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Effect of salinity change on free amino acid content in Pacific oyster

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ABSTRACT: In order to identify free amino acids (FAA) that are important as intracellular osmolytes in *Crassostrea gigas*, we investigated the change in FAA content in the mantle exposed to an abrupt decrease or increase in salinity. In hypo-osmotic adaptation, most FAA showed remarkable and synchronous decreases from 2 to 8 h, suggesting that the non-selective efflux of FAA was mainly responsible for the decrease in FAA. Taurine that accounted for approximately 80% of total FAA content contributed most significantly to the hypo-osmotic adaptation. In hyper-osmotic adaptation, significant increases in glycine, alanine, β-alanine, proline, arginine and taurine were observed. Of these, alanine showed an immediate increase that is important to short-term adaptation to hyper-osmolality, while taurine showed a slower and substantial increase that contributes to a long-term adaptation to hyper-osmolality.

KEY WORDS: **adaptation, cell volume regulation, *Crassostrea gigas*, free amino acid, mantle, osmolyte, oyster, salinity.**

INTRODUCTION

Some species of oyster live in habitats with various salinities. They thrive in inner bay areas where the salinity is lower than that of ocean water, in outer areas influenced by pelagic water and in estuarine environments where the salinity varies diurnally, seasonally and annually. For instance, the American oyster *Crassostrea virginica*, one of the most studied species of oyster, has a very wide range of optimal salinity, from 0.5 to 3.0%, and is therefore able to adapt to a wide range of environments.[1] Other oysters also have the ability to adapt to a wide range and/or to fluctuation of salinity, and the ability seems to depend on the species.[1–3]

To adapt to change or fluctuation in the salinity of ambient seawater, osmoconforming marine animals including oysters have mechanisms to adjust the concentration of intracellular osmolytes by which they regulate cell volume. Many articles have dealt with the osmoconforming mechanisms of bivalves, in which intracellular free amino acid (FAA) is demonstrated to contribute dominantly to intracellular osmolality and to cell volume regulation.[4] Studies on FAA as osmolytes in some species of oyster have also been reported. In the muscle of *C. virginica*, FAA accounted for 20.9% of the total osmotic effect in 785 mOsm/kg·H_2O, and, in particular, the content of taurine (Tau), alanine (Ala), glycine (Gly) and proline (Pro) changed significantly with salinity.[5] In the Portuguese oyster *Crassostrea angulata*, FAA made up an average of 30% of the total osmotically active substances in the adductor muscle of animals acclimated to both 1188 and 594 mOsm/kg·H_2O waters.[6]

The Pacific oyster *Crassostrea gigas* is the primary oyster species supporting shellfish industries around the world, accounting for an estimated 80% of the world oyster production.[7] C*rassostrea gigas* has been successfully introduced into various countries and areas.[7,8] Therefore, many studies have dealt with the biomass, industrial productivity and the biochemical composition of *C. gigas*,[9–11] whereas fewer physiological investigations have been reported. To the best of our knowledge, only one article dealing with salinity has appeared in the past decade, in which Calvo *et al.* showed that the optimal salinity of *C. gigas* was higher than that of *C. virginica*,[3] while none has dealt with the osmolyte system of *C. gigas*.

In the present study, as a first step towards understanding the osmoconforming mechanism

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of *C. gigas*, we measured FAA, which are known to be important osmolytes in marine bivalves, in the mantle of *C. gigas* exposed to abrupt changes in salinity. FAA that are important as osmolytes were identified and their role in the mechanism of osmo-adaptation is discussed.

MATERIALS AND METHODS

Animals and experimental procedures

Specimens of *C. gigas* at the age of about one and a half years, which had been cultured in Matoya bay, Japan, were maintained in artificial seawater (ASW; Marine Merit, Matsuda, Osaka, Japan) of 3.0% salinity (100% ASW) or 1.5% salinity (50% ASW) from 7 to 10 days with no feeding before exposure to abrupt changes in salinity. About 20 oysters were maintained in 50 L ASW with aeration, and water temperature was kept at 15°C. All experiments were performed in March 1999.

Oysters were exposed to an abrupt decrease or increase in salinity. In the first experiment, oysters acclimated to 100% ASW were transferred to 50% ASW (abrupt salinity decrease from 3.0% to 1.5%). In the second experiment, oysters acclimated to 50% ASW were transferred to 100% ASW (abrupt salinity increase from 1.5% to 3.0%). Part (about 10 mm long and 5 mm wide) of the shell edges of each specimen was chipped away in both experiments in order to ensure the free exchange of water between the inside and outside of the shells.

Three specimens were taken at 0, 2, 8, 24 and 72 h in the first experiment, and also at 48 h in the second experiment after exposure to the salinity changes. Approximately 100 mg tissue was taken from the edge of the mantle lobe of each specimen for analysis because it was thought to be the tissue exposed first and most directly to the change in ambient salinity. Hemolymph was taken from the pericardial space with a syringe.

Osmolality of hemolymph

The osmolality of the hemolymph and ASW were determined by freezing point depression using a Knauer semimicro osmometer (Knauer, Berlin, Germany).

Tissue water content

Approximately 1 g of sample tissue was blotted dry and its wet weight was determined. It was then dried at 120°C to constant weight and the water content was calculated.

Free amino acid analysis

Tissue samples were homogenized in 10 volumes (w/v) of 80% ethanol and centrifuged at 10 000 $\times g$ for 15 min. Samples of 50 mL of the supernatants were dried and analyzed for amino acids. The amounts of FAA in the tissues were determined by high-performance liquid chromatography (HPLC) on a Cosmosil $5C_{18}$AR-2 packed column (250 mm $\times$ 4.6 mm i.d., particle size 5 µm; Nacalai Tesque, Kyoto, Japan) by the method of Sato *et al.*[12] The amounts of Ala, arginine (Arg), aspartate (Asp), glutamate (Glu), Gly, Pro, β-alanine (β-Ala) and Tau, which had been reported as major FAA in *C. gigas* by Sato[13], were identified and determined.

Statistics

Statistical analysis was performed by *t*-test using EXCEL X (Microsoft), and $P < 0.1$ was used as the level of significance.

RESULTS

Change in osmolality

Figure 1 shows the changes in osmolality of the hemolymph. In the first experiment, oysters were exposed to an abrupt decrease in osmolality from 800 to 380 mOsm/kg·H_2O, and the average osmolality of the hemolymph showed a sharp decrease after 2 h. At 8 h after the exposure, the osmolality of the hemolymph was in equilibrium with that of the ambient seawater. In the second experiment, when oysters were exposed to an abrupt increase in osmolality from 380 to 800 mOsm/kg·H_2O, the hemolymph osmolality increased rapidly and reached equilibrium with the ambient osmolality after 8 h.

Water content

The water content of the mantle of oysters exposed to hypo-osmolality increased sharply from 80.9% at 0 h to 86.9% at 8 h, then decreased gradually to 80.4% at 72 h after the exposure, the same level as that at 0 h (Fig. 2a). However, the abrupt increase in osmolality of the ambient seawater resulted in a sharp decrease in water content of the mantle,

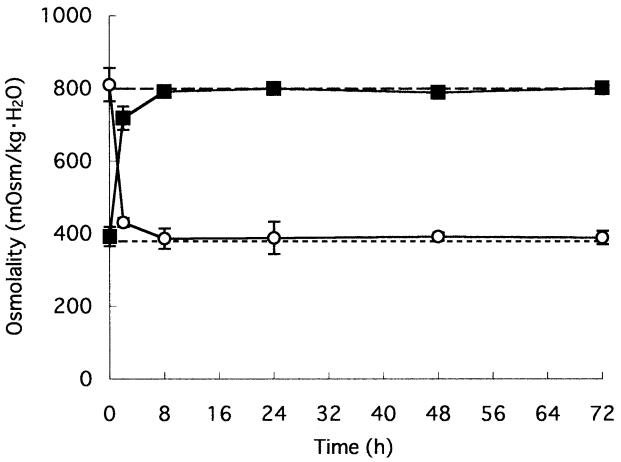

Fig. 1 Change in osmolality of the hemolymph in the Pacific oyster exposed to (○) an abrupt decrease in osmolality from 800 to 380 mOsm/kg·H_2O and (■) an abrupt increase in osmolality from 380 to 800 mOsm/kg·H_2O. Vertical bar, standard deviation ($n = 3$). (– – –), 100% artificial seawater and (- - -), 50% artificial seawater.

which decreased from 83.1% at 0 h to 77.7% at 24 h and remained at that level until 72 h (Fig. 2b).

Change in free amino acid content

In 100% ASW, Tau was the predominant FAA, accounting for about 80% of the total content of the eight FAA in the mantle. Gly, Ala, Asp and Glu were the major FAA in the remaining 20%, and β-Ala, Arg and Pro were minor (Table 1).

The exposure to 50% ASW resulted in a decrease in total FAA content from 391.1 μmol/g dry weight to 175.2 μmol/g dry weight at 72 h. The decrease was especially marked from 2 to 8 h. Because the amount of Tau was much greater than that of the other FAA, the change in total FAA content was mostly due to the change in Tau content. Although the other FAA, except for Pro, decreased significantly at 72 h in hypo-osmolality, their contribution to the total change was small due to their low contents.

The FAA content in the mantle of the oyster that acclimated to 50% ASW was 176.6 μmol/g dry weight, equivalent to 45.2% of that in the oyster acclimated to 100% ASW (Table 2). After transfer to 100% ASW, it remained almost constant for 24 h, then increased to 262.1 μmol/g dry weight at 72 h. As in the first experiment, the large amount of Tau significantly influenced the pattern of the change in total content, but some other FAA showed more remarkable increases.

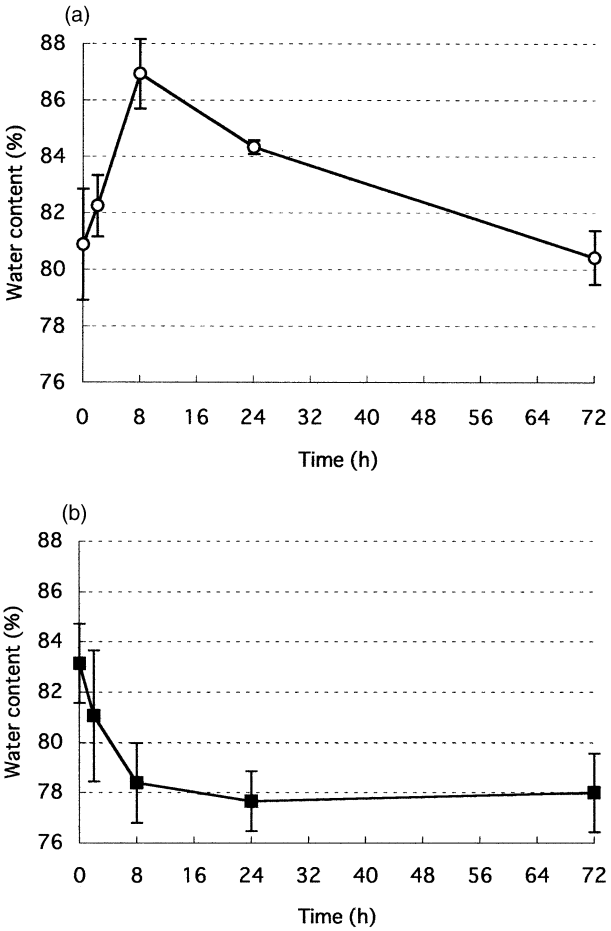

Fig. 2 Change in water content of the mantle of the Pacific oyster exposed to (a) an abrupt decrease in osmolality from 800 to 380 mOsm/kg·H_2O and (b) an abrupt increase in osmolality from 380 to 800 mOsm/kg·H_2O. Vertical bar, standard deviation ($n = 3$).

Gly, β-Ala, Pro and Arg increased especially from 24 to 48 h, and their contents at 72 h corresponded to 193.8%, 353.1%, 229.6% and 203.6% of that at 0 h, respectively. Ala was most sensitive to the hyper-osmolality, increasing immediately from 0 h to 2 h after the exposure to 100% ASW and again from 24 h to 48 h, and its content at 72 h corresponded to 270.9% of that at 0 h. Asp and Glu did not increase significantly, suggesting that they are not involved in the adaptation to hyper-osmolality.

DISCUSSION

In the present study, we confirmed that *C. gigas* is an osmoconformer,[14] because its hemolymph osmolality attained equilibrium with the ambient osmolality within 8 h after the change in osmolality

Table 1 Time course of change in free amino acid content in the mantle of the Pacific oyster transferred from 100 % artificial seawater to 50 % artificial seawater

	Acclimated to 100% artificial seawater (0 h)	Time after exposure to 50% artificial seawater			
		2 h	8 h	24 h	72 h
Taurine	304.9 ± 73.9 (100%)	346.9 ± 151.9 (113.0%)	209.1 ± 30.0* (68.6%)	191.0 ± 16.0** (62.6%)	129.0 ± 28.1† (42.3%)
Glycine	21.6 ± 5.0 (100%)	32.1 ± 21.9 (148.6%)	12.9 ± 5.1* (59.7%)	8.7 ± 3.8** (40.3%)	6.0 ± 0.9† (27.8%)
Alanine	17.2 ± 5.4 (100%)	28.8 ± 14.3 (167.4%)	8.1 ± 2.2** (47.1%)	9.1 ± 5.9* (52.9%)	8.2 ± 5.6** (47.7%)
Aspartate	15.6 ± 1.0 (100%)	14.4 ± 7.3 (92.3%)	17.5 ± 0.5 (112.2%)	13.6 ± 4.3 (87.2%)	11.5 ± 2.1** (73.7%)
Glutamate	13.3 ± 0.8 (100%)	17.1 ± 8.7 (128.6%)	11.3 ± 1.9* (85.0%)	9.3 ± 2.2** (69.9%)	9.1 ± 1.6† (68.4%)
β-Alanine	7.7 ± 4.2 (100%)	8.2 ± 4.2 (106.5%)	4.1 ± 0.4* (53.2%)	1.4 ± 0.2† (18.2%)	1.4 ± 0.7† (18.2%)
Proline	7.2 ± 4.1 (100%)	8.9 ± 4.7 (123.6%)	5.6 ± 2.6 (77.8%)	7.6 ± 5.3 (105.6%)	8.1 ± 2.1 (112.5%)
Arginine	3.6 ± 1.3 (100%)	4.8 ± 3.5 (133.3%)	3.0 ± 0.5 (83.3%)	1.4 ± 0.4† (38.9%)	1.9 ± 1.7** (52.8%)
Total	391.1 ± 59.7 (100%)	461.1 ± 211.3 (117.9%)	271.6 ± 40.4** (69.4%)	242.1 ± 13.1† (61.9%)	175.2 ± 41.3† (44.8%)

Values are in μmol/gram dry weight ± SD. Values in parentheses are the percentage of the value at 0 h. Significant differences from the value at 0 h are represented by $^{*}P < 0.1$, $^{**}P < 0.05$ and $\dagger P < 0.01$. $n = 3$.

Table 2 Time course of the change in free amino acid content in the mantle of the Pacific oyster transferred from 50 % artificial seawater to 100 % artificial seawater

	Acclimated to 50% artificial seawater (0 h)	Time after exposure to100% artificial seawater				
		2 h	8 h	24 h	48 h	72 h
Taurine	121.9 ± 28.8 (100%)	135.5 ± 17.3 (111.2%)	126.5 ± 17.4 (103.8%)	119.1 ± 41.3 (97.7%)	113.4 ± 27.5 (93.0%)	162.0 ± 21.1* (132.9%)
Glycine	6.4 ± 3.9 (100%)	5.5 ± 1.6 (85.9%)	7.6 ± 4.4 (118.8%)	6.0 ± 0.1 (93.8%)	14.6 ± 8.4 (228.1%)	12.4 ± 0.7** (193.8%)
Alanine	13.4 ± 5.6 (100%)	24.1 ± 8.1* (180.0%)	24.7 ± 6.7* (184.3%)	21.7 ± 4.4* (161.9%)	38.4 ± 12.9** (286.6%)	36.3 ± 7.5** (270.9%)
Aspartate	9.3 ± 3.5 (100%)	4.7 ± 1.9* (50.5%)	5.6 ± 0.4** (60.2%)	5.6 ± 1.6* (60.2%)	6.5 ± 2.2 (69.9%)	7.9 ± 1.6 (84.9%)
Glutamate	12.5 ± 3.6 (100%)	11.8 ± 1.2 (94.4%)	10.5 ± 2.1 (84.0%)	9.5 ± 4.9 (76.0%)	9.0 ± 4.0 (72.0%)	10.3 ± 2.3 (82.4%)
β-Alanine	3.2 ± 1.7 (100%)	3.4 ± 0.6 (106.3%)	5.1 ± 2.8 (159.4%)	5.0 ± 1.5 (156.3%)	7.6 ± 1.7** (237.5%)	11.3 ± 0.5† (353.1%)
Proline	7.1 ± 6.2 (100%)	8.0 ± 3.5 (112.7%)	12.2 ± 3.3 (171.8%)	5.1 ± 4.4 (71.8%)	16.5 ± 11.3 (232.4%)	16.3 ± 1.6** (229.6%)
Arginine	2.8 ± 0.9 (100%)	4.3 ± 1.1 (153.6%)	4.0 ± 1.1 (142.9%)	2.6 ± 0.5 (92.9%)	4.9 ± 1.9* (175.0%)	5.7 ± 1.3** (203.6%)
Total	176.6 ± 52.9 (100%)	197.3 ± 17.1 (111.1%)	196.2 ± 26.6 (111.1%)	174.4 ± 58.4 (98.8%)	210.8 ± 68.3 (119.4%)	262.1 ± 31.6** (148.4%)

Values are in μmol/gram dry weight ± SD. Values in parentheses are the percentage of the value at 0 h. Significant differences from the value at 0 h are represented by $^{*}P < 0.1$, $^{**}P < 0.05$ and $\dagger P < 0.01$. $n = 3$.

of the ambient seawater (Fig. 1). As shown in Fig. 2a,b, the change in water content of the mantle tissue was also caused by the change in osmolality of the ambient seawater, suggesting that the movement of water through the body wall was caused by the difference in osmolality between the tissue of the oyster and the ambient seawater. The decrease in osmolality and increase in water content of the mantle within 8 h after the exposure to hypo-osmolality were caused by the permeation of water into the oyster. Thereafter, the water content decreased gradually up to 72 h, while the osmolal-

ity did not change. These data indicate that the oyster released and/or metabolized ions and osmolytes in order to reduce the water content of the tissue and relieve the swelling caused by permeation of water. On exposure to hyper-osmolality, the increase in osmolality and decrease in water content of the mantle were caused by the permeation of water from the oyster to the ambient seawater. As the water content of the mantle tissue remained low for 72 h, it is speculated that the mantle tissue shrank in hyper-osmolality and did not return to the original state within at least 72 h. In other words, the oyster might not be able to adapt sufficiently to the abrupt increase in osmolality to which it was subjected in the present study. In its natural habitat, the oyster never experiences such an acute increase in salinity, while it may often experience an acute decrease in salinity caused by heavy rain or a rapid rise in river level.

The observed changes in FAA content are consistent with the changes in osmolality and water content of the mantle. In hypo-osmolality, FAA content decreased significantly within 8 h after the exposure, and then decreased gradually up to 72 h (Table 1). It is speculated that the decrease in FAA content led to the permeation of water to the ambient seawater and to the concomitant decrease in water content of the mantle. In hyper-osmolality, FAA content did not increase significantly within 48 h (Table 2) and the water content did not return to the original level within 72 h. Both of these data suggest the importance of FAA as osmolytes to regulate the tissue osmolality and water content.

In marine mollusks, Ala, Gly, Pro, Tau and Glu are typically the dominant FAA contributing to the intracellular osmolality and the osmoconforming process.[4] These FAA were also found to be dominant in *C. gigas* (Tables 1,2). Tau is an important osmolyte in various animals and generally the most abundant FAA component in mollusks.[15] In *C. gigas*, Tau accounted for approximately 80% of the total FAA content both in 100 and 50% ASW and decreased markedly in hypo-osmolality (Tables 1,2). These data suggest that the large amount of Tau not used in protein synthesis is accumulated to maintain the osmolality and to be released for adaptation to hypo-osmolality. In contrast, in hyper-osmotic adaptation, Tau showed a slow but substantial increase, contributing to relatively long-term adaptation. It is speculated that the slow increase in Tau is mainly due to the biosynthesis because Tau is not a constituent of proteins. In other species of bivalves, accumulation of Tau in hyper-osmotic adaptation also took longer than that of other FAA, probably due to the absence of Tau in proteins, which suggests that the source of Tau and its metabolic precursors is mainly the diet.[16] Conversely, it has been proposed that a major source of other FAA is protein degradation.[17–19]

Of the FAA other than Tau, all except Pro decreased significantly in hypo-osmolality. They thus contributed to hypo-osmotic adaptation, although the decreases in their amounts were much smaller than the decrease in Tau. Judging from the synchronous decreases in these FAA from 2 to 8 h in hypo-osmolality, the FAA seemed to be released non-selectively in *C. gigas*. In hyper-osmolality, significant increases in Gly, Ala, β-Ala, Pro and Arg were observed, especially from 24 to 48 h. Increases in Gly, Ala and Pro during the hyper-osmotic adaptation were also observed in the previous studies on bivalves.[4] Concerning the specific FAA increase, it is speculated that biosynthesis of FAA occurred during hyper-osmotic adaptation, in addition to protein degradation. Asp and Glu that did not increase in hyper-osmolality might be amino donors. Ala was most sensitive to the hyper-osmolality in *C. gigas*, increasing immediately from 0 to 2 h after the exposure to 100% ASW and again from 24 to 48 h. Generally, Ala requires less energy for its de novo synthesis than other FAA,[20] and there are many pathways for the synthesis of Ala via transamination to pyruvate. Ala is found as an osmolyte in various mollusks, possibly because it is advantageous in hyper-osmotic adaptation from the point of view of energy cost. In addition, in its natural habitat, as mentioned above, the oyster does not experience abrupt increases in salinity and does not need to increase FAA by a large amount in a short time. Therefore, Ala that showed a small but most rapid increase appears to be an important FAA as an osmolyte for immediate response to hyper-osmolality.

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