NAUPLIUS, Rio Grande, 5(2): 23-31, 1997.

CARBONIC ANHYDRASE ACTIVITY IN GILLS AND OTHER TISSUES OF THE ESTUARINE CRAB, *Chasmagnathus granulata* (DECAPODA, BRACHYURA, GRAPSIDAE).

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ABSTRACT

Activity of the carbonic anhydrase (CA) enzyme was studied in adult male crabs of the species *Chasmagnathus granulata*. CA activity was measured in different gills (3 to 8), hepatopancreas, muscle (claw) and hypodermis of crabs aclimated to 12 $^{o}/_{oo}$ salinity. The enzymatic activity was significantly ($p< 0.05$) higher in posterior gills (6 to 8) than in anterior ones (3 to 5). The CA activity was not significantly different ($p> 0.05$) among muscle, hypodermis and hepatopancreas, being their enzimatic activities lower than the CA activity of posterior gills.

In a second experiment, crabs were acclimated to 20 $^{o}/_{oo}$ salinity during 10 days, and then submitted to a 2.5 $^{o}/_{oo}$ salinity. The temporal course of AC activation in the posterior gills (6 a 8) was then determined at 0, 7, 24, 96 and 168 h after transference to the low salinity. CA activity at 7, 96 and 168 h was significantly ($p<0.05$) higher than the activity recorded at the beginning of the experiment. These results suggest the existence of both a short and long term aclimation response in *C. granulata* during ionic hyperegulation, similar to the activation profile of Na^+,K^+-ATPase exhibited by several crustacean species.

Keywords: carbonic anhydrase - gills - crabs

INTRODUCTION

The carbonic anhydrase (CA), which catalyzes the hydration or dehydration of CO_2, is a widely distributed enzyme within the animal and vegetal kingdoms (Bötcher and Siebers, 1993). In brachyuran crabs, a functional separation between anterior and posterior gills has typically been reported, having the anterior gills a predominant respiratory function, while the posterior ones retain mainly an osmoregulatory function (Péqueux, 1995). Estuarine crabs have been characterized as good ionic regulators, against a wide range of external salinities (Péqueux, 1995). Several authors have postulated a relevant function for gill cytosolic CA in ion-regulation, providing H^+ and HCO_3- as contraions to be exchanged by Na^+ and Cl^- (Henry, 1988; Bötcher and Siebers, 1993). Piller *et al.* (1995) showed a higher CA activity in posterior gills of crabs during hyperegulation at dilute salinities.

The grapsid crab *Chasmagnathus granulata* (Decapoda, Brachyura) lives in the meso and supralitoral zones of estuarine environments. It is distributed along southern Brazil, Uruguay and Argentine (Boschi, 1964). The populations living at Samborombon Bay (Argentine) are subjected to high daily salinity variations, due to tidal influence. *C. granulata* has been previously

characterized as a good hypo and hyperegulator crab in a wide range of salinities, concerning Na^+ and K^+ ions (Luquet *et al.,* 1992, Bromberg *et al.,* 1995). On the other hand, Nery and Santos (1993) showed carbohydrates mobilization in *C. granulata* after both hypo and hyperosmotic shocks.

This work was aimed at: (a) studying the activity of the CA in different tissues of *C. granulata* and (b) evaluating in the same species the temporal course of the CA induction after transference to low salinities.

MATERIALS AND METHODS

Adult males in stage C or initial D of the molting cycle (according to Drach and Tchernigovtzeff, 1967) were used. The mean weight of a representative sample was 14.21 ± 0.47 g (n=31). The crabs were collected at Faro San Antonio beach, southern point of Samborombón Bay (36° 18'S and 56° 48'W). Once in the laboratory, crabs were acclimated in water at 12 or 20 $^{o}/_{oo}$ salinity, at 20 °C. During this acclimation period, crabs were fed twice a week with rabbit food (protein 17 %, fat 3 %, fiber 15 %, on wet weight) and a photoperiod of 12L: 12D was maintained, according to previous works (Rodríguez y Lombardo, 1991; Monserrat *et al.,* 1991). Two experiments were carried out:

Experiment 1. The CA activity of gills 3 to 8 from crabs acclimated during two weeks at 12 $^{o}/_{oo}$ salinity was determined. Due to their small size, gills 1 and 2 were not studied. The CA activity of hepatopancreas, hypodermis and muscle (claw) were also determined in the same animals.

Experiment 2. The induction of CA activity of crabs submitted to 2.5 $^{o}/_{oo}$ salinity after an acclimation period of 10 days to 20 $^{o}/_{oo}$ salinity, was determined after 0, 7, 24, 96, and 168 h of transference to the low salinity. Only the CA activity of posterior gills (6 to 8) was determined.

Crabs from both experiments were crioanesthezied and the tissues dissected. The samples were frozen at -70 °C until analysis of CA activity.

CA activity protocol

The preserved tissues were homogenized (10 % W/V) in cold phosphate buffer, 0.25 M, pH 7.40. The homogenates were then centrifuged at 2.000 g, during 5 min at 4°C, according to the methodology cited by Giraud (1981). After centrifugation, the supernatant was employed as enzyme source.

The (delta)pH method (Henry, 1991) was employed to estimate the CA activity at 2.5°C, with some modifications. The reaction medium was made with manitol (225mM), sucrose (75mM) and tris-phosphate (10mM), being the pH adjusted to 7.40. In the enzyme activity determinations the following volumes were used: 7.5 ml of reaction medium, 0.1 ml of tissue homogenate and 1 ml of CO_2 saturated distilled water at 2.5°C. Figure 1 shows the employed device. Those values were chosen in order to ensure linear enzyme activity against time. The pH drop was measured during 30 s by means of a pHmeter (Hanna Instruments). A linear regression was adjusted to data. The non-enzymatic activity was estimated from the pH drop of the same reaction medium during 30 s, after adding 1 ml of CO_2 saturated distilled water at 2.5°C and 0.1 ml of

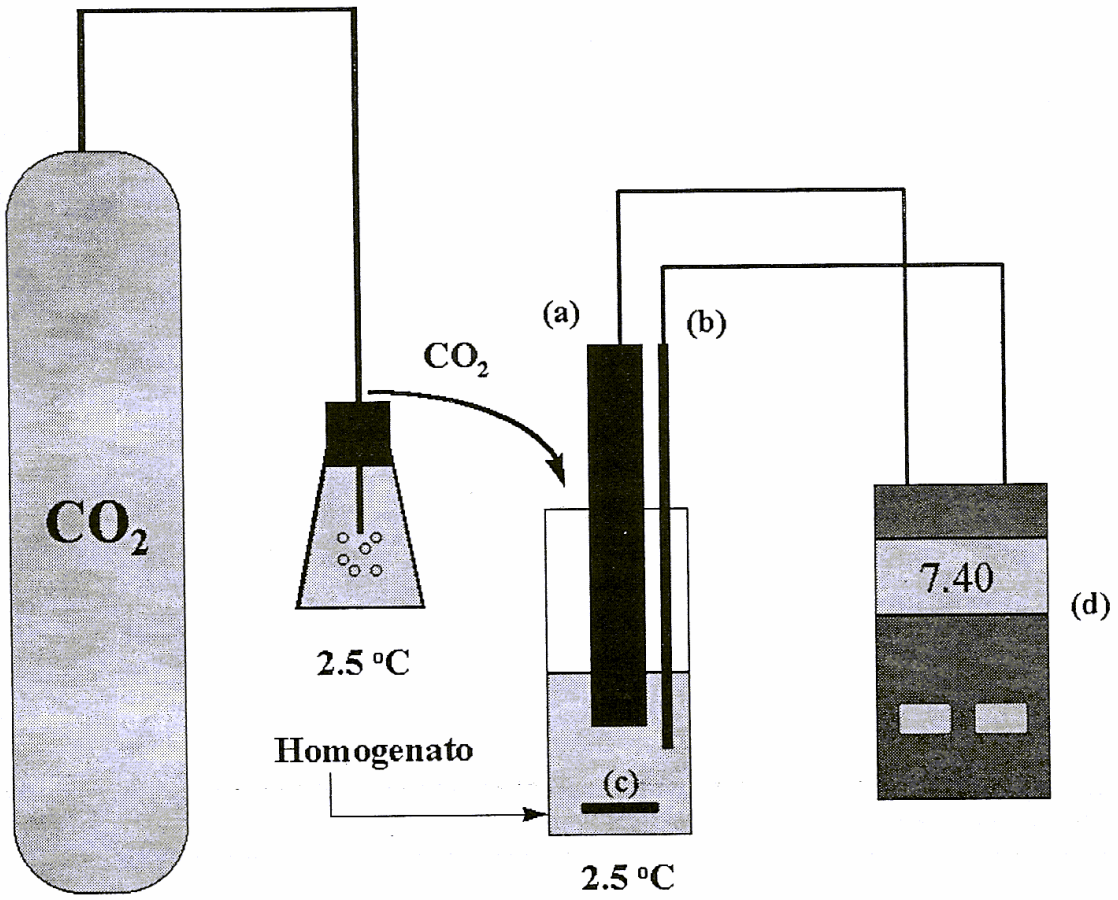

Figure 1. Schematic design of the apparatus employed to determine carbonic anhydrase activity (based upon the pH method of Henry, 1991, with modifications). **(a)** pH electrode. **(b)** temperature sensor. **(c)** magnetic stirrer. **(d)** pH meter.

phosphate buffer. The catalyzed reaction rate was then calculated as the difference between the enzymatic and non-enzymatic activities; a representative example can be seen in Figure 2.

In order to express the pH drop/min in terms of (delta) mmol H^+/min, the pH drop rate after adding 0.1 ml of HCl 0.1 N to the reaction medium was estimated. Total protein content was determined according Lowry *et al.* (1951), employing bovine serum albumin (Sigma Company) as standard. The CA activity was finally expressed as mmol H^+/min/mg of protein.

CA activity data were analyzed by means of a one way Anova, applying the Tukey test (α=0.05) for *a posteriori* comparisons. Normality and homocedasticity were previously checked by residual analysis (Montgomery, 1984).

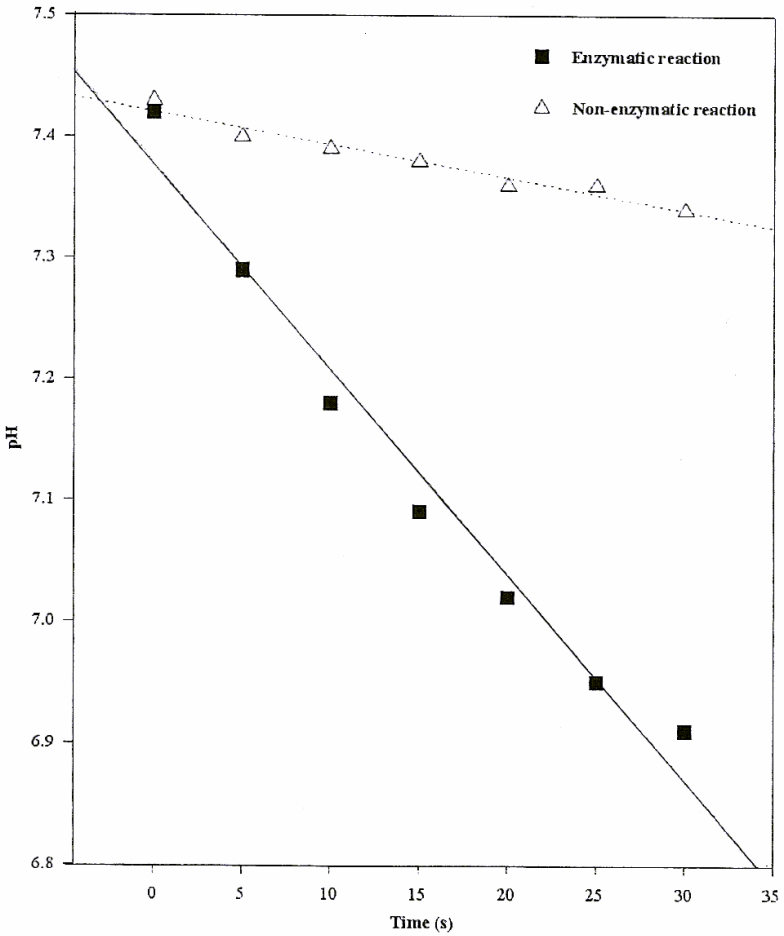

Figure 2. A typical pH record of the enzymatic (CA) and non-enzymatic activity.

RESULTS

Experiment 1. CA activity was significant higher ($p < 0.05$) in posterior gills (6 to 8) than in anterior ones (3 to 5). Comparisons among posterior gills showed that the CA activity was higher ($p < 0.05$) in gills 6 and 7 than in gill 8 (Figure 3). As shown in Figure 4, the CA activity of other tissues was significant ($p < 0.05$) lower than that of posterior gills; no significant differences ($p > 0.05$) were found among muscle, hypodermis and hepatopancreas.

Experiment 2. CA activity increased significantly ($p < 0.05$) 7 h after the transference of crabs to 2.5 $^{o}/_{oo}$ salinity (Figure 5), with respect to the activity registered at 0 h (T_0 group). After 24 h, CA activity decreased, to reach the same levels registered at the beginning of the experiment. After 96 and 168 h, the CA activity was again higher (514.3 %, $p < 0.05$) than T_0 group.

CA activity was significant ($p < 0.05$) higher at the salinities 2.5 and 12 $^{o}/_{oo}$ than at 20 $^{o}/_{oo}$. The corresponding mean values (± SE) can be seen in Figures 4 and 5.

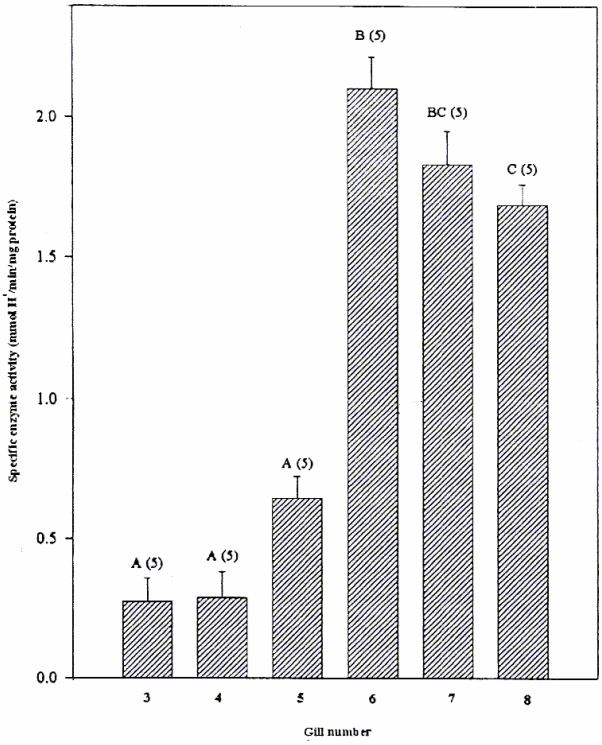

Figure 3. Mean anhydrase carbonic activity (1 SE) in different gills of *Chasmagnathus granulata* acclimated at 12 °/oo salinity. Values into brackets represent the number of analyzed samples, each sample was a pool from 4 to 6 crabs. Equal letters indicate no significant difference (P0.05) between mean values.

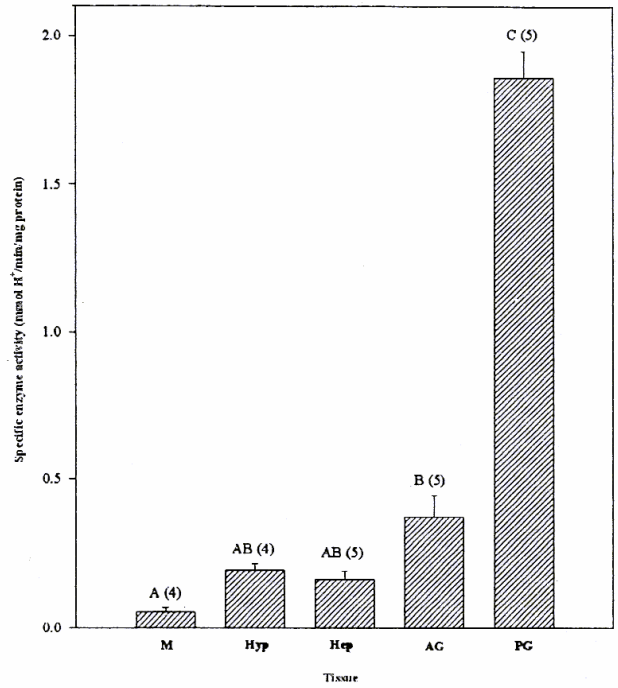

Figure 4. Mean anhydrase carbonic activity (1 SE) in several tissues of *Chasmagnathus granulata* acclimated at 12 °/oo salinity. Values into brackets represent the number of assayed crabs. Equal letters indicate no significant difference (P0.05) between mean values. **M**: muscle. **Hyp**: hypodermis. **Hep**: hepatopancreas. **AG**: anterior gills (3, 4 and 5). **PG**: posterior gills (6, 7 and 8).

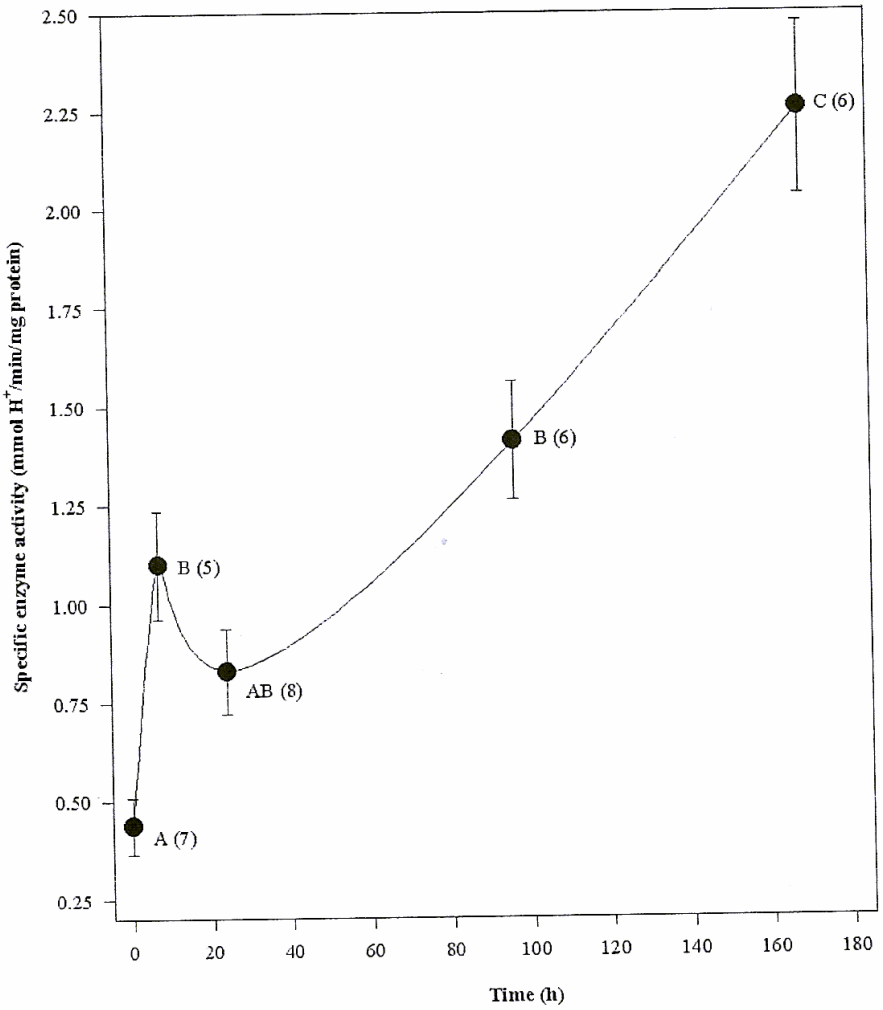

Figure 5. Mean anhydrase carbonic activity (1 SE) in posterior gills (6 to 8) of *Chasmagnathus granulata* maintained at 20 °/oo salinity during 10 days and transferred later to 2.5 °/oo salinity. Values into brackets represent the number of assayed crabs. Equal letters indicate no significant difference (P0.05) between mean values.

DISCUSSION

C. granulata showed a similar pattern of CA activity to the estuarine crab *Callinectes sapidus* (Henry and Cameron, 1982a), concerning the relative enzymatic activity observed among the studied tissues. *C. granulata* is clearly a strong ionic hyper-regulator at the assayed salinity of 12 °/oo (Luquet *et al.*, 1992). The relative high CA activity we have observed in their posterior gills has also been reported for other ionic hyperegulator crabs, such as *Callinectes similis* (Piller *et al.*, 1995) and *Eriocheir sinensis* (Olsowski *et al.*, 1995). On the contrary, *C. granulata* showed a CA activity profile quite different from that of *Cancer productus* (McMahon *et al.*, 1984). The CA activity of *C. productus*, a marine and stenohaline crab, was relatively homogenous when comparing among gills, being the hepatopancreas the organ with maximum enzyme activity. Therefore, the high CA activity registered in posterior gills (6-8) of the estuarine crab *C. granulata* is consistent with the main role assigned to the CA

in the ionic regulation carried out by the posterior gills of osmoregulator crustaceans (Henry, 1988; Bötcher and Siebers, 1993; Péqueux, 1995).

Considering the temporal course of CA activity induction, *C. granulata* showed several differences when compared to *C. sapidus.* While the former species showed a definitive increase in CA activity 96 h after transference to low salinity, the latter species showed the same response only after one week (Henry and Cameron, 1982b). Such difference could be assigned to the magnitude of the hypo-osmotic shock, since *C. sapidus* was transferred from the acclimation salinity to a salinity 3.2 times more diluted (28 to 8.75 $^{o}/_{oo}$) and *C. granulata* to a salinity 8 times more diluted (20 to 2.5 $^{o}/_{oo}$).

Some authors (Towle, 1981; Corotto and Holliday, 1996) have suggested that the short-term acclimation to salinity changes observed in crabs, could be related to a rapid recruitment of preexisting Na^{+}/K^{+} ATPase molecules, while the long-term acclimation certainly involves *de novo* synthesis. The same pattern could be assigned to CA. In this context, the transient increase of CA activity at 7 h after transference to low salinity could be explained as a short-term acclimation response, while the increase at 96 and 168 h would be likely linked to a long-term acclimation process. A new steady state in the haemolymphatic sodium concentration of *C. granulata* was reported after 168 h of transference from 20 $^{o}/_{oo}$ to more dilute salinities (Bromberg *et al.*, 1995). Therefore, the long-terrm change of CA activity could likely be involved in the new sodium equilibrium, among other compensatory responses such as Na^{+}/k^{+} ATPase induction and/or the reduction of body surface salt permeability (Robinson, 1994).

The long-term induction of CA activity, in terms of promoting *de novo* synthesis of the enzyme, could be regulated *in vivo* by several chemical factors. Biogenic amines, like dopamine and octopamine, have shown an effect concerning Na^{+} uptake (Lohrman and Kamemoto, 1987; Sommer and Mantel, 1991). Other factors, probably peptides of CHH/MIH/VIH family, have been recently reported as acting in ion-regulatory processes (Pierrot *et al.,* 1996). It is also possible that during the long-term acclimation to low salinity, another CA isozyme with a higher V_{max}, for example, was being expressed. To our knowledge, only one work has characterized the CA of crustaceans from a biochemical point of view (Bötcher *et al.,* 1994). Although some isozymes have been recognized, their selective induction during hyperegulatory processes has not been studied.

ACKNOWLEDGEMENTS

This work was supported by a grant from the University of Buenos Aires (UBACYT 94-97 programm). We wish to thank the Behaviour Physiology Lab. (University of Buenos Aires) for providing crabs.

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