SITE-SPECIFIC CLEAVAGE OF GLYCATED HUMAN SERUM ALBUMIN IN THE PRESENCE OF IRON

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Nonenzymatically glycated human serum albumin was incubated with ferric ion at 37°C in 0.2 M phosphate buffer, pH 7.4, up to 30 days. In the incubation mixture, amino acids, tyrosine and phenylalanine, were detected, suggesting site specific cleavage of glycated human serum albumin.

KEYWORDS nonenzymatic glycation; human serum albumin; tyrosine; phenylalanine; iron

Nonenzymatic glycation is a posttranslational modification of protein in vivo.[1] Ketoamine adduct, produced by rearrangement of Schiff's base between amino groups of lysine residue in proteins and glucose, has been reported to be decomposed gradually to carboxymethyllysine (CML) residue by oxidative process.[2] Since Dunn et al. identified CML in acid hydrolysates of lens proteins[3] or skin collagen[4] in vivo, much attention has been focused on the glycation and its relevance to oxidative stress in diabetes mellitus and aging. We also detected CML in the hydrolysates of glycated human serum albumin(G-HSA) in an in vitro study,[5] in which G-HSA was further incubated with or without iron for up to 30 days at 37°C. The ketoamine structure in G-HSA with iron was preferentially decomposed, compared with the sample without iron.

In this communication, we would like to discuss the identification of amino acids, tyrosine(Tyr) and phenylalanine(Phe), in the incubation medium of G-HSA with iron during 30 days. G-HSA was obtained by incubation of HSA with 1 M of glucose at 37°C for 7 days followed by dialysis and lyophilization as described previously.[5] G-HSA was further incubated with 100 μM Fe^{3+} as $Fe(NO_3)_3$-adenosine diphosphate(ADP)(1:16.6 M/M) complex in 0.2 M sodium phosphate buffer (pH 7.4) at 37°C for 15 and 30 days, and these were referred to as G-HSA-15(Fe) and G-HSA-30(Fe), respectively. As controls, HSA and G-HSA were incubated for 15 and 30 days without Fe^{3+}-ADP and referred to as HSA-15, HSA-30, G-HSA-15, and G-HSA-30, respectively. The reaction mixtures were applied on Sephadex G-25. Figure 1 shows the typical elution profiles of the incubation mixtures of G-HSA with or without Fe^{3+}-ADP, estimated at 280 nm on Sephadex G-25. The protein fractions eluted at void volume had been analyzed to assign CML residues in G-HSA.[5] An absorbance at 280 nm appeared at the fractions of low molecular weight substances (fraction number 75-100). Since these fractions were considered to contain sodium phosphate, Fe^{3+}-ADP, and erythronic acid (cleavage product of ketoamine adduct),[3] we looked upon this absorbance as due to ADP. These fractions were lyophilized and dissolved in water, and UV and fluorescence spectra were measured. As was expected, G-HSA-30(Fe) showed UV spectrum with maximum at 260 nm, which corresponds to ADP. G-HSA-30, however, showed UV spectrum with maximum at 275 nm. Both G-HSA-30(Fe)

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and G-HSA-30 showed fluorescence spectra of emission maxima at 305 nm exciting at 280 nm, though no fluorescence was observed during excitation at 305 nm. Lyophilized powder was dissolved in 0.02N HCl (1 mg/ml), and 10 μl were subjected to HPLC for amino acid analysis (Fig. 2). Two predominant peaks appeared at the time of 51.8 min and 55.3 min, corresponding to Tyr and Phe, respectively. The other amino acids' peaks were negligible. Cochromatography showed complete coincidence of two peaks with authentic Tyr and Phe, respectively. Therefore, we estimated the amounts of Tyr and Phe in the lyophilized powder (Table I). HSA incubated up to 30 days(HSA-30) in 0.2 M phosphate buffer essentially indicated absence of them; G-HSA-30, G-HSA-15(Fe) and G-HSA-30(Fe) showed significant amounts. The amounts of two amino acids in the sample with Fe^{3+}-ADP were about three times of those without Fe^{3+}-ADP. These results suggest site-specific cleavage at Tyr and Phe residues.

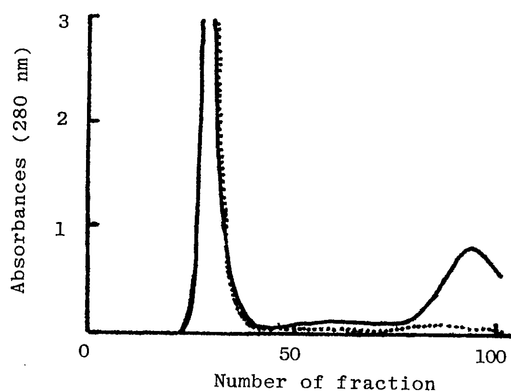

Fig.1 Elution Profiles on Sephadex G-25

4% of G-HSA(2 mole of glucose bound to 1 mole of HSA) was incubated with (——) and without (----) 100 μM Fe^{3+}-ADP[5] at 37°C for 30 days. 5 ml of incubation mixture was applied on the column(2.5x80 cm) equilibrated with 100mM ammonium acetate(pH 7.4). Each fraction is 5 ml.

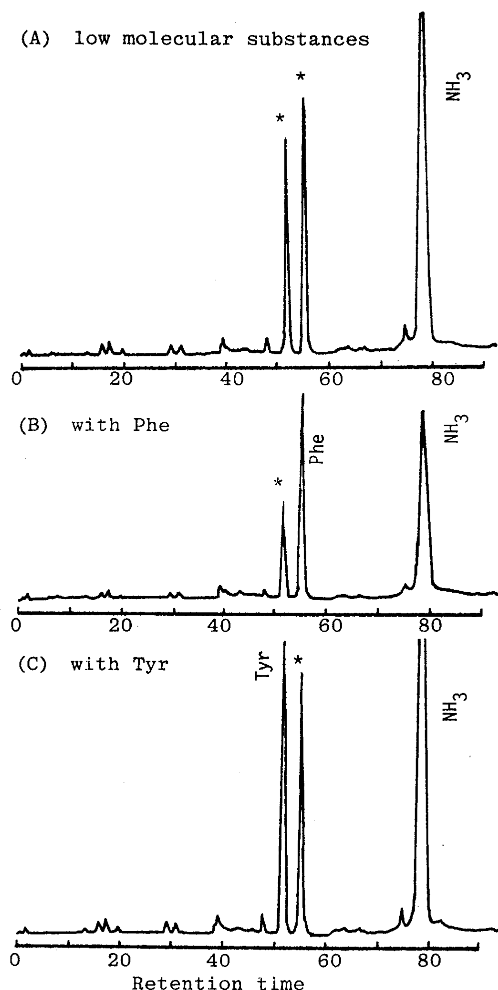

Fig.2 Elution Profiles on Amino Acid Analysis Column

The low-molecular weight substances obtained from Sephadex G-25 column were lyophilized and dissolved in 0.02N HCl (1 mg/ml), and aliquots(10 μl) were analyzed by Hitachi L-8500 Amino Acid Analyzer. (A)Low-molecular weight substances in G-HSA-30(Fe), (B) and (C)cochromatography of low-molecular weight substances in G-HSA-30(Fe) with authentic phenylalanine and tyrosine, respectively.

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Table I. Amounts of Amino Acids in Low-Molecular Weight Substances

	Tyr(n mol)[a]	Phe(n mol)[a]
HSA-15	0.40	0.45
HSA-30	0.40	0.42
G-HSA-15	1.55	1.62
G-HSA-30	6.6	5.20
G-HSA-15(Fe)	18.0	16.0
G-HSA-30(Fe)	15.7	20.3

a)Low-molecular weight substances (10 μg) were analyzed.

Rana and Meares reported that iron-EDTA attached to a protein cleaves peptide chain at sites determined by its proximity, in the presence of ascorbate and hydrogenperoxide.[6] Their plausible explanation is that highly nucleophilic oxygen species, such as peroxide coordinated to the iron-EDTA, attack a carbonyl carbon of peptide chain nearby. The presence of activated iron-oxygen complex by coordination with enediol structure, a tautomer of ketoamine, has been proposed by us in the study of lipid peroxidation caused by glycated polylysine in the presence of iron.[7] Glycated polylysine coordinates to iron even in the presence of phosphate buffer, generating superoxide anion. It is supposed that glycated HSA could be coordinated to iron, forming activated iron. Our proposed activated-iron in the glycated HSA could work essentially in the same way as hydrogenperoxide coordinated to iron-EDTA does. If so, some peptide fragments of high molecular weight could be produced. We have not investigated the presence of fragments yet, but we have observed the decrease in Tyr residue in the hydrolysate of protein obtained at void volume fraction on Sephadex G-25.[5] The adjacent sequence Tyr-Phe-Tyr is located at the residues 148-150 of HSA.[8] Amino acid residues in proximity to the glycated site of HSA, such as lysine 525[9] or lysine 199[10] in the three-dimensional structure, should give critical information.

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