[Chem. Pharm. Bull. 33(6)2438—2444(1985)]

Mutagenicity of Nondialyzable Melanoidins Prepared from Carbohydrates and L-Tryptophan before and after Nitrite Treatment

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(Received September 11, 1984)

Nondialyzable melanoidins were isolated from the reaction mixtures of carbohydrates or their derivatives (such as sugars, an osone, and furfurals) with L-tryptophan in 0.1 M phosphate buffer (pH 7.0) after 4 weeks at 37 °C or 1—2 h at 110 °C by dialyzing the mixtures against distilled water at 4 °C for 24 h. The melanoidins were tested for mutagenic activities toward *S. typhimurium* TA100 with and without nitrite treatment. None of these melanoidins was mutagenic without nitrite treatment, though the melanoidins from 3-deoxy-D-glucosone, furfural, 5-hydroxymethylfurfural, diacetyl and triose reductone were strongly mutagenic at the dose of 200 μg per plate after nitrite treatment under acidic conditions. However, the melanoidins from D-xylose, D-arabinose, D-glucose, D-fructose, maltose, lactose and sucrose induced less than twice as many his^+ revertant colonies as the negative control at the dose of 400 μg per plate after nitrite treatment. The mutagenic activities of the products were variable depending on the reaction conditions such as reaction time and reaction temperature. Nondialyzable substances from soy sauce and bean paste also showed weak mutagenic activities after nitrite treatment.

Keywords——nondialyzable melanoidin; carbohydrate; L-tryptophan; mutagenicity; nitrite treatment

Amino-carbonyl reaction occurs between amino compounds (ammonia, amines, amino acids, peptides, proteins) and carbonyl compounds containing sugar moieties and their derivatives.[1] Such reactions have also been observed in some natural and manufactured foods, resulting in discoloration and a decrease in their nutritive value.[2] Recently, it has been reported that several daily foodstuffs, *e.g.*, soy sauce and alcoholic beverages showed mutagenic activities after nitrite treatment, and promutagens were formed from some of their components *via* amino-carbonyl reaction.[3] Melanoidins, isolated as final products of the amino-carbonyl reaction, are nitrogenous brown polymers or copolymers and their physical properties such as pH, isoelectric point and molecular weight are variable depending on the reaction conditions.[4] They are known to have antioxidative[5] and antibacterial[6] properties. However, no data are available on their genotoxic effects.

In the present study, we isolated nondialyzable melanoidins from the reaction mixtures of carbohydrates and L-tryptophan and screened them for mutagenic activity toward *S. typhimurium* TA100.

Experimental

Materials and Chemicals——Carbohydrates (D-xylose, D-arabinose, D-glucose, D-fructose, maltose, lactose, sucrose) were purchased from Wako Pure Chemical Industries (Tokyo). 2-Furaldehyde (furfural) and 2,3-butanedione (diacetyl) were from Tokyo Kasei Kogyo Co. (Tokyo), and they were distilled under reduced pressure before use. 3-Deoxy-D-glucosone was prepared from D-glucose, 5-hydroxymethyl-2-furaldehyde(5-hydroxy-

methylfurfural) from sucrose and 2,3-dihydroxy-2-propenal (triose reductone) from D-glucose according to the methods of Kato,[7] Bonner *et al.*[8] and Euler *et al.*,[9] respectively. All other solvents and chemicals used were of reagent grade.

Apparatus and Operation Conditions——Dialysis of reaction mixtures was performed at 4 °C against distilled water using cellulose tubing (18/32, Union Carbide Co.). Gel filtration was carried out at 25 °C on Sephadex G-50 (medium, Pharmacia Fine Chemicals). Ultraviolet (UV) spectra were measured with a Hitachi 100-60 spectrophotometer.

Amino-Carbonyl Reaction of Carbonhydrate with L-Tryptophan——A carbonyl compound (0.1 mol) was dissolved in 500 ml of 0.1 M phosphate buffer (pH 7.0) containing L-tryptophan (0.05 mol) and the mixture was kept at 37 °C for 4 weeks in an incubator (method A). Moreover, in the cases of D-glucose, furfural and diacetyl, each compound was heated with L-tryptophan at 110 °C for 1—2 h in an autoclave (1.2 kg/cm^2) (method B).

Preparation of Nondialyzable Melanoidins——A reacted solution was poured into cellulose tubing and dialyzed at 4 °C for 24 h against distilled water. The outer solution was changed several times until the solution became colorless. Then the inner solution was lyophilized; the residue was transferred to a test tube with a tightly fitting plastic stopper and kept in a refrigerator.

Nitrite Treatment of Nondialyzable Melanoidins——Samples were treated with nitrite at pH 4.0 as described in the previous paper.[10] A lyophilizate was dissolved in 0.3 M acetate buffer (pH 4.0) at an appropriate concentration. The solution (0.4 ml) was mixed with 0.3 M $NaNO_2$ solution and incubated at 37 °C for 1 h under shielding from light. Then, 0.3 M ammonium sulfamate solution was added to the above solution to stop the reaction with nitrite. After 10 min, a 0.1 ml aliquot of the solution was used for mutagenicity assay.

Mutagenicity Assay——The method was essentially that of Ames *et al.*[11] with some modifications.[12] 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2) was used as a positive control. The numbers of revertant colonies given in figures and tables are the averages of three plates.

Gel Filtration of Nondialyzable Melanoidins on Sephadex G-50——The lyophilizate (10 mg) obtained from D-glucose and L-tryptophan was dissolved in 1 ml of 0.1 M phosphate buffer (pH 7.0) containing 0.1 M NaCl and applied to a column of Sephadex G-50 (i.d. 1.5 × 45 cm). Elution was carried out with the same buffer at a flow rate of 43 ml/h at room temperature.

Preparation of Nondialyzable Substances from Soy Sauce and Bean Paste——A 150-ml aliquot of soy sauce (brand A) was poured into cellulose tubing. A 70 g sample of bean paste (brand B) was suspended in 300 ml of boiling water, cooled and kept at room temperature for 10 min. The suspension was then centrifuged at 3000 rpm for 5 min and the supernatant was dialyzed against distilled water in the same manner as nondialyzable melanoidins from carbohydrates and L-tryptophan. Both inner solutions were lyophilized to obtain the nondialyzable substances.

Results and Discussion

The yields and mutagenic activities of nondialyzable melanoidins obtained from the reaction mixtures of various carbonyl compounds with L-tryptophan at 37 °C for 4 weeks are shown in Tables I and II, respectively.

Before nitrite treatment, no sample showed mutagenic activity toward *S. typhimurium* TA100 at a dose of 400 μg per plate, but after nitrite treatment the melanoidins from D-xylose, 3-deoxy-D-glucosone, furfural, 5-hydroxymethylfurfural, diacetyl and triose reductone induced more than twice as many his$^+$ revertant colonies as the negative control at the dose of 200 μg per plate. The melanoidins from diacetyl, furfural and 5-hydroxymethylfurfural showed clear dose-response curves (Fig. 1a—c).

The activity of the melanoidin from 5-hydroxymethylfurfural decreased at doses above 200 μg per plate. The mutagenic activities of the melanoidins prepared from diacetyl, furfural and D-glucose by heating at 110 °C for 1 and 2 h are shown in Table III.

After nitrite treatment, the melanoidin from D-glucose and L-tryptophan formed at 37 °C for 4 weeks induced 126 net his$^+$ revertant colonies at the dose of 200 μg per plate, while after heating at 110 °C for 1 h it showed 1325 net his$^+$ revertants at the same dose. It is apparent from Table III that the mutagenic activities of all the melanoidins prepared at 110 °C for 2 h were lower than those of the compounds prepared at 110 °C for 1 h. It appears that the mutagenicity of melanoidins is highly dependent upon the reaction conditions. The mutagenic activities and UV spectra of melanoidins prepared from D-glucose and L-tryptophan are shown in Fig. 2a and 2b.

TABLE I. Yield of Nondialyzable Melanoidins Obtained from the Reaction Mixtures of Carbohydrates and L-Tryptophan

Melanoidins from	Reaction conditions		Yield[b] (mg/5 mmol of carbohydrate)
	Temp. (°C)	Time[a]	
D-Xylose + Trp	37	4 w	66.9
D-Arabinose + Trp	37	4 w	39.1
D-Glucose + Trp	37	4 w	23.8
D-Fructose + Trp	37	4 w	38.7
Maltose + Trp	37	4 w	44.6
Lactose + Trp	37	4 w	56.4
Sucrose + Trp	37	4 w	57.8
3-Deoxy-D-glucosone + Trp	37	4 w	56.6
Furfural + Trp	37	4 w	189.7
5-Hydroxymethylfurfural + Trp	37	4 w	158.7
Diacetyl + Trp	37	4 w	95.8
Triose reductone + Trp	37	4 w	97.4
D-Glucose + Trp	110	1 h	5.7
D-Glucose + Trp	110	2 h	21.7
Diacetyl + Trp	110	1 h	44.2
Diacetyl + Trp	110	2 h	56.4
Furfural + Trp	110	1 h	64.5
Furfural + Trp	110	2 h	47.2

a) w, week; h, hour. *b*) The figures showed the mean values of two experiments. A mixture of a carbonyl compound (10 mmol) and L-tryptophan (5 mmol) was dissolved in 0.1 M phosphate buffer (pH 7.0, 50 ml) and kept at 37 °C for 4 weeks or at 110 °C for 1—2 h. The reaction mixture was dialyzed at 4 °C against distilled water and then lyophilized.

TABLE II. Mutagenic Activity of Nondialyzable Melanoidins Obtained from the Reaction Mixtures of Carbohydrates and L-Tryptophan Incubated at 37 °C

Melanoidins from	Dose (μg/plate)	TA100, His^+ revertants/plate	
		Untreated	Nitrite-treated
D-Xylose + Trp	200	145	313
D-Arabinose + Trp	400	136	275
D-Glucose + Trp	400	112	268
D-Fructose + Trp	400	136	224
Maltose + Trp	400	165	220
Lactose + Trp	400	152	252
Sucrose + Trp	400	148	213
3-Deoxy-D-glucosone + Trp	200	150	561
Furfural + Trp	200	137	841
5-Hydroxymethylfurfural + Trp	200	147	529
Diacetyl + Trp	200	143	957
Triose reductone + Trp	200	150	576
AF-2	0.01	735	
Control		135	142

A mixture of a carbonyl compound (0.1 mol) and L-tryptophan (0.05 mol) was dissolved in 500 ml of 0.1 M phosphate buffer (pH 7.0) and incubated at 37 °C for 4 weeks. Then, the reaction mixture was dialyzed at 4 °C against distilled water and lyophilized. The mutagenic activity of the residue was assayed on *S. typhimurium* TA100 before and after nitrite treatment at pH 4.0.

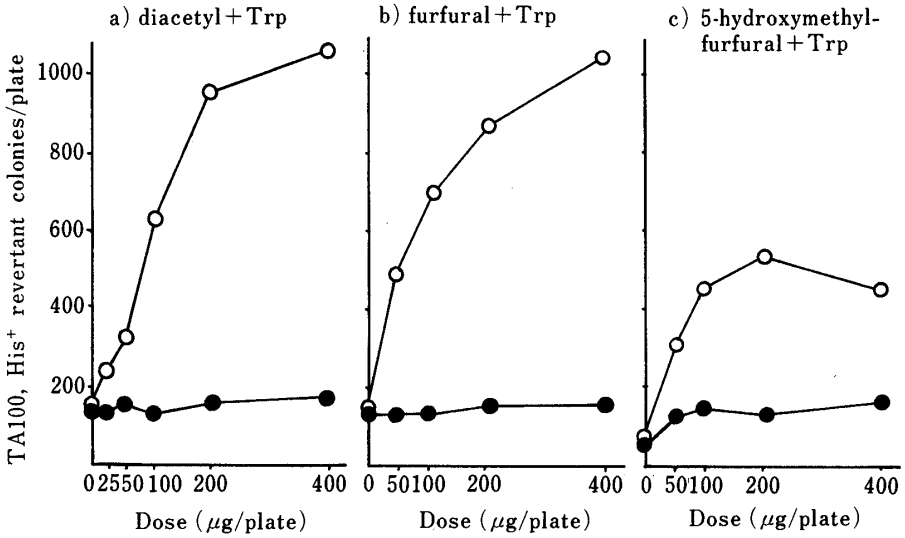

Fig. 1. Dose–Response Curves of Mutagenic Activity of Nondialyzable Melanoidins Obtained from the Reaction Mixtures of Carbonyl Compounds with L-Tryptophan

●—●, untreated; ○—○, treated with nitrite.
The preparation and the mutagenicity assay of each nondialyzable melanoidin were performed as described in Table II.

TABLE III. Mutagenic Activities of Nondialyzable Melanoidins Obtained from the Reaction Mixtures of Carbonyl Compounds and L-Tryptophan Heated at 110 °C

Melanoidins from	Reaction time (h)	Dose (μg/plate)	TA100, His^+ revertants/plate	
			Untreated	Nitrite-treated
Diacetyl+Trp	1	200	211	1616
Diacetyl+Trp	2	200	184	1124
Furfural+Trp	1	400	148	490
Furfural+Trp	2	400	156	427
D-Glucose	1	400	185	1488
D-Glucose	2	400	166	1193
AF-2		0.01	708	
Control			145	163

A mixture of a carbonyl compound (25 mmol) and L-tryptophan (12.5 mmol) was dissolved in 50 ml of 0.1 M phosphate buffer (pH 7.0) and heated at 110 °C for 1—2 h in an autoclave. Then, the reaction mixture was treated and assayed as described in the footnote to Table II.

The melanoidin prepared by method B exhibited 6-fold higher mutagenic activity than that prepared by method A at the dose of 400 μg. The absorbance at 217 nm decreased with increase of the reaction time and reaction temperature. Moreover, the peak of absorption shifted to 274 nm from 279 nm and a third peak appeared at 350 nm in the case of this melanoidin. The Sephadex G-50 gel filtration profiles of melanoidins prepared from D-glucose and L-tryptophan under 3 different conditions are shown in Fig. 3.

The melanoidin obtained by method A showed a single peak at a retention volume of 160 ml and the melanoidin prepared by method B (110 °C, 2 h) exhibited a single peak at 115 ml. Thus, the molecular weight of melanoidins may be proportional to the reaction time and reaction temperature. When each melanoidin was dissolved in distilled water (10 mg/ml),

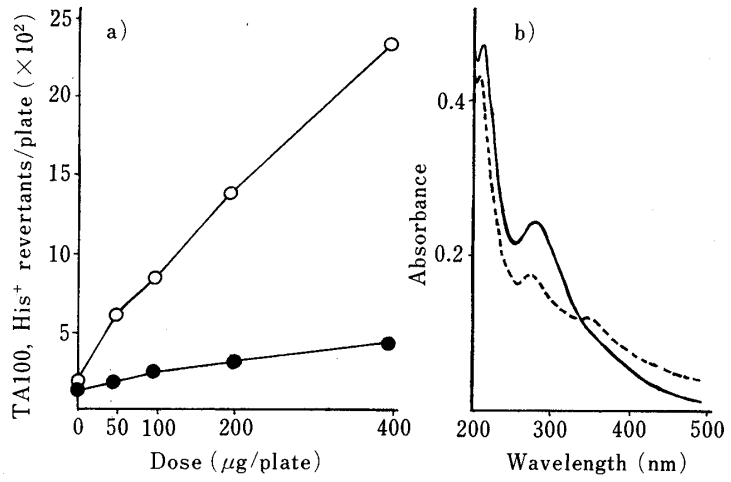

Fig. 2. Mutagenic Activity and UV Spectra of Nondialyzable Melanoidins Obtained from the Reaction Mixture of D-Glucose and L-Tryptophan

a) ●—●, melanoidins obtained at 37 °C for 4 weeks; ○—○, melanoidins obtained at 110 °C for 2 h.
b) ——, melanoidins obtained at 37 °C for 4 weeks; ------, melanoidins obtained at 110 °C for 2 h.
A 0.1% aqueous solution was used for measuring the UV spectra.

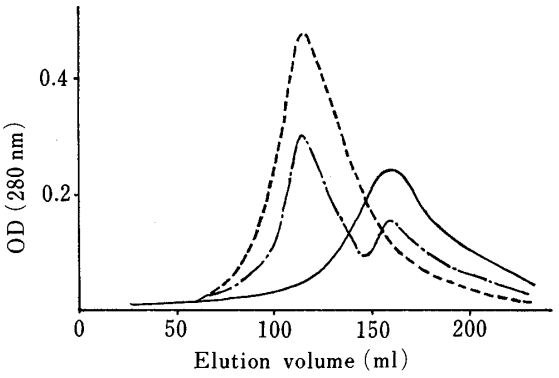

Fig. 3. Gel Filtration of Nondialyzable Melanoidins Obtained from the Reaction Mixtures of D-Glucose and L-Tryptophan on Sephadex G-50

——, melanoidins obtained at 37 °C for 4 weeks; —·—, melanoidins obtained at 110 °C for 1 h; ------, melanoidins obtained at 110 °C for 2 h.
A sample (10 mg) was dissolved in 1 ml of 0.1 M phosphate buffer (pH 7.0) containing 0.1 M NaCl and applied to a column (i.d. 1.5 cm × 45 cm). Elution was carried out with the same buffer at a flow rate of 43 ml/h at room temperature.

the solution exhibited pH 5.6—5.8, and on the filter paper, they showed positive reactions with ninhydrin and ammoniacal silver nitrate. The solution, moreover, discolored a 1% ethanol solution of 1,1-diphenyl-2-picrylhydrazil, a radical scavenger. The properties mentioned above are general characteristics of melanoidins.[13]

This study has shown that nondialyzable melanoidins are formed as nitrogenous products by dehydration, condensation and polymerization of various carbohydrates with L-tryptophan. Though they have no mutagenic activity toward *S. typhimurium* TA100 without nitrite treatment, some of them induced his^+ revertants after nitrite treatment. The melanoidins from pentoses (D-xylose, D-arabinose), hexoses (D-glucose, D-fructose) and disaccharides (maltose, lactose, sucrose) showed weak mutagenic activities. However, the melanoidins from 3-deoxy-D-glucosone, furfural and 5-hydroxymethylfurfural, which are important intermediate products in amino–carbonyl reactions, and the melanoidins from diacetyl and triose reductones, which are decomposition products of sugars, induced significant numbers of his^+ revertants after nitrite treatment. It may be presumed that amino or imino groups in the melanoidin molecule are modified to produce mutagens after nitrite treatment.

Recently, the mutagenicity of soy sauce treated with nitrite was studied by Lin *et al.*,[14] and the mutagenicities of β-carboline derivatives and tyramine were investigated by Ochiai *et*

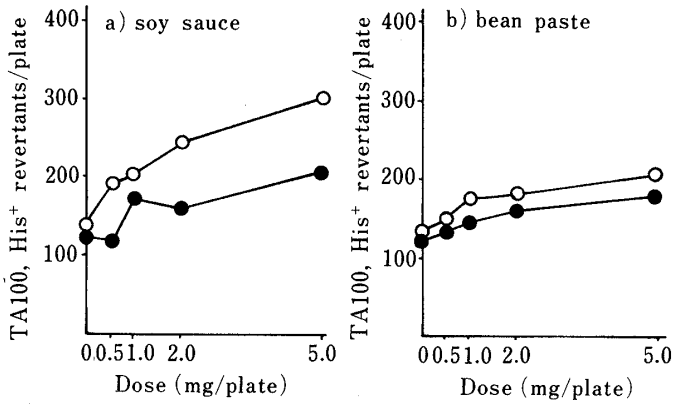

Fig. 4. Mutagenic Activity of Nondialyzable Substances Obtained from Daily Foods

●—●, untreated; ○—○, treated with nitrite.
The nondialyzable material was prepared from soy sauce or bean paste and assayed for mutagenicity before and after nitrite treatment as described in Experimental.

al.[15] It is well known that brewed foods contain amino–carbonyl reaction products such as 3-deoxy-D-glucosone and 5-hydroxymethylfurfural.[16] We were interested in the mutagenicity of nondialyzable substances (including melanoidin) in daily foods. The nondialyzable substances were isolated from soy sauce (brand A) and bean paste (brand B), which are major protein sources for Japanese people. They were tested for mutagenic activity toward *S. typhimurium* TA100 with and without nitrite treatment (Fig. 4).

After nitrite treatment, the number of net his^+ revertants of the nondialyzable substances of soy sauce increased from 86 to 156 at the dose of 5 mg per plate (equivalent to 0.41 ml of soy sauce). In the case of bean paste, the nondialyzable substance showed 56 and 89 net his^+ revertants at the same dose (equivalent to 0.58 g of bean paste) before and after nitrite treatment, respectively. Although the content and the mutagenic activity of the nondialyzable substances in daily foods can be expected to be variable depending on the brand, preparation method and storage conditions, it may be presumed that they can serve as precursors of mutagens.

The physical and chemical properties of nondialyzable products obtained from model systems, soy sauce and bean paste, are under study.

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