < 0.05$).

chitobiase activity would not be a limiting factor for copepod growth at low water pH, as opposed to energy availability.

Regarding temperature, maximum activity of *A. tonsa* chitobiase was observed at 30 and 35 °C. However, it is important to note that MUFNAG decomposes spontaneously at temperatures above 30 °C. Therefore, the increased fluorescence observed at the 30–35 °C range could also be a result from the combining effect of the enzyme activity and the spontaneous decomposition of MUFNAG. Nevertheless, these values are close to those found for the chitobiase of the copepod *Temora longicornis* (35 and 40 °C) (Baars and Oosterhuis, 2007). Other enzymes from copepods also show maximum activity at a similar range of temperature. For example, maximum activity of aspartate transcarbamylase in the copepod *Calanus helgolondicus* was achieved at 35 °C (Biegala and Bergeron, 1998). Also, the amylase of the copepod *H. viduus* shows its maximum activity at 30 °C (Dutta et al., 2006). However, *A. tonsa* chitobiase showed a maximum activity at lower temperatures than those observed for other species from different groups (Table 1) like the cladoceran *Daphnia magna* (45–50 °C) (Espie and Roff, 1995), the decapod *Uca pugilator* (50–60 °C) (Zou and Fingerman, 1999c) and the fungi *T. emersonii* (75 °C) (O'Connell et al., 2008).

The close taxonomic relation between these two copepod species can explain the observed similarity of *A. tonsa* and *T. longicornis* in respect to chitobiase response to temperature. Also, this could be associated with the similarity of environments where they live. Both species are planktonic and inhabit water with maxima temperatures not exceeding 30 °C. On the other hand, the difference between copepods and *U. pugilator* can be explained considering the fact that this crab can be subjected to higher temperatures during the air exposure period associated with the low-tide condition (Wilkens and Fingerman, 1965). Regarding the fungi *T. emersonii*, the extremely high optimum temperature (75 °C) for chitobiase maximum activity can be explained by the fact that this species is thermophilic (Arora et al., 1991).

According to findings discussed above for *A. tonsa* and other species, changes in chitobiase properties seems to be more associated with temperature than pH adaptation. However, enzyme adaptation as a function of salinity has never previously been evaluated, especially when coastal and estuarine species are considered.

The copepod *A. tonsa* is cosmopolitan, being more abundant in subtropical estuaries. This species is euryhaline, surviving in salinities ranging from 0 to 72 ppt (Cervetto et al., 1999). However, any life stage of *A. tonsa* is commonly found in environments with predominance of freshwater. Furthermore, nauplii *A. tonsa* show low survival rates in low salinities (Chinnery and Williams, 2004). In fact, larval stages of this copepod show maximum survival rates in salinities ranging from 15 to 25 (Cervetto et al., 1999; Tester and Turner, 1991). Also, it has been shown that growth rates are limited in salinities lower than 15 ppt (Chinnery and Williams, 2004) and higher than 30 ppt (Cervetto et al., 1999). Considering the important role of chitobiase in larval molting, it would be expected that maximum chitobiase activity could be observed in salinities ranging from 15 to 25 ppt. However, maximum enzyme activity was observed in freshwater, with significant decrease with increasing salinity

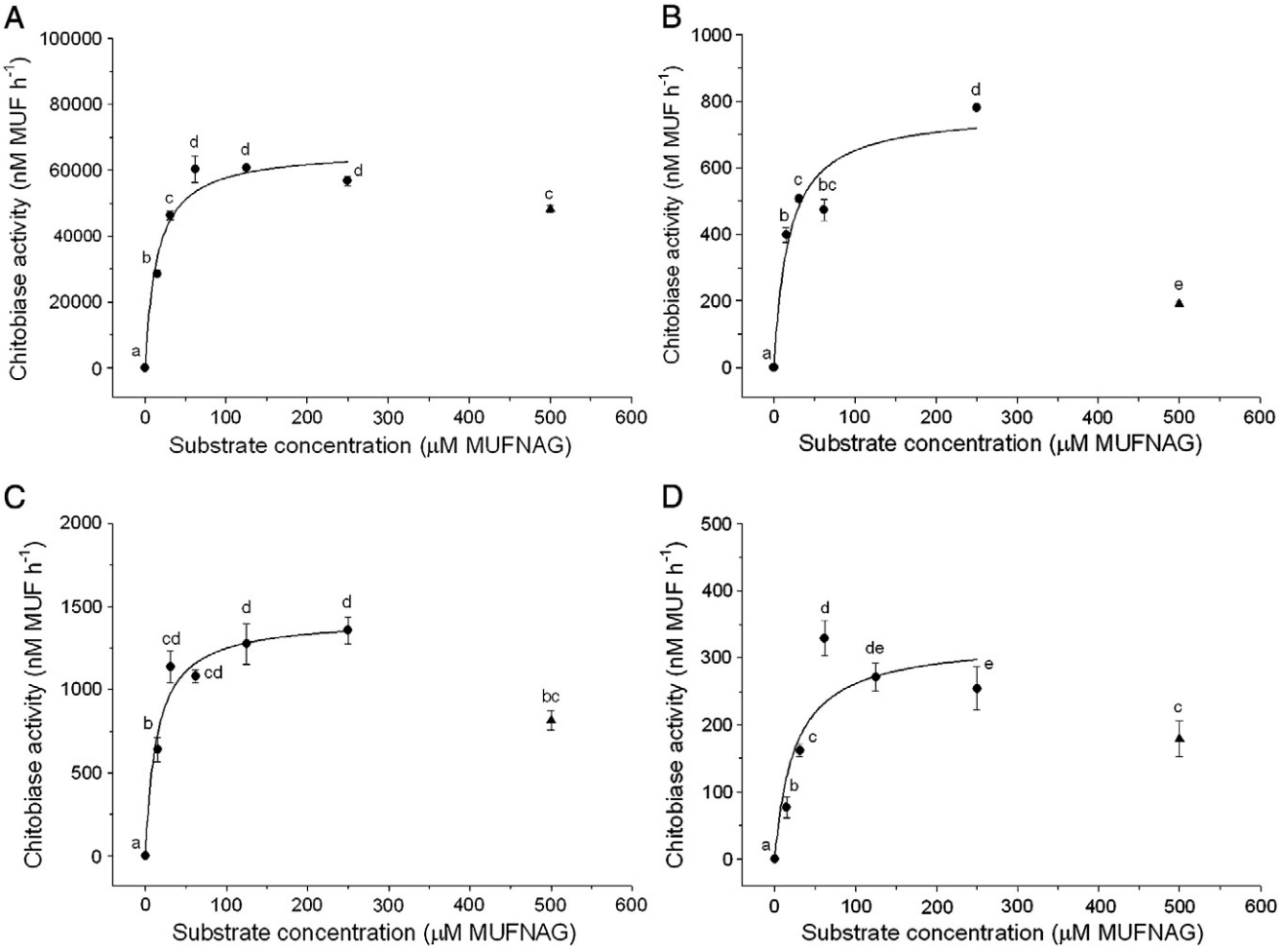

Fig. 3. Chitobiase activity of crustaceans as a function of substrate (MUFNAG) concentration. Enzyme affinity for the substrate (*Km*; μM MUFNAG) was calculated for (A) adult mysid *Metamysidopsis elongata atlantica* [$Km = 14.68$; $y = 66{,}140.0x/14.68 + x$ ($R^2 = 0.96$)], (B) nauplii barnacle *Balanus improvisus* [$Km = 18.19$; $y = 770.3x/18.19 + x$ ($R^2 = 0.93$)], (C) Decapoda zoea [$Km = 14.30$; $y = 1427.0x/14.30 + x$ ($R^2 = 0.97$)], and (D) Decapoda megalopa [$Km = 24.77$; $y = 326.5x/24.77 + x$ ($R^2 = 0.83$)]. ▲: inhibitory concentration. Data are expressed as mean ± standard error ($n = 4$). Different letters indicate significantly different mean values ($p < 0.05$).

(Fig. 2B). Despite the significant decrease in chitobiase activity as a function of increasing salinity, it is suggested that the observed enzyme activity in the range of 15 to 25 ppt, *i.e.*, 68 to 47% of that measured in freshwater, would not be a limiting factor for *A. tonsa* growth (molting) under these saline conditions. However, the extremely decreased enzyme activity observed in salinity 30 ppt, *i.e.*, only 33% of that measured in freshwater, suggests that chitobiase activity might be one of the limiting factors for copepod growth at 30 ppt salinity or higher.

Since chitobiase activity seems not to be the limiting factor for *A. tonsa* growth in low salinities, the reduced growth could be associated with the higher energy expenditure for osmoregulation at

Table 1
Chitobiase characteristics of different organisms according to enzyme affinity for substrate (*Km*; μM substrate) and optimum pH (OP pH) and temperature (OP T; °C). Temperature used to determine the *Km* is also given (*Km* T; °C). In all studies, methylumbelliferyl N-acetyl-ß-D-glucosaminide (MUFNAG) was used as substrate.

Organism	Km	OP pH	OP T	*Km* T	Reference
Bodo saltans and *Cyclidium* sp. (Protozoa)	<0.5			15	(1)
Aeromonas hydrophila (Bacteria)	>100			15	(1)
Bacteria	>100			25	(2)
Oxyrrhis marina (Dinophyta)	<0.2			25	(2)
Talaromyces emersonii (Fungi)	500	5.0	75	50	(3)
Daphnia pulicaria (Cladocera)	56.7			22	(4)
Daphnia magna (Cladocera)	61.5	5.5	45–50	20	(5)
Uca pugilator (Decapoda) epidermis	190	5–6	50–60	19	(6)
Uca pugilator (Decapoda) hepatopancreas	203	5–6	50–60	19	(6)
Temora (Copepoda)		6	35–40	25	(7)
Temora longicornis (Copepoda)	55	~8	55	25	(2)
Neocalanus plumchrus (Copepoda)		5–6		25	(8)
Acartia tonsa (Copepoda)	20.8	5–6	30–35	25	Present study

(1) Vrba et al. (1993); (2) Oosterhuis et al. (2000); (3) O'Connell et al. (2008); (4) Vrba and Machácek (1994); (5) Espie and Roff (1995); (6) Zou and Fingerman (1999c); (7) Baars and Oosterhuis (2007); (8) Sastri and Dower (2006).

these saline conditions. In fact, the copepod *A. tonsa* shows reduced free amino acid concentration, increased oxygen consumption and increased ammonia excretion as water salinity decreases (Farmer and Reeve, 1978). Findings from the present study and those reported in the literature indicate that optimum salinity for enzyme activity is not related to the environmental salinity for maximum survival and growth (Cervetto et al., 1999).

Taken altogether, results reported in the present study show that enzyme activity is influenced by water physicochemical parameters. The enzyme activity response to changes in water physicochemical parameters could reflect the species tolerance to particular environmental conditions. However, it is important to stress that our results were obtained changing *in vitro* the enzyme assay conditions (experimental medium pH, temperature and salinity). However, a better interpretation of the inter-relationships between water physicochemical parameters, maximum enzyme activity and animal development (survival and growth) would be possible using animals collected, acclimated or cultivated at different water pH, temperature and salinity.

According to Peters et al. (1998), modifications in enzyme affinity for its substrate (*Km*) can be associated with alterations in the enzyme kinetic properties to achieve different catalytic rates in response to changes in environmental conditions. Results from the present study indicate a significant variation in chitobiase *Km* values among species and life stages of crustaceans (copepod *A. tonsa*, mysid *M. elongata atlantica*, nauplii barnacle *B. improvisus*, Decapoda zoea, and Decapoda megalopa) collected in the Patos Lagoon estuary and the Cassino Beach. Chitobiase *Km* values ranged from 14.30 μM MUFNAG for Decapoda zoea to 24.77 μM MUFNAG for Decapoda megalopa. Despite this variation, values were closer amongst themselves than with those reported for organisms from the Northern Hemisphere, which vary from 55 μM MUFNAG for the copepod *T. longicornis* and 203 μM MUFNAG for the hepatopancreas of the crab *U. pugilator* (Table 1).

In fact, *Km* values determined for all organisms evaluated in the present study are lower than those reported for crustaceans from the Northern Hemisphere. Chitobiase *Km* for the copepod *A. tonsa* was found to be 20.77 μM MUFNAG, while it was reported to be 55 μM MUFNAG for the copepod *T. longicornis* (Oosterhuis et al., 2000). Furthermore, the *Km* values for chitobiases of the mysid *M. elongata atlantica* (14.67 μM MUFNAG) and the meroplanktonic larvae (barnacle nauplii *B. improvisus*, 18.19 μM MUFNAG; decapod zoea, 14.30 μM MUFNAG; decapod megalopa, 24.77 μM MUFNAG) were found to be much lower than those for the chitobiase of the epidermis (190 μM MUFNAG) and hepatopancreas (203 μM MUFNAG) of the decapod crab *U. pugilator* (Zou and Fingerman, 1999c). However, it is important to bear in mind that *Km* values reported in the present study were accessed at pH 7.7 while most of the studies which found *Km* values in the range of 50 to 60 μM MUFNAG for planktonic crustaceans were performed at pH 5–6. In this case, a possible relationship between water pH and enzyme affinity (*Km*) cannot be disregarded. Unfortunately, this relationship was not evaluated in the present study.

If one assumes that enzyme *Km* is not pH-dependent, differences observed in *Km* values among chitobiases from different crustacean species could to be related to the habitat occupied by the species. This statement is based on the fact that subtropical estuarine species from the South Atlantic analyzed in the present study showed *Km* values much lower than other crustacean species from the Northern Hemisphere. As observed for temperature, bigger differences in enzyme properties are found between crustaceans and other organisms from groups more phylogenetically distant like protozoans ($Km < 0.5$ μM MUFNAG), dinoflagelates ($Km < 0.2$ μM MUFNAG), and bacteria ($Km > 100$ μM MUFNAG). Furthermore, it is important to note that the concentration of substrate (500 μM MUFNAG) necessary to achieve 50% of the maximum activity (*Km*) for the fungi *T. emersonii* inhibited significantly the chitobiase activity of all crustaceans tested in the present study (Fig. 3).

Considering that chitobiase degrades chitin and is widely found in nature, results from the present study together with those reported in the literature show that this enzyme might be differentially evolved in each specific group of organism, and even within different ontogenetic stages of the same species, for a better adaptation to cope with its respective needs. For the aspartate transcarbamylase, it was shown a lack of genetic alteration from dipterans to mammals (Jones, 1980). However, the same pattern was not shown for chitobiase, and differences between the digestive and epithelial actions in insects and crustaceans or its extra corporal action in fungi and bacteria could be the origin of the differences found among each enzyme evaluated.

In turn, the differences observed in chitobiase affinity according to the developmental stage in crustaceans also deserve attention. For example, chitobiase affinity in decapod larvae living in the Patos Lagoon estuary was much higher for zoea (14.30 μM MUFNAG) than for megalopa (24.77 μM MUFNAG). It is important to note that zoea of the estuarine crab *Neohelice granulata*, the dominant decapod in the Patos Lagoon estuary, develops in the marine portion of the estuary at higher water salinity, while megalopa of this crab species is found in the freshwater portion of the estuary at lower water salinity. The ability of crab larval stages to cope with the characteristic salinities of the different estuarine areas is directly related to their osmoregulation capacity (Bianchini et al., 2008). It is also important to consider that maximum chitobiase activity in *A. tonsa*, the dominant copepod during periods of saltwater intrusion into the Patos Lagoon estuary (Montú, 1980), was shown in the present study to be inversely related to water salinity. Therefore, differences observed in *Km* values for decapod larval stages could be an adaptive mechanism to compensate the salinity effect on maximum enzyme activity. In decapod zoea, the lower maximum enzyme activity due to the higher water salinity in the marine portion of the estuary would be compensated by a higher enzyme affinity. On the other hand, the higher maximum enzyme activity associated with the lower salinity in the freshwater portion of the estuary would be compensated by a lower enzyme affinity. Taken altogether, these findings suggest that changes in chitobiase properties in planktonic crustaceans could be also related to specific characteristics of the animal life cycle.

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