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Structural and Enzymatic Studies on Glucans Synthesized with Glucosyltransferases of Some Strains of Oral Streptococci

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Water insoluble glucans, synthesized by different glucosyltransferases produced by cariogenic strains of *Streptococcus mutans* and *Streptococcus sanguis* have been investigated by chemical and physical methods. They are essentially α-linked and contain different proportions of terminal, 3-, 6-, and 3,6-linked glucopyranose residues. The $\overline{M}_w$ of the glucans vary within wide limits. The extent of hydrolysis with dextranase decreases with the content of 1→3 linkages.

It has been indicated by many authors that certain oral microorganisms are required for the initiation of carious lesions on the smooth surfaces of teeth, and that specific streptococci have the ability to synthesize extracellular polysaccharides of the dextran and levan type. The enzyme dextransucrase (α-1,6-glucan:D-fructose 2-glucosyltransferase, EC 2.4.1.5) is known to transfer a glucosyl moiety from sucrose to the growing chain of the glucose polymer, dextran.[1] Levan is synthesized by the enzyme levansucrase (β-2,6-fructan:D-glucose 6-fructosyltransferase, EC 2.4.1.10) which requires a fructofuranosidic group linked to the anomeric carbon of an aldosyl group as the fructosyl donor. Extracellular polysaccharides, such as those mentioned above, are thought to be important in enabling cariogenic organisms to colonize on smooth surfaces of the teeth, and function also as a storage of fermentable carbohydrate for continuous acid production.[2] Extracellular polysaccharides have been synthesized *in vitro*, using extracellular enzymes of caries-inducing streptococci of the species *sanguis* and *mutans*.[3–5] There are numerous reports concerning the hydrolysis of extracellular bacterial polysaccharides both *in vitro* and *in vivo* by different dextranase preparations.[6–11] The various dextranases used for this purpose were specific for α(1→6) linkages. However, polysaccharides synthesized *in vitro*, as well as the polysaccharides accumulated in dental plaques may differ considerably in structure and may, as this report

will reveal, contain other linkages than α(1→6) in the main chain. Therefore the failure to find a significant reduction in caries using the dextranase with the abovementioned specificity is not surprising.[11]

This report describes the physical, chemical, and biochemical characterization of some glucans synthesized *in vitro*. The glucans were synthesized with glucosyltransferases contained in culture fluid of *Streptococcus sanguis* (strain 804)[3] and *Streptococcus mutans* OMZ 176 (HS strain).[4] The correlation between the chemical structure of various glucans and the extent to which they are hydrolyzed by dextranases of various origin is reported.

EXPERIMENTAL

Materials

Dextranases 1 and 2 were crude samples obtained by growing *Penicillium funiculosum* CBS 17060 in media containing α-glucan 1 and 2, respectively. Dextranase 3 (21 U/mg) was a commercial sample, purchased from Worthington Biochemical Corporation. Dextranase 4 was prepared from a culture supernatant of *P. lilacinum* NRRL 896 grown on dextran as previously described.[9] Dextranase 5 from a culture fluid of *P. funiculosum* NRRL 1768 grown on dextran [6] was a gift from Dr. R. J. Fitzgerald, National Institute of Dental Research, Bethesda, Maryland, USA. A culture of *P. funiculosum* CBS 17060 was obtained from Centralbureau voor Schimmelcultures, Baar, The Netherlands.

Dextran T 40 ($\overline{M}_w$ 4×10^6) and T 2000 ($\overline{M}_w$ 2×10^6) are products of Pharmacia Fine Chemicals, Uppsala, Sweden. α-Glucans 1, 2, and 3 were kindly supplied by Dr. B. Krasse, Department of Odontology, University of Göteborg, Sweden. They were prepared with glucosyltransferases obtained from a culture medium of *Streptococcus sanguis*, which was used in a crude state, after purification by chromatography on hydroxylapatite or after further purification by isoelectric focusing.[3] α-Glucans 4 and 5 were synthesized with purified glucosyltransferases (with isoelectric points 5.6 and 5.0, respectively) from *S. mutans* OMZ 176 medium as previously described.[4]

Methods

Production of dextranase. *P. funiculosum* CBS 17060 was grown in a medium containing 1000 ml dist. water, 0.25 g $MgSO_4.7H_2O$, 20 g of the appropriate dextran, 0.10 g $FeSO_4.7H_2O$, 1.6 g $NaNO_3$, 0.044 g $ZnSO_4.7H_2O$, 1.0 g $(NH_4)_2SO_4$, 0.020 g $MnSO_4.4H_2O$, 0.50 g KH_2PO_4, and 0.005 g $CuSO_4$. The culture was grown for 5 – 8 days in submerged culture with air bubbling through the medium. The molds were then filtered through a 33 μ nylon net and washed twice with small amounts of distilled water. The combined filtrate and washings were stored frozen and used as a source of crude dextranase.

Determination of dextranase activity. The activity of dextranase was determined by the DNS method.[12] For routine testings of dextranase activity, 1 % dextran solution (or 1 % α-glucan suspension) was used which was prepared in 0.1 M potassium phosphate buffer, pH 6.0, containing 0.02 % sodium azide. The glucan suspension was then incubated with dextranase containing material at 45°C. 200 μl aliquots were removed at 0, 3, 6, 10, and 15 min. The samples were cooled in tap water and 300 μl of DNS reagent (3,5-dinitrosalicylic acid) was added. This mixture was kept in a boiling water bath for 15 min, and was subsequently cooled to room temperature. The solution was then centrifuged and the extinction of the supernatant was determined in Zeiss PMQ 11 spectrophotometer at 640 nm. The equivalents, in maltose values, were obtained from a maltose standard curve. (Maltose was used as reference instead of isomaltose because it was more readily available.) In order to obtain arbitrary activity units, the amounts of reducing sugars, expressed as maltose values, were divided by the reaction time and the protein concentration of the enzyme solution, giving mg maltose equivalents per 10 min per mg of protein. The protein concentration was determined by determining total nitrogen on extensively dialysed samples, using the Micro-Kjeldahl method.[13]

Methylation analysis. The α-glucan (10 mg) was dissolved in dry methyl sulphoxide (3 ml) in a 5 ml serum bottle provided with a rubber cap. The bottle was flushed with nitrogen and 2 M methylsulphinyl sodium in methyl sulphoxide (1 ml) was added with a syringe. The gelatinous solution was agitated in an ultrasonic bath (40 kc/s) for 10 min and kept at room temperature for 1 h. The solution was cooled in an ice bath until crystallization of methyl sulphoxide took place when methyl iodide (0.25 ml) was added. The resulting solution was stored for about 1 h with occasional stirring, poured into water (50 ml), dialyzed overnight and evaporated to dryness. The fully methylated α-glucan was then dissolved in cold 72 % sulphuric acid (1 ml) and kept at room temperature for 1 h. Distilled water (8 ml) was added and the solution hydrolyzed on a water bath (100°C) for 4 h. The mixture was then cooled, neutralized with barium carbonate, filtered and reduced with sodium borohydride. The resulting partially methylated alditols were converted into the corresponding alditolacetates by acetylation with acetic anhydride–pyridine and analyzed by GLC–mass spectrometry.[14]

Table 1. The results of the methylation analysis of various glucans.

Methyl glucose ethers	Glucan mol % 1	2	3	4	5	Mode of linkage
2,3,4,6-Tetra-O-Me	8.1	12.9	14.9	5.0	2.9	Gp-(1→
2,3,4-Tri-O-Me	82.4	62.1	52.2	40.0	2.9	→6)-Gp-(1→
2,4,6-Tri-O-Me	2.4	10.8	17.7	49.5	94.2	→3)-Gp-(1→
2,4-Di-O-Me	7.1	14.2	15.1	5.4	–	→6)-Gp-(1→ →3)

Molecular weight and viscosity determinations. Molecular weight determinations were performed by light scattering measurements using the Sofia Photogoniodiffusometer. In the evaluation of molecular weight, the measured scattered intensities were extrapolated to zero concentration and zero angle.[15] As the glucans were almost insoluble in water and slowly became turbid in M potassium hydroxide, the measurements were made in cadoxen, an aqueous solution of cadmium oxide–ethylene diamine which is also a good cellulose solvent.[16] Cadoxen was prepared as described by Henley.[17] The glucans gave clear, viscous, and stable solutions in cadoxen.

The refractive index increment needed for the molecular weight calculations was determined by dialyzing the solutions against the solvent and measuring Δn for each concentration of dialysand–dialysate.[17] As limited amounts of glucans were available a soluble dextran produced by *Leuconostoc mesenteroides* strain B 512 was used. The value $dn/dc = 0.196$ ml/g at 436 nm was obtained for dextran in cadoxen.

Table 2. Molecular weights and intrinsic viscosities of various glucans.

Glucan	$\overline{M}_w$	$[\eta]_{20}$ dl/g
1	7.3×10^6	4.9
2	4.7×10^6	9.6
3	3.1×10^6	9.0
4	24 000	0.37
5	70 000	0.46

Solutions of the glucan in cadoxen – water, 1:1, were centrifuged in Dandliker cells [18] at 57 000 *g* for 2 h before measuring the light scattering in the same cells. 30 – 40 min were required before the readings became stable, probably because of temperature gradients in the viscous solutions. The readings were thereafter reproducible over a period of several days. The evaluated molecular weights are given in Table 2.

Intrinsic viscosities [η] were also determined in cadoxen – water 1:1. The results are shown in Table 2.

RESULTS

GLC of the alditolacetates [19] prepared from completely hydrolyzed glucans, revealed that glucose was the only sugar component. The glucans were fully methylated by the Hakomori procedure,[20] hydrolyzed, and the mixture of methylated sugars obtained analyzed as their alditolacetates by GLC – MS.[14] The results are summarized in Table 1. Another glucan sample prepared using a crude glucosyltransferase from the supernatant of a culture of *S. mutans* OMZ 176 gave the same methylation analysis as glucan 5. The optical rotation of glucans 1 and 5 in 1 M potassium hydroxide were $[\alpha]_D^{20}$ +228° and $[\alpha]_D^{20}$ +199°, respectively.

The molecular weights of the glucans 1 – 3 (Table 2) are low compared to those of dextrans elaborated by *Leuconostoc mesenteroides*.[21] The intrinsic viscosities are considerably higher than the corresponding values for 96 native dextrans studied.[22] Rees and Scott [23] have recently provided evidence that α(1→3) linkages in the main chain confer rigidity and hence high viscosity. This applies also to the samples 4 and 5 (*S. mutans*). These are extremely low in molecular weight, however, and thus hardly comparable to the three glucans from *S. sanguis*.

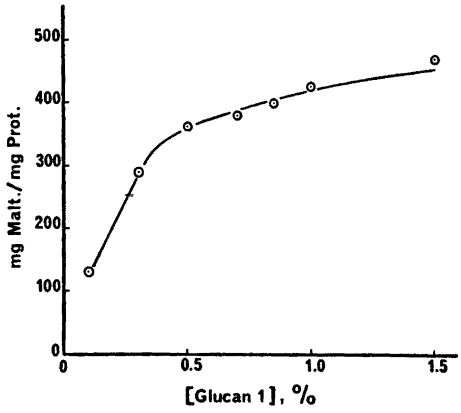

Fig. 1. Enzyme saturation curve with substrate. Glucan 1 was used as substrate The samples were hydrolyzed for 10 min at 45°C with dextranase 1.

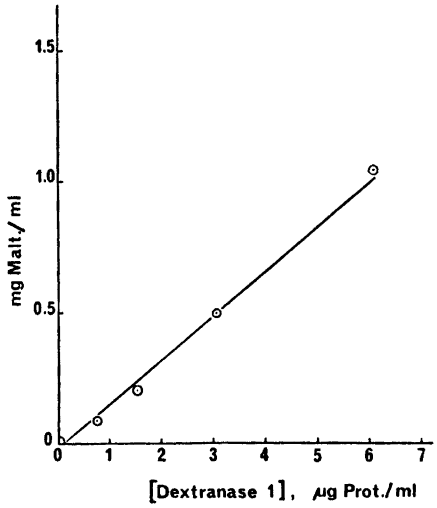

Fig. 2. The dependence of glucan 1 hydrolysis (mg maltose) on enzyme dextranase 1 concentration (expressed as µg protein/ml), for 10 min at 45°.

The enzymatic breakdown of the various glucans has been performed under substrate saturation conditions. Fig. 1 shows a Michaelis-Menten type of curve, using various concentrations of glucan 1 as the substrate and dextranase 1 as the enzyme. The reaction was run for 10 min. The saturation level of enzyme with substrate was achieved at about 1 % suspension of glucan 1. Similar results were also obtained with the other glucans, and therefore a substrate concentration of 1 % was used in all the following experiments. The hydrolysis of glucan 1 with dextranase 1 (expressed as mg maltose per ml of reaction mixture) is shown in Fig. 2. The hydrolysis of glucan 1 by dextranase 1 after different reaction times is seen in Fig. 3.

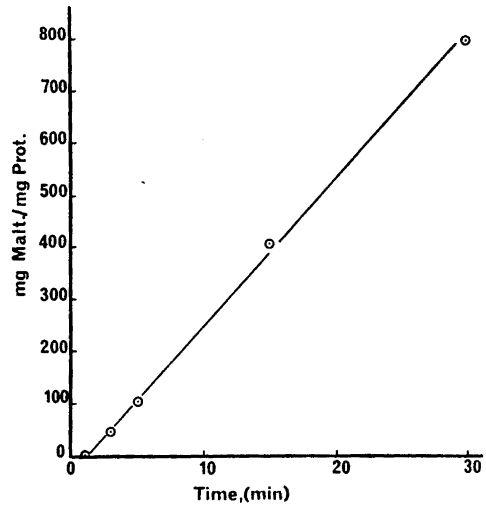

Fig. 3. The hydrolysis of glucan 1 with dextranase 1 at different reaction times and at 45°C.

All the different glucans were hydrolyzed under the conditions given above with dextranases from different sources. The 10 min hydrolysis values, recorded as mg maltose/mg protein, are given in Table 3. The reaction rates obtained for glucans must be regarded as semiquantitative, since they refer to substrates in suspension. It is seen that glucan 1, for instance, which is rather

Table 3. Hydrolysis values of the glucans by various dextranases.

Dextranase	Glucan 1	Glucan 2	Glucan 3	Glucan 4	Glucan 5	Dextran T 40
1	252	187	137	68	1	361
2	124	104	69	38	0	199
3	13	18	12	–	–	50
4	47	40	31	–	–	80
5	291	229	170	76	–	372

similar in structure to the soluble dextrans T 40 and T 2000, shows lower hydrolysis rates with all dextranases than the dextrans, probably due to the heterogeneous conditions.

The degradation rates of two dextrans of average molecular weights ($\overline{M}_w$ 4×10^4 and 2×10^6, respectively) by dextranase 1 are seen in Fig. 4. It can be observed that the hydrolysis decreases only slightly when the molecular weight is increased by two orders of magnitude. It is therefore concluded that the differences in molecular weights of the glucans 1 – 5 cannot account for the differences observed in their hydrolysis. From Tables 1 and 3 it is evident, however, that the degree of hydrolysis of the various glucans decreases with an increasing content of 1→3 linkages. Fig. 5 shows a plot of degradation rates of various glucans with respect to their content of all 1→3 linkages (non-(1→6)). Dextranase 1 was used for the hydrolysis. Dextran of $\overline{M}_w$ 2.0×10^6 was used as a control, as it contains a low percentage of 1→3 linkages, all of them branched residues. Analogous results were obtained with the other dextranases as shown in Table 3.

The growth of mold *P. funiculosum* on α-glucan 2, containing 10.8 % of 1→3 linkages in the main chain, did not seem to result in the production of

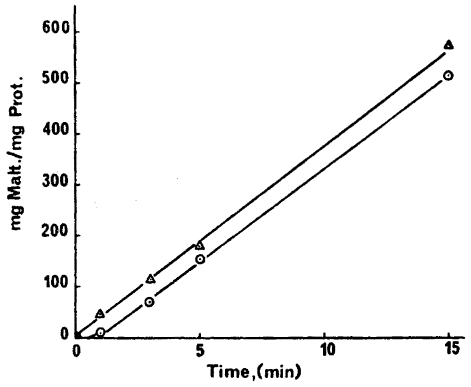

Fig. 4. The hydrolyses of dextran T 40, △ and dextran T 2 000 ○ with dextranase 1. The samples were hydrolyzed for 15 min at 45°C.

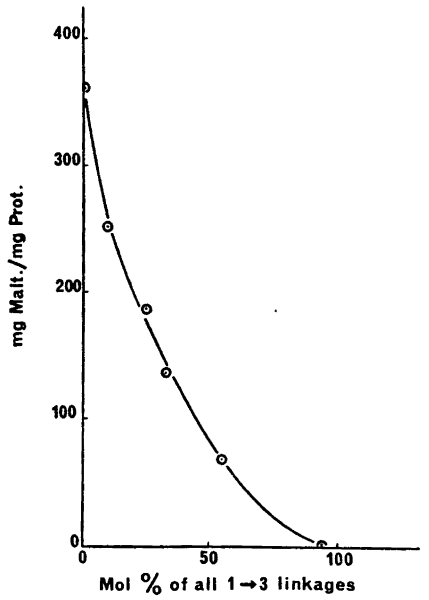

Fig. 5. Plot of hydrolyses of various glucans against their content (mol %) of total 1→3 linkages (*i.e.* in main chain plus branch linkages). Enzyme dextranase 1 was used for the hydrolyses. Dextran of $\overline{M}_w$ 2.0×10^6 was used as a control, as it contains no 1→3 linkages in the main chain.

enzymes capable of hydrolyzing 1→3 linkages. In this connection, it was unfortunate that owing to the limited amount of material, we were unable to grow *P. funiculosum* on glucans containing higher amounts of 1→3 linkages in the main chain.

DISCUSSION

On the basis of the high optical rotation, glucans 1 and 5 must be essentially α-linked, and it seems reasonable to assume that this also is true for the other glucans. Results from the methylation analysis (Table 1) show that all glucose residues are pyranosidic and either terminal or linked to the 3-, 6-, or 3,6-positions. Glucan 1 produced by crude glucosyltransferases from *S. sanguis* contains essentially only 1→6 linkages in the main chain. Glucan 5 produced by a highly purified glucosyltransferase from *S. mutans* contains essentially 1→3 linkages. The other glucans have intermediate structures.*

The compositions of glucans 1 – 3 are dependent on the purity of the enzyme preparations. Thus a glucan with a higher percentage of 1→3 linkages in the main chain was produced, using a highly purified glucosyltransferase from *S. sanguis* than when partially purified or crude enzyme preparations were used. Two glucosyltransferase preparations from *S. mutans* with different isoelectric points also produced polysaccharides with different chemical compositions. It is not possible at present to make a clear distinction concerning the specificities of the glucosyltransferases from their column elution pattern.[4] The possibility remains that the fractions may contain a mixture of two distinct enzymes, one synthesizing glucan chains with 1→3 linkages and the other 1→6 linkages.

The molecular weights of all the glucans tested were low in comparison to native soluble dextrans, while the intrinsic viscosities were high. These properties suggest restricted rotation and therefore more extended configuration, as compared to the soluble dextrans. The rigidity of the structure seems to be closely dependent on the proportion of non-(1→6) linkages in the main chain, as the correlation between $[\eta]$ and molecular weight increases with increasing mol % of 1→3 linkages in the main chain. The above methods of characterization do not, of course, provide any evidence of the structural homogeneity of the samples. Further studies on these aspects are in progress.

The synthesized glucans show different susceptibility to dextranases. From degradation studies (using dextranases of various origin), it was found that the extent of hydrolysis of the various glucans is dependent on the amount of 1→3 linkages present. Since our analyses indicated the presence of 1→3 linkages in the main chain of these glucans, we attempted to grow mold *P. funiculosum* on some of these glucans in the hope that an extracellular α-1,3-

* The experimental $\overline{M}_w$ of glucan 5 (70 000) might, in view of the general polydispersity, correspond to a $\overline{M}_n$ of about 30 000 – 40 000 (DP ~200). Hence the expected amount of 2,3,4,6-tetra-*O*-Me glucose would be ~0.5 %. The discrepancy between the experimental figure of 2.9 % could be due to degradation (4 – 5 linkages per molecule), inaccuracy in determining small amounts of methylation products by GLC, or the presence of a contaminating glucan. Whatever the reason may be, it does not alter the fact that the glucan 5 appears to be a predominantly (94.2 %) 1→3 linked glucan.

hydrolase would be induced in addition to the α-1,6-hydrolase. Our expectations were not fulfilled. One explanation may be that α-1,3-hydrolase is not a constitutive enzyme of this type of mold.

It has been shown by Bourne *et al.*[24] and by Hutson and Weigel [25] that branching in dextrans decreases their extent of hydrolysis by mold dextranases. It seems reasonable to assume that all non-α-(1→6) linkages between the D-glucose residues in the α-glucans should similarly decrease their extent of enzymatic hydrolysis.

It is anticipated that if an enzyme preparation is to have a therapeutic effect, it must contain hydrolases specific for 1→3 as well as for 1→6 linkages.

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