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Inhibition of Nitrosamine Formation by Nondialyzable Melanoidins

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The degradation of nitrite and the inhibition towards formation of carcinogenic nitrosamines by melanoidins produced from the glucose-glycine system were investigated at various conditions. The degradation of nitrite was highest at pH 1.2 (29%), when the ratio of melanoidins to nitrite was 1:3. The inhibition towards formation of nitrosamines by melanoidins had the same tendency as the degradation of nitrite, the inhibition also being highest at pH 1.2 (99%). In addition, melanoidins after nitrite treatment exhibited a little higher mutagenicity and much stronger desmutagenicity than those of the original melanoidins. The change of the structure of melanoidins after treating with nitrite was also investigated by HPLC and CP-MAS NMR.

The Maillard reaction is very important in food manufacturing, and its results can be either desirable or undesirable. Recently, research on the formation of several weak mutagens by the Maillard reaction at 100°C,[1] and on the isolation of many strong mutagens from cooked foods were reported.[2,3] Hitherto, it has been known that the factors which inhibit or modify the mutagens are presented in foods or in biological systems. The nitrosamines are generally produced by an electrophilic reaction of nitrite with the corresponding secondary amines. The biological effect which mostly concerns humans is carcinogenicity, and in order to prevent this, it is very important to inhibit the formation of nitrosamines. Much research has been done on the formation of nitrosamines in food[4,5] and in biological systems.[6,7] There has also been some investigations on the mutagenicity of the Maillard reaction products,[8] and on melanoidins produced from carbohydrate and L-tryptophan before and after nitrite treatment.[9]

However, there has been no report on the effects of melanoidins against mutagens. In the previous papers,[10,11] we found that melanoidins produced from glucose and glycine had desmutagenicity against several mutagens, but the details remained to be clarified.

In this paper, the inhibition towards formation of nitrosodiethylamine and nitrosopyrrolidine at various conditions by melanoidins was investigated, and the change of mutagenicity or desmutagenicity of melanoidins before and after nitrite treatment is also reported.

MATERIALS AND METHODS

Materials. Glucose, glycine, sodium nitrite ($NaNO_2$), sodium borohydride ($NaBH_4$) and sodium bicarbonate ($NaHCO_3$) were from Kanto Chemical Co., Tokyo. Diethylamine and pyrrolidine were from Tokyo Kasei Chemical Co., Tokyo. N-nitrosodiethylamine (NDEA) and N-nitrosopyrrolidine (NPYR) as standards were from Nakarai Chemical Co., Kyoto. Trp-P-1 was from Wako Pure Chem. Ind. Ltd., Tokyo, and S-9 was from Oriental Yeast Co., Ltd., Tokyo. All other chemicals used were of analytical reagent grade.

Preparation of nondialyzable and reduced melanoidins. Glucose (1 mol), glycine (1 mol) and $NaHCO_3$ (0.2 mol) were dissolved in 500 ml of deionized and distilled water (pH 6.8) and then refluxed in an oil bath at 95°C for

7 hr. The resulting brown solution was dialyzed against deionized and distilled water for 1 week and then lyophilized. The dried powder was extracted with diethyl ether, and the nondialyzable melanoidins were obtained. The reduced melanoidins were prepared by reducing the nondialyzable melanoidins (1.5 g) with $NaBH_4$ (2 g) in 200 ml of deionized and distilled water (pH 8.0) at room temperature for 24 hr, and then lyophilizing.

Formation of NDEA and NPYR. NDEA and NPYR were prepared from the secondary amines, *i.e.*, diethylamine or pyrrolidine and $NaNO_2$. 0.5 ml of 1 M $NaNO_2$ and 0.25 ml of 0.5 M diethylamine or pyrrolidine were dissolved in various buffer solutions [pH 1.2 (0.1 N HCl solution), pH 3.0, 4.2 and 6.1 (0.2 M citrate buffer)] and made up to 10 ml. After incubating at 37°C for 2 hr by shaking at 60 ~ 70 rpm, ammonium sulfamate (0.7 g) was added to stop the reaction. The pH of the reaction mixture was adjusted to 10 by 6 N NaOH or 6 N HCl. These reaction solutions were extracted three times with diethyl ether after adding sodium chloride (2 g). Anhydrous sodium sulfate was added for dehydration and, after filtration, the extract was concentrated *in vacuo* at room temperature.

GC-FTD analysis of NDEA and NPYR. The quantities of the nitrosamines formed were measured by a Shimadzu model 7A gas chromatograph equipped with a flame thermionic detector (FTD), a fused silica WCOT capillary column (50 × 0.25 mm i.d.) coated with PEG-20M being used. The column oven was programmed from 100 to 200°C at a rate of 4°C/min, and the injection port and detector temperature were maintained at 220°C. Nitrogen was used as the carrier gas at a flow rate of 1.5 ml/min with a split ratio of 1 : 25. Measurement of the peak area was done by a Shimadzu chromatopac integrator (model EI-B) connected to the gas chromatograph. One μl of the sample was injected and pyridine in methanol was used as an internal standard.

Degradation of nitrite by melanoidins. The degradation of nitrite by nondialyzable melanoidin and its reduced form was measured by the colorimetry method using Griess reagents.[12] Each melanoidin (10 mg) and $NaNO_2$ (34.5 mg) were mixed and incubated, before adding ammonium sulfamate. Then 1 ml of the sample, 5 ml of 2% acetic acid and 0.4 ml of Griess reagents were mixed. After 15 min, the color intensity was measured by a spectrophotometer at 520 nm.

Measurement of the discoloration of melanoidins after nitrite treatment. Nondialyzable melanoidin or its reduced form (10 mg) and $NaNO_2$ (34.5 mg) were dissolved in various buffer solutions (pH 1.2, 3.0, 4.2 and 6.1), and made up to 10 ml. After incubating at 37°C for 2 hr, ammonium sulfamate was aded, and the color intensity of the reaction mixture was measured at 470 nm.

Preparation of nitrite-treated melanoidins. Nondialyzable melanoidins (10 mg or 20 mg) and 0.5 ml of 1 M $NaNO_2$ or 1 ml of 3 M $NaNO_2$ were dissolved in a 0.1 N HCl solution (pH 1.2), made up to 10 ml, and then incubated at 37°C for 1 hr or 2 hr, respectively. In addition, nondialyzable melanoidin (20 mg) and 1 ml of 3 M $NaNO_2$ were dissolved in 0.2 M citrate buffer (pH 4.0), made up to 10 ml, and then incubated at 37°C for 1 hr. After incubating, ammonium sulfamate was added and the reaction solutions were dialyzed for 2 days, before being lyophilized to obtain the nitrite-treated melanoidin.

Mutation assay of melanoidins before and after nitrite treatment. The mutagenicity or desmutagenicity of melanoidins was assayed by a modification[13] of the method of Ames *et al.*,[14] using *Salmonella typhimurium* TA 100 in the absence of S-9 mix or *Salmonella typhimurium* TA 98 against Trp-P-1 in the presence of S-9 mix, respectively. The desmutagenic effect is expressed as the percentage loss of mutagenicity.

HPLC analysis of melanoidins. The nondialyzable melanoidins and nitrite-treated melanoidins were analyzed by gel permeation chromatography (HPLC), the analysis being performed by a Hitachi 638-30 liquid chromatograph under the following conditions: column, stainless steel (50 cm × 7.6 mm) prepacked with Asahi PAK GS-320; detector, Shodex RI SE-31 and a Hitachi spectrophotometer (model 100-50) at 280 nm. The column was eluted with 0.1 N NaCl containing 1 mM sodium azide at a flow rate of 0.5 ml/min, the operating pressure being kept at 28 kg/cm^2.

NMR spectroscopy. The nondialyzable melanoidins and nitrite-treated melanoidins were submitted to ^{13}C cross polarization-magic angle spinning (CP-MAS) NMR analysis. ^{13}C CP-MAS NMR spectra were recorded with a JEOL GX-270 NMR spectrometer operating at 67.8 MHz in the pulsed Fourier-transform mode. These spectra were recorded with 8,192 data points. The cross polarization time was 2 msec and the recyling time 5 sec. The number of scans was 2400 ~ 4700.

RESULTS AND DISCUSSION

Degradation of nitrite by melanoidins at various pH levels

Nitrosamines are known to be formed by the reaction of secondary amines with nitrosating agents. Generally, it has been considered that inhibition towards formation of nitrosamines was occurred by the degradation of nitrite itself, and the inhibition of nitrosation of secondary amines by chemicals having a reducing ability.

TABLE I. DECREASE IN NITRITE BY NONDIALYZABLE MELANOIDINS AT VARIOUS pH CONDITIONS

Melanoidins	Decrease in nitrite (%)			
	pH 1.2	pH 3.0	pH 4.2	pH 6.1
Nondialyzable melanoidins	29.0±3.1[a] 99.0±0.2[b]	27.8±2.9 95.1±0.6	5.3±1.0 61.9±8.4	1.7±0.8 21.4±1.9
Reduced melanoidins	26.0±3.7[a] 53.0±1.3[b]	19.2±5.1 48.5±1.6	6.8±2.4 22.4±0.5	3.7±1.1 4.2±2.2

[a] Melanoidins (10 mg) were treated with nitrite (34.5 mg) in 10 ml of each pH solution at 37°C for 2 hr.
[b] Melanoidins (10 mg) were treated with nitrite (276 μg) in 10 ml of each pH solution at 37°C for 1 hr.
Data are the average of three determinations ± standard deviation.

Table I shows the change in quantity of nitrite by nondialyzable and reduced melanoidins at various pH levels (pH 1.2, 3.0, 4.2 and 6.1). In the case of nondialyzable melanoidins, the quantity of nitrite decreased after the reaction, and the degradation degree was higher at acidic pH conditions, being highest at pH 1.2 (29%). From these results, it can be inferred that nitrite was degraded by melanoidins, but there is a possibility that this was due to the reaction between nitrite and the NH of melanoidins, and nitrite was decomposed to NO or HNO_3. In the case of reduced melanoidins, the same tendency as that of nondialyzable melanoidins was observed. Mirvish *et al.*[15] and Fan[16] have investigated the effects of ascorbic acid, α-tocopherol and other antioxidants against nitrite. In the case of ascorbic acid, when a concentration of 0.18% (w/v) was used against nitrite at a concentration of 0.07% (w/v), it was found that about 60% of nitrite was decomposed at pH 2.0 and 25°C after 30 min.[16] In the case of triose reductone,[17] an intermediate product of the Maillard reaction, the same tendency as that of ascorbic acid was observed, and the decomposing capacity of triose reductone against nitrite was similar to that of ascorbic acid. It was also postulated that the blocking of the nitrosation reaction by ascorbic acid was probably due to competition for the available nitrite, or nitrous anhydride. On the other hand, α-tocopherol, which possesses a chroman ring as well as the phenolic functional group, probably reacts in the same manner, causing reduction of the nitrite to nitric oxide. As shown in Table I, the degradation ability of nondialyzable melanoidins against nitrite was stronger than that of reduced melanoidins. From our previous study,[10] it is known that reduced melanoidins had only half the reducing ability in comparison with that of the original melanoidins. Ascorbic acid and triose reductone also have strong reducing ability, and consequently, reducing ability can be considered as a major factor in the degradation of nitrite. In our study, melanoidins were used at a concentration of 0.1% (w/v) against nitrite at a concentration of 0.34% (w/v); in this case, the decrease of nitrite was about 29% at pH 1.2 and 37°C after 2 hr. When nitrite was used at a lower concentration (approximately 0.003%, w/v), the decrease of nitrite was about 99% at pH 1.2 and 37°C after 1 hr. Thus, it can be concluded that nondialyzable melanoidins are very effective for the degradation of nitrite.

Inhibition of NDEA and NPYR formation by melanoidins

It was well known that compounds having reducing ability such as ascorbic acid, α-tocopherol, phenolic compound, glutathione and cysteine inhibit the formation of nitrosamines in model systems.[18]

Table II shows the inhibition percentage towards formation of NDEA and that of NPYR by melanoidins at various pH levels. In the case of the formation of NDEA, the inhibition was highest at pH 1.2 (99.1%) by

TABLE II. INHIBITION OF NITROSAMINE FORMATION BY MELANOIDINS AT VARIOUS pH CONDITIONS

Melanoidins		Inhibition (%)			
		pH 1.2	pH 3.0	pH 4.2	pH 6.1
Nondialyzable melanoidins	NDEA	99.1±0.4	99.0±0.2	30.9±7.6	66.7±4.6
	NPYR	60.6±1.4	82.8±1.9	34.8±7.6	55.9±3.2
Reduced melanoidins	NDEA	38.0±1.6	9.9±2.2	—	—
	NPYR	20.0±0.9	7.9±2.4	—	—

Melanoidins (10 mg) were treated with nitrite (34.5 mg) and diethylamine (9.1 mg) or pyrrolidine (8.9 mg) in 10 ml of each pH solution at 37°C for 2 hr. NDEA, *N*-nitrosodiethylamine; NPYR, *N*-nitrosopyrrolidine.
Data are the average of three determinations ± standard deviation.

nondialyzable melanoidins. On the other hand, in the case of NPYR, the inhibition was highest at pH 3.0 (82.8%). And the inhibition of nitrosamines formation by nondialyzable melanoidins was lowest at pH 4.2, however, further experiments are necessary in order to make clear this problem. The reduced melanoidins, which had half the reducing ability in comparison with nondialyzable melanoidins, showed the same tendency as the original melanoidins; however, the inhibitive effect on the formation of nitrosamines at various pH levels was lower than that of the original melanoidins. In the case of ascorbic acid, Mirvish *et al.*[15] have reported that when a concentration of 8.8% (w/v) was used against nitrite with a concentration of 2% (w/v) at pH 3 or 4 and 25°C for 1 hr, nitrosamines were inhibited by about 90%. In the case of α-tocopherol, Pensabene *et al.*[19] have reported that when a concentration of 0.05~0.1% (w/v) was used against nitrite with a concentration of 0.002% (w/v) at pH 6 and 52°C for 2 hr, nitrosamines were inhibited by about 66%. In this study, with melanoidins, at a lower ratio [1 (0.1%) : 3 (0.34%)] of melanoidins to nitrite and at pH 1.2, the blocking percentage was found to be 99.1%. From these results, it can be inferred that the reducing ability of melanoidins played an important role in the inhibition of nitrosamine formation.

However, the inhibition percentage of nitrosamine formation (approx. 60.6~99.1%) was much higher than the degradation of nitrite (approx. 29%). Therefore, it is necessary to elucidate the changes in the structure of melanoidins before and after nitrite treatment.

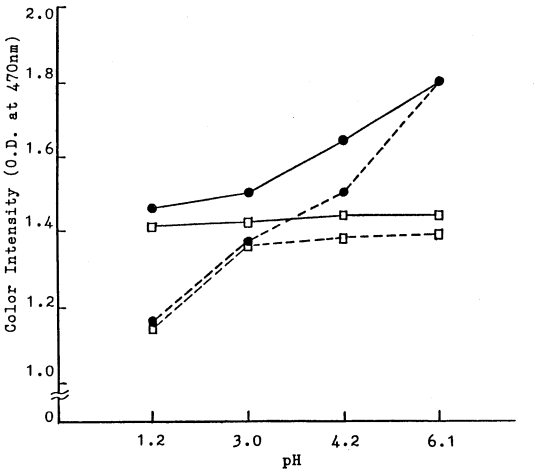

FIG. 1. Change in the Color Intensity of Melanoidins by the Reaction with Nitrite at Various pH Conditions.
Melanoidins (10 mg) were treated with nitrite (34.5 mg) in 10 ml of each pH solution at 37°C for 2 hr. ●—●, nondialyzable melanoidins + nitrite (control); ●---●, nondialyzable melanoidins + nitrite (after the reaction); □—□, reduced melanoidins + nitrite (control); □---□, reduced melanoidins + nitrite (after the reaction).

Discoloration of melanoidins by nitrite treatment at various pH levels

Figure 1 shows the change in color intensity before and after the nitrite treatment of nondialyzable and reduced melanoidins. The color intensities of both melanoidin solutions at various pH levels were decreased by the reaction with nitrite. The differences between the

color intensities of melanoidins before and after nitrite treatment were higher at acidic pH conditions, being highest at pH 1.2. From this result, it may be concluded that the melanoidin structure was changed by the reaction with nitrite.

Characteristics of melanoidins before and after nitrite treatment

Table III shows the mutagenicity and desmutagenicity of nondialyzable melanoidins, nitrite-treated melanoidins and the solution of melanoidins reacted with nitrite without dialysis by the Ames test.

TABLE III. CHANGES OF THE MUTAGENICITY AND DESMUTAGENICITY OF MELANOIDINS DERIVED FROM A D-GLUCOSE–GLYCINE SYSTEM

Melanoidins (2 mg/plate)	Mutagenicity*	Desmutagenicity**
	Revertants/plate (inhibition, %)	
Nondialyzable melanoidins	100 ± 5	208 ± 10 (63.6)
Nitrite-treated melanoidins[a]	121 ± 10	64 ± 3 (88.8)
	130 ± 7[d]	219 ± 4[d] (61.7)
Nitrite-treated melanoidins[b]	125 ± 7	105 ± 9 (81.6)
Nitrite-treated melanoidins[c]	118 ± 9	102 ± 6 (82.2)

* Mutagenicity was assayed by the preincubation method, using *S. typhimurium* TA 100 in the absence of S-9 mix. Data are the average of three determinations ± standard deviation. The revertants for distilled water as a control were 85 ± 5.

** Desmutagenicity against Trp-P-1 (0.1 μg/plate) was assayed by the preincubation method, using *S. typhimurium* TA 98 in the presence of S-9 mix. The revertants for Trp-P-1 as a control were 572 ± 6.

[a] Nondialyzable melanoidins (20 mg) were treated with nitrite (34.5 mg) in 10 ml of 0.1 N HCl solution (pH 1.2) at 37°C for 2 hr. After the incubation, nitrite-treated melanoidins were obtained by dialysis and lyophilization.

[b] Nondialyzable melanoidins (20 mg) were treated with nitrite (207 mg) in 10 ml of 0.1 N HCl solution (pH 1.2) at 37°C for 1 hr.

[c] Nondialyzable melanoidins (20 mg) were treated with nitrite (207 mg) in 10 ml of citrate buffer (pH 4.0) at 37°C for 1 hr.

[d] Nitrite-treated melanoidins (a) without dialysis (0.1 ml/plate).

The nitrite-treated melanoidins showed about 80% of inhibition against Trp-P-1, while that of the original melanoidins was only about 60%. On the other hand, the solution of melanoidins reacted with nitrite without dialysis produced a little higher mutagenicity and lower desmutagenicity (61.7%) than that of the originals.

The change in molecular weight of melanoidins before and after nitrite treatment was investigated by HPLC, the results revealing that nitrite-treated melanoidins showed a tendency to decrease in overall mean molecular weight (data not shown), detection of these two kinds of melanoidins by UV and RI showing similar patterns. In addition, the structure of melanoidins before and after nitrite treatment was studied by ^{13}C CP-MAS NMR (Fig. 2). While comparing the two spectra, it was observed that in the spectrum of nitrite-treated melanoidins, signals in the regions of 60 ~ 65 ppm and 100 ~ 140 ppm decreased, and signals in the region of 80 ~ 100 ppm mostly disappeared. The signals in the region of 60 ~ 140 ppm are considered to be the signals of aliphatic carbons bonded to

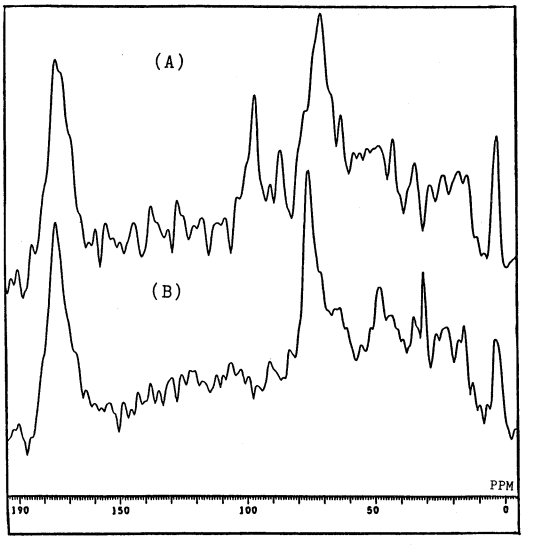

FIG. 2. ^{13}C CP-MAS NMR Spectra of D-Glucose–Glycine System for Nondialyzable Melanoidins before (A) and after (B) Nitrite Treatment.

an oxygen or nitrogen atom, and this region is also attributed to unsaturated carbons. When melanoidins were reacted with nitrite, these functional groups as well as the double bond of the unsaturated carbons were modified. Moreover, the signals in the region of $0 \sim 50$ ppm from aliphatic carbons also slightly decreased and it might be proposed that cleavage of the methylene or methyl groups of nondialyzable melanoidins by nitrite treatment occurred. Consequently, the molecular weight of melanoidins decreased, this proposition being supported by data on the aforementioned HPLC analysis of the molecular weight of melanoidins.

From all these results, it is assumed that the mutagenic and desmutagenic properties of melanoidins were governed by the change in structure of the melanoidins by nitrite treatment, and more studies are now in progress.

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