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Occurrence of free D-aspartate and aspartate racemase in the blood shell *Scapharca broughtonii*

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Summary. Substantial concentrations of D-aspartate were found in several tissues of *Scapharca broughtonii* together with approximately equal concentrations of L-aspartate. The foot and mantle extracts also contained an aspartate racemase activity. The formation of L-aspartate from the D-enantiomer by the foot extract was apparently slower than the reverse reaction, and this unbalance seemed to be due to the presence of an enzyme activity which rapidly converted L-aspartate to L-alanine. The possible role of D-aspartate in the anaerobiosis was discussed.

Keywords: Amino acids – D-Aspartate – Aspartate racemase – L-Alanine – *Scapharca broughtonii* – Bivalve

Introduction

The blood shell, *Scapharca broughtonii*, has been shown (Sato et al., 1987) to contain *N*-methyl-D-aspartate, which is well known for its neuroexcitatory action. The compound was isolated and crystallized from the muscle extracts of the organism. The bivalve remains unknown as to whether it contains D-aspartate which is structurally related to *N*-methyl-D-aspartate. On the other hand, D-aspartate has been found in several other species of marine bivalves, of which *Solemya reidi* has the highest content of the amino acid, at around 10 μmol/g fresh weight in the foot and gill (Felbeck, 1985; Felbeck and Wiley, 1987). The presence of aspartate racemase has been also found in this bivalve (Felbeck, 1985). This siutuation prompted us to examine *S. broughtonii* for the presence of D-aspartate, which might be involved in the metabolism of *N*-methyl-D-aspartate.

The present report shows that the foot and gill tissues of the bivalve contain substantial concentrations of D-aspartate together with approximately equal concentrations of L-aspartate, and that the foot and mantle also contain

an aspartate racemase activity. The presence of L-aspartate decarboxylase is also suggested.

Materials and methods

Materials

Living specimens of the blood shell *S. broughtonii*, being cultured in Miyagi Prefecture, Japan, were purchased from a fish farm and kept in sea water which was bubbled with air. They were opened and the foot, gills, adductors, mantles were removed. The foot was cut into two parts, the more intensely colored outside part (about 46% of the total weight) which we designated as foot 1, and the other less intensely colored inside part designated as foot 2. The mantles were also devided into two parts, the circular fringe part which we designated as mantle 1 and the other inner part designated as mantle 2. The tissues were washed in ice-cold saline and stored at −40°C until use. All the chemicals used were of analytical purity.

Extraction of tissue amino acids

The frozen tissues were homogenized with a Potter-Elvehjem homogenizer first in 5 volumes of 5 mM potassium phosphate buffer (pH 7.4) containing 25 mM KCl, then further homogenized after addition of 5 volumes of 16% perchloric acid. The homogenate was centrifuged for 20 min at 12,000 × g and the supernatant was neutralized with KOH. After centrifugation to remove the precipitated $KClO_4$, the supernatant was concentrated to dryness under reduced pressure at 40°C, and the residue was subjected to amino acid analysis with HPLC.

Enzyme assays

Crude extracts of the tissues were prepared by homogenizing the frozen tissues in 5 mM potassium phosphate buffer (pH 7.4) containing 25 mM KCl with a Potter-Elvehjem homogenizer, followed by centrifugation of the homogenates at 23,000 × g for 30 min. The supernatants were then dialyzed three times, about 5 h each time, against 100 volumes of 20 mM potassium phosphate buffer (pH 7.4). Enzyme reactions were carried out under conditions as described for individual experiments, and were stopped by the addition of methanol (4 volumes to 1 volume of the reaction mixture). After centrifugation (12,000 × g for 20 min at 4°C) to remove precipitated protein, the supernatant was concentrated to dryness under reduced pressure at 40°C, and the residue was subjected to amino acid analysis with HPLC.

HPLC analysis

The residue, resulting from the procedure for "Extraction of tissue amino acids" and "Enzyme assays" described above, was dissolved in water containing 100 μM L-cysteic acid (adjusted to about pH 7 with KOH) employed as the internal standard. The solution was subjected to HPLC after treatment with *o*-phthalaldehyde and *N*-acetyl-L-cysteine by the method of Aswad (1984) to convert the amino acids to fluorescent derivatives. The chromatography was performed essentially as previously reported (Watanabe et al., 1996) with some minor modifications, using a Shimadzu (Kyoto, Japan) HPLC system consisting of two Model LC-9A high-pressure pumps, a Model RF-10A fluorescence detector, a Model C-R6A Chromatopac integrator, a Model DGU-4A degasser and a J′ sphere-ODS-M80 column(4.6 × 250 mm)(YMC, Kyoto, Japan). Excita-

tion and emission wavelengths were 320 and 440nm, respectively. Solvents used were Solvent A: 50mM sodium acetate buffer (pH 5.53), Solvent B: methanol. Elution was performed with a linear gradient from 4.0% to 6.0% B in A for 60min at a flow rate of 1.2ml/min, followed by a linear gradient from 6% to 45% for further 60min at a flow rate of 0.8ml/ml.

Results

Under the conditions employed for HPLC analysis, the enantiomers of aspartate, glutamate, alanine and serine were all separated as shown in Fig. 1, and were identified by comparison with the behaviors of authentic amino acids.

Analysis thus conducted of the tissue extracts of *S. broughtonii* shows that most of the tissues examined contained D-aspartate at concentrations approximately equal to those of L-aspartate (Table 1). On the other hand, D/(D + L)

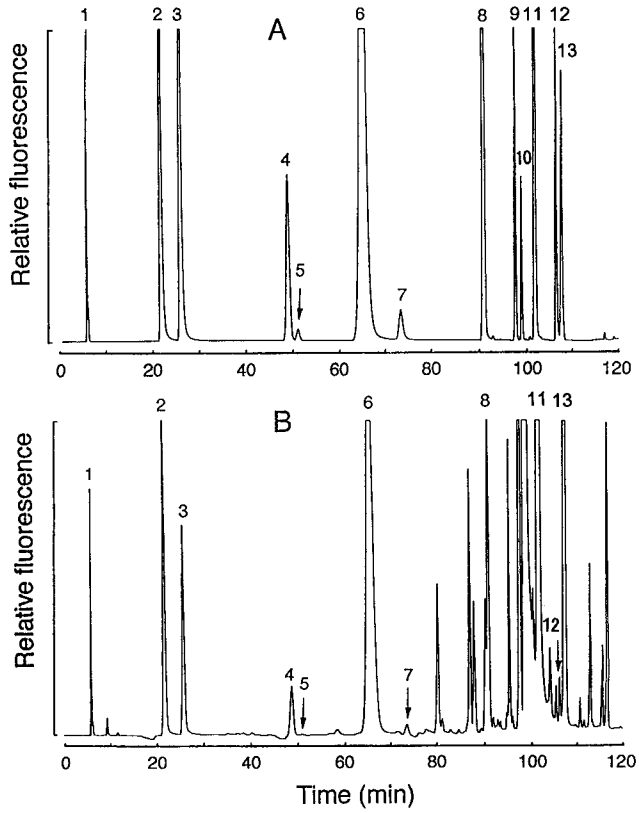

Fig. 1. A typical HPLC resolution of the derivatized D- and L-enantiomers of some amino acids. HPLC chromatograms of (**A**) authentic amino acids, and (**B**) an extract from a "foot 1" tissue of *S. broughtonii* described in "Materials" section. *1* L-cysteic acid; *2* D-aspartate; *3* L-aspartate; *4* L-serine; *5* D-serine; *6* L-glutamate; *7* D-glutamate; *8* glycine; *9* L-arginine; *10* D-arginine; *11* β-alanine; *12* D-alanine; *13* L-alanine

Table 1. Contents of free D- and L-amino acids in the tissues of *S. broughtonii*

Tissue	Aspartate			Glutamate			Alanine			Serine		
	D form (nmol/g fw)	L form (nmol/g fw)	D/(D + L) (%)	D form (nmol/g fw)	L form (nmol/g fw)	D/(D + L) (%)	D form (nmol/g fw)	L form (nmol/g fw)	D/(D + L) (%)	D form (nmol/g fw)	L form (nmol/g fw)	D/(D + L) (%)
Foot 1 (n = 5)	4,765 ± 1,146	4,443 ± 1,302	51.7 ± 8.0	108 ± 35	10,736 ± 1,267	0.99 ± 0.27	153 ± 95	11,304 ± 2,738	1.28 ± 0.50	16.8 ± 6.7	1,387 ± 414	1.19 ± 0.40
Foot 2 (n = 5)	1,184 ± 484	3,497 ± 2,380	29.9 ± 12.7	239 ± 62	18,480 ± 2,425	1.28 ± 0.27	321 ± 252	18,850 ± 4,368	1.54 ± 0.94	27.6 ± 22.0	2,127 ± 554	1.13 ± 0.79
Gill (n = 4)	2,280 ± 682	2,693 ± 854	46.0 ± 1.9	105 ± 74	2,963 ± 1,005	3.11 ± 1.50	60.8 ± 9.0	2,190 ± 1,148	3.18 ± 1.30	8.2 ± 3.0	585 ± 215	1.38 ± 0.20
Adductor (n = 5)	542 ± 97	976 ± 972	46.5 ± 38.2	176 ± 33	16,993 ± 2,809	1.04 ± 0.20	189 ± 91	16,524 ± 5,051	1.10 ± 0.30	33.9 ± 28.1	1,461 ± 1,052	2.42 ± 1.40
Mantle 1 (n = 5)	2,039 ± 662	2,453 ± 768	45.3 ± 2.6	61.2 ± 19.9	6,228 ± 2,507	1.03 ± 0.20	67.7 ± 19.8	5,942 ± 3,336	1.36 ± 0.60	18.3 ± 8.7	1,460 ± 684	1.23 ± 0.10

The contents and percentages are expressed as means ± SD.

of glutamate, alanine and serine were generally at around 1% and at most a few percent, indicating that the high ratio of D-enantiomer was unique to aspartate in this bivalve. The highest concentration of D-aspartate was found in the intensely colored part of the foot (foot 1) and was at around 5 μmol/g fresh weight. This concentration is lower than, but may be regarded as comparable to, the reported concentrations of the amino acid at around 10 μmol/g fresh weight (Felbeck, 1985; Felbeck and Wiley, 1987) in the foot of *S. reidi*, and is at about the same level as those found in the foot and gill of *Mytilus edulis* and *Modiolus capax* (Felbeck and Wiley, 1987).

To find out if D-aspartate is produced enzymatically from L-aspartate, extracts from the tissues given in Table 1 were assayed for this activity by

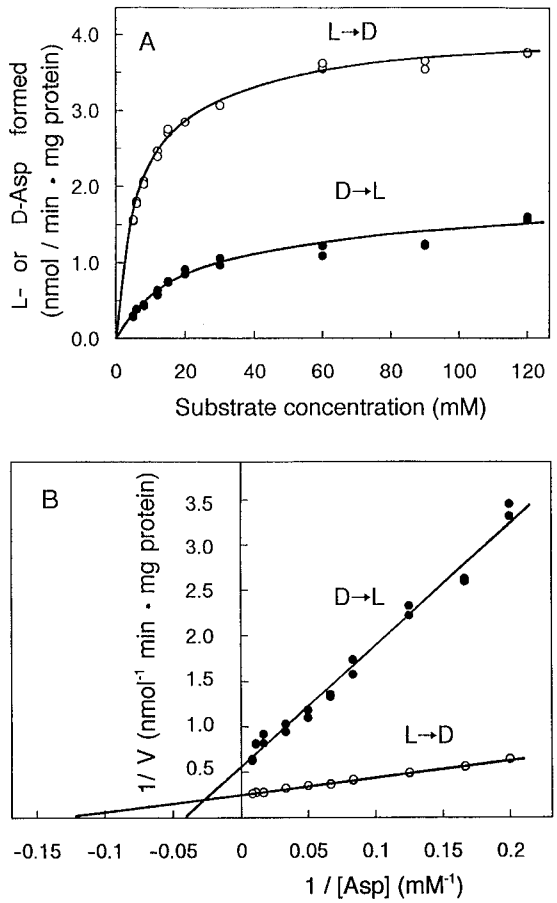

Fig. 2. Effect of substrate concentration on aspartate racemase activity in foot of *S. broughtonii*. The foot extract was prepared from two "foot 1" tissues described in "Maetrials" section, and a portion of the extract (1.39 mg protein) was incubated with an indicated concentration of D-aspartate or L-aspartate in 20 mM potassium phosphate buffer (pH 7.4) in a total volume of 0.5 ml at 37°C for 60 min. Preliminary experiments had shown that the reactions proceeded linearly with time up to 90 min. D-Aspartate and L-aspartate were determined when L-aspartate and D-aspartate, respectively, were the substrates. (○) formation of D-aspartate from L-aspartate; (●) formation of L-aspartate from D-aspartate

incubating the extracts with 100 mM L-aspartate in 20 mM potassium phosphate buffer (pH 7.4) at 37°C for 30 min. The major activity was found in foot 1 and was 69.1 ± 47.9 nmol/min per g fresh weight (mean ± SD, n = 4), which was 4.12 ± 1.20 nmol/min per mg of protein in terms of specific activity. The minor activity was observed in two of the four mantle 1 tissues, but the other two showed no detectable activity. All the other tissues examined were inactive despite the presence of D-aspartate as shown in Table 1. Since the enzyme showing this activity was expected to be a racemase, the extract from foot 1 was also tested for the activity catalyzing the reverse reaction to produce L-aspartate from the D-enantiomer. The activity was detected, but the reaction was apparently slower than the production of D- from L-enantiomer. Figure 2 compares the reaction velocities in the two directions at different substrate concentrations. The Lineweaver-Burk plots suggest that the two reactions

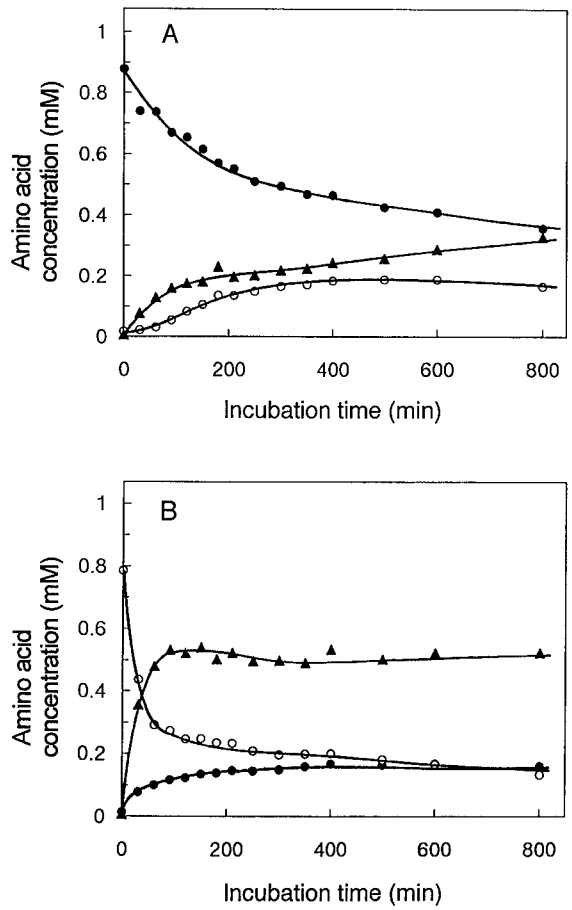

Fig. 3. Time course of production of (**A**) L-aspartate and L-alanine from D-aspartate, and (**B**) D-aspartate and L-alanine from L-aspartate, by the foot tissue extract. The foot extract was prepared from two "foot 1" tissues described in "Materials" section, and a portion of the extract (1.9 mg protein) was incubated with 1 mM D-aspartate and L-aspartate in (**A**) and (**B**), respectively, in 20 mM potassium phosphate buffer (pH 7.4) in a total volume of 0.5 ml at 37°C for different periods. (○) L-aspartate; (●) D-aspartate; (▲) L-alanine

subject to Michaelis-Menten kinetics, and the data yield apparent *Vmax* values of 4.00 and 1.81 nmol/min per mg protein and *Km* values of 7.60 and 24.1 mM for the L-to-D and D-to-L conversions, respectively.

To examine what caused this unbalance, the extract was tested for D-aspartate oxidase activity, which might decrease D-aspartate to interfere with the interconversion between the enantiomers, but the result was negative. Further examinations of the reaction systems revealed that the third amino acid, L-alanine, appeared during the reactions. When the time course of the production of L-aspartate and L-alanine from D-aspartate was examined with a low concentration of D-aspartate, suitable to follow its decrease, the total of the two L-amino acids produced was approximately equal to the decrease of the D-amino acid throughout the incubation period (Fig. 3A). In another experiment where L-aspartate was added as substrate, the results were similar in that the decrease of L-aspartate was approximately equal to the total increase of the other two amino acids, but differed markedly in that the production of L-alanine was far more rapid than in the previous experiment (Fig. 3B). The results suggest that the direct precursor of L-alanine was L-aspartate, which probably underwent decarboxylation, and this conversion affected the apparent velocities of the reactions catalyzed by the racemase. It is seen in Fig. 3B, that the concentrations of D- and L-aspartate were approaching to a same level with time after the increase of L-alanine slowed down. Figure 3A also shows a similar phenomenon, although less clearly. These findings leave little doubt on the presence of racemase and its involvement in the interconversion of D- and L-aspartate. Since no racemase activity was detected for glutamate and alanine, the racemase may be safely designated as aspartate racemase.

Discussion

The present data have shown that substantial concentrations of D-aspartate are present in several tissues of *S. broughtonii* together with approximately equal concentrations of L-aspartate, and that a racemase activity specific to aspartate is present primarily in the foot muscle, which also contains an enzyme activity to convert L-aspartate to L-alanine. With these findings, it appears very likely that D-aspartate is involved in the production of *N*-methyl-D-aspartate discovered by Sato et al. (1987) in this bivalve. Studies are in progress to test this hypothesis and elucidate the pathways involving these two compounds.

The observed rapid conversion of L-aspartate to L-alanine suggests that the conversion proceeds via a single step reaction which probably removes the β-carboxyl group of L-aspartate. Interestingly, *S. reidi* is reported to take up 4-^{14}C-D-aspartate added in sea water and release ^{14}C-CO_2 rapidly. Taken collectively, it is possible that the same sequence of reactions occurs in both bivalve species to yield L-alanine and CO_2 from D-aspartate.

S. broughtonii is known to tolerate exposure to low oxygen environment (Sato et al., 1987). Studies by Brooks et al. (1991) of anaerobiosis of bivalves including *Scapharca inaequivalvis* which is very closely related to *S.*

broughtonii have shown that L-aspartate is markedly consumed, while L-alanine is accumulated during the anoxia exposure, and the decrease of the former almost exactly matched the increase of the latter. Moreover, L-aspartate is supposed to be an established anaerobic energy substrate in euryoxic invertebrates (Zwaan et al., 1992). Then it may be safely considered that L-aspartate serves as an anaerobic energy substrate also in *S. broughtonii*. On this basis, the present results would mean that D-aspartate participates as a reserve form of L-aspartate, which is used in anaerobic energy metabolism.

References

Aswad DD (1984) Determination of D- and L-aspartate in amino acids mixtures by high-performance liquid chromatography after derivatization with a chiral adduct of *o*-phthalaldehyde. Anal Biochem 137: 405–409

Brooks SPJ, Zwaan A de, Thillart G van den, Cattani O, Cortesi P, Storey KB (1991) Differential survival of *Venus gallina* and *Scapharca inaequivalvis* during anoxic stress: covalent modification of phosphofructokinase and glycogen phosphorylase during aonoxia. J Comp Physiol B 161: 207–212

Felbeck H (1985) Occurrence and metabolism of D-aspartate in the gutless bivalve *Solemya reidi*. J Exp Zool 234: 145–149

Felbeck H, Wiley S (1987) Free D-amino acids in the tissues of marine bivalves. Biol Bull 173: 252–259

Sato M, Takahara M, Kanno N, Sato S, Ellington WR (1987) Isolation of a new opine, β-alanopine, from the extracts of the muscle of the marine bivalve mollusc, *Scapharca broughtonii*. Comp Biochem Physiol 88B: 803–806

Sato M, Inoue F, Kanno N, Sato Y (1987) The occurrence of *N*-methyl-D-aspartic acid in muscle extracts of the blood shell, *Scapharca broughtonii*. Biochem J 241: 309–311

Watanabe T, Kera Y, Matsumoto T, Yamada R (1996) Purification and kinetic properties of a D-amino-acid peptide hydrolyzing enzyme from pig kidney cortex and its tentative identification with renal membrane dipeptidase. Biochim Biophys Acta 1298: 109–118

Zwaan A de, Cortesi P, Thillart G van den, Brooks S, Storey KB, Roos J, Lieshout G van, Cattani O, Vitali G (1992) Energy metabolism of bivalves at reduced oxygen tensions. Sci Total Environ [Suppl]: 1029–1039

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