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Short communication

Modifying Action of Chitosan Oligosaccharide on 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx)-induced Mutagenesis

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The mutagenic activity of chitosan oligosaccharide and its antimutagenic effect against 2-amino-3,8-dimethylimidazo [4,5-*f*]quinoxaline (MeIQx) were investigated using the *Salmonella*/Ames test. No mutagenic activity was found in the *Salmonella typhimurium* strains TA 98 and TA 100, either with or without S9 activation. In contrast, chitosan oligosaccharide showed an inhibitory effect on the mutagenic activity of the cooked food mutagen, MeIQx, in the presence of S9. The influence of chitosan oligosaccharide on the genotoxicity of MeIQx was examined using a host-mediated assay in mice. The oligosaccharide was administered for 14 consecutive days (intragastric application at doses of 0.1 or 0.5 g/kg body wt) to mice. *S. typhimurium* TA 98 was given intravenously before an oral dose of MeIQx (4.5 mg/kg body wt.). The number of his$^+$ revertants were determined from the liver of mice. The intragastric application of oligosaccharide led to a 47% reduction in the number of mutants induced by MeIQx ($p<0.05$). These results suggested that chitosan oligosaccharide had antimutagenic properties against MeIQx *in vitro* and *in vivo*.

Keywords: 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), Antimutagenicity, Chitosan oligosaccharide, Host-mediated assay, *Salmonella typhimurium* TA 98.

Introduction

When proteinaceous foods, such as meat and fish are cooked, a family of heterocyclic aromatic amines is produced (Edenharder *et al.*, 1993). These amines are highly mutagenic in the *Salmonella*/reversion assay. They also induce DNA damage in mammalian cells (Aeschbacher and Tursky, 1991) and are potent multi-organ carcinogens in rodents (Ohgaki *et al.*, 1991). One of the most commonly occurring heterocyclic amines is 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), which was originally found in fried beef. MeIQx is mutagenic in both bacterial and mammalian cell mutation assays and carcinogenic in rodents (Kasai *et al.*, 1981). Long-term feeding studies in mice and rats resulted in a high incidence of lung and liver tumors (Ohagaki *et al.*, 1987; Kato *et al.*, 1988). The genotoxicity and carcinogenicity of MeIQx is believed to result from the metabolic conversion of MeIQx to electrophilic intermediates that can bind to DNA. The major activation step is N-hydroxylation catalyzed by cytochrome P4501A2 followed by conjugation of the N-hydroxy moiety to any of several leading groups, such as acetate or sulfate.

It has been suggested that the use of antimutagens and anticarcinogens in everyday life is the most effective procedure for preventing genetic disease and human cancer (Hannan *et al.*, 1989; Choi and Cho, 1999; Seo *et al.*, 1999). The majority of antimutagenic agents are natural compounds such as ascorbic acid, chlorophyll, tocopherol, ellagic acid, and vitamin A. The components of the diet such as fat, fiber, and plant flavonoids have been found to modify the genotoxicity of heterocyclic amines by altering their uptake from the gut lumen or their metabolism (Brennan-Craddock *et al.*, 1990; Aldrick *et al.*, 1993).

Amino sugars and their derivatives are known to represent an important group of biologically active compounds influencing the immune system of mammals and plants. Muramyldipeptide and several of its derivatives were reported to exhibit immunostimulating properties by the direct effect on lymphocytes and on macrophages (Chedid, 1983). They are also able to produce hyperthermia by acting on thermoregulation centers. Oligosaccharides of dipeptides were found to possess a hemoattractive effect on macrophages and

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antitumor activity against Ehrlich ascites carcinoma (Nishikawa *et al.*, 1976). Chitohexaose and its N-peracetylated derivatives were shown to inhibit the growth of murine cancer cells (Suzuki *et al.*, 1986; Kobayashi *et al.*, 1990). Glycolipids derived from chitooligosaccharides possessed immunostimulatory activity by inducing interleukin-1 and the tumor necrosis factor from immunocompetent cells. The compounds also increased the mean life of mice with Ehrlich carcinoma by 140-190% (Gorbach *et al.*, 1994).

We previously demonstrated the antitumor (Nam *et al.*, 1999) and cancer chemopreventive activities (Nam *et al.*, 2000) of chitosan oligosaccharide. In the present study, the mutagenic activity of chitosan oligosaccharide and its antimutagenic effect on MeIQx was further investigated by an *in vitro* experiment using *S. typhimurium* TA 98 and a host-mediated assay in mice.

Materials and Methods

Chemicals Two different molecular sizes of chitosan oligosaccharides, chitosan oligosaccharide I (COS I, 1000<Mr< 3000) and chitosan oligosaccharide II (COS II, 3000<Mr<5000), were kindly provided by the Kitto Life Co. (Seoul, Korea). 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) was purchased from Wako Pure Chemicals (Osaka, Japan), 4-nitro-*o*-phenylenediamine (NPD), sodium azide (NaN_3), 2-aminofluorene (2-AF), and benzo[a]pyrene (B[a]P) were obtained from the Sigma Chemical Co. (St. Louis, USA). Bacto agar and Oxoid nutrient broth No. 2 were purchased from Difco Laboratories (Detroit, USA) and Oxoid (Hants, UK), respectively. All solvents and other chemicals were reagent grade.

Preparation of S9 mixture Many mutagens need to be metabolized by the cytochrome P-450 dependent monooxygenase system before they elicit mutagenic activity (Kim *et al.*, 1999; Moon *et al.*, 1999). Mammalian hepatic microsomes, or 9,000 × g supernatants (S9) that contain this system, are commonly used for the activation of promutagens to mutagenic metabolites. The S9 mixture was prepared according to Maron and Ames (1983). Sprague-Dawley male rats received a combined intraperitoneal injection of phenobarbital and β-naphthoflavone. The treated rats were starved 12 h before they were sacrificed, then their livers were removed aseptically, minced, and homogenized in three volumes of 0.15 M KCl. The liver homogenate was centrifuged at 9,000 × g for 20 min, and the supernatant (S9 fraction) was stored as aliquots at -80°C. The preparation of S9 mixture was carried out according to the method of Maron and Ames (1983). The 0.1 M NADP, 1 M glucose 6-phosphate, $MgCl_2$-KCl salts, and 0.2 M sodium phosphate buffer (pH 7.4) were mixed with 10% S9 fraction.

Bacterial tester strains The *S. typhimurium* TA 98 and TA 100 strains, obtained from the Korean Collection of Type Cultures (Taejeon, Korea), were tested for the presence of the ampicillin resistance factor (R-factor) and cultured for 13-14 h before use.

Mutagenicity assay The *Salmonella* mutagenicity tests were performed essentially as described by Maron and Ames (1983). Chitosan oligosaccharide I and chitosan oligosaccharide II at concentrations of 0.01, 0.1, 1.0, and 2.0 mg in 100 μl of distilled water were tested with the *S. typhimurium* strains TA 98 and TA 100, with or without the addition of 0.5 ml of the S9 mixture. The positive control plates for TA 98 contained NPD and 2-AF. The positive control plates for TA100 contained NaN_3 and B[a]P. The plates for the negative control also contained 100 μl of H_2O both with and without the S9 mixture. A sample was considered mutagenic when the observed number of colonies was at least 2-fold above the spontaneous level.

***In vitro* antimutagenicity assay** The inhibitory effect of chitosan oligosaccharide on the mutagenic activity of MeIQx (5 ng per plate) was examined in a preincubation assay as outlined by Maron and Ames (1983). The preincubation assay was carried out as follows: chitosan oligosaccharide I, or chitosan oligosaccharide II and/or mutagen MeIQx dissolved in 0.1 ml water, were mixed with 0.5 ml S9 mix and 0.1 ml bacterial culture in a sterile tube and incubated in a shaking bath (low speed) for 20 min at 20°C. Tube contents were transferred to test tubes containing 2 ml of top agar. The tubes were vortexed and poured onto minimal agar plates. The revertant colonies were counted to determine the inhibitory effects, expressed as an inhibition rate.

Host mediated bacterial mutation assay Four-week-old male ICR mice were purchased from the Dae-han Laboratory Animal Research Center (Eumsung, Korea). Seven animals were housed in each cage. After a one-week acclimation period, chitosan oligosaccharide II or the vehicle alone were administered for 14 consecutive days (intragastric application at doses of 0.1 or 0.5 g/kg body wt).

The experiment was a modification of the method of Arni *et al.* (1977). Mice were treated intravenously with 0.1 ml of concentrated overnight culture (5×10^{10} colony-forming units/ml) of *S. typhimurium* TA 98. Immediately after the intravenous injection, the mice were orally dosed with 4.5 mg MeIQx/kg body wt in a 10 mM sodium acetate buffer, pH 4.2. Control mice received an equivalent volume of solvent (10 ml/kg body wt). One hour after treatment, the mice were killed, and the livers were aseptically removed. The livers were homogenized in 10 vols. of phosphate-buffered saline (PBS), pH 7.2. Bacteria were then separated from the unbroken cells and debris by centrifugation for 10 min at 100 × g. The resulting supernatant was centrifuged for 20 min at 2,500 × g in order to sediment the bacteria. The pellet was then resuspended in a 10 ml fresh nutrient broth medium and incubated for 1 h in a shaking incubator at 37°C. Bacteria were recovered by differential centrifugation and resuspended in 0.5 ml of PBS. The number of his^+ revertants were determined by adding 0.1 ml of a bacterial resuspension to 2.5 ml of top agar, which was then poured onto agar plates. The plates were incubated for 48 h at 37°C, and the number of colonies were counted to determine the inhibitory effects. Seven mice per group were treated, and the number of revertants recovered was determined from the mean result obtained from five replicate plates per liver.

Statistical analysis The data were analyzed for statistical significance using Student's t-test. P values less than 0.05 were considered to be significant.

Results and Discussion

Mutagenic activity As shown in Table 1, no mutagenic activity of chitosan oligosaccharide I and chitosan oligosaccharide II was detected when investigated on the *S. typhimurium* strains TA98 and TA100 both with and without the S9 mixture. The number of spontaneous revertants were normal, and the background growth was typical in all experiments.

***In vitro* antimutagenic activity** The inhibitory effects of chitosan oligosaccharide I and chitosan oligosaccharide II on the mutagenic activity of the MeIQx using tester strain *S. typhimurium* TA 98 were examined. It is evident from Table 2 that chitosan oligosaccharide I and II (at the concentration of 2 mg level) inhibited the mutagenic activity of MeIQx by 26.94% and 31.07%, respectively. The results showed that the oligosaccharides inhibited the mutagenicity of the cooked food mutagen, MeIQx, at a concentration dependent manner. This is in accordance with some previous studies where solvent extracts from fruit and vegetable residues (Edenharder *et al.*, 1994) and dichlorostearic acid (Vereskuns *et al.*, 1998) inhibits the MeIQx-induced mutagenicity using *S. typhimurium* TA 98. The present study suggested that chitosan oligosaccharide might possess antimutagenic components, which could reduce the mutagenic activity induced by MeIQx. The exact mechanisms by which chitosan oligosaccharide exerts its antimutagenic effect with respect to heterocyclic aromatic amines from cooked food are unknown. However, chitosan oligosaccharide may inhibit MeIQx conversion to a bacterial mutagen in a complex manner. This type of inhibition may depend on the inhibitor concentration being competitive at low and mixed or non-competitive at elevated concentrations. It indicates interactions with some components of the microsomal enzyme system, especially with the activation of MeIQx. Sousa *et al.* (1985) found a concentration dependent inhibition mechanism of mixed function oxidase by quercetin, an inhibitor of ethoxyresorufin metabolism. With aflatoxin B1, however, a competitive concentration independent inhibition mechanism was reported for fisetin, kaempferol, and rutin and a non-competitive one for morin (Francis *et al.*, 1989). From these results it may be concluded that multifactorial inhibition takes place with the competition of chitosan oligosaccharide and substrate being one mechanism. Inhibition of NADPH-cytochrome oxidase may be involved. Additional studies are required to determine the effect of chitosan oligosaccharide on various components of the microsomal enzyme system in order to elucidate the inhibition mechanisms more precisely.

Host mediated assay In the host mediated assay, the genotoxicity of mutagen in the livers of mice was measured by the detection of revertants of *S. typhimurium* that had previously been administered by a tail vein injection. In a preliminary study, B[a]P at doses of 50-200 mg/kg body wt was not mutagenic in the host mediated assay, presumably

Table 1. Mutagenic activities of chitosan oligosaccharide I (COS I) and chitosan oligosaccharide II (COS II) using *S. typhimurium* strain TA 98. The *Salmonella* mutagenicity test was performed using plate incorporation assay.

Samples	Dosage(per plate)	Revertants per plate[a]			
		Without S9 mixture		With S9 mixture[b]	
		TA 98	TA 100	TA 98	TA 100
H_2O	0.1 ml	26 ± 2.5[c]	154 ± 16.0	34 ± 7.8	146 ± 12.4
NPD[d]	10 μg	801 ± 47.1	-	-	-
NaN_3[e]	1 μg	-	426 ± 37.3	-	-
2-AF[f]	5 μg	-	-	259 ± 14.8	-
B[a]P[g]	5 μg	-	-	-	201 ± 23.3
COS I	0.01 mg	18 ± 3.9	120 ± 14.7	26 ± 3.7	114 ± 10.6
	0.1 mg	24 ± 3.1	151 ± 14.9	35 ± 5.6	103 ± 16.8
	1 mg	17 ± 1.8	107 ± 9.1	34 ± 3.2	145 ± 7.4
	2 mg	17 ± 2.8	141 ± 11.1	24 ± 2.2	134 ± 11.4
COS II	0.01 mg	19 ± 3.2	144 ± 13.6	23 ± 3.2	142 ± 24.8
	0.1 mg	26 ± 4.0	165 ± 19.6	23 ± 2.2	121 ± 11.8
	1 mg	24 ± 3.2	173 ± 12.5	31 ± 2.1	101 ± 9.2
	2 mg	17 ± 2.0	132 ± 10.1	30 ± 4.2	120 ± 8.9

[a]The revertants colonies induced by H_2O, NPD, NaN_3, 2-AF, B[a]P, and test samples were counted.
[b]0.5 ml S9 mixture/plate
[c]Values are mean ± SD (standard deviation) of three experiments, each plated in duplicate.
[d]NPD, 4-nitro-*o*-phenylenediamine, [e]NaN_3, sodium azide, [f]2-AF, 2-aminofluorene, [g]B[a]P, benzo[a]pyrene

Table 2. Inhibition of MeIQx-induced mutagenesis in *S. typhimurium* TA98 by chitosan oligosaccharide (COS I) and chitosan oligosaccharide II (COS II). The antimutagenic activity of oligosaccharide against MeIQx was examined in a preincubation assay.

Samples	Dosage (per plate)	Number of revertants per plate	Inhibition of mutagenesis (%)
MeIQx	5 ng	411.67 ± 23.67	
COS I	0.1 mg	363.22 ± 13.67*	11.77
	1 mg	332.78 ± 23.28*	19.16
	2 mg	283.78 ± 6.36**	31.07
COS II	0.1 mg	340.33 ± 14.84*	17.33
	1 mg	324.56 ± 17.24**	21.16
	2 mg	300.78 ± 11.10**	26.94

Values represent mean ± S.D. (standard deviation) of three experiments.
The values statistically significant as compared with control group (*: $p<0.05$, **: $p<0.01$).

Table 3. Effect of chitosan oligosaccharide II (COS II) on mutagenicity of MeIQx on *S. typhimurium* TA98 using a host-mediated assay in mice.

Samples (mg/kg body weight)	His^+ revertants/plate	% of inhibition
Control	256.11 ± 60.76	
100	157.68 ± 31.68	38.43
500	135.56 ± 29.26*	47.07

Values represent mean ± S.D. (Standard deviation).
The values statistically significant as compared with control group (*: $p<0.05$).

because of the high levels of detoxifying enzymes, such as epoxide hydratase, in the liver (results not shown). Treatment with MeIQx leads to a genotoxic response in the livers of control mice. Animals applied with chitosan oligosaccharide II exhibited a significantly ($p<0.05$) lower response when challenged with MeIQx than those applied with the control. Our results are consistent with those of Alldrick *et al.* (1995), who reported that consumption of caffeine led to a 47% reduction in the number of mutants induced by MeIQx using a host-mediated assay.

MeIQx is mutagenic in the bacterial genotoxicity assay and is carcinogenic in rats and mice (Kasai *et al.*, 1981). Oral administration of MeIQx leads to its binding to the DNA of major organs, with binding to liver DNA being of a similar order to that of 2-acetylaminofluorene (Alldrick and Lutz 1989). The biochemical basis of these toxicological effects lies in the metabolic conversion of MeIQx to electrophilic intermediates that can bind to DNA. Thus, it is possible that chitosan oligosaccharide can modify MeIQx mutagenicity by altering the enzymes and cofactors in the liver responsible for its activation. Overall our results suggested that chitosan oligosaccharide had antimutagenic activity against MeIQx *in vitro* and *in vivo*.

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