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CHANGES IN TISSUE WATER, LEUCINE AMINOPEPTIDASE ACTIVITY, NINHIDRINE POSITIVE SUBSTANCES AND PROTEIN CONCENTRATION IN *CRASSOSTREA RHIZOPHORAE* EXPOSED TO EXTREME SALINITIES

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ABSTRACT: In this work, we studied the variations of LAP enzymatic activity (E.C, 3.4.1.1), of protein concentration and of intracellular free aminoacid (FAA) concentration in the muscular tissues of three groups of *Crassostrea rhizophorae* exposed to a range of salinities from 5‰ (minimum tolerable) through 38‰ (usual) up to 60‰ (maximum tolerable). In the groups maintained at 60‰ and 5‰ salinity, LAP activity increased 55.7% and 17.45% respectively in comparison with the control group. As for FAA concentration, determined under the form of Ninhidrin Positive Substances (NPS), 60‰ salinity caused an accumulation of NPS, while 5‰ salinity caused their decrease. The higher LAP activity at 60‰ salinity coincided with a 20‰ decrease in protein concentration and with an increase in SPN concentration. Although these results confirm the LAP action model in the process of cellular volume regulation in response to a salinity increase, it was unexpected to verify a slight increase of LAP activity at 5‰ salinity, without significant variation of protein concentration. Our results confirm the importance of LAP in the process of cellular volume regulation in marine bivalves in response to a salinity increase, but the response of the enzyme in the hypoosmotic medium suggests the possibility that the enzyme is also associated to other metabolic functions, distinct from cellular volume regulation.

RESUMEN: Se estudió la variación de la actividad enzimática de LAP (E.C. 3.4.1.1), de la concentración de proteínas y de la concentración de aminoácidos intracelulares libres en el tejido muscular de *Crassostrea rhizophorae* sometidas a salinidades de 5‰ (mínima tolerable), 38‰ (habitual) y 60 ‰ (máxima tolerable). En los grupos mantenidos a 60‰ y 5‰, la actividad de LAP resultó incrementada en un 55,7% y en 17,45% respecto al grupo control. Para la concentración de aminoácidos intracelulares libres, determinados en la forma de sustancias positivas a la ninhidrina (SPN), un aumento en la salinidad resultó en la acumulación de SPN, mientras que la baja salinidad determinó su disminución. El mayor promedio de actividad a 60‰ coincidió con una disminución en la concentración de proteínas del 20% y con un incremento en la concentración de SPN Si bien estos resultados se ajustan al modelo de acción de LAP en la regulación del volumen celular ante un aumento en la salinidad, un hallazgo inesperado fue constatar un ligero aumento de actividad, sin variación significativa de la concentración de proteínas a 5‰. Los resultados obtenidos sustentan la generalización de la importancia que reviste LAP en el proceso de regulación del volumen celular en los bivalvos marinos ante el aumento de salinidad ambiental, pero la respuesta de la enzima en el medio hipoosmótico sugiere la posibilidad de que también se encuentre asociada a otras funciones metabólicas distintas a la regulación del volumen celular.

INTRODUCTION

When exposed to salinity variations in the surrounding water, marine mollusks modify the concentration of intracellular small organic molecules to maintain the cellular volume between appropriate physiological limits (PIERCE, 1982; JADHAV & LOMTE, 1990). These molecules are known as Ninhidrin Positive Substances (NPS) and are principally free aminoacids (FAA) and in less quantity ammonia, meltylamines, trimetylamines oxides, betaine and sarcosine (LANGE, 1972; LOOCKWOOD, 1976; BAGINSKI & PIERCE, 1977; IVANOVICI *et al.*, 1981; PIERCE, 1982).

Four main mechanisms have been proposed as major source of FAA and derived metabolites for osmoregulation in mollusks: (1) release of free aminoacids via protein degradation; (2) transamination reactions; (3) *de novo* synthesis and (4) uptake of dissolved aminoacids from the environment (SOMERO & BOWLUS. 1983).

It has been demonstrated that lysosomal and proteinase inhibitors prevent the accumulation of FAA in the gills of *Geunkensia demissa* leading to conclude that proteolysis was a major source of FAA for volume regulation in this mollusk (DEATON, 1987) and several studies in *Mytilus edulis*

(DEATON *et al*, 1984; KOEHN & HILBISH, 1987) have suggested that an important part of the FAA employed as osmotic effectors is released as a result of enzymatic activity of leucine aminopeptidase (LAP), an α-aminoacyl peptide hidrolase (EC.3.4.1.1) that catalizes the liberation of neutral and aromatic aminoacids from the N-terminus of di, tri and tetrapeptides which would be employed directly or after their conversion into osmotically active products by transamination reactions (KOEHN & HILBISH, 1987)

The importance of selection in determining the patterns of LAP variation in natural populations of *M. edulis* has been successfully demonstrated (KOEHN, & IMMERMAN, 1981; KOEHN & HILBISH, 1987). Although detailed biochemical studies of LAP have not been reported for other bivalves, population genetics studies on *M. galloprovincialis* and *Perna canaliculus* (GAERDNER & KATHIRAVETPILLA 1997), *Mercenaria mercenaria* (KOEHN *et al.*, 1980), *Geukensia demissa* (GARTHWAITE, 1986) *Crassostrea virginica* (BUROKER, 1983; ROSE, 1984) *C. rhizophorae* (NIRCHIO *et al.*, 1991) and *C. angulata* (MICHININA & REBORDINOS, 1997) support the hypothesis that LAP variation would be generally of selective importance in marine bivalves.

The mangrove oyster *Crassostrea rhizophorae* is closely related to *C. virginica*, the former replacing the latter in regions where there is no seasonal reduction of oceanic salinity (BUROKER, 1983). In spite of the fact that it lives in a very stable environment, the mangrove oyster is a strongly eurioyc mollusk and a wide range of tolerance to changes in environmental salinities has been reported (Poza & RODRIGUEZ, 1987). Early studies have revealed that in *C. rhizophorae* from La Restinga lagoon (Venezuela) LAP is determined by two polymorphic loci and that the genetic structure of the population seems to be associated to the selective effect of salinity (NIRCHIO *et al.*, 1991).

In this paper, the variations in LAP activity and in the concentration of intracellular proteins and ninhidrin positive substances of the mangrove oyster, *C. rhizophorae*, exposed to extreme tolerable salinities, are reported.

MATERIALS AND METHODS

Oysters were collected in La Restinga lagoon, Margarita Island, Venezuela and transported to the laboratory where epibionts were detached and valves were cleaned by brushing.

Oysters (6-7 cm total length) were divided into three groups of forty individuals each and placed in circular Plexiglas acuaria (60 l) provided with constant aeration. Water salinity was adjusted to 5. 38 and 60 ‰ and replaced daily.

Salinity of 38 ‰ was used as control since it was the environmental salinity at the time of collection; 5 ‰ and 60 ‰ are the minimum and the maximum salinity limits of tolerance reported for this species (POZA & RODRIGUEZ, 1987).

Ten individuals were taken daily from each aquarium, in order to determine the tissue water (TW) in the adductor muscle. The analysis continued until stabilization of TW values was achieved, taking this as a sign of acclimation. During the assay, oysters were not fed. To determine TW, the adductor muscle of each specimen was blotted with absorbent paper and weighed (wet weight), then samples were dried at 60° C overnight and weighed again. TW was calculated as the difference between wet and dry weight of the muscle as a percentage of the wet weight (CHEN & LIN, 1994).

To determine LAP activity, Ninhidrin Positive Substances concentration (NPS) and protein concentration, a portion of adductor muscle (50 ± 5 mg) was removed from each specimen and homogenized in 2 ml of double distilled and deionized water. Homogenates were then centrifuged at 3000 rpm for 15 min at room temperature and supernatant recovered and frozen until the analysis.

Total protein was determined by the Folin method with a standard Bovine Serum Albumine curve, enzymatic activity by the colorimetric method at 540-560 nm (SIGMA DIAGNOSTIC, 1986) and NPS by the following procedure: 0.5 ml from each supernatant was deproteinized adding 10 drops of 20% trichloroacetic acid with Pasteur pipet, left standing for 5 min at room temperature and centrifuged during 15 min at 3000 rpm. Fifty µl of each deproteinized sample, 450 µl of double distilled and deionized water and 0.5 ml of ninhidrin reagent were well mixed, placed in boiling water for 20 min and then cooled in chilled water. After cooling, 8 ml of 50% n-propanol was added to each tube and mixed. Absorbance (A570) was read using a blank water. SPN concentration was deduced from a calibration curve using a glicine standard. Data were expressed as µg of SPN/mg wet weight.

Statistical analysis was performed according to SOKAL & ROLFH (1969).

RESULTS AND DISCUSSION

Figure 1 shows the variation of TW in the muscle of *Crassostrea rhizophorae* at each salinity tested in relation with time of exposition. No statistical differences were found in the 38 ‰ control group (Fs=2.300; P>0.05) while in the 5 ‰ group an increase in TW was observed. TW decreased in the 60 ‰ group. Changes in TW in the extreme salinities were observed during the first 48 hours in the 5 ‰ group (Fs=19.336; P<0.001) and during the first 24 hours in the 60 ‰ group (Fs=11.489; P<0.001). After this time and until 96 hours, no differences were detected within each group (P>0.05). This was taken as an indication of acclimation.

At the end of the assay, TW, which represents interstitial and intracellular water (CHEN & LIN, 1994), increased 12,38% per oyster in the 5 ‰ treatment and decreased 6,48% in the 60 ‰ group. These results seem to indicate that the species is more efficient in the regulation of TW and hence cell volume regulation when exposed to hypersaline waters, probably as a result of the adaptation of this species to local hypersaline conditions. Actually, salinity levels in La Restinga lagoon range between 36.24 ‰ and 60.59 ‰ (CABRERA & PENOTH, 1988). Although the salinities tested are the extreme tolerable limits, it is not probable that under natural conditions this species confronts variations of salinity as pronounced and sudden as the ones observed here. As a matter of fact, there are no records of salinities below 36 ‰ in La Restinga lagoon.

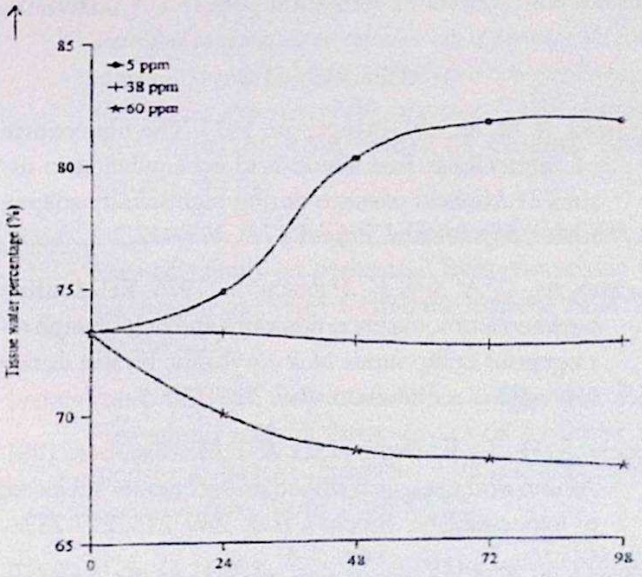


Figure 1: Tissue water variation in the muscle of *Crassostrea rhizophorae* during exposition at three experimental salinities.

Free aminoacids (FAA) are the main constituent of NPS (PENG *et al.*, 1994). Data obtained in several species of mollusks indicate that at least 95% of NPS composition in deproteinized adductor muscle samples correspond to FAA (LYNCH & WOOD, 1966; LIVINGSTONE *et al.*, 1979; IVANOVICI *et al.*, 1981). In most species of marine invertebrates studied so far, some combination of taurine, alanine, glycine, proline, aspartate and glutamate make up the portion of aminoacids pool employed for volume regulation (PIERCE, 1982).

Since FAA concentration is the result of uptake-release budget (LANGE, 1972), and that the biochemical action, cellular distribution, and response of enzyme activity to salinity changes support the interpretation of LAP playing a critical role in protein catabolism to provide the FAA employed as osmotic effectors for cellular volume regulation (KOEHN & HILBISH, 1987), it is reasonable to assume that maintaining this pool in appropriate levels under high salinity conditions requires an increment in the activity of the enzyme.

The mean activity of LAP, NPS concentration and protein concentration in each group is shown in figure 2. Analysis of variance revealed statistical differences for LAP activity (Fs=61.6; P<0.001) at different salinity, NPS concentration (Fs=20.368; P<0.001) and protein concentration (Fs=5.054; P<0.001). The SNK test shows that, in the case of LAP activity, the three means are different, while in the case of protein concentration differences were evident only between the 5 ‰ and 60 ‰ groups.

The highest detected mean of LAP activity in the 60 ‰ group coincided with a significant reduction of total protein concentration and with an increment in NPS concentration.

The correlation between salinity and NPS has been verified in *Mya arenaria* (VIRKAR & WEEB, 1970), *Crassostrea virginica* (HAND & STICKLE, 1977), *Modiolus demissus* (BAGINSKI & PIERCE, 1977) and *Mytilus edulis* (LIVINGSTONE *et al.*, 1979). As in the cited examples, data here presented show that in *Crassostrea rhizophorae*, an increment in environmental salinity results in the accumulation of intracellular NPS, while low salinity exposition determines its diminution.

Since LAP hydrolyzes N-terminus bonds of di, tri and tetrapeptides to cleave off free aminoacids, an increment of intracellular NPS in the oyster exposed to the highest

salinity (60 ‰) could be interpreted as a result of an increase in the LAP activity. This is supported by the fact that the increment of enzymatic activity and NPS were accompanied by a 20% protein decrease. Since the oysters had been deprived of food during the test period, it is most likely that the increase in NPS was due to increased protein catabolism. BISHOP *et al.* (1981) have pointed out that during hyperosmotic acclimation in *Mytilus* FAA accumulation is accompanied by a reduction of 10-15% in protein levels.

It has been suggested that, without excluding other processes, at least 43% of FAA accumulation after transfer to high salinity is the result of LAP related protein catabolism in *M. edulis* (DEATON *et al.*, 1984). Since LAP hydrolyzes small peptides and not proteins, the reduction of total protein levels supports the participation of other proteolytic enzymes that should provide the small peptides used as substrate for LAP.

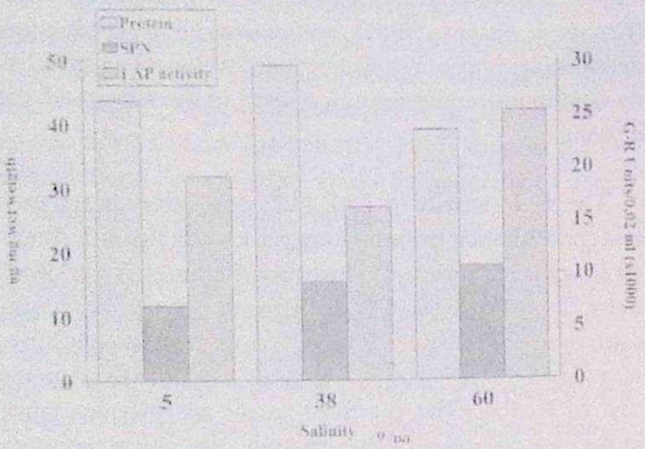


Figure 2. Lap activity (G-R Units/0.02 ml), ninhidrine positive substances (ug/mg wet weight) and protein concentration (ug/mg wet weight) in *Crassostrea rhizophorae* exposed to extreme salinities.

It was an unexpected finding to verify that LAP activity was also increased at 5 ‰, although in less proportion than at 60 ‰. Due to the fact that acclimation to a hyposaline environment requires a reduction of intracellular osmotic pressure by releasing aminoacids instead of incorporating them, and because NPS concentration in this group was significantly lower than at 38 ‰ and 60 ‰ (Fig 2), an increment of LAP activity does not seem to be convenient in oysters maintained at 5 ‰. This increase could be interpreted as part of an homeostatic mechanism to restore intracellular FAA that are released by direct and sustained excretion of aminoacids or ammonia, its catabolic product, in order to support a constant turnover rate, minimizing the effect of critical alterations of cell volume, or to cover other requirements of protein metabolism (e.g. synthesis).

It has been proved that a decrease in FAA concentration is parallel to an increment in ammonia excretion rate and, conversely, when the FAA pool increases, the loss of ammoniacal nitrogen is reduced. Consequently, variations in the FAA pool can be followed by changes in $NH_{3,4}$ excretion (LOCKWOOD, 1976; KOEHN & HILBISH, 1987). Unpublished data (GAVIRIA, 1989) indicate that $NH_{3,4}$ excretion *in C. rhizophorae* increases at low salinity. The increased $NH_{3,4}$ excretion in mollusks maintained in hypoosmotic environment has been interpreted as a result of aminoacid degradation instead of polymerization to intracellular peptides or proteins (BARTBERGER & PIERCE, 1976; LOCKWOOD, 1976).

Data presented are in agreement with the generalization (KOEHN & HILBISH, 1987) about LAP being an important enzyme in the process of cellular volume regulation of bivalves.

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