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Effects of high-salinity seawater acclimation on the levels of D-alanine in the muscle and hepatopancreas of kuruma prawn, *Marsupenaeus japonicus*

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ABSTRACT

Changes in D- and L-alanine contents were determined in the muscle and hepatopancreas of kuruma prawn *Marsupenaeus japonicus*, during acclimation from seawater containing 100% salinity to artificial seawater containing 150% salinity. In the hepatopancreas, contents of both amino acids increased by approximately threefold. The activity of alanine racemase, which catalyzes the interconversion of D- and L-alanine, also increased in the high-salinity seawater. In addition, the expression of the gene encoding alanine racemase increased in the hepatopancreas with an increase in the alanine racemase activity. These data indicate that the biosynthesis of D- and L-alanine is controlled by the gene expression level of alanine racemase, and D-alanine in the hepatopancreas functions as a major osmolyte for isosmotic regulation. In contrast, the content of D-alanine and alanine racemase activity did not change in the muscle during hyper-osmotic acclimation. Therefore, we suggest that D-alanine, which exists in the several tissues of *M. japonicus*, is considered to be utilized in some different physiological phenomena in different tissues.

1. Introduction

D-Amino acid is an important constituent of the peptidoglycan layer in the cell wall of microorganisms, and its existence has been considered to be unique to these organisms. However, several D-amino acids have been detected in many organisms, including mammals [1]. Among various animals, marine invertebrates, such as crustaceans and bivalve mollusk species, contain large amounts of free D-alanine in their tissues [2,3]. Although the contents of D-amino acids are in the concentration of nanomole per gram of tissue in mammals [1], some aquatic invertebrates have been found to contain free D-alanine in the concentration of up to 100 μmol/g wet weight or higher [4].

Free D-alanine in these animals has been considered to be biosynthesized by alanine racemase, which catalyzes the interconversion of D- and L-alanine [5–8]. We demonstrated the alanine racemase gene in the muscle and hepatopancreas of *Marsupenaeus japonicus* and the biosynthesis of D-alanine in both tissues by alanine racemase [9]. However, little is known about the metabolism and accumulation mechanisms of D-alanine in *M. japonicus*. In our previous paper, we reported that D-alanine was found in all tissues of *M. japonicus*, particularly in the muscle, heart, and gill in large amounts [10]. Although D-alanine was present at high levels in the muscle relative to the hepatopancreas, the activity of alanine racemase in the muscle was extremely low compared with that in the hepatopancreas. Moreover, the activity exhibited a twofold increase in the muscle after molting of the prawn, whereas no increase was detected in the hepatopancreas [10]. These finding suggested that D-alanine and alanine racemase were used in some essential physiological processes in the different tissues of *M. japonicus*, and their response to physiological conditions varied in each tissue.

Aquatic animals are known to acclimate to changes in environmental factors, such as external salinity, temperature, water pressure, and dissolved oxygen concentration [11,12]. Under hyperosmotic stress, several free amino acids increase as intracellular osmolytes in aquatic invertebrates [13–17]. Similarly, the levels of D- and L-alanine in crustaceans and bivalve mollusks both increased during acclimation to high salinity, indicating that D-alanine is also a major osmolyte for intracellular isosmotic regulation [18–21]. To identify the physiological functions of D-alanine and alanine racemase in these animals, it is necessary to clarify the roles of D-alanine and alanine racemase during acclimation to high-salinity seawater.

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In this paper, we describe the changes of D- and L-alanine in the muscle and hepatopancreas of *M. japonicus* during acclimation from seawater containing 100% salinity to artificial seawater containing 150% salinity. Moreover, to elucidate the accumulation mechanism of D-alanine, we evaluated changes in the enzyme activity and mRNA expression levels of alanine racemase in each tissue of *M. japonicus* over time depending on conditions. We discuss the physiological function of D-alanine and alanine racemase in intracellular osmoregulation.

2. Material and methods

2.1. Reagents and animals

Amino acid enantiomers were obtained from Sigma-Aldrich (St. Louis, MO, USA), Nacalai Tesque (Kyoto, Japan), and Wako Pure Chemical Industries (Osaka, Japan). As fluorescence derivatizing reagents, *o*-phthalaldehyde (OPA) was obtained from Wako. *N*-*tert*-butyloxycarbonyl-L-cysteine (Boc-L-Cys), tetrahydrofuran, and Acetonitrile of HPLC grade were also purchased from Wako. The reagents for real-time PCR analysis were obtained from Applied Biosystems (California, USA). All other chemicals were of analytical grade.

Live specimens of kuruma prawn *M. japonicus*, weighing 15–18 g, were obtained from Matsumoto Suisan Sadowara farm (Miyazaki, Japan). The animals were kept in a 60L laboratory glass tank supplied with aerated circulating 100% seawater (34‰) at 18 °C and were fed on commercial prawn pellets. After prawns were acclimated to 100% seawater in the tank for a week, the salinity in the rearing water was increased to150% (51‰) using an artificial seawater salt mixture (KOWA PETS, Ama, Japan). After 2 days in 150% artificial seawater, the tail muscle and hepatopancreas of the prawn were dissected, frozen in liquid nitrogen, and stored at −80 °C for analysis. Hemolymph osmolality was determined with an automatic micro-osmometer, Roebling Type 13 DR (Hermann Roebling, Berlin, Germany).

2.2. Analytical methods of D- and L-amino acids

Enantiomers of alanine were determined with the previously reported HPLC system [10]. Briefly, amino acids extracted from the muscle and hepatopancreas of *M. japonicus* were derivatized with OPA and Boc-L-Cys [22] and determined by HPLC system (Jasco, Tokyo, Japan) using a reversed-phase Shim-pack CLC-ODS column (250 × 4.6 mm I.D.; Shimadzu, Kyoto, Japan). Mobile phase A consisted of 50 mM potassium phosphate buffer (pH6.5), acetonitrile, and tetrahydrofuran (92:5:3, v/v/v), and mobile phase B consisted of the same reagents (45:50:5, v/v/v). Linear gradient elution was performed from 0% B to 25% B for 3 min, from 25% B to 31.2% B for 27 min, and from 31.2% B to 40.2% B for 35 min at a flow rate of 0.7 mL/min at 40 °C. Eluates were monitored fluorimetrically at 344 nm excitation and 443 nm emission wavelength. Total alanine and other amino acids were determined using an L-8900 amino acid analyzer (Hitachi High-Technologies, Tokyo, Japan).

2.3. Enzyme assay

Alanine racemase activity was assayed by D- and L-alanine determination using HPLC as described previously [9]. Alanine enantiomers were separated using an HPLC system (Jasco) equipped with a chiral column, Sumichiral OA-5000 (150 × 4.6 mm I.D.; Sumika Chemical Analysis Service, Osaka, Japan). As the mobile phase, 1 mM copper sulfate was used at a flow rate of 1 mL/min. At ambient temperature, D- and L-alanine eluted from the column were monitored at 254 nm as alanine–copper complexes. The enzyme activity was calculated from increases in D- or L-alanine content.

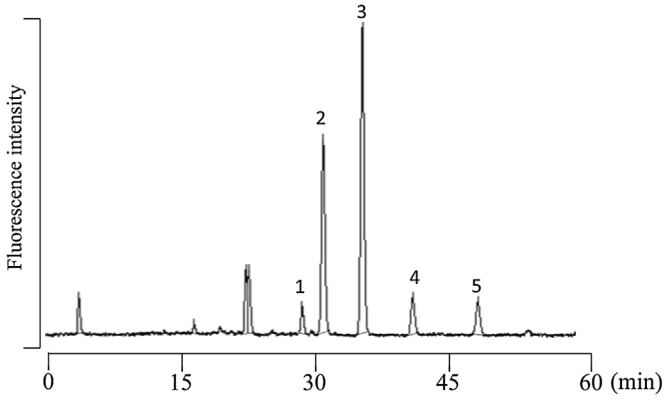

Fig. 1. Chromatogram of Boc-L-Cys-OPA derivatives of amino acids in the hepatopancreas of *M. japonicus* during acclimation from 100% seawater. Peaks: 1 = L-Glu; 2 = Gly; 3 = Tau; 4 = L-Ala; and 5 = D-Ala. Other peaks are not identified.

2.4. mRNA expression analysis

Quantification of mRNA level of *M. japonicus* alanine racemase was performed by two-step RT-PCR with a TaqMan probe. First-strand cDNA was synthesized from 2 μg total RNA from the muscle and hepatopancreas of the prawn using a High Capacity cDNA Archive kit with RT Primer mix. Primers and probes for alanine racemase and 18S rRNA were designed as described previously [9]. Real-time PCR was performed with an Mx3000P (Stratagene, California, USA). Thermal cycling conditions consisted of the initial steps for 2 min at 50 °C and then 10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing at 60 °C for 1 min.

Total RNA extracted from the hepatopancreas of *M. japonicus* was prepared as a standard. A calibration curve was constructed by measuring the levels of alanine racemase mRNA and 18S rRNA in a serial dilution of standard samples. 18S rRNA served as an internal positive control and a normalizing reference for individual expression level (alanine racemase/18S rRNA). Each value represents the mean and SD of three prawns obtained from triplicate determinations.

3. Results and discussion

3.1. Determination of D-amino acids in the muscle and hepatopancreas of M. japonicus

In our previous report, the Boc-L-Cys-OPA derivatives of D- and L-aspartate, D- and L-glutamate, D- and L-asparagine, D- and L-serine, D- and L-glutamine, glycine, L-arginine, D- and L-alanine, β-alanine, and taurine were separated within 80 min [10]. Of these amino acids, D-alanine was found in all tissues, D-aspartate was found in the nervous tissue and eye, and D-glutamate was found particularly in the testis of *M. japonicus*. Thus, we regarded these D-amino acids and other major components, glycine and taurine, as the targets of this study and tried to separate these amino acids within 60 min. As a result, we detected L-glutamate, glycine, taurine, and D- and L-alanine in the hepatopancreas (Fig. 1) and muscle (Fig. 2) of the prawn. Glycine and taurine were abundant in the hepatopancreas (Fig. 1), and glycine was most abundant in the muscle (Fig. 2).

To confirm the acclimation to the high-salinity seawater, the hemolymph osmolality in *M. japonicus* was determined (Table 1). The levels of hemolymph osmolality of prawns in 100% seawater