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STRUCTURAL CHARACTERISTICS OF NATIVE AND ENZYMICALLY FORMED DEXTRAN OF *S. sanguis* ATCC 10558

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ABSTRACT

The structure of the native dextran produced by *Streptococcus sanguis* ATCC 10558 was analyzed by g.l.c.–m.s. of the methylated alditol acetates derived from the polymer. The results indicate that the polymer contains D-glucosyl residues substituted at C-6 or C-3, or both, as well as unsubstituted D-glucosyl residues. These data are correlated with the proportion of C-6 and non-C-6 substitution as measured by p.m.r. Similar data are reported for the analysis performed on a dextran produced by action of a partially purified dextransucrase on sucrose. The proportion of D-glucosyl residues substituted at C-3 is diminished in this case. It is concluded that several enzymes are involved in the dextran synthesis.

INTRODUCTION

Dextrans are homopolysaccharides of D-glucose having as principal linkage[1] α-(1→6). These polymers have been shown to be produced by a spectrum of plants and bacteria, and their structures have been examined with an array of analytical techniques. Interest in these polymers has been stimulated by the observation that they are present as a component of dental plaque[2–4]. A study has been initiated on the dextran produced by *Streptococcus sanguis* ATCC 10558, which is one of the common oral bacteria and which can be considered to be a model for the carriogenic organisms, in conjunction with a study of a partially purified dextransucrase (sucrose:(1→4)-α-D-glucan:4-D-fructose-α-D-glucosyl-transferase, EC 2.4.1.5), which catalyzes the formation of dextran from sucrose. The results of mass-spectral analysis of the methylated alditol acetates derived from dextran samples, performed as described by Björndal *et al.*[5], were correlated with the p.m.r. spectra of these samples.

RESULTS AND DISCUSSION

Native polysaccharide. — The polysaccharide was isolated and purified from different growths of the organism on sucrose. All the preparations were poorly soluble in water and in dimethyl sulfoxide, and, in general, irradiation in a sonic bath

was required for solution in dimethyl sulfoxide, whereas blending was sufficient for water solution. Approximately 85% of the total dry mass could be accounted for as hexose as determined by anthrone[6]. Paper chromatographic analysis of acid hydrolyzates indicated that glucose was the only sugar present. Treatment of acid hydrolyzates with D-glucose oxidase converted the D-glucose into D-gluconic acid, which was removed by the addition of Dowex-1 (CO_3^{2-}) ion-exchange resin. When a large sample of the treated hydrolyzate was chromatographed, traces of D-fructose were detected.

Samples of polysaccharide were methylated according to the Sanford and Conrad[7] modification of the Hakamori[8] procedure. In one instance, two methylations were required to achieve complete methylation. Recovery of the methylated polysaccharide ranged between 78 and 104% of the theoretical value. The samples were hydrolyzed according to Lindberg[9] to yield the methylated aldoses, which were reduced with sodium borohydride to give the corresponding methylated alditols. These derivatives were acetylated with acetic anhydride–pyridine to yield the methylated alditol acetates. Recovery of these derivatives ranged bteween 50 and 107% of the theoretical value. The samples were then analyzed by g.l.c.–m.s. as described by Björndal *et al.*[5] on a column of OV-225 as the stationary phase.

The results of the analysis on four different samples of dextran are shown in Table I. The data indicate that complete methylation of the polysaccharide occurred, since incomplete methylation would have resulted in the appearance of a mixture of mono- and di-methyl derivatives. The data in Table I show a high degree of agreement for different preparations of the polysaccharide. Also, duplicate analyses of the

TABLE I

ANALYSIS[a] OF THE PRODUCTS OF METHYLATION

Sample	O-*Methyl*-D-*glucose*			
	2,3,4,6-[b]	*2,4-*[c]	*2,4,6-*[d]	*2,3,4-*[e]
1	15.7	14.8	15.6	53.9
1	16.3	16.9	14.1	52.7
2	17.2	18.1	12.8	51.9
3	15.7	14.6	12.1	57.6
4	16.2	16.5	13.5	53.8
Average	16.2±0.5	16.2±1.3	13.6±1.2	54.0±2.0

[a]The entire procedure was effected in duplicate on sample 1. The results are expressed in mol %. The average is given along with the standard deviation of the mean. [b]Terminal residues. [c]Branched residues. [d]Linkage (1→3). [e]Linkage (1→6).

same polysaccharide preparation indicate that the procedures involved are highly reproducible. Correspondence can be seen between the proportion of the 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (nonreducing termini) and 1,3,5,6-tetra-*O*-acetyl-di-*O*-methyl-D-glucitol (branch points), since each of these residues account

for an average of 16 mol % of the total polymer. The presence of the 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl derivative indicates that (1→3) linkages occur in the linear portion of the chain as well as at branch points. Ceska *et al.*[10] have also reported the presence of linear (1→3) bonds in studies on dextrans produced by glycosyl transferases obtained from oral bacteria. The major portion of the D-glucosyl residues in the polymer are substituted at C-6 and are represented by the 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl derivative.

Sidebotham *et al.*[11] have examined by periodate oxidation the structure of dextrans isolated from the culture fluids of a series of oral bacteria. On the basis of periodate consumed and formic acid produced, they estimated the percentage of (1→6), "(1→3)-like", and "(1→2)-like" linkages, and concluded that the dextran from *S. sanguis* 10558 consisted of 68% of (1→6)-linked D-glucosyl residues, whereas the percentage of "(1→3)-like" was only 9%. Our data indicate that a total of 30% of the D-glucosyl residues are substituted at C-3 (the sum of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl- and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl derivatives) and would be periodate-resistant residues. Thus, it is clear that the results obtained by periodate oxidation and by methylation are significantly different. However, Sidebotham *et al.*[11] observed some discrepancies in their own data and warn that the results of periodate oxidation alone must be interpreted cautiously.

The proportion of (1→6) linkages and non-(1→6) linkages can also be estimated by p.m.r. spectrometry. Pasika and Cragg[12] have assigned the peaks at τ 5.03 and 4.60 to the anomeric protons of (1→6)-linked residues and to ring positions, respectively. From the relative areas of these peaks obtained by integration, we have calculated the proportion of non-(1→6)linkages in three of the samples analyzed by methylation. These data, shown in Table II, indicate that approximately 25% of the residues appear to be substituted at positions other than C-6 as compared to ~30% of non-(1→6) linkages calculated from the methylation data in Table I. The integration of the p.m.r. spectra is rendered difficult by the width of the peaks observed. The line-broadening is most likely due to the extreme viscosity of the samples.

TABLE II

PROPORTION (%) OF NON-(1→6)-LINKAGES AS DETERMINED BY P.M.R.

Sample	*Non-(1→6)-linkage*	
	Observed[a]	*Estimated*[b]
2	25.6	30.9
3	22.8	26.7
4	24.3	30.0

[a]The proportions of non-(1→6)-linkages observed were calculated from the integrated peaks at τ 5.03 and 4.60. Samples were analyzed in deuterium oxide solution (*c* ~5.0). [b]The proportions of non-(1→6)-linkage estimated were derived from the results shown in Table I and represent the sum of the mol % of the 2,4-di-*O*-methyl-D-glucose and 2,4,6-tri-*O*-methyl-D-glucose derivatives.

These data suggest that the dextran produced by *S. sanguis* 10558 is a branched structure. Since 16% of the residues are at branch-points of the polymer, the average branch-length is ~6 D-glucosyl residues. However, no information concerning the fine structure of the polymer is currently available. It is possible that the polymer is essentially linear with alternate short branches extending from it. This type of structure has been proposed for the dextrans isolated from *Leuconostoc mesenteroidies* B-1397 (Ref. 13) and B-512 (Ref. 14). It is equally possible that the structure of the dextran of *S. sanguis* 10558 is analogous to that of glycogen.

Dextran produced by cell-free dextran sucrase. — This study was undertaken to ascertain whether the enzymic system is comprised of a single activity catalyzing the formation of both α-D-(1→3) and α-D-(1→6) linkages, or whether it is a complex of enzymes. If the dextran synthesis requires two or more enzymes, the structural properties of the dextran synthesized by purified-enzyme preparations should differ from those of the native polymer, assuming that the purification procedure separates the enzymic activities. The enzyme was obtained from cultures supplemented with D-glucose, and purified by chromatography on hydroxylapatite similar to that described by Carlsson *et al.*[15]. This represents a more than 150-fold purification of the crude enzyme to give specific activity of 5.8 units per mg of protein. The enzyme was used to synthesize dextran in a reaction mixture containing sucrose and phosphate buffer at pH 6, and the resulting dextran was subjected to methylation. The results of g.l.c.–m.s. analysis shown in Table III indicate that the same four methylated alditol acetates found in the study of the native dextran were obtained. The significant feature of the data is that the proportion of linear (1→3)linkages decreased from 13 to 6.5%. Since this difference is well outside the standard deviation (Table I), this decrease is significant and indicates an altered ability of the enzyme preparation to form linear (1→3) linkages; consequently, dextran sucrase is a complex of enzymes.

TABLE III
COMPARISON OF NATIVE AND CELL-FREE DEXTRAN[a]

Sample	O-*Methyl*-D-*glucose*			
	2,3,4,6-[b]	*2,4-*[b]	*2,4,6-*[b]	*2,3,4-*[b]
Native	16.2	16.2	13.6	54.0
Cell-free	17.4	18.7	6.7	57.2

[a]The proportions of *O*-methyl-D-glucoses are expressed in mol %. [b]See footnotes of Table I.

A similar suggestion has been made by Bailey *et al.*[16] who observed that the structural properties of dextran synthesized by frozen and unfrozen enzyme preparations are different, and proposed that one of the enzymes was sensitive to freezing and thawing. Ceska *et al.*[10] have studied the dextrans produced by enzymes from *S. sanguis* and *S. mutans* at several stages of purification. They also observed the change of some structural characteristics of the dextrans produced by these preparations

upon purification. In addition, they observed that purification of the *S. sanguis* enzyme resulted in a decrease in the proportion of (1→6) and an increase in the proportion of (1→3) linkages. This is in disagreement to our observations, since we have found a decrease in the proportion of (1→3) linkages. The reasons for this discrepancy are not clear. However, it appears that the enzymes obtained by Ceska *et al.*[10] in the fractionation differ in specificity from those observed in the present study. Fukui *et al.*[17] have reported a purification of dextran sucrase from *S. mutans* HS-6, and have indicated that the enzyme is homogeneous on disc-gel electrophoresis. The product formed by the purified enzyme was shown, by periodate oxidation, to contain more than 90% of α-(1→6) linkages. Other reports have indicated that multiple forms of dextran sucrase exist[18–20]; however, the nature of these forms has not been characterized, and it is not clear whether these enzymes are isozymes or dextran sucrases with different specificities.

The information available concerning the nature of the enzymes involved in dextran formation is inadequate. It is uncertain whether two or more dextran sucrases function, each one having a specificity for either (1→6) or (1→3) linkages, or whether there is one dextran sucrase that catalyzes the formation of one type of linkage, and a transglucosylase that catalyzes an intramolecular rearrangement to form the other type of linkage. This latter mechanism would be analogous to that observed in the synthesis of glycogen.

EXPERIMENTAL

Preparation of native dextran. — Cultures of brain heart infusion (BHI, 5 l each) supplemented with 1% sucrose were innoculated with BHI cultures (500 ml) of *Streptococcus sanguis* ATCC 10558. The cultures were maintained at 30° with light magnetic-stirring. The pH was maintained between 6.5 and 7.0 by addition of 2.5M sodium hydroxide. Growth was continued until acid production ceased and until the increase in turbidity, as measured by absorbance at 660 nm, stopped. The cells were harvested by centrifugation in a continuous-flow centrifuge at 4°. The supernatant fluid was adjusted to pH ~5 with glacial acetic acid, and the polysaccharide precipitated by the addition of ethanol (2 vol.) and potassium acetate (5 g). The mixture was kept overnight at 4°, the supernatant fluid siphoned off, and the precipitate collected by centrifugation. The polysaccharide was dissolved in water (300–400 ml) and buffered to pH 8 with 2M Tris. Pronase (5 mg, Sigma Chem. Co., St. Louis, Mo. 63178) was added with Chloramphenicol (1 mg), and the solution was maintained at 30° overnight in order to digest the protein. The polysaccharide was precipitated with ethanol at pH 5 and collected. The polysaccharide was dissolved and precipitated two more times, the final precipitate being dissolved in water (100–150 ml) and the solution lyophilized. Yields ranged between 1.5 g and 2.5 g for a 5-liter culture. Following acid hydrolysis (M hydrochloric acid for 90 min at 100°) of these preparations and paper chromatography of the products in 1-butanol–acetic acid–water (12:3:5, v/v) D-glucose was the only sugar observed.

Preparation of enzymically-formed dextran. — Dextran was prepared in a cell-free system from sucrose by action of dextran sucrase. The enzyme had been partially purified from the culture fluid of *S. sanguis* 10558, grown in BHI supplemented with 1% glucose. The enzyme was purified by adsorption on hydroxylapatite and eluted with increasing concentrations of potassium phosphate buffer, as described by Carlsson *et al.*[15].

The reaction mixture contained sucrose (500 μmol), phosphate buffer (500 μmol), Chloramphenicol (1 mg), and dextran sucrase (0.84 units, 5.8 units/mg) in a final volume of 5 ml. The reaction was monitored by measuring the formation of D-fructose from sucrose, and was terminated after 80 h, at which time 340 μmol of fructose had been released. The polysaccharide was isolated by repeated precipitation with ethanol and dialyzed against distilled water. It was further purified by repeated precipitation with ethanol, and lyophilized. The final yield was 53.2 mg and was equivalent to 327 μmol of D-glucose.

Methylation. — Dextran samples (10–100 mg) were dried *in vacuo* overnight at 40° in an Abderhalden pistol over phosphorus pentaoxide and suspended in anhydrous dimethyl sulfoxide. The native polysaccharides generally were difficult to dissolve and required irradiation in a sonic bath and warming to 40°. The dextran solution was added to 2M methylsulfinyl sodium in dimethyl sulfoxide[7,8] (20 ml) and stirred for 6 h at room temperature. The reaction mixture was cooled in an ice-bath, and methyl iodide (3 ml) was added dropwise with stirring, while the temperature was maintained between 20 and 25°. The mixture was stirred at room temperature for 30 min, poured into water (100 ml), and dialyzed overnight against running tap-water. After concentration, the methylated polysaccharides were extracted with chloroform.

The methylated product was hydrolyzed with 90% formic acid (3 ml) for 2 h at 100° and after removal of the formic acid in a rotary evaporator, hydrolysis was continued for 12 h with 0.25M sulfuric acid at 100° in a sealed tube[9] to give the methylated monosaccharides. Sulfate ions were removed as barium sulfate after neutralization with barium carbonate. The products were then treated for 2 h with an excess of sodium borohydride (100 mg) at room temperature to yield the methylated alditols. Dowex-50 (H^+) ion-exchange resin was added and the mixture filtered. The filtrate was evaporated to dryness and the borate ions were removed as methyl borate by repeated additions of methanol and M hydrochloric acid (1 drop), followed by evaporation to dryness. The methylated alditols were acetylated with acetic anhydride–pyridine (10 ml, 1:1, v/v) for 10 min at 100°. The solvent mixture was evaporated to dryness by evaporation after addition of toluene (5 ml).

The residues were analyzed by g.l.c.–m.s.[5] on columns (1.8 m) of 1.5% OV-225 (Applied Science Labs., Inc., State College, Pa. 16807) with a Perkin–Elmer model 990 gas chromatograph interfaced with a Dupont CEC-490 mass spectrometer, at a temperature program between 140 and 210°. The mol % was calculated for each sugar derivative by integrating the area under the gas-chromatogram curves, and correcting for the molecular weight.

Analytical procedures. — The total amount of hexose was determined by the

anthrone procedure[6]. D-Fructose was measured enzymically in a reaction mixture containing hexokinase (0.2 unit), phosphoglucose isomerase (0.2 unit), glucose 6-phosphate dehydrogenase (0.2 unit), ATP (0.2 μmol), NADP (0.2 μmol), and Tris buffer (pH 7.0, 50 μmol) in a volume of 1 ml. After 20 min, the absorbance at 340 nm was determined. P.m.r. spectra were recorded with a Brucker 90-MHz spectrometer by Fourier transform.

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