Formation of Hydroxyfuranone and Hydroxypyranone Derivatives with DNA-Breaking Activity in the Maillard Reaction of Glucose and Albumin under Physiological Conditions

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Formation of DNA breaking hydroxyfuranone and hydroxypyranone derivatives in the Maillard reaction of glucose and bovine serum albumin (BSA) under physiological conditions was investigated. A mixture of glucose and BSA was incubated at 37°C in water or in 1 M phosphate buffer (pH 7.4). The ethyl acetate/2-propanol extract of the reaction mixtures showed significant DNA breaking activity against supercoiled DNA especially in the presence of Fe(III) ion. Gas chromatography/mass spectrometry analysis of the mixture revealed the formation of DNA breaking hydroxyfuranones (HMF and DMHF) and hydroxypyranone (DDMP).

Key words glucose; bovine serum albumin (BSA); hydroxyfuranone; hydroxypyranone; Maillard reaction; DNA breaking

We have previously reported the formation of DNA breaking substances in the heated Maillard reaction mixtures of sugars and amino acids^{1,2)} and in soy sauce.³⁾ These substances were identified as 4-hydroxy-2-hydroxymethyl-5-methyl-3(2H)-furanone (HHMF)⁴⁾ and 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP)⁵⁾ formed in the Maillard reaction of glucose and glycine, 4-hydroxy-5-methyl-3(2H)-furanone (HMF),³⁾ 2,5-dimethyl-4-hydroxy-3(2H)-furanone (DMHF)⁶⁾ and 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone (HEMF)⁷⁾ in soy sauce (Fig. 1). All the components are known as fragrant components. They reduced metal ions and dissolved oxygen to generate active oxygen species including hydroxyl radical, which break DNA single strands.^{3–7)} These compounds were mutagenic *in vitro* against *Salmonella typhimurium* TA100 without metabolic activation and induced micronucleated reticulocytes *in vivo* in mouse peripheral blood.^{3,5–7)} In the present investigation, whether or not these DNA breaking compounds are produced in the Maillard reaction of glucose and proteins under physiological conditions was examined. Some of the DNA breaking substances were found to be readily produced in the reaction of glucose and albumin.

MATERIALS AND METHODS

Materials Glucose was obtained from Wako Pure Chemical Industries (Osaka, Japan). Bovine serum albumin (BSA) was obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Supercoiled pBR 322 DNA (1 mg/ml in 10 mM Tris-HCl pH 8 and 1 mM ethylenediaminetetraacetic acid (EDTA)) was a product of New England Biolabs (Beverly, MA, U.S.A.). DMHF was obtained from Tokyo Chemical Industry (Tokyo, Japan). HMF and DDMP were purified from soy sauce and a heated Maillard mixture of glucose and glycine, respectively, as described previously.^{3,5)} Other reagents were obtained from Wako.

Analytical Methods DNA Strand Breaking: DNA strand breaking activity against supercoiled DNA was assayed as described previously.^{2,8)} A 5-μl solution of supercoiled pBR 322 DNA at 20 μg/ml in 0.2 M phosphate buffer (pH 7.4) with or without 0.2 mM FeCl₃ was mixed with a 5-μl aqueous

Fig. 1. Structures of DNA Breaking Substances Formed in the Heated Maillard Reaction of Glucose and Glycine and in Soy Sauce

solution of the extract of the Maillard reaction mixture of glucose and BSA. The mixture was incubated at 37°C overnight. After addition of 1 μl of dye mixture containing 0.5% bromophenol blue, 0.5% xylene cyanol and 50% glycerol, the whole mixture was subjected to agarose gel electrophoresis; this was run at 4 V/cm for 2 h using a Mupid-2 submarine electrophoresis apparatus (Advance Company, Tokyo) on 0.7% SeaKem ME agarose (FMC BioProducts, Rockland, ME, U.S.A.). The running buffer used for the electrophoresis contained 45 mM Tris-borate (pH 8.3), 1 mM EDTA and 0.5 μg/ml ethidium bromide. The DNA bands were visualized by irradiation at 300 nm.

Gas Chromatography/Mass Spectrometry (GC/MS): GC/MS was carried out on a GCD GC/MS system (Yokogawa Analytical Systems, Tokyo). A DB-WAX capillary column (0.25 mm i.d. × 30 m, 0.25 μm film thickness) (Yokogawa Analytical Systems) was used. A 2-μl aliquot of the Maillard reaction mixture was directly injected. The carrier gas flowed at a rate of 1 ml/min. The oven temperature was maintained at 50°C for 1 min and raised to 240°C at a rate of 5°C/min, then held at 240°C for 11 min. The temperature of the injector and the detector was set at 250 and 240°C, respectively. The detector was operated at a mass range of 30–300 amu.

Extraction of Maillard Reaction Products of Glucose and BSA Glucose (10%, w/v) and BSA (5%, w/v) were dissolved in water and incubated at 37°C for the indicated period. The initial pH of the solution was 6.8 and was gradu-

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ally decreased during the reaction reaching to 5.8 after 1-week reaction, 4.6 after 2-month reaction and maintained constant thereafter. The Maillard reaction was carried out at neutral pH; glucose and BSA were incubated in 1 M phosphate buffer (pH 7.4) at the same concentration. In this case, the pH value of the reaction mixture was kept at 7.4 throughout the reaction.

A 5-ml aliquot of the reaction mixture incubated in water or in phosphate buffer was extracted with 5 ml of ethyl acetate/2-propanol (2:1, v/v), and the organic phase was evaporated to dryness followed by redissolving in 50 μ l of water. The aqueous solution of the extract was diluted with water to test for the DNA strand breaking activity.

RESULTS AND DISCUSSION

A solution of glucose and glycine in water was incubated at 37 °C for 3 months. The reaction mixture became slightly acidic (pH 4.6) after the reaction. The ethyl acetate/2-propanol extract of the reaction mixture was tested for the DNA breaking activity against supercoiled pBR 322 DNA. The supercoiled (form I) DNA is converted into nicked open

circular (form II) and/or linear (form III) DNA when the single strands were cleaved. Three forms of DNA can be separately detected in agarose gel electrophoresis.⁸⁾ While the 100-fold diluted extract showed little activity to break the DNA (Fig. 2A, lanes 2, 3), the undiluted extract cleaved the DNA to produce form II DNA (Fig. 2A, lane 4). When the 100-fold diluted extract was incubated with the DNA in the presence of Fe(III) ion, extensive DNA breaking activity was observed (Fig. 2B, lane 3). The undiluted extract converted all form I DNA into form II DNA in the presence of Fe(III) ion (Fig. 2B, lane 4). The unincubated extract of a mixture of glucose and BSA showed no activity even in the presence of Fe(III) ion (data not shown). These results showed the formation of DNA breaking substances that are extractable in an organic solvent in the reaction of glucose and BSA at 37 °C.

Solutions of glucose and BSA in water and in 1 M phosphate buffer (pH 7.4) were incubated at 37 °C for 1 week. The pH value of the reaction mixture in 1 M phosphate buffer was unchanged during the reaction. The ethyl acetate/2-propanol extract of each reaction mixture was incubated with the supercoiled DNA in the presence and absence of Fe(III) ion. In the absence of Fe(III), the extract of the mixture incu-

Fig. 2. Agarose Gel Electrophoresis of Supercoiled pBR 322 DNA Incubated with an Ethyl Acetate/2-Propanol Extract of the Maillard Reaction Mixture of Glucose and BSA

A: Supercoiled pBR 322 DNA was mixed in 0.1 M phosphate buffer (pH 7.4) with water alone (lane 1), 10000-fold diluted (lane 2), 100-fold diluted (lane 3) or undiluted (lane 4) extract of the Maillard reaction mixture. The Maillard reaction was carried out in water for 3 months. B: Supercoiled pBR 322 DNA was mixed in the phosphate buffer containing 0.1 mM Fe(III) ion with the same test solutions as shown in A. C: Supercoiled pBR 322 DNA in the phosphate buffer (lanes 1—3) or that containing 0.1 mM Fe(III) ion (lanes 4—6) was mixed with water (lanes 1 and 4) or with the undiluted extract of the Maillard reaction mixture. The Maillard reaction was carried out incubated in water (lanes 2 and 5) or in 1 M phosphate buffer (pH 7.4) (lanes 3 and 6) for 1 week. All the mixtures were incubated at 37 °C overnight and subjected to agarose gel electrophoresis. Electrophoretic positions of form I and II DNA are indicated.

Fig. 3. GC Chromatogram of the Maillard Reaction Mixture of Glucose and BSA

A mixture of glucose and BSA was incubated in water at 37 °C for 3 months and a 2 μ l aliquot was directly injected to GC/MS. The positions of the peaks of authentic DMHF, HMF and DDMP are indicated.

Fig. 4. MS of the Peaks in the GC/MS Chromatogram in Fig. 3 and the Peaks in GC/MS Chromatogram of Authentic Samples

A, B and C: MS of the peaks at 25, 26.5 and 29.5 min in GC chromatogram of the Maillard mixture (Fig. 3), respectively. D, E and F: MS of the peaks in GC chromatograms obtained from authentic DMHF, HMF and DDMP, respectively.

bated in water caused few DNA strand breaks (Fig. 2C lane 2), whereas the extract of the mixture incubated in 1 M phosphate buffer caused significant DNA strand breaks (lane 3). The activity of both the extracts was enhanced by addition of Fe(III) (lanes 5 and 6). Enhanced breaking activity of these extracts by Fe(III) ion was characteristic of the reported DNA breaking substances produced in the heated Maillard reaction and soy sauce.³⁻⁷⁾

GC/MS of the Maillard reaction of glucose and BSA in water was carried out. Authentic samples of DMHF, HMF and DDMP were eluted at retention times of 25, 26.5 and 29.5 min, respectively, by gas chromatography (Fig. 3) and their mass spectra were obtained (Fig. 4D-F). A mixture of glucose and BSA was incubated in water at 37°C for 2 months and an aliquot of the reaction mixture was analyzed on GC/MS. Many peaks were detected by gas chromatography (Fig. 3) and peaks appeared whose retention times were the same as those of DMHF, HMF and DDMP. These peaks were identified as those of DMHF, HMF and DDMP, respectively, by comparing the mass spectra (Fig. 4A-C) with those of authentic samples (Fig. 4D-F). Thus DMHF, HMF and DDMP with DNA breaking activity were formed in the Maillard reaction of glucose and BSA at neutral pH ranges and 37°C.

It is known that glycosylated proteins produced in the Maillard reaction of glucose and BSA under physiological conditions^{9,10)} generate active oxygen species. It may be important that organic-solvent-extractable low-molecular-weight substances generated active oxygens. The mechanisms underly-

ing the DNA breaking activity of these substances have been examined.^{3,4,6)} Hydrolyzed form with the enediol and ketol structures may reduce a trace amount of Fe(III) ion into Fe(II) ion and oxygen into superoxide. Superoxide may dismutate into hydrogen peroxide, which may in turn generate hydroxyl radical by Fenton type reaction. Hydroxyl radical may be a final active form causing DNA strand breaks. The present results suggest the *in vivo* formation of the DNA-breaking hydroxyfuranone- and hydroxypyranone-compounds in the Maillard reaction of glucose and proteins.

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