Variations in D-Aspartate Content with Season and Part of *Hizikia fusiformis*

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D-Aspartate content in the marine alga *Hizikia fusiformis* was determined in various seasons and different parts of the alga. D-Aspartate content varied seasonally; it was high from late autumn to early spring and low in summer. The content also differed among various parts of the alga; it was generally high in the adhesive root and main branch, while the content in the blade was less than half that in the adhesive root. The content in the receptacle which was obtained from the August sample showed a level similar to that in the adhesive root and the main branch. These results suggest that free D-aspartate may play an important role in the early stage of growth of *H. fusiformis*.

Key words: D-amino acid, D-aspartate, seasonal variation, alga, *Hizikia fusiformis*

It used to be generally accepted that D-amino acids found in living organisms except some bacteria are anomalous. However, over the last few decades, it has been recognized that D-amino acids are present either in the free or combined form with other molecules in various animals,¹⁾ plants,^{2,3)} and human tissues,^{4–8)} and recently the biochemical and physiological function of D-amino acids has been the focus of increasing attention in various research fields.

On the other hand, those have been very few studies on the D-amino acids in marine organisms. Free D-aspartate has been found in cephalopods,^{9–11)} bivalves,^{12,13)} and marine macroalgae.¹⁴⁾ In addition, D-alanine has been found in bivalves.¹⁵⁾ With respect to the physiological roles of D-amino acids, D'Aniello and Giuditta⁹⁾ suggested that D-aspartate in cephalopods might have a specific neural function, and Matsushima *et al.*¹⁵⁾ suggested that D-alanine in some marine bivalves plays a part in osmoregulation.

Studies on free L-amino acids, but not D-amino acids, in marine algae have been extensive and thorough. Previously, we were the first to isolate free D-aspartate from the marine macroalgae *Hizikia fusiformis*, and reported that this amino acid occurred in several other species of marine macroalgae.¹⁴⁾ D-Aspartate is present in high concentrations in Phaeophyta, particularly *H. fusiformis*, which is a popular edible alga in Japan. Thus it is important to elucidate the physiological and nutritional function of D-aspartate in this alga.

In this paper, we report variations in D-aspartate content with season and part of *H. fusiformis*, and discuss the physiological role of D-aspartate in this alga.

Materials and Methods

Chemicals and Alga

All chemicals, including D- and L-aspartate, *o*-phthalaldehyde (OPA), and *N*-acetyl-L-cysteine (NAC) of reagent grade, were purchased from Wako Pure Chem. Ind., Ltd. (Osaka, Japan).

Sample alga, *H. fusiformis*, was collected from Okkirai Bay (Iwate Prefecture, Japan), during the middle ten days of every month from 1992 to 1993.

Preparation of Acidic Amino Acid Solution from Alga

The algae were divided into parts, *i.e.*, whole, adhesive root, main branch, blade, and receptacle. Each part was rinsed well in fresh water. In particular, extraneous matter associated with the adhesive root, such as different species of algae, shells, sand and other materials were carefully removed with tweezers.

Fifty or twenty-five grams (wet weight) of each sample was used for extraction of the acidic amino acids. The sample was finely sliced and homogenized with 3 vol. (v/w) of 99% ethanol in an Ystral disperser. The homogenate was centrifuged at $10,000 \times g$ at 0°C for 20 min. The precipitate was again extracted with the same volume of 80% ethanol. Both ethanolic extracts were mixed and evaporated to dryness under reduced pressure at 45–50°C. The residue was dissolved in a small amount of distilled water and rinsed with a sufficient amount of diethyl ether to remove pigments and fatty materials. This extract was evaporated to dryness and dissolved in 30 ml of distilled water.

Five milliliters of the extract was loaded on a Dowex 1-X8 (100–200 mesh, acetate form) column (30 ml vol.). After the column was washed with 500 ml of distilled water, adsorbed acidic amino acids were eluted with 500 ml of 4N acetic acid. The eluate was evaporated to dryness under similar conditions as above to remove acetic acid and the residue was dissolved in 10 ml of deionized water.

Reversed-Phase HPLC Amino Acid Analysis

The sample solution was analyzed using the chiral HPLC amino acid analysis method described by Sato *et al.*¹⁶⁾ Briefly, the sample solution was passed through a Millipore filter (GV; pore size, 0.22 μ m), then 10 μ l of the filtrate was derivatized with 60 μ l of 50 mM sodium borate and 40 μ l of OPA-NAC solution at room temperature. The derivatized sample was immediately applied to a reversed-phase column (TSK-ODS 80T, Tosoh Co., Tokyo, Japan) HPLC system (Jasco model LC-800). Materials were eluted from the column with the mixture of solvent A (50 mM sodium acetate buffer, pH 5.4) and solvent B (acetonitrile) at 40°C. The gradient was set as follows: 0–10 min, 0–10% solvent B in solvent A; 10–20 min, 10% solvent B in solvent A. The flow rate was 1.0 ml/min throughout. Fluorometric detection was performed at an excitation wavelength of 348 nm and an emission wavelength of 450 nm.

Measurement of Nitrogen in Ethanolic Extracts

The nitrogen content of the ethanolic extracts from alga was measured using ninhydrin methods.

Results and Discussion

Seasonal Variation of D-Aspartate Content in *H. fusiformis*

Alga samples were collected each month for a year, and the contents of D- and L-aspartate in the whole alga were

measured by the chiral HPLC amino acid analysis method. Details of the alga samples examined are shown in Table 1, and the chromatograms of standard DL-amino acids and sample extracts are shown in Fig. 1. The total extractive nitrogen in the sample extract was relatively high from winter to spring, and thereafter it decreased gradually, remaining relatively constant from summer to autumn (Table 1). The chromatogram of whole alga showed three major peaks corresponding to D- and L-aspartate and L-glutamate (Fig. 1B). The chromatogram of the extract hydrolyzed with 6N HCl showed a similar pattern (Fig. 1C), and the D-aspartate content hardly changed from its original form. This indicates that D- and L-aspartate in the alga extract are of the free form.

On the other hand, the D-aspartate content varied greatly with the seasons. As shown in Fig. 2, the D-aspartate content in *H. fusiformis* was high from October to March, and low from May to September. The content in August was the lowest and less than half that in February or March. The content increased drastically from around October. The L-aspartate content changed in the same manner, although

the magnitude of change was less than that of the D-isomer. Consequently, the ratio of D/D + L was generally high (max. 0.6) except in winter when the D-aspartate content was high, while it was low (min. 0.3) when the D-aspartate content was low.

In the sea near Sanriku, Iwate Prefecture, *H. fusiformis* germinates in summer and grows from autumn to spring, exhibiting rapid growth particularly in early spring. The D-aspartate content increased during the early growth period in this alga.

Variation of D-Aspartate Content with Alga Part

The alga was divided into three or four parts, i.e., the adhesive root, main branch, blade, and receptacle (only for the August sample), and the D-aspartate content of each part was measured by the same method as described above. The D-aspartate content was highest in the adhesive root followed by the main branch throughout all seasons,

Fig. 1. Details of alga samples, *H. fusiformis*

Sampling date	Aver. length* (cm)	Cont. extractive-N (μ g/g fresh sample)
Apr. 15, 92	20	76
May 26	17	54
Jun. 21	20	43
Jul. 20	25	56
Aug. 17	28	44
Sep. 13	13	61
Oct. 13	6	77
Nov. 11	8	92
Dec. 14	10	95
Jan. 14, '93	10	81
Feb. 25	15	84
Mar. 22	18	88

* Values are means of 10 specimens.

Fig. 1. Chromatograms of OPA-NAC derivatives of standard DL-amino acids and extract of *H. fusiformis*.

A, standard DL-aspartate and DL-glutamate; B, whole alga extract; C, hydrolyzed whole alga extract with HCl. Reversed-phase HPLC was performed as described in the text. 45.5 pmol of each standard amino acid was injected. The extract from alga was hydrolyzed with 6N HCl at 110°C for 20 h.

Fig. 2. Seasonal variation of D-aspartate content in *H. fusiformis*.

Fig. 3. Variation of D-aspartate content with alga part.

Table 2. Variation of the D-aspartate content with alga size

Month	Contents of D-aspartate (nmol/g fresh sample)			
	Size	5 cm >	10–15 cm	20 cm <
Jan.		251	240	261
Apr.		166	158	175
Aug.		90	100	112
Oct.		240	242	257

whereas the content in the blade was almost less than half that in the adhesive root (Fig. 3). The receptacle, which was examined only in the August sample, showed a value similar to that of the adhesive root and main branch.

Variation of D-Aspartate Content with Alga Size

H. fusiformis in each collection batch was classified according to size (length), i.e., less than 5 cm, from 10 to 15 cm and more than 20 cm, and the D-aspartate content of the whole alga was compared among the size groups. As shown in Table 2, no distinct difference in the content among the alga size groups was found throughout all seasons.

These results suggest that D-aspartate plays an integral role in the growth of *H. fusiformis*, particularly in the germination and early growth stage. It was suggested by Ogawa *et al.*^{2,17)} that D-alanine and D-aspartate might play a role in the development of pea seedlings. It is well known that the contents of many free L-amino acids in marine algae show seasonal variations,¹⁸⁾ and are considered to affect the growth of algae. However, details of the mechanisms are not clear.

To elucidate the physiological roles of D-aspartate, it is also necessary to study the various enzymes involved in metabolism of this amino acid. However, despite much effort, we have not yet identified enzymes such as racemase or transaminase which produce D-aspartate. Activity of D-aspartate oxidase was found in the aqueous extract of this alga, though at very low levels (data not shown). It is difficult to purify enzymes from Phaeophyta because the concentration of protein in these algae is generally very low. We will study this problem further in the near future.

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