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A trial to determine D-amino acids in tissue proteins of mice

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Summary. Eleven neutral amino acids and two acidic amino acids in tissue proteins of mouse kidney, liver and brain were analyzed for the presence of D-enantiomers. The proteins were hydrolyzed with HCl for 6 h. Of the thirteen amino acids investigated, the presence of D-enantiomers of serine, alanine, proline, aspartate and glutamate (including asparagine and glutamine) was shown in the hydrolysates. However, the level of D-enantiomers were not significantly higher than that of 6-h hydrolysate of serum albumin examined as a control protein. Serum albumin was shown to contain no D-amino acid residues.

Keywords: Amino acids – D-Amino acid – Hydrolysis – Tissue protein

Introduction

D-Amino acids are generally thought to be unnatural in vertebrates, whereas the presence of D-amino acids were reported on microorganisms, arthropods, molluscs and plants. However, we have detected considerable amounts of free neutral D-amino acids in human sera (Nagata et al., 1987) and mouse tissues (Nagata et al., 1989), where the activity of D-amino acid oxidase was low or none. D-Amino acid oxidase (EC 1.4.3.3) is a flavoprotein widespread in many animal tissues, and catalyzes oxidative deamination of neutral free D-amino acids (Meister and Wellner, 1963). The free neutral D-amino acids have been revealed to be serine, alanine and proline in mouse tissues (Nagata et al., 1992c). As to D-amino acids in animal peptides or proteins, dermorphin, a highly potent opioid heptapeptide isolated from the skin of arboreal frog, contains D-alanine (Broccardo et al., 1981), and the D-configuration was essential to the pharmacological activity (Broccardo et al., 1981). Spontaneous chemical racemization of L-aspartates is known to occur with time in metabolically inactive long-lived proteins in humans such as lens (Masters et al., 1977), teeth dentin (Helfman and Bada, 1976) and myelin (Shapira and Jen Chou, 1987).

In the present study, we investigated metabolizing tissue proteins in mouse kidney, liver and brain in search of the presence of D-amino acid residues, as an attempt to elucidate the source of the above free D-amino acids. In order to determine D-amino acids in a peptide chain, the peptide was hydrolyzed with HCl for various time lengths. The presence of a D-amino acid in the native peptide was obtained by extrapolation of rate of the D-enantiomer to L-enantiomer to 0-h hydrolysis, since the rate increases with time length of hydrolysis owing to the chemical racemization caused by acid hydrolysis. Serum albumin was employed as a control protein to assess the rate of chemical racemization caused by hydrolysis.

Materials and methods

Preparation of tissue proteins and hydrolysis

Eight weeks old ddY male mice were used. The mice were killed by bleeding from axillary vessels under anaesthesia with diethyl ether. After rinsing with phosphate-buffered saline [150 mM NaCl/10 mM sodium phosphate buffer (pH 7.4)], the kidney, liver and brain were minced into small pieces, and homogenized as whole organs, each with four volumes of the saline in a glass homogenizer in an ice bucket, at 1000 rpm for 1 min. The homogenate was centrifuged at 16000 g for 60 min at 4°C. To 0.2 ml of the supernatant extract, cold trichloroacetic acid solution was added to make a final concentration of 5%. The precipitates of centrifugation at 3000 g for 5 min was dissolved in 0.1 ml of H_2O and washed two times with 1.0 ml of acetone. The resulting white pellet was suspended in 0.2 ml of H_2O. The top of a glass tube containing 0.1 ml of the suspension and 6 M HCl was sealed with flame under a reduced pressure, and then, the proteins in the tube were hydrolyzed at 110 ± 0.2°C for 6 h in an aluminum block. 100 μg of purified crystalline serum albumin (from human, Sigma, St. Louis, MO, USA) was dissolved in 0.1 ml of H_2O, and hydrolyzed as above for 6, 16 and 24 h.

Resolution of D- and L-enantiomers

The hydrolysates were derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA, Pierce, Rockford, IL, USA) according to the method of Marfey (1984). The FDAA-amino acids were analyzed by HPLC for the resolution of D- and L-enantiomers as previously described (Nagata et al., 1992b), using a reversed-phase column, Nova-Pak C18 (150 × 3.9 mm ID, Waters, Milford, MA, USA), and a Tosoh (Tokyo, Japan) gradient HPLC system. Amounts of D- and L-enantiomers of amino acids, and the D/L ratios were calculated based on the peak areas obtained by a Chromato-Integrator (D-2500, Hitachi, Tokyo, Japan), and on the standard curves (Nagata et al., 1992b).

All chemicals used were chromatography or analytical grade.

Results

As a result of hydrolysis, no D-enantiomer of threonine, methionine, valine, tyrosine, leucine, isoleucine and phenylalanine was detected in both the tissue proteins and serum albumin. In the hydrolysates of serum albumin, the amounts of D-enantiomers of serine, alanine, proline, aspartate and glutamate (including asparagine and glutamine) increased with time length of hydrolysis, whereas the amounts of L-enantiomers were maximum in the 16- or 6-h hydrolysate (Table 1). The result suggests that the increase of D-enantiomers was caused by chemi-

Table 1. Amounts of D- and L-enantiomers of serine, alanine, proline, aspartate and glutamate detected in 6, 16 and 24-h hydrolysates of serum albumin

Hydrolysis (h)	Amino acids (nmol/mg)									
	Ser		Ala		Pro		Asp		Glu	
	D	L	D	L	D	L	D	L	D	L
6	0.300	208	1.92	534	1.44	209	13.9	747	11.1	1067
16	0.450	323	4.84	786	5.99	340	35.8	729	22.6	1086
24	0.675	259	8.28	686	7.40	286	33.1	459	22.9	665

Results from a typical experiment are shown

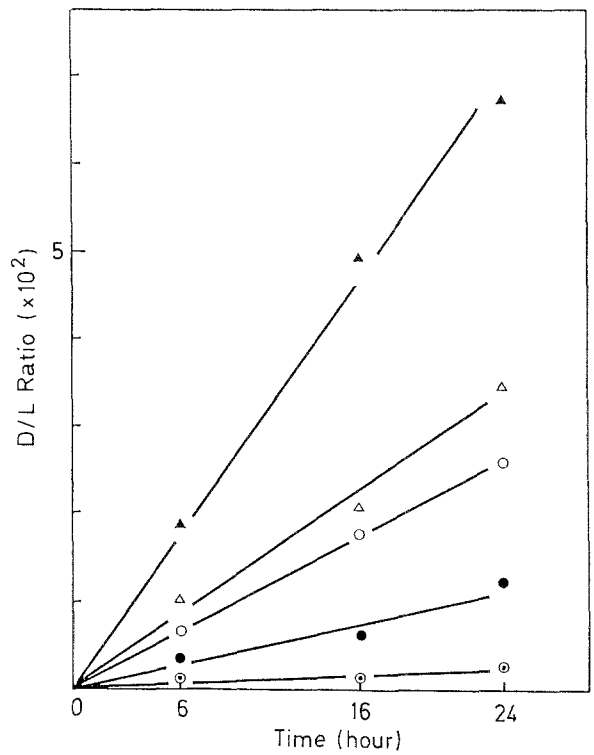

Fig. 1. D/L Ratios of serine (⊙), alanine (●), proline (○), aspartate (▲) and glutamate (△) in 6, 16 and 24-h hydrolysates of serum albumin. Results from a typical experiment are shown

cal racemization. It has been shown that serum albumin contains no D-amino acid residues based on that all the D/L ratios of serine, alanine, proline, glutamate and aspartate at 0-h hydrolysis were zero by extrapolating the D/L ratios at 6, 16 and 24-h hydrolysis to 0-h (Fig. 1). Hence, it was indicated that serum albumin could be used as a control protein to assess the rate of chemical racemization.

The D/L ratios of 6-h hydrolysates of tissue proteins from mouse kidney, liver and brain (cerebrum and cerebellum) were compared with that of the 6-h hydrolysate of serum albumin (Table 2). None of serine, alanine, proline, as-

Table 2. D/L Ratios of serine, alanine, proline, aspartate and glutamate in 6-h hydrolysates of tissue proteins from mouse kidney, liver, cerebrum and cerebellum, and of serum albumin

Tissue	D/L Ratio ($\times 10^2$)				
	Ser	Ala	Pro	Asp	Glu
Kidney	0	0.52 ± 0.14	1.24 ± 0.23	2.48 ± 0.32	1.43 ± 0.17
Liver	0	0.82 ± 0.22	1.26 ± 0.37	2.53 ± 0.40	1.26 ± 0.41
Cerebrum	0.65 ± 0.26	0.69 ± 0.07	1.06 ± 0.02	2.65 ± 0.25	1.25 ± 0.01
Cerebellum	0.19 ± 0.02	0.72 ± 0.18	1.18 ± 0.17	2.34 ± 0.09	1.23 ± 0.09
Albumin	0.23 ± 0.19	0.37 ± 0.03	0.85 ± 0.14	1.98 ± 0.18	1.01 ± 0.13

Values are the mean ± S.D. for three animals, or three hydrolysates (serum albumin)

partate and glutamate (including asparagine and glutamine) of the tissue proteins gave a significantly higher D/L ratio than albumin. Therefore, it has been strongly suggested that the tissue proteins from mouse kidney, liver and brain contain no D-amino acids.

Discussion

The present method to determine D-amino acids in proteins by extrapolating the D/L ratios at 6, 16 and 24-h hydrolysis to the ratio at 0-h may be the most convenient and accurate one. Proteolytic enzymes solely digest peptide bonds between amino acids of L-configuration. Hence, peptides including a D-amino acid residue are left without being digested by the enzymic treatment. The materials investigated in the present experiment, however, were mixtures of tissue proteins, and so, it seems impossible to be digested completely even at peptide bonds between L-amino acids. Other means utilising ^{13}C NMR and capillary electrophoresis are able to analyze for D- and L-amino acids in a whole peptide without hydrolysis. At the present time, those methods are not available for a protein of large size.

Free D-serine was found recently in mouse (male, 8 weeks old) cerebrum and cerebellum. The D-serine concentration was surprisingly high in the cerebrum (D/L = 0.44), whereas it was low in the cerebellum (D/L = 0.07) (Nagata, 1992). Since the transport of L-serine through the blood-brain barrier is slow (Oldendorf and Szabo, 1976) and the D-enantiomer of an amino acid is less permeable than the L-enantiomer (Oldendorf, 1973), free D-serine in mouse brain may not be derived from the diet. Nor does it seem to have originated from the brain tissue proteins as suggested in the present study, in spite that racemization of L-aspartate and L-serine in human myelin basic protein was reported (Shapira and Jen Chou, 1987). The life span of mice may be too short for the detection of amino acid racemization. The tissue proteins from kidney and liver in a mutant mouse lacking D-amino acid oxidase (Nagata et al., manuscript in preparation) as well as the serum proteins from patients with renal diseases (Nagata, 1992) were also investigated since the presence of considerable amounts of free D-serine, D-alanine and D-proline was found in those tissues (Nagata et al., 1992a;

1992c). However, no D-amino acid was detected in those proteins either. The present results suggest that the source of free D-serine, D-alanine and D-proline in mice may not be the tissue proteins.

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