A Glucose-Diglycine Condensation Product Participating in Oxygen-Dependent Browning

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An unknown compound (UC) was isolated as a main product in addition to 1-deoxy-1-glycino-D-fructose (FG) in the browning reaction between glucose and diglycine. UC was ninhydrin active and reduced ferricyanide at pH 7.0 and liberated diglycine by acid hydrolysis. From the results of elemental analysis and molecular weight determination, UC was suggested to be an Amadori compound, 1-deoxy-1-diglycino-D-fructose (FGG). An aqueous solution of FGG darkened more rapidly than a glucose + diglycine mixture in the presence of oxygen. The oxidative browning of FGG was hastened by 30–40 times by the addition of Fe^{2+} but that of the glucose + diglycine mixture was not affected by the metal. Furthermore, the FGG solution absorbed atmospheric oxygen appreciably, whereas the glucose + diglycine mixture absorbed no oxygen. In the browning reaction of glucose with triglycine, the similar intermediates (FG, UC, etc.) were detected. The degradation of peptide linkage was observed in both cases.

It is well known that many foodstuffs such as soy sauce and miso, etc., which contain a fair amount of peptides darken during processing and storage. This darkening (nonenzymic browning) was shown to be partially due to a sugar-peptide reaction (Okuhara *et al.*, 1971). However, studies on amino-carbonyl browning reaction in foodstuffs or model systems have concentrated on amino acids, amines, and proteins (Hodge, 1953; Reynolds, 1965; Talley and Porter, 1968) and not on peptides. Although as intermediates of the browning reaction, some kinds of sugar-amino acid condensation products were isolated in sugar-amino acid model systems (Gottschalk, 1952; Horn *et al.*, 1968), little was reported in the sugar-peptide system. Recently Prey and Petershofer (1968) and Chuyen *et al.* (1973) identified some pyrazinones from glyoxal-peptide reactions, but the relation of these pyrazinones to the browning reaction remains uncertain.

On the other hand, it is also well recognized that atmospheric oxygen has a significant effect on the darkening of soy sauce (Hashiba, 1974). As to oxygen-dependent browning (oxidative browning), many investigations have been done with respect to ascorbic acid (Joslyn, 1957; Clegg, 1964; Kurata and Sakurai, 1967). Also, in sugar-amino acid systems, Kato (1963) identified glucosone formed by oxidative browning degradation of *N*-D-glucoside. However, the sugar-peptide oxidative browning reaction has not been investigated.

In order to study the sugar-peptide browning reaction that occurs in contact with air, isolation and identification of an intermediate between glucose and diglycine were conducted and the effects of atmospheric oxygen on the browning of the intermediate were investigated in this paper.

EXPERIMENTAL SECTION

Browning Reaction of Glucose and Diglycine. One hundred milliliters of a mixture solution containing glucose (40%), diglycine (20%), and lactic acid (2%) at pH 5.0 was placed into a rubber-plugged 150-ml erlenmeyer flask and held at 50° for 1 month.

Analysis of Amino Acids. Amino acids and related compounds were analyzed with a Hitachi KLA-5 automatic amino acid analyzer using a neutral and acidic analyzer column (0.9 × 50 cm) with a buffer flow rate of 90 ml/hr. The buffer change from pH 3.25 to 4.25 was made at an effluent volume of 85 ml. The peaks of amino acids were identified by (i) the elution volume in comparison with the results obtained for authentic amino acid and (ii) the increase in a peak resulting from the addition of an authentic amino acid to the samples.

Paper Electrophoresis. Paper electrophoresis was done with a voltage of 40 V/cm at 30° for 50 min on Whatman filter paper No. 1 (46 × 57 cm) using a volatile buffer solution consisting of pyridine-acetic acid-water (1:10:289, v/v, pH 3.6).

Preparation of Standard 1-Deoxy-1-glycino-D-fructose (FG). The procedure of Hodge and Fisher (1963) was used.

Preparative Ion Exchange Chromatography. Dowex 50W-X4 (200–400 mesh, Na^+ form) was packed in a column (1.8 × 150 cm). The temperature of the column was controlled at 30° by running warm water through the jacket. The resin was equilibrated with 0.23 *M* sodium citrate buffer (pH 3.25). A sample was charged on the column and eluted by the same buffer with a flow rate of 180 ml/hr. The effluent was collected in 10-ml fractions. The amino acid content of each fraction was determined by the ninhydrin method (Rosen, 1957). The buffer change (pH 3.25 to 4.25) was made as glycine emerged (elution volume, 1050 ml). The prepared amino acids were identified by the amino acid analyzer.

Paper Chromatography. Whatman filter paper No. 1 was used in a butanol-acetic acid-water (4:1:1, v/v) system.

Isolation of Unknown Compound (UC) [1-Deoxy-1-diglycino-D-fructose (FGG)]. One hundred milliliters of the glucose-diglycine browned mixture, diluted to 500 ml with distilled water, was passed over Dowex 50W-X4 (H^+ form, 100–200 mesh), packed in a 3.5 × 50 cm column. The flow rate during absorption was about 15 ml/min. After the resin was washed with 4 l. of distilled water, absorbed compounds were eluted with 6 l. of 0.1 *N* ammonia collecting in 20-ml fractions. The fractions, positive for both ninhydrin and phenol-H_2SO_4 (Smith, 1956) tests, were pooled (*ca.* 600 ml). Two hundred milliliters of the pooled fractions was applied to the preparative ion exchange chromatograph. The effluent between 1310 and 1390 ml was collected as FGG. Three such preparations obtained from the preparative chromatography were combined and charged on a 2.5 × 70 cm column of Dowex 50W-X4 (H^+ form, 100–200 mesh). After the resin was washed with 3 l. of distilled water to remove citrate, absorbed FGG was eluted with 0.1 *N* ammonia collecting in 20-ml fractions. These fractions were tested on paper chromatography and the fractions containing FGG were collected and lyophilized. A little hygroscopic white powder was obtained (*ca.* 500 mg): mp 119–120° dec; $[\alpha]^{20}D$ −44° (0.5%, in water); λ_{max} (H_2O) (pH 5.0) 189.5 nm; ν_{max}

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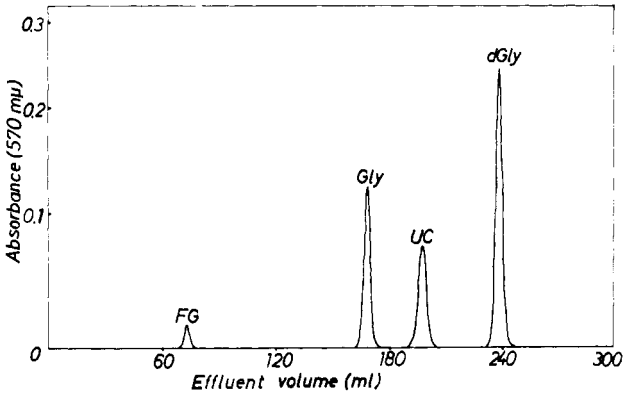


Figure 1. Effluent peaks obtained with a glucose–diglycine browned mixture; neutral and acidic analyzer column of Hitachi KLA-5, 570-mμ trace only.

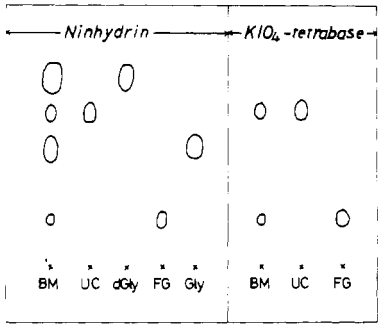


Figure 2. Paper electrophoresis of a glucose–diglycine browned mixture (BM).

(KBr) 3100–3500 s, 1680 s, 1600 m, 1395 s, 1300 m, 1080 s, 920 w, 830 w, 780 m cm^{-1}. *Anal.* Calcd for $C_{10}H_{18}N_2O_8$: C, 40.82; H, 6.12; N, 9.52; neut equiv 294.3. Found: C, 40.55; H, 6.25; N, 9.30; neut equiv 295.8.

Attempts to crystallize FGG were unsuccessful. The high purity of this preparation was indicated by its emergence in the amino acid analyzer as a single symmetrical peak at an effluent volume of 197 ml (at a position between alanine and cystine), and uncontaminated by FG, glycine, or diglycine. When examined by paper chromatography, the product appeared as a single spot at R_f 0.15, at the identical position whether detected by ninhydrin or KIO_4–tetrabase reagent (Yoda, 1952). In the same system, glycine and diglycine appeared at R_f 0.25, glucose at 0.26, and fructose at 0.30.

Hydrolysis of FGG. Thirty milligrams of FGG was dissolved in 10 ml of 1 *N* H_2SO_4 and heated for 3 hr at 100°.

Conditions of Oxidative Browning. Two millimoles of compounds was dissolved in 10 ml of water (containing 1% lactic acid, pH 5.0). One-milliliter aliquots were placed into two test tubes (1.5 × 16 cm) and (a) one was plugged with rubber (under aerobic conditions, headspace volume of 28 ml) and (b) another was sealed under vacuum (under anaerobic conditions, <0.1 mmHg). Both samples were held for 2 weeks at 37° and the color increase (ΔE_{555}) was measured colorimetrically. The difference of ΔE_{555} between methods a and b was caused by atmospheric oxygen. The author defined this difference as oxidative browning and defined ΔE_{555} of b as nonoxidative browning in this experiment.

Measurement of the Amount of Oxygen Uptake. Oxygen absorbed was measured by a Warburg manometer; a sample containing 0.2 *M* of compounds with or without 40 ppm of Fe^{2+} was placed into a vessel (total volume, 2 ml) and shaken at 37° for 2 hr.

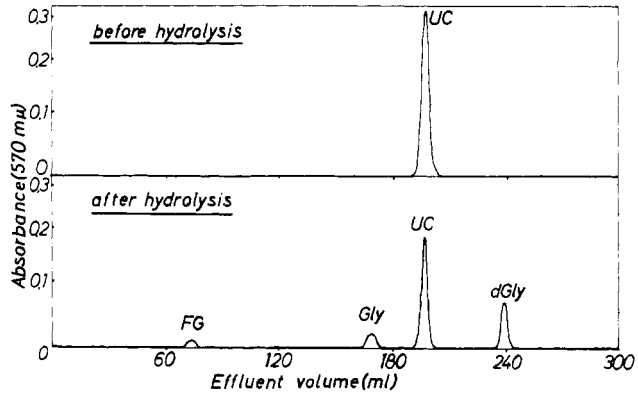


Figure 3. Effluent peaks obtained with UC hydrolyzed by 1 *N* H_2SO_4 at 100° for 3 hr; neutral and acidic analyzer column of Hitachi KLA-5, 570-mμ trace only.

RESULTS AND DISCUSSION

Examinations of the Glucose–Diglycine Browned Mixture. The browned mixture of glucose and diglycine was decolorized by three successive treatments with active carbon (2% w/v) and examined with the amino acid analyzer. Figure 1 illustrates that appreciable amounts of an unknown compound (UC), FG, and glycine were produced as reaction products. Glycine was suggested to be liberated from diglycine by cleavage of the peptide bond in the browning reaction. When a solution containing only diglycine was stored under the same conditions (50°, for 1 month), no compounds except diglycine were detected. Diglycine degradation occurred after the 3-months' storage. Chuyen *et al.* (1973) also reported the cleavage of peptide chains in the reaction between peptides and glyoxal. It is interesting that the degradation of diglycine occurred in such a mild condition by the browning reaction with glucose.

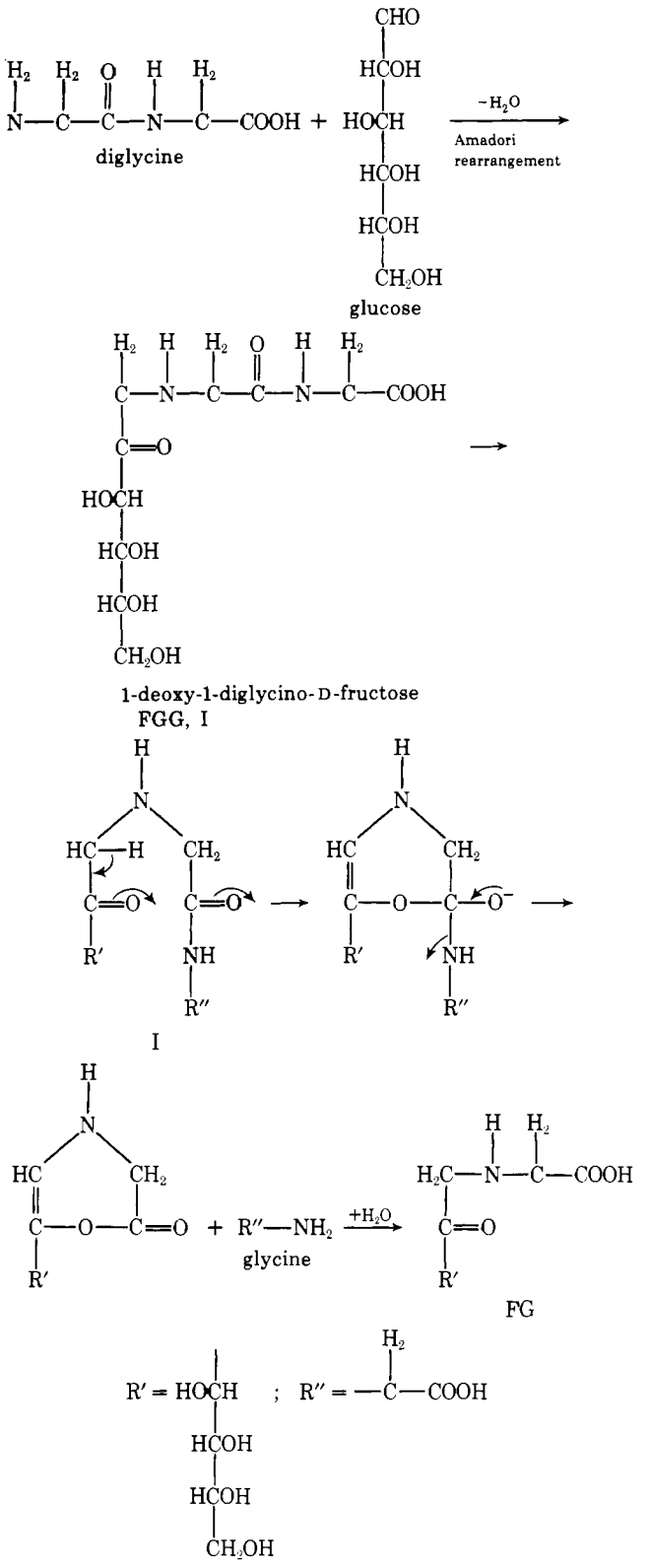
The browned mixture was examined by paper electrophoresis, and amino compounds and sugars were detected by the ninhydrin and the KIO_4–tetrabase methods, respectively (Figure 2). The successful separation of the compounds shows that UC is positive for both reagents like FG.

Determination of UC. UC was positive to both triphenyltetrazolium chloride (TTC) and ninhydrin tests and reduced ferricyanide at pH 7.0, but did not reduce dichlorophenol–indophenol at pH 3.5. Such properties of UC were very similar to those of Amadori compounds.

The hydrolysis products of UC were examined with the amino acid analyzer (Figure 3). Diglycine was liberated from UC as a major product. When the total reducing sugars, aldoses, and ketoses in UC were determined by the methods of Ting (1956) and Horn *et al.* (1968), the value for sugar was found to be the same at 55° as at 100°. Since UC reduced all the ferricyanide at 55°, this suggests all the sugar to be in the form of a ketose and none in the form of an aldose. Furthermore, the content of the sugar and the amino compound in UC, calculated as fructose (by Somogyi-Nelson's method; Nelson, 1944) and as diglycine (by the ninhydrin method), respectively suggested that fructose combined with diglycine in a 1:1 ratio. Conceivably, the structure of UC might be proposed as an Amadori rearrangement product, 1-deoxy-1-diglycino-D-fructose (FGG) from the experimental results.

Stability of FGG. In order to investigate the process of the liberation of glycine from the glucose–diglycine browning reaction, the aqueous solution of 0.1 *M* FGG (containing 1% lactic acid, pH 5.0) was stored for 1 month at 50°. FGG was less stable than diglycine and degraded to FG and glycine, whereas diglycine was not decomposed under the same conditions. Thus, the cleavage process of

Scheme I



the peptide bond in diglycine was suggested as shown in Scheme I.

Examination of a Glucose-Triglycine Browned Mixture. To investigate a sugar-tripeptide browning reaction, the glucose-triglycine system was studied by means of the same procedures as those used in glucose-diglycine systems. The browned mixture of glucose and triglycine was examined by the amino acid analyzer (Figure 4). Similar results as shown in Figure 1 were obtained; that is, the

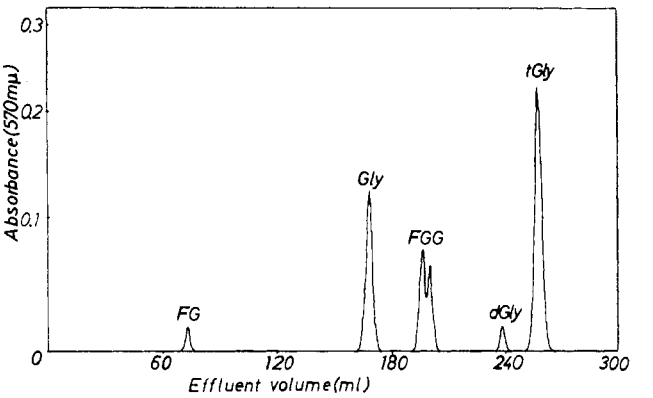


Figure 4. Effluent peaks obtained with a glucose-triglycine browned mixture; neutral and acidic analyzer column of Hitachi KLA-5, 570-mμ trace only.

Table I. Comparison of the Effect of Oxygen on the Browning of the Solutions of Fructose-Diglycine and Related Compounds

	Browning (ΔE_{555})[a]			
	Nonoxidative		Oxidative	
Solutions[b]	Without Fe^{2+}	With Fe^{2+}[c]	Without Fe^{2+}	With Fe^{2+}[c]
FGG	0.088	0.092	0.008	0.342
Glc + dGly	0.009	0.010	0.000	0.001
FG	0.010	0.015	0.006	0.333
Glc + Gly	0.001	0.001	0.000	0.000

[a] Samples were held at 37° for 2 weeks. [b] The concentration of all the compounds in the solutions was 0.2 *M*. Abbreviations used are: Glc, glucose; dGly, diglycine(glycyl-glycine); Gly, glycine; FG, 1-deoxy-1-glycino-D-fructose; FGG, 1-deoxy-1-diglycino-D-fructose. [c] The concentration of Fe^{2+} was 40 ppm.

degradation of triglycine was observed and FG, glycine, FGG, and diglycine were produced. A substance eluted at about 200 ml could not be identified. However, it is supposed to be a glucose-triglycine condensation product based on the elution order, positive reaction to ninhydrin, phenol-sulfuric acid, and reduction of ferricyanide at pH 7.0.

Effect of Oxygen on the Browning of FGG. The browning of an FGG solution during storage in or out of contact with air was investigated and compared with that of glucose + glycine, FG, and glucose + diglycine solutions. As shown in Table I, the oxidative browning of FGG was remarkable.

When 40 ppm of Fe^{2+} was added to the solutions, the oxidative browning of FGG accelerated very significantly and FG also showed a strong ability to brown. On the other hand, little browning was observed in the glucose + diglycine and glucose + glycine. At first, FGG developed a light red color and changed to a red brown color on prolonged storage. The browning of FGG in the presence of oxygen was 50–100 times as great as that of glucose + diglycine or glucose + glycine. It is suggested that FGG is an important precursor in the browning in the presence of air.

For reference, the browning of the solutions in the absence of oxygen was also shown in Table I. The browning of FGG was significant and peptide (diglycine) showed stronger activities than amino acid (glycine) for browning.

Oxygen Uptake of FGG. The oxygen uptake in browning reaction was studied with only furfural (Dunlop *et al.*, 1946), but the autoxidation of Amadori compounds has

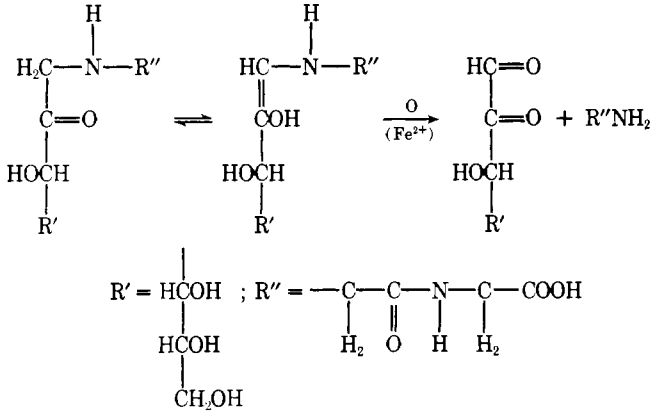
Table II. Comparison of the Oxygen Uptake of the Solutions of Fructose–Diglycine and Related Compounds

Solutions[b]	Oxygen uptake[a] Without Fe^{2+}	With Fe^{2+}
FGG	8.3	45.1
Glc + dGly	0.2	0.3
FG	2.2	36.1
Glc + Gly	0.2	0.2

[a] Microliters/2 ml of solution per 2 hr, determined by a Warburg manometer. [b] See the footnotes to Table I for the other experimental conditions and abbreviations.

not been reported. In order to correlate oxygen uptake with the oxidative browning, an FGG solution was shaken in an oxygen atmosphere at 37° in a Warburg apparatus to measure the amount of oxygen absorbed. Table II shows that the FGG solution absorbed a greater amount of oxygen than a glucose + diglycine solution.

When 40 ppm of Fe^{2+} was added, although the oxygen uptake of the glucose + diglycine solution did not increase appreciably, that of the FGG solution increased 5–6 times. Similar results were obtained in the case of FG, that is, remarkable oxygen uptake of FG was observed whereas glucose + glycine absorbed no oxygen. The experimental results in Table II agreed fairly well with those in Table I. Thereby, the effectiveness of oxygen on the browning of FGG was again proved. Referring to the review by Hodge (1955), the process of oxygen uptake by FGG is assumed as follows.



A strong enhancing effect of oxygen and Fe^{2+} on the browning of the sugar-amine system was well known (Hashiba *et al.*, 1970). However, the substances depending on oxygen in browning have not been fully clarified (Umemoto *et al.*, 1970). The color in the solutions of FGG and FG, the Amadori compounds, increased remarkably in the presence of oxygen and Fe^{2+} as shown in Tables I and II. Therefore, Amadori compounds, important intermediates of sugar–amine reactions, are presumed to participate in oxidative browning.

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