Carbohydrate Research, 185 (1989) 239–248
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

USE OF ^{13}C-N.M.R. SPECTROSCOPY FOR THE QUANTITATIVE ESTIMATION OF 3-*O*- AND 3,6-DI-*O*-SUBSTITUTED D-GLUCOPYRANOSYL RESIDUES IN α-D-GLUCANS FORMED BY THE D-GLUCOSYLTRANSFERASES OF *Streptococcus sobrinus*

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(Received February 8th, 1988; accepted for publication in revised form, August 5th, 1988)

ABSTRACT

The ^{13}C-n.m.r. spectra of the three α-D-glucans from *Streptococcus sobrinus* and the dextran from *Leuconostoc mesenteroides*, which differ widely in the ratios of ω (terminal, nonreducing) D-glucopyranosyl groups: 3-:6-:3,6-linked D-glucopyranosyl (Glc) residues, were measured in 0.5M NaOH at 22°. The C-1 signals of 3-*O*-substituted Glc in a linear sequence, 6-*O*-substituted Glc in a linear sequence, 3,6-di-*O*-substituted Glc in a (1→6)-linked sequence, and Glc attached to O-3 of 3,6-di-*O*-substituted Glc were distinguished from each other. The C-3 signal of 3,6-linked Glc appeared downfield by 0.6 to 1.0 p.p.m. compared to the C-3 signal of 3-linked Glc in a linear sequence. The C-6 signals of ω-terminal, 3-linked, 6-linked, and 3,6-linked Glc were also assigned. The C-2 signal of 3-linked Glc in a linear sequence appeared separately, at 73.76 p.p.m. Based on these assignments, the various D-glucopyranosyl residues of the *S. sobrinus* α-D-glucans were quantitatively estimated from the signal areas of the C-2 atom of 3-linked Glc, the C-3 atom of 3-linked and 3,6-linked Glc, the C-6 atom of 6-linked and 3,6-linked Glc, and the C-6 atom of the ω-Glc groups and 3-linked Glc residues. The figures thus derived for the linkage ratios were close to those obtained by methylation analysis.

INTRODUCTION

Among the mutans group of streptococci, the serotype *b*, *c*, *e*, and *f* strains secrete at least two distinct D-glucosyltransferases and a D-fructosyltransferase, and the serotype *a*, *d*, *g*, and *h* strains secrete three D-glucosyltransferases[1,2]. These D-glucosyltransferases, when isolated, individually synthesize α-D-glucans that differ in such physicochemical properties as water-solubility, and in the ratios of terminal, nonreducing (ω) groups:3-:6-:3,6-linked D-glucopyranosyl (Glc) residues. These enzymes act cooperatively *in vivo* to synthesize water-insoluble and yet adhesive polysaccharides[3,4], whereas the α-D-glucan separately synthesized by each enzyme is not "adhesive". The adhesive polysaccharides enable the organism to adhere to tooth surfaces, and to induce dental caries[5,6].

0008-6215/89/$ 03.50

Few D-fructans of *Streptococcus mutans*[7] and *Streptococcus salivarius*[8,9] have been chemically studied, because of the heat-labile properties of D-fructofuranosides, which may decompose while being converted into derivatives suitable for analysis by g.l.c. Recently, a ^{13}C-n.m.r. study directly determined the structure of the D-fructan of *S. mutans*, which is composed solely of β-D-(2→1)-linked D-fructofuranosyl residues[10]. As for D-glucans of *S. mutans*, the water-insoluble D-glucans contain a high proportion of α-D-(1→3) linkages, and the soluble D-glucans have α-D-(1→6) linkages in a high proportion. The D-glucans synthesized by each of three D-glucosyltransferases from *Streptococcus sobrinus* 6715 (the mutans group serotype *g*) were studied in detail by methylation analysis[11], and reported to be (1→3)-α-D-glucan (IG), (1→6)-α-D-glucan with ω- and 3,6-linked Glc (SG1), and (1→6)-α-D-glucan with ω-, 3-, and 3,6-linked Glc (SG2).

Several D-glucans from *S. mutans, Leuconostoc mesenteroides*, and related organisms were also analyzed by ^{13}C-n.m.r. spectroscopy[12–19]. In these D-glucans, some were found to contain α-D-(1→3) and α-D-(1→6) linkages, the ratio of which was estimated from the areas of the signals of anomeric carbon atoms[17,19]. However, in cases of α-D-glucans having 3,6-linked branch-points, the ratios of ω-:3-:6-:3,6-:linked Glc have not yet been determined by n.m.r. alone, because 3- and 3,6-linked Glc could not be distinguished from each other by n.m.r. spectroscopy.

Herein are presented the ^{13}C-n.m.r. spectra of the three α-D-glucans from *S. sobrinus*, measured under the same conditions. Signals due to 3- and 3,6-linked Glc were assigned, and then the ratios of all the different D-glucopyranosyl residues of the α-D-glucans were estimated from the areas of, at most, four specific signals.

RESULTS AND DISCUSSION

Three D-glucosyltransferases[11], having pI values of 3.9, 4.9, and 5.5, were purified from the culture supernatant liquor of *S. sobrinus* 6715 in 0.4, 6.3, and 1.8% yield, respectively. The respective specific activities were 31.4, 25.1, and 46.3 IU per mg of protein, when the D-glucan-synthesizing activity was assayed in the presence of primer dextran. In this study, the enzymes having pI 3.9, 4.9, and 5.5 corresponded to the enzymes previously purified and characterized[20–22], and the physicochemical properties of the three enzymes had been summarized by Mukasa[1]. To measure ^{13}C-n.m.r. spectra, SG1, IG, and SG2 were synthesized by the enzymes having pI 3.9, 4.9, and 5.5, respectively, in the absence of the primer. SG1 (0.80 g), IG (0.35 g), and SG2 (1.13 g) were formed from sucrose (2.5 g) in 68, 30, and 95% yield, respectively. These D-glucans were composed solely of D-glucose, as judged by their n.m.r. spectra (see Figs. 1B, 1C, 2).

The ^{13}C-n.m.r. spectra of α-D-glucans dissolved in 0.5M NaOH were measured, because the IG was clearly soluble in the alkaline solution. However, in the solution, the saccharides possessing reducing terminals decomposed gradually. Therefore, all samples used were reduced with $NaBH_4$ to convert the reducing

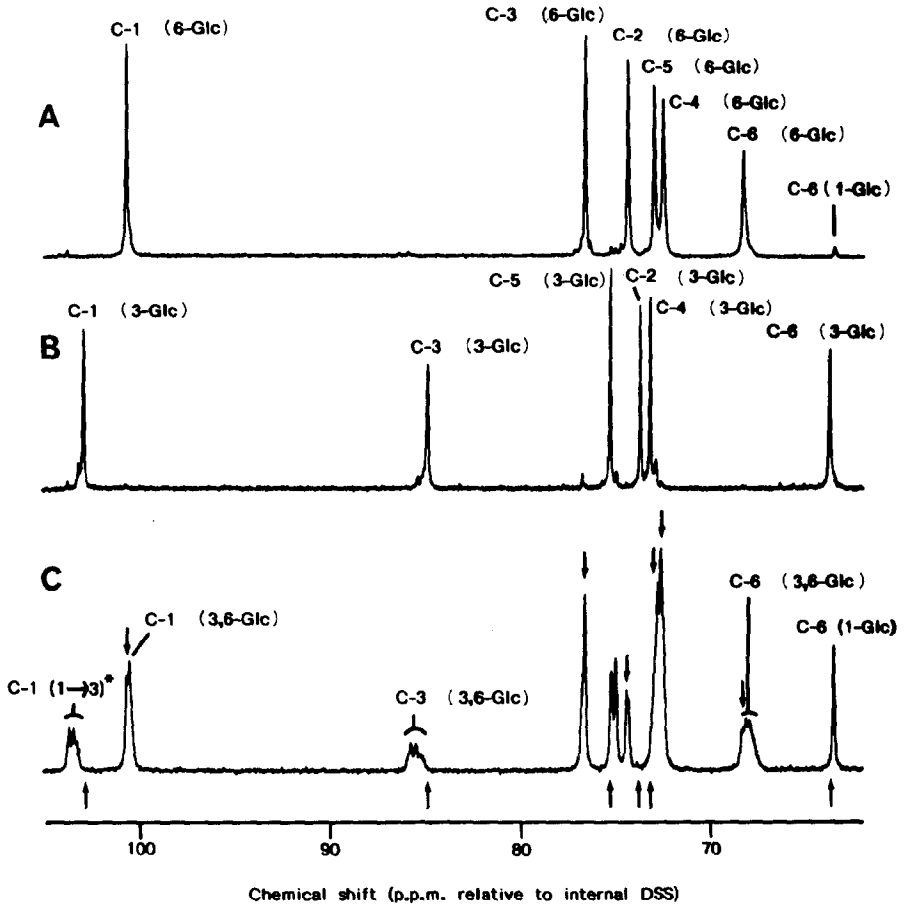

Fig. 1. ^{13}C-N.m.r. spectra of (A) dextran T70, (B) *S. sobrinus* IG, and (C) *S. sobrinus* SG1. The D-glucans were reduced with $NaBH_4$ prior to the measurement. 6-Glc, 3-Glc, *etc.*, represent 6-, 3-linked α-D-glucopyranosyl residues, *etc.* C-1 (1→3)* represents all the C-1 of Glc attached to O-3 of the next residue, except those of 3-linked Glc in a sequence. In C, arrows (↓ and ↑) show the positions of signals of 6-linked Glc in a sequence (A) and 3-linked Glc in a sequence (B), respectively.

terminals into D-glucitol residues. The reduced samples were stable, judging from the observation that their n.m.r. spectra no longer changed during the measurements.

The assignments for the ^{13}C-n.m.r. spectra of linear (1→6)-α-D-glucan and (1→3)-α-D-glucan were initially made by Colson *et al.*[13], although the assignments for C-4 and C-5 of (1→6)-α-D-glucan were left uncertain. Gagnaire and Vignon[16] made clear the assignments for the C-4 and C-5 atoms by using a selective proton-irradiation technique. Furthermore, the C-6 signal of the nonreducing (ω) terminal group was first assigned by Usui *et al.*[12]. Based on these assignments, the signals of *L. mesenteroides* dextran T70 (see Fig. 1A and Table I) and *S. sobrinus* IG (see Fig. 1B, and Table I), which respectively mainly contain α-(1→6) and α-(1→3)

TABLE I

^{13}C-CHEMICAL SHIFTS OF DEXTRAN T70 AND THREE ENZYMICALLY SYNTHESIZED α-D-GLUCANS (IG, SG1, AND SG2)[a], WITH RELATIVE SIGNAL AREAS FOR SG2

Dextran T70	*IG*	*SG1*	*SG2*		*Assignment*
p.p.m.	*p.p.m.*	*p.p.m.*	*p.p.m.*	*Area[b] (%)*	
		103.62, 103.49, 103.38	103.76	3.52	C-1 (1→3)*[c]
	102.88		102.85		C-1 (3-linked Glc)
100.63		100.63	100.66[d]	13.30	C-1 (6-linked Glc)
		100.49			C-1 (3,6-linked Glc)
		85.76, 85.49	85.89	3.22	C-3 (3,6-linked Glc)
	84.89		84.87		C-3 (3-linked Glc)
76.66		76.66	76.68	13.52	C-3 (6- & 1-linked Glc)
	75.33		75.32	3.52	C-5 (3-linked Glc)
		75.24			
		75.02	75.07	1.90	
74.40		74.43	74.44	12.14	C-2 (6-linked Glc)
	73.36		73.75	2.10	C-2 (3-linked Glc)
	73.23				C-4 (3-linked Glc)
73.04			73.07	30.13	C-5 (6-linked Glc)
		72.82			
72.56		72.65	72.61		C-4 (6-linked Glc)
68.34		68.30	68.37[e]	12.74	C-6 (6-linked Glc)
		68.14, 67.96			C-6 (3,6-linked Glc)
	63.79		63.78	3.90	C-6 (3-linked Glc)
63.49		63.54	63.59		C-6 (1-linked Glc)

[a]IG, SG1, and SG2 were synthesized by purified D-glucosyltransferases of *S. sobrinus* 6715, and all the D-glucans were reduced with $NaBH_4$ prior to the measurement of spectra. [b]Relative to total signal area. [c]All the C-1 of Glc in α-(1→3) linkage, except the C-1 of 3-linked Glc in a sequence. [d]Overlapped signals of the C-1 of 6- and 3,6-linked Glc. [e]Overlapped signals of the C-6 of 6- and 3,6-linked Glc.

linkages, were assigned. In the case of the ^{13}C-n.m.r. spectrum of 3-branched (1→6)-α-D-glucan, Ito and Schuerch[23] clearly assigned the signals to ω-, 6-, and 3,6-linked Glc by measuring, in D_2O, the spectra of synthetic (1→6)-α-D-glucans having different degrees of branching, and by comparing the chemical shifts and the signal intensities. According to their assignments, the signals of *S. sobrinus* SG1 (see Fig. 1C and Table I), which contains ω-, 6-, and 3,6-linked Glc, were determined.

One of the aims of the present study was to determine the relative positions among the signals of ω-, 3-, 6-, and 3,6-linked Glc (see Table I), because the signals of 3- and 3,6-linked Glc had not been precisely distinguished. Therefore, the spectra (see Fig. 1A, B, and C) of dextran T70, IG, and SG1, which were separately measured under the same conditions, were carefully compared with each other. In the region for signals of glycosidically linked C-1 (100 to 104 p.p.m.), the C-1 atom of 6-*O*-substituted Glc and 3,6-di-*O*-substituted Glc in a (1→6)-linked sequence resonated at 100.63 and 100.49 p.p.m., respectively. The C-1 signal of 3-*O*-sub-

stituted Glc in a linear sequence appeared at 102.88 p.p.m., whereas the C-1 signal of Glc attached to O-3 of 3,6-di-*O*-substituted Glc appeared at 103.38 to 103.62 p.p.m. The C-2 atom of 3-linked Glc resonated separately at 73.76 p.p.m. The C-3 signals of 3- and 3,6-linked Glc appeared at 84.89 p.p.m. and at 85.49 to 85.76 p.p.m., respectively. The C-6 signals of the 6-linked (68.30 to 68.34 p.p.m.) and 3,6-linked Glc (67.96 to 68.14 p.p.m.) were also distinguishable, although they partly overlap. The C-6 signals of 6-unsubstituted (ω- and 3-linked) Glc were found closely grouped at 63.49 to 63.54 p.p.m. and at 63.79 p.p.m., respectively, but were certainly distinguishable. After determining the sorts of residues, their ratio was estimated based on the areas of some distinct signals. In the spectrum of SG1 (see Fig. 1C), the areas of the C-3 signal of 3,6-linked Glc, the C-6 signals of 6- and 3,6-linked Glc, and the C-6 signal of ω-linked Glc were respectively, 5.01, 10.23, and 6.33% of the total area. The area of the C-6 signal of 6-linked Glc was calculated to be 5.22% by subtracting the area (5.01%) of the signal of substituted C-3 from that (10.23%) of the signal of substituted C-6. Thus, the ratios of ω-:6-:3,6-linked Glc were estimated to be 6.33:5.22:5.01; that is, SG1 consists of 38.2, 31.5, and 30.3% of ω-, 6-, and 3,6-linked Glc, respectively. These values are fairly compatible with the 38.2, 35.7, and 26.1% of ω-, 6-, and 3,6-linked residues determined by methylation analysis (see Table II), which was repeated in the present study, because the D-glucans previously analyzed[11] had been synthesized by degraded forms of each D-glucosyltransferase, whereas the D-glucans in this study were synthesized by means of the native form of each enzyme.

The spectrum of SG2 from *S. sobrinus* (see Fig. 2) was similarly analyzed. The signals of C-6 of ω-linked Glc, C-1, C-2, C-3, C-5, and C-6 of 3-linked Glc, C-1, C-2, C-3, C-4, C-5, and C-6 of 6-linked Glc, C-3 of 3,6-linked Glc, and C-1 of the Glc attached to O-3 of 3,6-linked Glc were observed (see Table I), showing that the D-glucan consisted of four kinds of D-glucopyranosyl residue. Areas of the C-2 signal (73.75 p.p.m.) of 3-linked Glc, the C-3 signals (84.87 to 85.89 p.p.m.) of 3- and 3,6-linked Glc, the C-6 signals (68.37 p.p.m.) of 6- and 3,6-linked Glc, and the C-6 signals (63.59 to 63.78 p.p.m.) of ω- and 3-linked Glc were respectively 2.10, 3.22, 12.74, and 3.90% of the total areas. Successively, the signal areas of C-3 of 3,6-linked Glc (1.12%), C-6 of 6-linked Glc (11.62%) and C-6 of ω-linked Glc

TABLE II

MOL % OF METHYLATED D-GLUCITOL ACETATES FROM SG1 AND SG2 (REDUCED WITH $NaBH_4$)

Acetate of methyl ether	*Mol % of acetate from*	
	SG1	*SG2*
2,3,4,6-tetra-	38.2	13.5
2,4,6-tri-		16.4
2,3,4-tri-	35.7	62.8
2,4-di-	26.1	7.2

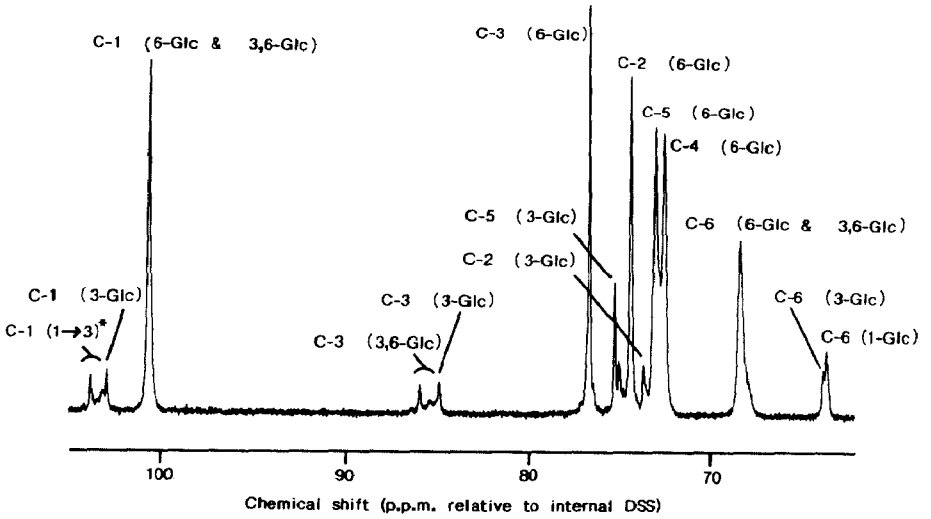

Fig. 2. ^{13}C-N.m.r. spectrum of SG2 from *S. sobrinus*. The D-glucan was reduced with $NaBH_4$ prior to the measurement. Abbreviations in this Figure are defined in the legend to Fig. 1.

(1.80%) were derived from these four areas by subtraction. The ratios of ω-:3-:6-:3,6-linked Glc were estimated to be 1.80:2.10:11.62:1.12; that is, SG2 consists of 10.8, 12.6, 69.8, and 6.7% of ω-, 3-, 6-, and 3,6-linked Glc, respectively. The corresponding values obtained by methylation analysis were 13.5, 16.4, 62.8, and 7.2% (see Table II), in fairly good agreement with the values obtained by n.m.r. spectroscopy.

In the present study, the ratios among D-glucosyl residues were directly estimated from the ^{13}C-n.m.r. spectra of α-D-glucans, as already described. Previously, Usui *et al.*[14] had estimated the ratio of (1→3) to (1→6) linkages from the ^{1}H-n.m.r. spectra of *S. mutans* α-D-glucans. Meyer *et al.*[24] also calculated the ratio from ^{1}H-n.m.r. data of α-D-glucans. By ^{13}C-n.m.r. spectroscopy, Colson *et al.*[17] estimated the proportions of (1→3) and (1→6) linkages of *S. mutans* α-D-glucans from the relative areas of the C-1 and C-6 signals. They further assigned, in a 68-MHz spectrum of soluble α-D-glucan from *S. mutans* OMZ175, the signals at 102.3 and 102.8 p.p.m. to C-1 of 3-linked Glc in a sequence and to C-1 of 3,6-linked Glc, respectively, and they estimated the ratio of the two D-glucosyl residues from the C-1 signal areas. However, it was found in the present study that all the signals due to C-1 of ω-, 3-, 6-, and 3,6-linked Glc which were attached to O-3 of the next 3- and 3,6-linked Glc appear at ~103 p.p.m. In detail, C-1 of 3-linked Glc in a sequence resonate at 102.88 p.p.m. (see Table I). The C-1 atoms of ω-linked Glc of IG resonates at 103.2 to 103.7 p.p.m. (see Fig. 1B), and that of ω-linked Glc of reduced nigerose at 103.84 p.p.m. (data not shown). The C-1 atoms of ω-, 6-, and 3,6-linked Glc attached to O-3 of the next 3,6-linked Glc resonated at 103.38 to 103.62 p.p.m. in the spectrum of SG1 (see Fig. 1C and Table I). Furthermore,

in the spectrum of SG2 (see Fig. 2 and Table I), all the C-1 signals of Glc in a (1→3) linkage appeared at 103.0 to 103.9 p.p.m., except those of 3-linked Glc in a sequence (at 102.85 p.p.m.). Similarly, all the signals due to C-1 of the four residues attached to O-6 of the next 6-linked Glc and 3,6-linked Glc appeared at ~100 p.p.m. Therefore, the C-1 signal areas could not be used for the quantitative estimation of D-glucosyl residues. The signal at 102.8 p.p.m. measured by Colson *et al.*[17] should be assigned not only to C-1 of 3,6-di-*O*-substituted Glc attached to O-3 of the next residue but also to all the C-1 atoms of Glc in (1→3) linkages, except those of 1,3-linked Glc in a sequence, and the signals at ~100 p.p.m. should be assigned not only to C-1 of 6-linked Glc in a sequence but also to C-1 of 3,6-di-*O*-substituted Glc attached to O-6 of the next residue.

In the quantitative analysis of the spectrum of the *S. sobrinus* SG2 (see Table I), the sum of the areas of the C-6 signals of 6- and 3,6-linked Glc, at ~68 p.p.m., and of ω- and 3-linked Glc, at 63 p.p.m., comprised 16.64% of the total area. The sum of the areas of the C-3 signals of 3- and 3,6-linked Glc, at 85 p.p.m., and of ω- and 6-linked Glc, at 76.68 p.p.m., was 16.74%, and the sum of the areas of the C-1 signals (at 100 and 103 p.p.m.) was 16.82%. These values are each very close to one sixth of the total area, indicating that the spectrum was so measured that the area of each signal was proportional to the number of carbon atoms of the corresponding type in the D-glucan molecule.

Preliminary experiments were carried out using reduced dextran T70 in order to find conditions suitable for measuring ^{13}C-n.m.r. spectra quantitatively. The completely proton-decoupled spectra measured with acquisition times of >1.0 s and pulse delays of >1.0 s closely agreed with each other in regard to the relative areas of signals, and also agreed with the proton-decoupled spectrum with suppressed n.O.e. and with an acquisition time of 1.0 s and a pulse delay of 7.0 s. These observations indicated that the spin–lattice relaxation times (T_1) of the polysaccharide were short enough, as previously observed in the case of *L. mesenteroides* dextran[25], and that the n.O.e. on each carbon atom was approximately equal under the conditions used in this study.

A major source of error in the quantitative estimation of ^{13}C-n.m.r. spectra lay in the inaccuracy of integration by cutting and weighing of pieces of signals on paper. The difference between the absolute total areas, determined by the integration, of the two same spectra on separate sheets was maximally 5%; whereas, in methylation analysis, the decrease of the yield of the methylated derivatives from D-glucan could be a major source of error. The estimated ratios of the derivatives in duplicate methylation procedures varied by several percent. Therefore, the differences between the ratios of D-glucopyranose residues estimated by n.m.r. spectroscopy and by methylation analysis would be due to the respective inaccuracy of these two analytical methods. Although apparent differences were observed in some residues in this study, the general features of the ratios estimated by the two independent methods resembled each other well.

EXPERIMENTAL

Purification of glucosyltransferases. — *S. sobrinus* strain 6715 was cultured in a chemically defined medium[26] as previously reported[27]. The organisms were removed from the culture supernatant liquor by centrifugation. After being cooled below 0°, the supernatant liquor was mixed with ethanol (1 vol.) precooled to −40°. The precipitate was collected, dissolved in distilled water, and the solution dialyzed against 20mM imidazole·HCl buffer, pH 6.2, containing 0.1mM phenylmethylsulfonyl fluoride (PMSF), 0.1% of Triton X-100, and 0.001% of merthiolate. Insoluble materials were removed by centrifugation, and the dialyzate was applied to a column of DEAE-Sepharose, as reported[1]. The three kinds of D-glucosyltransferase were separately eluted at ~0.05, 0.12, and 0.18M in a linear concentration-gradient of NaCl. The first eluate, at 0.05M, which mainly contained the enzyme synthesizing SG2, was diluted with distilled water (1 vol.), to decrease the ionic strength, and rechromatographed on a column of DEAE-Sepharose equilibrated with 50mM Tris·HCl buffer, pH 7.2, containing 0.1mM PMSF and 0.001% of merthiolate. The enzyme was eluted with a linear gradient of 0 to 30mM NaCl in the buffer. The purified enzyme had a pI value of 5.5.

The second eluate, which contained the enzyme synthesizing IG, was mixed with distilled water (2 vol.), and the solution applied to a column equilibrated with 50mM sodium acetate buffer, pH 5.5, containing 50mM NaCl, 0.1mM PMSF, and 0.001% of merthiolate. Elution was conducted with 50 to 100mM NaCl (linear gradient) in the buffer. The enzyme having pI 4.9 was obtained.

The third eluate, which contained the enzyme synthesizing SG1, was diluted two-fold, and the solution applied to the column with 50mM Tris·HCl buffer, pH 7.2, containing 0.1mM PMSF and 0.001% of merthiolate. The column was washed with the buffer and then with 50mM sodium acetate, pH 4.5, containing 70mM NaCl and 0.1mM PMSF. The enzyme was eluted with a 70 to 120mM NaCl gradient in the acetate buffer, and it has pI 3.9. Enzyme activities synthesizing water-insoluble and soluble D-glucans were measured as previously reported[20]. One unit of the activity is defined as the amount of the enzyme incorporating, into D-glucan, 1 μmol of D-glucose from sucrose per min. Protein was assayed by the method of Lowry *et al.*[28]. Three enzyme preparations obtained were electrophoretically shown to be free from each other. Antiserum to each enzyme was prepared as reported[6], and used for double immunodiffusion analysis[29]. The three enzymes purified here were immunologically unrelated to each other.

Preparation of D-glucans. — Each of the enzyme preparations and 2.5 g of sucrose were incubated for 10 to 14 d at 37° in 50 mL of 0.1M sodium phosphate buffer, pH 6.5, containing 0.01% of merthiolate. Water-insoluble IG synthesized by the enzyme (15.6 IU) having pI 4.9 was collected from the reaction mixture by centrifugation, washed three times with distilled water, and lyophilized. Soluble SG1 and SG2 synthesized by the pI 3.9 and 5.5 enzymes (2.7 and 8.0 IU, respectively) were precipitated by addition of ethanol (3 vol.), collected, washed three

times by dissolving in distilled water and precipitating with ethanol, dialyzed against water, and lyophilized.

Reduction of saccharides. — Commercial dextran T70 (Pharmacia, Uppsala, Sweden) of *L. mesenteroides* and the three α-D-glucans of *S. sobrinus* (100 mg each) were dissolved in 5 mL of 0.5M NaOH containing 200 mg of $NaBH_4$ at 0°. After being kept for 7 d at 4°, the solutions were made neutral with 4M HCl, exhaustively dialyzed against water, and lyophilized. Nigerose (Sigma, St. Louis, MO, U.S.A.) was reduced for 7 d at ~20° with $NaBH_4$ in distilled water. The solution was mixed with Bio-Rad AG 50W-X8 cation-exchange resin (Richmond, CA, U.S.A.) until the pH of the solution became <4, and the suspension was filtered. The filtrate was dried *in vacuo* in a centrifugal evaporator (Yamato model RD-41, Tokyo, Japan), and then mixed with methanol and dried three times to remove boric acid by co-distillation of its methyl ester.

N.m.r. spectroscopy. — Reduced saccharides (30 to 60 mg) were dissolved in 0.6 mL of 0.5M NaOH containing 10% (v/v) of D_2O and 1% (w/v) sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), ultrasonicated, and poured into 5-mm (o.d.) tubes. ^{13}C-N.m.r. spectra were recorded at 67.9 MHz with a JEOL JNM-GX-270 spectrometer operated in the Fourier-transform mode, with complete proton-decoupling, at 22 ±1°. The spectra were recorded with ~100,000 scans, a digital resolution of 0.24 Hz, a pulse angle of 90°, and an acquisition time of 1.0 s in a pulse interval of 2.0 s. This interval was long enough, based on the spin–lattice relaxation times (T_1) of 63 to 250 ms reported for the carbon atoms of *L. mesenteroides* dextrans[25], to measure signals quantitatively. Field-frequency locking was provided by D_2O. Chemical shifts are expressed as p.p.m. relative to the internal standard of DSS. Relative areas of signals were determined by excising and weighing the pieces of signals plotted on paper.

Methylation analysis. — Reduced polysaccharides (2 mg) were permethylated[30], the products converted into partially methylated D-glucitol acetates, and these analyzed by g.l.c., as previously described[11].

ACKNOWLEDGMENT

The author is grateful to Dr. H. Mukasa, Professor of Chemistry, National Defense Medical College, for valuable discussions.

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