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Antimutagenicity of a partially fractionated Maillard reaction product

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The Maillard reaction product was prepared by refluxing glucose (0·5 M) and tryptophan (0·5 M) at pH 11·0 and 100°C for 10 h. It was further fractionated into different molecular weights (MW) below 1000, MW 1000–5000, MW 5000–10 000, MW 10 000–30 000, MW 30 000–50 000 and MW above 50 000, by means of membrane filters. The characteristics and antimutagenicity of each glucose–tryptophan (GT) fraction were investigated. The trends of browning, reducing power, antioxidative activity and antimutagenicity of each GT fraction were generally of the order of MW above 5000 > unfractionated > MW below 1000. The antimutagenicity of each GT fraction exhibited a suitable correlation with these characteristics. The reducing power and antioxidative activity of each GT fraction could be concluded to play an important role in terms of its antimutagenicity. Additionally, an inhibitory mechanism of GT was due to a desmutagenic effect but not a bio-antimutagenic effect.

INTRODUCTION

The Maillard reaction, between carbonyl and amino compounds, is one of the most important reactions occurring in foods during processing and storage. This reaction has been previously studied, from various aspects, in foods, and numerous reviews are available (Namiki, 1988; Monnier, 1989; O'Brien & Morrissey, 1989). The antimutagenicity of Maillard reaction products (MRPs) has been previously studied in different model systems using amino acids and sugars (Chan *et al.*, 1979; Kato *et al.*, 1985; Yamaguchi & Iki, 1986; Kong *et al.*, 1989; Yen *et al.*, 1992). The inhibitory effect of MRPs on mutagens is, however, still not completely understood.

The antimutagenicity of twelve MRPs prepared from four amino acids (glycine, lysine, tryptophan and arginine) and three sugars (glucose, xylose and fructose) as combinations was investigated in our previous study (Yen *et al.*, 1992). Among the twelve combinations, the MRPs obtained from glucose and tryptophan had stronger antimutagenic effect against the mutagenicity of 2-amino-3-methylimidazo[4,5-f]quinoline, 2-amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole and 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole toward *Salmonella typhimurium* TA98. The antimutagenic effects of MRPs also correlated with their antioxidative activity and reducing power. The antimutagenicity of the partially fractionated glucose–tryptophan browning reaction product has been investigated here for the purpose of further understanding the antimutagenic property and antimutagenic mechanism of MRPs.

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MATERIALS AND METHODS

Materials

Glucose and tryptophan were supplied by Sigma Chemical Co. (St Louis, MO). The chemicals, 2-amino-6-methyldipyrido(1,2-a:3',2'-d)imidazole (Glu-P-1) and 2-amino-3-methylimidazo(4,5-f)quinoline (IQ) were obtained from Wako Pure Chemical Co. (Tokyo). All other reagents used were of guaranteed grade.

Preparation and fractionation of the Maillard reaction product

The Maillard reaction product was prepared by dissolving glucose (0·5 M) and tryptophan (0·5 M) in potassium phosphate buffer (0·1 M, pH 11·0), and then heating at 100°C for 10 h under reflux in an oil bath. The glucose–tryptophan Maillard reaction product was further fractionated with Diaflo ultrafiltration membranes (Amicon Division, W. R. Grace and Co., Danvers, MA) into six fractions: molecular weight (MW) below 1000, MW 1000–5000, MW 5000–10 000, MW 10 000–30 000, MW 30 000–50 000 and MW above 50 000. Each fraction was collected and the yield was calculated after lyophilization. The browning, reducing power, antioxidative activity and antimutagenicity of each fraction were determined.

Browning intensity

The browning extent of the Maillard reaction product was determined, after an appropriate dilution with distilled water, by measuring the absorbance at 420 nm with a Hitachi spectrophotometer (Model U-2000).

Reducing power

The reducing power of the Maillard reaction product was determined as previously described by Oyaizu (1986). After a dilution (1000 times) with distilled water, the Maillard reaction product (0·1 ml) was mixed with phosphate buffer (5·0 ml, 2·0 M, pH 6·6) and 1% potassium ferricyanide (5 ml); then the mixture was incubated at 50°C for 20 min. A portion (5 ml) of 10% trichloroacetic acid was added to the mixture and then centrifuged at 3000 rpm for 10 min. The upper layer solution (5 ml) was mixed with distilled water (5 ml) and 0·1% ferric chloride (1 ml), and the absorbance was measured at 700 nm. The higher absorbance of the reaction mixture inferred a higher reducing power.

Measurement of absorbance increase at 234 nm

The Maillard reaction mixture (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 for the purpose of accelerating the oxidation. A control without the Maillard reaction mixture was run in parallel. Both before and 15 h after incubation, the substrate–Maillard reaction mixture (0·2 ml) was solubilized in absolute methanol (2 ml). The methanol (6 ml, 60%) in water was added and the absorbance at 234 nm was measured. The antioxidative activity (AOA) was calculated according to the method previously described by Lingnert *et al.* (1979). AOA = (ΔA 234 of control − ΔA 234 of sample)/ΔA 234 of sample. An AOA value equal to 1 implied the strongest antioxidative activity of the sample.

Assay for antimutagenic effects

The antimutagenic effect of each sample was examined according to the Ames method (Maron & Ames, 1983). The histidine-requiring strains of *Salmonella typhimurium* TA98 and TA100 were kindly supplied by Professor B. N. Ames (University of California, Berkeley, USA). The S9 mix was prepared from Sprague–Dawley male rats treated with Aroclor 1254 (a product of Organ Teknika Co., Switzerland) by the method of Ames *et al.* (1975). An aliquot of sample (2 mg) was added to the mixture of an overnight culture of *Salmonella typhimurium* TA98 or TA100 (0·1 ml), and a mutagen dissolved in dimethylsulphoxide (0·1 μg IQ/ml; 0·5 μg Glu-P-1/ml, and S9 mix (0·5 ml, containing 0·77 mg of the S9 fraction as protein). The entire mixture was preincubated at 37°C for 20 min before molten top agar (2 ml) was added; the mixture was poured onto a minimal agar plate. The his^+ revertant colonies were counted after incubation at 37°C for 48 h. Each assay was performed in triplicate and the data presented were the means of at least two experiments. The dosage of each sample tested in this study exhibited no mutagenicity and no lethal effect to the bacteria. The mutagenicity of each mutagen in the absence of the Maillard reaction product was expressed as 100. A lower percentage of revertants of sample compared to control meant a stronger antimutagenicity of sample (Francis *et al.*, 1989).

Bio-antimutagenicity assay

The bio-antimutagenicity test was conducted as previously described by Sato *et al.* (1987). *Salmonella typhimurium* TA98 and TA100 were cultured overnight. Bacterial suspension (5 ml) was washed twice by centrifugation with a cold M/15 phosphate buffer (PB) at pH 7·0 and resuspended in 4 ml of cold PB. To 3 ml of this bacterial suspension, 5 μg IQ/0·5 ml or 25 μg Glu-P-1/0·5 ml for TA 98, 1000 μg IQ/0·5 ml or 1000 μg Glu-P-1/0·5 ml for TA100 and 3 ml of S9 mix were added, and the mixture was incubated at 37°C for 1 h with slow shaking. The treated bacteria were washed twice by centrifugation with cold PB and resuspended in cold PB. The Ames tests were performed with or without glucose–tryptophan on the treated bacteria. The amount of glucose–tryptophan was 2·0 mg/plate. Another 1 ml of the bacterial suspension was used for determination of spontaneous mutation.

Statistical analysis

The correlations between the antimutagenicity and the characteristics of the Maillard reaction product were calculated as prescribed by Duncan's multiple range test (Duncan, 1955).

RESULTS AND DISCUSSION

Characteristics of fractionated glucose–tryptophan browning product

The percentage yields for glucose–tryptophan fractions with MW below 1000, precipitate, MW 1000–50 000 and MW above 50 000 are shown in Table 1 to be respectively, 69·2%, 16·2%, 7·5% and 7·0%. The fraction of MW 30 000–50 000 had the largest amount in the range of MW 1000–50 000. Each glucose–tryptophan fraction exhibited a different degree of browning, reducing power and antioxidative activity, and were in the order, MW above 5000 > unfractionated > MW below 1000. The substances with higher browning, reducing power and antioxidative activity were clearly indicated by this result to be included in the fractions with MW > 5000, especially for the fractions with MW 5000–10 000 and MW 30 000–50 000. This result is also in agreement with the previous report of Yamaguchi *et al.* (1981) that a high molecular weight of melanoidin prepared from xylose and glycine achieved a higher browning, reducing power and antioxidative activity.

Table 1. Percentage, browning intensity, reducing power and antioxidative activity of Maillard reaction product prepared by heating glucose and tryptophan at 100°C and pH 11·0 for 10 h

Samples[a]	% of total weight	Browning intensity[b]	Reducing power[c]	AOA[d]
Unfractionated	100·0	0·842	0·341	0·78
MW below 1000	69·2	0·536	0·287	0·63
MW 1000–5000	0·0	—	—	—
MW 5000–10000	1·8	2·358	0·664	0·96
MW 10000–30000	1·2	1·878	0·510	0·89
MW 30000–50000	4·5	2·540	0·726	0·97
MW above 50000	7·0	1·333	0·499	0·91
Precipitate	16·2	—	—	—

[a] Maillard reaction product was fractionated into different molecular weights (MW) by membrane filter.
[b] Browning intensity: absorbance at 420 nm.
[c] Reducing power: absorbance at 700 nm.
[d] AOA: capacity to inhibit the formation of conjugated dienes in linoleic acid. An AOA of 1 corresponds to the strongest antioxidative activity.

Antimutagenicity of fractionated glucose–tryptophan browning product

The mutagenicity of each glucose–tryptophan fraction was evaluated in the preliminary study. No mutagenicity was found for each glucose–tryptophan fraction in the dosage of 2 mg/plate towards *Salmonella typhimurium* TA98 and TA100, either with or without S9 mix, and, as a result, the revertants of the sample were less than twice that of spontaneous revertants (data not shown). This dosage was therefore used for further study of antimutagenicity of each glucose–tryptophan fraction. The inhibitory effect of fractionated glucose–tryptophan on the mutagenicity of IQ and Glu-P-1 towards *S. typhimurium* TA98 and TA100 is shown in Table 2. The antimutagenicity of each glucose–tryptophan fraction was generally in the order of MW above 5000 > unfractionated > MW below 1000. All fractions with MW above 5000 showed a significant inhibition effect on mutagenicity of IQ and Glu-P-1. The MW 30 000–50 000 fraction was therefore selected for the purpose of studying the dosage response relationship of its antimutagenicity and compared with unfractionated and the MW fraction below 1000. The antimutagenic effect of each sample can be seen from Fig. 1 to have increased with increase in dosage. The MW 30 000–50 000 fraction demonstrated a stronger antimutagenicity than other samples; it inhibited approximately 90% mutagenicity of IQ and Glu-P-1 towards *S. typhimurium* TA98 at the dosage of 0·5 mg/plate. The antimutagenic effect of each sample, as applied to both test strains TA98 and TA100, was in the order, MW 30 000–50 000 > unfractionated > MW below 1000. Similar results in the antimutagenicity of fractionated

Table 2. Inhibition of the mutagenicity of IQ and Glu-P-1 by the Maillard reaction products prepared from glucose and tryptophan to *Salmonella typhimurium* TA98 and TA100 in the presence of S9 mix

Sample	His$^+$ revertants/plate[a]			
	TA98		TA100	
	IQ (0·01 μg)	Glu-P-1 (0·05 μg)	IQ (0·01 μg)	Glu-P-1 (0·05 μg)
Control	1612 ± 91 (100)[b]	1801 ± 182 (100)	1389 ± 178 (100)	1783 ± 93 (100)
Spontaneous revertants	35 ± 4 (2·2)	35 ± 4 (1·9)	122 ± 15 (8·8)	122 ± 15 (8·8)
Unfractionated	301 ± 33 (18·7)	263 ± 37 (14·6)	782 ± 75 (56·3)	429 ± 52 (24·1)
MW below 1000	736 ± 95 (45·7)	858 ± 66 (47·6)	1153 ± 25 (83·0)	936 ± 91 (52·5)
MW 5000–10000	37 ± 3 (2·3)	36 ± 4 (2·0)	184 ± 12 (13·2)	214 ± 21 (12·0)
MW 10000–30000	73 ± 3 (4·5)	55 ± 12 (3·1)	294 ± 63 (21·2)	294 ± 56 (16·5)
MW 30000–50000	41 ± 5 (2·5)	34 ± 3 (1·9)	199 ± 21 (14·3)	165 ± 17 (9·3)
MW above 50000	51 ± 3 (3·2)	49 ± 5 (2·7)	158 ± 79 (11·4)	165 ± 16 (9·3)

[a] To each plate was added 2·0 mg of different fraction powders and 0·1 ml of mutagen (IQ or Glu-P-1) which was dissolved in DMSO. The control was with mutagen but without sample. The No. of spontaneous revertants was determined without sample and mutagen. Data are the means ± SD of six plates.
[b] Values in parentheses are percentages relative to control value (100%)..

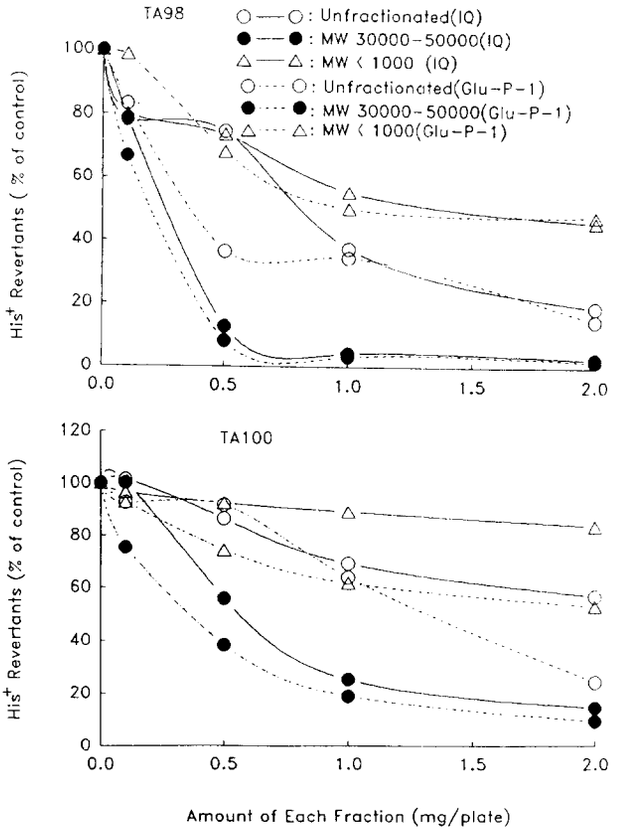

Fig. 1. Effect of glucose–tryptophan browning reaction product on the activity of 2-amino-6-methyldipyrido(1,2-a: 3',2'-d)imidazole (Glu-P-1) and 2-amino-3-methylimidazo(4,5-f)-quinoline (IQ) to *Salmonella typhimurium* TA98 and TA100 in the presence of S9 mix. Spontaneous revertants for TA98 and TA100 were 35 ± 4 and 122 ± 15, respectively. Revertants for IQ (0·01 μg/plate) and Glu-P-1 (0·05 μg/plate) to TA98 and TA100 were 1612 ± 91, 1801 ± 182 and 1389 ± 178, 1782 ± 93 (n = 6), respectively.

Table 3. Correlation[a] between antimutagenicity and browning intensity, reducing power and antioxidative activity of Maillard reaction product prepared from glucose and tryptophan at 100°C and pH 11·0 for 10 h with different molecular weight fractions

	TA98		TA100	
	IQ	Glu-P-1	IQ	Glu-P-1
Browning intensity[b]	−0·85	−0·82	−0·87	−0·87
Reducing power[b]	−0·89	−0·85	−0·87	−0·88
Antioxidative activity[b] (AOA)	−0·99	−0·98	−0·99	−0·97

[a] Significant correlation: $P < 0{\cdot}05$.
[b] See Table 1 for descriptions.

glucose–glycine browning products were previously reported by Kim *et al.* (1986), again exhibiting the trend as being in the order, MW above 5000 > unfractionated > MW below 1000.

The antimutagenicity of the Maillard reaction product is not yet completely understood. The mutagenic action is initiated or promoted by means of an active oxygen, free radical, and carbonyl compounds induced by peroxidation. Some antioxidants have therefore been reported to possess antimutagenic activity because they could scavenge a free radical or induce antioxidative enzymes (Hochstein & Atallah, 1988). Melanoidin has been shown by Hayase *et al.* (1989) to be able to remove some active oxygen, i.e. hydroperoxide, hydroxyl radical and superoxide. This could possibly be due to the specific structure of melanoidin or a result of the reductone, pyrrole compound and amine group containing antioxidative activity and metal chelating ability. The antimutagenicity of fractionated glucose–tryptophan can be seen from Table 3 to be well correlated with its browning, reducing power and antioxidative activity. The reducing power and antioxidative activity of the glucose–tryptophan browning product has therefore played an important role with regard to its antimutagenicity.

Antimutagenic mechanism of glucose–tryptophan browning product

The MW 30 000–50 000 fraction was used for investigating the antimutagenic mechanism of the glucose–tryptophan browning product. The mutagenesis of IQ and Glu-P-1 is shown in Fig. 2 to have been significantly reduced by the MW 30 000–50 000 fraction in the desmutagenic test but not in the bio-antimutagenic test, and the inhibitory effect of the glucose–tryptophan browning product has been clearly demonstrated by this result to be a desmutagenic effect but not a bio-antimutagenic effect. This means the browning product could directly inactivate mutagen or metabolic enzyme for the purpose of inhibiting the mutagenicity before the DNA reacted with mutagen. The DNA however, could not be repaired by treatment with browning product if it was damaged by a mutagen (Osawa, 1990).

A suitable correlation between antimutagenic activity and antioxidative activity of the glucose–tryptophan browning product is shown in Table 3. A desmutagenic effect of glucose–tryptophan could therefore be possibly partially due to the inhibition of mutagen (Hayatsu *et al.*, 1988). The antimutagenicity of a glucose–tryptophan browning product could possibly be caused by inactivation of metabolic enzymes as a result of the mutagenicity of IQ and Glu-P-1 being required for the metabolic enzyme to be activated. The antimutagenic effect of glucose–tryptophan browning product could also be due to an adsorption effect. Humic acid, for example, was previously reported as having an adsorption effect on mutagen and also reduces the mutagenicity (Sato *et al.*, 1987). Also purified cellulose of high molecular weight can irreversibly adsorb mutagens, i.e. 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole, 3-amino-

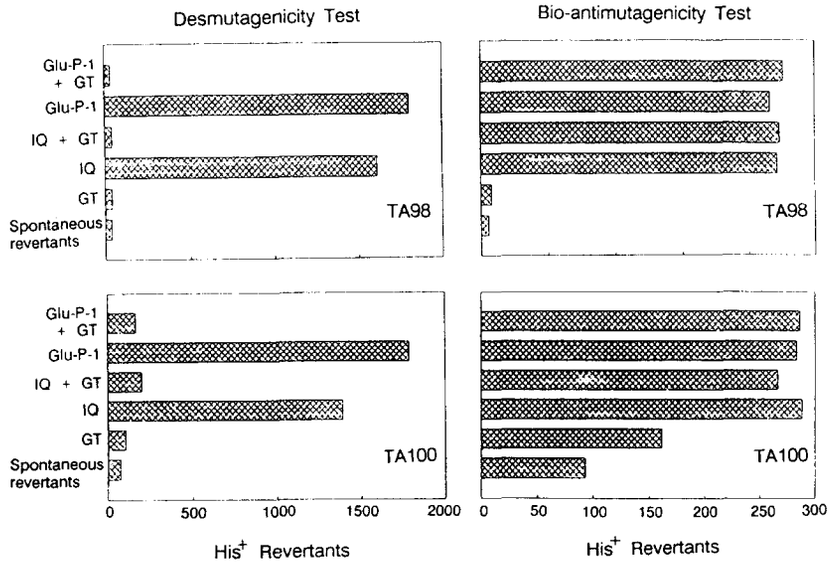

Fig. 2. Bio-antimutagenic and desmutagenic effect of glucose–tryptophan browning reaction product toward *Salmonella typhimurium* TA98 and TA100.

1-methyl-5H-pyrido[4,3-b]indole and Glu-P-1, and inhibits their mutagenicities (Kada *et al.*, 1984). The carbonyl compound in melanoidin could, on the other hand, also modify the free radical coming from heat-induced mutagens (Kim *et al.*, 1986). The inhibitory effect of the glucose–tryptophan browning product towards the mutagenicity of IQ and Glu-P-1 might therefore originate in many of these functions.

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