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INCORPORATION OF GLYCINE CARBON ATOMS INTO MELANOIDIN POLYMERS

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Incorporation of C atoms of 1-[^{14}C]-glycine and 2-[^{14}C]-glycine into melanoidin polymers (>3500 Da) was studied for the reaction of glycine and D-glucose.

Key words: glucose, glycine, melanoidin polymers.

The melanoidin reaction (Maillard reaction) plays an important role in the production of food products [1, 2] and is currently being extensively studied as a process occurring in humans [3, 4]. The Maillard reaction occurs betwen reducing sugars and amines (amino acids, proteins, amines, amides) in acidic, neutral, or basic medium with heating. The reaction mechanism is exceedingly complicated. As a result, a variety of products is formed [1, 2]. Despite extensive research, the formation mechanism of these products is still unknown. Our goal was to investigate the features of glycine C atom incorporation into melanoidin polymers formed by reaction of 1-[^{14}C]-glycine and 2-[^{14}C]-glycine with glucose.

Glycine preparations labeled with radioactive C of the same specific activity ($3.0 \cdot 10^8$ Bq/g) were used in the experiments. The reaction between 1-[^{14}C]-glycine or 2-[^{14}C]-glycine and D-glucose was carried out in phosphate buffer (pH 5.8 or 8.0) at 100°C for 5 h. Melanoidin polymers (>3500 Da) were purified of low-molecular-weight impurities by dialysis. The degree of ^{14}C incorporation and C/N and ^{14}C/N ratios were investigated for the melanoidin polymers. Figure 1 shows plots of the dialysis data for melanoidin products formed at pH 5.8.

Dialysis of the melanoidin polymers showed that the degree of incorporation of glycine C atoms into the melanoidin polymers (>3500 Da) was significantly greater if these products were formed at pH 8.0 (1 or 2) than at pH 5.8 (3 or 4). This phenomenon is explained by the fact that lowering the pH increases the number of protonated amino groups on glycine and decreases their ability to react with glucose.

The first step of the Maillard reaction between glucose and glycine must be examined. In this instance, the aldose carbonyl condenses with the amine of the amino acid to form the *N*-substituted aldosylamine via nucleophilic attack of the amino-acid amine on the electrophilic carbonyl of the sugar. The resulting product loses quickly water and transforms into a Schiff base that cyclizes into the aldosylamine. Then, an Amadori rearrangement forms 1-amino-1-deoxy-2-fructose. It is assumed that the Amadori rearrangement product itself is a key intermediate in the formation of melanoidin. Oxidation of this product forms an α-ketone that catalyzes Strecker decomposition of glycine [5]: R–CO–CO–R′ + H_2N–CH_2–COOH→ H–CHO + CO_2 + R–CH(NH_2)–CO–R′.

This decomposition forms from 1-[^{14}C]-glycine H–CHO and $^{14}CO_2$; from 2-[^{14}C]-glycine, H–^{14}CHO and CO_2.

The dialysis scheme shows that incorporation of labeled C into melanoidin poylmers is much greater for 2-[^{14}C]-glycine than for 1-[^{14}C]-glycine. This indicates that mainly the methylene C of glycine is incorporated into melanoidin polymers, apparently through formaldehyde, which forms via decomposition of the amino acid.

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TABLE 1. Elemental Analysis of Dialyzed Melanoidin Polymers (MP) (>3500 Da)

Melanoidin polymers (>3500 Da)	C/N	^{14}C/N
From D-glucose (0.015 M) + 1-[^{14}C]-glycine (0.015 M), pH 5.8 (phosphate), 100°C, 5 h	11.5	0.7
From D-glucose (0.015 M) + 2-[^{14}C]-glycine (0.015 M), pH 5.8 (phosphate), 100°C, 5 h	11.2	1.1
From D-glucose (0.015 M) + 1-[^{14}C]-glycine (0.015 M), pH 8.0 (phosphate), 100°C, 5 h	9.3	0.7
From D-glucose (0.015 M) + 2-[^{14}C]-glycine (0.015 M), pH 8.0 (phosphate), 100°C, 5 h	9.7	1.1

TABLE 2. Incorporation of Glycine C Atoms into Melanoidin Polymer (MP) (>3500 Da) upon Reaction of Dialyzed MP with 2-[^{14}C]-Glycine (MP:Glycine Ratio 300 mg:0.1 mmol, Glycine Sp. Radioactivity $3.0 \cdot 10^6$ Bq/g)

Starting MP (>3500 Da)	Reaction conditions	Final MP radioactivity (>3500 Da), cpm/mg
From D-glucose (0.03 M) + glycine (0.03 M), pH 5.8 (phosphate); 100°C, 5 h	pH 8.0 (phosphate), 100°C, 5 h	420
From D-glucose (0.03 M) + glycine (0.03 M), pH 8.0 (phosphate); 100°C, 5 h	pH 5.8 (phosphate), 100°C, 5 h	170
From D-glucose (0.03 M) + glycine (0.03 M), pH 5.8 (phosphate); 80°C, 240 h	pH 5.8 (phosphate), 100°C, 5 h	90
From D-glucose (0.03 M) + glycine (0.03 M), pH 8.0 (phosphate); 80°C, 240 h	pH 8.0 (phosphate), 100°C, 5 h	130

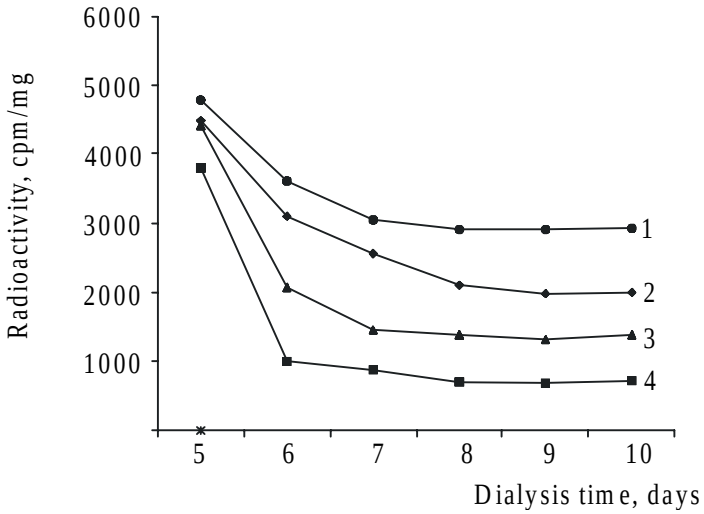

Fig. 1. Final dialysis stage of melanoidin products, starting radioactivity of dialyzed mixtures $3.37 \cdot 10^6$ Bq/g. Product from: 2-[^{14}C]-glycine and D-glucose (pH 8.0) (1), 1-[^{14}C]-glycine and D-glucose (pH 8.0) (2), 2-[^{14}C]-glycine and D-glucose (pH 5.8) (3), 1-[^{14}C]-glycine and D-glucose (pH 5.8) (4). Dialysis through a regenerated cellulose membrane (SPECTRA/POR®3, membrane can retain compounds with molecular weight >3500 Da). Distilled water (5 L each) was replaced regularly after 12 h, 15-18°C.

Considering the mechanism of Strecker decomposition of α-amino acids, it can be proposed that the carboxyl C atom of the amino acid should not be incorporated into the melanoidin polymers. However, it is incorporated (Fig. 1, 2 and 4) and rather extensively at that. Therefore, the carboxyl C of the amino acid is actively incorporated into melanoidin polymers, supposedly via the Amadori rearrangement product. Interesting results were obtained frm a study of the polymeric products of the Maillard reaction between D-glucose and L-alanine [6]. A polymeric fraction (MW ~16,000) was found if labeled glucose

and alanine were used in the Maillard reaction. ^{13}C NMR found that C1 and C2 of alanine were incorporated into the polymer whereas C1 of glucose was found as a labeled methyl.

Elemental analysis of the melanoidin polymers (>3500 Da) prepared by us indicated that the C/N ratio, one of the principal parameters of these polymers, was less for the products formed at pH 8.0 than that for those formed at pH 5.8 (Table 1). This phenomenon, where the C/N ratio of the melanoidin polymer decreases with increasing pH, was also found for the reaction product of glucose with alanine [7].

The ^{14}C/N ratio reflects the degree of incorporation of N and labeled C of glycine into the melanoidin polymers (Table 1). For glycine, the C/N ratio = 1.7. In all polymeric products studied by us, the degree of incorporation of glycine C atoms was less. Furthermore, autoradiography did not detect radioactive glycine in the acid hydrolysates of these melanoidin polymers. Therefore, glycine was incorporated into the polymer not as a unit of the molecular chain but as fragments. These data led to the conclusion that ~65% of the methylene C and ~41% of the carboxyl C from glycine were incorporated under the employed conditions into the melanoidin polymers.

The melanoidin polymers can react with an additional amount of glycine under conditions differing from those under which they were prepared (Table 2). For example, the melanoidin polymer reacts at pH 5.8 with glycine at pH 8.0; the melanoidin polymer reacts at 80°C with glycine at 100°C, etc. The change of pH has a greater effect on incorporation of labeled C of glycine into the polymer than the temperature (Table 2).

The molecular weight of the nonradioactive analogs of melanoidin polymers were determined by synthesizing them from equimolar amounts of glycine and nonradioactive D-glucose under analogous conditions (pH 5.8 and 8.0, 100°C) with purification from low-molecular-weight impurities by dialysis. The yields of high-molecular-weight fractions (>3500 Da) were greater at pH 8.0 than at pH 5.8. The molecular weights were determined by osmometry. For polymers prepared at pH 8.0, MW = 5100 ± 17%; at pH 5.8, 4700 ± 21%. The wide scatter of the data is apparently explained by the high polydispersion of the analyzed fractions.

EXPERIMENTAL

Preparation of ^{14}C-Glycine Working Solutions. We used chemically pure D-glucose and glycine labeled with radioactive C at the carboxyl (1-[^{14}C]-) and methylene (2-[^{14}C]-) groups purified by preparative chromatography on Whatmann 3 paper (*n*-butanol:glacial acetic acid:water, 4:1:5). The radiochemical purity was monitored by autoradiography. The specific radioactivities of the 1-[^{14}C]-glycine and 2-[^{14}C]-glycine preparations were the same at $3.0 \cdot 10^8$ Bq/g. The radiochemical purity was 100%. Working solutions of 1-[^{14}C]-glycine and 2-[^{14}C]-glycine with radioactivity $3.0 \cdot 10^6$ Bq/g each were prepared from these solutions by dilution with nonradioactive chemically pure glycine.

Preparation of ^{14}C-Melanoidin Polymers. The reaction between D-glucose and labeled glycine was carried out at 100°C for 5 h in phosphate buffer (0.05 M, pH 5.8 or 8.0) in a quartz vessel equipped with a reflux condenser. The reaction mixtures consisted of equimolar amounts of D-glucose and labeled glycine (0.015 mol each) dissolved in buffer (20 mL). After the reaction was complete, the reaction mixture was quickly cooled to room temperature, transfered to a volumetric flask (100-mL), and diluted to the mark with distilled water. The resulting solution (50 mL aliquot) was placed in a bag of regenerated cellulose for dialysis (SERVA, SPECTRA/POR®3, catalog No. 44184.01, average diameter 28.6 mm, membrane capable of retaining compounds with MW > 3500 Da). Dialysis was performed against distilled water under strictly identical conditions for 10 d. The distilled water (5 L) was changed regularly every 12 h. The dialysis temperature was 15-18°C. Samples (0.2 mL) were collected daily from the dialysate. The radioactivity of the melanoidin product and the concentration of dry compounds were measured. The radioactivity was measured on an LKB RackBeta II scintillation counter in hydrophilic Bray cocktail [8]. The concentration of dry substance in the sample was determined gravimetrically by evaporating a sample placed on a thin aluminum foil at 70°C. Two parallel experiments were performed for each variation (D-glucose + 1-[^{14}C]-glycine and D-glucose + 2-[^{14}C]-glycine at pH 5.8 and 8.0). The difference between the corresponding data for these experiments was less than ±2%. Average values are reported.

Analysis of ^{14}C-Melanoidin Polymers. Water was removed from the dialyzed melanoidin products by lyophilization. Ignition in a rapid stream of oxygen determined the total C content in these preparations [9]. The content of ^{14}C was determined by wet ashing with $^{14}CO_2$ absorption by monoethanolamine:methylcellulosol (9:1) and measurement of its radioactivity [10]. The N content was measured by a modified Kjeldahl method [11]. Dialyzed melanoidin polymers were hydrolyzed (6 N HCl,

24 h). The hydrolysates were analyzed for labeled glycine content by autoradiography. Nonradioactive melanoidin polymers (>3500 Da) were prepared analogously from D-glucose and glycine. Their reaction with labeled glycine preparations were studied.

Molecular weights of melanoidin polymers were determined by synthesizing their analogs from equimolar amounts of glycine and nonradioactive D-glucose (pH 5.8 and 8.0, 100°C), purifying them from low-molecular-weight impurities via dialysis through a membrane of regenerated cellulose (membrane can retain compounds with molecular weight >3500 Da), and lyophilizing. Molecular weights were determined by osmometry using a Dogadkin osmometer with a membrane of regenerated cellulose (see above). Aqueous solutions (0.001%) of melanoidin polymers were studied [12]. Five parallel experiments were performed for each variation with three determinations for each experiment. The results were processed using the Excel computer program.

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