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A new mannoheptaose containing α and β-(1→2) linkages isolated from the mannan of *Torulaspora delbrueckii*: ELISA inhibition studies

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Abstract

Torulaspora delbrueckii starin IFO 0955 was examined with respect to its structural and serological properties of the cell wall mannan (Td-0955-M). Td-0955-M revealed significant reactivities with sera from a commercially available factor serum kit (Candida Check) in ELISA. Td-0955-M was investigated for its chemical structure by acetolysis under conventional and mild conditions. NMR and GC techniques were used as analytical techniques. The mannooligosaccharide fractions eluted from a Bio-Gel P-2 column were found to consist of Man(α1-2)Man, M2, Man(α1-2)Man(α1-2)Man and Man(β1-2)Man(α1-2)Man, M3, Man(α1-2)Man(β1-2)Man(β1-2)Man(α1-2)Man, M5, and a new mannoheptaose, which possesses the structure, Man(α1-2)Man(β1-2)Man(β1-2)Man(β1-2)Man(β1-2)Man(α1-2)Man, M7. The results of the inhibition ELISA showed that the M7 oligosaccharide significantly inhibited the reactivities in the Td-0955-M-factor serum systems.

Keywords: *Torulaspora delbrueckii*; Mannan; Manno-oligosaccharides; Antigenic epitopes; Serology; Yeast

1. Introduction

The yeast *Torulaspora delbrueckii* has been commercially used in the bread-making industry.[1] In the evolutionary tree comprising 10 fungal species, *T. delbrueckii* and *Saccharomyces cerevisiae* seem to be very closely related.[2] Several workers investigated the antigenic characteristics of the yeast cells,[3–5] and the ^{1}H NMR spectroscopy of the yeast cell wall mannan.[5,6] However, little is known about the detailed structural and serological properties of the mannan of *T. delbrueckii*.

A commercially available kit including 10 polyclonal factor sera, 'Candida Check' (Iatron, Tokyo, Japan),[7] has been a good tool for identifying the viable cells of seven medically important *Candida* species[8] in clinical speciments and the results of the structural determination of 10 antigenic factors have been summarized.[9] During the course of the investigation of the mannan of *T. delbrueckii*, we have detected a new α- and β-(1→2) linkage containing mannoheptaose which is similar to the mannopentaose obtained from the mannan of *Pichia pastoris* cells.[6,10] Here, we report the chemical structure of the mannan of the *T. delbrueckii* strain IFO 0955 determined by NMR and GC analyses and the identification of antigenic factors by using the inhibition ELISA.

Abbreviations: NMR, nuclear magnetic resonance; GC, gas–liquid chromatography; COSY, two-dimensional ^{1}H–^{1}H correlated spectroscopy; NOESY, two-dimensional nuclear Overhauser enhancement and exchange spectroscopy; 2D-HOHAHA, two-dimensional homonuclear Hartmann–Hahn spectroscopy; ELISA, enzyme-linked immunosorbent assay.

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2. Methods

2.1. Culture conditions

The *T. delbrueckii* strain IFO 0955 was obtained from the Institute for Fermentation (Osaka, Japan). This

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strain was precultivated in yeast extract containing Sabouraud medium (YSLM) at 27 °C for 24 h on a rotary shaker (150 rpm) as previously described.[11,12] The culture (5 mL) was then inoculated into fresh YSLM and cultured at 27 °C for 48 h on a shaker. After harvesting by centrifugation at 5000 rpm, the cells were washed three times with saline. The washed cells were then dehydrated with large volumes of acetone.

2.2. Preparation and acetolysis of mannan

The mannan was extracted according to a previously published procedure.[13] Briefly, a combination of hot-water extraction and short-term precipitation with Fehling solution was used. The mannan fraction obtained from the *T. delbrueckii* strain IFO 0955 was designated as Td-0955-M. The yield of Td-0955-M was 9.3% on a dry weight basis of the whole cells. The acetolysis of mannan was conducted by essentially the same method as that used for the acetolysis of the mannan of the *P. pastoris* strain IFO 0948.[10] Briefly, 200 mg of Td-0955-M was first converted into its O-acetyl derivative in accordance with a previous description.[14] The O-acetylated mannan was then subjected to acetolysis under conventional and mild conditions as follows: for conventional conditions,[15] the O-acetylated mannan was dissolved in 100 mL of a 10/10/1 (v/v) mixture of acetic anhydride, acetic acid, and H_2SO_4, and the resultant solution was kept at 40 °C for 12 h. The O-acetylated mannooligosaccharide mixture was cooled in ice-water, and extracted from this mixture with chloroform and de-O-acetylated with sodium methoxide in methanol. For mild conditions, the acetolysis was carried out using a 100/100/1 (v/v) mixture of acetic anhydride, acetic acid, and H_2SO_4 at 40 °C for 36 h. The de-O-acetylated mannooligosaccharide mixture was dissolved in 2.0 mL of water and applied onto a column of Bio-Gel P-2 (2.5 × 100 cm). The fractions were eluted with water (0.25 mL/min), and aliquots of the eluates, 3.0 mL each, were obtained. The appropriate carbohydrate fractions were combined and lyophilized after concentration in vacuo. Abbreviations of the obtained di-, tri-, penta-, and heptaoligomannosides are M2, M3, M5, and M7, respectively.

2.3. Methylation analysis

Methylation of the oligosaccharides was performed as previously described.[16] GC of the *O*-methyl-*O*-acetyl-D-mannitols was performed using a glass column (3 mm × 200 cm) containing 3% OV-210 on Supelcoport (100–200 mesh) at 185 °C with N_2 as the carrier gas at a flow rate of 20 mL/min.

2.4. Nuclear magnetic resonance (NMR)

The ^1H NMR spectra were recorded using a JEOL JNM-GSX 400 spectrometer at 400 MHz. Each sample was dissolved in D_2O at 1% (v/v), and the determinations were conducted at 45 °C; acetone was used as the internal standard (2.217 ppm).[17] 2D-HOHAHA experiment was performed as described previously.[18] The molar ratio of the side chains in the mannan was calculated based on the peak dimensions of the corresponding H-1 signals in the NMR spectrum. COSY and NOESY were performed as described in a preceding paper.[19]

2.5. Enzyme-linked immunosorbent assay (ELISA)

The ELISAs of Td-0955-M were conducted as previously described[18] using factor sera 1, 4, 5, 6, 8, 9, 11, 13, 13b, and 34 of Candida Check (lot no. R-260), rabbit polyclonal antibodies against *Candida* cells purchased from Iatron (Tokyo, Japan). It was shown that the adherence behavior of mannan to a plastic surface was dominated by the hydrophobic properties of the peptide moiety of the mannan.[20] The Td-0955-M had strongly reacted with factor sera 1, 4, 5, and 6 (data not shown). Inhibition ELISAs were conducted as mentioned above except for pretreatment of the factor sera using the manno-oligosaccharides obtained from Td-0955-M by acetolysis under mild conditions as inhibitors.[12] Namely, the factor serum diluted 10-fold with phosphate-buffered saline containing 0.1% (v/v) Tween 20 (PBST) were mixed with a known quantity (0.2 μmol/0.12 mL) of inhibitors dissolved in PBST. The inhibitor solutions were diluted with the factor serum solutions (0.12 mL each) diluted 20-fold with PBST and were preincubated for 1 h at 37 °C prior to the addition of 0.1 mL aliquots to the wells of the microtiter plates. The inhibition ratio (%) was calculated using the formula: Inhibition (%) $= (1 - A/B) \times 100$, where A and B are the optical densities at 492 nm with and without inhibitor, respectively. All samples were tested in triplicate for each experiment.

2.6. Other methods

The total carbohydrate was determined using the phenol–sulfuric acid method with D-mannose as the standard.[21] The total phosphate was determined by the molybdate method of Ames and Dubin, using KH_2PO_4 as the standard.[22]

3. Results and discussion

3.1. Structural studies of mannan

The structures of the cell wall polysaccharides, especially the mannans, can be directly used in yeast taxo-

nomy. The discovery of new mannan structures is evidence of the fact that distinct features are revealed in individual species of yeast. Fig. 1 shows the elution profiles of the acetolyzate of Td-0955-M obtained under conventional and mild conditions. Acetolysis under the conventional conditions (Fig. 1A) yielded a large amount of mannose in addition to the manno-oligosaccharides, from biose to heptaose, designated as M2 (19.08 mg), M3 (16.81 mg), M5 (34.22 mg), and M7 (10.46 mg). The major oligosaccharides were M2 and M5. However, as shown in Fig. 1B, acetolysis under mild conditions yielded manno-oligosaccharides, from biose to heptaose, designated as M2 (5.69 mg), M3 (5.33 mg), M5 (56.34 mg), and M7 (10.30 mg). The major oligosaccharides were M5 and M7. These results show that acetolysis under mild conditions is superior for studying the structure of Td-0955-M. From the percentage of each mannose unit, we estimated the molar ratios of the side chains of the mannan.

Fig. 2 shows the anomeric region of the ^{1}H NMR spectra of Td-0955-M. The anomeric chemical shifts indicate the presence of the β-(1→2)-linked oligomannosyl residues because of the chemical shifts of 4.781, 4.843 and 4.883 ppm corresponding to the β-(1→2)-linked mannopyranose unit.[10,23] As the β-mannose units show characteristic H-5 signal at about 3.5 ppm, we also determined the configuration of each mannose unit from a 2D-HOHAHA experiment (data not shown).[18] This mannan contains no phosphate groups based on the following two negative results; one is the absence of phosphodiesterified oligosaccharides, based on the lack of the ^{1}H NMR H-1 signal at about 5.55 ppm corresponding to the 1-*O*-α-phosphorylated mannose unit;[23,24] also no phosphate was found upon analysis by the method of Ames and Dubin (data not shown). The mannan did not contain Man(α1-2)Man(α1-3)Man units corresponding to the 5.375 ppm signal which can be observed in the mannans of *Candida albicans*.[11]

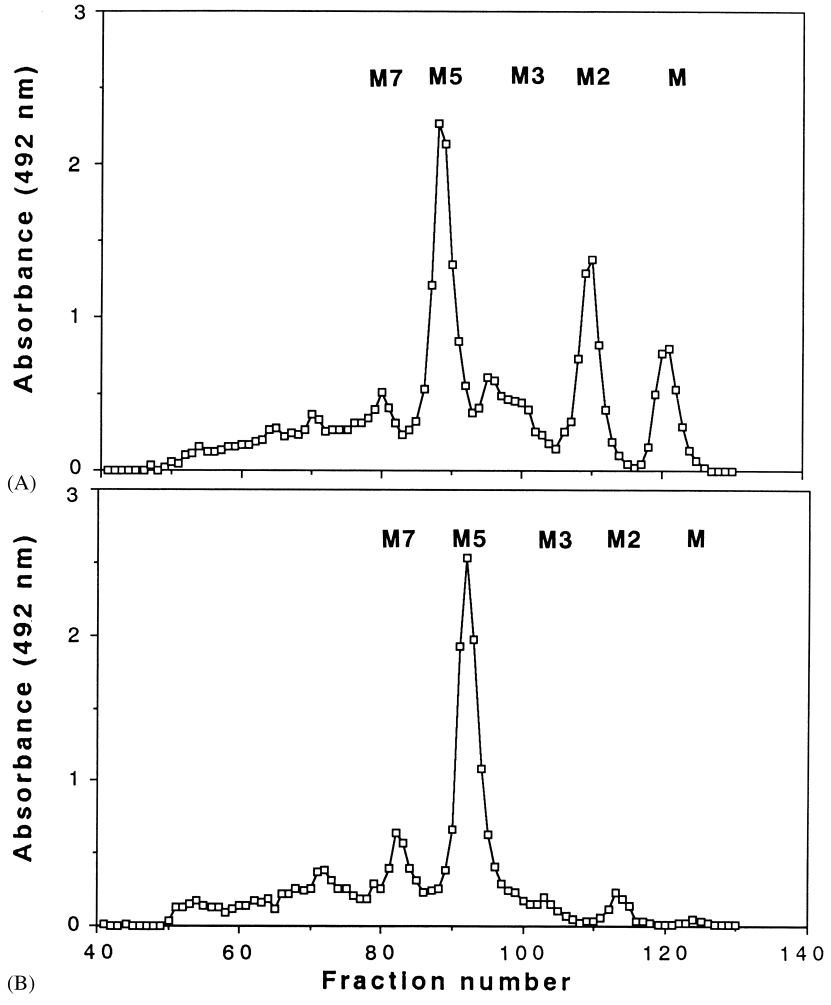

Fig. 1. Elution patterns of oligosaccharides obtained from Td-0955-M by acetolysis under conventional (A) and mild (B) conditions using a Bio-Gel P-2 column. (A) Acetolysis was performed with $(CH_3CO)_2O$–CH_3COOH–H_2SO_4 (10:10:1, by volume) at 40 °C for 12 h. (B) Acetolysis was performed with $(CH_3CO)_2O$–CH_3COOH–H_2SO_4 (100:100:1, by volume) at 40 °C for 36 h. M, M2, M3, M5, and M7 indicate mannose, mannobiose, mannotriose, mannopentaose, and mannoheptaose, respectively.

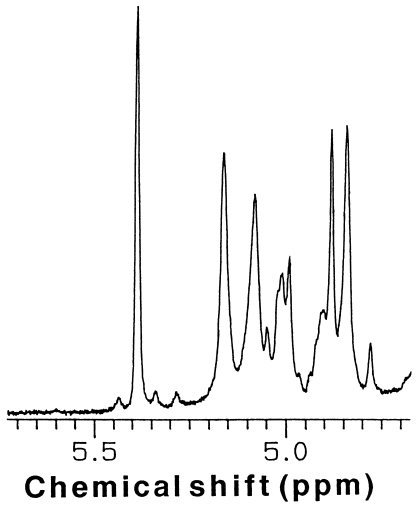

Fig. 2. ^{1}H NMR spectra of Td-0955-M. Spectra were recorded with a JEOL JNM-GSX 400 spectrometer in D_2O at 45 °C, using acetone (2.217 ppm) as the standard.

Fig. 3 shows the ^{1}H NMR spectra (H-1 region) of the manno-oligosaccharides obtained by acetolysis under mild conditions. Assignment of the anomeric proton chemical shifts for the oligosaccharides from M2 to M5 was based on the findings of Cohen and Ballou[17] and Kobayashi and co-workers[10] (Table 1). The oligosaccharides obtained using the mild conditions were identified as M2; Man(α1-2)Man, M3; a mixture of Man(α1-2)Man(α1-2)Man, Man(β1-2)Man(α1-2)Man, and M5; Man(α1-2)Man(β1-2)Man(β1-2)Man(α1-2)Man. As M7 shows an additional new signal at 4.985 ppm, we examined the structure of M7. Methylation analysis of M7 gave only 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetyl mannitol (1 mol) and 3,4,6-tri-*O*-methyl-1,2,5-tri-*O*-acetyl mannitol (6 mol) using GC. Therefore, it is clear that M7 contains six linear 2-*O*-substituted mannose units. A sequential assignment study of the H-1 and H-2 signals of M7 was performed to confirm the structure using a previously described method.[19,25] For this purpose, M7 was analyzed by a combination of COSY and NOESY (Fig. 4). The cross-peaks from C′ to F′ indicate the inter-residue H-1–H-2′ connectivities between each of the mannose units. Since H-2 of the mannose unit with an H-1 signal at 5.376 ppm has no NOE cross-peak, it is apparent that this mannose unit corresponds to the nonreducing terminal group (Man-G) of this oligosaccharide. The H-1 and H-2 signals of M7 were sequentially assigned from the H-1 of the Man-A, A-A′-B-B′-C-C′-D-D′-E-E′-F-F′-G (Table 1). Thus, we found the presence of a new mannoheptaose, M7, Man(α1-2)Man(β1-2)Man(β1-2)Man(β1-2)Man-(β1-2)Man(α1-2)Man, in the mannan Td-0955-M of the *T. delbrueckii* strain IFO 0955.

3.2. Possible structure of Td-0955-M

Based on the molar ratio (Fig. 1B) and the dimensions of the ^{1}H NMR signals (Fig. 3) of the oligosaccharides obtained by mild acetolysis, we proposed the chemical structure of Td-0955-M as shown in Fig. 5.

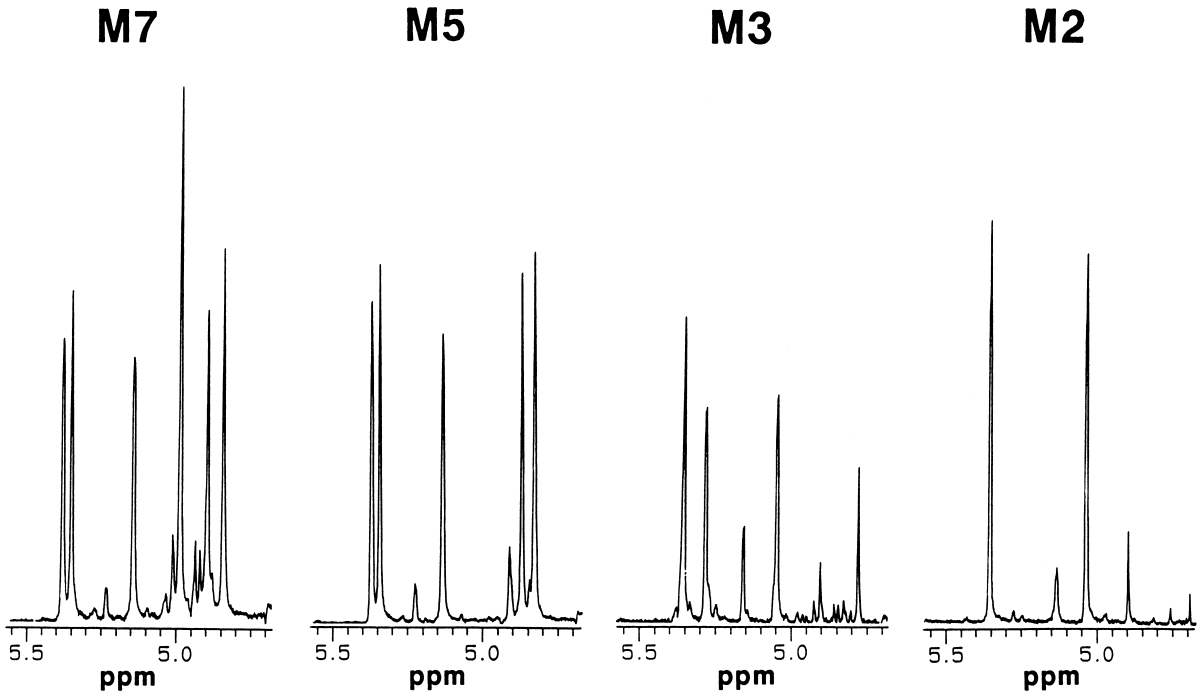

Fig. 3. ^{1}H NMR spectra of D-manno-oligosaccharides obtained from Td-0955-M by acetolysis under mild conditions. Spectra were recorded as shown in Fig. 2. M2–M7 are designated as in the legend of Fig. 1.

Table 1
^{1}H chemical shifts of oligosaccharides obtained from Td-0955-M by acetolysis under mild conditions

Oligosaccharide	Sugar residue								Chemical shift (ppm)						
	G	F	E	D	C	B	A		G	F	E	D	C	B	A
M2						M(α1-2)M		H-1						5.039	5.367
M3					M(α1-2)M(α1-2)M			H-1					5.043	5.280	5.351
					M(β1-2)M(α1-2)M			H-1					4.774	5.152	5.351
M5			M(α1-2)M(β1-2)M(β1-2)M(α1-2)M					H-1			5.377	4.876	4.835	5.140	5.349
M7	M(α1-2)M(β1-2)M(β1-2)M(β1-2)M(β1-2)M(α1-2)M							H-1	5.376	4.985	4.985	4.894	4.843	5.141	5.348
								H-2	4.056	4.306	4.364	4.383	4.243	4.306	3.962

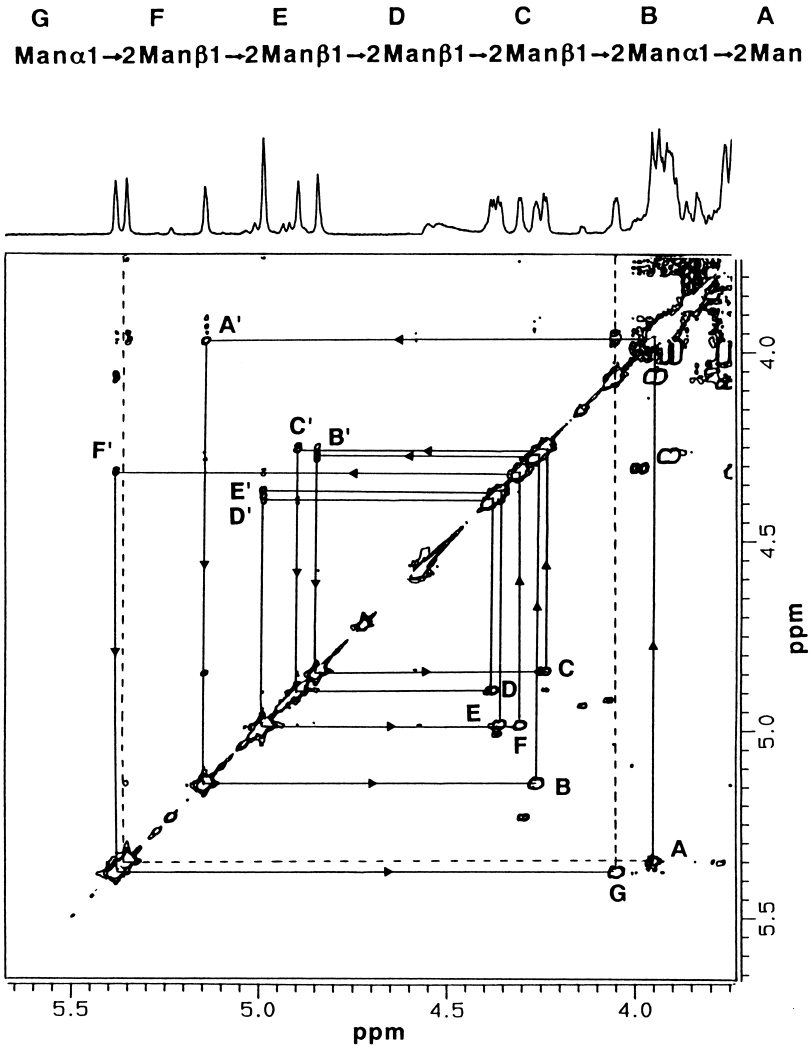

Fig. 4. Sequential connectivities of mannose units of M7. The right side of the diagonal shows COSY, and the left side of the diagonal shows NOESY. Primed letters indicate inter-residue H-1–H-2′ NOE cross-peaks and unprimed letters signify the H-1–H-2 correlated cross-peak of a reducing terminal mannose unit (Man-A) and A′ indicates the inter-residue NOE cross-peak between the H-2 of Man-A and H-1 of the adjacent mannose unit (Man-B). Arrows indicate the direction of the sequential connectivity from the reducing terminal unit to the nonreducing terminal unit.

3.3. Serological studies

The inhibitory activity of M2, M3, M5, and M7 on the reactivities of factors 1, 4, 5, and 6 sera with Td-0955-M on ELISA is shown in Fig. 6. The inhibitory activity of M2 is poor with all four factors sera. However, the three oligosaccharides, M3, M5, and M7, exhibited inhibitory activities. Increases in the inhibitory activities with factors 1, 4, and 5 sera were observed in the order M2, M3, M5, and M7. For factor 6 serum, the increases in the inhibitory activities were observed in the order M2, M3, and M5 = M7. These results indicate that M5 and M7 in Td-0955-M mainly behave as the reactive elements against all the factors 1, 4, 5, and 6 sera. It is also possible to say that the factor 1 serum recognizes the length of the parts involving the α- and β-(1→2)-linked oligomannosyl residues in addition to the α-1,2-linked ones.[12,18,26,27] As the α-1,6-branched side chain corresponding to the factor 4 epitope was not detected in Td-0955-M,[28] the reactivity against factor 4 serum appears that the antibodies against factors 5 and 6 in the serum react with each of the epitope parts described below.[4,18,28] The factor 5 serum recognizes both the two and four middle β-(1→2)-linked manno-oligosaccharides[9,12,29] and the length of the manno-oligosaccharides involving the α- and β-(1→2) linkages, and also the factor 6 serum recognizes both the minimum essential structure, Man(β1-2)Man(α1-,[9,29] and the length of the oligosaccharides. These new findings will be very useful in consideration of the structural heterogeneity of the antigenic factors.

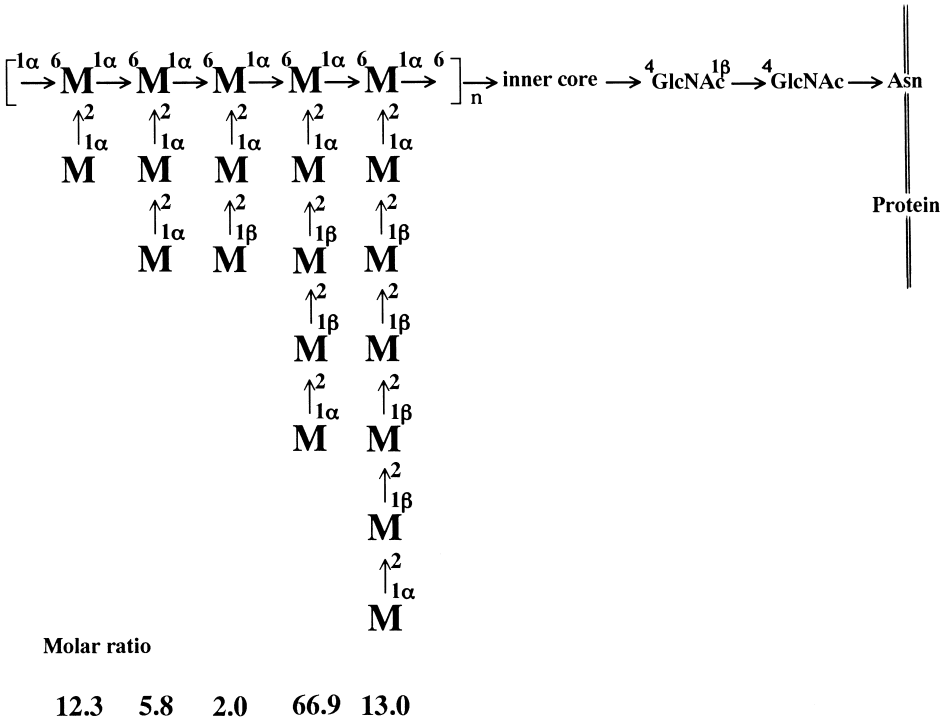

Fig. 5. Possible structure of Td-0955-M. M denotes a D-mannopyranose unit. The values are calculated from the molar ratio (Fig. 1B) and the dimensions of the ^{1}H NMR signals (Fig. 3) of the oligosaccharides obtained by acetolysis under mild conditions.

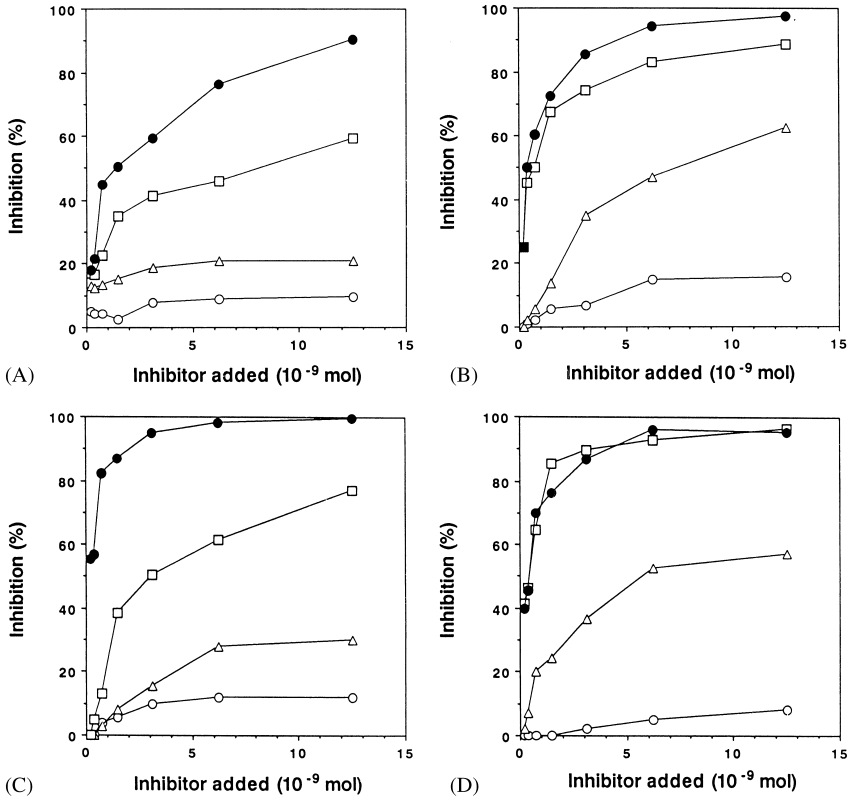

Fig. 6. Inhibition ELISA test of manno-oligosaccharides. M2 (○), M3 (△), M5 (□), and M7 (●), obtained from Td-0955-M by acetolysis under mild conditions, were used on Td-0955-M-factor sera, 1 (A), 4 (B), 5 (C), and 6 (D) systems.

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