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Note

Autoxidative degradation of Amadori compounds in the presence of copper ion

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Amadori compounds are well known as important intermediates in the browning reaction of amino acids and reducing sugars during food processing[1,2]. The same reaction of reducing sugars and proteins under physiological conditions produces glycated proteins that may contribute to the development of pathologies associated with diabetes and aging in vivo[3–5]. Glycation gives Amadori derivatives, and thus may cause the denaturation, polymerization, cross linking, and insolubilization of tissue proteins in vivo. Oxidative changes of glycated proteins leading to the formation of cross-linked products[6–8], the degradation of their sugar moieties[9–11], and oxygen radical production by their autoxidation[12–13] have been observed in vitro.

We previously reported that ascorbic acid causes the fragmentation of proteins and the oxidative degradation of their histidine residues in the presence of copper ion[14–16]. This oxidative damage was due to oxygen radicals formed by a one electron reduction through the ascorbic acid–copper ion–oxygen complex. More recently, we showed that the browning solution prepared from amino acids and glucose caused the oxidative fragmentation of bovine serum albumin in the presence of copper ion[17], and found that the oxidative damage was dependent on the Amadori product. This fragmentation also seemed to be caused by the action of oxygen radicals, presumably generated in the autoxidation of Amadori compounds.

In the present work, we have investigated in detail the oxidative degradation of Amadori compounds through complexes with copper ion. These degradations may also proceed autoxidatively, much as with ascorbic acid and a metal ion. To clarify the oxidative processes four different Amadori compounds, namely fructose-β-alanine* (FA), fructosephenylalanine (FP), N^2-Boc-N^6-fructoselysine (FL), and fructose-*p*-toluidine (FT), were used. The first experiment was to determine whether the decomposition of Amadori compounds is catalyzed by cupric ion at 40°. As shown in Fig. 1, FA was not degraded at all in aqueous solution in the absence of cupric ion, but some degradation was observed in phosphate buffer at pH 7.2. However, the addition of cupric ion to FA

* A more systematic name for FA would be *N*-(1-deoxy-D-fructos-1-yl)-β-alanine. Similar names could be constructed for FP, FL, and FT.

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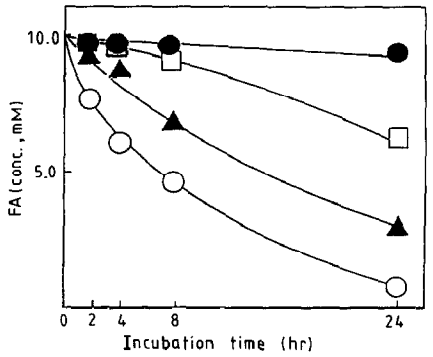

Fig. 1. Effect of cupric ion on the oxidative degradation of fructose-β-alanine: ○, 500μM cupric ion, phosphate buffer; ▲, 50μM cupric ion, phospate buffer; □, phosphate buffer, no Cu^{2+} added; ●, distilled water, no Cu^{2+} added.

in phosphate buffer accelerated the degradation of FA proportionally to the concentration of cupric ion. The rate was such that FA was completely decomposed in the presence of 500μM cupric ion during 24 h. The degradation of Amadori compounds in phosphate buffer without cupric ion has been observed previously for N^2-formyl-N^6-fructoselysine by M.U.Ahmed *et al*[10,11]. A trace of metal ion in the phosphate buffer was apprently responsible. The reaction mixture containing FA and cupric ion was analyzed by h.p.l.c. using an anion-exchange column. Figure 2 shows that the FA (peak 3) was degraded to products **1** and **2**, which were identified as D-*arabino*-hexos-2-ulose and

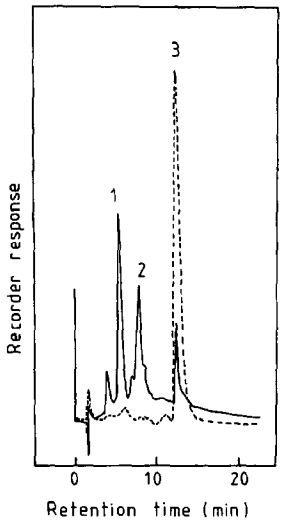

Fig. 2. Oxidative degradation of fructose-β-alanine (FA) by the action of 100μM cupric ion in phosphate buffer. H.p.l.c. analysis (see Experimental) of the reaction mixture at zero time (-----) and after incubation for 24 h (——). Peak 1, D-*arabino*-hexosulose; peak 2, β-alanine; peak 3, FA.

β-alanine, respectively, by comparison of their retention times with those of authentic samples. Furthermore, the identification of compound **1** was carried out by the following two methods. First, the collected peak 1 was concentrated, reduced, and acetylated by usual procedures to give alditol acetates. The mixture of acetates when submitted to g.l.c. gave two equivalent major peaks of glucitol and mannitol, and minor peaks of erythritol and arabinitol (data not shown). Although glucitol and mannitol could be derived from either fructose or D-*arabino*-hexosulose, the dragradation product of the Amadori compound would not be fructose but D-*arabino*-hexosulose. If 3-deoxy-D-*erythro*-hexosulose (3-deoxy-D-glucosone) were formed by this oxidation, 3-deoxyglucitol resulting from its reduction would be detected on g.l.c., with a retention time between those of arabinitol and mannitol, but no such peak was observed.

The second method depended on the identification of the quinoxaline derivative of D-*arabino*-hexosulose, which was prepared from the reaction mixture mentioned in the Experimental section. The quinoxaline derivative gave only one peak on h.p.l.c., and this peak was collected and acetylated. The acetylated quinoxaline was repeatedly passed through the same h.p.l.c. to give a pure material. Its ^{1}H-n.m.r. spectrum contained four acetyl signals, at δ 1.96, 2.07, 2.09, and 2.14. The molecular weight by f.a.b.-m.s. was 418 [$(M + 1)^+$ at m/z 419]. These data were as expected for a 2-(1,2,3,4-tetraacetoxybutyl)-quinoxaline, and thus supported the conclusion that peak 1 in Fig. 2 was D-*arabino*-hexosulose, not 3-deoxy-D-*erythro*-hexosulose.

The time course of the degradation of FA and the formation of **1** and **2** is shown in Table I. After incubation of FA with cupric ion for 24 h at 40°, the concentrations of β-alanine and D-*arabino*-hexosulose in the reaction mixture reached 7.8 and 8.8mM, respectively, showing that the conversion of FA into these products was almost quantitative. The other three Amadori compounds showed a behavior similar to that of FA (Table I).

TABLE I

Oxidative degradation of Amadori compounds and formation of amino acids and aldosulose

Incubation time[a] *(h)*	*Fructose-β-alanine*			*Fructose-*p*-toluidine*			*Fructose-phenylalanine*			N^2-*Boc*-N^6-*fructoselysine*		
	FA	*β-Ala*	*Aldos*[b]	*FT*	p-*T*	*Aldos*[b]	*FP*	*Phe*	*Aldos*[b]	*FL*	*Boc-Lys*	*Aldos*
	(conc.,mM)			*(conc.,mM)*			*(conc.,mM)*			*(conc.,mM)*		
0	10.0	0	0	2.0	0	0	2.0	0	0	10.0	0	0
2	7.7	0.9	0.8	1.5	0.5	0.5	1.0	0.9	1.0	6.7	3.8	3.0
4	6.0	1.9	2.1	1.3	0.7	0.9	0.2	1.7	1.8	3.6	5.1	4.1
8	4.6	4.0	4.4	1.0	1.1	1.2	0	1.7	1.9	1.8	6.4	5.3
24	0.6	7.8	8.8	0.3	1.6	1.6	0	1.7	1.9	0	7.0	6.9

[a] Conditions: Fructose-β-alanine (FA), N^2-Boc-N^6-fructoselysine (FL) (each 10mM, with $CuSO_4$ 100μM), fructose-*p*-toluidine (FT), and fructose-phenylalanine (FP) (each 2mM, with $CuSO_4$ 50μM) were incubated in phosphate buffer, pH 7.2, for 24 h at 40°. [b] Aldosulose (D-*arabino*-hexos-2-ulose).

The oxidation reactions were markedly inhibited by the addition of chelating agents such as EDTA and diethylenetriaminepentaacetic aid (DTPA). Radical scavengers and enzymes related to the elimination of the oxygen radical, however, did not suppress these degradations. Thus, the observed processes may involve a redox reaction of the Amadori compounds (AH_2) with cupric ion, and autoxidative regeneration of the latter as follows:

$$AH_2 + Cu(II) \rightarrow AH^{\cdot} + Cu(I) + H^+$$
$$Cu(I) + O_2 \rightarrow Cu(II) + O_2^{\cdot}$$
$$AH^{\cdot} + Cu(II) \rightarrow A + Cu(I) + H^+$$
$$A + H_2O \rightarrow \text{amino acid} + \text{D-}\textit{arabino}\text{-hexosulose}$$

The cleavage of the compounds probably proceeds through complexation of their enol forms with cupric ion. In the final step the oxidation product (A) is hydrolyzed to amino acids and aldosulose, which undergoes some degradation to lower aldose derivatives. Oxygen radicals formed secondarily in these processes may attack adjacent protein molecules to produce site-specific oxidative damage.

EXPERIMENTAL

Preparation of Amadori compounds and D-arabino-*hexosulose.* — Fructose-β-alanine (FA), fructose-*p*-toluidine (FT), fructosephenylalanine (FP) and N^2-Boc-N^6-fructoselysine (FL) were prepared by reaction of glucose with β-alanine[18], *p*-toluidine[19], phenylalanine[20], and N^2-Boc-lysine[7], respectively. D-*arabino*-Hexos-2-ulose (D-glucosone) was prepared from glucose phenylosazone[21].

Reaction conditions. — All reactions were carried out in 10mL of 0.067M phosphate buffer, pH 7.2, containng one of the Amadori compounds (10 mM FA or FL, 2mM FT or FP) and cupric sulfate (0–500μM) for 24 h at 40°.

Analytical methods. — FA and its decomposition products were determined on a Toyo Soda High Speed Liquid Chromatograph HLC-803D, using a Develosil NH_2-5 column (4.6 × 150 mm) eluted with 4:1 v/v acetonitrile–water, flow rate 2 mL/min, with u.v. monitoring at 210 nm. Other Amadori compounds (FT, FP, and FL) and their degradation products were analyzed using a Develosil ODS-5 (4.6 × 250 mm) column with 0.05M ammonium acetate (AA)–methanol (M) as eluent. The conditions were FT, 2:1 AA–M, flow rate 0.8 mL/min, detection with u.v. at 240 nm; FP, 19:1 AA–M, flow rate 0.8 mL/min, detection with u.v. at 254 nm; FL, 19:1 AA–M, flow rate 1.0 mL/min, detection with u.v. at 210 nm. Aldosulose was also determined colorimetrically as the 2,4-dinitrophenylosazone[20], which corresponded to the osazone of authentic D-*arabino*-hexosulose. Conditions for g.l.c. of the alditol acetates were: apparatus, Shimadzu Gaschromatograph GC-9A; column, 5% Silicone GE-XE-60 on Chromosorb W(AW) (0.3 × 200 cm); N_2 flow rate, 30 mL/min; temperature (column), 140–210° at 2°/min; (detector, FID), 230°. ^{1}H-n.m.r. spectra were taken with a Jeol JNM-FX200 spectrometer, on solutions in $CDCl_3$, with Me_4Si as internal standard. F.a.b. mass spectroscopy was performed on a Jeol JMS-DX303 mass spectrometer.

Preparation of the quinoxaline derivative. — A solution (5 mL) of Amadori

compound (15mM) and cupric sulfate (100μM) in phosphate buffer, pH 7.4, was added to 15 mL of 50mM *o*-phenylenediamine in phosphate buffer, and the mixture was incubated at 40° for 90 min. The residue remaining after concentration of the reaction mixture was dissolved in Me_2SO and submitted to h.p.l.c. (column, Develosil ODS-5, 8 × 250mm; solvent, 8:2:1 0.1% TFA–MeOH–CH_3CN; flow rate, 1.5 mL/min; detector, u.v. at 320 nm). A single peak (quinoxaline derivative) was obtained, in addition to the reagent peak. The quinoxaline-containing fractions were collected and freeze dried, and the residue was acetylated with acetic anhydride in anhydrous pyridine to give a product showing a single peak on h.p.l.c. (ODS-5 column, 2:1:1 0.1% TFA–MeOH–CH_3CN, 2.0 mL/min).

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