β -Aspartylurea and 4-Methylproline in Urine of Rats Fed Ozonated Casein

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It has been proved in our laboratory that rats fed ozonated casein that did not contain aromatic amino acid residues besides a very small amount of phenylalanine residues had no significant unfavorable change compared with those fed casein and that ozonated casein supplemented with amino acids lost by ozonolysis has nearly the same biological value as that of casein. But some unusual ninhydrin-positive substances existed in urine of rats fed ozonated casein. Two of them, β -aspartylurea (major component) and 4-methyl-proline (minor), were identified. It was confirmed that β -aspartylurea was formed during ozonolysis of casein in aq. solution that contained urea to solubilize casein and, therefore, present originally in ozonated casein ingested by rats. The origin of 4-methylproline is still obscure.

Aromatic amino acid residues of proteins are decomposed by ozonolysis.^{1–4)} Ozonated protein containing a very small amount of aromatic amino acid residues may be useful for therapy of patients with inborn errors of aromatic amino acid metabolism such as phenylketonurea. We have prepared ozonated casein in which about 85% of the phenylalanine and all of the other aromatic amino acid residues in original casein were decomposed, and proved by feeding experiments with rats that the ozonated casein supplemented with amino acids lost by ozonolysis has nearly the same biological value as that of casein, although the true digestibility was significantly lower.^{5–7)} Histopathological examination of some organs of rats fed ozonated casein did not show any significant deterioration compared with those fed casein. The levels of several plasma enzymatic activities were also normal, but some unusual ninhydrin-positive substances were detected on an amino acid chromatogram of urine of rats fed ozonated casein (UN-1 and -2 in Fig. 1). Those peaks with higher intensity at OD_{440nm} than at OD_{570nm} were not detected in urine of rats fed casein. This paper describes the identification of two unusual components, β -aspartylurea

(UN-1) and 4-methylproline (UN-2), in urine of rats fed ozonated casein and their origin.

Experimental

General methods. For paper chromatography (PC) we used *n*-BuOH–AcOH–H₂O (4:1:2). High voltage paper electrophoresis (HVE) was done at pH 3.6 (pyridine–AcOH–H₂O, 1:20:200, 50 v/cm). The amino acid analysis was done with a model 835 Hitachi high speed amino acid analyzer, using Hitachi #2619 custom ion-exchange resin (2.6 mm i.d. $\times$ 250 mm). An MCI® 835-PF-Kit was used under the conditions for physiological fluid analysis. NMR spectra were taken on a Bruker AM500 (in D₂O). FDMS spectra were measured with a JEOL-JMS OISG-2.

Animals and diets. Ozonated casein was prepared by the method described in detail previously.^{5,6)} Sixty g of casein (AlacidTM, New Zealand) was dissolved in 8 M urea solution (2 l). About 0.3% O₃ in an O₂ stream that was generated by passing extra dry O₂ through a Nihon Ozone Model 0-3-2 laboratory ozonator was bubbled into the solution at a rate of about 100 l/hr for 20 hr. Hydrogen peroxide solution (30%, 60 ml) was added to the ozonated solution to assure the oxidative decomposition of the ozonide and the mixture was dialyzed against running tap-water (Viskase Corp, UC 36-32-100 seamless cellulose tubing). Solid ammonium sulfate was added to the dialyzed solution. The precipitate formed by 0.8 saturation of ammonium sulfate was collected by centrifugation, dissolved in water and dialyzed against running tap-water.

Lyophilization of the dialyzed solution gave ozonated casein (yield, 42%). About 85% of phenylalanine and all of other aromatic amino acids were oxidized, principally to aspartic acid, under these conditions. Methionine and cystine were also oxidized mainly to methionine sulfone and cysteic acid, respectively. The amino acid composition of the ozonated casein is shown in Table I (the 4th column of the table shows the content of each amino acid in 80 g of ozonated casein). Since a very large amount of urea was used to solubilize the casein, residual urea in the final product was measured by the biacetylmonooxime method.⁸⁾ The content of urea in the final product was 1.6 g/100 g ozonated casein.

Male weanling rats of the Sprague Dawley strain (about 50 g, Japan SLC Comp. Ltd.) were housed in individually suspended cages at $23 \pm 1^\circ\text{C}$ in a temperature-controlled room operated on a 12 hr cycle of light (8.00 AM–8.00 PM) and dark. The animals were fed a 25% casein diet for 4 days before experimental feeding and were then divided into 8% casein diet (8C-AA) and 8% ozonated casein diet (8OC-AA) feeding group (6 rats each). The animals were assigned so that each group had nearly the same mean initial body weight (about 68 g). The amino acid composition of 8C-AA and 8OC-AA diets is shown

Table I. AMINO ACID COMPOSITION OF THE EXPERIMENTAL DIETS (g/kg diet)

	8% Casein supplemented with amino acids (8C-AA)		8% Ozonated casein supplemented with amino acids (8OC-AA)	
	Content	Supplement	Content	Supplement
Asp	4.76	3.21	7.97	
Thr	2.94		2.33	0.61
Ser	3.77		3.28	0.49
Glu	15.05		14.26	0.79
Pro	6.66		5.10	1.56
Gly	1.26		1.18	0.08
Ala	2.01		1.96	0.05
Val	4.44		3.87	0.57
Cys	0.56		0	0.56
Met	2.45	0.66	0	3.11
Ile	3.50		2.72	0.78
Leu	6.30		5.29	1.01
Tyr	3.81		0	3.81
Phe	3.57		0.47	3.10
Trp	1.08		0	1.08
Lys	5.33		3.93	1.40
His	1.87		0	1.87
Arg	2.74		1.92	0.82

Methionine which is the first limiting amino acid of casein was added.

Each amino acid content in two diets was made equal by addition of free amino acid.

in Table I. Each rat was housed in a metabolic cage to collect urine and feces for 3 days each during days 5 to 7 and 12 to 14. Urine was collected in 10 ml of $2\text{N H}_2\text{SO}_4$.

Identification of β -aspartylurea (UN-1). Urine of rats fed 8OC-AA diet contained two unusual ninhydrin-positive compounds which were eluted just before serine (UN-1) and immediately after alanine (UN-2), respectively, on the amino acid chromatogram (Fig. 1). Both UN-1 and -2 showed higher absorption at $\text{OD}_{440\text{nm}}$ than at $\text{OD}_{570\text{nm}}$ and were neutral compounds, because they were adsorbed on a column of Dowex 50 (H^+), but not on Dowex 50 (NH_4^+) and Dowex 1 (AcO^-) and did not migrate on HVE. UN-1 was decomposed easily by acid (1 N HCl, 100°C , 1 hr) or nitrite (40% NaNO_2 , 60°C , 2 hr) treatment, but UN-2 was stable under these conditions. A part of the pooled urine of the 8OC-AA group was put on a column of Dowex 50 (H^+ , 100 ml), which was thoroughly washed with H_2O (1 l), and the compounds absorbed were eluted with $2\text{N NH}_4\text{OH}$. Fractions of 80 ml each were collected. Fractions 2 and 3 were combined, concentrated *in vacuo* and put on a column of Dowex 1×4 (AcO^- , 10 ml), which was thoroughly washed with H_2O . The effluent and H_2O wash containing basic and neutral amino acid fractions were concentrated *in vacuo* and put on a column of Dowex $50\text{W} \times 4$ (NH_4^+ , 10 ml). The effluent and H_2O wash from the column, which contained the neutral amino acid fraction, was used for preparative PC. Neutral amino acid fraction of urine of 8OC-AA group contained a large amount of methionine sulfone.⁵⁾ UN-1 moved very slowly and was found in a band below methionine sulfone on PC. The UN-1-containing band was eluted with H_2O and the eluate was treated with a small amount of active carbon. The sparingly soluble UN-1 was crystallized out from the concentrated aqueous solution. UN-1 gave only aspartic acid and ammonia after acid hydrolysis, indicating that UN-1 was composed of aspartic acid, and a ninhydrin-negative substance that gave ammonia by acid hydrolysis (1 N HCl, 110°C , 1 hr, in a sealed tube). NMR spectrum of UN-1 had nearly the same splitting pattern corresponding to the $-\text{CH}_2-\text{CH}-$ system as that of asparagine. NMR of UN-1 δ_{DSS} (D_2O): 3.03 (dd, $J=7.3$, 17.9 Hz, 1H), 3.11 (dd, $J=4.2$, 17.8 Hz, 1H), 4.06 (dd, $J=4.3$, 7.3 Hz, 1H). NMR of asparagine δ_{DSS} (D_2O)⁹⁾: 2.87 (dd, $J=7.1$, 17.0 Hz), 2.92 (dd, $J=5.0$, 17.0 Hz), 4.00 (dd, $J=5.0$, 7.1 Hz). It was shown that UN-1 was a derivative of asparagine by these results and the color reaction with ninhydrin on amino acid chromatogram ($\text{OD}_{440\text{nm}} > \text{OD}_{570\text{nm}}$). FDMS spectrum indicated that UN-1 was β -aspartylurea. FDMS of UN-1 m/z (rel. int): 198 ($\text{M}^+ + \text{Na}$, 18.5%), 176 ($\text{M}^+ + 1$, 100), 131 (176 – COOH , 46.4), 131 ($\text{M}^+ - \text{COOH}$, 53.2), 87 (CONHCONH_2 , 11.2). The amount of β -aspartylurea excreted in urine was estimated by assuming that β -aspartylurea gives the same color constant as that of asparagine at 440 nm on the amino acid chromatogram.

Ozonated casein was hydrolyzed enzymatically to see whether the β -aspartylurea residue was present in the

Fig. 1. Part of the Amino Acid Chromatogram of Urine of Rats Fed 8% Ozonated Casein Supplemented with Amino Acids (8OC-AA) Diet.

See the text for the conditions of the amino acid analysis. UN-1, β -aspartylurea; UN-2, 4-methylproline.

ozonated casein, for the β -aspartylurea residue was easily hydrolyzed to an aspartyl residue by acid hydrolysis as described above. Two ml of 2% ozonated casein solution in 0.1 M borate buffer containing 5 mM CaCl_2 (pH 7.5) was incubated with Pronase (Calbiochem) solution in the same buffer (0.3 mg/2 ml) for various times at 40°C. The peak with the same elution time and color ($\text{OD}_{440\text{nm}} > \text{OD}_{570\text{nm}}$) as those of β -aspartylurea was recognized on the amino acid chromatogram of the reaction mixture incubated for 30 min. The behavior of the product on PC and HVE was also consistent with that of β -aspartylurea. It was confirmed, therefore, that β -aspartylurea was originally present in ozonated casein taken by rats. The formation of β -aspartylurea during ozonolysis of aromatic amino acids was proved by ozone oxidation of 3% tyrosine solution in 8 M urea (100 ml). Tyrosine was chosen as a model compound because it was the most abundant aromatic amino acid in casein (Table I, the 2nd column) and decomposed rapidly by ozonolysis. One ml of the ozonated solution was treated with a column of Dowex 1

(AcO^- , 1 ml) to adsorb a large amount of aspartic acid formed during ozonolysis. The effluent and H_2O wash (10 ml) from the column was placed on a column of Dowex 50 (H^+ , 1 ml), which was thoroughly washed with H_2O to remove urea. The eluate from the column with 2 N NH_4OH (5 ml) gave the peak of β -aspartylurea on the amino acid chromatogram. Neither of ozonolysis of aqueous solution of tyrosine nor aspartic acid solution in 8 M urea gave a peak corresponding to β -aspartylurea.

Identification of 4-methylproline (UN-2). UN-2 was not affected by HCl or NaNO_2 treatment as described above. Part of the pooled urine of rats fed 8OC-AA diet was put on a column of Dowex 50 (H^+ , 100 ml), which was washed with H_2O and eluted with 2 N NH_4OH . The eluate gave two isatin-positive spots on PC. One was proline and the other had a higher R_f value than proline, which was separated from proline by preparative PC. The isatin-positive band with a higher R_f value than proline was eluted with H_2O . The eluate gave two peaks of UN-2 and

pipecolic acid on the amino acid chromatogram. The NMR spectrum of the mixture gave a strong doublet of a secondary methyl group at δ 1.08 and some other small signals besides peaks corresponding to pipecolic acid. The base peak on FDMS spectrum (m/z , 130) of the mixture corresponded to pipecolic acid + H, but the spectrum showed a de-methyl fragment (m/z 114) that could not arise from pipecolic acid. The presence of methyl group on NMR spectrum and de-methyl fragment from m/z 129 on FDMS spectrum suggested that the mol. wt. of UN-2 was also 129. The stability to nitrite treatment, color reaction with ninhydrin ($OD_{440\text{nm}} > OD_{570\text{nm}}$), positive reaction with isatin and the spectral results indicated that UN-2 was one of the monomethylprolines. NMR of the mixture of UN-2 and pipecolic acid δ_{DSS} (D_2O): besides the signals assigned to pipecolic acid [1.63 (m), 1.86 (m), 2.20 (m), 3.02 (m), 3.40 (m), 3.56 (dd, $J=3.3, 11.5$ Hz)], 1.08 (d, $J=6.7$ Hz), 4.13 (t, $J=8.8$ Hz), 4.20 (dd, $J=4.5, 9.4$ Hz) and other complex signals overlapped with those of pipecolic acid. FDMS of the mixture of UN-2 and pipecolic acid m/z (rel. int.): 130 ($M^+ + 1$, 100%), 129 (M^+ , 21.9), 114 ($M^+ - CH_3$, 10.7), 85 (130-COOH, 20.2), 84 ($M^+ - COOH$, 25.6). As the presence of a secondary methyl group indicated that UN-2 was one of 3-, 4-, or 5-methylproline, all of them were prepared for comparison with UN-2. 4-Methylproline (racemate) was prepared from methacrolein and diethylacetamidomalonate by the method of Dalby *et al.*¹⁰⁾ The NMR spectrum showed that synthesized 4-methylproline was a mixture of *cis* and *trans* isomers.¹¹⁾ NMR of synthesized 4-methylproline δ_{DSS} (D_2O): 1.08 (d, $J=6.7$ Hz, 6H), 4.13 (t, $J=8.8$ Hz, 1H, *cis* $\alpha\text{-CH}$), 4.20 (dd, $J=4.5, 9.4$ Hz, 1H, *trans* $\alpha\text{-CH}$), besides complex methylene protons of *cis* and *trans* isomers at 1.62 (1H), 1.95 (1H), 2.26 (1H), 2.39 (2H), 2.54 (1H), 2.89 (2H), 3.47 (1H) and 3.56 (1H). 3- And 5-methylproline (both racemate) were prepared from crotonaldehyde or methylvinylketone, respectively, instead of methacrolein. Chromatographic behavior and NMR spectrum of UN-2 agreed with those of 4-methylproline. Since NMR spectrum of UN-2 showed α -protons of both *cis* and *trans* isomers at δ 4.13 and 4.20, respectively, it was proved that UN-2 was a mixture of diastereoisomers. 4-Methylproline has not yet been detected in hydrolyzate of ozonated casein by hydrochloric acid or Pronase at this stage.

Results and Discussion

Two unusual ninhydrin-positive compounds, β -aspartylurea and 4-methylproline, were identified in urine of rats fed ozonated casein. They were not detected in urine of rats fed casein. It was proved that β -aspartylurea residues were formed from aromatic amino acid residues of casein during ozonolysis of casein solution containing high concentration

of urea to solubilize the protein and, therefore, existed in ozonated casein itself ingested by rats. It is considered that nearly all or a part of β -aspartylurea ingested is excreted into urine without degradation in the body, although the degree of hydrolysis of β -aspartylurea in rat body, if any, is unknown at this stage. The amount of β -aspartylurea excreted in urine was about 50 μmol (8.8 mg)/day/rat, which corresponded to about 1.0% of ozonated casein and 7.4% of aspartic acid in the diet ingested. A study of the mechanism of β -aspartylurea formation during ozonolysis of aqueous solutions of protein containing urea is in progress. Although the biological value of ozonated casein is nearly the same as that of the original casein, its true digestibility is significantly lower than casein.⁵⁻⁷⁾ Since it was demonstrated in this experiment that β -aspartylurea was not digested in the rat body and excreted in urine in relatively high concentration, the existence of β -aspartylurea residues in ozonated casein may be responsible for its low digestibility to some extent. Hydrolysis of β -aspartylurea residues of ozonated casein by a mild deamidation reaction¹²⁾ and feeding experiments of rats with the preparation will be presented in a subsequent paper.

4-Methylproline, which was a minor component and a racemate, has not yet been detected in the hydrolyzate of ozonated casein. The origin of racemic 4-methylproline found in urine of rat fed ozonated casein is, therefore, still obscure. The amount of 4-methylproline excreted in urine was about 2.5 μmol (0.3 mg)/day/rat.

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