Carbohydrate Polymers **16** (1991) 341–353

Structure of the Linear, Low Molecular Weight Dextran Synthesized by a D-Glucosyltransferase (GTF-S3) of *Streptococcus sobrinus*

Norman W. H. Cheetham

School of Chemistry, The University of New South Wales, PO Box 1, Kensington, Sydney, 2033, Australia

Morey E. Slodki

Northern Regional Research Centre, Agricultural Research Service, US Department of Agriculture, Peoria, Illinois 61604, USA

Gwen J. Walker

Institute of Dental Research, 2 Chalmers Street, Sydney, NSW 2010, Australia

(Received 25 March 1990; revised version received 1 June 1990; accepted 8 June 1990)

ABSTRACT

Four extracellular α-D-glucosyltransferases (GTF) have been separated from cultures of Streptococcus sobrinus *strains grown in continuous culture. Three of the GTF synthesized soluble dextrans from sucrose, and one of these enzymes, GTF-S3, catalysed the production of a low molecular weight, linear dextran. Methylation analysis and high field proton NMR spectroscopy on the intact S3 glucans confirmed that these dextrans were small (dp 20–30) and linear, with the majority of chains terminated with a sucrosyl moiety. Enzymic hydrolysis, followed by analytical and semi-preparative HPLC, led to the isolation of only linear oligosaccharides, one of which was identified as 6^G-glucosylsucrose.*

The results are accommodated by the two-site insertion mechanism for dextran synthesis proposed by Robyt et al. *(1974) (Arch. Biochem. Biophys., **165**, 634).*

Carbohydrate Polymers 0144-8617/91/$03.50 — © 1991 Elsevier Science Publishers Ltd, England. Printed in Great Britain

INTRODUCTION

The structural features of the extracellular α-D-glucans synthesized by the mutans group of streptococci are closely related to the growth conditions of the microorganism (Walker *et al.*, 1983). Such dependency is due to the variation in production of the various D-glucosyltransferases (GTF) involved in the conversion of sucrose into α-D-glucan (Walker *et al.*, 1984; 1990).

We have recently (Walker *et al.*, 1990) described the isolation of four extracellular GTF from *Streptococcus sobrinus* strains grown under defined conditions in the chemostat. The limitations imposed by batch culture were thereby avoided. Growth in continuous culture, under defined or reproducible conditions, permitted the effects of different fermentable carbohydrates, growth rate, pH and Tween 80 to be fully documented. One of the four GTF (GTF-I) synthesized insoluble D-glucan in which (1→3)-α-linked sequences preponderated. Three of the GTF synthesized soluble dextrans from sucrose, and one of these enzymes, GTF-S3, catalysed the production of a low molecular weight, linear dextran. Although this enzyme generally accounted for only 3% of the total GTF activity, its contribution rose two to three-fold when the growth medium contained Tween 80. GTF-S3 activities of up to 90 U litre^{-1} were obtained at high growth in the chemostat, giving a productivity of 36 U g^{-1} litre^{-1}. This glucosyltransferase is most likely involved in the synthesis of dextrans of low molecular weight that have been separated from the soluble glucans synthesized by culture filtrates of several subspecies of *Streptococcus mutans* (Inoue & Koga, 1979; Sund *et al.*, 1987).

We now present a detailed analysis of the structure of S3-dextrans. They were synthesized from sucrose by GTF-S3 preparations from *S. sobrinus* strain K1-R and the dextranase-deficient mutant strain 6715-13-201.

MATERIALS AND METHODS

Carbohydrates

Native dextran from *Leuconostoc mesenteroides* NRRL B-512(F) was a gift from Dr Allene Jeanes, and S3-dextrans were synthesized as described previously (Walker *et al.*, 1990). 6^{G}-Glucosylsucrose, isolated from sugar cane juice, was kindly provided by Dr Julian Gagolski. A

generous sample of chemically synthesized, linear, (1→6)-α-D-glucan (Ruckel & Schuerch, 1967) was a gift from Professor Conrad Schuerch.

Enzymes

GTF-S3 was separated from culture filtrates of *S. sobrinus* grown in medium supplemented with Tween 80 (0·5%) as previously described (Walker *et al.*, 1990). The column chromatography of hydroxyapatite (Fig. 1) yielded preparations of specific activity 34 U mg^{-1}.

Glucodextranase (EC 3.2.1.70) and isomaltodextranase (EC 3.2.1.94) from Biocon (Japan) Ltd were provided by Dr Barry McCleary, and *Penicillium funiculosum* QM 474 *endo*dextranase (EC 3.2.1.11) was prepared as described previously (Walker, 1972).

Enzymic hydrolysis of dextrans

Portions of the dextrans (100 mg) were incubated at 35°C with dextranase (2·7 U) for 48 h in digests (10 ml) containing sodium citrate buffer (15 mM). The concentration of reducing sugars (Dygert *et al.*, 1965) and soluble carbohydrate (Van Handel, 1965) was determined in a sample, and then the limit of hydrolysis by *endo*dextranase and isomaltodextranase was expressed as apparent conversion into iso-maltose,

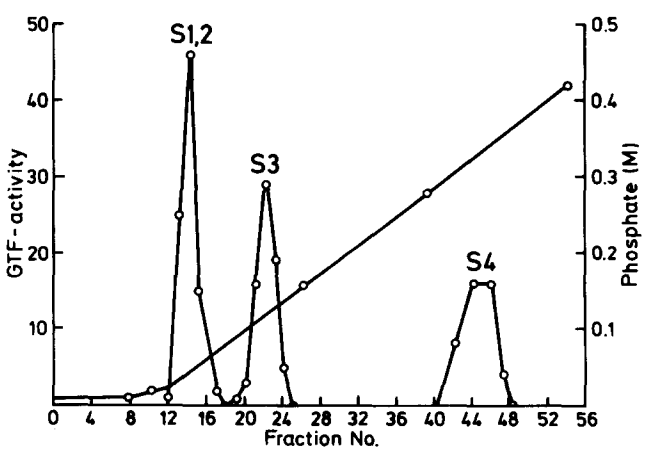

Fig. 1. Separation on hydroxyapatite of three extracellular D-glucosyltransferases of *S. sobrinus* 6715-13-201 that catalyze the synthesis of soluble dextrans. Peak fractions (21–23) of GTF-S3 were combined and incubated with sucrose to synthesize S3-dextran.

and the hydrolysis by glucodextranase was calculated as the conversion into glucose. The remainder of each digest was deionized with Amberlite MB-3 resin, then filtered and evaporated in preparation for chromatography.

Chromatography

A qualitative separation of the products of dextranase activity was made on Whatman No. 3 MM paper in 44:36:20 nitromethane:ethanol:water (Thoma & French, 1957) by descending pc for 28 h. Reducing sugars were revealed by the silver nitrate–sodium hydroxide dip procedure, and non-reducing oligosaccharides containing a fructose residue were detected with urea–phosphoric acid reagent (Wise *et al.*, 1955).

Quantitative separations were made by HPLC using (i) a Dextropak cartridge held in a radial compression system RCM100 (Waters Assoc.) and (ii) in Aminex HPX87P column (cation-exchanger in the Pb^{2+} form) from Bio-Rad Laboratories, California. In each case the eluting solvent was water, at room temperature for the Dextropak and at 85°C for the Aminex column.

The non-reducing oligosaccharides with Dextropak retention times (RT) 3·6 and 5·9 min were isolated by combining the appropriate fractions collected from a number of injections (100 μl) on to the Dextropak column.

Gel permeation chromatography

Molecular weights of S3-dextrans were compared with those of a series of pullulan standards (P82, Showa Denko K.K., Tokyo). Two columns (300 × 7·5 mm) TSK-Gel 3000 P.W. and 4000 P.W. (Toyo Soda Co. Ltd, Tokyo) were employed in series. The eluting solvent was water at a flowrate of 0·6 ml min^{-1}. All HPLC was performed using a refractive index detector (ERC-7510-Erma Optical Works, Tokyo).

NMR analyses

Proton NMR analyses were performed at 500 MHz in D_2O on a Brüker AM500 spectrometer, operating in the Fourier-transform mode.

Methylation analysis

Prior to methylation, $NaBD_4$ (4 mg, minimum 98 atom %D) was added slowly, over the course of 1 h, to the dextrans (2 mg) dissolved in water

(2 ml). After 16 h, excess borodeuteride was decomposed by the addition of washed Dowex AG5W-X1 (50–100 mesh, H form) cation exchange resin. The resin was removed with the aid of a membrane syringe filter, and the samples were evaporated to dryness. Boric acid was removed by successive evaporation from dry methanol (5 × 1 ml). The reduced dextrans were methylated and after hydrolysis and derivatization, were analysed by capillary gas–liquid chromatography–mass spectrometry (GC-MS) (Slodki *et al.*, 1986). Quantification of 1-*d*-1,2,3,4,5-penta-*O*-methyl alditol acetate and per-*O*-acetylated aldonitriles was based on capillary GC flame ionization response (computer-assisted integration). Experiments with known oligosaccharides demonstrated that the penta-*O*-methyl alditol acetate exhibited an 18% higher FID response than the tetra-*O*-methyl aldonitrile acetate. The integrations were corrected accordingly.

RESULTS

Enzymic hydrolyses

The major products of hydrolysis with *P. funiculosum* dextranase were glucose, iso-maltose and iso-maltriose. These were derived from the linear regions of the (1→6)-α-glucan chains, and amounted to 87 and 77% of the total products from S3-dextran and B-512(F) dextran, respectively (Table 1). In addition, oligosaccharides containing a (1→3)-α-linkage were obtained from the vicinity of the branch point in B-512(F) dextran (Taylor *et al.*, 1985), and unknown oligosaccharides with different retention times were obtained from S3-dextrans. A comparison of the oligosaccharide products from the two dextrans after separation by paper chromatography, disclosed that those from S3-dextrans were non-reducing.

The action of *exo*dextranases on most dextrans is limited because of their inability to hydrolyse or bypass secondary linkages. Even B-512(F) dextran, with less than 5% of (1→3)-α-branch linkages, was hydrolysed no more than 20–30% (Table 2). Chemically synthesized (1→6)-α-glucan, which has about 2% of other linkages, was more susceptible to degradation by *exo*dextranase, and S3-dextrans were almost completely hydrolysed (Table 2). This indicated that S3-dextrans are virtually free from the branch linkages normally found in bacterial dextrans. Separation by pc of the products of hydrolysis of S3-dextrans with gluco- and iso-maltodextranases yielded glucose and iso-maltose, respectively. When duplicate papers were dipped in urea–phosphoric acid reagent,

TABLE 1
HPLC Separation of the Products of Hydrolysis of Dextrans with *P. funiculosum* Endodextranase

Standards[a]	*RT (min)*	*Products from dextran hydrolysates*			
		S3-dextran[b]		*B-512(F) dextran*	
		RT	*Area (%)*	*RT*	*Area (%)*
Glc	1·71	1·71	17·4	1·71	17·4
IM_2	1·91	2·02	64·8	2·03	58·5
Sucr		2·41	1·5		
IM_3	2·82	2·75	4·7	2·84	0·9
G-Sucr	3·40	3·60	6·8		
B_4	4·17	4·09	0·5	4·17	8·8
IM_4	4·47				
IM_2-Sucr	5·90	5·93	3·5		
B_5-1	6·50			6·46	4·6
B_5-2	7·80			7·82	2·7
IM_5	8·15				

[a]Glc — glucose, IM_2 — iso-maltose, IM_3 — iso-maltotriose etc., B_4 — branched oligosaccharide, D.P. 4 etc., G-Sucr — glucosylsucrose, IM_2-Sucr — iso-maltosylsucrose.
[b]Synthesized with GTF-S3 from *S. sobrinus* 6715-13-201.

TABLE 2
A Comparison of the Limit of Enzymic Hydrolysis of Three Dextrans

Enzyme	*Hydrolysis (%)*		*Synthetic α-(1→6)-glucan*
	S3-dextran	*B-512(F) dextran*	
Glucodextranase[a]	83	21	35
Isomaltodextranase[b]	98	31	68
Endodextranase[b]	123	122	

[a]Calculated as conversion into glucose.
[b]Calculated as apparent conversion into iso-maltose.

dried and heated for 3 min at 90°C, blue spots corresponding to sucrose and 6^G-glucosylsucrose were among the products from both *exo*dextranases. This procedure also revealed that 6^G-glucosylsucrose was a product of hydrolysis of S3-dextrans with *endo*dextranase. Thus, it seemed likely that the oligosaccharides separated by HPLC from the

products of enzymic hydrolysis of S3-dextrans were 6^G-glucosylsucrose and 6^G-iso-maltosylsucrose.

Acid hydrolysis of S3-dextrans

Determination of fructose and total monosaccharides (Van Handel, 1965; 1967) in hydrolysed S3-dextrans by the 'cold' and 'hot' anthrone methods respectively revealed that they contained 3·5% of fructose, by weight.

Mild, partial acid hydrolysis of the oligosaccharides derived from S3-dextrans by *endo*dextranase action, followed by separation on Dextropak, yielded products expected from oligosaccharides having a terminal sucrose linkage. The oligosaccharide with RT 3·6 min, suspected to be 6^G-glucosylsucrose, gave iso-maltose and fructose, while the product with RT 5·93 min gave iso-maltotriose and fructose. These results were confirmed on the Aminex column, where traces of glucose were also found in the acid hydrolysates.

Methylation analysis

S3-dextrans from strain K1-R and the D-mutant gave identical results (Table 3). The absence of dimethylsugars confirmed that the dextrans were linear, and the proportion of 2,3,4,6-tetra-*O*-methyl to 2,3,4-tri-*O*-methyl ethers indicated a dp of 20–21. By contrast with quantitative incorporation of deuterium into known oligosaccharides, the extent of deuterioreduction obtained suggests that about three-quarters of the S3-dextran chains may be non-reducing.

TABLE 3
Methylation Analyses of Deuterioreduced S3-Dextrans

Component[a]	*Mol% in preparation*	
	Strain K1-R	*Strain 6715-13-201*
2,3,4,6-Glc	5·0	5·9
2,3,4-Glc	93·7	92·8
1,2,3,4,5-Glc OH (1-d_1)	1·3 (0·26)[b]	1·3 (0·22)[b]

[a]2,3,4,6-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, etc. After hydrolysis of the $NaBD_4$-reduced per-*O*-methylated dextrans, sugars were analysed by GC as per-*O*-acetylated aldonitriles, and the alditol as the mono-*O*-acetate. (Identification by GC-MS.)
[b](Calculated) as mol fraction of tetra-*O*-methyl ether.

Gel-permeation chromatography showed that the S3 dextrans had an average molecular weight somewhat below 5300 daltons (the smallest pullulan standard), i.e. dp ≈ 25–30.

NMR analysis

Methylation analysis and enzymic hydrolysis indicated the presence of terminal sucrosyl units on most but apparently not all S3 chains. NMR analysis also revealed the presence of a small percentage of reducing end groups (Cheetham *et al.*, 1991*a*) in S3 dextrans from strains K1-R and 6715-13-201. As sonication had been used to dissolve these dextrans after their initial precipitation, it was decided to prepare and isolate from strain K1-R, a 'native' S3 dextran which had not been subjected to such vigorous treatment. Isolation involved gel-permeation chromatography on a TSK G5000PW column (21·5 × 600 mm) to separate fructose and buffer salts from the S3 dextran, followed by freeze-drying and D_2O exchange for NMR examination. The anomeric region of the proton NMR spectrum of this S3 dextran (Fig. 2(a)) from strain K1-R reveals a small doublet centred at 5·42 ppm (J = 4 Hz). This is close to but distinct from the resonance position of the anomeric protons of the (1 → 3)-α-D-glucosyl residues in other, branched dextrans (such as that from NRRL B-512 (F)) which occurs at 5·29 ppm (J = 4·1 Hz).

Figure 2(b) shows the spectrum of dextran T_{10}, a commercially available, low molecular weight (≈ 10000) derivative of dextran NRRL B-512(F). In addition to the resonances at ≈ 5·29 ppm (due to (1 → 3)-α-D-glucosyl side-chains) doublets at 5·22 ppm and 4·65 ppm show the presence of reducing end groups. These are absent from the S3 dextran spectrum (and disappear when dextran T_{10} is reduced with sodium borohydride (Cheetham *et al.*, 1991*b*). Overall, Fig. 2 confirms

(a) the absence of branching in S3 dextrans,
(b) the presence of a sucrose residue terminating the S3 chain,
(c) the small dp of the S3 dextrans. This was calculated from integration of the anomeric resonances. As there is a maximum of one terminal sucrosyl moiety per S3 chain,

$$\text{Maximum dp} = \frac{\text{total area of anomeric proton resonances in the polysaccharide}}{\text{area of the glycosyl anomeric proton resonances in the sucrose moiety}}$$

For the S3 dextran from K1-R shown in Fig. 2, the maximum dp was ≈ 29, (MW ≈ 4600). As no attempt was made to ensure complete relaxation of the anomeric protons, the accuracy of this figure could be in

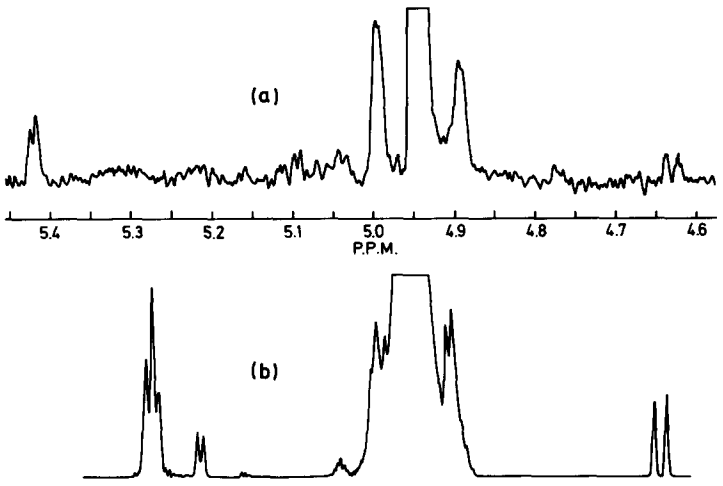

Fig. 2. The anomeric regions of the 500 mHz proton NMR spectra of (a) S3-dextran, (b) dextran T_{10}.

doubt. Results from the gel-permeation chromatography (see above) are close to this value, however, and methylation analysis of (sonicated) S3 dextrans yields a dp of 20–21. In the light of the present results we need to reduce, to ≈ 20–30, our original estimate of dp ≈ 60 (10–12 K daltons), for S3 dextrans (Cheetham *et al.*, 1991*a*). That estimation was based on the ratio of 'reducing' anomeric proton areas to total anomeric proton areas and on the assumption that all of the chain ends were reducing. Thus, the number of end groups actually present was under-estimated, and as a consequence the molecular weight was over-estimated. The gel-permeation column system used in the present study was more appropriate for small oligosacchsaccharides than that used previously (Cheetham *et al.*, 1991*a*).

Figure 3 shows the anomeric regions of the NMR spectra of (a) the non-reducing oligosaccharide (RT 3·6 min on Dextropak) derived from endodextranase activity on S3 dextran, and (b) that of an authentic sample of 6^G-glucosylsucrose. The spectra are identical, except for the presence of very small anomeric proton signals from the reducing end of iso-maltotriose which remains as a contaminant from the isolation of (a).

DISCUSSION

From a study of dextrans synthesized by 96 strains of bacteria (Jeanes *et al.*, 1954), only one dextran was found to contain more than 0·02% of

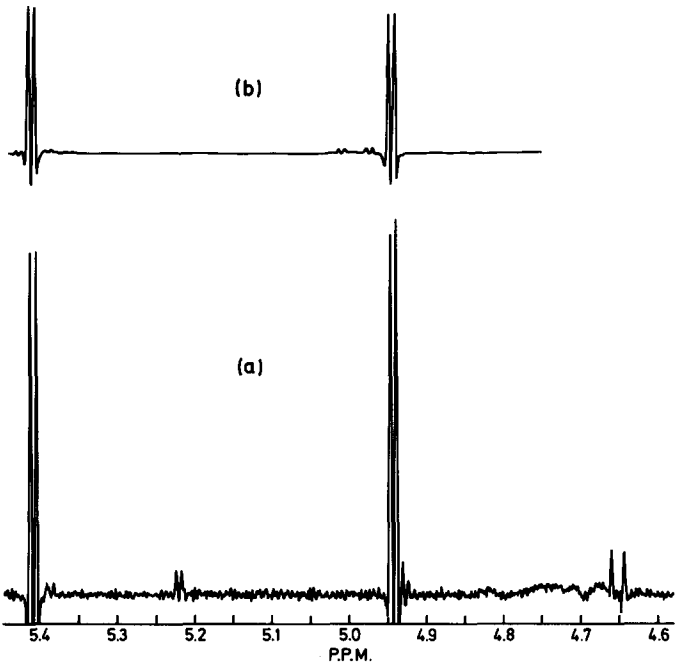

Fig. 3. The anomeric regions of the 500 mHz proton NMR spectra of (a) the non-reducing oligosaccharide derived from endodextranase activity on S3 dextran, (b) an authentic sample of 6^G-glucosylsucrose. The small doublets at 4·65 and 5·22 ppm in (a) arise from the free reducing group of a small amount of isomaltotriose.

fructose. *S. viridans* NRRL B-1351 converted sucrose into a dextran of low molecular weight (MW 54300) with a fructose content of 0·27%. The molecular weights of the other native dextrans are so high (B512(F) dextran (Jeanes, 1966) has MW $40–50 \times 10^6$), that a single fructose unit per chain would escape detection, the concentration of fructose being lower than the limiting value of any analysis. Little or no information can normally be obtained on the character of the 'reducing end' of most native dextrans.

Hehre (1966) suggested that the small size of dextran molecules from the *S. viridans* strain arose from synthesis by an uncommon type of dextransucrase, and he noted that inherent catalyst specificity had not hitherto been counted among the factors that influence the molecular weight of dextrans. The molecules of B-1351 dextran lacked a reducing group, having instead a non-reducing β-D-fructofuranosyl unit. Hehre concluded that the dextran owed both its low molecular weight and its fructosyl end group to an enzyme able to use sucrose as an acceptor substrate much more readily than the usual dextransucrase.

In the present study we have shown that *S. sobrinus* GTF-S3 is also an uncommon type of dextransucrase, for it synthesizes a dextran with a far lower molecular weight and with a different structure from those produced by the other two GTF-S type enzymes released by *S. sobrinus*. Results from gel filtration, NMR and methylation analysis all indicated that S3-dextrans have an average DP of 20–30. The release of 6^G-glucosylsucrose and 6^G-iso-maltosylsucrose by degradation with specific *exo*dextranases proved that most of the chains terminate with a non-reducing fructosyl residue. We found no evidence for $(1\rightarrow3)$-α-branch linkages in any S3-dextran.

Oligosaccharides containing fructosyl residues were also well resolved by pc and HPLC, from the products of *endo*dextranase action on S3-dextrans. By contrast, oligosaccharides of the iso-malto series were the only products separated from enzymic hydrolysis of low molecular weight dextrans produced by *S. mutans* sp. (Hanada & Takehara, 1987; Yamashita *et al.*, 1988). In those studies, the dextrans may have been synthesized with GTF preparations that contained traces of invertase or *endo*dextranase. Both enzymes would be capable of removing the fructosyl end group, and many *S. mutans* strains produce appreciable amounts of dextranase (Walker *et al.*, 1981) in batch culture. Our preparations of GTF-S3 were free from *endo*dextranase and they produced identical S3-dextran, whether the GTF was isolated from continuous culture of strain K1-R or from the dex-mutant 6715-13-201.

The presence or absence of fructose in dextran may also depend on the mechanism of action (Robyt *et al.*, 1974) of the particular dextransucrase involved. If the chains grow by insertion of a glucose residue at the reducing end of the polysaccharide, the resulting dextran would not retain a fructose residue.

Two possible mechanisms could explain the presence of fructose on S3 chain ends:

1. The two-site insertion mechanism of Robyt *et al.* (1974), as described above. High concentrations of sucrose were employed in the synthesis of S3 dextrans. Fructose is released as the chain is extended, but is a poor acceptor. Sucrose is likely to be much more efficient because of its glucosyl moiety, and chain growth would terminate when the 'final' acceptor is sucrose. In this case sucrose is acting as an acceptor in a fashion analogous to that of the ^{14}C-labelled maltose used by Robyt and Walseth (1978).
2. Certain dextransucrases may function by transfer of glucosyl residues from sucrose to the glucosyl residue of an initial sucrose acceptor, with further glucose residues becoming attached to the

non-reducing end of the growing chain. These unusual dextransucrases would synthesize dextran chains that should contain the fructose residue of the original sucrose acceptor.

Because *S. sobrinus* GTF-S3 reacts with sucrose in the absence of a dextran primer, its linear product may serve as a primer for the primer-dependent GTF-S1 and GTF-I. The latter enzyme is activated (Germaine *et al.*, 1974) to produce $(1\rightarrow3)$-α-glucan in the presence of small amounts of dextrans or $(1\rightarrow6)$-α-glucans with dp>8. Furthermore, dextrans with little or no branching are the most efficient stimulators (Walker & Schuerch, 1986) or GTF-I activity.

ACKNOWLEDGEMENT

This work was supported, in part, by a grant from the National Health and Medical Research Council of Australia.

REFERENCES

Cheetham, N. W. H., Walker, G. J., Pearce, B. J., Fiala-Beer, E. & Taylor, C. (1991*a*). *Carbohydr. Polym.*, **14**, 3–16.

Cheetham, N. W. H., Fiala-Beer, E. & Walker, G. J. (1991*b*). *Carbohydr. Polym.*, **14**, 149–58.

Dygert, S., Li, L. H., Florida, D. & Thoma, J. A. (1965). *Anal. Biochem.*, **13**, 367–74.

Germaine, G. R., Chludzinski, A. M. & Schachtele, C. F. (1974). *J. Bacteriol.*, **120**, 287–94.

Hanada, N. & Takehara, T. (1987). *Microbios*, **50**, 147–52.

Hehre, E. J. (1966). *J. Biol. Chem.*, **222**, 739–50.

Inoue, K. & Koga, T. (1979). *Infect. Immun.*, **25**, 922–8.

Jeanes, A. (1966). *Encycl. Polymer Sci. Technol.*, **4**, 805–24.

Jeanes, A., Haynes, W. C., Wilham, C. A., Rankin, J. C., Melvin, E. H., Austin, M. J., Cluskey, J. E., Fisher, B. E., Tsuchiya, H. M. & Rist, C. E. (1954). *J. Amer. Chem. Soc.*, **76**, 5041–52.

Robyt, J. F. & Walseth, T. F. (1978). *Carbohydr. Res.*, **61**, 433–45.

Robyt, J. F., Kimble, B. K. & Walseth, T. F. (1974). *Arch. Biochem. Biophys.*, **165**, 634–40.

Ruckel, E. R. & Schuerch, C. (1967). *Biopolymers*, **5**, 515–23.

Slodki, M. E., England, R. E., Plattner, R. D. & Dick, W. E. (1986). *Carbohydr. Res.*, **156**, 199–206.

Sund, M.-L., Ericsson, C., Linder, L. E. & Branting, C. (1987). *Caries Res.*, **21**, 1–9.

Taylor, C., Cheetham, N. W. H. & Walker, G. J. (1985). *Carbohydr. Res.*, **137**, 1–12.

Thoma, J. A. & French, D. (1957). *Anal. Chem.*, **29**, 1645–8.
Van Handel, E. (1965). *Anal. Biochem.*, **11**, 266–71.
Van Handel, E. (1967). *Anal. Biochem.*, **19**, 193–4.
Walker, G. J. (1972). *J. Dent. Res.*, **51**, 409–14.
Walker, G. J. & Schuerch, C. (1986). *Carbohydr. Res.*, **146**, 259–70.
Walker, G. J., Pulkownik, A. & Morrey-Jones, J. G. (1981). *J. Gen Microbiol.*, **127**, 201–8.
Walker, G. J., Morrey-Jones, J. G., Svensson, S. & Taylor, C. (1983). Chemical senses. In *Glucosyltransferases, Glucans, Sucrose and Dental Caries*, ed. R. J. Doyle & J. E. Ciardi. I.R.L. Press, Washington, DC, p. 179–87.
Walker, G. J., Brown, R. A. & Taylor, C. (1984). *J. Dent. Res.*, **63**, 397–400.
Walker, G. J., Cheetham, N. W. H., Taylor, C., Pearce, B. J. & Slodki, M. E. (1990). *Carbohydr. Polym.*, **13**, 399–421.
Wise, C. S., Dimler, R. J., Davis, H. A. & Rist, C. E. (1955). *Anal. Chem.*, **27**, 33–6.
Yamashita, Y., Hanada, N. & Takehara, T. (1988). *Biochem. Biophys. Res. Commun.*, **150**, 687–93.