Novel Decomposition of Amadori Compound Catalyzed by Copper Ion

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A novel decomposition of Amadori compound from glucose catalyzed by copper ion is discussed. The Amadori compounds prepared from glycylglycylhistidine and histidyltyrosine were oxidatively decomposed in the presence of copper ion to free peptide, N^α-formyl peptide, and the sugar moieties of glucosone and arabinose. The formation of N^α-formyl peptide was especially dependent on the concentrations of copper ion. Copper(II)-catalyzed autoxidative decomposition of Amadori compound to free peptide and glucosone has been clarified in our previous study, but the formation of N^α-formyl peptide and arabinose was a novel pathway confirmed in this study. The results indicated the complexity of metal ion-catalyzed oxidation of Amadori derivatives in glycated protein, and this novel pathway may be useful for studying the amino-carbonyl reaction related with diabetic complications and aging.

INTRODUCTION

Nonenzymatic glycation is a well-known posttranslational protein modification. This modification has a severe effect on the structure of proteins, changing their functional and biological properties. The sugar concentration and the biological half-life of the protein are important in the level of protein modification; long-lived proteins are eventually glycated more extensively.

Glycation of protein produces an important intermediate, the Amadori compound. By oxidative degradation of the Amadori compound under physiological conditions, N-(carboxymethyl)lysine (CML) or 3-(N-lysino)lactic acid (Ahmed et al., 1986, 1988) was confirmed. On the other hand, advanced glycation end products, pentosidine and pyrraline, were also identified recently (Sell and Monnier, 1989; Grandhee and Monnier, 1991; Dyer et al., 1991; Hayase et al., 1989). Each of these products was found in good proportion with aging, diabetes, and cataracts. The results indicated the potential role of the amino-carbonyl reaction *in vivo* in the pathogenesis of diabetic complications and the aging process.

On the other hand, we have confirmed that glucosone was a main product formed from copper(II)-catalyzed autoxidation of the Amadori compound (Kawakashi et al., 1991), and glucosone was a strong oxygen radical generator to induce the oxidative damages of protein in the presence of copper ion (Cheng et al., 1992). N-Benzoylhistidine was clarified as being degraded to asparate *via* a 2-oxoimidazolone intermediate by reaction with the Amadori compound–copper ion system (Cheng and Kawakishi, 1993) or the glucosone–copper ion system (Cheng et al., 1992). However, the oxidation mechanism of histidine residue in glycated protein is not clearly understood now.

To clarify the oxidation process of histidine residue in glycated protein, glycated His-containing di- or tripeptides were used as a model of glycated protein in the present study. Glycated His-containing di- or tripeptides were decomposed in different pathways mediated by copper ion, but the histidine residue in glycated His-containing di- or tripeptide was hardly oxidized. However, a novel decomposition of the Amadori compound, by formation of N^α-formyl peptide and arabinose, was confirmed. The different decompositions of His-containing Amadori compounds and the negligible oxidation on its histidine residues were considered as being the result of the

coordinate form and the binding intensity of the copper ion to each peptide.

EXPERIMENTAL PROCEDURES

Reagent. L-Histidyl-L-tyrosine (His-Tyr) and glycyl-L-leucyl-L-tyrosine (Gly-Leu-Tyr) were purchased from Sigma Chemical Co. Carnosine (β-Ala-His) and glycylglycyl-L-histidine (Gly-Gly-His) were obtained from Peptide Institute Inc. (Osaka). Glucose and CuSO$_4$·5H$_2$O were purchased from Wako Pure Chemical Industries, Ltd. (Osaka). All other reagents were of the highest grades commercially available.

Preparation and Isolation of Amadori Compounds. The reaction mixtures of peptide (20 mg) and glucose (200 mg) in 4 mL of methanol were refluxed in a water bath (80–90 °C) with stirring for 45 min, and then 0.3 mL of acetic acid was added and refluxed for about another 45 min. The reaction mixture was concentrated, dissolved in 1 mL of H$_2$O, and then isolated with reversed-phase HPLC on a Develosil ODS-5 column (8 × 250 mm). Chromatographic conditions were as follows: The reaction mixture was eluted at a rate of 2.0 mL/min with 95/5 (v/v) ammonium acetate/methanol solution (AA/M) for histidyltyrosine; 1.5 mL/min with 99/1 (v/v) AA/M for glycylglycylhistidine; 1.0 mL/min with 99/1 (v/v) AA/M for carnosine; and 2.5 mL/min with 100/35 (v/v) AA/M for glycylleucyltyrosine. The elutions were monitored at 210 nm. Isolated Amadori compounds were identified by ^{1}H NMR and FAB–mass and stored at –20 °C until used.

Reaction Conditions of Amadori Compound with Copper Ion. The oxidation of each glycated peptide (Amadori compound) mediated by copper ion was carried out at 37 °C. The reaction mixtures contained 5 mM glycated peptide and 50 or 500 μM CuSO$_4$ in 67 mM phosphate buffer (pH 7.2). The reactions were initiated by the addition of copper ion and stopped by the addition of EDTA solution (0.1 mM). The oxidation products from Amadori compounds were isolated by HPLC and identified by ^{1}H NMR and FAB–mass.

Determination of Acetylated Sugar Derivatives Formed in Oxidation System. The reaction mixture (5 mL) containing 5 mM glycated peptide (Amadori compound) and 50 μM CuSO$_4$ for glycated histidyltyrosine or 500 μM CuSO$_4$ for glycated glycylglycylhistidine was incubated at 37 °C for 6 h in 67 mM phosphate buffer (pH 7.2) and then passed through a Dowex 50-X8 (H form, 10 × 60 mm) column to separate the sugar fraction products (unadsorbed parts) from the amino acid derivatives (adsorbed parts) by eluting the column with 50 mL of distilled water. The eluate was lyophilized and dissolved in 3 mL of distilled water and then reduced with NaBH$_4$ (pH 9) for 2 h and neutralized to pH 7 by addition of Amberlite IR-120 resin, followed by filtration and lyophilization. Furthermore, the residue was acetylated with acetic anhydride in anhydrous pyridine (1/1). The acetylated sugar products were analyzed with HPLC on a Develosil ODS-5 column (8 × 250 mm) monitored

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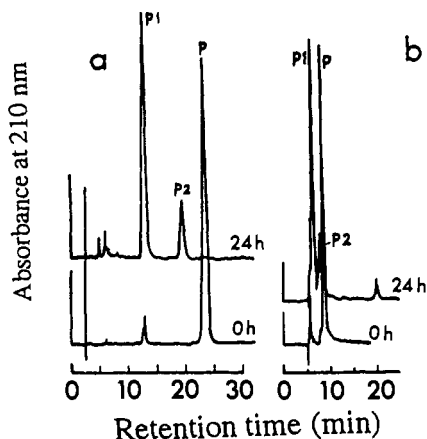*J. Agric. Food Chem.*, Vol. 42, No. 3, 1994 **701**

Figure 1. HPLC profiles of the decomposition of glycated peptides mediated by copper ion. Glycated peptides were prepared as described under Experimental Procedures. The reactions were carried out at 37 °C for 24 h. The mixtures contained 5 mM glycated peptide and 50 μM copper ion for glycated histidyltyrosine (a) or 500 μM copper ion for glycated glycylglycylhistidine (b) in phosphate buffer (67 mM, pH 7.2). The HPLC was performed according to that described under Experimental Procedures. P, P1, and P2 represent glycated peptide and oxidized products 1 and 2, respectively.

at 210 nm and eluted at a rate of 1.5 mL/min with H$_2$O (0.1% TFA, v/v)/acetonitrile (2/1, v/v) solution.

On the other hand, the acetylated sugar products were also submitted to GC–mass analyses on a JEOL JMS-DX 705 mass spectrometer coupled to an HP 7745 gas chromatograph. The GC column was a 15 m × 0.25 mm (i.d.) fused silica capillary with a 0.2-μm DB-1 phase. Sample introduction was made by on-column injection, and the column temperature was programmed from 130 to 200 °C at 5 °C/min. The mass spectrometer was run in scan mode for compound identification, and the mass spectra were recorded in electron impact mode.

Fast Atom Bombardment–Mass Spectrometry (FAB–Mass). FAB–mass was performed using a JEOL JMS-DX 705 mass spectrometer. The sample was dissolved in glycerol, and 1 nmol in 0.5 μL of matrix was deposited on a stainless steel probe tip and placed in the ion source, where it was bombarded with a beam of xenon atoms from a JEOL neutral atom gun (5 keV, 2-A cathode current, 10-mA emission).

Nuclear Magnetic Resonance Spectrometry (NMR). A NMR spectrum using a JEOL JNM-FX 200 spectrometer was taken in D$_2$O with dioxane as the internal standard.

RESULTS AND DISCUSSION

Oxidative Decomposition of Amadori Compounds Catalyzed by Copper Ion. In the previous study on the copper(II)-catalyzed oxidation of glycated protein, the fragmentation of protein, especially the selective degradation of its histidine residue, was confirmed (Cheng et al., 1991). However, in the present model system, glycated His-containing di- or tripeptides were autoxidatively decomposed but the histidine residue, which is contained in the peptide, was not degraded. Among them, the Amadori compounds of glycylleucyltyrosine and β-alanylhistidine were decomposed into free peptide and glucosone (data not shown); this pathway has been proven in our previous study (Kawakishi et al., 1991). However, the Amadori compounds of glycylglycylhistidine and histidyltyrosine were oxidized into free peptide, N$^\alpha$-formyl peptide, glucosone, and arabinose. The decomposition of Amadori compound into N$^\alpha$-formyl peptide and arabinose is a novel pathway being confirmed in the present study.

As shown in Figure 1, glycated histidyltyrosine (a) and glycated glycylglycylhistidine (b) were oxidized into two new main products, which are referred to as P1 and P2,
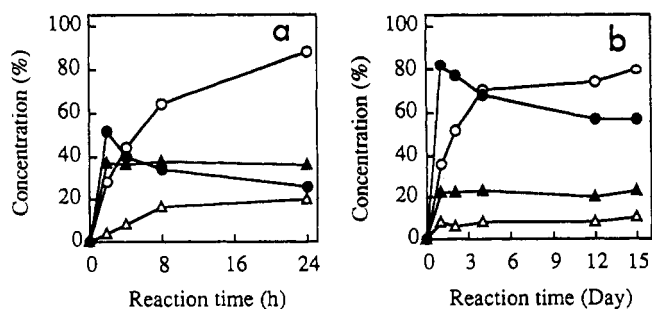

Figure 2. Copper(II) concentration-dependent formation of P2 during the oxidation of glycated histidyltyrosine (a) and glycated glycylglycylhistidine (b). The reactions were carried out at 37 °C. The mixture contained 5 mM glycated peptide and 50 μM (○, P1; △, P2) or 500 μM (●, P1; ▲, P2) copper ion in phosphate buffer (67 mM, pH 7.2). The concentrations (%) of P1 and P2 were calculated as [peak area of P1 or P2]/[peak area of glycated peptide (5 mM, 0 h)].
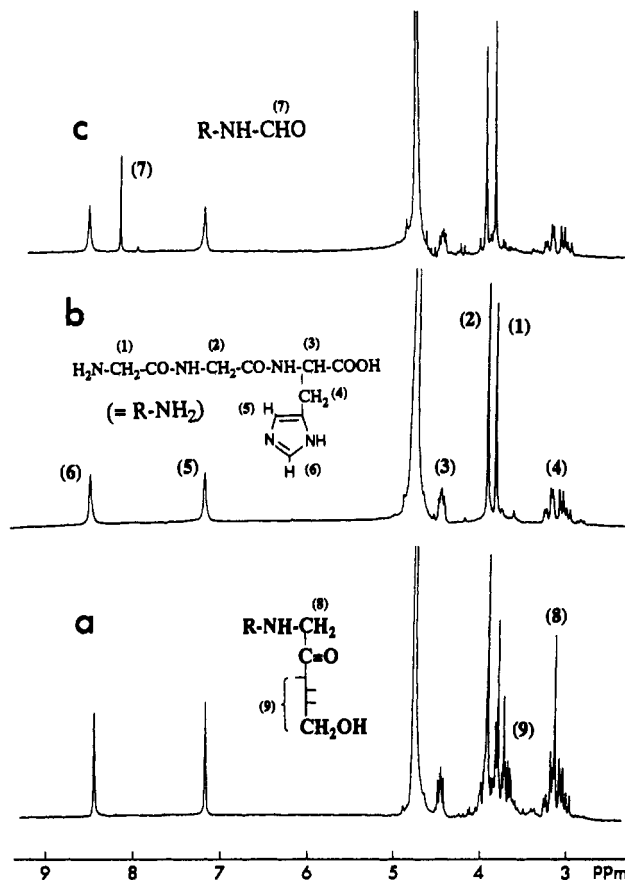

Figure 3. ^{1}H NMR profiles of glycated glycylglycylhistidine (a), P1 (b), and P2 (c).

respectively. Each of them was formed as a function of reaction time.

On the other hand, P2 was formed depending on the concentrations of copper ion. As shown in Figure 2, a significant formation of P2 from glycated histidyltyrosine (a) or glycated glycylglycylhistidine (b) was detected in the presence of 500 μM copper ion compared with that in the 50 μM copper ion system.

These oxidized products (P1, P2) were isolated by reversed-phase HPLC and then analyzed by ^{1}H NMR and FAB–mass methods. P1 was identified as the original peptide decomposed from the glycated peptide by cleaving the C^1–N Amadori bond, and P2 was the N$^\alpha$-formyl peptide formed by cleaving the C^1–C^2 bond from the Amadori compound. Figure 3 shows the ^{1}H NMR results of glycated glycylglycylhistidine (a), P1 (b), and P2 (c).
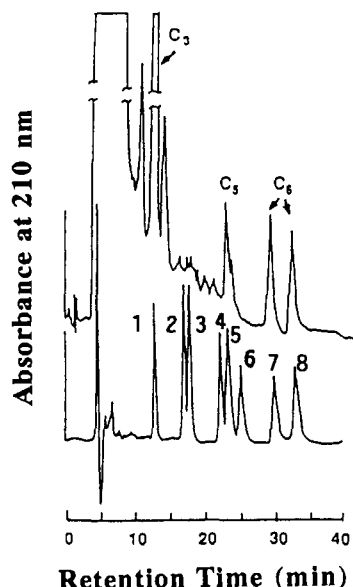
Figure 4. Determination of the sugar moieties formed in glycated glycylglycylhistidine–copper ion system. The preparation and determination of acetylated sugar derivatives were as described under Experimental Procedures. Peaks 1–8 represent the acetylated glycerol, threitol, erythritol, ribitol, arabinitol, xylitol, glucitol, and mannitol, respectively.

The multiple peaks (5H) of the glucose part in glycated glycylglycylhistidine appeared around 3.86 ppm and a signal peak (2H) appeared at 3.20 ppm, which disappeared in the oxidized products (P1, P2). However, a new signal (1H) at 8.22 ppm in P2 was detected. This signal was proposed from the aldehyde proton on RNH–CHO. On the other hand, through the determination of their molecular weight by FAB–mass analyses, the molecular ions (M + 1) of the glycated glycylglycylhistidine, P1, and P2 were 432, 270, and 298, respectively. On the basis of these results, P1 and P2 was identified as the original peptide and the N^{α}-formyl peptide formed from the copper(II)-catalyzed oxidation of the glycated glycylglycylhistidine. On the other hand, the same result was confirmed in the glycated histidyltyrosine–copper ion system.

Determination of the Sugar Moieties Formed in the Reaction System. Accompanying the formation of the original and N^{α}-formyl peptides, the sugar moieties in the reaction system were also investigated. The isolation of sugar moieties, the preparation of acetylated sugar products, and the analytic conditions of HPLC are described in detail under Experimental Procedures. As shown in Figure 4, the upper and lower profiles represent the acetylated sugar moieties formed from the glycated glycylglycylhistidine and the standard acetylated sugar mixtures (from C3 to C6), respectively. Compared with the results of the standard acetylated sugar mixtures, the two peaks eluting at 30 and 33 min were glucitol and mannitol, respectively. They were deduced as the reduced products of glucosone by NaBH₄. The peak eluting at 23 min had the same retention time with acetylated arabinitol; it was proposed to derive from arabinose formed by cleaving the C^1–C^2 bond of the Amadori compound. Moreover, the C3 moiety (glycerol) was also detected as a main product in the mixture, which was suggested as a secondary degradation product from glucosone since no peptide-bound C3′ moiety was detected in the reaction system. A similar result was detected in the glycated histidyltyrosine–copper ion system.

On the other hand, analyses of the acetylated sugar moieties were also performed by GC–mass as described
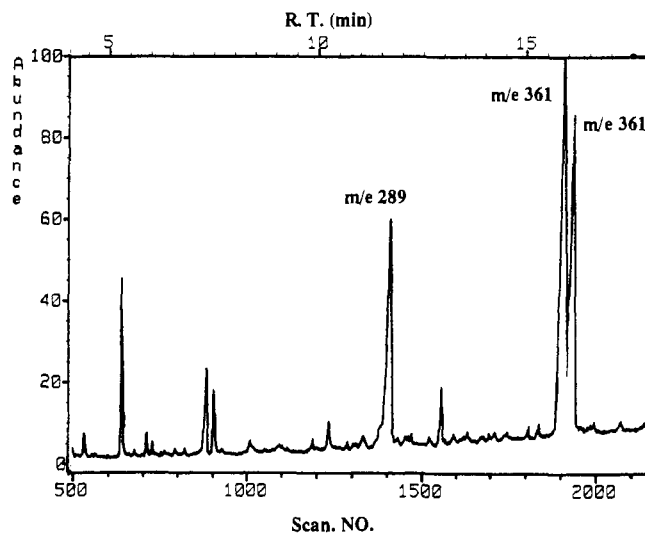

Figure 5. GC–mass of total ion chromatogram of acetylated sugar products formed from oxidation of Amadori compound: m/e 361 (M_{6C}–CH_2OAC); m/e 289 (M_{5C}–CH_2OAC).

under Experimental Procedures. Figure 5 shows the total ion chromatogram of the acetylated sugar products. The molecular weight of each main peak was determined by EI scan mode. Since the base peaks in the spectra of all alditol acetates are formed by cleavage of the alditol chain, as shown below, m/e 361 (M – CH_2OAC) and four other primary fragments are formed from hexitol hexaacetate (A). Similarly, m/e 289 (M – CH_2OAC) and three other

(A)   (B)

primary fragments are formed from pentitol pentaacetate (B). On the other hand, the acetylated C5 and C6 sugar derivatives also have the same retention time with authentic acetylated arabinitol, glucitol, and mannitol by gas chromatography.

Furthermore, in another analysis system using o-phenylenediamine to trap α-dicarbonyl compound which formed from the Amadori compound, the quinoxaline derivative of glucosone was detected as a main product, as reported in our previous paper (Kawakishi et al., 1991). Therefore, the glucitol and mannitol in the present system are reduced products from glucosone by NaBH₄, not from glucose and mannose. On the basis of these facts, it can be said that arabinose and glucosone were the main sugar products generated from copper(II)-mediated oxidation of the Amadori compound.

Metal ion-catalyzed oxidative damage to protein is an important process concerning diseases and aging. As described above, many oxidized products, such as CML and glucosone, were generated from the oxidation of the Amadori compound. However, the present study clarified a novel decomposition pathway of the Amadori compound mediated by copper ion. This result suggested that the oxidation of the Amadori compound in glycated protein may be very complicated. The arabinose formed might take part in the Maillard reaction further and induce a secondary modification of protein.

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