Side-Chain Structure of Cell Surface Polysaccharide, Mannan, Affects Hypocholesterolemic Activity of Yeast

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We previously reported that *Kluyveromyces marxianus* YIT 8292 exhibited more potent hypocholesterolemic activity than other yeasts containing *Saccharomyces cerevisiae*. To clarify the reason for the higher hypocholesterolemic activity, we examined the side-chain structure of cell surface polysaccharide, mannan, of *K. marxianus* YIT 8292. The result shows that *K. marxianus* YIT 8292 had shorter α-(1,2)-linked oligomannosyl side chains and lower phosphate content in mannan than *S. cerevisiae*. The association between its structural features and hypocholesterolemic activity was investigated by comparing the hypocholesterolemic activities of *S. cerevisiae* mannan mutants in rats fed a high-cholesterol diet. *S. cerevisiae mnn5* mutant with deficiencies in the phosphorylation and elongation of mannan side chains showed higher hypocholesterolemic activity than the wild-type strain. These results show that the side-chain length and phosphate contents of mannan affect hypocholesterolemic activity.

KEYWORDS: *Kluyveromyces marxianus*; mannan; side chain; cell wall; cholesterol

INTRODUCTION

Yeasts have diverse fermentation activities and therefore have been used for the production of many kinds of fermented foods such as bread, beer, wine, and fermented milk (*1*). Dried yeasts (usually heat inactivated) have also been used as a nutritional supplement because whole yeast cells are rich in vitamin B, dietary fiber, and protein (*1*). Three species of yeast, *Saccharomyces cerevisiae*, *Candida utiliz*, and *Kluyveromyces marxianus*, are widely used as dried yeasts (*1*). Recently, the beneficial effects of dried yeasts on health have been investigated (*2−11*). In our previous study comparing the hypocholesterolemic activities of 81 yeast strains (*8*), we found that the hypocholesterolemic activities of the yeasts varied remarkably between strains, with *K. marxianus* YIT 8292 exhibiting more potent hypocholesterolemic activity than the other yeasts containing *S. cerevisiae* (baker's yeast and brewer's yeast). We have also found that cell wall polysaccharides are the major active components of *K. marxianus* YIT 8292 (*9*). Rats fed cell wall polysaccharide-enriched fractions had increased fecal sterol excretion and cecal concentration of short-chain fatty acids (SCFA) (*9*). Thus, the cell wall polysaccharides of *K. marxianus* YIT 8292 can exert hypocholesterolemic activity through the suppression of intestinal cholesterol absorption, the interruption of the enterohepatic circulation of bile acids, and/or the production of SCFA. Our previous paper has shown that daily intake of *K. marxianus* YIT 8292 crude cell wall fraction decreases serum total cholesterol and

LDL-cholesterol levels in hypercholesterolemic subjects at doses of 3.0 and 4.0 g/day (*10*).

The cell wall of yeast is mainly composed of dietary fiber such as β-glucan and mannan (*12*). Mannoprotein, a mannan-rich glycoprotein, surrounds the inner layer consisting of β-(1,3) glucan, β-(1,6) glucan, and a small amount of chitin. It has been reported that the proportions of β-glucan and mannan within the cell wall and the structures of those polysaccharides differ according to the strain (*13−15*). We therefore suggest the possibility that the structural and/or compositional differences of cell wall polysaccharides among yeast strains are involved in the variation of hypocholesterolemic activities.

The structural differences in the outer chain of N-linked mannan among other cell wall polysaccharides are well characterized and utilized for the serological classification of yeasts. The mannan structures of several yeasts such as *S. cerevisiae*, *S. italicus*, *C. albicans*, *C. kefyr*, and *K. lactis* have been identified (*13, 14*). A long branched α-(1,6)-linked mannose backbone structure has been found in a wide variety of yeast species, suggesting it is a general feature of most yeasts. These chains are usually branched with α-(1,2)-linked mannoses, which are then extended with a variety of genus- or species-specific linkages. Mannan of *K. lactis* is closely analogous to that of *S. cerevisiae*, except that it lacks phosphorylated side chains and instead possesses *N*-acetylglucosamine substituents on some of the mannnotetrarose units (*13, 16*). Ballou documented that *K. marxianus* and *K. dobzhanskii* have α-mannan structurally similar to that of *K. lactis* (*13*). On the other hand, Kobayasi showed that *C. kefyr* (the imperfect state of *K. marxianus*), strain IFO 0586, has a comblike structure consisting of an α-(l,6)-linked backbone and

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short α-(l,2) branches (*17*, *18*). Its side-chain structure differs from that of *K. lactis* and *S. cerevisiae* by a lack of phosphate and terminal α-(1,3)-linked mannose units.

Ballou and colleagues isolated and characterized several mannan mutants of *S. cerevisiae* X2180-1A to investigate the mechanism of mannan synthesis (*13*, *14*, *19*). The *mnn4* and *mnn1* mutants lack the mannosyl-phosphate side chain and the terminal α-(1,3)-mannose residues of the outer chain portion, respectively. The *mnn5* mutant is defective in the addition of the second α-(l,2)-linked mannose. These mutants can be used to study the effects of the side-chain lengths and phosphate groups of mannan on function.

In the present study, we analyzed the mannan structure of *K. marxianus* YIT 8292. The association between its structural features and hypocholesterolemic activity was investigated by comparing the hypocholesterolemic activities of *S. cerevisiae* mannan mutants in rats fed a high-cholesterol diet.

MATERIALS AND METHODS

Yeast and Cultivation. *S. cerevisiae* X2180-1A (*MATα SUC2 mal mel gal2 CUP1*) and its mannan mutants *mnn4* (LB6-5D *MATα mnn4-1 SUC2 mal CUP1*), *mnn1 mnn4* (LB 97-3C *MATα mnn1 mnn4*), and *mnn5* (LB65-5D *MATα mnn5*) were obtained from the American Type Culture Collection (ATCC). The other strains were obtained from the culture collection of the Yakult Central Institute for Microbiological Research (Tokyo, Japan).

All strains were grown in liquid culture medium containing 0.5% yeast extract, 1% polypeptone, 3% glucose, 0.2% KH_2PO_4, 0.5% K_2HPO_4, and 0.05% $MgSO_4 \cdot 7H_2O$ (*8*). Cultures were grown aerobically at 30 °C for 24 h and harvested by centrifugation.

Crude Cell Wall Fraction and Mannan Preparation. Crude cell wall fraction was prepared as described previously (*9*). Mannan was extracted by autoclaving at 120 °C for 90 min in 0.1 M citrate buffer at pH 7.0 and was further purified by selective precipitation of mannoprotein with borate and hexadecyltrimethylammonium bromide (Cetavlon) (*14*).

Cell Surface Hydrophobicity (CSH) Assay. CSH of crude cell wall was determined by hydrophobic interaction chromatography as described previously (*20*). Yeast crude cell wall was washed twice with 100 mM sodium acetate buffer (pH 4.2) and resuspended in the same buffer to a final concentration of 15 mg/mL. Phenyl Sepharose 6 Fast Flow gel (GE Healthcare, Piscataway, NJ) was packed in a disposable chromatography column (0.6 cm × 3.7 cm) (Biospin empty column; Bio-Rad, Richmond, CA) to a volume of 1.0 mL and equilibrated with 100 mM sodium acetate buffer (pH 5.0) containing 125 mM NaCl. Yeast cell suspensions (50 μL) were then applied to the column and eluted by gravity flow with 3 mL of buffer containing NaCl. The OD_{660} of the eluent was measured, and CSH was determined by using the following equation: CSH (%) = $100(A_{applied} - A_{eluent})/A_{applied}$, where $A_{applied}$ is the OD_{660} of 0.1 mL of the cell suspension diluted with 3 mL of elution buffer and A_{eluent} is the OD_{660} of the eluent.

Animals and Diets. Five-week-old male Wistar rats were obtained from Clea Japan, Inc. (Tokyo, Japan). All animal experiments were performed in accordance with the guidelines of the Ethical Committee for Animal Experiments of Yakult Central Institute for Microbiological Research (Tokyo, Japan). The rats were housed individually in stainless steel cages and kept in an isolated room at a controlled temperature (24 ± 1 °C) and humidity (60 ± 5%). Lighting was maintained on a 12 h light–dark cycle (lights on from 8 a.m. to 8 p.m.). The rats were fed a commercial solid diet (MF; Oriental Yeast Co. Ltd., Tokyo, Japan) during the acclimatization period of 7 days. After this period, they were randomly divided into groups (*n* = 8/group) and fed the powdered diets described next. All treatment groups started with similar mean body weights ranging from 176 to 179 g.

The high-cholesterol diet consisted of (g/kg) casein, 223; sucrose, 578; mineral mix, 40; vitamin mix, 10; soy oil, 10; lard, 100; choline bitartrate, 1.5; cholesterol, 5.0; sodium cholate, 2.5; and cellulose, 30 (*9*). Each cell wall fraction was added to the high-cholesterol diet at a concentration of 3.5% (w/w). To equalize the contents of fiber, protein, and fat in each diet, the constituents of the cell wall fraction (**Table 1**) replaced cellulose, casein,

Table 1. Composition of Crude Cell Wall Fractions Isolated from *S. cerevisiae* X2180-1A, Its *mnn* Mutants, and *K. marxianus* YIT 8292[a]

| | *S. cerevisiae* | | | | |
	wild type[b]	*mnn4*	*mnn1 mnn4*	*mnn5*	KM
dietary fiber (%)	44.1	41.4	43.7	41.1	46.6
crude protein (%)	49.5	52.0	49.7	52.5	44.2
crude fat (%)	2.82	2.89	2.90	2.82	4.04
ash (%)	1.69	1.73	1.74	1.69	3.09
other components[c] (%)	1.88	1.93	1.94	1.88	2.13

[a] Each result is expressed as the mean value of two replicates. [b] Wild type, *S. cerevisiae* X2180-1A; KM, *K. marxianus* YIT 8292. [c] Components excluding dietary fiber, protein, fat, and ash.

and lard, respectively. Their moisture, ash, and other components replaced sucrose.

The rats were given a high-cholesterol diet with or without 3.5% crude cell wall fraction isolated from *S. cerevisiae*, the mutants of *S. cerevisiae mnn1*, *mnn1 mnn4*, *mnn5*, or *K. marxianus* YIT 8292 ad libitum for 14 days. Food intake and body weight were measured three times or once a week, respectively. The rats were then anesthetized with an intraperitoneal injection of sodium pentobarbital (Nembutal, Abbot Laboratories, Chicago, IL) at 10 a.m. on the last day of the experimental period, and nonfasting blood samples were collected from the abdominal aorta. The blood from each rat was put into a plastic tube containing heparin, and the plasma was separated by centrifugation. Each liver was perfused with 0.9% NaCl, removed, and weighed. Liver lipids were extracted according to the method of Folch et al. (*21*).

General Analytical Procedures. The total carbohydrate content of mannan was measured by using the phenol–sulfuric acid method (*22*). Sugar compositions of mannan and crude cell wall fraction were analyzed by high-performance liquid chromatography (HPLC) after 1 h of 4 M trifluoroacetic acid (TFA) hydrolysis at 80 °C and methanolysis (5% HCl–methanol, 100 °C for 2 h) combined with 4 M TFA hydrolysis (80 °C, 1 h), respectively, followed by labeling with 1-phenyl-3-methyl-5-pyrazolone (PMP) (*23*). Total phosphate was determined according to the method of Ames and Dubin with KH_2PO_4 as the standard.

Approximately 50 mg of mannan was acetolyzed as described previously (*24*). A 10:10:1 (v/v/v) mixture of $(CH_3CO)_2O$, CH_3COOH, and H_2SO_4 was used for preferential cleavage of the α-(1,6) linkage. The O-acetylated mannooligosaccharide mixture was extracted from the reaction mixture with $CHCl_3$ and deacetylated in 0.2 M sodium methoxide in methanol. One-tenth of the resultant mannooligosaccharide mixture was pyridylaminated and then analyzed by HPLC with a size exclusion chromatographic column (TSKgel α-2500, Toso, Tokyo, Japan) (*25*). The other part of the acetolysis product was applied to a column packed with Bio-Gel P2 (Bio-Rad). The biose and triose fractions were isolated and then identified by NMR analysis.

^{1}H and ^{13}C NMR (DEPT 135) spectra were recorded with a FT-NMR spectrometer (ECA-500, JEOL, Tokyo, Japan) for solutions in D_2O at 30 °C with acetone as the internal standard. 2D-NMR (HSQC, HMQC, HMBC, COSY) spectra were measured with a FT-NMR spectrometer (ECA-500, JEOL) for solutions in D_2O at 60 °C with acetone as the internal standard.

The methylation of mannan was performed according to the method of Ciucanu and Kerek (*26*). The methylated product was hydrolyzed with 4 M TFA at 121 °C for 1 h, reduced with $NaBH_4$, and acetylated. The alditol acetates of methylated sugars were identified by GC-MS, performed on a Hewlett-Packard 6890A gas chromatograph connected to a JMS-700 V mass spectrometer (JEOL). Gas chromatography of *O*-methyl-*O*-acetyl-D-mannitols was performed using a SE52 glass capillary column (30 m × 0.25 mm i.d.) at 200 °C using He as the carrier gas at a flow rate of 0.7 mL/min. The MS detection conditions were as follows: interface temperature, 270 °C; ionization mode, EI+; electron energy, 70 eV.

Total cholesterol, triglycerides, and phospholipids in the plasma and liver were enzymatically measured on two replicates with commercial kits (Determiner TC555, Kyowa Medics, Tokyo, Japan; Triglyceride E test, Wako Pure Chemical Industries, Tokyo, Japan; and Phospholipid B test, Wako Pure Chemical Industries). HDL-cholesterol was measured

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with a commercial kit (Determiner HDL, Kyowa Medics, Tokyo, Japan). Dietary fiber, crude protein, crude fat, and ash were measured according to the methods of the Association of Official Analytical Chemists (27).

Statistical Analysis. Data are presented as mean ± SD. Statistical significance was determined using the Tukey test, and $p < 0.05$ was considered to be statistically significant.

Table 2. Composition of *K. marxianus* YIT 8292 Mannan[a]

	K. marxianus YIT 8292 mannan
chemical composition	
total carbohydrate (g/g)	0.73 ± 0.02
total phosphate (μmol/g)	50 ± 2
sugar composition	
mannose (%)	98 ± 1
glucose (%)	1 ± 0
glucosamine (%)	0.5 ± 0.0

[a] Each result is expressed as the mean value of three replicates.

RESULTS AND DISCUSSION

Analysis of Mannan Structure Isolated from *K. marxianus* YIT 8292. The results of compositional analysis of *K. marxianus* YIT 8292 mannan are given in **Table 2**. It contained a large amount of carbohydrate, 98% of which was mannose. The phosphate content was 50 μmol/g, which is lower than that of *S. cerevisiae* X2180-1A (230 μmol/g) or baker's yeast (*S. cerevisiae* YIT 10025) (200 μmol/g).

^{1}H NMR, ^{13}C NMR DEPT 135, HSQC, HMBC, and COSY NMR spectra of intact *K. marxianus* YIT 8292 mannan are shown in **Figure 1**. Two major signals were detected in the anomeric region at approximately 5.09 and 5.01 ppm in the ^{1}H NMR and were labeled H1A and H1B, respectively. Two major anomeric carbon signals, 102.4 and 98.5 ppm, were assigned as C1B and C1A from cross peaks at 5.01/102.4 and 5.09/98.5 ppm, respectively, in the HSQC spectrum. The chemical shifts of H2A and C2A were identified from the presence of cross peaks at 5.09/4.01 ppm in the COSY spectrum and at 4.01/78.9 ppm in the
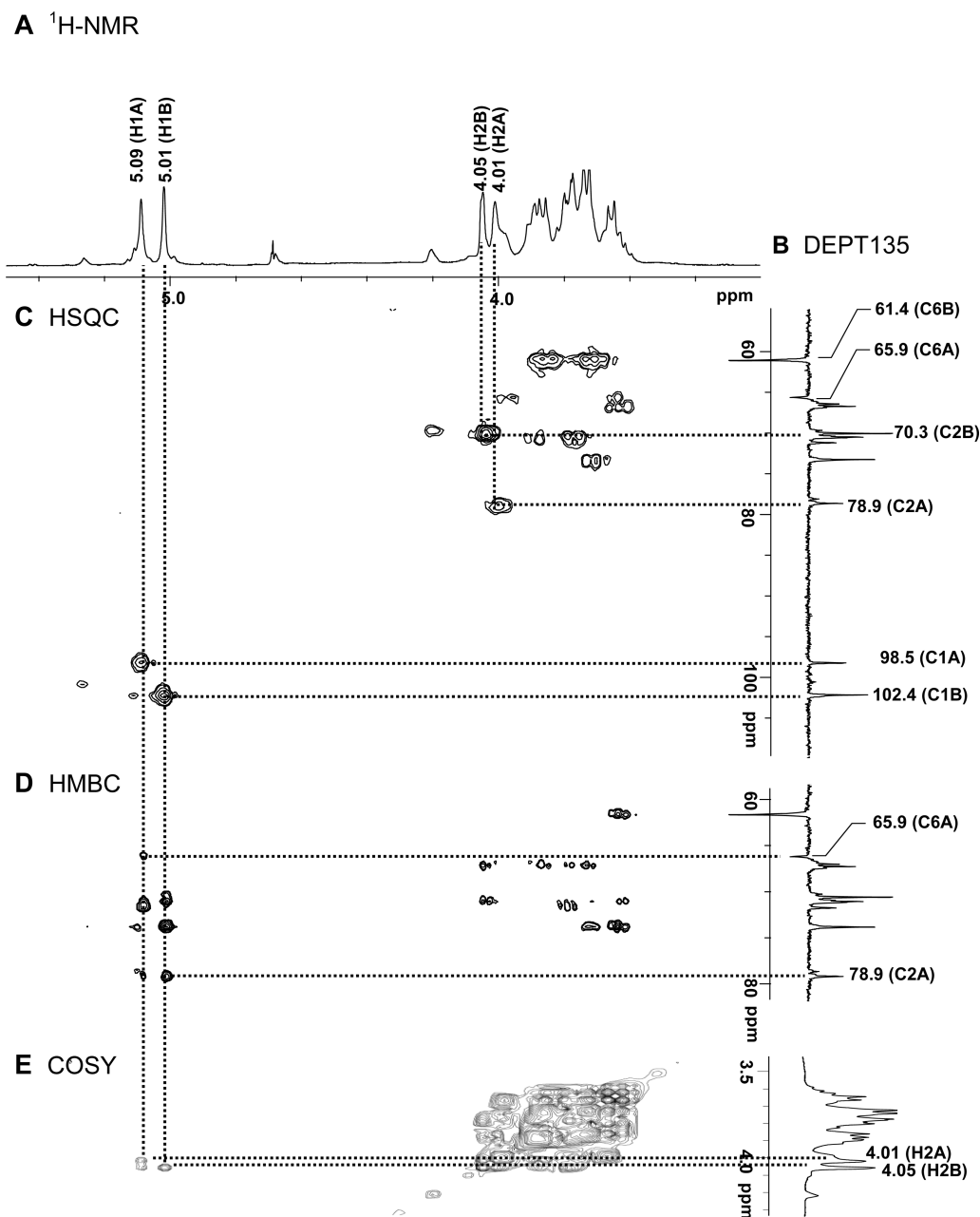

Figure 1. ^{1}H NMR (**A**), ^{13}C NMR DEPT 135 (**B**), HSQC (**C**), HMBC (**D**), and COSY (**E**) spectra of intact *K. marxianus* YIT 8292 mannan.

Table 3. ^{1}H and^{13}C NMR Chemical Shifts of Intact Mannan Isolated from *K. marxianus* YIT 8292 and Its Acetolysis Products

oligosaccharide	sugar residue			proton	chemical shift (ppm)			carbon	chemical shift (ppm)		
	C	B	A		C	B	A		C	B	A
acetolysis-released triose	Manα1→2Manα1→2Manα			H1	4.99	5.25	5.32	C1	102.3	100.7	92.6
				H2	4.01	4.06	3.88	C2	70.5	78.6	79.4
intact mannan			↓$_6$	H1		5.01	5.09	C1		102.4	98.5
	Manα1→2Manα			H2		4.05	4.01	C2		70.3	78.9
			↓^{1}					C6		61.4	65.9

Table 4. Methylation Analysis of *K. marxianus* YIT 8292 Mannan

| partially methylated mannitol acetate	linkage	molar ratio
2,3,4,6-tetra-*O*-methyl	Man 1→	1.00
2,4,6-tri-*O*-methyl	→3 Man 1→	0.36
3,4,6-tri-*O*-methyl	→2 Man 1→	0.36
2,3,4-tri-*O*-methyl	→6 Man 1→	0.07
3,4-di-*O*-methyl	→2,6 Man 1→	0.71
4-*O*-methyl	→2,3,6 Man 1→	0.07

HSQC spectrum. H2B and C2B were assigned as 4.05 and 70.3 ppm, respectively, from cross peaks at 5.01/4.05 ppm in the COSY spectrum and at 4.05/70.3 ppm in the HSQC spectrum. In the HMBC spectrum, it was suggested that an α-(1,2) linkage was present between residues A and B from a cross peak between H1B (5.01 ppm) and C2A (78.9 ppm). The DEPT 135 spectrum shows two inverted methylene signals (61.4 and 65.9 ppm). In the HMBC, the methylene signal (65.9 ppm), which is a downfield shift, correlated with the signal at 5.09 ppm (H1A), indicating the presence of an α-(1,6)-linked backbone. Therefore, it was suggested that *K. marxianus* YIT 8292 mannan consists mainly of the partial structure shown in **Table 3**. In the methylation analysis of *K. marxianus* YIT 8292 mannan, the major methylation products 2,3,4,6-tetra-*O*-methylmannitol and 3,4-di-*O*-methylmannitol made up approximately 70% (mol/mol) of the total, which is consistent with the above-mentioned structure (**Table 4**).

K. marxianus YIT 8292 mannan was subjected to acetolysis, which selectively cleaves backbone α-(1,6) linkages, to obtain the oligosaccharides corresponding to the side chains. The proportions of biose (M$_2$), triose (M$_3$), tetraose (M$_4$), and pentaose (M$_5$) in the resultant total oligosaccharides were 76.5, 11.8, 8.8, and 2.9%, respectively, on a molar basis (**Figure 2**). The composition of mainly short side chains is similar to that of *S. cerevisiae* mnn5 mutant and *C. kefir* IFO 0586 (*17–19*), but different from that of *S. cerevisiae* and *K. lactis* (*16*).

M$_2$ and M$_3$ were isolated and then identified by NMR analysis. The ^{1}H NMR and ^{13}C NMR data of M$_2$ are identical to those of authentic Man-α-(1,2)-Man. The major component of M$_3$ was identified as Man-α-(1,2)-Man-α-(1,2)-Man by HMQC, HMBC, and COSY NMR spectra (**Figure 3**; **Table 3**). The chemical shifts are consistent with those in previous papers (*17, 18*).

The presence of M$_3$ in the acetolysis products and 3,4,6-tri-*O*-methylmannitol in the methylation products indicates that *K. marxianus* YIT 8292 mannan also contains the internal α-(1,2)-linked mannose unit. The weak cross peak observed at 5.26/100.8 ppm in the HSQC spectrum of intact *K. marxianus* YIT 8292 mannan (**Figure 1**) was deduced to correspond to the internal mannose unit from the NMR data of *C. kefir* IFO 0586 (*17, 18*). The cross peaks in the HSQC and HMBC spectra of *K. marxianus* YIT 8292 mannan were identical to those observed in *S. cerevisiae* mnn5 mutant (spectrum not shown). These findings show that *K. marxianus* YIT 8292 possesses a comblike structure comprising an α-(1,6)-linked backbone substituted with a large number of short side chains composed of α-(1,2)-linked mannose.

Mannan of *K. lactis* is closely analogous to that of *S. cerevisiae* except that it lacks phosphorylated side chains and instead
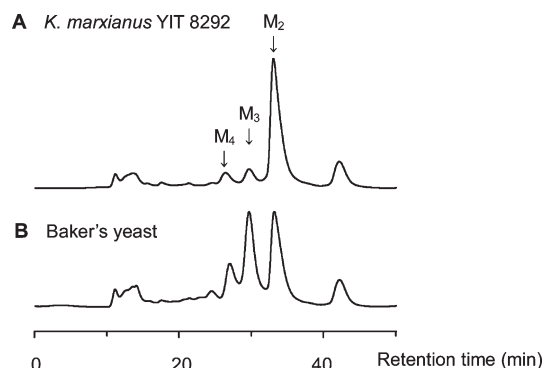
Figure 2. HPLC chromatogram of pyridylamino derivatives of acetolysis products of *K. marxianus* YIT 8292 mannan (**A**) and baker's yeast (*S. cerevisiae* YIT 10265) mannan (**B**).

possesses *N*-acetylglucosamine substituents on some of the mannnotetrarose units (*15*). Ballou described that *K. marxianus* has α-mannan structurally similar to that of *K. lactis* (*13, 16*). The results of our structural analysis show that the cell surface polysaccharide, mannan, of *K. marxianus* YIT 8292 consists of a backbone of α-(1,6)-linked residues with short α-(1,2)-linked branches, but lacks terminal α-(1,3)-mannose residues. It has a comblike structure with shorter side-chain lengths and a lower number of phosphate groups than that of *S. cerevisiae*. These structural characteristics are quite similar to those reported for *C. kefyr* IFO 0586 (*17, 18*), which is the imperfect state of *K. marxianus*, and mannan mutants *mnn1 mnn4* and *mnn5* of *S. cerevisiae*. The discrepancy concerning the mannan structure of *K. marxianus* may be attributed to the existence of intraspecific variations in mannan structure.

Comparison of Hypocholesterolemic Activities in Rats and Cell Surface Hydrophobicity of *S. cerevisiae* X2180-1A and Its Mannan Mutants. In our previous study, which compared the hypocholesterolemic activities of 81 yeast strains, we found that *K. marxianus* YIT 8292 exhibits greater hypocholesterolemic activity compared with other yeasts containing *S. cerevisiae* (*8*). We also found that cell wall polysaccharides are the major active components of *K. marxianus* YIT 8292 (*9*). On the basis of these previous findings, we suggest that the structural features of mannan side chains of *K. marxianus* YIT 8292 may be involved in its potent hypocholesterolemic activity. This possibility was confirmed by comparing the hypocholesterolemic activities of mannan mutants derived from *S. cerevisiae* X2180-1A in rats fed a high-cholesterol diet. Schematic structures of N-linked mannan outer chain of *S. cerevisiae* mannan mutants are shown in **Figure 1A** on the bases of previous papers (*13, 14*). The nutritional composition of each crude cell wall is shown in **Table 1**. Their dietary fiber contents were very similar. The hydrolysates of these cell walls had an approximate composition of glucose and mannose in the ratio of 1:1. These data suggest that no important difference in β-glucan and α-mannan contents exists.

Food intake and body weight gain did not differ among groups (**Table 5**). Plasma triglycerides, phospholipids, and HDL-cholesterol
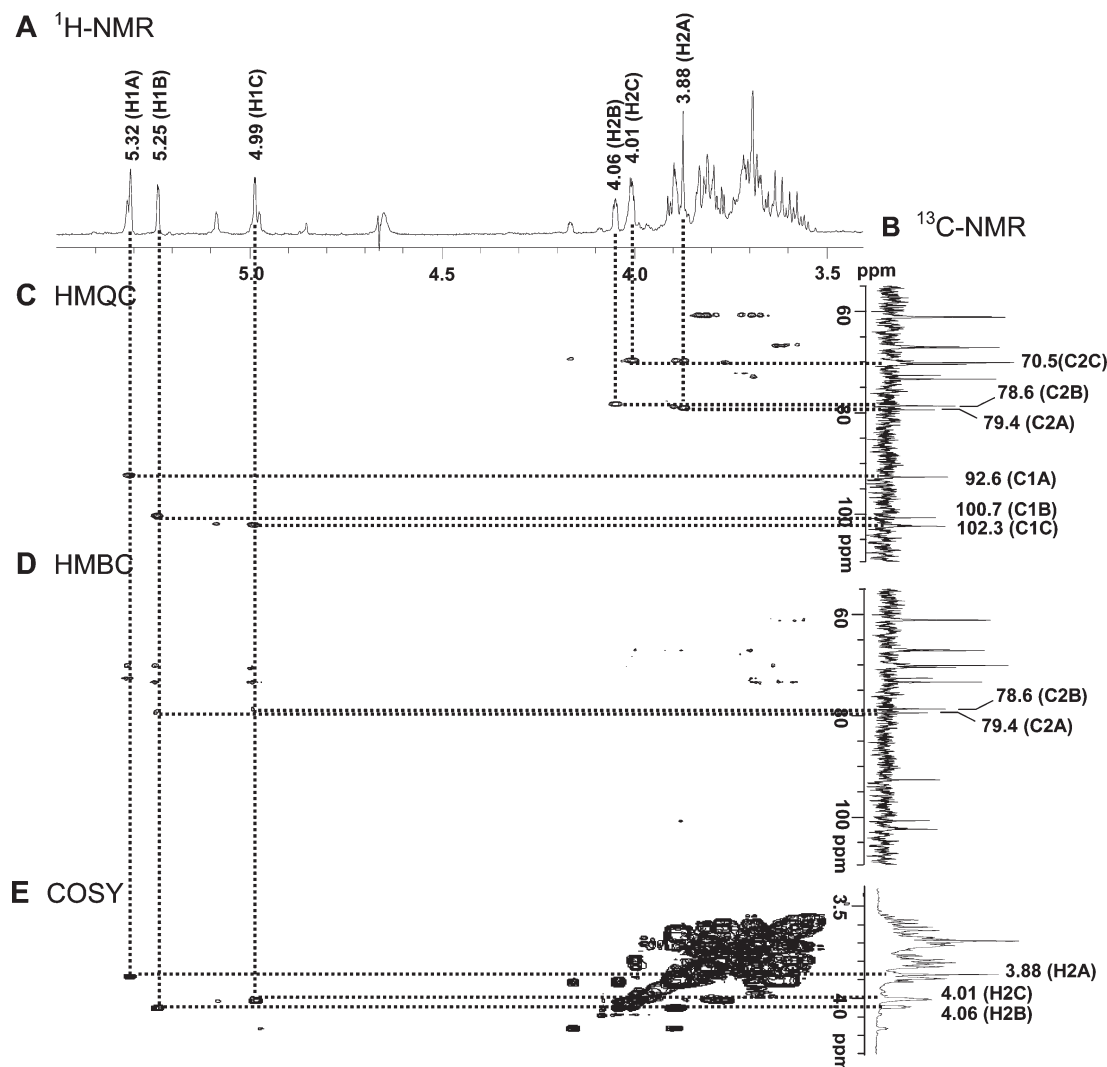
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Figure 3. ^{1}H NMR (**A**), ^{13}C NMR (**B**), HMQC (**C**), HMBC (**D**), and COSY (**E**) spectra of triose obtained from acetolysis product of *K. marxianus* YIT 8292 mannan.

Table 5. Effects of Crude Cell Wall Fractions Isolated from *S. cerevisiae* X2180-1A, Its *mnn* Mutants, and *K. marxianus* YIT 8292 on Body Weight Gain, Food Intake, Plasma Lipids, and Liver Lipids in Rats Fed a High-Cholesterol Diet[a]

		S. cerevisiae				
	control	wild type	mnn4	mnn1 mnn4	mnn5	KM
body weight gain (g/14 days)	94.5 ± 9.2 a	90.1 ± 8.2 a	86.2 ± 9.4 a	91.5 ± 7.2 a	85.0 ± 6.6 a	87.0 ± 6.6 a
food intake (g/14 days)	254 ± 22 a	252 ± 15 a	236 ± 20 a	247 ± 12 a	233 ± 10 a	238 ± 15 a
plasma lipids (mg/dL)						
HDL-cholesterol	11.4 ± 1.3 a	16.7 ± 2.3 a	17.6 ± 2.1 a	18.3 ± 2.3 a	17.4 ± 2 a	16.7 ± 1.9 a
triglycerides	136 ± 44 a	191 ± 75 a	228 ± 81 a	150 ± 89 a	153 ± 51 a	162 ± 83 a
phospholipids	156 ± 24 a	159 ± 17 a	164 ± 24 a	137 ± 31 a	148 ± 16 a	145 ± 28 a
liver weight (dry g)	5.9 ± 0.4 a	5.6 ± 0.3 ab	5.3 ± 0.6bc	5.4 ± 0.3 bc	5.1 ± 0.3 bc	5.0 ± 0.4 c
liver lipids (mg/g)						
triglycerides	160 ± 17 a	169 ± 17 a	163 ± 17 a	158 ± 20 a	153 ± 15 a	150 ± 13 a
phospholipids	126 ± 4 a	124 ± 14 a	118 ± 13 a	123 ± 13 a	117 ± 4.5 a	114 ± 11 a

[a] The rats were given a high-cholesterol diet with or without 3.5% crude cell wall fraction isolated from *S. cerevisiae*, *mnn1*, *mnn1 mnn4*, *mnn5*, or *K. marxianus* YIT 8292 for 14 days. Each value is the mean ± SD ($n = 8$). Mean values within a row not sharing a common letter are significantly different ($p < 0.05$, Tukey test). Wild type, *S. cerevisiae* X2180-1A; KM, *K. marxianus* YIT 8292.

levels did not change significantly (**Table 5**). Liver dry weight was decreased by feeding the crude cell wall of *mnn4*, *mnn1 mnn4*, and *mnn5*, but not by wild type of *S. cerevisiae* (**Table 5**). Feeding crude cell wall isolated from *mnn4* mutant, which possesses phosphate-deficient mannan (*13, 14*), significantly decreased the total cholesterol levels in the plasma and liver, whereas the crude cell wall of the wild-type strain did not (**Figure 4**). In a comparison

of mannan mutants with different side-chain lengths, the hypocholesterolemic activity showed a tendency to decrease as the side-chain lengths shortened (**Figure 4**). The hypocholesterolemic activity of *mnn5* mutant, lacking the addition of the second α-(1,2)-linked mannosyl residue to the mannan side chains (*13, 14, 19*), was higher than that of the wild-type strain and approximately equal to that of *K. marxianus* YIT 8292. These results indicate that the side-chain
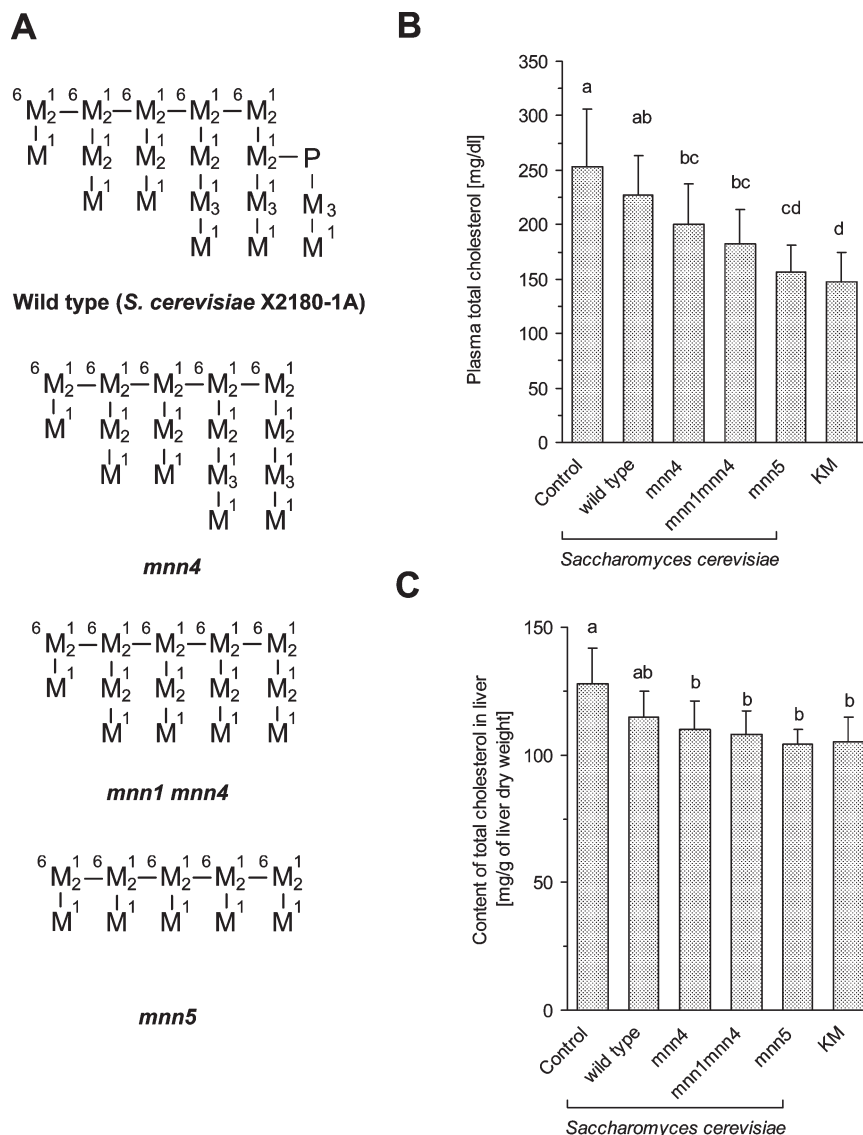
A

$$^6M_2{-}^1{}^6M_2{-}^1{}^6M_2{-}^1{}^6M_2{-}^1{}^6M_2{-}^1$$

Wild type (*S. cerevisiae* X2180-1A)

mnn4

mnn1 mnn4

mnn5

B

C

Figure 4. Effects of crude cell wall fractions isolated from *S. cerevisiae* X2180-1A, its mannan mutants, and *K. marxianus* YIT 8292 on total cholesterol levels in plasma (**B**) and liver (**C**). Each value is the mean ± SD (*n* = 8). Values not sharing a common letter are significantly different (*p* < 0.05, Tukey test). Wild type, *S. cerevisiae* X2180-1; KM, *K. marxianus* YIT 8292.

structure of cell surface mannan has an impact on the hypocholesterolemic activity of yeast. The results also suggest that the structural characteristics of mannan side chains of *K. marxianus* YIT 8292, low levels of phosphate, and short side chains are responsible, at least in part, for its potent hypocholesterolemic activity.

In the present paper, the effect of mannan side-chain structures on CSH was also investigated. CSH was determined by measuring the affinity of crude cell wall to the hydrophobic resin as described previously (*20*). As shown in **Figure 5**, CSH of all mannan mutants, which possesses phosphate-deficient mannan, was higher than that of wild-type strain. CSH of *mnn5* mutant, lacking the addition of the second α-(1,2)-linked mannosyl residue to the mannan side chains (*12, 13, 18*), was not significantly different from that of the *mnn4* mutant (*p* = 0.051), but significantly higher than those of the wild-type strain and *mnn1 mnn4* mutant. This result shows that deficiencies in the phosphorylation and the addition of the second α-(1,2)-linked mannosyl residue increase CSH. The increase of the CSH may explain part of the reason for the potent hypocholesterolemic activities of *mnn5* mutants.
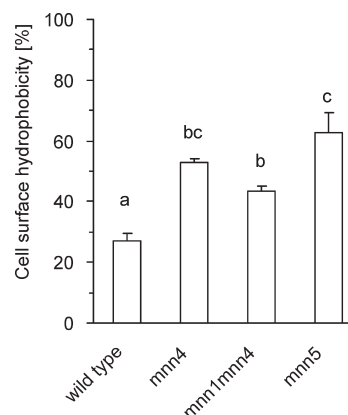

Figure 5. Cell surface hydrophobicity of *S. cerevisiae* X2180-1A and its mannan mutants.

At present, it is not clear why the side-chain length and phosphate contents of mannan affect hypocholesterolemic activities. It is well-known that some dietary fibers exhibit

hypocholesterolemic activity. The mechanisms by which dietary fibers elicit their hypocholesterolemic effect have been proposed (*28*). One of the primary actions is to reduce cholesterol uptake and dietary fat in the intestine. It has been proposed that the viscosity associated with soluble fibers interferes with key physiological events in the cholesterol absorptive process. These interference mechanisms include direct binding of sterols within the intestinal lumen, interference with the diffusion of cholesterol toward the epithelial cell surface, and/or antagonization of the cholesterol emulsification process. Dietary fibers also interfere with the enterohepatic circulation of bile acids, resulting in an increase in the conversion of cholesterol to bile acids in the liver (*28*). Another mechanism is the effect of short-chain fatty acids (SCFA) on cholesterol metabolism. SCFA are products of the colonic bacterial fermentation of dietary fiber. Several studies have shown that the suppressive effect of certain dietary fibers on the plasma cholesterol level was at least partly due to the inhibition of cholesterol biosynthesis caused by SCFA (*28*). We previously reported that feeding cell wall fraction isolated from *K. marxianus* YIT 8292 increased the fecal excretion of acidic and neutral sterols and the cecal SCFA concentration (*9*). These observations suggest that cell wall polysaccharides of *K. marxianus* YIT 8292 exerted a cholesterol-lowering effect through the several mechanisms described above. Thus, we speculate that the side-chain length and phosphate contents of mannan may affect the interaction with cholesterol and/or bile acids and the fermentability of cell wall polysaccharides. The association of CSH with hypocholesterolemic activity among *S. cerevisiae* mannan mutants may imply partial involvement of CSH in the cholesterol lowering (e.g., through direct binding of cholesterol). Further studies are necessary to demonstrate these possibilities.

In conclusion, mannan of *K. marxianus* YIT 8292 has a comblike structure with short side chains of α-(1,2)-linked mannopyranose residues and low phosphate content. Our results show that these structural characteristics have an impact on hypocholesterolemic activity.

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