ELSEVIER

Carbohydrate Research 275 (1995) 433–440

CARBOHYDRATE RESEARCH

Note

Inactivation of two D-glucosyltransferases from Serotype c *Streptococcus mutans* 103220T by 1,2-epoxy-3-(α-D-glucopyranosyl)propane

Sophie Bombard, Magali Maillet, Marie-Louise Capmau *

L.R.M.A., Université Paris VI, 15 rue de l'Ecole de Médecine, F-75270 Paris Cedex 06, France

Received 14 March 1994; accepted in revised form 14 December 1994

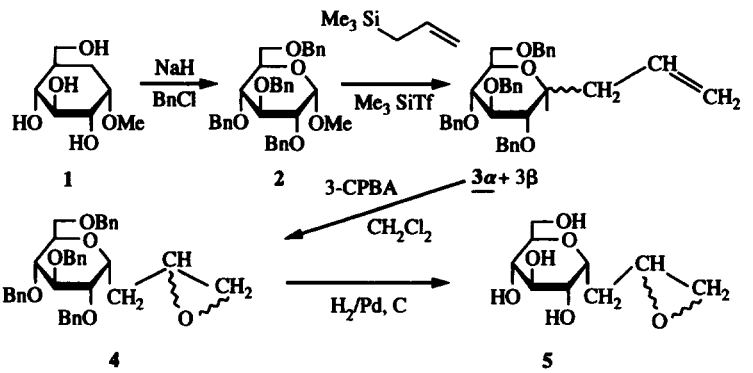
Keywords: Serotype c *Streptoccocus mutans*; Glucosyltransferase inactivation

The α-D-glucosyltransferases (Gtase, EC 2.4.1.5) produced by various species of oral streptococci are of considerable interest owing to their production of extracellular glucans from dietary sucrose [1]. These glucans are shown to play a key role in the formation of dental plaque [2,3] and the subsequent development of dental caries [4]. Therefore, the glucosyltransferases (Gtases) from serotype c *Streptococcus mutans*, which are the most prevalent in humans, have become one of the potential targets for the prevention of dental caries [5,6]. Informations on the catalytic site, as well as the study of inhibitors, would promote the development of anti-caries agents. Accordingly, 1,2-epoxy-3-(α-D-glucopyranosyl)propane (**5**) has been synthesized and tested as a potential active-site directed irreversible inhibitor of the two glucosyltransferases Gtase I and Gtase II from the serotype c *Streptococcus mutans* 103220T.

The ß anomer of **5** has already been described [7]. The α anomer could be theoretically obtained from two precursors, namely 3-(2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl)-1-propene (**3**) and 3-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl)-1-propene [8]. Although the latter compound could be obtained by allylation of penta-*O*-acetyl-D-glucose in good yield, this approach was abandoned because, after epoxidation, elimination of the protecting groups did not preserve the epoxide function. Accordingly, the precursor **3**, was prepared following the methodology of Hosomi et al. [9]. A modification of the reaction temperature gave a more useful proportion of α anomer despite a

* Corresponding author.

Elsevier Science Ltd.
SSDI 0008-6215(95)00045-3



Scheme 1.

lower global yield (80% considering the recovered starting material). Compound **5**, obtained in 70% yield by 3-chloroperoxybenzoic acid epoxidation of **3** followed by catalytic debenzylation, is a 3:2 mixture of two stereoisomeric epoxides, a new asymmetric center being created at C-2 by epoxidation (Scheme 1).

Epoxide **5** proved to be an effective inhibitor of Gtases I and II obtained from *Streptococcus mutans* 103220T according to a purification procedure of Baba et al. [10]. Kinetic inhibition studies showed that 10 mM **5** inhibited 50% of the activity of the two Gtases. Compound **5** was also able to inactivate the two enzymes in a time-dependent manner. The Gtase activity of the enzyme was not restored by gel-filtration of the enzyme-inhibitor complex on Sephadex G-25. Furthermore, inactivation of the two Gtases by **5** appeared to be specific since treatment of the enzymes by 20 mM 1,2-epoxy-3-phenoxypropane during 10 min did not alter the Gtase activity.

The inhibition of the two Gtases was examined over a concentration range of **5**. At each concentration, an exponential inactivation rate of the Gtases was observed. Semilogarithmic plots of residual activity as a function of time inactivation for various concentrations of **5** were linear (Fig. 1 for Gtase I), indicating that in any case the inactivation process follows pseudo-first-order kinetics. It may be thus deduced that the inactivation event is preceded by the formation of a dissociable enzyme–inhibitor complex before inactivation, [Eq. (1)], where E*I is a reversible complex between enzyme and inhibitor; E − I is the inactivated enzyme with covalently bound inhibitor, and k_1, k_{-1}, and k_2 represent the rate constant for each step.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E*I \xrightarrow{k_2} E - I \qquad (1)$$

When the inhibitor concentration is greatly in excess as compared with the enzyme concentration (and assuming that k_2 is much slower than k_{-1}), the model is described by Eq. (2) (ref. [11]).

$$k_{obs} = k_2/(1 + K_I/[I]) \qquad (2)$$

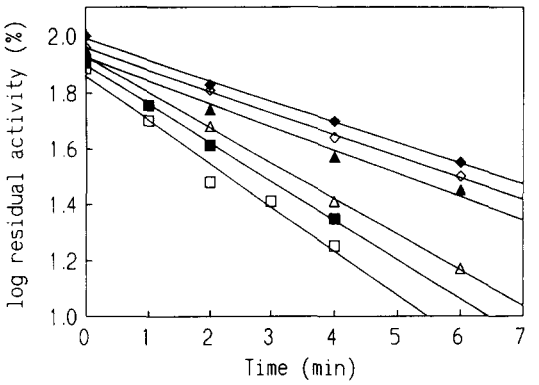


Fig. 1. Semi-logarithmic plot of Gtase I inactivation by varying concentrations of **5**. The enzyme (10 mIU) was treated with 1.43 (♦), 1.66 (◇), 2 (▲), 2.5 (△), 3 (■) and 4 mM (□) of **5**. Incubations and assays were carried as described in Experimental.

In the reciprocal form, we obtain Eq. (3), where K_I is the dissociation constant of the E*I complex, k_{obs} is determined from the equation $\ln(E_t/E_0) = -k_{obs}t$, E_t is the enzyme activity remaining at time t, and E_0 is the original enzyme activity at t_0 with the same inhibitor concentration.

$$1/k_{obs} = K_I/([I]k_2) + 1/k_2 \quad (3)$$

A double-reciprocal plot of k_{obs} as function of inhibitor concentration was linear (as shown in Fig. 2 for Gtase I). Values of k_{obs}, k_2 and K_I were determined from linear regression curves evaluated by the least-squares method. We obtained K_I values of 12.8 ± 0.4 and 7.4 ± 0.2 mM and k_2 values of 1.6 ± 0.2 min^{-1} and 1.2 ± 0.1 min^{-1} for Gtase I and Gtase II, respectively. The values of K_I are lower for GTase II (7.4 mM) than for Gtase I (12.8 mM). This cannot be related to differences in affinity (K_m) of 1 and 4.5 mM for sucrose. Epoxide **5** has a relatively good affinity for the two Gtases.

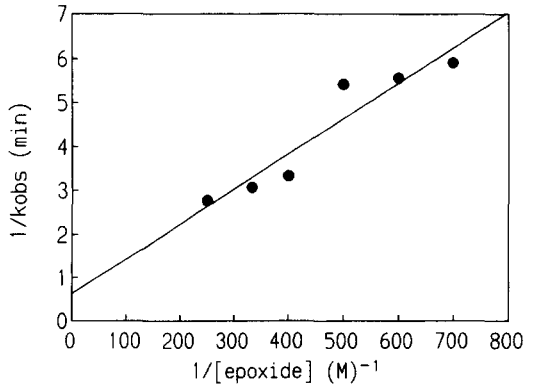


Fig. 2. Double reciprocal plot of the rate of Gtase I inactivation obtained from Fig. 1, as a function of concentration of **5**. Values of K_I and k_2 were estimated from the intersects of the abscissa and ordinate.

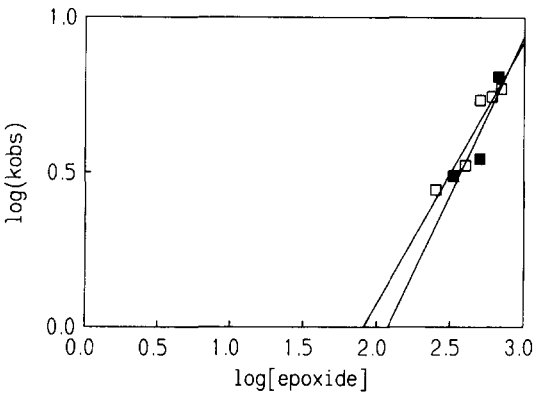


Fig. 3. Replot of $\log(k_{obs})$ values from Fig. 1 (□) and rate constants inactivation of Gtase II (■) by epoxide **5**, as a function of log[epoxide]. The Hill plots for Gtase I and Gtase II had slopes of 0.86 and 1 respectively indicated an 1:1 stoichiometry of epoxide binding to each enzyme.

Indeed, K_I has the same order of magnitude as K_m for sucrose, in agreement with the observation that the D-fructosyl moiety of sucrose is not important for the fixation of sucrose: analogs of sucrose where the D-fructosyl moiety is replaced by a fluorine [12,13] or a 4-nitrophenyl group [14] are substrates for the Gtases. The k_2 values of the two Gtases are relatively close and one or two magnitude order higher than those for the inactivation of glucoside hydrolases by various epoxides [15].

The inactivation-rate curves of several epoxide concentrations were also used to determine the stoichiometry of the binding of **5** to the two Gtases [16]. Fig. 3 is a replot of $\log(k_{obs})$ vs. log [**5**] for the inactivation of Gtase I and Gtase II. The slopes of these plots are 0.86 and 1 respectively, suggesting that the inactivation of the Gtases was caused by the binding of one mole of epoxide / mole of enzyme.

Gtase has been shown to be composed of two functional domains: an amino terminal domain, for the binding and the hydrolysis of sucrose, and a carboxyl-terminal domain for the binding of the dextran in formation [17,18]. The inactivation could then occur in one of these two domains. If indeed **5** binds at the hydrolysing site of Gtase I and Gtase II, sucrose (which is the natural substrate), should prevent the binding of **5** to the enzyme and, thus, should decrease the rate of inactivation by **5**. Sucrose at 3, 10 and 20 mM concentrations was effective in preventing the inactivation of the two Gtases (Fig. 4). If **5** binds at the carboxyl terminal glucan binding-domain, inactivation of the two Gtases without DT10 should be enhanced. Gtase I and Gtase II were incubated with 1.5, 2, or 3 mM **5** with or without 0.2 mg/mL of DT10. The rates of inactivation (k_{obs}) were similar in the incubations with or without DT10. These sucrose protection experiments suggested that **5** is reactive at the active site of the sucrose-binding domain. On the contrary, DT10 did not protect the two Gtases againts the inactivation by **5**, showing that it does not interfere with the carboxyl terminal glucan-binding domain.

The results of the inhibition studies suggested then that 1,2-epoxy-3-(α-D-glucopyranosyl)propane reacts irreversibly with specific amino acids residues of the sucrose binding-domain of the active site of the two Gtases. Epoxides have already been used to

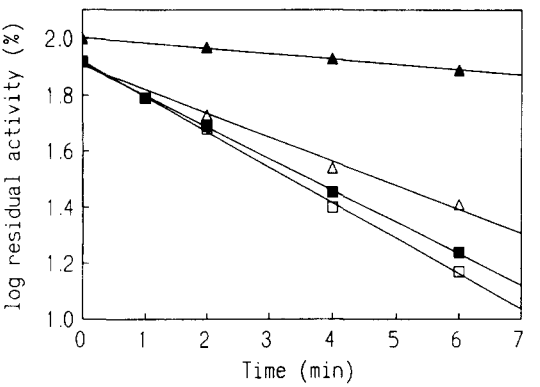


Fig. 4. Protection of Gtase I by 3 (■), 10 (△) and 20 mM (▲) sucrose against inactivation by 2.5 mM epoxide and inactivation in absence of protectant (□). Incubations and assays were carried as described in Experimental.

inactivate various glucoside hydrolases binding irreversibly to a carboxylate residue [15]. From previous studies of the Gtase system, a mechanism of synthesis of D-glucans by the Gtases involving the formation of a glucosyl-enzyme in transfer of the D-glucosyl group from sucrose to an acceptor has been proposed [19,20]. In addition, a glucosyl-enzyme complex, compatible with a ß-D-glucosyl ester linkage to an aspartic or glutamic residue of a Gtase from *Streptococcus sobrinus* has been characterized [21]; an active-site peptide containing a catalytic Asp residue has also been isolated from two *Streptococcus sobrinus* glucosyltransferases [22] and an aspartic residue was shown to be essential for the sucrose-binding active site by mutagenesis [23]. Thus, as epoxides are known to react with a carboxylate residue, and since an Asp residue seems to be implicated in the catalytic mechanism of the Gtases, our results gave further evidence that a carboxylate residue in the sucrose-binding domain is essential for the activity of the Gtases.

1. Experimental

Material.—Streptococcus mutans 103220 T, a typical strain of serotype c, was isolated from human carious lesions and was selected from the culture collection of the Institut Pasteur (CIP). It was cultured three to four times and stored at −80°C in BHI with 20% glycerol. [U-^{14}C]sucrose and [U-^{3}H-fructose]sucrose with specific activities of 17.6 GBq/mmol and 462.5 GBq/mmol respectively, were purchased from New England Nuclear, and diluted with unlabelled sucrose before use to 0.74 Gbq/mmol. DEAE-cellulose (DE52) and CM-cellulose (CM52) were purchased from Whatman (France); commercial Dextran T10 (DT10) from Pharmacia; 1,2-epoxy-3-phenoxypropane and methyl α-D-glucopyranoside from Aldrich Chemical Co. Ltd.

General methods.—Optical rotations were measured with a Perkin–Elmer 241 polarimeter. ^{1}H and ^{13}C NMR spectra were recorded with a Brucker AC 200 spectrome-

ter, and the IR spectra with a Perkin–Elmer 1720 spectrophotometer. Reactions were monitored by TLC on Silica Gel 60-F_{254}, (E. Merck) with detection by UV light or charring with H_2SO_4. For preparative flash and normal column chromatographies, Amincon Silicagel Si 6–35 mesh and Silicagel 60 70–230 mesh were respectively used.

*Methyl 2,3,4,6-tetra-*O*-benzyl-α-*D*-glucopyranoside* (**2**).—Methyl α-D-glucopyranoside (**1**, 2 g, 10.3 mmol) in benzyl chloride (50 mL) was treated with NaH (19.8 g) and the temperature gradually raised until the reaction started. More methyl α-D-glucopyranoside (**1**, 18 g, 92.7 mmol) in benzyl chloride (80 mL) was added dropwise to the reaction mixture at reflux temperature. After 5 h and cooling, MeOH (30 mL) was added. The mixture was poured in cold water and extracted with $CHCl_3$. The residue was purified by flash chromatography on silica gel using CH_2Cl_2 as eluent to give **2** (36 g, 63%): $[\alpha]_D^{21}$ + 19.6° (*c* 2.82, $CHCl_3$), lit [24]. $[\alpha]_D^{25}$ + 18.7° ($CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 7.4–7.2 (m, 20 H, Ar), 5–4.4 (m, 9 H, H-1 and 8-H, PhCH_2), 4–3.9 (dd 1 H), 3.7–3.5 (m, 5 H), 3.3 (s, 3 H, OCH_3); m/z = 572 $(M + 18)^+$ 100%.

*3-(2,3,4,6-Tetra-*O*-benzyl-α-*D*-glucopyranosyl)-1-propene* (**3**).—Compound **2** (4.03 g, 7.2 mmol) in dry MeCN (17.5 mL), was cooled to 0° C. Under Ar, allyltrimethylsilane (2.74 mL, 17.2 mmol) and trimethylsilyl triflate (0.837 mL, 4.3 mmol) were added to the solution with a syringe. The mixture was stirred for 10 h at 0° C and 5 h at room temperature and then diluted with CH_2Cl_2 (50 mL) and poured into a saturated solution of $NaHCO_3$ (30 mL). The layers were separated and the aqueous phase was extracted with CH_2Cl_2 (20 mL). The organic layers were combined and dried (Na_2SO_4) and evaporation under reduced pressure gave crude **3** (4.325 g). Purification on a silica gel column (4:1, pentane–EtOAc) afforded two fractions. The first (0.525 g) contained a mixture of α and ß anomers (with less than 5% of ß). The last eluted component (1.295g, 45%) was the pure α anomer described by Hosomi et al. [9]. $[\alpha]_D^{21}$ + 31.9° (*c* 1.52, $CHCl_3$), lit. [9]. $[\alpha]_D^{21}$ + 32.1° (*c* 0.97, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 7.36–7.29 (m, 20 H, Ar), 5.89–5.75 (m, 1 H, H-2), 5.15–4.73 (m, 2 H, H-1 and 8 H, PhCH_2), 4.16–4.09 (m, 1 H, H-1′), 3.71–3.59 (6 H, H-2′,H-3′,H-4′,H-5′,H-6′), 2.49–2.45 (m, 2 H, H-3); Anal. Calcd for $C_{37}H_{40}O_5 \cdot 0.5\ H_2O$: C, 77.48; H, 7.15. Found: C, 77.5; H, 7.18. Starting material **2** (25%) and its anomer (10%) were recovered.

*1,2-Epoxy-3-(2,3,4,6-tetra-*O*-benzyl-α-*D*-glucopyranosyl)propane* (**4**).—The pure α anomer of **3**, (1.15 g, 2.04 mmol) was dissolved in anhydrous freshly distilled CH_2Cl_2 (24 mL). 3-Chloroperoxybenzoic acid (1.3 g, 7.5 mmol) was added, and the mixture was stirred at reflux for 5 h. After cooling, the organic solution was washed with 5% $NaHCO_3$ (3 × 10 mL) and H_2O (2 × 10 mL). The organic layer was dried (Na_2SO_4). After concentration, the residue was separated on a silica gel column with 3:2 pentane–EtOAc as eluent resulting in an isomeric mixture of 1,2-epoxy-3-(2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl)propane (**4**) (0.82 g, 70%); ^{1}H NMR ($CDCl_3$): δ 7.16–7.12 (m, 20 H, Ar), 4.73–4.31 (m, 8 H, PhCH_2), major isomer 4.2 (m, 0.6 H, H-1′), minor isomer 4.08 (m, 0.4 H, H-1′), 2.88 (m, 1 H, H-2), major isomer 2.64 (dd, 0.6 H, H-1a), minor isomer 2.56 (dd, 0.4 H, H-1a), major isomer 2.38 (dd, 0.6 H, H-1b), minor isomer 2.3 (dd, 0.4 H, H-1b), 1.85–1.75 (m, 2 H, H-3);^{13}C NMR ($CDCl_3$): 26.61 (C-3),50.2, 49.77, 47.59, 46.27 (C-1,-2). Anal. Calcd for $C_{37}H_{40}O_6 \cdot 0.5\ H_2O$: C, 75.38; H, 6.96. Found: C, 75.29; H, 6.89.

*1,2-Epoxy-3-(α-*D*-glucopyranosyl)propane* (**5**).—Compound **4** (0.482 g, 0.83 mmol)

was dissolved in 1:1 MeOH–EtOH (20 mL) and 10% Pd–C (70 mg) was added. The product was hydrogenolysed in a Paar apparatus under 4.13 10^5 Pa for 30 h. After filtration and elimination of the solvent, the product was dissolved in H_2O (5 mL) and purified on a C_{18} cartridge (Millipore) to give **5** (0.125 g, 70%). TLC (7:3 CH_2Cl_2–MeOH) showed the two isomers (R_{f_1}: 0.35; R_{f_2}: 0.29); ν_{max} 3060 cm^{-1} (epoxide); ^{13}C NMR (D_2O): 32.47, 32.89 and 33.74 (C-1,-2). Anal. Calcd for $C_9H_{16}O_6 \cdot H_2O$: C, 45.37; H, 7.56. Found: C, 45.49; H, 7.58.

Growth and harvesting of bacteria.—*Streptococcus mutans* 103220 T was cultured for 18 h in 3.6 L medium in a 4 L fermentation vessel (Biolafitte) as previously described by Mohan et al. [25].

Purification of glucosyltransferases.—All purification procedures were carried at 4°C. They followed the purification steps, described by Baba et al. [10] for a Gtase from *Streptococcus mutans* MT8148. The crude enzyme was applied to a column of DEAE-cellulose (pH 5). The majority of Gtase activity was eluted as a single band in the wash-through peak. Residual Gtase activity (7%) was then eluted by the NaCl gradient together with the total fructosyltransferase activity (13%). The active fractions were then applied to a CM-cellulose column (pH 7.5). Two peaks of Gtases activity were separated with the NaCl gradient. They were eluted at 0.08 M (Gtase I) and 0.11 M NaCl (Gtase II). They were recovered in a yield of 21% and 5% with an specific activity of 30 IU/mg and 60 IU/mg of protein, respectively. Gtase I has a MW of 150,000 and an isoelectric point of 8, and Gtase II a MW of 135,000 and an isoelectric point of 8.8. They showed a K_m value for sucrose of 1 and 4.5 mM and their activities were enhanced by 5 and 100 fold respectively in the presence of Dextran T10. Both enzymes synthesized a water-soluble D-glucan from sucrose as shown by its coloration by the periodic acid-Schiff reagent within the electrophoresis gels [26].

Assay for enzyme activity.—All the activities were measured by the determination of the amount of radioactivity incorporated in MeOH-insoluble glucans from [U-^{14}C]sucrose. The reaction mixture contained 0.1 M potassium phosphate buffer pH 6 (KH_2PO_4/K_2HPO_4), 30 mM [U-^{14}C]sucrose, 0.2 mg/mL DT10 and 8–10 mIU Gtase activity, in a total volume of 65 μL. The reaction was stopped by adding MeOH (1 mL). The radioactivity incorporated into the polysaccharides was estimated by the filter paper method [27] with the following modifications: the total glucan synthesized was filtered on glass fibre disks GFA (Whatman) and washed with MeOH (3 × 5 mL). The radioactivity retained on the disks was determined with a liquid-scintillation spectrometer.

One unit of enzyme (IU) was defined as the amount of glucosyltransferase catalysing the incorporation of 1 μmol of glucose from sucrose per min under the above conditions.

Determination of K_m *value.*—The substrate saturation kinetics of the two Gtases were determined by the method of Lineweaver and Burk [28]. The enzyme (10 mIU) was treated with 0.5–50 mM sucrose containing 1.48 kBq of [U-^{14}C]sucrose in the 0.1 M phosphate buffer pH 6 at 37°C. The incubation and the estimation of glucan synthesized were described earlier.

Determination of the inactivation rate constants.—For the time-dependent experiment, Gtase (8–10 mIU) in 0.1 M potassium phosphate buffer pH 6, was treated with

varying concentrations of **5** (1.43 to 4 mM final concentrations) and incubated at 37°C with 0.2 mg/mL of DT10 in a vol of 55 μL. At the indicated time, [U-^{14}C]sucrose was added at 30 mM final concentration and the residual activity was determined as described above. In the experiments without DT10, Gtases were incubated only with 2, 2.5 or 3 mM **5**. At the end of the incubation, 0.2 mg/mL of DT10 and 30 mM [U-^{14}C]sucrose (final concentration) were added. For the inhibitions without preincubation time (t_0), Gtase was added at the end.

Sucrose protection experiments to determine active-site specificity of the inhibitor were carried as follows: Gtase (10 mIU) was incubated with 2.5 mM **5** in the presence of 0.2 mg/mL of DT10 and increasing sucrose concentrations (3, 10 or 20 mM). The residual Gtase activity was determined as described above.

Acknowledgement

We thank Mrs Geneviève Aubert for her technical assistance.

References

[1] W.J. Loesche, *Microbiol. Rev.*, 50 (1986) 353–380.
[2] S. Hamada, T. Koga, and T. Ooshima, *J. Dent. Res.*, 63 (1984) 407–411.
[3] T. Koga, Y. Toda, I. Mora, and S. Hamada, *Zentralbl. Bacteriol. Microbiol. Hyg., Ser. A*, 269 (1988) 492–500.
[4] T. Koga, H. Asakawa, N. Okahashi, and S. Hamada, *J. Gen. Microbiol.*, 132 (1986) 2873–2883.
[5] M. Addy, M.A. Slayne, and W.G. Wade, *J. Appl. Bacteriol.*, 73 (1992) 269–278.
[6] P.D. Marsh, *Caries Res.*, 27 (suppl.1) (1993) 72–76.
[7] M.L. Shulman, S.D. Shiyan, and A.Y. Khorlin, *Biochim. Biophys. Acta.*, 445 (1976) 169–181.
[8] D. Horton and T. Miyake, *Carbohydr. Res.*, 184 (1988) 221–229.
[9] A. Hosomi, Y. Sakata, and H. Sukurai, *Carbohydr. Res.*, 171 (1987) 223–232.
[10] T. Baba, T. Ogawa, N. Okahashi, T. Yakushiji, T. Koga, M. Morimoto, and S. Hamada, *Carbohydr. Res.*, 158 (1986) 147–155
[11] R. Kitz and I.B. Wilson, *J. Biol. Chem.*, 237 (1962) 3245–3249.
[12] W.R. Figures and J.R. Edwards, *Carbohydr. Res.*, 48 (1976) 245–253.
[13] T.J. Grier and R.M. Mayer, *Arch. Biochem. Biophys.*, 212 (1981) 651–659.
[14] T.P. Binder and J.F. Robyt, *Carbohydr. Res.*, 124 (1983) 287–299.
[15] P.B. Hoj, E.B. Rodriguez, J.R. Iser, R.V. Stick, and B.A. Stone, *J. Biol. Chem.*, 266 (1991) 11628–11631, and references cited therein.
[16] H.M. Levy, P.D. Leber, and C.M. Ryan, *J. Biol. Chem.*, 238 (1963) 3654–3659.
[17] H. Abo, T. Matsumura, T. Kodama, H. Ohta, K. Fukui, K. Kato, and H. Kagawa, *J. Bacteriol.*, 173 (1991) 989–996.
[18] C. Kato and H.K. Kuramitsu. *FEMS Microbiol. Lett.*, 72 (1990) 299–302.
[19] J.F. Robyt and P.J. Martin, *Carbohydr. Res.*, 113 (1983) 301–315.
[20] G. Mooser, D. Shur, M. Lyou, and C. Watanabe, *J. Biol. Chem.*, 260 (1985) 6907–6915.
[21] G. Mooser and K.R. Iwaoka, *Biochemistry*, 28 (1989) 443–449.
[22] G. Mooser, S.A. Hefta, R.J. Paxton, J.E. Shively, and T.D. Lee, *J. Biol. Chem.*, 266 (1991) 8916–8922.
[23] C. Kato, Y. Nakano, M. Lis, and H.K. Kuramitsu, *Biochem. Biophys. Res. Commun.*, 189 (1992) 1184–1188.
[24] M.E. Tate and C.T. Bishop, *Can. J. Chem.*, 41 (1963) 1801–1805.
[25] S.B. Mohan, B.M. Newman, and J.A. Cole, *FEMS Microbiol. Lett.*, 5 (1979) 69–72.
[26] A.C. Kenny and J.A. Cole, *FEMS Microbiol. Lett.*, 16 (1983) 159–162.
[27] G.R. Germaine, C.F. Schachtele, and A.M. Chludzinski, *J. Dent. Res.*, 53 (1974) 1355–1360.
[28] H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, (1934) 658–666.