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Structural studies of the antigen III cell wall polysaccharide of *Trichosporon domesticum*

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Abstract

Cell wall and soluble polysaccharides that reacted with *Trichosporon domesticum* factor III serum were isolated from the type strain of *T. domesticum*. The fractions contained *O*-acetyl groups, which contributed to the serological reactivity. The antigenic structure was characterized by chromatographic and spectroscopic methods. The polysaccharide has an α-(1→3)-D-mannan backbone with hetero-oligosaccharide side chains consisting of a 2-*O*-substituted β-D-glucuronic acid residue bound to O-2 of the mannose residue, β-D-xylopyranosyl residues located in the middle of the side chain, and a nonreducing terminal α-L-arabinopyranosyl residue bound to O-4 of xylose. The mannan backbone is O-acetylated at O-6 of the mannose residues. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Trichosporon domesticum*; Antigenic polysaccharide; Chemical structures

1. Introduction

The genus *Trichosporon* is a medically important yeast that includes a number of species causing deep-seated and superficial infections.[1,2] *Trichosporon* species have also been reported as the causative agents of summer-type hypersensitivity pneumonitis (SHP), which is the most prevalent type of hypersensitivity pneumonitis in Japan.[3]

The genus *Trichosporon* was reclassified by Guého et al.[4] in 1992 and again by Sugita et al.[5] in 1994. As presently classified, this genus includes approximately 20 species.

Ikeda et al.[6] conducted antigenic analyses of *Trichosporon* species and prepared three specific antisera: factors I, II, and III. It was shown that the *Trichosporon* species could be divided into at least four distinct groups: serotypes I, II, III, and I–III (reacting with both factors I and III). Each group was correlated with clusters on a molecular phylogenetic tree based on the large subunit rDNA sequences.[7,8] The standard strains of serotypes I and II were re-identified as *T. cutaneum* and *T. asahii* var. *asahii*, respectively, using the new taxonomy. The standard strain of serotype III was isolated from a SHP patient's home and described as a new species, *T. domesticum*, by Sugita et al.[9]

About 15% of *Trichosporon* isolates from SHP patients' homes belong to serotype III.[7] Ando et al.[10] reported that SHP patients developed symptoms of SHP by inhaling serotype antigen of the strain isolated from their home.

Cell wall or capsular polysaccharides are responsible for the serological specificities of many yeasts.[11] Gorin and Spencer[12] reported

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the chemical structure of the arabinoxylomannan of *T. cutaneum* RS1. However, the new taxonomic position of the isolate according to the current revised criteria is not known. The serotype of the isolate is also uncertain. Recently, Mizobe et al.[13] reported the structure of the glucuronoxylomannan (GXM) antigen from the serotype II strain (*T. asahii*). However, the structure and the serological reactivity of the polysaccharide from *T. domesticum* have not been reported.

In this paper, we report the antigenic activity of the cell wall polysaccharide (CWPS) and soluble polysaccharide (SPS) from *T. domesticum*, and analyze the chemical structure of the polysaccharide fraction containing the antigen III epitope.

2. Results

Chemical and serological analysis of the constituent carbohydrates of CWPS and SPS.— Both the crude polysaccharides, CWPS and SPS, were separated into four fractions (F-1, F-2 (F-2-1 and F-2-2), F-3 and F-4) on a DEAE-Toyopearl 650M column (Fig. 1(A and B)). Minimal protein was detected as the absorbance at 280 nm. The sugar components and the mole percentages of these fractions are shown in Table 1. All the fractions contained arabinose, xylose, mannose, and glucose. Glucuronic acid was identified after hydrolysis of the carboxyl-reduced product. These carbohydrates were also identified on thin-layer chromatography (TLC) plates. CWPS F-4 (C-F-4) and SPS F-4 (S-F-4) were substantially different from the other fractions, containing more arabinose, xylose, and glucuronic acid and less glucose. The molar ratio of the sugar components of C-F-4 was very similar to that of S-F-4. Both the F-4 fractions were also differentiated from the other fractions by the presence of *O*-acetyl groups in the ^{1}H NMR analysis (signal at 2.17 ppm). The H-1 region of the ^{1}H NMR spectrum of C-F-4 was almost identical to that of S-F-4. The molar proportions of sugar–*O*-acetyl groups were 5–6:1 and 2–3:1 in C-F-4 and in S-F-4. To determine the antigenic activity of these fractions for antigenic factor III, the slide agglutination–inhibition test was carried out (Table 1). C-F-4 and S-F-4 showed inhibitory activity, whereas none of the remaining fractions showed inhibitory activity. Both the F-4 fractions became inactive after deacetylation. Since C-F-4 and S-F-4 have antigenic activity and they resemble each other, C-F-4 was analyzed after the microheterogeneous fractions were removed by gel-filtration using a Sepharose CL-4B column. A highly polymerized polysaccharide with an estimated size of about 2000 kDa was obtained. The major component sugars of the purified C-F-4 were arabinose, xylose, mannose, and glucuronic acid in an approximate molar ratio of 1:3:3:1.

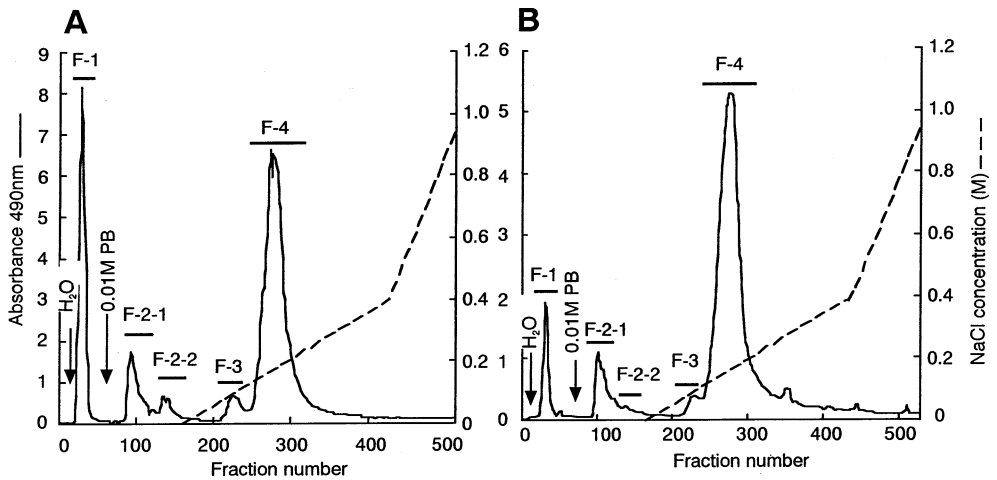


Fig. 1. Ion-exchange chromatography of polysaccharides from *T. domesticum* on DEAE-Toyopearl 650M. (A) Cell wall polysaccharide; (B) soluble polysaccharide.

Table 1
Sugar components and antigenic activity of the polysaccharide fractions obtained from *T. domesticum* by DEAE-Toyopearl 650 M column chromatography

	Fraction	Mole percentage of sugar components [a]						*O*-Acetyl group	Amount required for inhibition [c] (μg/mL)
		Ara	Xyl	Man	Gal	Glc	GlcA [b]		
Cell wall PS	F-1	0.9	2.8	17.9		78.4		−	$1.6\times10^4<$
	F-2-1	0.5	6.6	37.9	1.0	53.9	0.1	−	$1.6\times10^4<$
	F-2-2	0.8	9.3	46.8	4.6	38.4	0.1	−	$2.0\times10^3<$
	F-3	1.9	7.7	34.2	1.3	53.6	1.3	−	$2.0\times10^3<$
	F-4	11.8	35.5	39.5		2.8	10.4	+	1.0×10^3
Soluble PS	F-1	2.7	5.9	39.6		51.2	0.6	−	$1.6\times10^4<$
	F-2-1	0.7	8.2	46.1	0.5	44.2	0.3	−	$1.6\times10^4<$
	F-2-2	0.5	11.3	62.1	4.7	21.4		−	$4.0\times10^3<$
	F-3	4.0	16.9	68.2	1.3	9.0	0.6	−	$1.6\times10^4<$
	F-4	11.8	36.7	37.5	0.9	2.5	10.6	+	5.0×10^2

[a] The alditol acetates were identified by their retention times relative to xylitol pentaacetate.
[b] GlcA was determined by GLC and measured by a colorimetric method.
[c] The amounts of inhibitor required for a one-fourth reduction in the agglutination reaction between factor III serum and *T. domesticum* cell antigen.

Table 2
Methylation analysis of de-O-acetylated and carboxyl-reduced C-F-4 polysaccharide [a]

O-methyl alditol acetate	Molar ratio	Mode of linkage
1,5-Di-*O*-acetyl-2,3,4-tri-*O*-methyl-arabinitol	1.0	Nonreducing terminal Ara*p*
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol	0.12	Nonreducing terminal Glc*p*
1,4,5-Tri-*O*-acetyl-2,3- and 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-xylitol	2.6	4-*O* and 2-*O*-substituted Xyl*p*
1,2,5-Tri-*O*-acetyl-3,4,6-tri-*O*-methyl-glucitol	1.3	2-*O*-substituted Glc*p*
1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl-mannitol	0.53	3-*O*-substituted Man*p*
1,4,5-Tri-*O*-acety1-2,3,6-tri-*O*-methyl-glucitol	0.18	4-*O*-substituted Glc*p*
1,2,3,5-Tetra-*O*-acetyl-4,6-di-*O*-methyl-mannitol	1.4	2,3-di-*O*-substituted Man*p*

[a] The methylated alditol acetates were identified by their retention times relative to 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol.

Structural analysis of antigenic polysaccharide C-F-4.—Methylation analysis of the de-O-acetylated and carboxyl-reduced C-F-4 polysaccharide showed the presence of 2,3,4-tri-*O*-methyl arabinopyranose, 2,3- or 3,4-di-*O*-methyl xylopyranose, 2,4,6-tri-*O*-methyl- and 4,6-di-*O*-methyl mannopyranose, and 3,4,6-tri-*O*-methyl glucopyranose (Table 2). The results were compared with those for the methylation of an unreduced sample. The 3,4,6-tri-*O*-methyl glucopyranose was absent in the unreduced sample and appeared in the reduced sample, as a result of the conversion of glucuronic acid to glucose. To assign the substitution in the xylopyranose, the methylated polysaccharide was reduced with sodium borodeuteride. There were prominent peaks in the gas chromatography–mass spectrometry (GLC–MS) of the partially methylated alditol acetates of the internal xylose at 117, 118, 189, and 190 m/z, proving the presence of both 2,3- and 3,4-di-*O*-methylxylitol acetate.

The neutral and acidic oligosaccharides were obtained by hydrolysis of the de-O-acetylated C-F-4 (0.5 M TFA, 95 °C, 5 h). The gel-filtration profile of the acidic fraction on the Bio-Gel P-2 is shown in Fig. 2. The modes of linkage of the three acidic oligosaccharide fractions, AF-6, AF-7, and AF-8, were determined by NMR spectroscopy (Table 3). The α

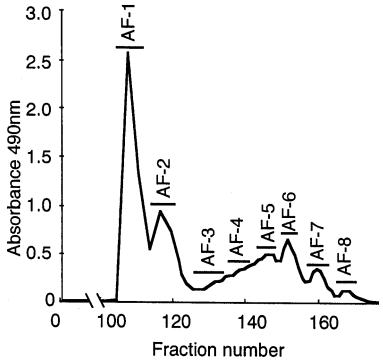
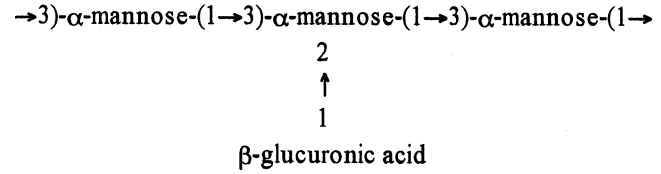


Fig. 2. Filtration profile of acidic oligosaccharides obtained by partial acid hydrolysis of purified C-F-4 under Condition A (0.5 M TFA, 95 °C, 5 h) on Bio-Gel P-2.

and β configurations of mannose and glucuronic acid were identified by the values of $J_{1,2}$ and the chemical shifts ($J_{1,2}$ 7.8 Hz for the β configuration of glucuronic acid).[14] The correlation signal for glucuronic acid H-1/mannose[a] C-2 (4.48/81.2 ppm) observed in the heteronuclear multiple-bond coherence (HMBC) spectrum of AF-8 indicated the linkage of glucuronic acid to O-2 of mannose. The phase-sensitive nuclear Overhauser effect spectroscopy (NOESY) spectra of AF-7 and AF-6 showed correlation signals for glucuronic acid H-1/mannose[a] H-1 and mannose[a] H-1/mannose[b] H-3, indicating that the glucuronic acid is linked to the nonreducing terminal mannose residue of the oligosaccharides (Table 3). The molar proportions of mannose–glucuronic acid of the AF-2 polysaccharide were 3.4:1, suggesting that the major repeating basic unit was



Weaker acid hydrolysis of the de-O-acetylated C-F-4 (0.1 M TFA, 95 °C, 1 h) and subsequent size-exclusion chromatography (Bio-Gel P-2) gave neutral mono- and disaccharides, and a partially hydrolyzed polysaccharide. The monosaccharide contained arabinose and xylose in a ratio of 1:2.4. The specific rotation of this fraction was measured, and compared with that of a mixed solution of D- or L-arabinose and D-xylose for the D and L configurations, respectively. The specific rotation, $[\alpha]_D = +32°$, suggested that the monosaccharide from C-F-4 consists of L-arabinopyranose and D-xylopyranose. The disaccharide contained arabinose and xylose in a ratio of 1:1. NMR spectroscopy was used to determine the linkage position of each residue. The assigned proton and carbon signals in the disaccharide are shown in Table 4. In the phase sensitive NOESY spectrum, there was a correlation peak for the proton signals for arabinose H-1/xylose H-4 (4.38/3.77 ppm). In the HMBC spectrum, three-bond heteronuclear connectivity was observed across the internal oxygen (H–C–O–C), xylose C-4/arabinose H-1 (79.1/4.38 ppm). The vicinal $J_{1,2}$ coupling constant of the arabinose was 7.6 Hz, and the correlation signal for arabinose H-1/arabinose H-3 and/or H-5 was observed in the NOESY spectrum. The data

Table 3
Structure and chemical shifts of acidic oligosaccharides

Fraction	Mode of linkage [a]	Assignment of H-1 region [b] (ppm)			
		GA	M[a]	M[b]	M[c]
AF-8	β-GA(1→2)M[a]	4.476 4.568	α 5.270 β 4.920		
AF-7	β-GA(1→2)α-M[a](1→3)M[b]	4.480	5.246	α 5.131 β 4.868	
AF-6	β-GA(1→2)α-M[a](1→3)α-M[b](1→3)M[c]	4.502	5.249	5.107	α 5.133 β 4.866

[a] M, mannose, GA, glucuronic acid.
[b] NMR spectra of solutions in D_2O were recorded at 90 °C. Chemical shifts are reported in ppm relative to external DSS.

Table 4
Chemical shifts (ppm) for the neutral disaccharide and the mannan

	Reporter group		Disaccharide [a]		α-(1→3)-mannan [b]
			Ara	Xyl	Man
^{1}H	H-1	α	4.38	5.16	5.11
		β		4.57	
	H-2	α	ca. 3.54	ca. 3.54	4.21
		β		3.25	
	H-3	α	3.64	ca. 3.76	3.99
		β		3.55	
	H-4	α	3.92	nd [c]	3.77
		β		ca. 3.76	
	H-5	α	ca. 3.63	nd	3.80
			3.94	nd	
		β		3.37	
				4.04	
	H-6	α			3.74
					3.88
^{13}C	C-1	α	104.45	94.61	104.60
		β		99.12	
	C-2	α	73.27	74.01	72.31
		β		76.65	
	C-3	α	74.92	nd	80.87
		β		76.61	
	C-4	α	70.76	nd	68.84
		β		79.09	
	C-5	α	68.76	nd	76.17
		β		65.66	
	C-6	α			63.75

[a] NMR spectra were recorded at 75 °C.
[b] NMR spectra were recorded at 65 °C.
[c] Not determined.

indicated that the arabinose H-1 is axial. The linkage of the disaccharide was identified as the 4-*O*-α-L-arabinopyranosyl-D-xylopyranose (Fig. 3). This structure was confirmed by methylation analysis of the disaccharide. The prominent peaks at 118 and 189 *m*/*z* in the mass spectrum of the partially methylated derivative of the reducing terminal xylose revealed the presence of 2,3-di-*O*-methylxylitol acetate. The partially hydrolyzed polysaccharide was further hydrolyzed for 3 h under the same conditions (0.1 M TFA, 95 °C). After hydrolysis, the polysaccharide fraction was obtained by Bio-Gel P-2 gel-filtration. The ^{1}H NMR spectra (H-1 region) of the unmodified and modified C-F-4 polysaccharides are shown in Fig. 4. A longer period of hydrolysis resulted in the disappearance of the signals from 4.6 to 4.8 ppm and at 4.40 ppm, and shifted the signal from 4.57 to 4.51 ppm, because of the removal of β-D-xylose and α-L-arabinose and the appearance of the nonreducing terminal β-D-glucuronic acid residue. The strength of the signals from 5.0 to 5.4 ppm varied very little.

Based on these data, it was assumed that the purified C-F-4 possesses an α-(1→3)-

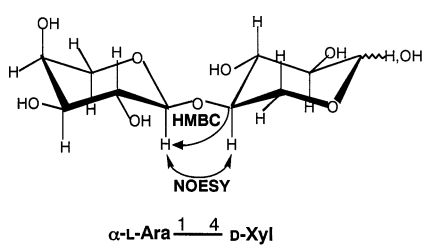


Fig. 3. Linkage assignments of a neutral disaccharide obtained from the purified C-F-4 by interglycosidic connectivity observed in the HMBC and NOESY spectra.

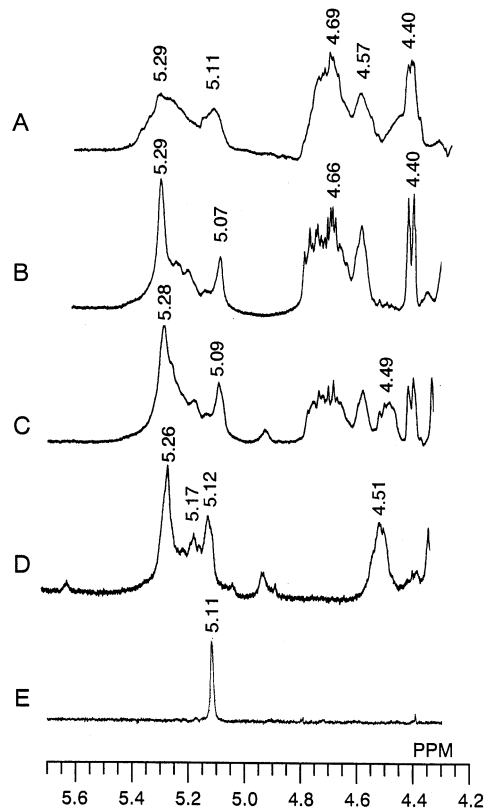


Fig. 4. ^{1}H NMR spectra (H-1 region) of non-modified and modified C-F-4 polysaccharide fractions. (A) Purified C-F-4; (B) de-O-acetylated C-F-4; (C) 1 h hydrolysis with 0.1 M TFA at 95 °C; (D) further hydrolysis for 3 h; (E) controlled Smith degradation of C-F-4.

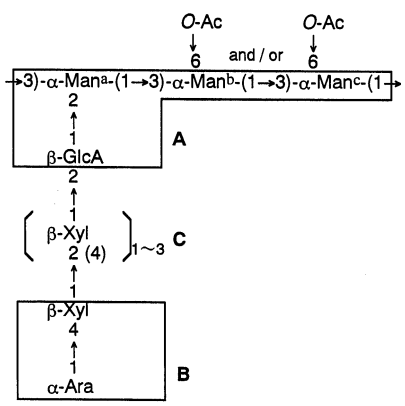


Fig. 5. A possible major repeating structure of the antigenic polysaccharide from *T. domesticum* M 9401. The repeating unit is composed of D-mannose (Man), D-glucuronic acid (GlcA), D-xylose (Xyl), and L-arabinose (Ara).

linked mannan backbone. To confirm this, controlled Smith degradation of C-F-4 was carried out. As shown in Fig. 4(E) and Table 4, α-(1→3)-mannan was obtained.

O-Acetyl groups are essential for the antigenic activity of C-F-4 for factor III. To determine the carbon position at which the *O*-acetyl groups are linked, NMR spectra of the polysaccharides were compared before and after de-O-acetylation. The C-6, carbonyl carbon, and acetyl proton signals of the acetylated mannose residue were assigned at 66.7, 176.7, and 2.15 ppm, respectively. On the heteronuclear multiple-quantum coherence (HMQC) spectrum (70 °C), a correlation was observed between the C-6 (66.7 ppm) of the mannose residues, in which an anomeric proton was observed at 5.10 ppm, and the H-6 of the same mannose residue at 4.45 and 3.99 ppm. In the HMQC spectrum of de-O-acetylated C-F-4, these correlation signals disappeared. These results suggest that the acetyl group located at O-6 of the mannose residue which is not replaced by glucuronic acid.

3. Discussion

This study suggested that the antigenic polysaccharide from *T. domesticum* that reacts with specific factor III serum has the structure shown in Fig. 5. The major repeating unit consists of eight sugar residues, and *O*-acetyl groups are mainly linked to the O-6 of mannose residues. Methylation analysis of de-O-acetylated C-F-4 polysaccharide before and after reduction showed that the nonreducing terminal residue was predominantly arabinose. Glucuronic acid was mainly substituted at O-2 and there were three or four xylose residues in the middle of the side chains. The analyses of the acidic oligo- and polysaccharides obtained by 0.5 M TFA hydrolysis showed that the β-D-glucuronic acid residue was attached to the O-2 of the backbone mannose and the major basic repeating unit was structure **A** (Fig. 5). The disaccharide in structure **B** was obtained by 0.1 M TFA hydrolysis. The methylation analysis using sodium borodeuteride showed that (1→4)-linked and (1→2)-linked xylose were present, although the ratio of the linkages is unknown. At least one of the remaining xylose residues could have a (1→2) linkage where the nonreducing terminal arabinose residue links to the O-4 of the xylose residue. We do not have any direct evidence for structure **C**. However, the removal of neutral sugar residues (arabinose

and xylose) from the side chain resulted in the appearance of a nonreducing terminal β-D-glucuronic acid residue (Fig. 4(D)). The double-quantum filtered COSY (DQF-COSY) spectrum of the de-O-acetylated polysaccharide showed the existence of three or four different neighboring β-D-xylose residues. Since the number of 1,2,3-*O*-substituted mannose residues obtained by the methylation analysis exceeded that estimated from the structure shown in Fig. 5, the xylose residue could be linked to mannose[b] or mannose[c]. Using NMR spectroscopy, Cherniak et al.[15] identified six structural motifs in GXM from *Cryptococcus neoformans* based on the replacement of an α-(1→3)-mannotriose with variable quantities of 2-*O*-β- and 4-*O*-β-xylopyranosyl and 2-*O*-β-glucopyranosyluronic acid. The basic motif is the same as structure **A** in Fig. 5. The three signals at 5.26, 5.19, and 5.12 ppm correspond to the anomeric protons of mannose residues a, b, and c, respectively. When a β-xylose residue attaches to O-2 of the mannose[b] residue, the chemical shift of the anomeric proton of the mannose[b] residue should be lower than that of the mannose[a] residue.[15] However, lower chemical shifts were not observed in de-O-acetylated C-F-4. When a β-xylose residue attaches to O-2 of the mannose[c] residue, the chemical shift of the anomeric proton of the mannose[c] residue should be the same as that of the mannose[b] residue at 5.19 ppm.[14] In de-O-acetylated C-F-4, however, another chemical shift at 5.12 ppm was observed. These data suggest that O-2 of mannose residues b and c in C-F-4 are not substituted.

The partially hydrolyzed polysaccharide (Fig. 3(D)) has structure **A** shown in Fig. 5. After reduction of the polysaccharide, the signal at 5.12 ppm increased, while the signal at 5.17 ppm disappeared (data not shown). The disappearing signal may correspond to the anomeric proton of a mannose residue that is correlated with the carboxyl group of glucuronic acid. These data support the structure shown in Fig. 5.

Antigenically active O-acetylated polysaccharide fractions were obtained from CWPS and SPS isolated from *T. domesticum*. The activity was lost after de-O-acetylation. The inhibitory activity of S-F-4 was twice that of C-F-4. The difference in the inhibitory activities corresponds quite well to the number of *O*-acetyl groups; there were approximately twice as many in S-F-4. However, partial deacetylation of S-F-4 resulting in the same number of *O*-acetyl groups as in C-F-4 resulted in the disappearance of the antigenic activity (data not shown). These results suggest that the antigenic activity depend on more than the number of *O*-acetyl groups.

Mizobe et al.[13] purified the serotype-specific GXM antigen of serotype II *T. cutaneum* TIMM 1318, which is now a synonym for *T. asahii*. GXM does not contain arabinose and is 80% mannose. The serotype II-specific GXM contains an α-(1→3)-mannan backbone bound at O-2 or O-2 and O-4 to short side chains of (1→4)-linked mannose, (1→2)-linked xylose, and nonreducing terminal glucuronic acid residues. Although they do not mention *O*-acetyl groups, the chemical structure of the antigenic polysaccharides of *T. asahii* and *T. domesticum* are considerably different. This structural difference should contribute to the serological specificity.

4. Experimental

Strain used.—*T. domesticum* M 9401, which is our standard serotype III strain for antigenic analysis, was used to determine the chemical structure of the antigenic polysaccharide. *T. cutaneum* IFO 1198 (serotype I) and *T. asahii* var. *asahii* IFO 0174 (serotype II) were used as adsorbing antigens for preparing factor III serum.

Immunological analysis

Preparation of antisera. M 9401 cells grown in modified Sabouraud broth (2% glucose, 0.5% yeast extract, 1% polypeptone) at 27 °C for 2 days were heated at 100 °C for 1 h. The washed cells were suspended in 0.5% formalinized saline at a concentration equivalent to the McFarland no. 10 standard. Male Japanese white rabbits were injected intravenously with 0.5, 1, 2, and 4 mL of this suspension at 4-day intervals until a high agglutination titer was obtained.

Preparation of factor sera and slide agglutination-inhibition test. The procedure described

by Nishikawa et al.[16] was used. Factor III serum was prepared from anti-*T. domesticum* M 9401 serum by adsorption with *T. cutaneum* IFO 1198 (serotype I) and *T. asahii* var. *asahii* IFO 0174 (serotype II). A slide agglutination–inhibition test using factor III serum and the *T. domesticum* M 9401 cell antigen system was performed to determine the antigenicity of the polysaccharide fraction. Factor III serum was incubated with an equal volume of polysaccharide inhibitor for 2 h at 37 °C, and then the quantitative slide agglutination test was performed. The concentration of inhibitor required for a one-fourth reduction in the agglutination reaction was determined.

Analytical method.—Total carbohydrate content was determined by the phenol–H_2SO_4 method.[17] Protein was assayed by the method of Lowry et al.[18] using BSA as a standard. Uronic acid was measured by a carbazole H_2SO_4 method[19] using glucuronic acid as a standard.

Preparation of cell wall and soluble polysaccharides.—*T. domesticum* M 9401 cultures were grown in yeast nitrogen base broth (Difco Laboratories, Detroit, MI., USA) containing 2% glucose, 1% casamino acids, and 100 μg/mL streptomycin at 27 °C for 5 days with shaking. Cells were separated by centrifugation and CWPS was extracted by the method of Raschke and Ballou.[20] The washed cells were suspended in 0.02 M sodium citrate buffer (pH 7.0) and autoclaved at 121 °C for 90 min. CWPS was precipitated from the supernatant by adding 2 vols of EtOH and 1/10 vol of 10% AcONa solution. The precipitate was dissolved, dialyzed against water, and lyophilized.

SPS was obtained from the culture supernatant.[21] Briefly, after concentration, 9 vols of EtOH and 1/10 vol of 10% AcONa solution were added. The precipitate was dissolved, dialyzed against water, and lyophilized. Both crude polysaccharides were treated with pronase (Kaken Pharmaceutical Co., Tokyo. Japan) at 40 °C for 24 h.

Purification of CWPS and SPS by ion-exchange column chromatography.—The deproteinized polysaccharide was purified by DEAE-Toyopearl 650M (Tosoh Co., Tokyo, Japan) column chromatography. Unbound material was eluted with water at a flow rate of 3.5 mL/min and bound polysaccharide was eluted with 0.01 M potassium phosphate buffer (PB), pH 7.4, and then eluted with linear gradients of 0–0.4 and 0.4–1.0 M NaCl in 800 and 380 mL of 0.01 M PB, respectively. The total carbohydrate content in the effluents was determined by the phenol–H_2SO_4 method.

Qualitative and quantitative analysis of the constituent carbohydrate of the polysaccharide.—The polysaccharide was hydrolyzed in 0.5 M H_2SO_4 at 100 °C for 18 h. The hydrolysate was neutralized with $BaCO_3$ and analyzed by TLC on a silica gel and cellulose plates with (40:11:19) *n*-butanol–EtOH–water and (2:1) phenol–1% ammonia solvent systems, respectively. The hydrolysate was reduced with $NaBH_4$ and then acetylated with (1:1) Ac_2O–pyridine. Alditol acetate was analyzed with a Shimadzu gas chromatograph GC-7A (Shimadzu Co., Ltd., Kyoto, Japan) equipped with a flame-ionization detector in a glass column containing 3% ECNSS-S on a Gas Chrom Q at 190 °C. Nitrogen was used as the carrier gas at 40 mL/min. To identify uronic acid, the carbohydrate was converted into its corresponding carboxyl-reduced product and analyzed by gas chromatography (GLC) as described for alditol acetate.

Sepharose CL-4B gel-filtration chromatography.—The polysaccharide was applied to a Sepharose CL-4B column equilibrated with 0.02 M 2-amino-2-hydroxymethyl-1,3-propanediol (Tris)–0.3 M NaCl buffer (pH 8.0) and eluted at a flow rate of 18 mL/h; 3.0 mL fractions were collected. The molecular weight of the polysaccharide was determined by comparison with dextran markers (Amersham Pharmacia Biotech, Uppsala, Sweden).

NMR analysis.—^{1}H NMR spectra were recorded with a GSX-400 NMR spectrometer (JEOL, Tokyo, Japan) for solutions in 99.9% D_2O, at 90 °C; sodium 3-(trimethylsilyl)-1-propanesulfonate (DSS) was the external standard. ^{13}C NMR spectra and two-dimensional (2D) NMR spectra were recorded with a JNM-LA500 NMR spectrometer (JEOL).

Carboxyl reduction.—The acidic sugars were converted into the corresponding neutral sugars according to the method of Taylor et al.[22] The carboxyl groups in the polysaccha-

ride were reduced by the addition of 1-(3-dimethylaminopropyl) - 3 - ethylcarbodiimide hydrochloride maintained at pH 4.7 and $NaBH_4$.

Partial and complete deacetylation.—The polysaccharide was de-O-acetylated completely using 0.1 M NaOH at 37 °C for 6 h following the method of Bhattacharjee et al.[23] Partial deacetylation was performed in 0.2 M carbonate–bicarbonate buffer, pH 9, at 37 °C for 2 h.

Methylation analysis.—The polysaccharides were methylated by the method of Hakomori[24] as described by Hellerqvist et al.[25] The partially methylated alditol acetate was analyzed by GLC and GLC–MS with a JMX-DX302 (JEOL). GLC was performed using the conditions described above. Mass spectra (75 eV) were recorded with an ion-source temperature of 193 °C, an accelerating voltage of 3 kV, and an ionizing current of 300 μA.

Partial acid hydrolysis.—The polysaccharide was hydrolyzed under the following two conditions: (A) 0.5 M trifluoroacetic acid (TFA) for 5 h at 95 °C; (B) 0.1 M TFA for 1–3 h at 95 °C. In Condition A, the partially hydrolyzed products were separated into neutral and acidic fractions by chromatography on an AG1-X2 column eluted with water and then with 10% formic acid.

Bio-Gel P-2 gel-filtration chromatography.—The partially hydrolyzed products obtained by hydrolysis under Condition B, and the neutral and acidic carbohydrates obtained under Condition A were applied to a 130 × 2.5 cm Bio-Gel P-2 column equilibrated with a (500:5:2) water–pyridine–AcOH solvent system[21] and eluted at a flow rate of 10 mL/h; 2.0 mL fractions were collected.

Optical rotation.—Samples were dissolved in water and measured at 589 nm with a DIP-140 polarimeter (Japan Spectroscopic Co., Ltd.) at rt.

Controlled Smith degradation of polysaccharide.—The polysaccharide was oxidized with 0.03 M sodium metaperiodate.[21] The oxidized solution was dialyzed against water, reduced with $NaBH_4$, and then hydrolyzed with 0.6 M HCl at rt for 7–50 h.

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References

1. Nahass, G. T.; Rosenberg, S. P.; Leonardi, C. L.; Penneys, N. S. *Arch. Dermatol.* **1993**, *129*, 1020–1023.
2. Walsh, T. J. *Infect. Dis. Clin. North Am.* **1989**, *3*, 43–52.
3. Shimazu, K.; Ando, M.; Yoshida, K.; Araki, S. *Am. Rev. Respir. Dis.* **1984**, *130*, 407–411.
4. Guého, E.; Smith, M. T.; de Hoog, G. S.; Billon-Grand, G.; Christen, R.; Batenburg-van der Vegte, W. H. *Antonie van Leeuwenhoek* **1992**, *61*, 289–316.
5. Sugita, T.; Nishikawa, A.; Shinoda, T. *J. Gen. Appl. Microbiol.* **1994**, *40*, 397–408.
6. Ikeda, R.; Yokota, M.; Shinoda, T. *Microbiol. Immunol.* **1996**, *40*, 813–819.
7. Nishiura, Y.; Nakagawa-Yoshida, K.; Suga, M.; Shinoda, T.; Guého, E.; Ando, M. *J. Med. Vet. Mycol.* **1997**, *35*, 45–52.
8. Sugita, T.; Makimura, K.; Nishikawa, A.; Uchida, K.; Yamaguchi, H.; Shinoda, T. *Microbiol. Immunol.* **1997**, *41*, 571–573.
9. Sugita, T.; Nishikawa, A.; Shinoda, T.; Yoshida, K.; Ando, M. *J. Gen. Appl. Microbiol.* **1995**, *41*, 429–436.
10. Ando, M.; Sakata, T.; Yoshida, K.; Yamasaki, H.; Araki, S.; Onoue, K.; Shinoda, T. *J. Allergy Clin. Immunol.* **1990**, *85*, 36–44.
11. Fukazawa, Y.; Kagaya, K.; Shinoda, T. *Curr. Top. Med. Mycol.* **1995**, *6*, 189–219.
12. Gorin, P. A. J.; Spencer, J. F. T. *Can. J. Chem.* **1967**, *45*, 1543–1549.
13. Mizobe, T.; Ando, M.; Yamasaki, H.; Onoue, K.; Misaki, A. *Clin. Exp. Allergy* **1995**, *25*, 265–272.
14. Dutton, G. G. S.; Paulin, M. *Carbohydr. Res.* **1980**, *87*, 107–117.
15. Cherniak, R.; Valafar, H.; Morris, L. C.; Valafar, F. *Clin. Diagn. Lab. Immunol.* **1998**, *5*, 146–159.
16. Nishikawa, A.; Sekine, T.; Ikeda, R.; Shinoda, T.; Fukazawa, Y. *Microbiol. Immunol.* **1990**, *34*, 825–840.
17. Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. *Anal. Chem.* **1956**, *28*, 350–356.
18. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265–275.
19. Galambos, J. T. *Anal. Biochem.* **1967**, *19*, 119–132.
20. Raschke, W. C.; Ballou, C. E. *Biochemistry* **1972**, *11*, 3807–3816.
21. Ikeda, R.; Matsuyama, H.; Nishikawa, A.; Shinoda, T.; Fukazawa, Y. *Microbiol. Immunol.* **1991**, *35*, 125–138.
22. Taylor, R. L.; Shively, J. E.; Conrad, H. E. *Methods. Carbohydr. Chem.* **1976**, *7*, 149–151.
23. Bhattacharjee, A. K.; Jennings, H. J.; Kenny, C. P.; Mertin, A.; Smith, I. C. P. *J. Biol. Chem.* **1975**, *250*, 1926–1932.
24. Hakomori, S. *J. Biochem.* **1964**, *55*, 205–208.
25. Hellerqvist, G. C.; Lindberg, B.; Svensson, S. *Carbohydr. Res.* **1968**, *8*, 43–55.