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A GLUCOMANNAN FROM *Candida utilis* METHYLATION ANALYSIS AND FRAGMENTATION ANALYSIS BY CONTROLLED ACETOLYSIS OF THE GLUCOMANNAN

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ABSTRACT

A new chemotype glucomannan isolated from *Candida utilis* mutant was further studied with the aid of methylation analysis and fragmentation analysis by controlled acetolysis. Thus, it was revealed that the glucomannan has an α-(1→6)-linked D-mannosyl backbone partially substituted with side chains of one, two, three, or four D-mannosyl units connected by α-(1→2) linkages; moreover, it has an additional side chain in which D-glucose residues are linked through an α-(1→6) linkage at the nonreducing ends of four D-mannosyl units. Isolation of 3,4-di-*O*-methyl-D-mannose, 2-*O*-α-D-mannopyranosyl-D-mannose, *O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-D-mannose, *O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-D-mannose, *O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-D-mannose, 6-*O*-α-D-glucopyranosyl-D-mannose, and *O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-D-mannose afforded direct evidence of the aforementioned conclusion. A probable structure of the repeating unit of the glucomannan is presented.

INTRODUCTION

In a previous study[1], ten oligosaccharides from partial acid hydrolyzates of the *Candida utilis* glucomannan were isolated. Especially, isolation of 6-*O*-α-D-glucosyl-D-mannose, obtained as the sole oligosaccharide containing D-glucose, afforded direct evidence that *C. utilis* mannan is a new chemotype glucomannan, and suggested that D-glucose is exclusively located at the nonreducing ends there of. Four of the six oligosaccharides larger than a disaccharide had both α-(1→2) and α-(1→6)

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linkages, and the latter linkage was always present at the reducing end. These results differ from those reported by Peat *et al.*[2], who used baker's yeast mannan. Consequently, the question arises as to whether or not the glucomannan has a fundamental structure common to yeast mannan, in which the units of an α-(1→6)-linked backbone are substituted with side chains connected by α-(1→2) linkages. Therefore, we attempted to examine the structure in detail with the aid of methylation analysis and fragmentation analysis by controlled acetolysis. Methylation analysis is commonly used as a method of obtaining evidence for highly branched structures, as well as modes of linkages of yeast mannans, ever since Haworth and coworkers[3] examined baker's yeast mannan and found it to be a highly branched molecule. Acetolysis is an efficient method for use in studies on the structure of yeast mannans which have a (1→6)-linked backbone, because the (1→6)-mannosidic linkage is much more labile than other mannosidic linkages to acetolysis[4]. For this reason, much information has been obtained from the partial degradation of mannans by acetolysis[5-8]. Our present results have shown that the glucomannan has a fundamental structure common to yeast mannan.

RESULTS

Methylation analysis. — The glucomannan was methylated three times by the method of Hakomori[9]. The methoxyl content and the value of specific optical rotation of the methylated product were 43.1% and +86.5° (*c* 1.0, chloroform), respectively. Hydrolysis of the fully methylated glucomannan yielded the methylated sugars shown in Table I. Moreover, the di-*O*-methyl sugar was separated by preparative paper chromatography, and crystallized from acetone; m.p. 97°, $[\alpha]_D$ +10° (*c* 0.5, water). The properties of this sugar were identical with those of 3,4-di-*O*-methyl-D-mannose, which was obtained from baker's yeast mannan by the same

TABLE I

HYDROLYSIS PRODUCTS FROM METHYLATED *Candida utilis* GLUCOMANNAN

Fraction[a]	*Yield (mg)*	*Components identified*[b]	*Composition of tri-O-methyl fraction (%)*[c]	*Molar ratio*
Tetra-*O*-methyl	83	2,3,4,6-tetra-*O*-methyl-D-mannose 2,3,4,6-tetra-*O*-methyl-D-glucose		1
Tri-*O*-methyl	186	3,4,6-tri-*O*-methyl-D-mannose 2,3,4-tri-*O*-methyl-D-mannose	58 42	1.4 1
Di-*O*-methyl	64	3,4-di-*O*-methyl-D-mannose		0.9

[a]Fractionated by paper chromatography with solvent *C*. [b]Identified by gas–liquid chromatography as methyl *O*-methyl-D-glycosides and their *O*-trimethylsilyl derivatives. [c]Measured by gas–liquid chromatography as trimethylsilyl derivative of methylated methyl glycoside.

TABLE II

GEL FILTRATION AND PREPARATIVE PAPER CHROMATOGRAPHY OF THE ACETOLYZATES OF THE *Candida utilis* GLUCOMANNAN

Gel filtration[a]			*Preparative paper chromatography*[b]
Fraction No.	*Yield (g)*	*Sugar component*	*Yield (g)*
32–44	0.34	higher oligosaccharide	[c]
45–54	0.98	higher oligosaccharide pentasaccharide	
55–58	1.41	higher oligosaccharide pentasaccharide	 0.62
59–61	1.60	pentasaccharide tetrasaccharide	0.28 0.36
62–66	3.46	tetrasaccharide trisaccharide	0.33 0.28
67–70	1.44	trisaccharide disaccharide	0.72 0.33
71–74	0.91	disaccharide glucose, mannose	0.32
75–81	1.84	glucose, mannose	

[a]On Sephadex G-25 (superfine). [b]Only required amount was fractionated. [c]Not examined.

procedures. These results suggested that the glucomannan has a highly branched structure, and the branched residue has (1→2) and (1→6) linkages.

Fractionation of the deacetylated acetolyzates. — The glucomannan was acetolyzed according to the method of Lee and Ballou[7] for 6 d at 25°. The acetolysis products were deacetylated, and fractionated into eight fractions by gel filtration, and five of them were further fractionated into di-, tri-, tetra-, and penta-saccharide fractions by preparative paper chromatography. The oligosaccharides larger than pentasaccharide gave indistinct spots on paper chromatograms, and so they were not subjected to further examination. Table II shows the results. Each fraction was refractionated by chromatography on a column of charcoal-Celite into two main fractions, one of which was a manno-oligosaccharide, and the other was a mixture of a manno- and a glucomanno-oligosaccharide. The herero-oligosaccharide was detected as it was resistant to α-D-mannosidase. These results are shown in Table III.

Structural analysis of the oligosaccharide. — The results of the structural analyses of the isolated oligosaccharides are summarized in Table IV.

TABLE III

CHARCOAL–CELITE COLUMN CHROMATOGRAPHY OF THE OLIGOSACCHARIDE FRACTIONS OBTAINED FROM GEL FILTRATION AND PREPARATIVE PAPER CHROMATOGRAPHY

Oligo-saccharide fraction	*Column size (cm); weight of charcoal (g); solvent used for elution (mL)*	*Yield (mg)*	*Sugars in complete acid hydrolyzate*	*Sugars in α-D-mannosidase hydrolyzate*	*Oligo-saccharide number*
Di-saccharide	2 x 15; 7; water (400),	580	M[a]	M	1
	0-2% EtOH (800)	60	M,G[b]	disaccharide	5
Tri-saccharide	1.8 x 20; 10; water (1400),	300	M	M	2
	5% EtOH (400)	60	M,G	M, trisaccharide	2,7
Tetra-saccharide	1.8 x 20; 10; water (2700),	290	M	M	3
	5% EtOH (200)	40	M,G	M, tetrasaccharide	3,8
Penta-saccharide	2.8 x 25; 20; 1% EtOH (800),	280	M	M	4
	1% EtOH(1300),	150	M,G	M, tetrasaccharide, pentasaccharide	4,6,9
	1-2% EtOH (2000)	110	M,G	M, pentasaccharide	4,6

[a]M = D-mannose. [b]G = D-glucose.

(1) Oligosaccharides 1 and 2. Characterization of oligosaccharides 1 and 2 was performed by comparison of their properties and mixed melting point with those of authentic specimens obtained in the previous study[1].

(2) Oligosaccharides 3 and 4. Oligosaccharide 3 (d.p. 4, $[\alpha]_D$ +61°) afforded only mannose on hydrolysis with α-D-mannosidase (paper-chromatographic examination). On methylation by the method of Hakomori[9], followed by methanolysis, the methanolyzate gave a mixture of trimethylsilyl derivatives that showed peaks for silylated methyl 2,3,4,6-tetra-*O*-methyl- and methyl 3,4,6-tri-*O*-methyl-D-mannoside in gas–liquid chromatography. Therefore, oligosaccharide 3 was characterized as being *O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-D-mannose. Oligosaccharide 4 was characterized in the same manner as oligosaccharide 3.

(3) Oligosaccharide 5. This sugar, d.p. 2, was hydrolyzed to afford glucose and mannose on treatment with α-D-glucosidase (from brewers' yeast), but it was not hydrolyzed by α-D-mannosidase or by emulsin. On methylation analysis, oligo-

TABLE IV

STRUCTURAL ANALYSIS OF THE OLIGOSACCHARIDES ISOLATED FROM ACETOLYZATES OF *Candida utilis* GLUCOMANNAN

Oligo-saccharide	D.p.	R_M[a]	M_G[b]	*Hydrolyzates from methylated products*	*Properties*	*Proposed structure*[c]
1	2	0.71	0.63	[d]	m.p. 135–136° (sugar alcohol)	M→$_2$M
2	3	0.44	0.51		m.p. 161–162	M→$_2$M→$_2$M
3	4	0.28		2,3,4,6-tetra-*O*-Me-mannose, 3,4,6-tri-*O*-Me-mannose	$[\alpha]_D$ +61° (*c* 1.0, H_2O)	M→$_2$M→$_2$M→$_2$M
4	5	0.19		2,3,4,6-tetra-*O*-Me-mannose, 3,4,6-tri-*O*-Me-mannose	$[\alpha]_D$ +61° (*c* 1.1, H_2O)	M→$_2$M→$_2$M→$_2$M→$_2$
5	2	0.68	0.49	2,3,4,6-tetra-*O*-Me-glucose, 2,3,4-tri-*O*-Me-mannose		G→$_6$M
6	5	0.19		2,3,4,6-tetra-*O*-Me-glucose, 2,3,4-tri-*O*-Me-Mannose, 3,4,6-tri-*O*-Me-mannose	$[\alpha]_D$ +88° (*c* 1.0, H_2O) acetolysis major products glucose, M→$_2$M→$_2$M→$_2$M	G→$_6$M→$_2$M→$_2$M→$_2$

[a]Paper-chromatographic mobility (developed twice by the ascending method with solvent *A*; relative to mannose). [b]Paper-electrophoretic mobility (relative to glucose). [c]M =)-α-D-Man*p*-(1-; G = α-D-Glc*p*-(1-. [d]Not examined.

saccharide 5 gave methyl 2,3,4,6-tetra-*O*-methylglucoside and methyl 2,3,4-tri-*O*-methylmannoside. These data, and R_M and M_G values were in agreement with those of 6-*O*-α-D-glucopyranosyl-D-mannose, which was obtained in the previous study[1].

(4) Oligosaccharide 6. The pentasaccharide fractions (see Table III), containing gluco-manno pentasaccharide (total, 260 mg) were dissolved in 0.2M acetate buffer (pH 4.0, 20 mL) containing NaCl (26 mg), and incubated with α-D-mannosidase solution (8 mL) for 24 h at 37° under a covering of toluene. Oligosaccharide 6 was isolated from the hydrolyzates by preparative paper chromatography (140 mg).

On complete acid hydrolysis, and quantitative paper chromatographic examination, this saccharide gave D-mannose and D-glucose in the ratio of 4:1, and the glucose unit was located at the nonreducing end, because the compound was not hydrolyzed by α-D-mannosidase. On methylation analysis, methyl 2,3,4,6-tetra-*O*-methyl-D-glucoside, methyl 2,3,4-tri-*O*-methyl-D-mannoside, and methyl 3,4,6-tri-*O*-methyl-D-mannoside were detected. These data and the high value of the specific optical rotation (+88°) indicated that oligosaccharide 6 had a structure of the type Glc-Man-Man-Man-Man containing α-(1→6) and α-(1→2) linkages. Partial acetolysis followed by paper chromatographic examination of the saccharide showed glucose and a tetrasaccharide as major components. Accordingly, the glucosyl group at the nonreducing end of oligosaccharide 6 is linked through an α-(1→6) linkage to an α-(1→2)-linked mannotetraose, because it had been reported that the (1→6)-glucosidic linkage is also much more labile to acetolysis than the other linkages[4,10]. Oligosaccharide 6 was partially hydrolyzed with 0.05M sulfuric acid for 3 h at 100°, and paper chromatographic examination was performed. The paper chromatogram, stained with aniline hydrogenphtalate[11], showed mono- to penta-saccharides, but, when the same chromatogram was stained with alkaline triphenyltetrazolium chloride (TTC)[12], no tri- to penta-saccharides were detected. [Partial acid hydrolyzates of Glc-Man-Man-Man-Man should contain such tri- to penta-saccharides as Glc-Man-Man, Glc-Man-Man-Man, and the original one]. These observations indicated that all of the mannosidic linkages are (1→2) linkages, because the TTC reagent cannot detect 2-*O*-substituted saccharides. Thus, oligosaccharide 6 was characterized as *O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-D-mannose.

DISCUSSION

The results of methylation analysis of the *Candida utilis* glucomannan (see Table I) were basically in agreement with the predominant structural features of the mannan from a *Candida* species reported by Yu *et al.*[13]. In each case, the polysaccharide had (*a*) a chain of (1→2)-linked D-mannose units and (*b*) branching by substitution at O-2 and O-6 of the D-mannose units. However, the presence of methylated glucose and a high proportion (23%) of 2,3,4-tri-*O*-methyl-D-mannose on methylation analysis were not consistent with the results for other *Candida* species mannans. If these methylated sugars originated only from a 6-*O*-α-D-glucopyranosyl-D-mannose unit, the proportion of 2,3,4,-tri-O-methyl-D-mannose should be < 10%, because the ratio of D-mannose to D-glucose as the component sugars of the glucomannan has been estimated to be 23:2[1]. Therefore, the high percentage of the tri-*O*-methylmannose suggests the existence of (1→6)-linked backbone units lacking side chains, and the sequence of consecutive backbone units having no side chain may be less than two, because, in the partial acid hydrolyzates of the glucomannan, the largest oligosaccharide having consecutive (1→6) linkages was *O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→6)-*O*-α-D-mannopyranosyl-

(1→6)-D-mannose.

Fragmentation analysis by controlled acetolysis clarified the location of D-glucose residues and the length of side chain of the glucomannan. Deacetylated acetolyzates of the glucomannan gave four clear spots of oligosaccharide, namely, di-, tri-, tetra-, and penta-saccharides on a paper chromatogram. When the log α' [$\alpha' = R_F/(1-R_F)$, ref.14] values of the four oligosaccharides were plotted against the degree of polymerization, a linear relationship was observed. This result indicated that these oligosaccharides belong to a homologous series. However, preliminary examination by acid hydrolysis and α-D-mannosidase digestion revealed that each oligosaccharide fraction includes manno-oligosaccharides and glucomanno-oligosaccharide, and so, more-detailed fractionation was conducted (see Tables II and III). Finally, nine kinds of oligosaccharide were detected in the acetolyzates of the glucomannan, and six of them were isolated and characterized (see Table IV).

Oligosaccharides 1, 2, 3, and 4 were respectively the di-, tri-, tetra-, and pentasaccharide of α-(1→2)-linked D-mannose series. These results suggested that the side chains have mainly α-(1→2) linkages and that their lengths are distributed from one to four, because it is already accepted that the acetolyzates of yeast mannans contain nearly intact side chains [on account of the preferential cleavage of the (1→6)-linked backbone[8]]. The average length of the side chains was estimated to be 2–3, from the results of the methylation analysis.

Seperation of oligosaccharide 6 indicated that the D-glucose residue is exclusively located at the end of the longest side-chain. Moreover, the isolation of this sugar seems to elucidate why, on partial acid hydrolysis, 6-*O*-α-D-glucopyranosyl-D-mannose was isolated as the sole oligosaccharide containing glucose. It has already been reported[10] that an α-(1→6)-glycosidic is much more stable against acid hydrolysis than an α-(1→2)-glycosidic linkage. It was considered, accordingly, that preferential cleavage had occurred at the α-(1→2) linkage adjacent to the glucose–mannose linkage of the longest side-chain, and yielded the disaccharide.

Oligosaccharides 7 and 8 could not be purified, but were confirmed to contain glucose and mannose, and to be resistant to α-D-mannosidase. Therefore, oligosaccharides 7 and 8 may belong to the same homologous series as oligosaccharides 5 and 6. Low yields of these saccharides suggest that oligosaccharides 5, 7, and 8 may be secondary acetolysis products of oligosaccharide 6.

Oligosaccharide 9, found in the pentasaccharide fraction, gave mannose and a tetrasaccharide on α-D-mannosidase digestion, and, consequently, compound 9 may be a complex pentasaccharide having branching or a mannosylglucose unit at the nonreducing side. Chiura *et al.*[15] reported that *C.utilis* extracellular glucomannan has glucose residues distributed at the inner part of the side chain. The possibility cannot be ignored that the cell-wall glucomannan may sometimes have such a side chain.

From the facts described, a probable structure of the repeating unit (structure A in Fig. 1) for the *Candida utilis* glucomannan is presented. Structure B in Fig. 1 also appears possible, but it may be difficult to satisfy the average-length require-

ment of side chains (2–3) that was estimated from the results of methylation analysis.

EXPERIMENTAL

Materials. — The glucomannan used was purified *via* the copper complex, as described in the previous article[1], in which the properties of the purified glucomannan were presented. Standard, partially methylated derivatives of D-mannose for gas–liquid chromatography were obtained in the previous study[1]. α-D-Mannosidase was prepared, and purified, from the liver of *Turbo cornutus* according to the method of Muramatsu and Egami[16].

General methods. — Evaporations were conducted under diminished pressure at 40–45°. The melting points are not corrected. Optical rotations were measured with a Nippon Bunko Model DIP-SL polarimeter. Paper chromatography was carried out by the multiple ascending method on Toyo No.50 filter paper with the following solvent systems: (*A*) 6:4:3 (v/v) 1-butanol–pyridine–water[17]; (*B*) 8:2:1 (v/v) ethyl acetate–pyridine–water[18]; and (*C*) butanone–water azeotrope[19]. Quantitative and preparative paper chromatography were respectively performed on Toyo No.51 filter paper with solvent *B* (descending), and No. 527 paper with solvent *A* or *C* (ascending). The zone corresponding to the desired compound was excised, eluted with water, and the solution evaporated. Paper electrophoresis was carried out on Toyo No.51 filter paper at 15 V/cm for 3 h in 0.1M borate buffer (pH 9.8). Aniline hydrogenphthalate[11] was used for detection of saccharides. The degrees of polymerization (d.p.) of the oligosaccharides were determined by the method of Peat *et al.*[20]. Gas–liquid chromatography was conducted on a Yanagimoto Model G-8 gas chromatograph fitted with a flame-ionization detector, at a gas flow-rate of 20–30 mL of N_2 per min. Analysis of the methylated methyl glycosides was achieved at 190° in a stainless-steel column (225 x 0.3 cm) packed with 10% of poly-DEGS on Diasolid (80–100 mesh). For the trimethylsilyl derivatives of methylated methyl glycosides, chromatography was conducted at 160° in a stainless-steel column (200 x 0.3 cm) packed with 5% of poly-DEGS on Chromosorb W (80–100 mesh). Complete

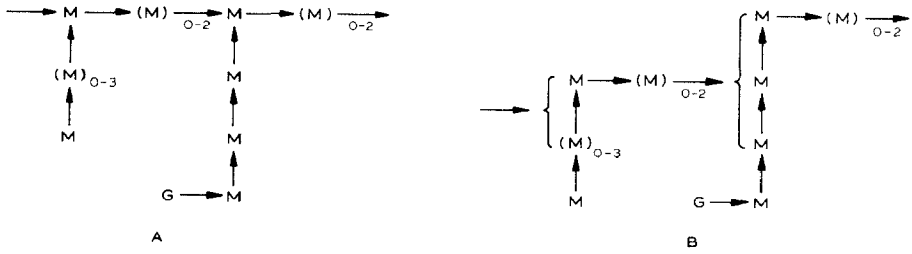

Fig. 1. Probable structures of the repeating unit of *Candida utilis* glucomannan. M and G indicate D-mannopyranosyl and D-glucopyranosyl residues, respectively. The symbols → and ↑ stand for α-(1→6)- and α-(1→2)-glycosidic linkages, respectively.

acid hydrolysis, partial acetolysis, and reduction of the oligosaccharides were performed as reported previously[1]. Methylation of the oligosaccharides was carried out, on a small scale, according to the method of Hakomori[9]. Trimethylsilyl derivatives of the partially methylated methyl glycosides were prepared by the method of Sweeley *et al.*[21] as modified by Yamakawa *et al.*[22].

Methylation analysis. — The glucomannan was methylated three times by the method of Hakomori[9] as described by Kato and Matsuda[23]. The methylated glucomannan (1 g) was hydrolyzed with 72% sulfuric acid (11 mL) for 2 h at room temperature and then with 8% sulfuric acid (155 mL) for 4 h in a boiling-water bath. After neutralization of the acid with barium carbonate, the solution was evaporated to dryness (0.8 g). The hydrolyzates of the methylated glucomannan were separated into tetra-, tri-, and di-*O*-methyl fractions by preparative paper chromatography with solvent system *C*. A part of each fraction (10 mg) was heated in a sealed tube with 0.85 M methanolic hydrogen chloride (0.2 mL) for 1 h at 100°, cooled, and freed of solvent and acid over sodium hydroxide. The methyl *O*-methyl-D-glycosides and their *O*-trimethylsilyl derivatives were analyzed by gas–liquid chromatography. The molar ratios of the methyl glycosides were calculated from the yields of tetra-, tri-, and di-*O*-methyl fractions and the composition of the tri-*O*-methyl fraction.

Acetolysis of the glucomannan. — Air-dried glucomannan (20 g) was suspended in a cold mixture of glacial acetic acid (115 mL), acetic anhydride (115 mL), and 98% sulfuric acid (11.5 mL). The mixture was kept for 6 d at 21°, and poured into ice–water (2 L), and the pH adjusted to 4 with sodium hydrogencarbonate. The acetolyzate was extracted with chloroform, and the extract was dried (anhydrous sodium sulfate), and evaporated to a syrup (32 g). The mixture of acetates was deacetylated with 0.05M methanolic sodium methoxide (300 mL) for 24 h at 0°. After evaporation, the products were dissolved in water, treated with Dowex 50-W (H^+), and Dowex-1 (OH^-) ion exchange resins, and then evaporated to dryness (13 g).

Gel filtration of the deactylated acetolyzate. — The deacetylated acetolyzate was divided into six portions, and a solution of each in water (5 mL) was placed on the top of a column (3 x 150 cm) of Sephadex G-25 (Superfine), eluted with water, and the eluate collected in 10-mL fractions. Thus, the acetolyzate was fractionated into eight fractions. Each oligosaccharide of the fraction was separated by preparative paper chromatography. The results are given in Table II.

Charcoal–Celite column chromatography of oligosaccharide fractions.— A solution (10%) of the oligosaccharides obtained from gel filtration and preparative paper chromatography was applied to a column containing equal amounts of charcaol (Takeda Pharmaceutical Co.) and Celite (No.545). The compounds were eluted stepwise, with water and then 1–5% ethanol, and the eluate was collected in 100-mL fractions. A portion of each fraction was evaporated, and the components were examined by complete acid hydrolysis and α-D-mannosidase digestion. Table III shows the results.

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