# Isolation and Characterization of the Most Antimutagenic Maillard Reaction Products Derived from Xylose and Lysine

Gow-Chin Yen,* Chi-Fai Chau, and Jen-Dan Lii

Department of Food Science, National Chung Hsing University, 250 Kuokuang Road, Taichung, Taiwan, Republic of China

The Maillard reaction products were prepared by refluxing D-xylose and L-lysine at 100 °C and pH 9.0 for 10 h and then fractionated into various molecular weight (MW) ranges by membrane filters. The most antimutagenic effect toward 2-amino-3-methylimidazo[4,5-*f*]quinoline was observed in the fractions of MW 30 000–50 000 and MW 50 000–100 000 toward TA98 and MW 50 000–100 000 toward TA100, whereas the fraction of MW 50 000–100 000 showed the strongest activity against 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole toward TA98 or TA100 at the dose of 0.8 mg/plate. Fractions with MW above 30 000 showed inhibitory effect to 4-nitroquinoline *N*-oxide and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine on TA100. No significant correlations ($P > 0.05$) were observed between the antimutagenicity of each fraction and the browning intensity, reducing power, and antioxidative activity. The fraction with MW 50 000–100 000 was further separated by Sephadex G-100 and HPLC, and the peak GIIa exhibited the strongest antimutagenic effect with molecular weight about 63 000.

## INTRODUCTION

In the past two decades, numerous mutagens from some foods have been detected and caused remarkable concern about their actual involvement in carcinogenesis, whereas an antimutagenic effect has also been observed in the same food and confused people (Aeschbacher 1990; Sugimura and Wakabayashi, 1990). The Maillard reaction or the nonenzymatic browning reaction is an actual example among them. Many strong mutagens, including 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1), 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2), 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), and 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]imidazole (Trp-P-2), have been isolated from pyrolyzates of amino acids and proteins and from charred parts of fishes and meats (Sugimura et al., 1977; Yamamoto et al., 1978; Kasai et al., 1980). However, few reports indicated that Maillard reaction products (MRPs) in food might have antimutagenic property, including glucose–lysine, glucose–glycine, sucrose–glycine, glucose–tryptophan, and xylose-lysine systems (Chan et al., 1982; Kato et al., 1985b; Kim et al., 1986; Yamaguchi and Iki, 1986; Yen and Lii, 1992a,b). Kong et al. (1989) also studied the antimutagenicity of amino–carbonyl mixtures of phenylalanine or cysteine and glucose or fructose in the presence of malic acid. Kim et al. (1986) studied the desmutagenic effects of melanoidins from a D-glucose and glycine system and claimed the fraction of molecular weight (MW) above 5000 showed the strongest activity of all fractions (i.e., MW below 1000, MW between 1000 and 5000 and MW above 5000).

Recently, Yen et al. (1992) investigated the antimutagenicity of 12 MRPs prepared from four amino acids (glycine, L-lysine monohydrochloride, tryptophan, and L-arginine monohydrochloride) and three sugars (glucose, xylose, and fructose) and showed that MRPs obtained from xylose and lysine have the strongest antimutagenic effect against the mutagenicity of IQ, Glu-P-1, and Trp-P-1 toward *Salmonella typhimurium* TA98; the antimutagenic effect of MRPs was also correlated to their antioxidative activity and reducing power. In a further study (Yen and Lii, 1992a), the effect of reaction conditions on the antimutagenicity of MRPs prepared from xylose and lysine was studied to find the optimum reaction condition for preparation of stronger antimutagenic MRPs. Three reaction condition combinations exhibited over 70% inhibitory effect to IQ toward *S. typhimurium* TA98 and TA100.

The objectives of this study were to further fractionate the strongest antimutagenic compound(s) in xylose–lysine MRPs, to characterize their properties, and to realize the relationship between antimutagenicity and other characteristics of xylose–lysine MRPs.

## MATERIALS AND METHODS

**Materials.** D-Xylose, L-lysine monohydrochloride, 4-nitroquinoline *N*-oxide (NQNO), and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) were obtained from Sigma Chemical Co. (St. Louis, MO). 2-Amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) were purchased from Wako Pure Chemical Co. (Tokyo).

**Preparation of Xylose–Lysine Maillard Reaction Products (XL MRPs).** The XL MRPs with the most antimutagenicity were prepared under the optimal reaction condition selected in the previous study (Yen and Lii, 1992a). D-Xylose (1.0 M) and L-lysine monohydrochloride (2.0 M) were dissolved in potassium phosphate buffer (0.1 M, pH 9.0) and refluxed at 100 °C in an oil bath for 10 h.

**Fractionation of XL MRPs by Membrane Filters.** The XL MRPs was fractionated with membrane filters (Diaflo ultrafilter, Amicon Division, W. R. Grace and Co., Danvers, MA) into five fractions: MW below 10 000, MW 10 000–30 000, MW 30 000–50 000, MW 50 000–100 000, and MW above 100 000. Each fraction was freeze-dried and diluted into four concentrations: 25, 17, 11, and 8 mg/mL. The mutagenicity and growth inhibitory effect of each fraction were determined toward *S. typhimurium* TA98 and TA100. The dose of each fraction with no mutagenicity and growth inhibitory effect was selected for evaluation of the antimutagenicity. The mutagens used were two direct-acting mutagens, NQNO (0.1 μg/plate) and MNNG (0.5 μg/plate), and two indirect-acting mutagens, IQ (0.1 μg/plate for TA98 and 0.5 μg/plate for TA100) and Glu-P-1 (0.05 μg/plate for TA98 and 0.5 μg/plate for TA100) which require S9 mixture for metabolic activation. The most antimutagenic fraction of XL MRPs was selected for further separation by gel filtration.

* Author to whom correspondence should be addressed.

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After proper dilution with distilled water, the browning intensity, reducing power, and antioxidative activity of each fraction were determined.

**Solvent Extraction.** The most antimutagenic fraction obtained by membrane separation was freeze-dried and extracted with 250 mL of petroleum ether, chloroform, methanol, acetone, *n*-hexane, and water, respectively. Each extract was evaporated to dryness in a rotary evaporator under vacuum at 35 °C, except the water extract was freeze-dried. The unextracted sample (freeze-dried) and each solvent extract were redissolved in 20 mL of phosphate buffer (0.01 M, pH 7.4). The aliquots were filter-sterilized (0.2 μm, Millipore), and the antimutagenicity was determined.

**Separation of the Most Antimutagenic Fraction of XL MRPs by Gel Filtration.** Five milliliters of the most antimutagenic fraction of XL MRPs obtained by membrane filter was applied to a Sephadex G-100 column (2.6 × 100 cm) and eluted with distilled water at a flow rate of 1.0 mL/min; 10 mL of eluate was collected per tube, and 0.1 mL of each tube fraction was used to perform antimutagenic assay. According to the preliminary antimutagenic results, fractions of tube numbers 44 and 45, 46–50, and 51–54 were combined into groups GI, GII, and GIII, respectively. Each subfraction was freeze-dried and diluted to 10 and 1 mg/mL for determination of the mutagenicity and antimutagenicity toward *S. typhimurium* TA98 and TA100.

**Purification of the Most Antimutagenic Subfraction of XL MRPs by HPLC.** A Hitachi (Hitachi, Ltd., Tokyo) high-performance liquid chromatograph was used; it consisted of a Model L-6000 pump, a Rheodyne Model 7125 syringe loading injector with 100-μL loop, a Model L-3000 photodiode array detector, and a D-6000 HPLC manager. A LiChrosorb RP-18 column (7 μm, 250 × 10 mm i.d., E. Merck) was used in this analysis. The mobile phase was methanol/water/acetic acid 400:200:3 (v/v/v) with 1.5 mL/min at ambient temperature. One hundred microliters of the most antimutagenic subfraction obtained by gel filtration was injected. Each peak fraction was collected, and the mutagenicity toward *S. typhimurium* TA98 and TA100 was determined. The dose of each peak with no mutagenicity was selected for the antimutagenic assay.

**Estimation of Molecular Weight of the Most Antimutagenic Fraction.** To estimate the molecular weight of the most antimutagenic fraction, the elution curve by Sephadex G-100 column was compared with the one established with a calibration kit solution containing ribonuclease A (MW 13 700), chymotrypsinogen A (MW 25 000), ovalbumin (MW 43 000), bovine serum albumin (MW 67 000), and blue dextran 2000 (MW 2 000 000) (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). The molecular weight of the most antimutagenic fraction purified by HPLC was also estimated by the same method.

**Browning Intensity.** After a dilution (1000 times) with distilled water, the browning intensity of samples was determined by measuring the absorbance at 420 nm with a Hitachi Model L-2000 spectrophotometer.

**Reducing Power.** The reducing power of samples was determined according to the method of Oyaizu (1986) with potassium ferricyanide, and the absorbance was measured at 700 nm. A high OD value indicates a high reducing power.

**Antioxidative Activity.** The sample (0.1 mL) was mixed with linoleic acid emulsion (2 mL, 10 mM, pH 6.5) in the test tubes and placed in darkness at 37 °C to accelerate the oxidation. After 15 h of incubation at 37 °C, substrate–MRP was added in absolute methanol (2 mL). Then, methanol in water (6 mL, 60%) was added, and the absorbance of the reaction mixture at 234 nm was measured. The antioxidative activity (AOA) was calculated according to the method described by Lingnert et al. (1979): AOA = ($\Delta A_{234}$ of control − $\Delta A_{234}$ of sample)/$\Delta A_{234}$ of sample. An AOA value equal to 1 indicates the strongest antioxidative activity of sample.

**Mutagenicity Assay.** The mutagenicity of MRPs were determined according to the Ames test with a 20-min preincubation at 37 °C (Maron and Ames, 1983). The histidine-requiring strains of *S. typhimurium* TA98 and TA100 were kindly supplied by Dr. B. N. Ames (University of California—Berkeley). The S9 mix (Organ Teknika Co.) was prepared from Sprague-Dawley male rats treated with Aroclor 1254 according to the Ames method (Ames et al., 1975). Diluted MRPs (0.1 mL) were added to the overnight-cultured *S. typhimurium* TA98 or TA100 (0.1 mL) and S9 mix (0.5 mL) or phosphate buffer (0.1 mL) in place of S9 mix. The entire mixture was preincubated at 37 °C for 20 min before molten top agar (2 mL) was added; the mixture was poured on a minimum agar plate. The $His^+$ revertant colonies were counted after incubation at 37 °C for 48 h. Assay of each sample was determined in triplicate plates per run, and data presented are means ± SD of three determinations. Two runs for a single experiment were performed to validate the reproducibility. The mutagenicity is expressed as the number of revertants per plate at a given concentration of each sample. In this mutagenicity testing, the result was recognized as positive when the number exceeds twice the number of spontaneous revertants (Ames et al., 1975). If the revertants were less than half the number of spontaneous revertants, the sample was considered to have a growth inhibitory effect toward spontaneous revertants.

**Antimutagenicity Assay.** The antimutagenic effect of each fraction of XL MRPs was assayed according to the Ames method described under Mutagenicity Assay except for the addition of mutagen before preincubation. The mutagens used were NQNO (0.1 μg/plate for TA100) and MNNG (0.5 μg/plate for TA100) (direct-acting mutagens) and IQ (0.1 μg/plate for TA98 and 0.5 μg/plate for TA100) and Glu-P-1 (0.05 μg/plate for TA98 and 0.5 μg/plate for TA100), which required S9 mix for metabolic activation. Mutagen (0.1 mL) was added to the mixture of strain and sample with S9 mix for IQ or with phosphate buffer (0.1 M, pH 7.4) for NQNO. The mutagenicity of each mutagen in the absence of MRPs is expressed as 100%. A smaller percentage of revertants of sample to the revertants of control indicates stronger antimutagenicity of the sample (Francis et al., 1989).

**Statistical Analysis.** The interactions between the treatments and the comparison of the means obtained for each group were calculated as described by Duncan (1955). Statistical differences at $P < 0.05$ were considered to be significant.

## RESULTS AND DISCUSSION

**Mutagenicity of Fractionated XL MRPs by Membrane Filter.** In the preliminary study, the mutagenicity and growth inhibitory effect of each fraction of XL MRPs with various molecular weight ranges toward *S. typhimurium* TA98 and TA100 were evaluated. Mutagenicity was observed in some fractions, i.e., MW below 10 000 (for TA98, −S9), or growth inhibition of spontaneous revertants was found in some fractions, i.e., MW 50 000–100 000 or MW above 100 000 (for TA98, +S9), at the dosage greater than 1.7 mg/plate (data not shown). If mutagenicity or growth inhibitory effect was present in the sample, the results of the antimutagenicity assay thereafter would be influenced and confused due to the increase or decrease in numbers of revertants of *S. typhimurium*. The doses of 1.1 and 0.8 mg/plate were therefore selected for comparative base of the antimutagenicity of each fraction.

**Antimutagenicity of Fractionated XL MRPs by Membrane Filter.** The antimutagenic effect of each XL MRP fraction on the indirect-acting mutagens, IQ and Glu-P-1, is shown in Tables I and II. The fractions of MW 30 000–50 000 and MW 50 000–100 000 toward TA98 and MW 50 000–100 000 toward TA100 showed the stronger antimutagenic effect against IQ at the dose of 0.8 mg/plate, whereas the strongest inhibition effect occurred in the fraction of MW 30 000–50 000 toward TA98 or TA100 at the dose of 1.1 mg/plate (Table I). On the other hand, the fraction of MW 50 000–100 000 showed the strongest antimutagenic effect against Glu-P-1 toward TA98 or TA100 at the dose of 1.1 and 0.8 mg/plate (Table II). These results are in agreement with the desmutagenic effects of melanoidins from a glucose and glycine system previously reported by Kim et al. (1986), but the size of membranes selected in our study was clearly to show the molecular weight range of the strongest antimutagenic component(s).

**Table I. Effect of Fractions of Xylose–Lysine Maillard Reaction Products (MRPs) with Various Molecular Weight Ranges on the Mutagenicity of IQ to *S. typhimurium* TA98 and TA100 in the Presence of S9 Mix**

| MRP[a] | dose, mg/plate | His$^+$ revertants/plate[b] (percentage) | |
|---|---|---|---|
| | | TA98 | TA100 |
| unfractionated | 0.8 | 2156 ± 10 (91.0)$^{bc}$ | 1159 ± 54 (91.5)$^{bc}$ |
| | 1.1 | 1986 ± 20 (83.8)$^{d}$ | 1050 ± 35 (82.9)$^{de}$ |
| | 1.7 | 1766 ± 58 (74.5)$^{ef}$ | 754 ± 35 (59.5)$^{h}$ |
| | 2.5 | 1212 ± 21 (51.2)$^{i}$ | 188 ± 13 (14.9)$^{k}$ |
| MW below 10 000 | 0.8 | 2360 ± 55 (99.6)$^{a}$ | 1200 ± 36 (94.8)$^{ab}$ |
| | 1.1 | 2100 ± 10 (88.6)$^{c}$ | 1240 ± 25 (97.9)$^{a}$ |
| | 1.7 | 1805 ± 55 (76.2)$^{e}$ | 1220 ± 64 (99.5)$^{ab}$ |
| | 2.5 | 1661 ± 45 (70.1)$^{g}$ | 1201 ± 16 (94.9)$^{ab}$ |
| MW 10 000–30 000 | 0.8 | 1964 ± 25 (82.8)$^{d}$ | 1218 ± 57 (96.2)$^{ab}$ |
| | 1.1 | 1934 ± 8 (81.6)$^{d}$ | 1059 ± 8 (83.6)$^{de}$ |
| | 1.7 | 1699 ± 98 (71.7)$^{fg}$ | 1008 ± 70 (79.6)$^{ef}$ |
| | 2.5 | 1402 ± 86 (59.2)$^{h}$ | 853 ± 45 (67.3)$^{g}$ |
| MW 30 000–50 000 | 0.8 | 1490 ± 110 (62.9)$^{h}$ | 997 ± 38 (78.7)$^{ef}$ |
| | 1.1 | 901 ± 80 (38.0)$^{j}$ | 721 ± 5 (56.9)$^{h}$ |
| | 1.7 | 600 ± 4 (25.3)$^{k}$ | 527 ± 23 (41.6)$^{i}$ |
| | 2.5 | 219 ± 21 (9.2)$^{m}$ | 519 ± 22 (40.9)$^{i}$ |
| MW 50 000–100 000 | 0.8 | 1482 ± 56 (62.6)$^{h}$ | 847 ± 33 (66.9)$^{g}$ |
| | 1.1 | 1136 ± 100 (48.0)$^{i}$ | 823 ± 30 (65.0)$^{g}$ |
| | 1.7 | 370 ± 5 (15.6)$^{l}$ | 431 ± 14 (34.0)$^{j}$ |
| | 2.5 | 105 ± 10 (4.4)$^{n}$ | 414 ± 27 (32.7)$^{j}$ |
| MW above 100 000 | 0.8 | 2222 ± 7 (93.8)$^{b}$ | 1255 ± 27 (99.1)$^{a}$ |
| | 1.1 | 2164 ± 29 (87.1)$^{bc}$ | 1109 ± 57 (87.6)$^{cd}$ |
| | 1.7 | 1195 ± 27 (50.4)$^{i}$ | 1084 ± 3 (85.6)$^{d}$ |
| | 2.5 | 885 ± 60 (37.4)$^{j}$ | 976 ± 55 (77.1)$^{f}$ |
| control | | 2369 ± 33 (100.0)$^{a}$ | 1266 ± 28 (100.0)$^{a}$ |
| spontaneous revertants | | 46 ± 2 | 152 ± 11 |

[a] MRPs were prepared by heating xylose (1.0 M) and lysine (2.0 M) at 100 °C and pH 9.0 for 10 h and then fractionated by membrane filters. IQ (0.1 μg/plate for TA98 and 0.5 μg/plate for TA100) was preincubated with or without MRPs at 37 °C for 20 min. [b] Control plates were with mutagen but without MRPs. Spontaneous revertants were obtained without MRPs and mutagen. Data are the mean ± SD of three plates. Values in parentheses are percentages relative to control value (100%). Values in a column with different superscripts are significantly different ($P < 0.05$).

**Table II. Effect of Fractions of Xylose–Lysine Maillard Reaction Products (MRPs) with Various Molecular Weight Ranges on the Mutagenicity of Glu-P-1 to *S. typhimurium* TA98 and TA100 in the Presence of S9 Mix**

| MRP[a] | dose, mg/plate | His$^+$ revertants/plate[b] (percentage) | |
|---|---|---|---|
| | | TA98 | TA100 |
| unfractionated | 0.8 | 553 ± 3 (98.1)$^{b}$ | 260 ± 8 (76.5)$^{d}$ |
| | 1.1 | 465 ± 9 (82.6)$^{c}$ | 239 ± 1 (70.1)$^{e}$ |
| | 1.7 | 169 ± 2 (29.9)$^{klm}$ | 154 ± 4 (45.2)$^{h}$ |
| | 2.5 | 323 ± 4 (57.3)$^{e}$ | 126 ± 5 (36.9)$^{j}$ |
| MW below 10 000 | 0.8 | 262 ± 2 (46.5)$^{fg}$ | 319 ± 4 (93.5)$^{b}$ |
| | 1.1 | 245 ± 18 (43.4)$^{ghi}$ | 310 ± 5 (91.1)$^{b}$ |
| | 1.7 | 126 ± 3 (22.4)$^{mn}$ | 307 ± 12 (90.2)$^{b}$ |
| | 2.5 | 38 ± 2 (6.8)$^{lmn}$ | 235 ± 1 (68.9)$^{e}$ |
| MW 10 000–30 000 | 0.8 | 416 ± 1 (73.8)$^{d}$ | 316 ± 3 (93.0)$^{b}$ |
| | 1.1 | 206 ± 4 (36.6)$^{ijk}$ | 286 ± 5 (83.9)$^{c}$ |
| | 1.7 | 136 ± 1 (24.2)$^{lmn}$ | 237 ± 1 (69.8)$^{e}$ |
| | 2.5 | 146 ± 5 (25.9)$^{lmn}$ | 216 ± 5 (63.4)$^{f}$ |
| MW 30 000–50 000 | 0.8 | 416 ± 1 (73.8)$^{d}$ | 193 ± 5 (56.7)$^{g}$ |
| | 1.1 | 291 ± 7 (51.6)$^{ef}$ | 161 ± 4 (47.3)$^{h}$ |
| | 1.7 | 237 ± 6 (42.0)$^{ghi}$ | 156 ± 3 (45.8)$^{h}$ |
| | 2.5 | 38 ± 2 (6.7)$^{o}$ | 96 ± 1 (28.1)$^{k}$ |
| MW 50 000–100 000 | 0.8 | 257 ± 9 (45.7)$^{fgh}$ | 131 ± 5 (38.3)$^{ij}$ |
| | 1.1 | 174 ± 9 (30.8)$^{jkl}$ | 102 ± 3 (29.8)$^{k}$ |
| | 1.7 | 145 ± 8 (25.8)$^{lmn}$ | 95 ± 3 (27.9)$^{k}$ |
| | 2.5 | 121 ± 7 (21.4)$^{n}$ | 75 ± 3 (22.0)$^{l}$ |
| MW above 100 000 | 0.8 | 462 ± 1 (82.0)$^{c}$ | 320 ± 1 (93.8)$^{h}$ |
| | 1.1 | 240 ± 2 (42.6)$^{e}$ | 267 ± 12 (78.3)$^{d}$ |
| | 1.7 | 215 ± 1 (38.1)$^{hij}$ | 189 ± 1 (55.4)$^{g}$ |
| | 2.5 | 127 ± 9 (22.5)$^{mn}$ | 141 ± 1 (41.3)$^{i}$ |
| control | | 563 ± 77 (100.0)$^{a}$ | 341 ± 29 (100.0)$^{a}$ |
| spontaneous revertants | | 26 ± 3 | 100 ± 5 |

[a] MRPs were prepared by heating xylose (1.0 M) and lysine (2.0 M) at 100 °C and pH 9.0 for 10 h and then fractionated by membrane filters. Glu-P-1 (0.05 μg/plate for TA98 and 0.5 μg/plate for TA 100) was preincubated with or without MRPs at 37 °C for 20 min. [b] Descriptions are the same as in Table I. Values in a column with different superscripts are significantly different ($P < 0.05$).

The antimutagenic effect of each XL MRP fraction on the activity of direct-acting mutagens, NQNO and MNNG, is shown in Table III. For the unfractionated XL MRPs, the antimutagenic effect was not observed against NQNO at the dosage less than 1.7 mg/plate, but the slightly enhancing effect for the mutagenicity of NQNO was found at the dosage of 2.5 mg/plate ($P < 0.05$). The mutagenicity of NQNO toward TA100 was slightly affected by the two fractions of MW below 30 000. It is interesting to note that the antimutagenic effect of fractionated XL MRPs on NQNO increased with decreasing dose of fractions with MW above 30 000. The enhancing and inhibitory effects of fractionated XL MRPs on the mutagenesis of IQ to TA100 were present counteractively in the above fractions but exhibited different dose response. The inhibitory effect was observed at the lower dose and increased slowly with increasing dose if mutagenesis enhancing effect had not occurred. However, the enhancing effect appeared at higher dose and increased sharply so as to interfere with the inhibitory effect. For the same ranges of test dose of fractions, only antimutagenicity against MNNG was observed, obviously because the enhancing effect was very slight even at the higher test dose (2.5 mg/plate). The mutagenicity of NQNO toward TA98 was not reduced by all fractions at the dose below 1.7 mg/plate except the fraction of MW 10 000–30 000 at the dose of 1.7 mg/plate (only 8.2% inhibitory effect, data not shown). These results are in agreement with the descriptions about the effect of XL MRPs against the mutagenicity of NQNO toward TA98 (Yen and Lii, 1992a). For the mutagenicity test, strain TA98 detects various frameshift mutagens but TA100 detects mutagens that cause base-pair substitutions (Maron and Ames, 1983). NQNO and MNNG directly induce both types of mutations (Kushi and Yoshida, 1987; Watanabe et al., 1988), whereas IQ and Glu-P-1 induce them with the activation by S9 mix (Matsukura et al., 1981; Ohgaki et al., 1984). The heterocyclic amines were converted to their hydroxyamino derivatives by cytochrome P-450s in S9 mix, and the derivatives exhibited the mutagenicity in the absence of S9 mix (Kato and Yamazoe, 1987). These results indicate that there are at least two kinds of antimutagenic substances in XL MRPs. One (compounds I) kind is presented principally in the fraction of MW below 30 000, but the other (compounds II) is presented in the fraction of MW above 30 000. Many antimutagenic substances with various molecular weights were included in each kind. The antimutagenic compounds I showed the inhibition for both frameshift and base-pair substitution mutations of indirect-acting mutagens, IQ or Glu-P-1, described in our previous study (Yen and Lii, 1992a). However, the antimutagenic compounds II also showed inhibition to base-pair substitution mutation of direct-acting mutagen, NQNO and MNNG, besides the inhibition for both frameshift and base-pair substitution mutation of indirect-acting mutagen. Compared with their total antimutagenic effect toward IQ and Glu-P-1, antimutagenic compounds II showed stronger

**Table III. Effect of Fractions of Xylose–Lysine Maillard Reaction Products with Various Molecular Weight Ranges on the Mutagenicity of NQNO and MNNG to *S. typhimurium* TA100 in the Absence of S9 Mix**

| MRP[a] | dose, mg/plate | His$^+$ revertants/plate[b] (percentage) | |
|---|---|---|---|
| | | NQNO | MNNG |
| unfractionated | 0.8 | 1280 ± 45 (100.8)[efgh] | 120 ± 2 (42.5)[i] |
| | 1.1 | 1300 ± 51 (102.4)[cdef] | 114 ± 2 (40.4)[i] |
| | 1.7 | 1210 ± 55 (95.2)[hi] | 130 ± 4 (46.1)[h] |
| | 2.5 | 1444 ± 41 (113.7)[a] | 147 ± 5 (52.1)[g] |
| MW below 10 000 | 0.8 | 1180 ± 25 (92.9)[i] | 228 ± 4 (80.9)[c] |
| | 1.1 | 1180 ± 25 (92.9)[i] | 192 ± 6 (68.1)[e] |
| | 1.7 | 1270 ± 45 (100.0)[efgh] | 172 ± 2 (61.0)[f] |
| | 2.5 | 1320 ± 62 (103.9)[cde] | 167 ± 5 (59.2)[f] |
| MW 10 000–30 000 | 0.8 | 1358 ± 2 (106.9)[bcde] | 245 ± 5 (86.9)[b] |
| | 1.1 | 1372 ± 27 (108.0)[bc] | 213 ± 2 (75.5)[d] |
| | 1.7 | 1330 ± 72 (104.7)[cde] | 200 ± 1 (70.9)[e] |
| | 2.5 | 1410 ± 15 (111.0)[ab] | 190 ± 17 (67.4)[e] |
| MW 30 000–50 000 | 0.8 | 366 ± 16 (28.8)[m] | 98 ± 2 (34.6)[k] |
| | 1.1 | 964 ± 25 (75.9)[k] | 78 ± 6 (27.5)[l] |
| | 1.7 | 1246 ± 27 (98.1)[fghi] | 97 ± 3 (34.4)[k] |
| | 2.5 | 1290 ± 60 (101.6)[defg] | 149 ± 4 (52.8)[g] |
| MW 50 000–100 000 | 0.8 | 340 ± 10 (26.8)[m] | 107 ± 3 (37.9)[jk] |
| | 1.1 | 848 ± 12 (66.8)[l] | 110 ± 3 (39.0)[ij] |
| | 1.7 | 1066 ± 66 (83.9)[j] | 105 ± 8 (37.1)[jk] |
| | 2.5 | 1300 ± 11 (102.4)[cdef] | 114 ± 0 (41.5)[ij] |
| MW above 100 000 | 0.8 | 998 ± 28 (78.6)[k] | 107 ± 1 (37.9)[jk] |
| | 1.1 | 1214 ± 2 (95.6)[hi] | 135 ± 7 (47.9)[h] |
| | 1.7 | 1219 ± 41 (96.0)[ghi] | 121 ± 2 (42.9)[i] |
| | 2.5 | 1362 ± 16 (107.2)[bcd] | 153 ± 5 (54.8)[g] |
| control | | 1270 ± 30 (100.0)[efgh] | 282 ± 18 (100.0)[a] |
| spontaneous revertants | | 140 ± 10 | 54 ± 3 |

[a] MRPs were prepared by heating xylose (1.0 M) and lysine (2.0 M) at 100 °C and pH 9.0 for 10 h and then fractionated by membrane filters. NQNO (0.1 μg/plate) or MNNG (0.5 μg/plate) was preincubated with or without MRPs at 37 °C for 20 min. [b] Descriptions are the same as in Table I. Values in a column with different superscripts are significantly different ($P < 0.05$).

**Table IV. Comparison in Browning Intensity, Antioxidative Activity, and Reducing Power of Xylose–Lysine Maillard Reaction Products (MRPs) with Various Molecular Weight Ranges**

| MRP[a] | yield, % | browning[b] | AOA[c] | reducing power[d] |
|---|---|---|---|---|
| unfractionated | | 2.219 | 0.904 | 2.690 |
| MW below 10 000 | 56.8 | 0.170 | 0.209 | 1.430 |
| MW 10 000–30 000 | 2.1 | 0.308 | 0.349 | 1.834 |
| MW 30 000–50 000 | 2.7 | 2.309 | 0.902 | 2.668 |
| MW 50 000–100 000 | 3.5 | 2.130 | 0.915 | 2.741 |
| MW above 100 000 | 34.9 | 2.144 | 0.906 | 2.831 |
| blank[e] | | 0.000 | 0.000 | 0.037 |

[a] XL MRPs were prepared by heating xylose (1.0 M) and lysine (2.0 M) at 100 °C, pH 9.0, for 10 h and then fractionated by membrane filters. [b] Each fractionated sample was diluted to 1000-fold (1.0 mg/mL). Absorbance was measured at 420 nm. [c] AOA = ($\Delta A_{234}$ of control − $\Delta A_{234}$ of sample)/$\Delta A_{234}$ of control. [d] Absorbance at 700 nm. [e] Blank was without sample.

activity than antimutagenic compound I. On the basis of these results, the fraction of MW 50 000–100 000 was selected to perform the separation of the strongest antimutagenic compound by gel filtration and further purification by HPLC.

**Some Characteristics of Fractionated XL MRPs and the Relationship to Antimutagenicity.** The changes in browning intensity, antioxidative activity, and reducing power are shown in Table IV. The yield of fractions with MW below 10 000 and MW above 100 000 had the large amount in total fractions. The levels of browning intensity of the fractions increased with increasing molecular weight below 50 000 but decreased slightly above that. The same trend was also observed in antioxidative activity, while the strongest antioxidative activity appeared at the fraction of MW 50 000–100 000. The levels in reducing power increased with increasing molecular weight range for all fractions.

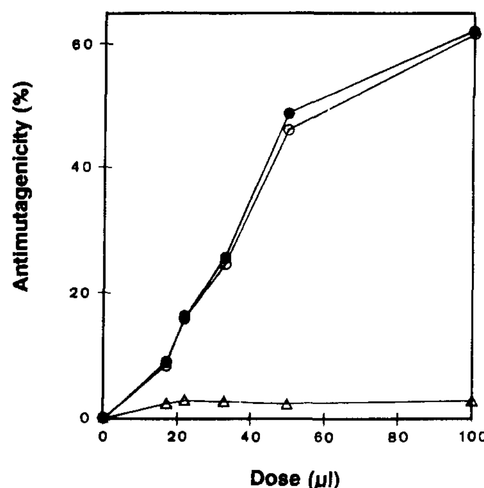


Figure 1. Antimutagenicity of various solvent extracts from the MW 50 000–100 000 fraction of xylose–lysine Maillard reaction products. (●) Unextracted sample; (○) water extract; (△) petroleum ether extract. For details, see Materials and Methods.

The antimutagenicity of XL MRPs against IQ, Glu-P-1, and NQNO was not significantly associated with the levels of the other measured characteristics of XL MRPs according to the calculated coefficients of correlation of antimutagenicity and other characteristics ($P > 0.05$). The results of XL MRP system is different from the glucose–glycine (GG) and glucose–tryptophan (GT) systems. Significantly positive correlations were observed for both GG and GT MRPs between the antimutagenicity against IQ or Glu-P-1 and reducing power and antioxidative activity of reaction solution (Kim et al., 1986; Yen and Lii, 1992b).

**Polarity of XL MRPs.** The antimutagenicity of the unextracted fraction, the water extract, and the petroleum ether extract of the fraction of MW 50 000–100 000 is shown in Figure 1. Results from the chloroform, methanol, acetone, and *n*-hexane extracts (data not shown) were similar to those of the petroleum ether extract and exhibited no obvious antimutagenicity. Furthermore, similarly obvious antimutagenicity was observed in the unextracted fraction and water extract, suggesting that the antimutagenic compound(s) in the fraction of MW 50 000–100 000 is (are) with polar property.

**Separation of the Fraction of MW 50 000–100 000 by Sephadex G-100.** The elution prifiles of the fraction of MW 50 000–100 000 by Sephadex G-100 and antimutagenicity of each subfraction are shown in Figure 2. Two peaks between fractions 44 and 54 were obtained by measuring the absorbance at 420 nm. However, antimutagenicity was observed starting from fractions 44 to 56, and the strongest activity appeared at fraction 48. The eluate was combined into three groups (GI, GII, and GIII) according to antimutagenicity. The dose–response relation of antimutagenicity of these groups is shown in Table V. The combined fractions 46–50 (GII) exhibited the strongest antimutagenic effect against IQ. GII was therefore subjected to further purification by HPLC. The molecular weight range of GII estimated from the elution profile with the calibration kit was found to be 61 000 < MW < 63 000. The antimutagenicity of glucose–glycine MRPs

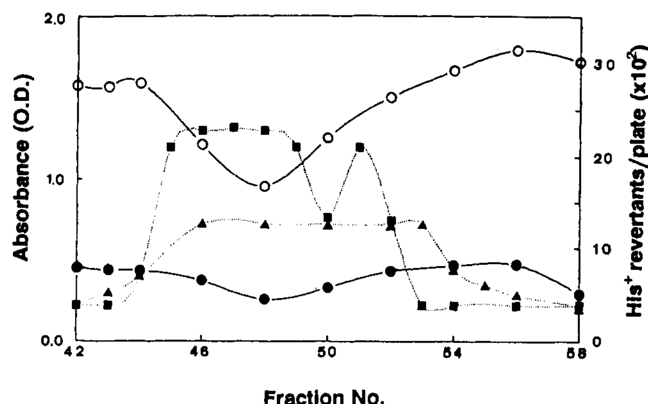


**Figure 2.** Permeation chromatography and antimutagenicity of MW 50 000–100 000 fraction of xylose–lysine Maillard reaction products by Sephadex G-100. (○) His+ revertants of TA98; (●) His+ revertants of TA100; (▲) absorbance at 280 nm; (■) absorbance at 420 nm; column size, 2.6 × 100 cm; flow rate, 1.0 mL/min; fraction size, 10 mL/tube.

**Table V. Effect of Each Gel Subfraction of MW 50 000–100 000 Xylose–Lysine Maillard Reaction Products (MRPs) on the Mutagenicity of IQ to *S. typhimurium* TA98 and TA100 with S9 Mix**

| MRP[a] | dose,[b] mg/plate | His+ revertants/plate[c] TA98 | TA100 |
|---|---|---|---|
| GI (no. 44–45) | 0.1 | 1850 ± 85 (88.1)[d] | 700 ± 52 (93.3) |
| | 1.0 | 1200 ± 90 (57.1) | 490 ± 40 (65.3) |
| GII (no. 46–50) | 0.1 | 1350 ± 90 (64.3) | 488 ± 22 (65.1) |
| | 1.0 | 900 ± 82 (42.9) | 380 ± 35 (50.6) |
| GIII (no. 51–54) | 0.1 | 2020 ± 180 (96.1) | 748 ± 45 (99.7) |
| | 1.0 | 1540 ± 85 (73.3) | 660 ± 25 (88.0) |
| control | | 2100 ± 22 (100.0) | 750 ± 35 (100.0) |
| spontaneous revertants | | 38 ± 3 | 160 ± 8 |

[a] Sample of each subfraction was collected by Sephadex G-100 and then freeze-dried for test. [b] Freeze-dried sample of each subfraction was diluted to 100- and 1000-fold (1.0 and 0.1 mg/plate). [c] IQ (0.1 μg/plate) was preincubated with or without MRP at 37 °C for 20 min. Control plates were with mutagen but without MRP. Spontaneous revertants were obtained without MRP and mutagen. Data are the mean ± SD of three plates. [d] Values in parentheses are percentage relative to control value (100%).

prepared at pH 6.8 and 95 °C for 7 h has been previously studied by Kato et al. (1985a) and indicates the mean molecular weight of nondialyzable melanoidin was 7000, which was determined by gel permeation chromatography HPLC. The molecular weight of MRPs with the strongest antimutagenicity varied with each kind of amino acid system and reaction conditions of preparation.

**Purification of Subfraction GII of XL MRPs by HPLC.** The GII was subjected to reversed-phase HPLC and separated into three peaks, GIIa, GIIb, and GIIc (data not shown). The strongest antimutagenic effect was observed in peak GIIa (Table VI). Peak GIIa inhibited 58.2 and 54.1% of mutagenicity of IQ toward *S. typhimurium* TA98 and TA100, respectively, at the dosage of 1.0 mg/plate. The molecular weight of peak GIIa, estimated from the elution profile of the calibration kit, was about 63 000.

**Conclusions.** There are at least two kinds of antimutagenic substances in XL MRPs. The most antimutagenic fraction of XL MRPs was observed mainly in the fraction of MW 50 000–100 000. Characterization of the strongest antimutagenic component in XL MRPs prepared with optimum condition has been achieved and shown to be a polar property, having molecular weight about 63 000. Gel filtration and HPLC appeared to be good methods to obtain the fraction from XL MRPs which are strongly antimutagenic. The mutagenicity of heat-induced mutagens once caused remarkable concern about their actual involvement in carcinogenesis, but it seems gradually to be less serious than first thought due to their own weak carcinogenicity, their inactivation by other food components, and their low levels observed in actual food. Since the antimutagenicity of MRPs is observed in some amino acid and sugar systems, the actual role of heat-induced mutagens in carcinogenesis should be taken into account with regard to both food preparation and intake. The information of this study provides an important prerequisite for further investigation to isolate antimutagenic compounds and to determine their molecular structures.

**Table VI. Effect of Each HPLC Peak of Xylose–Lysine Maillard Reaction Products (MRPs) Subfraction GII on the Mutagenicity of IQ to *S. typhimurium* TA98 and TA100 with S9 Mix**

| MRP[a] | dose,[b] mg/plate | His+ revertants/plate[c] TA98 | TA100 |
|---|---|---|---|
| GIIa | 0.1 | 1290 ± 19 (61.2)[d] | 443 ± 21 (61.7) |
| | 1.0 | 880 ± 119 (41.8) | 337 ± 4 (45.9) |
| GIIb | 0.1 | 1943 ± 12 (92.2) | 738 ± 12 (100.5) |
| | 1.0 | 1810 ± 111 (85.9) | 625 ± 39 (86.8) |
| GIIc | 0.1 | 2011 ± 120 (95.4) | 728 ± 50 (99.2) |
| | 1.0 | 1920 ± 90 (91.1) | 680 ± 59 (92.6) |
| control | | 2107 ± 67 (100.0) | 734 ± 35 (100.0) |
| spontaneous revertants | | 38 ± 4 | 153 ± 3 |

[a] Sample of each peak collected by HPLC was freeze-dried for test. [b] Freeze-dried sample of each peak collected by HPLC was diluted to 100- and 1000-fold (1.0 and 0.1 mg/plate). [c] IQ (0.1 μg/plate) was preincubated with or without MRPs at 37 °C for 20 min. Control plates were with mutagen but without MRPs. Spontaneous revertants were obtained without MRP and mutagen. Data are the mean ± SD of three plates. [d] Descriptions are the same as in Table V.

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