Distribution of Free D-Aspartic Acid and D-Aspartate Oxidase Activity in the Tissues of *Octopus vulgaris*

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The occurrence of free D-aspartic acid was measured in various tissues and organs of the octopus *Octopus vulgaris* by means of chiral HPLC amino acid analysis. This amino acid was detected in appreciable quantities in almost all of the tissues examined (muscle, renal appendage, ventricle, branchial heart, gill, crop, stomach, cecum, intestine, salivary gland, brain, and eye) except the hepatopancreas and gonad. In particular, the amount of D-aspartic acid in the intestine was 5- to 10-fold that in other tissues. The ratios of D/L were 2 or more in brain, eye, and intestine, but were less than 1 in the other tissues. D-Aspartate oxidase (EC 1.4.3.1) activity was found in various digestive organs and in the muscle of the octopus, but it was not detected in the brain, eye, gill, salivary gland, or gonad. The enzyme activity was highest in the cecum (1.3 U/g tissue) and was relatively high in the renal appendage, hepatopancreas, and intestine (0.8, 0.6 and 0.5 U/g tissue, respectively). The oxidizing activity towards L-aspartic acid was only slightly detected in all of the tissues examined. These results imply that the D-aspartic acid in octopus tissues originates mainly from intestinal bacteria and feed, and that the D-aspartate oxidase secreted from the cecum controls the accumulation of this amino acid.

It has generally been observed that the proteins and free amino acid pools of almost all living organisms except some bacteria are composed of L-amino acids, thus D-amino acids have been thought of as being unnatural. However, D-amino acids have been found either in the free form or combined with other molecules in several animals¹⁾ and plants.^{2,3)}

In recent years, the occurrence of D-aspartic acid and its biochemical and physiological role in mammals have gained increasing attention in various research fields. Helfman and Bada^{4,5)} reported that D-aspartic acid accumulates in human tooth enamel and dentine with aging, and similar results have been recognized in the human eye lens,⁶⁾ brain,^{7,8)} and aortic elastin.⁹⁾ D-Aspartic acid has also been identified as an important neurotransmitter in nervous tissue.¹⁰⁾ Furthermore, there have been some discussions concerning Alzheimer's disease¹¹⁻¹⁴⁾ and lesions of the red nucleus in guinea pigs.¹⁵⁾ In addition, this amino acid was found in various tissues of rat,¹⁶⁾ chicken embryo,¹⁷⁾ mouse kidney,¹⁸⁾ and amphibians.¹⁹⁾ Free D-aspartic acid has also been found in marine organisms, such as the nervous

organs of cephalopods,^{20,21)} bivalves,²²⁾ and some species of marine macroalgae.²³⁾ However, the physiological function of D-aspartic acid in marine organisms is still unclear.

D-Aspartate oxidase (EC 1.4.3.1) is a peroxisomal flavoenzyme catalyzing the deaminative oxidation of dicarboxylic D-amino acids, and this enzyme activity was discovered in rabbit kidney in 1949 by Still *et al.*²⁴⁾ Since then, such enzyme activity has been found in octopus hepatopancreas,²⁵⁾ various mammalian tissues,²⁶⁾ and in amphibian tissues.¹⁹⁾ However, the physiological function of the enzyme remains uncertain.

In this paper, we report on the distribution of free D-aspartic acid and D-aspartate oxidase activity in the various tissues of *Octopus vulgaris*. Our findings differed in several respects from the results of the previous report.²⁰⁾

Materials and Methods

Animals and Tissues

Live specimens of *Octopus vulgaris* weighing 1-2 kg were obtained from a fisherman in Okirai Bay, Iwate Prefecture.

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The muscle and various organs were rapidly dissected and rinsed with ice-cold water. The digestive organs, such as the stomach, crop, cecum, and intestine, were cut open and the inner matter was carefully washed away. Then these organs were kept frozen at -20°C until analysis.

Determination of D-Aspartic Acid

Homogenates were routinely prepared from 1–5 g tissue with 3 vol. of 1 N perchloric acid. Homogenization was carried out at temperatures below 4°C for 10 min, using an Ystral disperser. The supernatant obtained after centrifugation at 10,000 g for 20 min was neutralized with 10 N KOH. After standing for 1 to 2 hours in the cold, the precipitate was removed by centrifugation at 8,000 g for 10 min. The supernatant was evaporated to dryness under reduced pressure at $45\text{--}50^{\circ}\text{C}$ and the residue was dissolved in 15 ml of distilled water. A 5 ml sample of the extract was loaded on a Dowex 1 (100–200 mesh, acetate form) column (30 ml vol.). After the column had been washed with 500 ml of distilled water, adsorbed acidic amino acids were eluted with 500 ml of 4 N acetic acid. The eluate was evaporated to dryness under similar conditions to remove acetic acid and the residue was dissolved in 10 ml of deionized water. This sample solution was analyzed by means of the chiral HPLC amino acid analysis described by Sato *et al.*²⁷⁾ A Jasco model LC-800 HPLC system was used for measurement of D-aspartic acid, and fluorometric analysis was performed with an excitation wavelength of 348 nm and an emission wavelength of 450 nm.

Enzyme Assay

Crude enzyme solution for the assay of D-aspartate oxidase activity was prepared by homogenizing each tissue with 3 vol. of 50 mM Tris-HCl buffer containing 2 mM EDTA, 1 mM 2-mercaptoethanol and 10 mM flavin adenine dinucleotide (FAD) (pH 8.0) and by centrifuging at 10,000 g for 30 min at 0°C . The supernatant was dialyzed overnight against the same buffer with three changes to eliminate the endogenous amino acids and salts. The enzyme solution was stored at 0°C and used within several days.

The activities of D-aspartate oxidase and D-amino acid oxidase were assayed according to the method of Watanabe *et al.*²⁸⁾ except for the use of 30 μmol (final concentration) D-aspartic acid or D-alanine as substrate.

One unit of these enzymes was defined as the

amount of enzyme producing 1 μmol of H_2O_2 per min at 40°C under the reaction conditions. The enzyme activity of the tissue was expressed in units per gram of fresh tissue.

Results and Discussion

Distribution of D-Aspartic Acid in Octopus Tissues

Concentrations of D- and L-aspartic acid were measured in 14 tissues of octopus and the inner matter of two digestive organs (stomach and intestine) by means of chiral HPLC amino acid analysis.

Figure 1 shows chromatograms of standard DL-amino acids (Fig. 1A) and some samples, obtained by means of HPLC. The chromatogram of muscle extract showed three major peaks corresponding to D-, L-aspartic acids, and L-glutamic acid (Fig. 1B). That of the muscle extract hydrolyzed with 6 N HCl at 110°C for 20 hours also showed a similar pattern with an unknown peak (Fig. 1C). The chromatogram of brain extract showed four major peaks corresponding to D-, L-aspartic acids and D-, L-glutamic acids (Fig. 1D). Similar results were obtained in other tissues extracts (data not shown), but no D-glutamic acid peak was detected except in the brain extract. A large part of the D-aspartic acid probably occurs in a free form in octopus tissues. The distribution of D-aspartic acid in the tissues of octopus is summarized in Table 1. D-Aspartic acid was detected in almost all of the tissues examined, but not clearly in the gonad or hepatopancreas. Comparable levels of D-aspartic acid concentration (3–9 $\mu\text{mol/g}$ fresh tissue) were found in muscle, brain, eye, and almost all digestive organs except the intestine, where a significantly high concentration (50 $\mu\text{mol/g}$ tissue) of it was detected. D'Aniello and Giuditta^{20,21)} searched for D-amino acids in the organs of cephalopods by means of an assay method based on the use of specific oxidase and reported that D-aspartic acid was present in thier nervous system (2–15 $\mu\text{mol/g}$ fresh tissue) but not in non-nervous organs. The disparity in results may be due to the use of different assay methods.

The concentration of D-aspartic acid in octopus tissues was significantly higher than that in various tissues of rats¹⁶⁾ and chicken embryo,¹⁷⁾ in mouse kidney (mutant strain lacking D-amino acid oxidase and its control strain),¹⁸⁾ in human brain (normal and Alzheimer)^{11–14)} and in kidney and liver of amphibians,¹⁹⁾ whereas it was of a similar level in several marine bivalves.²²⁾ The ratios of

Fig. 1. Chromatograms of OPA-NAC derivatives of standard DL-amino acid and extracts of octopus tissues.

A: standard DL-aspartic acid and DL-glutamic acid, B: muscle extract, C: hydrolyzed muscle extract with HCl, D: brain extract.

Reversed-phase HPLC was performed on a column (250×4.6 mm) of TSK-ODS 80T (5 μm particle size) at 40°C. Materials were eluted from the column with the mixture of solvent A (50 mM sodium acetate buffer, pH 5.4) and solvent B (acetonitrile). The gradient was set as follows: 0–10 min, 0–10% solvent B in solvent A; 10–20 min, 10% solvent B in solvent

Table 1. Distribution of D-aspartic acid in the tissues of *Octopus vulgaris*

Tissue	D-Asp ¹⁾ (μmol/g tissue)	D/L ratio
Muscle (arm)	5.7±2.8	0.9
Brain*	4.8±1.4	2.0
Eye*	4.9±1.7	1.9
Ventricle	2.7±0.6	0.4
Branchial heart	2.9±1.0	0.1
Gill	3.2±0.9	0.4
Gonad	ND ²⁾	—
Salivary gland	3.2±2.5	0.1
Crop	6.1±2.7	0.7
Stomach	3.8±1.8	0.3
Content of the stomach*	6.8±2.2	0.5
Cecum	8.5±0.9	0.1
Intestine	51.2±4.9	2.5
Content of the intestine*	5.2±1.6	0.5
Hepatopancreas	Tr ³⁾	—
Renal appendage	4.8±1.7	0.9

¹⁾ Values are means ± SD obtained from two (*) or four specimens.

²⁾ Not detected.

³⁾ Below the level of 1.0 μmol/g wet tissue.

Table 2. D-Aspartate oxidase activity in the tissues of *Octopus vulgaris*

Tissue	DAO activity ¹⁾ (Unit/g tissue)
Muscle (arm)	0.08
Brain*	ND ²⁾
Eye*	ND
Ventricle	0.24
Branchial heart	0.09
Gill	ND
Gonad	ND
Salivary gland	ND
Crop	0.16
Stomach	0.10
Cecum	1.29
Intestine	0.52
Hepatopancreas	0.60
Renal appendage	0.84

¹⁾ Values are means obtained from one (*) or three specimens.

²⁾ Not detected.

A. The flow rate was 1.0 ml/min throughout. Fluorometric detection was performed with an excitation wavelength of 348 nm and an emission wavelength of 450 nm. An amount of 45.5 pmol of standard amino acids was injected. The extracts from octopus tissues were hydrolyzed with 6 N HCl at 110°C for 20 hours.

Table 3. Oxidizing activity towards various substrates in tissues of *Octopus vulgaris*

Tissue	Substrate	Activity ¹⁾ (U/g tissue)					
		D-Asp.	L-Asp.	D-Glu.	L-Glu.	D-Ala.	L-Ala.
Cecum		1.29	— ²⁾	1.97	—	0.15	—
Intestine		0.52	—	0.81	—	Tr ³⁾	—
Hepatopancreas		0.60	—	0.58	—	Tr	—
Renal appendage		0.84	—	0.88	—	0.80	—

¹⁾ Values are means obtained from three specimens.

²⁾ Not detected.

³⁾ Below the level of 0.05 U/g tissue.

D-aspartic acid to its L-form in octopus tissues were less than 1 in many tissues examined, but were about 2 or more in brain, eye, and intestine.

D-Aspartic acid was also detected in the inner matter of stomach and intestine of octopus (Table 1). The inner matter consisted mainly of sardines given to the octopus as fishing bait; however, D-aspartic acid was only slightly detected in whole sardine extract (below 0.1 μ mol/g sample). On the other hand, some marine bivalves and crustaceans which are feed for the octopus also contained D-aspartic acid (1–10 μ mol/g, unpublished). These results suggest that at least a part of the D-aspartic acid in the octopus tissues originates from intestinal bacteria and feed. By contrast, Nagata and Akino²⁰⁾ reported that D-amino acids in mouse tissues were not of microbial origin since these amino acid concentrations did not differ between germ-free mice and specific pathogen-free mice. In addition, Matsushima *et al.*²⁰⁾ suggested that D-alanine in a species of bivalve was dependent on the alanine racemase of the bivalve tissues. The presence of aspartate racemase in octopus tissues is still doubtful.

D-Aspartate Oxidase Activity in Octopus Tissues

D-Aspartate oxidase activity was detected in muscle, ventricle, branchial heart, crop, stomach, cecum, intestine, hepatopancreas, and renal appendage, but not in brain, eye, gill, gonad, or salivary gland (Table 2). Enzyme activity was highest in the cecum (1.3 U/g tissue) and was relatively high in renal appendage (0.8 U/g), hepatopancreas (0.6 U/g), and intestine (0.5 U/g), but in other tissues and organs it was only about one-fifth to one-tenth of that in the cecum. In mammals, D-aspartate oxidase activity is high in the kidney and liver,²⁰⁾ whereas the activity in octopus was significantly high in the cecum. In octopus, additionally, the lack of D-aspartate oxidase was observed in the brain, which is one of its main

sources in mammals. This finding agreed with the results of D'Aniello and Giuditta.²⁰⁾ Thus, the distribution of D-aspartate oxidase activity in octopus tissues differed from that in mammals.

The oxidizing activities of the extracts of cecum, hepatopancreas, and renal appendage in octopus towards some substrates were determined (Table 3). These tissue extracts showed apparently higher activity towards D-glutamic acid than towards D-aspartic acid (Table 3). According to Hamilton,²⁰⁾ D-aspartic acid is generally the best substrate for the mammalian enzyme with the reaction rate of D-glutamic acid at approximately one-tenth that of D-aspartic acid. Furthermore, in amphibian liver, it has been reported that the oxidizing activity towards D-glutamic acid was 15–33% of that towards D-aspartic acid.¹⁰⁾ Thus, it is probable that the substrate specificity of D-aspartate oxidase varies according to the animal. On the other hand, oxidizing activity towards D-alanine was also observed in the extracts of cecum and renal appendage of octopus, but slight activity towards L-amino acids such as L-aspartic acid, glutamic acid, and alanine was found in all of the tissues examined.

The physiological role of D-aspartic acid and D-aspartate oxidase in organisms is still unclear. There are some reports that D-aspartic acid accumulates in human tissues with aging and senescence.^{4–9)} Shapira *et al.*¹¹⁾ and Fisher *et al.*^{12–14)} suggested that D-aspartic acid in the human brain is related to Alzheimer's disease. On the other hand, in marine organisms, D'Aniello and Giuditta^{20, 21)} suggested that this amino acid might have a specific neural function, and Nagahisa *et al.*²³⁾ suggested that D-aspartic acid might be involved in the sporulation of marine macroalgae. In this work, it seems likely that at least some of the D-aspartic acid in octopus tissues originated from intestinal bacteria and feed, but its physiological function was not clear. Hamilton²⁰⁾ has sug-

gested that there was some other physiological substrate for the enzyme because D-aspartic acid did not generally seem to be toxic to animals.

The possible presence of D-aspartic acid in marine organisms and its involvement in metabolism should not be overlooked.

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