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Structures of Water-Soluble α-D-Glucans Synthesized from Sucrose by Glucosyltransferases Isolated from *Streptococcus sobrinus* Culture Filtrates

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ABSTRACT

From three strains of Streptococcus sobrinus *-K1-R, -6715-13-201 and -6715-13-27, grown in continuous culture under a variety of defined conditions, three different glucosyltransferases (GTF): GTF-S1, GTF-S3 and GTF-S4, were isolated. The enzymes were used to synthesize, from sucrose, soluble α-D-glucans S1, S3 and S4 respectively, of quite different structure. Methylation analysis, NMR spectroscopy and enzymic hydrolysis were used to determine the structural features of the S1, S3 and S4 polysaccharides. The GTF-S1-type enzymes synthesized highly branched (up to ≈ 34%)* glucans having single *-(1→3)-α-linked D-glucosyl residues attached to approximately one in two of the -(1→3)-α-linked D-glucosyl units of the main chain. The GTF-S3 enzymes produced low molecular weight linear -(1→3)-α-linked glucans.*

The GTF-S4 enzymes, released from strains K1-R and 6715-13-201 only in the presence of the surfactant Tween 80, form S4 glucans with branching ranging from ≈ 8 to 11%. Enzymic hydrolysis with an endo-*(1→3)-α-D-glucanase was used to show that side chains consisting of a sequence of -(1→3)-α-linked-D-glucosyl residues are attached to -(1→6)-α-linked-D-glucosyl residues of the backbone, in S4 glucans from strains K1-R and 6715-13-201. Side chains of single -(1→3)-α-linked glucosyl residues are also present in these S4 glucans, as shown by NMR studies.*

Strain 6715-13-27 released GTF-S4 enzymes in the absence of Tween 80. The S4 glucans formed by these enzymes have the single side chains

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almost exclusively, and undergo very limited hydrolysis with the -(1→3)-α-glucanase.

INTRODUCTION

A great deal of the information on the chemistry of the extracellular glucans of oral bacteria has resulted from studies on the enzymes involved in their synthesis. However the literature, particularly that prior to 1982, concerning the structure of glucans produced by oral streptococci is somewhat confusing (Walker & Jacques, 1987). Investigators tended to use different bacterial strains, growth media, growth conditions and purification procedures for the glucosyltransferases (GTF). It is now recognised that these variables must be reported if a correlation between structural details of glucan and the GTF profile of the organism is to be considered.

The authors' previous paper (Walker *et al.*, 1990) details the effect of a variety of growth conditions on the rate of production of GTF by strains K1-R and 6715-13-201 of *Streptococcus sobrinus*. In general, GTF production increased with the growth rate, and was greatly stimulated by the nonionic surfactant Tween 80. At low growth rate, the predominant GTF, GTF-S1, catalysed the synthesis of a highly branched soluble dextran, S1. At high growth rate, GTF-I, which catalysed the synthesis of a water-insoluble -(1→3)-α-linked-D-glucan, became the major enzyme, and GTF-S3, were also produced. When supplemented with Tween 80, strains K1-R and 6715-13-201 showed decreased production of GTF-S1, increased production of GTF-S3 and GTF-I, and released a new enzyme, GTF-S4, at all dilution rates. The GTF-S4 produced a viscous, soluble dextran designated S4 from sucrose. In addition, *S. Sobrinus* strain 6715-13-27 was grown at high and low dilution rates in the absence of Tween 80, and was found to produce large amounts of GTF-S4 under both growth conditions. However, production of GTF-I was low at both high and low dilution rates, in contrast to GTF-I production in strains K1-R and 6715-13-201, where GTF-I was the predominant enzyme at high growth rates. The structural details of a range of S1, S3 and S4 dextrans are reported here. The diversity of polysaccharide structural types produced by the various enzymes serves to reinforce the importance of maintaining, and reporting, the precise conditions used for continuous culture, which is preferred to batch culture for studies such as these. In batch culture the environment changes progressively with time. This could result in different GTF profiles depending on the sampling time chosen.

EXPERIMENTAL

Growth of the microorganisms

S. sobrinus strains were grown in continuous culture (Walker *et al.*, 1990), under glucose limitation in a complex medium. The pH was controlled at 6·5, and anaerobic conditions were maintained with a gas mixture of nitrogen and carbon dioxide (19:1 v/v). Culture filtrates from each steady state were obtained by centrifuging for 10 min at 12 000*g*.

Separation of GTF, e.g. for strain 6715-13-27

Cell-free culture filtrates were concentrated, and the proteins were precipitated with saturated ammonium sulphate as described previously (Walker *et al.*, 1990). The GTF were then separated by column chromatography on hydroxyapatite. At low growth rate (Fig. 1(a))

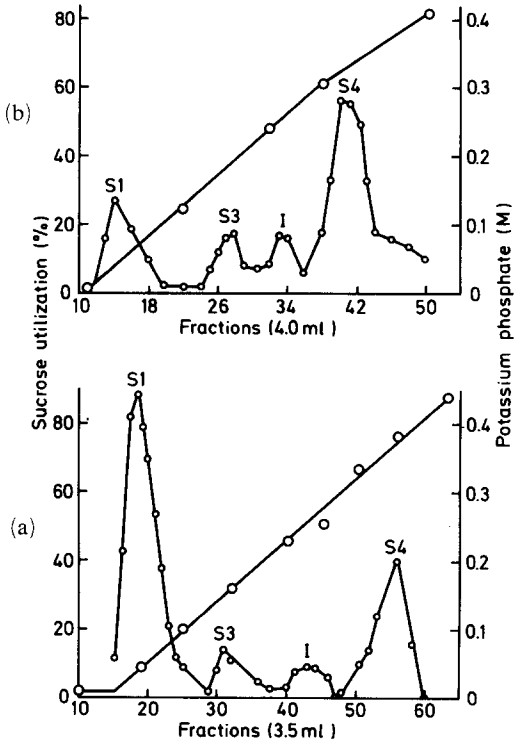

Fig. 1. Separation by hydroxyapatite chromatography of GTF from *S. sobrinus* strain 6715-13-27, grown: (a) at low growth rate. $D = 0{\cdot}075\ h^{-1}$; (b) at high growth rate. $D = 0{\cdot}42\ h^{-1}$.

($D = 0{\cdot}075\ h^{-1}$) the major GTF, eluted with 0·05 M phosphate, was the enzyme GTF-S1 that synthesized highly branched dextran, while at high growth rate (Fig. 1(b)) ($D = 0{\cdot}42\ h^{-1}$), the mutant 6715-13-27 produced maximal amounts of a different enzyme GTF-S4, which was eluted at a phosphate concentration of 0·35–0·40 M.

Enzymic synthesis of an S4-glucan

S. sobrinus 6715-13-27 released a small proportion (3–4%) of GTF-I, the enzyme that catalyses the synthesis of -(1→3)-α-linked-D-glucan, at low and high growth rates. This enzyme was eluted from the hydroxyapatite columns ahead of GTF-S4 at a phosphate concentration of 0·25 M. Nevertheless, it was possible that small amounts of GTF-I were still draining from the column while elution of GTF-S4 began. To reduce this possibility, GTF-S4 to be used for the synthesis of S4-glucan was prepared by two chromatographic steps. The sample was first loaded onto a Trisacryl-M-DEAE column, and the GTF-S4 peak was collected in the void volume (McCabe, 1985). The enzyme was then separated from possible traces of GTF-I by chromatography on hydroxyapatite. The GTF-S4 was then incubated with sucrose in the absence of primer and the resulting S4-glucan was precipitated by the addition of ethanol (2 vol.), dissolved in water, and reprecipitated with methanol.

Methylation analysis was carried out essentially by the method of Harris *et al.* (1984), using potassium hydride to generate the methylsulphinyl carbanion from DMSO. A Shimadzu gas chromatograph GC-9A (Shimadzu Corp., Tokyo) fitted with a flame ionization detector, an SGE Unijector control module, and an SGE bonded-phase vitreous silica capillary column, 25M, i.d. 0·33 mm, film 0·5 μm, BP10 phase. (Scientific Glass Engineering, Ringwood, Victoria, Australia), was used, together with a Hewlett-Packard 3390A integrator. High performance liquid chromatography was carried out on a system consisting of a Rheodyne 7125 injector (Rheodyne Inc., Berkley, California), a Waters M6000 pump (Waters Division of Millipore, Milford, Massachusetts) and a TSK-gel G5000PW gel-permeation column (GPC) $(7{\cdot}5 \times 600\ mm)^2$ (Toyo Soda Manufacturing Co., Tokyo). The solvent was Milli-Q water at 0·8 ml/min flow rate, or 0·02M ammonium formate, pH 5·0 at the same flow rate. An ERMA (ERMA Optical Works, Tokyo) model ERC-7510 refractive index detector was utilized. Pullulan standards (Shodex P82-Showa Denko KK, Japan) were used to calibrate the GPC column. A -(1→3)-α-linked-D-glucanase from *Cladosporium resinae* was isolated from the fungus and purified by DEAE Sephadex A50 and hydroxyapatite (Biogel HTP) column chromatography (Pearce *et al.*, 1990).

Typically, -(1→3)-α-linked-D-glucanase hydrolysis of the various S1 and S4 dextrans was carried out on 50 mg glucan in 2 ml 0·05 M citrate buffer, pH 6; 4–6 ml enzyme (containing a total of 11·5 units) at 35° for up to 13 days. The percentage of hydrolysis was found by using the anthrone reagent (Van Handel, 1965) for total carbohydrate concentration, neocuproine for reducing power determination (Dygert *et al.*, 1965). Glucose oxidase was used for glucose determination (Dahlqvst, 1961). Proton NMR spectra were run in D_2O at 85°C with either a Brüker CXP-300 or Brüker AM500 spectrometer operating in the Fourier-transform mode at 300 MHz and 500 MHz respectively. External acetone (2·2 ppm) was the reference.

RESULTS AND DISCUSSION

S1 Dextrans

In previous work the authors showed that S1 dextrans are hydrolysed to a limited extent (≈ 45%) by *endo*dextranases (Taylor *et al.*, 1990), indicating a high degree of branching. This was readily confirmed by methylation analysis and proton NMR. Methylation analysis of S1 dextrans yielded a range of 22–29% branching (Table 1), based on tetramethyl glucose, and from 31–39% (average 35) based on dimethyl glucose.

TABLE 1
Analytical Data for S1 Dextrans, and Growth Conditions for the Production of their Respective GTF-S1 Enzymes

Code	*Strain*[a]	*Per cent branching (methylation*[b]*)*	*Per cent branching (NMR)*	*Per cent -(1→3)-α-glucanase hydrolysis*[c]	*Dilution rate* (h^{-1})	*Tween 80 (0·5%)*
SD7	201	25(36)	30	4·4	0·05	+
SD9	27	25(34)	30	0·8	0·075	—
SD17	27	22(31)	27	7·7	0·075	+
SD20	K1-R	29(39)	34	—	0·075	+
SD21	K1-R	27(35)	28	—	0·075	+

[a] *S. sobrinus* strains 6715-13-201, 6715-13-27, and K1-R.
[b] Based on the amount of tetramethyl glucose (dimethyl glucose values are shown in brackets).
[c] Expressed as apparent conversion to D-glucose.
+, Tween present; —, Tween not present.

These values could not be improved, either by repeated use of methylating reagents on the one sample, or by repeating the complete methylation procedure on a fresh sample. Experiments to check possible loss of the volatile tetramethyl glucose (using a pure crystalline standard) proved negative. Response factors (effective carbon response) for partially methylated alditol acetates were used (Sweet *et al.*, 1975). For the same S1 dextrans, integration of the NMR peaks due to the anomeric protons yielded α-1→3-branching between 27 and 34% (Table 1). The integrals for the -(1→6)-α- and -(1→3)-α- anomeric protons were used (Pasika & Cragg, 1963; Meyer *et al.*, 1978). Comparison of methylation and NMR estimates of the degree of branching shows that methylation underestimates branching for these highly-branched polysaccharides by ≈ 5 percentage points, based on the amount of tetramethyl glucose, assuming the NMR method to give the more accurate result.

The authors have already concluded that essentially all the S1 side chains are single glucosyl stubs. This was deduced from the structures of a series of oligosaccharides released by *endo*dextranase activity on S1 dextrans (Taylor *et al.*, 1990). It was also confirmed by NMR analysis. Figure 2(a) shows the resonances for the anomeric protons of SD9, the

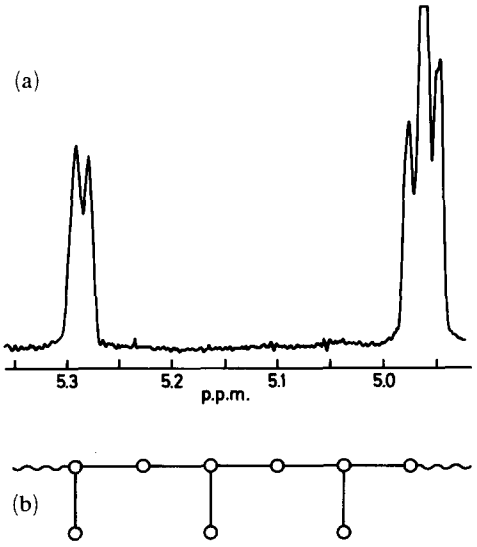

Fig. 2. (a) The anomeric proton resonances of the 500 mHz NMR spectrum of an S1 type glucan (SD9) synthesized by *S. sobrinus* strain 6715-13-201. The low field doublet is attributed to single D-glucopyranosyl units linked -(1→3)-α- to the main chain. The higher field resonances in SD9 arise from the α-1→6-linked regions of the main chain, the complexity arising from neighbouring group effects. (b) Idealized structure for an S1 type glucan, which accounts for the spectrum in (a).

doublet at ≈ 5·3 ppm being due to the -(1→3)-α-linked units. The fact that they are doublets is a reflection that essentially a single type of -(1→3)-α-linked residue is present, and that these residues are, for the most part, evenly spaced along the backbone chain. This leads to the idealised structure shown in Fig. 2(b). For significant amounts of other side chain distributions, one would expect to see a more complex pattern in both -(1→3)-α- and -(1→6)-α-linked regions (Meyer *et al.*, 1978) due to neighbouring group effects. The (1→3)-α-linked anomeric region of dextran NRRL-B1351, which has been shown to have single glucose residue side chains (Taylor *et al.*, 1985) for example, also shows only a simple doublet (Cheetham *et al.*, 1990). Despite the similar degrees of branching exhibited by the S1 polysaccharides, Table 1 shows that SD7, SD9 and SD17 dextrans are attacked to different extents by the *endo*-(1→3)-α-linked-D-glucanase from *Cladosporium resinae*.

The degree of hydrolysis of S1 dextrans by -(1→3)-α-glucanase was greatest on those polysaccharides from organisms grown in the presence of Tween 80 (Table 1) though the data are limited. Thus the S1 polymers from Tween 80 conditions (SD7 and SD17) appear to have a very small percentage of -(1→3)-α-linked sequences as side chains, rather than in the main chain. The reasoning for this conclusion is as follows:

Gel permeation chromatography of the S1 dextrans yielded profiles typified by SD7. A minor peak eluting near the column void volume was attributed to all species larger than the column pore size. The majority of material emerged as a broad peak whose maximum lay between ≈ 50000 and 100000 Daltons, indicating considerable size polydispersity.

High and low molecular weight fractions of SD7 eluted from the column were collected and subjected to methylation and NMR analysis. The two fractions yielded virtually identical results by each technique, indicating structural homogeneity. Gel permeation chromatography profiles of the S1 polysaccharides before and after hydrolysis by the *endo*-(1→3)-α-glucanase were also very similar, reflecting the small amount of enzymic hydrolysis shown in Table 1. Had the -(1→3)-α-linked sequences been in the main chain, one would expect a considerable reduction in molecular weight on hydrolysis by the -(1→3)-α-glucanase, and this was not evident.

As will be shown for S4 dextrans, significant regions of -(1→3)-α-linked sequences give rise to quite complex resonance patterns, while disaccharide side chains of the type D-glc-(1→6)-α-D-glc-(1→3)-α- in the presence of single -(1→3)-α-glucosyl stubs, yield a triplet in the -(1→3)-α-anomeric region of the NMR spectrum (Cheetham *et al.*, 1990). Such disaccharide side chains also give rise to a different oligosaccharide

series when the polysaccharide is treated with an *endo*dextranase (Taylor *et al.*, 1985). It can be concluded, therefore, that most of the branch-points are single-(1→3)-α-D-glucose stubs in SD7, SD9 and other S1 dextrans. A typical structure for an S1 dextran based on the above evidence is shown in Fig. 3 region (a), i.e. alternating branched and unsubstituted backbone glucosyl units, predominate. As on average, there is one -(1→3)-α-D-glucose (branching) residue for each unsubstituted glucose residue (region a) then for two such adjacent, substituted main chain residues (region b), there must be on average two unsubstituted residues occurring elsewhere (region c, Fig. 3), and so on for three residues etc. The possibility that some extended -(1→3)-α-linked side chains (region d, Fig. 3), could have been added by some contaminating GTF-I was considered, as in the presence of Tween 80, GTF-I levels are very high. However it is most unlikely, as the GTF-S1 and GTF-I peaks on hydroxyapatite are well separated (Fig. 1).

Figure 3 also shows the origin of the branched oligosaccharides which results from the hydrolysis of S1 dextrans by *endo*dextranases. The detailed discussion of the formation of these oligosaccharides can be found in the authors' previous paper (Taylor *et al.*, 1990).

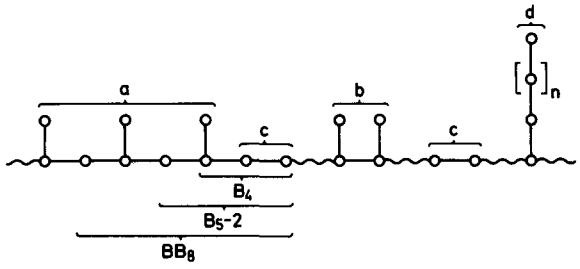

Fig. 3. Proposed structure for an S1-type dextran:

(i) o—o represents -(1→6)-α-linked-D-glucosyl units.

(ii)  represents -(1→3)-α-linked-D-glucosyl units.

Most of the molecule consists of single glucosyl side chains on alternate main chain glucosyl residues (region a). Some unsubstituted regions of the main chain (region c) must occur to account for the (limited) attack of endodextranases. Regions of fully substituted main chain units must occur (region b) to allow for region c and still account for the observed degree of branching (≈ 30%). Small amounts of -(1→3)-α-linked sequences (region d) are present in most S1 types (see text). The B_4, B_5-2 and BB_8 are branched oligosaccharides shown to arise from endodextranase digestion of S1 dextrans (Taylor *et al.*, 1990).

S3 Dextrans

S3 dextrans are hydrolysed by *endo*dextranase to the same extent as that from the well-known *L. mesenteroides* strain NRRL-B512(F). Methylation analysis revealed negligible branching. Some tetramethyl glucose from the non-reducing end of the small main chain was detected. The proton NMR also revealed the free anomeric resonances from the reducing end of the main chain. Comparison of integrals of the anomeric signals from the reducing end unit with the total anomeric signals yielded a DP of 60, i.e. an $M_n \approx 10000$ Daltons. Gel permeation chromatography confirmed that the molecular weight was small, in the region 10–12 thousand Daltons. Thus S3 dextrans are small linear polymers.

S4 Dextrans

All S4 dextrans examined were of high molecular weight (>400000 Daltons) most of the material eluting as a peak near the GPC column void volume. Table 2 shows analytical data for six S4 dextrans, the first three of which are from strains requiring the presence of Tween 80 for GTF-S4 activity to be expressed. These polysaccharides show a marked difference between the degree of branching as determined by methylation analysis, and that determined by NMR e.g. SD5 yields 10% branching from methylation analysis and apparently 17·7% from NMR integrals. This is despite good agreement between amounts of tetramethyl and dimethyl glucose. The reason for the difference becomes clear when one examines the anomeric -(1→3)-α-linked region of the NMR spectrum of SD5 before (Fig. 4(a)) and after (Fig. 4(b)) -(1→3)-α-glucanase digestion. Some of the lower field overlapping resonances disappear as a result of the enzymic treatment, showing that they were derived from -(1→3)-α-linked sequences.

The middle doublet is resolved after enzyme digestion, and is assigned to short (perhaps two glucosyl units) -(1→3)-α-sequences from the inner portion of the longer sequences. These inner portions would be present both before and after enzyme digestion, as the -(1→3)-α-glucanase cannot remove all residues close to the branch point. The higher field doublet is essentially unchanged, and arises from single -(1→3)-α-linked-D-glucosyl stubs. The authors have shown (Cheetham *et al.*, 1990), that single -(1→3)-α-linked-D-glucosyl stubs can be distinguished from two-unit glucosyl side chains by proton NMR in the anomeric regions. The structural origins of signals in the anomeric -(1→3)-α-linked regions of SD5 are proposed in Fig. 4. Methylation analysis (Table

TABLE 2
Analytical Data for S4 Dextrans, and Some Growth Conditions for their Respective GTF-S4 Enzymes

Code strain		*Per cent branching methylation*		*NMR*[c]		*Change in trimethyl glucose*[d]	*Per cent -(1→3)-α-glucanase digestion*	*Dilution rate (h^{-1})*	*Tween 80*
SD4	201	8[a]	11[b]	16·6[a]	13·2[b]	13(4)	15·7	0·05	+
SD5	K1-R	10	14	17·7	14·7	13(4)	14·7	0·45	+
SD6	201	11	13	14·9	14·5	15(3)	14·3	0·45	+
SD10	27	10	13	8·0	8·8	8(2)	9·1	0·075	—
SD15	27	8	9	8·0	8·8	3(2)	5·9	0·42	—
SD16	27	8	—	10·7	—	—	4·3	0·42	—

[a]Parent, i.e. before -(1→3)-α-glucanase digestion.
[b]After -(1→3)-α-glucanase digestion.
[c]Apparent branching, as resonances from -(1→3)-α-linked sequences cannot be quantitatively distinguished from -(1→3)-α-linked branch points.
[d]Values for molar per cent of 2,4,6-tri-O-methyl-D-glucose from methylation analysis before and after (brackets) digestion with -(1→3)-α-glucanase.
+, Tween present; —, Tween not present.

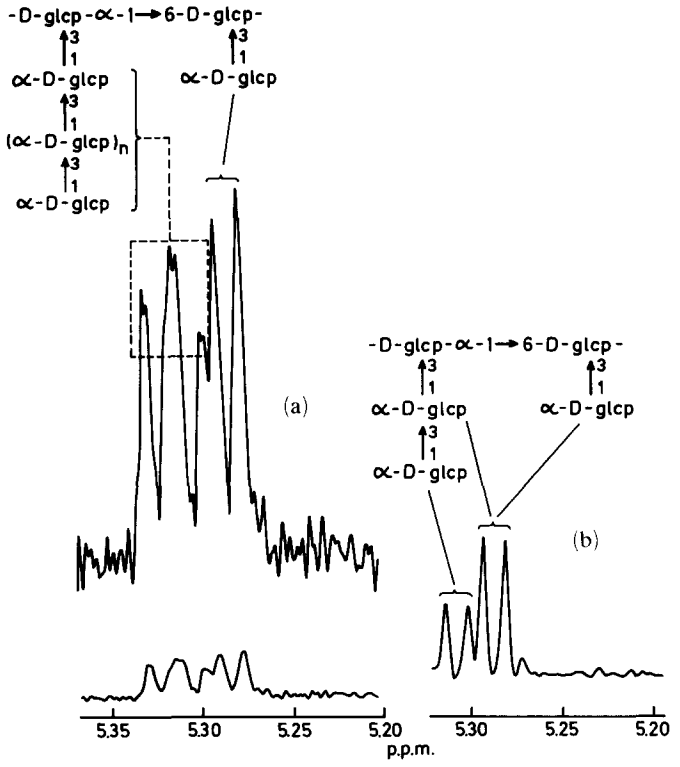

Fig. 4. The anomeric proton resonances at 500 mHz of the S4-type dextran SD5: (a) before a -(1→3)-α-glucanase digestion; (b) after a -(1→3)-α-glucanase digestion. The structural origins of the resonances are also shown:

(i) o—o represents -(1→6)-α-linked-D-glucosyl residues.

(ii)  represents -(1→3)-α-linked-D-glucosyl residues.

2) before -(1→3)-α-D-glucanase digestion showed substantial amounts of 2,4,6-tri-*O*-methyl-D-glucose to be present, which also indicates -(1→3)-α-linked sequences. After -(1→3)-α-glucanase digestion the amount of the trimethyl glucose in the modified polysaccharide was greatly reduced (Table 2) and methylation analysis now agrees quite well with the NMR result. The SD15 shows the lowest amount of -(1→3)-α-glucosidic linkages. Gel permeation chromatography of digested samples revealed little change if any, in the retention time (9·5 min) of the major, high molecular weight peak. Small amounts of much lower molecular weight material (17·0 min) and buffer salts (19·2 min, approximately, total permeation) were present. Overall, the gel permeation showed that

the -(1→3)-α-linked sequences were attached as side chains, rather than inserted in the main chain, and the methylation analysis of the enzyme-digested polysaccharides confirmed this (Table 2). A structure consistent with the analytical results from SD4, SD5 and SD6 is presented in Fig. 5. The three S4 dextrans SD10, SD15 and SD16 were produced by GTF-S4 enzymes of the mutant strain 6715-13-27 grown in the absence of Tween 80 (Table 2). The extent of branching shown by methylation and by NMR analysis were similar. They were digested to a lower extent than SD3, SD5 and SD6 by -(1→3)-α-glucanase, and methylation analysis (Table 2) revealed lower amounts of 2,4,6-tri-*O*-methylglucose. Gel permeation profiles before and after -(1→3)-α-glucanase activity showed similar behaviour to the other S4 types, i.e. no discernible decrease in molecular weight occurred. The NMR patterns in the -(1→3)-α-linked anomeric regions show a smaller proportion of signals (relative to those of SD4, SD5 (Fig. 4) and SD6) due to -(1→3)-α-linked sequences (Fig. 6(a)) and these decrease further after digestion with -(1→3)-α-glucanase (Fig. 6(b)). The mutant 6715-13-27 thus produces S4 dextrans similar in size and structure to those from the other strains examined, but with fewer and/or shorter -(1→3)-α-linked side chains. It was thought possible that the 1→3-linked sequences in S4 dextrans from strains K1-R and 6715-13-201 arise from some contaminating GTF-I. This enzyme can add -(1→3)-α-linked glucosyl sequences as side chains to a -(1→6)-α-linked-D-glucosyl backbone. The GTF-I elutes immediately before the GTF-S4 peak, and in the presence of Tween 80 is present in high amounts (Walker *et al.*, 1989). However we believe that the GTF-S4 preparations are free of GTF-I activity. GTF-S4 enzymes were isolated from fractions eluting close to the GTF-I peak, and from fractions well removed from it. The respective S4 polysaccharides were of virtually

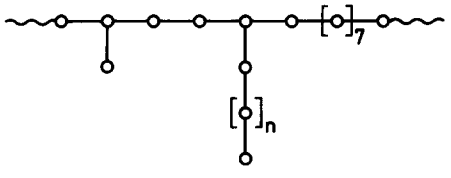

Fig. 5. Structural features of an S4-type dextran, SD5:

(i) o—o represents -(1→6)-α-linked-D-glucosyl residues.

(ii)  represents -(1→3)-α-linked-D-glucosyl residues, n ≈ 3–4.

The frequency of each glucosyl type is as shown, for each of the 20 glucose residues.

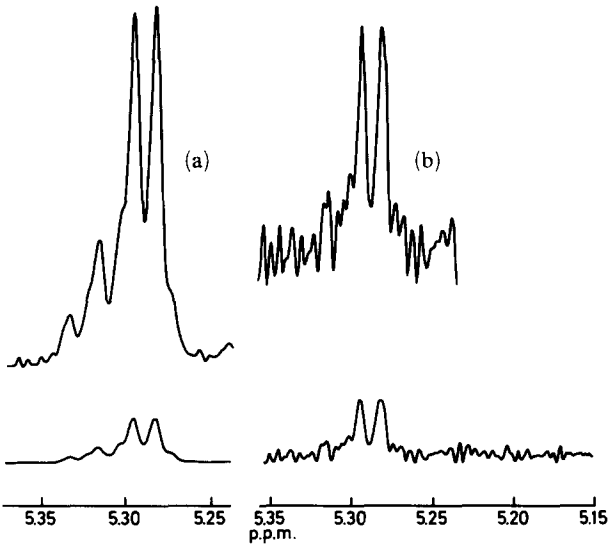

Fig. 6. The anomeric proton resonances at 500 mHz of the S4-type dextran SD15: (a) before -(1 → 3)-α-glucanase digestion; (b) after -(1 → 3)-α-glucanase digestion.

identical structure when examined by both methylation and NMR analysis.

The GTF-S4 from mutant 27 is even less likely to be contaminated by GTF-I, as the latter enzyme is present in such low amounts (Fig. 1), and an extra chromatographic step (see 'Experimental') was employed.

It would therefore appear that GTF-S4 enzymes are capable of synthesizing -(1 → 6)-α-linked-D-glucose backbones, to which they attach single glucosyl units and also -(1 → 3)-α-linked sequences.

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