Confirmation of D-aspartic acid in the novel dipeptide β -aspartylglycine isolated from tissue extract of *Aplysia kurodai*

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A novel *o*-phthalaldehyde-reactive compound was found in the h.p.l.c. chromatogram of *Aplysia kurodai* extract. This compound was isolated by ion-exchange chromatography and preparative high-voltage paper electrophoresis. It was shown by optical-rotatory-dispersion spectrum and optical-resolution h.p.l.c. analysis that this compound consisted of equimolar amounts of D-aspartic acid and glycine. This compound resisted cleavage in the Edman reaction. This peptide was inferred to be β -D-aspartylglycine, and this was confirmed by synthesis. β -D-Aspartylglycine was detected in all tissues of *Aplysia kurodai*, with especially high concentrations in body wall (skin and muscle) and gill.

INTRODUCTION

We have previously described the presence of *N*-methyl-D-aspartic acid in the muscle of the blood shell *Scapharca broughtonii* (Sato *et al.*, 1987). In the subsequent study on the distribution of D-aspartic acid (which seems to be the precursor of *N*-methyl-D-aspartic acid) in molluscs by optical-resolution h.p.l.c. analysis, we detected an unidentified peak in the acidic area before DL-aspartic acid in the tissue extract of *Aplysia*. It was isolated by ion-exchange chromatography and preparative paper electrophoresis. The structure of this compound was inferred to be β -D-aspartylglycine (β -D-Asp-Gly), and this was confirmed by synthesis.

The present paper deals with the isolation and identification of β -D-Asp-Gly from the tissues of *Aplysia kurodai*.

EXPERIMENTAL

Materials and reagents

All chemicals such as L- and D-aspartic acid, *o*-phthalaldehyde, *N*-acetyl-L-cysteine, phenyl isothiocyanate etc. in reagent grade were purchased from Wako Pure Chemicals Co., Tokyo, Japan. The reverse-phase column material TSK-ODS 80T (particle size 5 μ m; Toso Co., Tokyo, Japan) was packed into a 250 mm $\times$ 4.6 mm (internal diameter) stainless-steel column in our laboratory by the conventional slurry-packing technique.

Paper electrophoresis

Amino acids and related compounds were detected routinely by ninhydrin development after separation by high-voltage paper electrophoresis at pH 1.9 (Sato *et al.*, 1987).

Amino acid analysis

Amino acids were analysed by h.p.l.c. after reaction with phenyl isothiocyanate to produce the phenylthiocarbamoyl derivatives. Formation of derivatives and h.p.l.c. analysis were conducted in the manner described by Sato *et al.* (1988) except that the buffer pH was 5.4 instead of 5.0.

Optical-resolution analysis of DL-aspartic acid by h.p.l.c.

Chromatography was conducted in an h.p.l.c. system consisting of the following components: two Jasco 880-PU pumps, a Jasco 801-SC system controller, a Jasco 850-AS autosampler, a Jasco 210-FP spectrofluorimeter, an 865-CO column oven at $40 \pm 0.1^\circ\text{C}$ and a Sic Chromatocorder 12 data processor. The elution solvent systems were (solvent A) 50 mM-sodium acetate buffer, pH 5.4, and (solvent B) acetonitrile. The solvent proportions were set as follows: 0–10 min, 0–10% (v/v) solvent B in solvent A; 10–20 min, 10% (v/v) solvent B in solvent A. After this, a washing step was programmed to 60% (v/v) solvent B in solvent A for 5 min. The formation of amino acid derivatives was carried out by the method of Nimura & Kinoshita (1986). To 10 μ l of amino acid solution was added 60 μ l of 0.1 M-sodium borate and they were mixed well. Then 40 μ l of *o*-phthalaldehyde/*N*-acetyl-L-cysteine solution (8 mg of *o*-phthalaldehyde and 10 mg of *N*-acetyl-L-cysteine in 1 ml of methanol) was added, and the whole was mixed well and left to stand for 2 min. Then 10 μ l of the derivative was injected for h.p.l.c. analysis.

RESULTS

Isolation of β -D-Asp-Gly from *Aplysia*

Four living specimens of *Aplysia kurodai* were collected in Okkirai Bay, Iwate Prefecture, Japan, in June 1988. The foot and parapodium muscle (600 g) were used for ethanolic extraction by a previously published method (Sato *et al.*, 1979). The extract was applied on to a Dowex 50W-X8 (H^+ form) column (4 cm $\times$ 50 cm) and washed thoroughly with 20 litres of deionized water. The bound material was eluted by 4 litres of 2 M- NH_3 solution and fractionated into 1-litre portions. Each portion was concentrated by flash evaporation and subjected to high-voltage paper electrophoresis and h.p.l.c. analysis. The fractions containing the unknown compound that gave a peak before D-aspartic acid in the optical-resolution h.p.l.c. analysis (Figs. 1a and 1b) were combined and concentrated to dryness. The residue was dissolved in a small volume of deionized water and loaded on a Dowex 1-X8 (acetate form) column (1.5 cm $\times$ 25 cm). The column was washed thoroughly with 2 litres of deionized

Fig. 1. Chromatography of standard amino acids (a), acidic fraction of extract from parapodium muscle of *Aplysia* (b), isolated β -D-Asp-Gly (c) and HCl hydrolysate of β -D-Asp-Gly (d).

Chromatography was on a 250 mm $\times$ 4.6 mm (internal diameter) column of TSK-ODS 80T (5 μ m particle size) at 40 $^{\circ}$ C. The mobile phase was formed from 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% (v/v) solvent B in solvent A; 10–20 min, 10% (v/v) solvent B in solvent A. The flow rate was 1 ml/min. Fluorimetric detection was with an excitation wavelength of 348 nm and an emission wavelength of 450 nm. A 33.3 pmol amount of each standard amino acid was injected.

water and then eluted successively with 500 ml each of 0.1 M-, 0.15 M- and 0.2 M-acetic acid. The eluate was collected in 10 ml fractions and checked by paper electrophoresis and h.p.l.c. analysis as stated above. After elution of aspartic acid (fractions 44–64), the unknown compound was detected in fractions 75–105 with small amounts of other material. The fractions containing the unknown compound were combined and

Fig. 2. Optical-rotatory-dispersion spectrum of β -D-Asp-Gly isolated from *Aplysia*.

β -D-Asp-Gly was dissolved in water at a concentration of 0.35 mg per 3 ml, and its optical-rotatory-dispersion spectrum was measured at 23 $^{\circ}$ C with a Jasco model ORD-J-20A instrument.

it was finally purified by preparative paper electrophoresis on Toyo filter paper no. 526 (pH 1.9; 1000 V; 16.7 V/cm for 5 h). This compound gave a blue colour like that obtained with asparagine on development with ninhydrin and it was crystallized from aqueous ethanol (yield 2.5 mg).

Identification of β -D-Asp-Gly

The isolated compound consisted of equimolar amounts of aspartic acid and glycine as judged from the amino acid analysis after hydrolysis with 6 M-HCl at 110 $^{\circ}$ C for 20 h. The D-configuration of aspartic acid was strongly suggested by optical-rotatory-dispersion spectrum analysis: $[M]_{300} = -1177^{\circ}$, $[M]_{250} = -1827^{\circ}$, $[M]_{230} = -2801^{\circ}$, $[M]_{220} = -3613^{\circ}$, $[M]_{210} = -4527^{\circ}$ and $[M]_{205} = -3897^{\circ}$ (Fig. 2). It was also confirmed by optical-resolution h.p.l.c., as shown in Figs. 1(c) and 1(d). This compound gave neither aspartic acid phenylthiohydantoin derivative nor glycine phenylthiohydantoin derivative during attempted cleavage by the Edman reaction. From all the above data, the isolated compound was inferred to be β -D-Asp-Gly.

Synthesis of β -D-Asp-Gly and comparison with the compound isolated from *Aplysia kurodai*

N-Benzoyloxycarbonyl-D-aspartic acid α -ethyl ester prepared from D-aspartic acid (1.33 g) by the method of Kovacs *et al.* (1963) was combined with glycine by the general method of Yajima *et al.* (1967) to produce N-benzoyloxycarbonyl- β -D-Asp-(OEt)-Gly. The protecting groups were removed by the usual procedure and β -D-Asp-Gly was purified by ion-exchange chromatography on both a Dowex 50-X8 (H^{+} form) column (2.6 cm $\times$ 12 cm) and a Dowex 1-X8 (acetate form) column (2.2 cm $\times$ 17 cm). Colourless crystals were obtained by crystallization from aqueous ethanol (yield 1.4 mg). On h.p.l.c. analysis this compound showed a single peak with the same retention time as the compound isolated from *Aplysia*. The data of the optical-rotatory-dispersion spectrum of the synthesized compound are $[M]_{300} = -1020^{\circ}$, $[M]_{250} = -1620^{\circ}$, $[M]_{230} = -2641^{\circ}$, $[M]_{220}$

Table 1. Concentration of β -D-Asp-Gly in tissues of *Aplysia kurodai*

Tissue	β -D-Asp-Gly content (μ mol/g of tissue)	D-Asp content (μ mol/g of tissue)	100 $\times$ D-Asp/DL-Asp ratio (%)
Parapodium (skin)	20.93	3.47	73.90
Parapodium (muscle)	19.37	3.88	34.09
Gill	15.04	0.26	48.02
Mid-gut gland	0.28	0.03	5.42
Intestine	0.93	0.04	7.66
Mouth	5.08	0.65	36.02
Stomach	3.81	0.58	38.00
Gizzard	4.31	1.72	45.14
Ganglion and nerves	3.73	1.59	90.54
Purple dye fluid	Trace	Trace	—

$= -3213^\circ$, $[M]_{210} = -4108^\circ$ and $[M]_{205} = -3694^\circ$, agreeing well with the values obtained with the compound isolated from *Aplysia*.

Distribution of β -D-Asp-Gly in *Aplysia* tissues

Tissues of *Aplysia kurodai*, collected in May 1989, were homogenized in 3 vol. (v/w) of 1 M-HClO₄ in an Ystral disperser and then centrifuged at 10000 g for 20 min. The supernatant was neutralized with 10 M-KOH and centrifuged at 10000 g for 20 min. The final supernatant was diluted and used for h.p.l.c. analysis after derivative formation with *o*-phthalaldehyde/*N*-acetyl-L-cysteine. High β -D-Asp-Gly concentrations were detected in body-wall tissue (both skin and muscle of parapodium) and gill, as shown in Table 1. β -D-Asp-Gly was also detected in all other tissues analysed, including digestive and nervous tissues. There are high concentrations of D-aspartic acid, which seems to be a precursor of β -D-Asp-Gly, in all tissues of *Aplysia*.

DISCUSSION

In this study a novel acidic dipeptide, β -D-aspartylglycine (β -D-Asp-Gly), was isolated from extracts of parapodium and foot of *Aplysia kurodai*. This compound was confirmed to be distributed in all tissues of this gastropod mollusc. It is, however, noteworthy that high concentrations of β -D-Asp-Gly were contained in the external tissues in direct contact with sea-water. The low yields of β -D-Asp-Gly actually obtained compared with the high concentrations in parapodium measured by h.p.l.c. analysis can be attributed to incomplete separation from tissue extract and/or to seasonal variation.

β -Asp-Gly has been identified previously in human urine (Buchanan *et al.*, 1962; Gejyo *et al.*, 1978), in collagen (Pisano *et al.*, 1966), in caecal contents of mammals (Welling & Groen, 1978; Welling, 1982) and also in tissues of *Aplysia californica* (McCaman & Stetzler, 1984). However, there is no information in these reports on the configuration of aspartic acid of β -Asp-Gly. Since the configuration of aspartic acid of β -Asp-Gly occurring in *Aplysia kurodai* has now been confirmed to be the D-form, it is necessary to reconsider the configuration of aspartic acid of mammalian β -Asp-Gly from a comparative viewpoint.

Synthesis of β -Asp-Gly in the ganglion of *Aplysia californica* has been studied by radioactive tracer

experiments (McCaman & Stetzler, 1985). In these experiments a rapid incorporation of [¹⁴C]glycine from a sea-water medium into β -Asp-Gly was observed, but only a little of [¹⁴C]aspartic acid (configuration not stated). It might have been more rapidly incorporated if D-aspartic acid had been used in these experiments.

With regard to mammalian β -Asp-Gly, it has been pointed out that β -Asp-Gly would be metabolized by a number of strains of bacteria present in the intestinal tract of animals (Welling, 1982). It follows that the configuration of aspartic acid of mammalian β -Asp-Gly needs to be determined, since bacteria can metabolize D-amino acids. And, in the case of humans, there could be some relationship between D-aspartic acid and β -Asp-Gly, which have both been detected in the blood of uraemic patients (Nagata *et al.*, 1987; Gejyo *et al.*, 1978).

As shown in the present and previous studies, there are appreciable amounts of D-amino acids, especially D-aspartic acid, and related compounds in marine molluscs and other invertebrates (Preston, 1986; Felbesk & Wiley, 1987; Sato *et al.*, 1987). The metabolism and physiological role of D-amino acids seem to merit further study.

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