DNA Breaking by Maillard Products of Glucose–Amino Acid Mixtures Formed in an Aqueous System

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The Maillard product of glucose and an amino acid (Ala, Arg, Cys, Gly, His, Lys, and Pro) in an aqueous solution heated at 100 °C for 3 h cleaved plasmid supercoiled DNA to give single-strand breaks. DNA breaking activity of the product of Gly increased with the increase of heating time of the aqueous mixture, and the breaking activity was maintained during the heating. It is unlikely that metal ions or oxygen-derived radicals participated in the breaking. DNA breaking activity may be due to some polymeric substance(s). Sequencing analysis of the DNA fragment suggested that the breaking was induced at each nucleoside residue and preferentially at the guanosine residues of GC sequences.

INTRODUCTION

It is well-known that Maillard reaction takes place during cooking and processing of foodstuffs and plays an important role in the production of flavor and brownish color in foods. Maillard reaction of sugar–amino acids in several model systems produces various substances including Schiff bases, ketoamines, deoxyosones, melanoidins, furfurals, and so on (Fujimaki *et al.*, 1986). Generation of free radicals such as dialkylpyrazine radicals (Mitsuda *et al.*, 1965; Namiki *et al.*, 1973; Namiki and Hayashi, 1975; Hayashi *et al.*, 1977) and singlet oxygen (Bordalen, 1984) has been demonstrated. Maillard reaction produces genotoxic substances that are mutagenic to *Salmonella* bacterium and that induce chromosome aberrations in Chinese hamster ovary cells and gene conversion in *Saccharomyces cerevisiae* (Yoshida and Okamoto, 1980; Mihara and Shibamoto, 1980; Powrie *et al.*, 1981; Spingarn *et al.*, 1983; Shinohara *et al.*, 1983; Kim *et al.*, 1991). Imidazoquinoline and imidazoquinoxaline heterocyclic amine mutagens in cooked foods are suggested to be formed as a consequence of the reaction of creatine or creatinine with the free radicals generated in Maillard reaction (Pearson *et al.*, 1992).

In the previous paper we have demonstrated that Maillard reaction of glucose–amino acids in a solid system heated at 200 °C for 5 min produces substances having DNA breaking activity together with those generating singlet oxygen and chemiluminescence, and DNA breaking activity may not be related to singlet oxygen or chemiluminescence (Hiramoto *et al.*, 1993). It is worthwhile to investigate whether the Maillard reaction of glucose–amino acid in an aqueous system, which may take place generally during food cooking, produces DNA breaking activity. In this paper, we will describe the generation of DNA breaking activity in Maillard reaction of glucose and amino acids in an aqueous system heated at 100 °C and the site-specific breaking by the products.

MATERIALS AND METHODS

Materials. Glucose, arginine hydrochloride (Arg), cysteine hydrochloride (Cys), glycine (Gly), glutamic acid monosodium salt (Glu), methionine (Met), phenylalanine (Phe), proline (Pro), tryptophan (Trp), and tyrosine (Tyr) were obtained from Wako Pure Chemical Industries (Osaka). Alanine (Ala) was from Nippon Rikagaku-Yakuhin Co. (Tokyo).

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Plasmid pBR 322 DNA (Sutcliffe, 1978) [1 mg/mL in 10 mM Tris-HCl (pH 8.0), 1 mM ethylenediaminetetraacetic acid (EDTA)] was obtained from Takara-Shuzo Co. (Kyoto). A singly 5′-end-labeled DNA fragment composed of 377 basepairs was obtained from pBR 322 DNA (Maxam and Gilbert, 1980). Thus, pBR 322 DNA (7 μg) was digested with *Eco*RI (Takara-Shuzo Co.) to obtain the linearized DNA. The 5′-end of the fragment was labeled with ^{32}P by means of T4 polynucleotide kinase (Takara-Shuzo Co.) and [γ-^{32}P]ATP (222 TBq/mmol) (Amersham/Japan Co., Tokyo). The labeled fragment was digested with *Bam*HI (Takara-Shuzo Co.) into singly 5′-end-labeled 377- and 3984-basepair DNA fragments, which were subsequently separated by agarose gel electrophoresis using 1% agarose (SeaKem ME agarose, FMC BioProducts, Rockland, ME). The gel was run at 4 V/cm for 1 h using a buffer system composed of 40 mM Tris-acetate (pH 8.3), 1 mM EDTA, and 0.5 μg/mL ethidium bromide. The band corresponding to the 377-basepair fragment visualized by irradiation at 300 nm was extracted with 500 μL of 10 mM Tris-HCl (pH 8.0)/1 mM EDTA, and the fragment was precipitated with ethanol to be redissolved into 50 μL of the same buffer for use.

Catalase [EC 1.15.1.6] and superoxide dismutase (SOD) [EC 1.15.1.1] were from Sigma Chemical Co. (St. Louis, MO). 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was from Labotec Co. (Tokyo). *N*-*tert*-Butyl-α-phenylnitrone (PBN) and 2,2,6,6-tetramethylpiperidine (TEMP) were from Aldrich Chemical Co. (Milwaukee, WI). Sephadex and blue dextran were from Pharmacia LKB (Uppsala, Sweden).

Maillard Reaction of Glucose–Amino Acid in an Aqueous System. The reaction was carried out as follows unless otherwise stated. A mixture of 1 g of glucose and 1 g of Ala, Arg, Cys, Gly, Glu, His, Lys, Met, Phe, Pro, Trp, or Tyr was added to about 5 mL of water. The solution or the suspension was neutralized at pH 7 and made up to 10 mL with water. The solution or the suspension was placed in a glass tube with a screw cap and heated at 100 °C for 3 h unless otherwise specified. The solutions of Ala, Gly, Glu, His, and Lys turned reddish brown, and those of Arg, Cys, and Pro turned slightly yellow or orange. No visible particulates appeared in these solutions, and the solutions (0.2 g product/mL) were immediately used. The suspensions of Met, Phe, Trp, and Tyr could not be solubilized even after heating, and the supernatants were used.

DNA Breaking Activity of the Maillard Products of Glucose–Amino Acid. The DNA breaking activity of each Maillard product in the heated aqueous solution was estimated as described using plasmid supercoiled DNA (Hiramoto *et al.*, 1993). Briefly, a mixture of 8 μL of the solution containing Maillard product at 0.2 g/mL, 1 μL of 1 M phosphate buffer (pH 7.4), and 1 μL of a solution of supercoiled pBR 322 DNA dissolved in sterilized water at 0.1 mg/mL was incubated at 37 °C overnight. The pH of the mixture was kept at 7.1 throughout the incubation when checked by the large-scale reaction mixture.

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To investigate the effects of chelating agents and active oxygen radical scavengers, 5 μL of the solution of the Maillard product, 3 μL of the solution of the chelating agents or the scavengers in water [methanol for butyl hydroxyanisole (BHA)] at the indicated concentrations and with the pH value adjusted to 7, 1 μL of the buffer, and 1 μL of the supercoiled DNA solution were mixed, and the mixture was incubated similarly.

To investigate the effect of oxygen, 5 μL of the solution containing the Maillard product at 0.2 g/mL with and without 0.2 M sodium dithionite and 5 μL of the solution containing 20 μg/mL supercoiled DNA in 0.2 M phosphate buffer (pH 7.4) that had been purged with purified nitrogen gas for 15 min to remove dissolved oxygen were mixed in a tube under nitrogen atmosphere; the mixture was put into a tightly stoppered tube and was incubated similarly.

After the reaction mixture was mixed with 1 μL of a dye mixture containing 0.5% bromophenol blue, 0.5% xylene cyanol, and 50% glycerol, it was placed on 0.7% agarose. The gel was run at 4 V/cm for 2 h using a buffer system composed of 45 mM Tris-borate (pH 8.3), 1 mM EDTA, and 0.5 μg/mL ethidium bromide. The DNA band visualized was photographed through an orange filter.

Site-Specific Damage of a Singly 5′-End-Labeled DNA Fragment by the Maillard Product of Glucose–Gly. A mixture of 50 μL of the solution of the Maillard product (0.2 g/mL), 10 μL of 1 M phosphate buffer (pH 7.4), 5 μL of the singly 5′-end-labeled 377-basepair DNA fragment, and 35 μL of the sterilized water was incubated at 37 °C overnight. The labeled DNA fragments were recovered by gel filtration through a column of Sephadex G-50 (1.2 mL) and subsequent ethanol precipitation. The labeled fragments were analyzed according to the method of Maxam and Gilbert (1980). The fragments were dissolved into 100 μL of 1 M piperidine. An aliquot of 50 μL was lyophilized immediately as the non-piperidine-treated fragments. Another aliquot of 50 μL was heated at 90 °C for 30 min and lyophilized as the piperidine-treated fragments. The fragments were dissolved into 10 μL of formamide containing 0.5% bromophenol blue, 0.5% xylene cyanol, and 1 mM EDTA, and 2 μL of the solution was placed on a 8% polyacrylamide gel containing 8 M urea. The gel was run at 70 W and 2.4 kV·h using 90 mM Tris-borate (pH 8.3) and 2 mM EDTA. Autoradiography was carried out at −80 °C with an intensifying screen. The breaking sites were identified by comparing the bands to those of the DNA fragments cleaved by formic acid treatment (for the bands cleaved at purine bases) and hydrazinolysis (for the bands cleaved at pyrimidine bases).

Chemiluminescence of the Maillard Products. Chemiluminescence was measured as described previously (Hiramoto *et al.*, 1993) on a Lumi-Counter 1000 machine (Nition Co., Chiba). An aliquot of 2 mL of each Maillard product diluted in 0.1 M phosphate buffer (pH 7.4) at a concentration of 1 mg/mL was placed in a glass cell (14 mm i.d.), and photons emitted at 37 °C for 10 min were counted unless otherwise specified. The background number of photons emitted from the solvent alone was subtracted.

Electron Spin Resonance (ESR) Spectra. The mixture of 50 μL of the solution of the Maillard product of Gly, Cys, or Tyr (0.1 g of glucose and 0.002 g of Tyr/mL), 50 μL of ethanol, and 2 μL of TEMP was incubated at 37 °C overnight. ESR spectra were obtained on a Varian E-4 EPR spectrometer. The instrumental conditions were as follows: field setting, 338.5 mT; scan range, 10 mT; microwave power, 10 mW; modulation amplitude, 0.1 mT.

RESULTS

A mixture of glucose–amino acid (Ala, Arg, Cys, Gly, Glu, His, Lys, Met, Phe, Pro, Trp, or Tyr) in water at 0.1 g/mL each was heated at 100 °C for 3 h. The conditions were mild as compared to those for food cooking. No visible precipitates appeared in the reaction mixtures of Ala, Arg, Cys, Gly, Glu, His, Lys, and Pro, whereas the reaction mixtures of Met, Phe, Trp, and Tyr were turbid throughout the reaction because the solubilities of these amino acids were lower. The mixtures of Ala, Gly, Glu, His, and Lys turned reddish brown, and those of Arg, Cys, and Pro turned slightly yellow or orange.

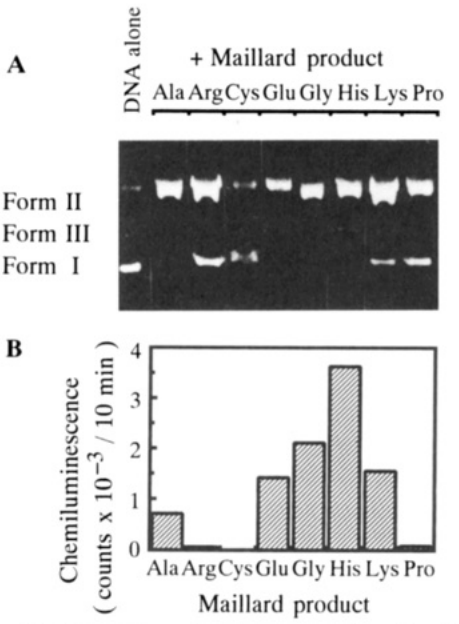


Figure 1. DNA breaking activity (A) and chemiluminescence (B) of Maillard products of glucose–amino acid in water. (A) Supercoiled pBR 322 DNA was incubated with the Maillard product (0.2 g/mL) at pH 7.1 and 37 °C overnight, and the mixture was subjected to agarose gel electrophoresis. Forms I, II, and III indicate a supercoiled form, a nicked open circular form, and a linear form of the DNA, respectively. (B) Chemiluminescence emitted at pH 7.4 and 37 °C for 10 min was measured.

DNA breaking activity of the Maillard products was examined using a plasmid supercoiled DNA, which is converted into a nicked open circular form and/or a linear form by single-strand breaking (Ueda *et al.*, 1985). After the Maillard products were incubated with plasmid supercoiled pBR 322 DNA at pH 7.1 and 37 °C overnight, the reaction mixtures were subjected to agarose gel electrophoresis (Figure 1A). The Maillard products from soluble amino acids Ala, Gly, Glu, and His at 0.1 g/mL effectively converted a supercoiled form (form I) into a nicked open circular form (form II), indicating that the products cleaved DNA to give single-strand breaks. In contrast, the Maillard products from soluble amino acids Arg, Cys, Lys, and Pro at 0.1 g/mL showed weaker activity. As controls, DNA breaking activity of the heated solution of glucose, Ala, Arg, Cys, Glu, Gly, His, Lys, or Pro alone and nonheated solution of the mixture of glucose and Ala, Arg, Cys, Glu, Gly, His, Lys, or Pro was examined, and they were quite inactive. The Maillard products from less soluble amino acids Met, Phe, Trp, and Tyr showed little activity (data not shown). Hence, Maillard reaction of glucose–amino acid in an aqueous system produced DNA breaking activity depending on the amino acids, their solubilities, and their concentrations. The solutions of the Maillard products of Ala, Gly, Glu, His, and Lys emitted strong chemiluminescence at pH 7.4 and 37 °C, whereas those of Arg, Cys, and Pro emitted little or no chemiluminescence (Figure 1B). The supernatants of the Maillard reaction mixtures of Phe and Tyr emitted significant chemiluminescence (data not shown).

DNA breaking activity of the Maillard product of Gly increased dose-dependently between 0.02 and 0.2 g/mL (Figure 2). Generation of DNA breaking activity of the Maillard mixture of Gly increased with the increase of heating time of the mixture (Figure 3A), indicating that DNA breaking activity generated was kept stable during heating. Chemiluminescence similarly increased with the

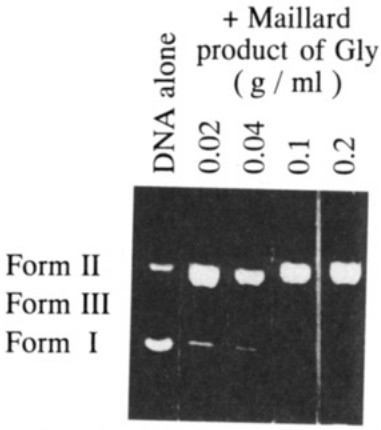


Figure 2. Dose-dependent DNA breaking activity of the Maillard product of glucose–Gly formed in water. Supercoiled pBR 322 DNA was incubated with the Maillard product at the indicated concentrations as described in Figure 1, and the mixture was subjected to agarose gel electrophoresis.

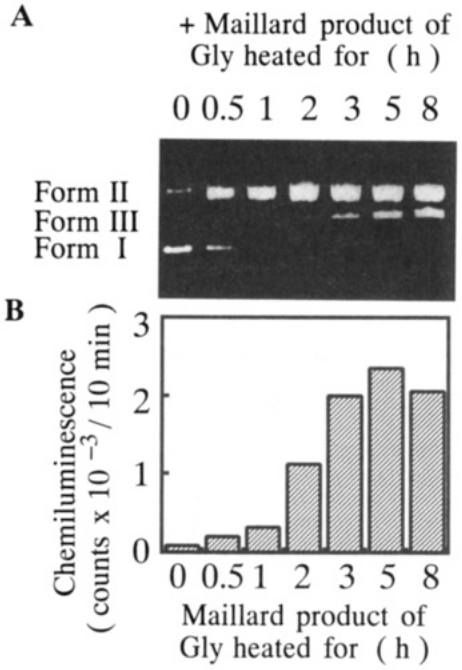


Figure 3. Effect of heating time of a solution of glucose–Gly on the generation of DNA breaking activity (A) and chemiluminescence (B). A mixture of glucose–Gly was heated at 100 °C for the indicated periods. Supercoiled pBR 322 DNA was incubated with the Maillard product for the DNA breaking activity, and the chemiluminescence of the product was measured as described in Figure 1.

increase of heating time up to 5 h, but it decreased thereafter (Figure 3B). When part of the water of the reaction mixture was replaced by dioxane, generation of DNA breaking activity was little affected (Figure 4A), but generation of chemiluminescence remarkably increased (Figure 4B). The result indicates that the amount of water in the Maillard reaction little affected the generation of DNA breaking activity but greatly affected the generation of chemiluminescence. When the Maillard reaction was performed in the presence of 10 mM BHA or 0.1–0.5 M 2-mercaptoethanol, DNA breaking activity was similarly generated, but the reaction in the presence of 1–5 M 2-mercaptoethanol produced less DNA breaking activity. In contrast, generation of chemiluminescence was completely inhibited by 0.1 M 2-mercaptoethanol.

An ESR spin trapping technique was employed for detection of active oxygen radicals in Maillard products:

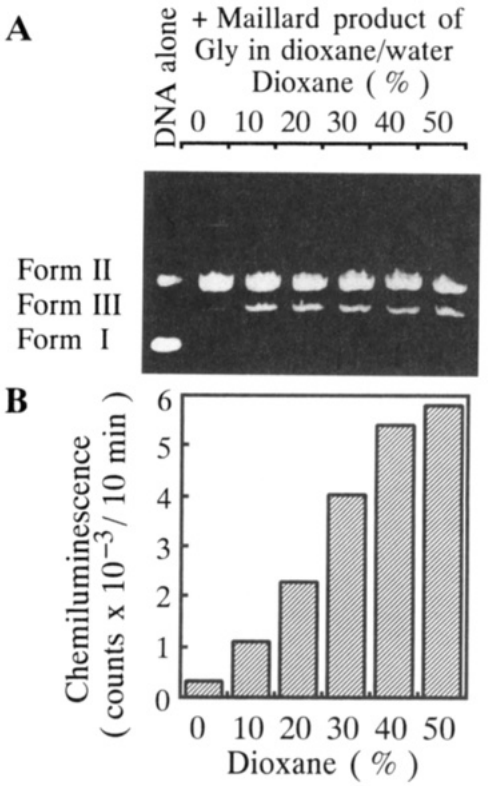


Figure 4. DNA breaking activity (A) and chemiluminescence (B) of the Maillard product of glucose–Gly formed in dioxane/water. A mixture of glucose–Gly in dioxane/water was heated at 100 °C for 3 h. The mixture was evaporated in vacuo at room temperature to dryness, and the residue was redissolved into water at the same concentration. The DNA breaking activity (A) and chemiluminescence (B) were measured as described in Figure 1.

DMPO and PBN for the trap of hydroxyl radical and superoxide anion (Finkelstein *et al.*, 1980; Harbour *et al.*, 1974) and TEMP for the trap of singlet oxygen (Lion *et al.*, 1976; Moan and Wold, 1979). No ESR signals of the DMPO and PBN adducts were observed in the incubation of the Maillard products of Gly, Cys, and Tyr with the spin trappers. ESR signals of 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO) with a_N values of 1.67–1.68 mT were observed when TEMP was incubated with the Maillard product of Gly (Figure 5A) and with the product of Tyr (Figure 5C) but not with the product of Cys (Figure 5B). It was found that the Maillard reaction in an aqueous system produced substances that generated singlet oxygen (Kurosaki *et al.*, 1989).

The effect of metal chelating agents and active oxygen scavengers (Halliwell and Gutteridge, 1989) on DNA breaking by the Maillard product of Gly was investigated (Figure 6). EDTA and DTPA at high concentrations little affected the breaking (lanes 2 and 3). SOD and catalase were slightly inhibitory (lanes 4 and 5). Hydroxyl radical scavengers mannitol, ethanol, and iodide were slightly effective (lanes 6–8). Singlet oxygen scavenger azide was not inhibitory (lane 9). Antioxidant BHA (lane 10), spin trapping agents DMPO and PBN (lanes 11 and 12), and sulfhydryl compounds 2-mercaptoethanol and Cys (lanes 13 and 14) were not inhibitory. To examine whether the product of Gly was active to cleave DNA even in the absence of molecular oxygen, the product was incubated with DNA after removal of dissolved oxygen by purging with nitrogen gas and also in the presence of dithionite. DNA was effectively cleaved in the incubation in the absence of molecular oxygen (data not shown). Hence, it

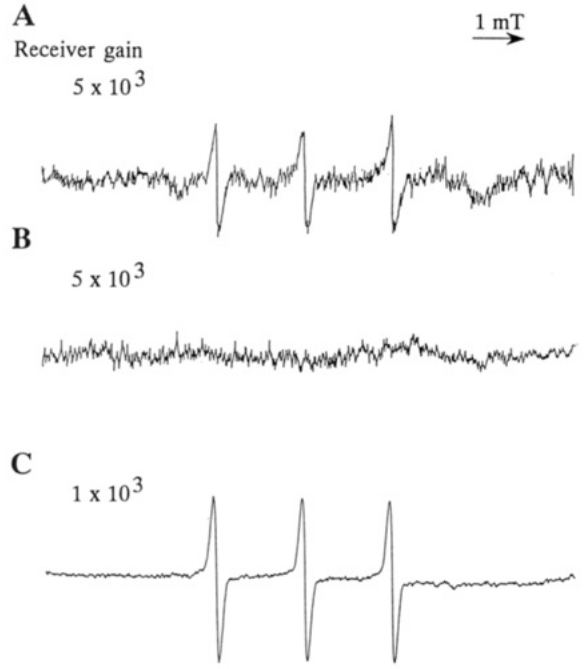


Figure 5. ESR spectra of the mixture of the Maillard product of Gly (A), Cys (B) or Tyr (C) with TEMP.

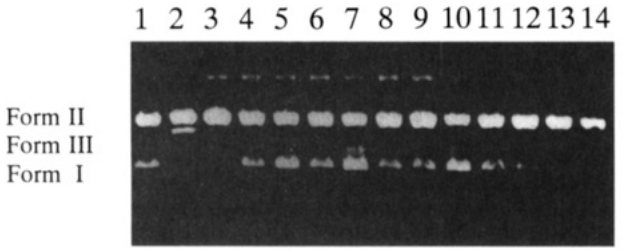


Figure 6. Effect of metal chelating agents and scavengers of active oxygen radicals on the DNA breaking by the Maillard product of glucose–Gly in water. Supercoiled pBR 322 DNA was incubated with the Maillard product (lane 1) and in the presence of 0.1 M EDTA (lane 2), 0.1 M DTPA (lane 3), 0.1 mg/mL SOD (lane 4), 0.1 mg/mL catalase (lane 5), 0.1 M mannitol (lane 6), 0.1 M potassium iodide (lane 7), 0.1 M sodium azide (lane 8), 10% ethanol (lane 9), 0.1 M BHA (lane 10), 0.1 M DMPO (lane 11), 0.03 M PBN (lane 12), 0.1 M 2-mercaptoethanol (lane 13), or 0.1 M Cys (lane 14), and the mixture was subjected to agarose gel electrophoresis as described in Figure 1.

is unlikely that DNA breaking activity of the product of Gly was due to active oxygen radicals.

When the Maillard product of Gly was dialyzed against water, DNA breaking activity was scarcely lost. On gel filtration of the product through a column of Sephadex G-10, DNA breaking activity appeared at the void volume and continuously eluted even after the bed volume (Figure 7A). A similar profile was obtained on gel filtration through a column of Sephadex G-25. On gel filtration through a column of Sephadex G-100, DNA breaking activity appeared around the bed volume together with brownish color and chemiluminescence (Figure 7B). A similar profile was obtained on gel filtration through a column of Sephadex G-50. It is likely that DNA breaking activity was due to polymeric substance(s) with relatively high molecular weights.

Sites and properties of the DNA damage induced by the Maillard product of Gly were investigated using a sequencing technique (Maxam and Gilbert, 1980). The 5′-end-^{32}P-labeled 377-basepair fragment of pBR 322 DNA was used as a substrate. When the fragment was incubated

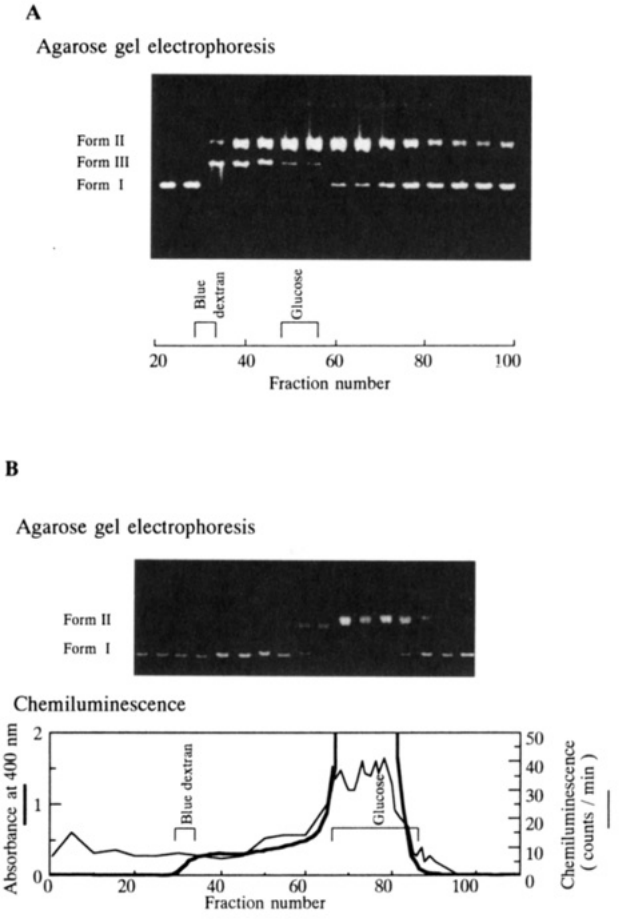
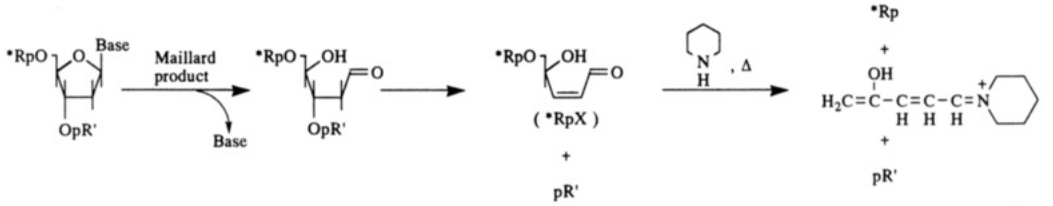


Figure 7. Gel filtration of the Maillard product of glucose–Gly formed in water. A portion of 0.2 mL of the Maillard solution was loaded on a column (9 mm i.d. × 1 m) of Sephadex G-10 (A) or G-100 (B). The column was eluted with 0.15 M sodium chloride, and each 1-mL fraction was collected. Absorbance at 400 nm, chemiluminescence emitted at 37 °C for 1 min, and the DNA breaking activity of each fraction were measured. The void and bed volumes of the columns were determined by blue dextran and glucose, respectively.

with the Maillard product overnight, labeled oligonucleotide fragments were detected as expected. The labeled oligonucleotide fragments shown in Figure 8 (lane 1) indicated that the cleavage of the phosphodiester linkage occurred at each nucleoside residue when compared to the fragments obtained by the purine nucleoside damage and alkaline treatment (Figure 8, lane 3) and those formed by the pyrimidine nucleoside damage and alkaline treatment (Figure 8, lane 4). The more intensely labeled oligonucleotide fragments appeared as if the cytidine residues of GC sequences were preferentially cleaved. But the fragments became about 1 base unit shorter on alkaline treatment (Figure 8, lane 2), indicating that the guanosine residues of GC sequences were preferentially damaged. It is likely that the damage was induced at each nucleoside residue and preferentially at the guanosine residue of the GC sequence, and the phosphodiester bond at the 3′-position of the damaged nucleoside residues was cleaved by a β-elimination mechanism to leave the labeled oligonucleotide fragments composed of the damaged nucleoside residues without phosphate at the 3′-position (*RpX) (Scheme 1). On alkaline treatment, the damaged nucleoside residues may be removed from the fragments, leaving the fragments composed of the nucleoside residues with 3′-phosphate termini (*Rp) that had been adjacent to the damaged nucleoside residues.

Scheme 1. Proposed Mechanisms of the 5′-End-Labeled DNA Fragment Cleavage by the Maillard Product of Glucose–Gly (Asterisks Indicate the 5′-End Labeled)



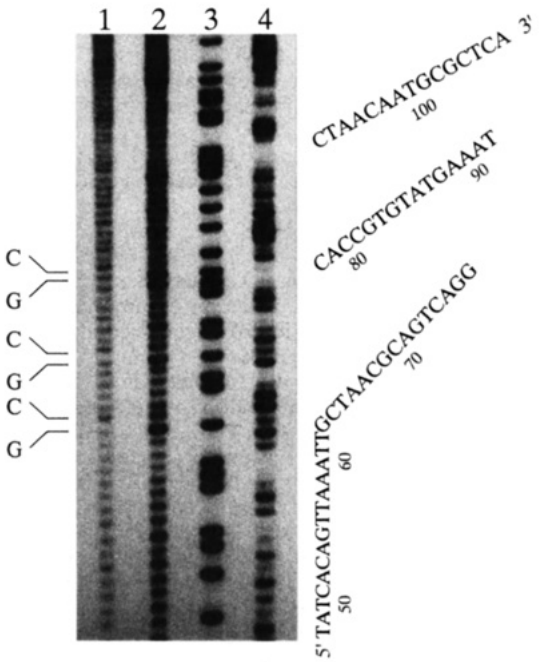


Figure 8. Autoradiogram of polyacrylamide gel electrophoresis of a singly 5′-end-labeled DNA fragment incubated with the Maillard product of glucose–Gly. (Lane 1) The singly 5′-end-labeled 377 basepair pBR 322 DNA fragment was incubated with the Maillard product of Gly. (Lane 2) The DNA fragment was incubated with the Maillard product and subsequently treated with piperidine at 90 °C for 30 min. (Lane 3) The DNA fragment was treated with formic acid and subsequently with piperidine (GA damage). (Lane 4) The DNA fragment was treated with hydrazine and subsequently with piperidine (CT damage). The control DNA fragment and that treated with piperidine remained at the top of the gel.

DISCUSSION

The DNA breaking activity was generated in the Maillard reaction of glucose–amino acid both in a solid system (Hiramoto *et al.*, 1993) and in an aqueous system shown in the present paper. In the Maillard reaction in a solid system heated at 200 °C for 5 min, most amino acids generated DNA breaking activity. In the Maillard reaction in an aqueous system heated at 100 °C, generation of DNA breaking activity was greatly dependent on amino acids or their solubilities. In both cases the generation of DNA breaking activity did not correlate to the generation of chemiluminescence.

It is well-known that a variety of compounds that generate free radicals induce DNA strand breaking. For example, thiol compounds including Cys (Rosenkranz and Rosenkranz, 1971), reductones including ascorbic acid (Chiou, 1983) and polyphenols (Shirahata *et al.*, 1985), sugars including sugar phosphates (Morita and Komano, 1983), peroxides including hydrogen peroxide (Inoue and Kawanishi, 1987) and lipid hydroperoxides (Morita *et al.*, 1983; Ueda *et al.*, 1985), and bleomycin (Sugiura and Kikuchi, 1978) induced DNA breaking when they were mixed with metal ions. Hydroxyl radical generated by interaction with metal ions is responsible for the breaking activity. Singlet oxygen can also induce DNA breaking (Decuyper-Debergh *et al.*, 1987; Wefers *et al.*, 1987; Mascio *et al.*, 1989; Devasagayam *et al.*, 1991). Enediyne antibiotics including neocarzinostatin (Saito *et al.*, 1989) and hydrazines and diazonium salts (Augusto, 1993) cleave DNA strands via carbon-centered radicals.

Characteristics of the DNA breaking activity of the Maillard products were found to be unique. It is readily conceivable that DNA breaking activity of the Maillard products may be due to active oxygen radicals or other radical species. However, participation of active oxygen radicals in the breaking by the product of Gly was found to be small. A singlet oxyen scavenger little inhibited the DNA breaking. Hydroxyl radical or related active oxygen radicals that may be generated by interaction with contaminated metal ions may participate little in the DNA breaking, because metal chelating agents at high concentrations little inhibited the DNA breaking. Removal of molecular oxygen did not prevent the DNA breaking.

It was suggested that the breaking was induced at each nucleoside residue and preferentially at the guanosine residues of GC sequences. The fragments (*RpX) with damaged nucleoside residues bearing no phosphate at the 3′-position may be derived from the nucleoside damage and subsequent β-elimination of the 3′-phosphate group (Scheme 1). With subsequent alkaline treatment, the damaged nucleoside X may be removed from *RpX to leave *Rp. The damage of purine residues by the nonradical mechanisms may be essential for the subsequent β-elimination of the phosphate groups. The fragment (*RpX) is similar to the fragments formed by the 4′-hydrogen abstraction by bis radical 1,4-benzyne, which is converted into Rp on subsequent alkaline treatment (Christner *et al.*, 1992).

ABBREVIATIONS USED

BHA, butyl hydroxyanisole; DTPA, diethylenetriaminepentaacetic acid; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; EDTA, ethylenediaminetetraacetic acid; ESR, electron spin resonance; PBN, *N-tert*-butyl-α-phenylnitrone; SOD, superoxide dismutase; TEMP, 2,2,6,6-tetramethylpiperidine; TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl.

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