Journal of Chromatography B, 879 (2011) 3283–3288

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

# HPLC determination of the distribution of D-amino acids and effects of ecdysis on alanine racemase activity in kuruma prawn *Marsupenaeus japonicus*☆

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ARTICLE INFO

*Article history:*
Received 10 December 2010
Accepted 20 April 2011
Available online 28 April 2011

*Keywords:*
D-Alanine
Alanine racemase
D-Glutamate
D-Amino acid
Aquatic invertebrate
Testis
*Marsupenaeus japonicus*

ABSTRACT

The distribution of D-amino acids was examined on several tissues of kuruma prawn *Marsupenaeus japonicus*. D-Alanine was found in all tissues, and the ratio of D-alanine to total alanine ranged from 18.7 to 43.7% depending on the tissues. Of these tissues, muscle, heart, and gill contained a relatively large amount of D-alanine. Nervous tissue and eye, on the other hand, contained a large amount of D-aspartate. D-Glutamate was specifically detected in testis. The percentage of D-glutamate to total glutamate was over 50% in testis, suggesting the existence of the biosynthetic enzyme in this tissue. The changes of alanine racemase activity were determined in the muscle and hepatopancreas of *M. japonicus* before and after molting. The activity after molting increased twice in the muscle. On the other hand, it was not changed in the hepatopancreas. These data suggest that D-alanine plays an important role in the muscle during ecdysis. However, the free D-alanine level in the muscle was not changed significantly before and after ecdysis. From these data, several D-amino acids are considered to be utilized in some essential physiological phenomena in the different tissues of the prawn.



## 1. Introduction

D-Amino acids have long been considered to be unique to microorganisms, which utilize some D-amino acids to construct the peptidoglycan layer of their cell wall. However, several D-amino acids have been detected in many organisms including mammals because of the advance of analytical techniques in the last two decades.

In mammals, free D-serine, D-aspartate, and D-alanine have been found and investigated for their physiological functions and pathological significances. D-Serine is found in mammalian brain and thought to be the neuromodulator of the *N*-methyl-D-aspartate subtype of the glutamate receptor [1–5]. D-Aspartate is present in the nervous and neuronal endocrine tissues of mammals [6–8]. The level of D-aspartate in rat testis increases significantly in amount during sexual maturity and appears to accelerate the testosterone synthesis by stimulating the gene expression of steroidogenic acute regulatory protein in Leydig cells [9]. D-Alanine is widely found in mammalian tissues [10]. The immunohistochemical study clarified that D-alanine is localized to insulin secreting β-cells in pancreas, indicating that it would be involved in the regulation of the blood glucose level [11]. Under these circumstances, D-amino acids in mammals are localized to specific tissues and thought to play significant roles in each tissue. The contents of D-amino acids are low in mammals but some aquatic invertebrates such as marine crustaceans and some molluskan species have been found to contain a large amount of free D-amino acids [12].

In aquatic invertebrates, D-aspartate was found in the brain of octopus *Octopus vulgaris* [13] and some bivalve mollusks [14]. Moreover, D-alanine was the most abundant and widely distributed among crustaceans and bivalve mollusks [15,16]. These aquatic invertebrates are known to acclimate to environmental changes such as external salinity, temperature, water pressure, and dissolved oxygen concentration [17]. Along with the hyper-osmotic stress, several free amino acids have been well known to increase as intracellular osmolytes in aquatic invertebrates, and the tissue levels of D- and L-alanine in crustaceans and bivalve mollusks were both increased during high salinity acclimation, suggesting that D-alanine is also a major osmolyte for intracellular isosmotic regulation [18–21]. Free D-alanine in these animals has been considered to be biosynthesized by alanine racemase which catalyzes the interconversion of D- and L-alanine or to be incorporated from external media or from symbiotic bacteria. In our previous report, we demonstrated that alanine racemase gene existed in the mus-

☆ This paper is part of the special issue "Analysis and Biological Relevance of D-Amino Acids and Related Compounds", Kenji Hamse (Guest Editor).

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doi:10.1016/j.jchromb.2011.04.026

cle and hepatopancreas of *Marsupenaeus japonicus*, and D-alanine in these tissues are biosynthesized by alanine racemase [22]. However, the metabolism and accumulation mechanisms of D-alanine in *M. japonicus* remain unknown. To reveal the physiological functions of D-alanine and alanine racemase, the anatomical distribution of D-alanine and factors which affect postnatal change of D-alanine content or alanine racemase activity need to be elucidated in *M. japonicus*.

In the present study, we examined the occurrence of D-amino acids in several tissues of *M. japonicus*. We also examined the changes of alanine racemase activity in the muscle and hepatopancreas of *M. japonicus* before and after molting and discussed the physiological function of D-alanine and alanine racemase in relation to the ecdysis of this species.

## 2. Experimental

### 2.1. Reagents and animals

The amino acid enantiomers were obtained from Sigma–Aldrich (St. Louis, MO, USA), Tokyo Kasei (Tokyo, Japan), Nacalai Tesque (Kyoto, Japan) and Wako Pure Chemical Industries (Osaka, Japan). As the fluorescence derivatizing reagents, *o*-phthalaldehyde (OPA) was obtained from Wako, and 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was from Tokyo Kasei. *N*-*tert*-Butyloxycarbonyl-L-cysteine (Boc-L-Cys), methanol (MeOH), trifluoroacetic acid (TFA), citric acid monohydrate, and boric acid were from Wako. Acetonitrile (MeCN) of HPLC grade was purchased from Nacalai Tesque. All other chemicals were of analytical grade.

Live specimens of male and female kuruma prawn *M. japonicus*, weighing 15–18 g, were obtained from a local prawn farm in Oita Prefecture, Japan. The animals were kept in a laboratory 60 L glass tank supplied with aerated circulating seawater of 15 °C and were fed on commercial prawn pellets. Prawns were sampled in their intermolting stage for controls and within 15 h after molting for molting samples. Each tissue was dissected individually from five individuals and stored at −80 °C prior to analysis.

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

To prepare the tissue extract, each tissue was homogenized with a 10-fold excess of 8% perchloric acid. After centrifugation at 12,000 × *g* for 20 min at 4 °C, the supernatant was neutralized with solid potassium bicarbonate and centrifuged to eliminate the pellet. Amino acids in these tissue extracts including D-amino acids were derivatized with OPA and Boc-L-Cys [23] and determined by high-performance liquid chromatography (HPLC) system (Jasco, Tokyo, Japan) using a reversed-phase Shim-pack CLC-ODS column (250 mm × 4.6 mm I.D.; Shimadzu, Kyoto, Japan). A mobile phase A consisted of 50 mM potassium phosphate buffer (pH 6.5), acetonitrile, and tetrahydrofuran (92:5:3, v/v) and a mobile phase B consisted of the same reagents (45:50:5, v/v). A linear gradient elution was carried out from 0% B to 73% B within 85 min at a flow-rate of 0.7 mL/min at 40 °C. Eluates were monitored fluorimetrically at 344 nm and 443 nm for excitation and emission wavelength, respectively. A 10 μL sample after derivatization was injected onto the HPLC system.

### 2.3. Two-dimensional HPLC determination of D- and L-glutamate

Enantiomers of glutamate were determined by the previously reported two-dimensional HPLC system [24]. Briefly, amino acids in the testis of *M. japonicus* were derivatized with a fluorescence labeling reagent, NBD-F, and the reaction mixture was subjected to the HPLC system (NANOSPACE SI-2 series, Shiseido, Tokyo, Japan) combining a microbore-reversed-phase column (monolithic ODS column, 1000 mm × 0.53 mm I.D., Shiseido) and a narrowbore-enantioselective column (Sumichiral OA-2500S, 250 mm × 1.5 mm I.D., self-packed, material was from Sumika Chemical Analysis Service, Osaka, Japan). By a microbore-ODS column, fraction of NBD-glutamate was isolated, and the D- and L-enantiomers were separated and determined by a narrowbore-enantioselective column. Fluorescence detection of the NBD-amino acids was carried out at 530 nm with excitation at 470 nm. In order to reverse the elution order of the enantiomers, a Sumichiral OA-2500R column (250 mm × 1.5 mm I.D. self-packed, material was from Sumika Chemical Analysis Service) was used.

### 2.4. Enzyme assay

Alanine racemase activity was assayed by determining D- and L-alanine contents with HPLC. Crude enzyme solution was prepared as follows. The muscle and hepatopancreas of *M. japonicus* were homogenized with 10 volumes of 10 mM Tris–HCl buffer (pH 8.0) containing 50 mM KCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 20 μM pyridoxal 5′-phosphate (PLP). The homogenate was centrifuged at 12,000 × *g* for 20 min and the supernatant was fractionated with ammonium sulfate. The precipitate obtained from 30 to 70% saturation was dissolved into a minimal volume of 0.1 M Tris–HCl buffer containing 1 mM EDTA, 0.2 mM PMSF, and 20 μM PLP, and dialyzed overnight against the same buffer. The dialyzate was centrifuged at 12,000 × *g* for 20 min and the supernatant was utilized for enzyme assay.

The enzyme reaction mixture contained 100 mM Tris–HCl buffer, pH 8.5, 200 mM D- or L-alanine, and enzyme solution. After incubation for 30 min at 37 °C, an aliquot of the reaction mixture was deproteinized with 0.6 M perchloric acid. Following centrifugation at 14,000 × *g* for 2 min, the supernatant was neutralized with 0.6 M potassium bicarbonate and centrifuged as above. The resulting supernatant was injected into HPLC.

Alanine enantiomers were separated using an HPLC system (Jasco) equipped with a chiral column, Sumichiral OA-6000 (150 mm × 4.6 mm I.D.; Sumika Chemical Analysis Service). As the mobile phase, 2 mM copper sulfate was used at a flow rate of 1 mL/min. At an ambient temperature, D- and L-alanine eluted from the column were monitored at 254 nm as alanine–copper complex. Ten microliter of reaction mixture was subjected to HPLC. Enzyme activity was calculated from the increase of D- or L-alanine content.

## 3. Results and discussion

### 3.1. Determination of D-amino acids in several tissues of M. japonicus

The Boc-L-Cys-OPA derivatives of D-, L-aspartate, D-, L-glutamate, D-, L-asparagine, D-, L-serine, D-, L-glutamine, glycine, L-arginine, D-, L-alanine, β-alanine and taurine were separated within 80 min (Fig. 1). We have already reported the D-alanine content in the muscle and hepatopancreas of *M. japonicus* [22]. To clarify the physiological functions and metabolism of D-alanine in *M. japonicus*, we determined the distribution of D-alanine content in several tissues of *M. japonicus*. The dissected tissues were heart, intestine, stomach, hepatopancreas, gill, nervous tissue, eye, muscle, testis, and ovary. Together with D-alanine, several other D-amino acids were also determined simultaneously in the same tissues.

The amounts of free D- and L-alanine in these tissues are shown in Fig. 2. D-Alanine occurred in all tissues but the ratio of D-alanine to total alanine varied from 18.7 to 43.7% depending on the tissues. Of these tissues, muscle, heart, and gill were abundant in D-alanine.

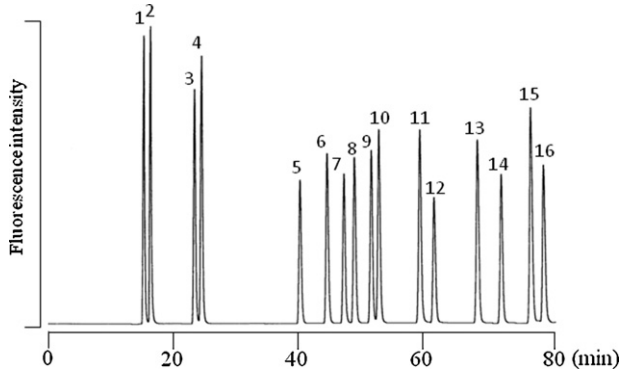


**Fig. 1.** Chromatogram of Boc-L-Cys-OPA derivatives of standard amino acids. Peaks: 1 = L-Asp; 2 = D-Asp; 3 = L-Glu; 4 = D-Glu; 5 = L-Asn; 6 = D-Asn; 7 = L-Ser; 8 = L-Gln; 9 = D-Ser; 10 = D-Gln; 11 = Gly; 12 = L-Arg; 13 = L-Ala; 14 = β-Ala; 15 = D-Ala; 16 = Tau.

On the other hand, only a small amount of D-alanine was observed in nervous tissue and eye.

Alanine racemase activity and the expression level of alanine racemase gene in hepatopancreas were both higher than in muscle [22]. However, D-alanine content was lower in hepatopancreas than in muscle. We have also detected the gene expression of alanine racemase in all these tissues except for gills (data not shown). These data suggest that D-alanine accumulated in gills is incorporated from exogenous origin. Hard clam which was reared in seawater containing D-alanine incorporated a large amount of D-alanine from seawater in gills [20]. Thus, the components contained in gills of prawns were considered to be influenced by its surroundings. In addition to gills, digestive organs such as intestine and stomach are also possible to be affected by external D-amino acids which derived from feeds or symbiotic bacteria. Thus, the content of D-alanine in these three tissues may not be dependent on the gene expression of alanine racemase and is not necessarily reflect its functions. To reveal the physiological functions of D-alanine in these tissues, further investigations are necessary for each tissue.

D-Aspartate is another D-amino acid present in *M. japonicus* (Fig. 3). Nervous tissue and eye contained a rather large amount of D-aspartate. The contents of D-aspartate in these tissues were almost the same level as that of D-alanine but the ratio of D-form to total aspartate was much higher than that of D-alanine. Thus, the data strongly suggest that D-aspartate in these tissues is biosynthesized by aspartate racemase. The presence of D-aspartate as an endogenous amino acid in nervous and endocrine tissues of invertebrates and vertebrates is well known. In rat testis, D-aspartate levels increase immediately before birth and during sexual maturity [9]. D-Aspartate is localized in Leydig cells of rat testis, suggesting its implication in hormonal processes and in steroidogenesis [7]. In *M. japonicus*, however, D-aspartate was not detected in testis but was found in ovary and hepatopancreas. It is possible that the levels of D-aspartate vary in the gonads during maturation. Thus, it is interesting to carry out another experiment to determine the D-aspartate content in the ovary during the life span of the prawn. D-Aspartate was also detected in small amount in intestine. Although it was speculated to derive from external origins, the D-enantiomer ratio to total aspartate was high and almost the same as that in nervous tissue and eye.

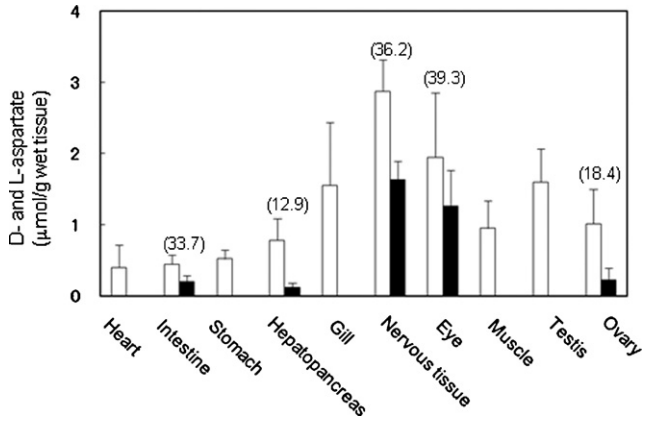


**Fig. 3.** Distribution of D- and L-aspartate in several tissues of *M. japonicus*. Closed column shows D-aspartate and open column L-aspartate. Values represent means and SD for five prawns. Percentages of D/(D + L) are shown in parentheses.

D-Glutamate was the third D-amino acid found in *M. japonicus*. This D-amino acid was specifically detected in testis and surpassed the content of L-form (Fig. 4). The percentage of D-glutamate to total glutamate was shown to be 52.7%, suggesting the existence of biosynthetic enzyme in this tissue (Fig. 5). The existence of D-glutamate has been rarely reported in animals, thus, we also confirmed the peak of D-glutamate in testis using a two-dimensional HPLC determination system (Figs. 6 and 7). It is considered that the enzyme which biosynthesizes D-glutamate is glutamate racemase or D-amino acid specific aminotransferase. D-Alanine and alanine racemase also exist in the testis of the prawn as described above. Thus, it is expected that D-glutamate in testis is synthesized by D-glutamate aminotransferase utilizing D-alanine as an amino group donor. In microorganisms, D-glutamate is known to be an essential component of the peptidoglycan layer of the cell wall structure and biosynthesized by glutamate racemase [25]. However, no investi-

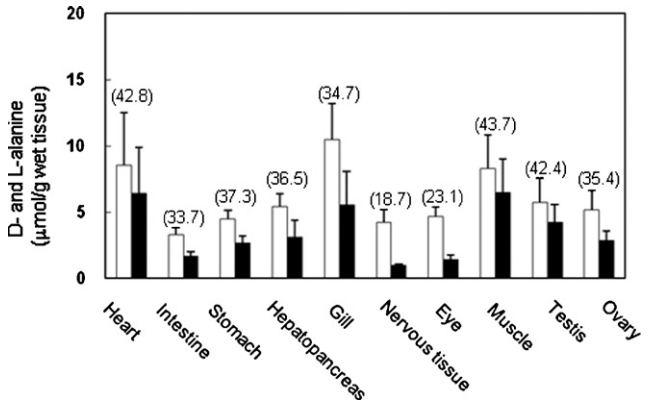


**Fig. 2.** Distribution of D- and L-alanine in several tissues of *M. japonicus*. Closed column shows D-alanine and open column L-alanine. Values represent means and SD for five prawns. Percentages of D/(D + L) are shown in parentheses.

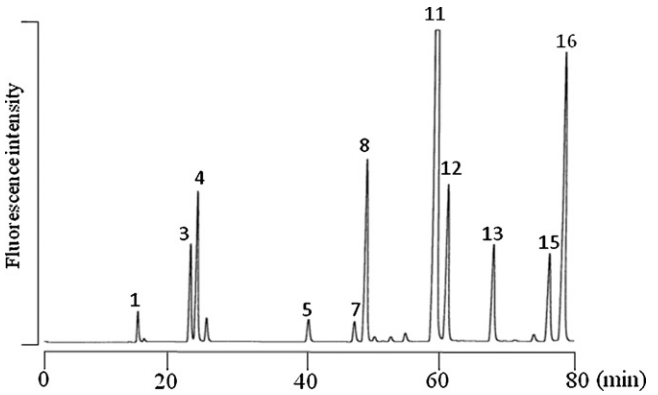


**Fig. 4.** Chromatogram of Boc-L-Cys-OPA derivatives of amino acids in the testis of *M. japonicus*. Peaks as in Fig. 1.

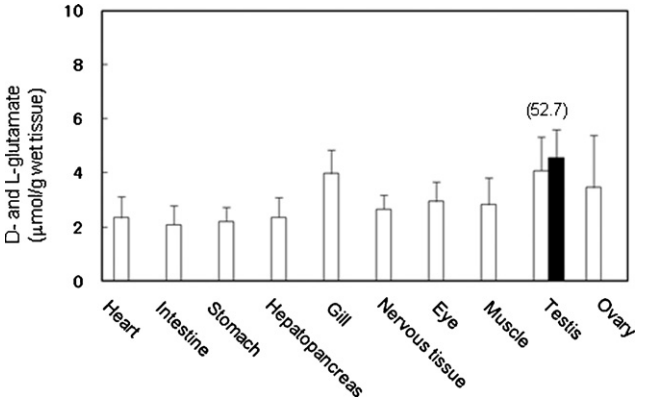


**Fig. 5.** Distribution of D- and L-glutamate in several tissues of *M. japonicus*. Closed column shows D-glutamate and open column L-glutamate. Values represent means and SD for five prawns. Percentages of D/(D + L) are shown in parentheses.

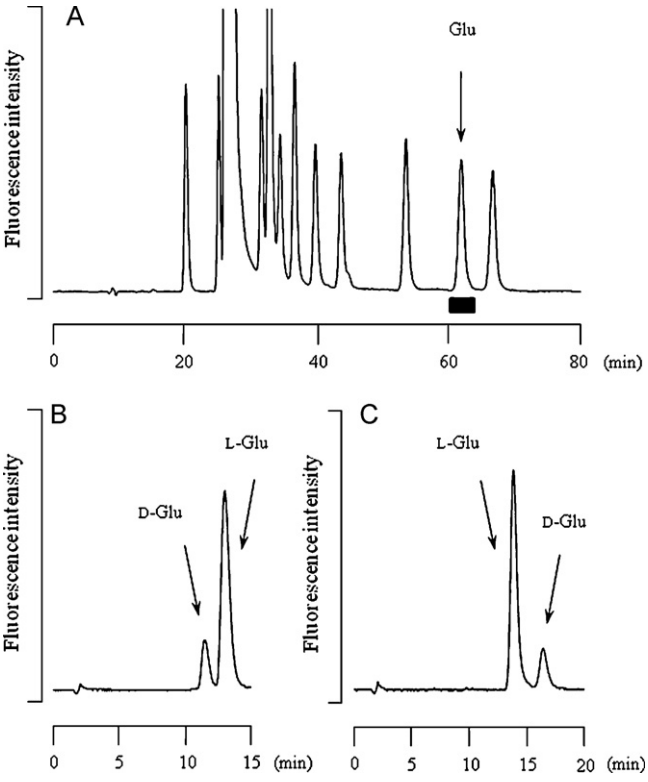


**Fig. 6.** Separation of 10 hydrophilic amino acids (His, Asn, Ser, Gln, Arg, Asp, Gly, *allo*-Thr, Glu and Thr) as their NBD derivatives using a microbore-ODS column (A) and the continuously interlinked enantiomer separation of NBD-Glu using a narrowbore-Sumichiral OA-2500S column (B). The mixtures of NBD-derivatized D- and L-amino acids in a molar ratio of 1:4 were analyzed. The fraction of NBD-Glu indicated by a bar was on-line collected to a loop device and transferred to the narrowbore-enantioselective column. Elution order of the enantiomers was reversed by using a narrowbore-Sumichiral OA-2500R column (C).

gation has been performed on this D-amino acid in animals. Solving the physiological functions of D-glutamate in the testis of the prawn may provide new information on the role of D-amino acids in animal kingdom.

D-Serine which is found in mammals was not detected in the tissues of *M. japonicus* in the present experiments. It may be possible to detect small quantities of other D-amino acids which we could not find in this study by highly sensitive determination method [26].

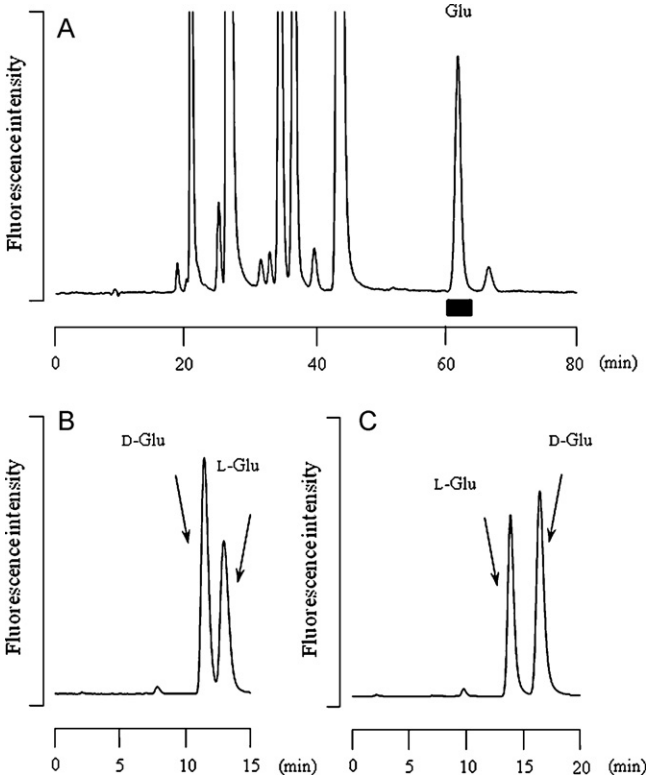


**Fig. 7.** Separation of amino acids in the testis of *M. japonicus* as their NBD derivatives using a microbore-ODS column (A) and the continuously interlinked enantiomer separation of NBD-Glu using a narrowbore-Sumichiral OA-2500S column (B). The fraction of NBD-Glu indicated by a bar was on-line collected to a loop device and transferred to the narrowbore-enantioselective column. Elution order of the enantiomers was reversed by using a narrowbore-Sumichiral OA-2500R column (C).

### 3.2. Effects of ecdysis on alanine racemase activity

Alanine racemase activity was determined in the muscle and hepatopancreas of *M. japonicus* before and after molting (Fig. 8). Enzyme activity was calculated from the increase of D- or L-alanine content for both L to D and D to L directions. The activity after molting increased twice in the muscle for both directions. On the other hand, it did not significantly rise in the hepatopancreas. It is considered that the carapace which softens in ecdysis, may be sensitive to environmental salinity change compared with that during intermolting period. In euryhaline macrurans such as *M. japonicus*, it is possible that a large amount of D-alanine is used for the maintenance of physiological conditions in the muscle during and just after molting until the shell hardening. According to this estimation, we also determined free D- and L-alanine contents in the muscle and hepatopancreas after molting (Fig. 9). However, the D-alanine level in these tissues was not changed significantly before and after ecdysis, although the level after molting might be underestimated as mentioned above.

In crustaceans such as *M. japonicus*, it has already been confirmed that free D-alanine is a major component of osmolytes responsible for the isosmotic regulation or cell volume regulation during hyper- or hypo-salinity acclimation. In crayfish *Procabarus clarkii* acclimated from freshwater to seawater, D-alanine largely increased in the muscle for this purpose [19]. Moreover, the most interesting behavior of D-alanine was shown in Japanese mitten crab *Eriocheir japonicus*. This species is a strong osmoregulator and post-larval juvenile crabs migrate upstream from brackish water area and grow up in freshwater river for 3–5 years [21]. In estuaries during their downstream spawning

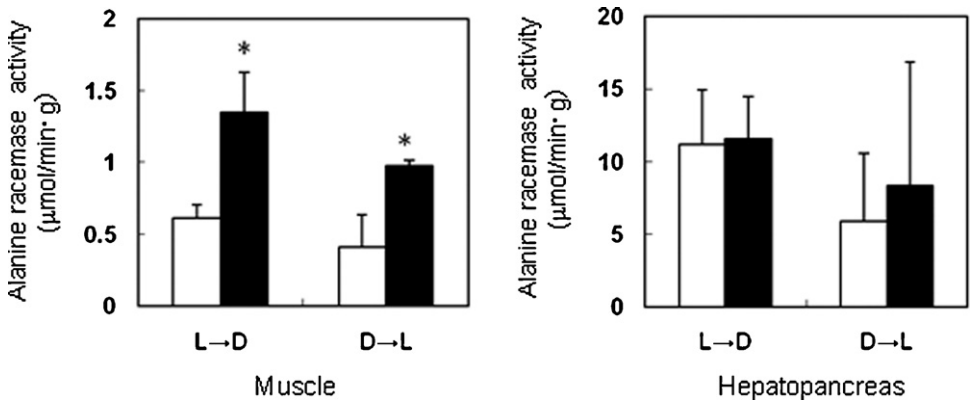


**Fig. 8.** Effect of ecdysis on alanine racemase activities in the muscle and hepatopancreas of *M. japonicus*. Values represent means and SD for five prawns. Closed column shows the activity in prawns after molting and open column that in the control. Values represent means and SD for five prawns. Significant (*<0.05) from the control prawns.

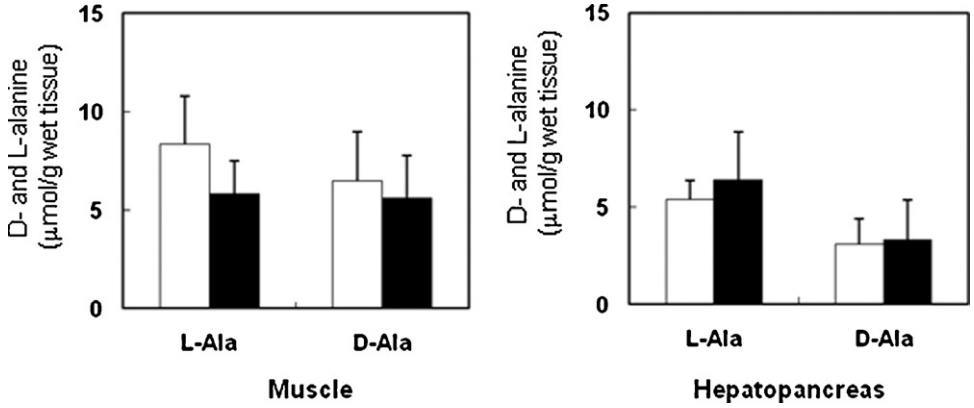


**Fig. 9.** Effect of ecdysis on D- and L-alanine contents in the muscle and hepatopancreas of *M. japonicus*. Closed column shows the content in prawns after molting and open column that in the control. Values represent means and SD for five prawns.

migration after maturation, they accumulate large amount of D- and L-alanine and uptake much inorganic ions for osmoregulation. After reaching the sea, the crabs increase more D- and L-alanine and glycine in place of harmful inorganic ions for the muscle cell. Thus, D- and L-alanine in this species are responsible to acquire salinity tolerance during spawning migration to the sea.

In this study, there was no change of D-alanine content just after molting of *M. japonicus*, although the activity of the biosynthetic enzyme significantly increased in the muscle. This contradiction may lead a speculation in which the increase of alanine racemase activity is a provision or defense against the environmental salinity increase during the soft and salt permeable carapace stage after molting. Another hypothesis is that D-alanine produced in the muscle by alanine racemase might be utilized to synthesize some bound form component in the prawn. D-Amino acid is known to exist in peptides produced in some animals, such as dermorphin of *Phyllomedusa sauvagei* [27] and hyperglycemic peptide of *Homarus americanus* [28]. In the fungus *Tolypocladium niveum*, alanine racemase is a key enzyme in the biosynthesis of cyclosporine A [29]. Thus, it may be possible that D-alanine is used for the synthesis of an important component which is necessary for the physiological changes during ecdysis in the muscle of *M. japonicus*. In our investigations, the molting samples were collected within 15 h after molting but the stage of control samples could not be specified. Thus, to solve the inconsistency between D-alanine content and alanine racemase activity during ecdysis, tissue samples should be collected in each life stage of the prawn and investigated in detail.

## 4. Conclusion

We demonstrated the distribution of D-amino acids in the several tissues of *M. japonicus*. D-Alanine was detected in all tissues and in particular high concentration of D-alanine was found in the heart, muscle, and gill. Nervous tissue and eye contained a large amount of D-aspartate. D-Glutamate was exclusively found in testis and the ratio of D-glutamate to total glutamate was shown to be 52.7%, indicating it is produced enzymatically in this tissue. The increase of alanine racemase activity was detected in the muscle of *M. japonicus* just after molting. It is suggested that D-alanine plays an important role in the muscle during ecdysis. However, the free D-alanine level in the muscle was not changed significantly before and after ecdysis. From these data, several D-amino acids in the prawn tissues are considered to play some important roles in different physiological phenomena such as ecdysis and osmoregulation in different tissues in the prawn.

## Acknowledgements

This work has been supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and technology of Japan [18780161 to N.Y., 14360121 and 16380141 to H.A.]. The authors give thanks to Shiseido Co., Ltd. (Tokyo, Japan) for the technical support (2D-HPLC analysis).

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