Effects of Seawater Acclimation on the Levels of Free D- and L-Alanine and Other Osmolytes in the Japanese Mitten Crab *Eriocheir japonicus*

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(Received April 19, 1999)

During artificial seawater acclimation in the laboratory of freshwater Japanese mitten crab *Eriocheir japonicus*, glycine and L-proline other than D- and L-alanine increased significantly in the muscle of the immature group, while no significant increase in glycine and L-proline was found in the mature group. Total inorganic ions in the muscle of the mature crabs increased significantly along with the salinity increments and reached the levels of estuarine specimens captured during downstream migration. In the immature group, inorganic ions also increased during salinity stress but the increase was far lower than those in the mature group. These data suggest that immature crabs cannot tolerate high ionic concentrations in muscle and have to increase glycine and L-proline during seawater acclimation. After adjustment of salinity tolerance, mature crabs incorporate inorganic ions in muscle and their cell volumes are likely regulated mainly by D- and L-alanine and inorganic ions.

Hemolymph ion concentrations and osmolalities increased largely during seawater acclimation for both immature and mature crabs and reached the same levels as those for estuarine and sea specimens in the natural environment. These data suggest that no special adjustment of hemolymph osmoregulatory capability occurs along with the maturation.

Key words: D-alanine, L-alanine, crustacean, *Eriocheir japonicus*, hemolymph, muscle, osmoregulation, seawater acclimation

As we proposed previously,^{1–4)} free D-alanine accumulated in large amount in the tissues of various crustaceans and mollusks is thought to be the most potent osmolyte for intracellular isosmotic regulation. The levels of this specific amino acid are well regulated in the tissues of these invertebrates through alanine racemase action.^{5,6)} Several species of crustaceans have been known to be strong osmoregulators and to migrate freely between freshwater and seawater. A representative of these euryhaline crustaceans is the Japanese mitten crab *Eriocheir japonicus* which inhabits the river for growth and maturation and migrates downstream to the sea for mating and spawning. A closely related Chinese counterpart *E. sinensis* has long been employed for the studies on the tissue isosmotic regulation as a classical model animal from the early 1950s.^{7–12)} These catadromous species are known to exhibit an extremely high capability to regulate intracellular and extracellular osmolality. Thus, we were interested in the mechanisms underlying the powerful hyper-osmoregulation of this species with special reference to the physiological functions of free D-alanine.

In the previous paper,¹³⁾ we reported that the Japanese mitten crab accumulates only D- and L-alanine and inorganic ions in muscle during maturation in the river and during

downstream migration to estuaries, and accumulates more D- and L-alanine and glycine in place of deleterious inorganic ions in the sea. We concluded from these data that D- and L-alanine are not simple osmolytes but play an important role in the adjustment of salinity tolerance prior to and during downstream migration toward the sea.

In this paper, we describe the changes of D- and L-alanine and other osmolytes in muscle and hemolymph of Japanese mitten crab captured in the river during artificial seawater acclimation in the lab, and we compare the changes with those during downstream spawning migration in the natural environment.

Materials and Methods

Materials

Japanese mitten crabs were captured at 30 km upstream from the mouth of the Kushida River (Mie Prefecture, Japan) once a month from April to October, 1995. Fifteen individuals in each month except for October ($n=9$) were transported alive to the laboratory. Estuarine ($n=5$) and sea ($n=10$) specimens were captured in October at the mouth of the Kushida River and coastal shallow regions, respectively. Crabs were caught using commercial crab

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traps. The ratio of male to female was almost the same in all groups. All the crabs captured in the river were in inter-moult stage.

Seawater Acclimation

Animals were kept in a laboratory tank (60 l) supplied with aerated circulating water at ambient temperature. Animals used for the seawater acclimation experiments were initially maintained in freshwater for 3 to 5 days after capture. Salinity in rearing water was then increased to full-strength seawater level using an artificial seawater salt mixture. At the 1st day, crabs were transferred to 50% seawater (17 ppt) tank, salinity was then increased to 67% seawater level at the 2nd day and 75% seawater level at the 5th day. After that, salinity was elevated gradually to full-seawater level (35 ppt) over 4 days and the crabs were kept in seawater for 3 days. Animals were not fed during the acclimation period. Five individuals were sampled at freshwater, 50% seawater, and full-seawater stages. All the contents of amino acids and inorganic ions were expressed as $\mu\text{mol/g}$ wet weight, because only a several percent decrease of muscle moisture content was found during seawater acclimation.

Preparation of Extracts

Tissues were dissected from individuals obtained from each seawater acclimation stage and from estuary and sea. A perchloric acid extract of each tissue was prepared from a one gram sample as described previously.^{2,14} Hemolymph was drawn from the pericardial cavity by a syringe and extracted by an equal volume of 8% perchloric acid.

Derivatization and HPLC Determination of D- and L-Amino Acids

Precolumn derivatization of amino acids with (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC) was carried out as previously described.^{13,14} FLEC-derivatized amino acids were separated and determined by a Shimadzu LC-6A HPLC system (Shimadzu, Kyoto) according to the previous method.^{13,14}

Determination of Inorganic Ions and Osmolality

Ion chromatography was performed using a HPLC with ion chromatography system (Shimadzu, Kyoto). Sodium, potassium, and chloride ions were determined according to the manufacturers instructions. Hemolymph osmolality was determined with an automatic micro-osmometer (Roebing Type 13 DR, Berlin) on the whole hemolymph after filtration with 0.45 μm filter and appropriate dilution with HPLC grade water.

Statistical Analysis

Statistical comparisons of the results were carried out using one-way ANOVA followed by post-hoc comparison of means (Duncan's multiple range test). All data were expressed as means $\pm$ SD.

Results

Changes of D- and L-Alanine and Other Osmolytes in Muscle during Salinity Stress

Monthly captured freshwater specimens were acclimat-

Table 1. Average body weight and carapace width of four groups of Japanese mitten crab

	Immature <i>n</i> =45	Mature <i>n</i> =54	Estuary <i>n</i> =5	Sea <i>n</i> =10
Body weight (g)	64 $\pm$ 36 ^a	85 $\pm$ 45 ^b	139 $\pm$ 26 ^c	108 $\pm$ 40 ^{bc}
Carapace width (mm)	51 $\pm$ 7 ^a	57 $\pm$ 7 ^b	65 $\pm$ 4 ^c	59 $\pm$ 6 ^{bc}

Immature and mature crabs were captured in the river from April to June and from July to October, respectively. Estuarine and sea specimens were caught at the mouth of the river and in a coastal shallow region, respectively, during downstream spawning migration in October.

Means with the same horizontal line that do not share a common superscript letter were significantly different ($p < 0.05$ or less).

ed to 50% and full-strength seawater in each month. Female crabs with matured gonads emerged from July and all females attained maturity in the September, October, and estuarine specimens. Females captured in the sea ($n=5$) bore eggs without exception. In consideration of their gonad maturation and a turning point of D- and L-alanine levels, the monthly data were combined into two groups, immature (April to June) and mature (July to October). Table 1 shows body weights and carapace widths for these groups. Mean values of body weight and carapace width became larger significantly along with maturation ($p < 0.05$ for body weight and $p < 0.001$ for carapace width) and were largest in the estuarine specimens. The mature and estuarine crabs showed no significant difference in these values from the sea specimens.

Figure 1 shows the D- and L-alanine, glycine, and L-proline data in muscle with those of the estuarine and sea specimens for comparison. Both D- and L-alanine in muscle increased with increasing the salinity, although the significant increase of both alanines was restricted only in the full-seawater acclimated specimens. In both immature and mature groups, D- and L-alanine levels after seawater acclimation did not differ significantly from those of the estuarine specimens but were significantly lower than those of the sea specimens. Total alanine levels of the seawater-acclimated immature and mature groups were 62.5 ± 18.6 and $67.7 \pm 20.0 \mu\text{mol/g}$, respectively, which were almost the same as those of the estuarine specimens ($63.1 \pm 23.5 \mu\text{mol/g}$) but significantly ($p < 0.001$) lower than those of the sea specimens ($82.9 \pm 7.8 \mu\text{mol/g}$). The percentage of D-alanine to total alanine of the seawater-acclimated, estuarine, and sea specimens showed rather small change and fell into the range of 31.4–34.0%. Concentrations of L-alanine vs. D-alanine were plotted for each individual crab ($n=114$) irrespective of freshwater, seawater-acclimated, or estuarine and sea specimens (Fig. 2). High correlation was found between D- and L-alanine levels ($r=0.942$). The linear regression line was as follows: [L-alanine] = $1.60 \times [\text{D-alanine}] + 12.5$.

In contrast to the increase of D- and L-alanine, glycine increased only in the immature group during seawater acclimation and no substantial increase was found in the mature group. These glycine levels in the seawater-acclimated immature crabs were, however, significantly lower than those in the specimens captured in the sea. L-Proline also increased significantly in the immature crabs during salinity stress and reached the level of the sea specimens. L-Proline showed an increasing tendency even in the mature

Fig. 1. Changes of D- and L-alanine, glycine, and L-proline in muscle during artificial seawater acclimation of Japanese mitten crab.

Data for the estuarine ($n=5$) and sea specimens ($n=10$) were shown for comparison. FW, freshwater; HSW, 50% seawater; SW, full-strength seawater. Significant (* $p<0.05$; ** $p<0.01$; *** $p<0.001$) when compared with each freshwater control. Significant (# $p<0.05$; ## $p<0.01$; ### $p<0.001$) when comparing the full-seawater acclimated and estuarine crabs with the sea specimens. All data are represented in terms of mean $\pm$ SD for 15 and 18 individuals in each acclimation stage for immature and mature group, respectively.

Fig. 2. Correlation between D- and L-alanine levels ($\mu\text{mol/g}$ wet weight) in the muscle of Japanese mitten crab.

All 114 data are for the immature and mature groups, estuarine, and sea specimens shown in Table 1.

group while the increase was not significant.

The changes of all major amino acid osmolytes and inorganic ions are shown in Fig. 3. In spite of the increase of D- and L-alanine or glycine and L-proline (Fig. 1), changes of the other amino acid levels were small and the total amino acid changes were not significant statistically during seawater acclimation in either group, due to a large decline of arginine (insignificant) along with the salinity increments. Total amino acid levels of the seawater-acclimated speci-

Fig. 3. Changes of major amino acids and inorganic ions in muscle during artificial seawater acclimation of Japanese mitten crab.

SD bars were shown only for total amounts. Refer to the legend of Fig. 1 for salinity and significance.

mens, however, exhibited no significant difference from the sea specimens for both immature and mature groups. The levels were significantly lower in the estuarine specimens, due to a large decrease of arginine.

Total inorganic ions increased significantly during seawater acclimation. The levels for the seawater-acclimated crabs of the mature group were almost the same as those for the estuarine specimens and much higher than those for the sea specimens. Ionic concentrations in seawater-acclimated immature crabs were almost the same as those in freshwater mature crabs which showed no difference from the sea specimens in ionic concentrations. The ionic com-

Fig. 4. Hemolymph total amino acids, inorganic ions, and osmolalities during artificial seawater acclimation of Japanese mitten crab.

Refer to the legend of Fig. 1 for salinity and significance. FAA, free amino acids.

position in muscle was rather unique; much higher sodium than potassium ions were found even in the freshwater specimens for both immature and mature groups. The sums of free amino acids and inorganic ions were 617 ± 93 , 708 ± 88 , 628 ± 78 , and 634 ± 63 $\mu\text{mol/g}$ wet weight for the seawater-acclimated immature and mature, estuarine, and sea specimens, respectively, and revealed to be insignificant.

Changes of Osmotic Concentrations and Osmolalities in Hemolymph during Seawater Acclimation

Hemolymph osmolytes and osmolalities changed much more remarkably during seawater acclimation (Fig. 4). Although the total free amino acids were low and declined slightly along with the acclimation, total inorganic ions elevated significantly with increasing salinity. No difference was found between the estuarine and the seawater-acclimated specimens of both immature and mature groups. As was the case for muscle (Fig. 3), the sea specimens contained significantly fewer inorganic ions compared with the seawater-acclimated specimens. Hemolymph osmolality also increased with increasing salinity in both immature and mature groups, although the osmolalities were rather low for freshwater and half-seawater specimens in the mature group. No significant difference of osmolalities was observed among the seawater-acclimated, estuarine, and sea specimens. Osmolalities as a function of external osmotic concentrations are shown in Fig. 5 as the mean values for both immature and mature crabs. The increment of hemolymph osmolalities was almost linear with increasing the external salinity but the slope was rather low from freshwater to seawater.

In the other tissues, heart muscle also accumulated D- and L-alanine significantly during seawater acclimation and their contents surpassed those in the estuarine and sea specimens (data not shown). In hepatopancreas, gills, and nervous tissues, both D- and L-alanine were small in amount and did not increase during salinity stress. The changes of total free amino acids, however, differed be-

Fig. 5. Effects of external salinity on the hemolymph osmolality during seawater acclimation of Japanese mitten crab.

Individual numbers were 33 for each freshwater (FW), half-seawater (HSW), and seawater (SW) acclimated specimens. Osmolalities for FW and HSW crabs were significantly lower than those for the specimens captured in the sea (Sea).

tween the immature and mature crabs during seawater acclimation (data not shown).

Discussion

Japanese mitten crab is a typical euryhaline catadromous crustacean and known to survive even when a crab inhabiting freshwater is transferred directly into full-seawater, and *vice versa*. This potential capability is typically seen in the small concentration differences of total osmolytes in muscle (Fig. 3) and hemolymph (Figs. 4 and 5) between freshwater and seawater-acclimated crabs. Freshwater crabs kept high osmolalities in hemolymph which accounted for 68% of seawater-acclimated counterparts (Fig. 5). In the natural environment as we reported previously,¹³ the crab accumulated only D- and L-alanine as well as inorganic ions in muscle during maturation before downstream migration. During migration, the crab accumulated more D- and L-alanine and inorganic ions around the estuarine regions. After reaching the sea, however, glycine was also accumulated in large amounts with a concomitant decrease of inorganic ions, as seen in the sea specimens in Figs. 1 and 3. No proline increase was found during downstream migration.

In contrast to the downstream migration in the natural environments, seawater acclimation carried out artificially in the lab gave some different data (Figs. 1 and 3). In the muscle of the immature group, D- and L-alanine, glycine, and L-proline increased significantly during seawater acclimation but the glycine and L-proline increase was restricted only in the immature group (Fig. 1). In this respect, *E. sinensis*, a close relative of the Japanese species, has been reported to accumulate large amounts of alanine, glycine, proline, and glutamate + glutamine in muscle during artifi-

cial seawater acclimation.⁷⁻¹²⁾ Our data shows that the increase of glycine and proline, especially glycine, is limited only in the immature group of Japanese mitten crabs. In addition, in the natural environment as described above, glycine increased only in the sea specimens and no glycine and proline increase was found during the course of the migration to the sea. Thus, the difference of amino acid response between *E. sinensis* and *E. japonicus* may stem from their maturation stages.

The increase of these amino acids in muscle was eliminated by the decrease of arginine and no large increase was found in total amino acids during seawater acclimation (Fig. 3). Large increase was, however, significant only in the ionic concentrations. There was almost no difference of total inorganic ions between the seawater-acclimated immature crabs and freshwater mature crabs. This suggests the absorption of inorganic ions probably into extracellular space of muscle from hemolymph along with the maturation of this crab. This is in agreement with the data that sodium ions surpassed potassium ions even in the muscle of freshwater specimens. This fact has also been reported for *E. sinensis*.⁹⁾ These data suggest that the immature crab does not yet acquire salinity tolerance and cannot incorporate large amounts of inorganic ions from hemolymph into muscle. Thus, the immature crab might have to increase glycine and L-proline as well as D- and L-alanine in muscle during seawater acclimation. Mature crabs, on the contrary, are thought to acquire salinity tolerance for incorporating inorganic ions in large amounts into muscle and need the increase of only D- and L-alanine as amino acid osmolytes. It is not clear, however, the reason why seawater-acclimated mature crabs did not accumulate glycine in muscle in contrast to the sea specimens in the natural environment.

Irrespective of the maturation stages, D- and L-alanine were the only common amino acid osmolytes increased during salinity stress. High correlation between the contents of D- and L-alanine (Fig. 2) suggests that alanine racemase catalyzes the interconversion between the two enantiomers. Alanine racemase activity assayed according to the previous method^{5,6)} was highest in the muscle of mitten crab of all crustaceans thus far examined.⁵⁾ Thus, this high activity of alanine racemase may be responsible for the rapid interconversion between D- and L-alanine in this euryhaline crab.

In hemolymph, the increase of inorganic ions was significantly large during seawater acclimation and did not differ between the immature and mature groups (Fig. 4). Ionic concentration and hemolymph osmolality of the seawater-acclimated specimens, irrespective of the immature or mature group, were almost the same as those of the estuarine and sea specimens (Fig. 4), indicating no special adjustment of the capability for the hemolymph extracellular osmoregulation is needed during maturation in the river. The crabs kept hyperosmoticity in lower salinity and hypoosmoticity in higher salinity environments, suggesting the strong capability of extracellular osmoregulation in this crab. The isoionic point was high at about 700 mOsm/kg water (Fig. 5).

In conclusion, it was apparent that the response of amino acid osmolytes and inorganic ions to salinity increase differed between the immature and mature crabs. After

maturation, muscle isosmotic pressure of this outstanding euryhaline species and thus the muscle cell volume may be regulated by the incorporation of inorganic ions from hemolymph during seawater acclimation and during downstream spawning migration. They may have to accumulate only D- and L-alanine rather than inorganic ions to cope with the extracellular salinity increase. In contrast, the immature crab having no salinity tolerance cannot tolerate the high ion concentrations in muscle and may have to accumulate glycine and L-proline as well as D- and L-alanine. Thus, the glycine and/or L-proline increase is thought to occur only above half-seawater salinity level during seawater acclimation of the immature crab and only in the sea during downstream migration of the mature crab. Therefore, the artificial seawater acclimation experiment using the immature animals may lead to some different mechanisms from those occurring in nature. It is clear, however, that D-alanine together with L-alanine is a key osmolyte during maturation,¹³⁾ during seawater acclimation for both immature and mature crabs, and during downstream migration in the natural environment.¹³⁾ These data also gave rise to an interest in the mechanism for acquiring the muscle salinity tolerance during the maturation of this competent euryhaline species.

Acknowledgments This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan (07660278) and by a grant from the Ito Gyogaku Foundation. We wish to thank Ms Ai Yamada for her technical assistance. We also thank Mr. Yukio Suzuki for collecting the freshwater crab samples.

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