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SOME PROPERTIES OF AN ENDODEXTRANASE INHIBITOR FROM CONTINUOUS CULTURES OF *STREPTOCOCCUS SOBRINUS*

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Cell-free filtrates of *Streptococcus sobrinus*, cultured at low growth rate in the chemostat, contain a dextranase inhibitor that can completely inhibit the activity of *S. sobrinus* endodextranase. The range of conditions under which inhibition occurs, and the situations in which enzyme activity can reappear, have been examined in continuous cultures of strain 6715-13WT and the dextranase-deficient mutant 6715-13-201. A purified preparation of the inhibitor was specific for *S. sobrinus* dextranase, having no action on dextranases from other oral streptococci. The percentage inhibition of *S. sobrinus* dextranase varied with the enzyme concentration, and the complete inhibition of low amounts of enzyme indicated a very tight bond between the inhibitor and the enzyme.

KEY WORDS: Dextranase, dextranase inhibitor, *Streptococcus sobrinus*, tight-binding inhibition.

INTRODUCTION

The importance of the adhesion of bacteria to host surfaces as a first step in the pathogenesis of many infectious diseases is well established. Among the mutans group of streptococci, *S. sobrinus* is strongly implicated as a prime pathogen in dental caries,[1] and its ability to colonise on tooth surfaces[2] is greatly dependent on the structure of the glucans that are synthesized from sucrose. Glucans with predominantly α-(1 → 3)-linked sequences are water-insoluble and adhesive,[3] and these properties are important for the accumulation of *S. sobrinus* in dental plaque.[4,5] Non-cariogenic strains of oral streptococci convert sucrose into soluble, non-adhesive glucans having up to 90% of α-(1 → 6)-linked sequences, as in dextrans.[5]

Cariogenic strains of *S. sobrinus* and *S. mutans* produce extracellular endodextranase,[6,7] an enzyme that can alter the balance of the α-(1 → 6)- and α-(1 → 3)-linked chains, which determines the solubility and adhesiveness of the mixed-linkage glucans. Removal of most of the α-(1 → 6)-linked sequences by hydrolysis with dextranase gives a product that is more adhesive due to the increase in the proportion of α-(1 → 3)-linked sequences. Several species of mutans streptococci produce a dextranase inhibitor,[8] and this protein may also indirectly affect the structure of the glucans, by permitting the accumulation of those α-(1 → 6)-linked glucans that are characteristic products of non-cariogenic streptococci.

Information on the type of inhibition of *S. sobrinus* dextranase is necessary before a systemic approach can be made towards devising the most effective inhibitors for

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the dextranases found in dental plaque. Increasing attention is being directed towards the inhibition of target enzymes,[9] and tight-binding inhibitors show promise as therapeutic agents that have great specificity.[10,11] In this paper we describe the production and properties of the endogenous dextranase inhibitor of *S. sobrinus*. We have found that a characteristic of the inhibitor is its tight-binding, pH-independent inhibition kinetics.

MATERIALS AND METHODS

S. sobrinus strain K1-R, its derivatives 6715-13WT and the dextranase-deficient mutant 6715-13-201 were kindly provided by Dr. R.J. Fitzgerald, Veterans Administration Hospital, Miami, and a culture of *S. sobrinus* OMZ176 was received from Professor B. Guggenheim, University of Zurich. Strains of *Streptococcus cricetus* and *Streptococcus rattus* were from Dr. D.D. Zinner, University of Miami, and *Streptococcus downei* MFe28 were provided by Professor R.R.B. Russell, London Hospital Medical College.

Continuous culture of the streptococci, the preparation of dialysed cell-free filtrates, the assays of dextranase activity and the determination of the dry weight of cells were carried out as described previously.[12]

Quantitative assays of free dextranase inhibitor activity were made using *S. sobrinus* K1-R endodextranase[13] and dextran 2000 (Sigma Chemical Co.). One international unit (U) of dextranase inhibitor activity was defined as the amount required to reduce the activity of two international units of dextranase by 50%. The assay mixtures (0.5 ml), which usually contained 7 mU of dextranase and approximately 3.5 mU of inhibitor, were incubated with dextran (7.5 mg) in 0.05 M-sodium citrate buffer (pH 6.0) for 30 min at 35°C. The experiments, and the determinations, were generally repeated at least twice.

Qualitative surveys of oral streptococci for extracellular dextranase inhibitors were carried out by heating samples of concentrated cell-free filtrates in 2% sodium dodecyl sulphate (SDS) for 1 min at 100°, and applying them to SDS-polyacrylamide (7.5%) slab gels (1 mm) containing blue dextran (0.5%). After electrophoresis for 2 h at 100 V the gels were washed with 0.05 M-sodium citrate buffer (pH 6.0) and then incubated for a day at 35° in a solution (20 ml) of *S. sobrinus* K1-R dextranase (50 mU) in 0.2 M-sodium citrate buffer (pH 6.0) containing Triton X-100 (1%). The dextranase degraded all the blue dextran except in the regions where the reaction was inhibited, and dextranase inhibitor was thus located as blue bands on a clear background (Figure 1). As little as 0.15 mU of total inhibitor activity could be revealed in the sample applied to the gel. Inhibitors with activity against other dextranases were detected simply by incubation of identical gels with the appropriate enzyme.

Purification of Dextranase Inhibitor

A culture of *S. sobrinus* 6715-13-201 was grown in the chemostat at a dilution rate (D) of $0.35\,h^{-1}$ and pH 6.0. Fresh cell-free filtrate (1 litre) was concentrated to 100 ml in a hollow fibre concentrator fitted with an H1P10 cartridge (Amicon Corporation). Saturated ammonium sulphate (pH 6.3) was added to 75% of saturation, and the mixture was left at 4°C for 4 h, and then centrifuged for 10 min at 12,000 × *g*.

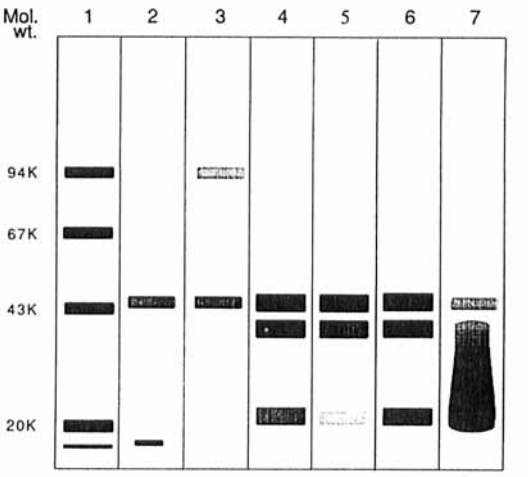

FIGURE 1 Separation of dextranase inhibitors from concentrated cell free filtrates by electrophoresis on SDS-polyacrylamide gels containing blue dextran. The inhibitors were detected after the gels were incubated with dextranase. Lane 1, Molecular weight markers; lane 2, *S. sobrinus* 6715-13 WT; lane 3, *S. cricetus* AHT; lanes 4 and 5, mutant 201 grown at pH 6 and pH 7 respectively; lanes 6 and 7, mutant 201 fresh and after storage respectively.

The pellet was washed with 75% saturated ammonium sulphate, then dissolved in 27 ml of sodium citrate buffer (0.05 M, pH 6.0) and dialysed against the same buffer.

A sample containing 44.5 U of dextranase inhibitor activity was dialysed for 1 h against potassium phosphate buffer (10 mM, pH 6.5), and then applied to a column (1.5 × 26.5 cm) of hydroxyapatite (Bio-Gel HTP) prepared in the same buffer. The column was eluted with 40 ml of the same buffer, and then a linear gradient of potassium phosphate buffer was applied at a flow rate of 10 ml per hour. Separation of the proteins was carried out at 5°C.

The fractions were assayed by the quantitative method, and those containing dextranase inhibitor activity were combined and dialysed against distilled water. Further purification was achieved by isoelectric focusing for 72 h at 5°C in an LKB column (110 ml) with a density gradient of glycerol, a pH gradient of 3–7 and a terminal voltage of 300 V. After draining the column, peak activity was found in the fraction with pH 3.8, and the inhibitor was recovered in 74% yield. Peak fractions were pooled and immediately set to dialyse for 24 h against three changes of 0.05 M-sodium citrate buffer (pH 6.0). The dialysed inhibitor was stable to freeze-drying, but the solution lost 25% of the inhibitor activity after storage at 4°C for 3 weeks.

Protein was determined with the Bio-Rad assay,[14] and the increase in specific activity following each fractionation step is shown in Table I.

RESULTS

Dextranase Inhibitor in Culture Filtrates of Mutans Streptococci

Determination of dextranase inhibitor activity in continuous cultures of *S. sobrinus* revealed that the release of inhibitor was markedly strain-dependent.[13] The

TABLE I
Purification of *S. sobrinus* dextranase inhibitor

Purification step	Inhibitor activity (Units)	Inhibitor activity (Yield, %)	Specific activity (Units mg protein^{-1})
Cell-free filtrate (1 l)	106	100	0.1
0–75% $(NH_4)_2SO_4$	89	84	3.2
Hydroxyapatite chromatography	37	35	30
Isoelectric focusing	27	26	100

"dextranase-negative" strain 6715-13-201 (mutant 201) was the best source of dextranase inhibitor at low growth rates. Comparison of the mutant with the wild type 6715-13WT (Table II) showed that the mutant was exceptional in producing dextranase inhibitor under all growth conditions tested in the chemostat. The productivity increased up to 7-fold as the dilution rate was raised from 0.05 to 0.45 h^{-1}. By contrast, the release of inhibitor by strain 6715-13WT fell to zero at high growth rate, in accord with the pattern established previously in continuous cultures of several strains of *S. sobrinus*.[13] Thus, active dextranase could always be found in cell free filtrates of *S. sobrinus* wild type strains cultured at high growth rate.

The assays of dextranase inhibitor and dextranase could be subject to error if both proteins were free in culture filtrates from the same condition of growth. It was therefore necessary to test this possibility by separating the enzyme and inhibitor from culture filtrates before determining their activities. For this experiment strain K1-R was grown at pH 6.5 under glucose limitation at low (0.075 h^{-1}) and high (0.45 h^{-1}) dilution rates. Cell-free filtrates (190 ml) were concentrated with hollow fibres, and the proteins were precipitated with ammonium sulphate (see Methods) prior to chromatography on hydroxyapatite. After the samples (5 ml) were applied, the columns were washed with one bed volume of 0.01 M potassium phosphate buffer (40 ml) and collection of the numbered fractions (Figure 2) began when the reservoir containing 0.40 M potassium phosphate buffer was connected to the gradient mixer. Assay of the fractions (4 ml) showed that the sample derived from the slowly growing culture (Figure 2a) contained only free inhibitor, which was eluted with 0.01 M phosphate in 96% yield. Dextranase was eluted, with 0.05 M phosphate, only from

TABLE II
Effect of growth rate (D) on the activity and apparent productivity of free dextranase inhibitor released by *S. sobrinus* strain 6715-13WT and the mutant strain 6715-13-201 grown at pH 6.5 under glucose limitation

	Strain 6715-13WT				Mutant 6715-13-201			
D (h^{-1})	Dry wt (gl^{-1})	Activity (Ul^{-1})	(Ug^{-1})	Productivity ($Ug^{-1}h^{-1}$)	Dry wt (gl^{-1})	Activity (Ul^{-1})	(Ug^{-1})	Productivity ($Ug^{-1}h^{-1}$)
0.05	0.75	23	31	1.5	0.76	184	242	12
0.30	1.10	19	17	5.2	0.79	100	126	38
0.45	1.14	0	0	0	0.89	162	182	82
Batch	1.03	0	0	0	0.87	159	183	

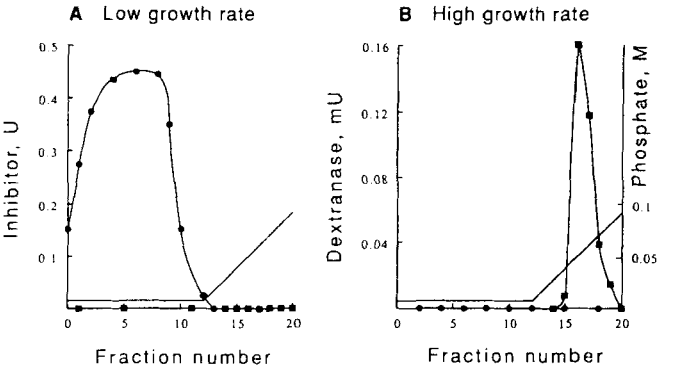

FIGURE 2 The effect of growth rate on the release of dextranase (■) and dextranase inhibitor (●) by *S. sobrinus* K1-R grown at A, D 0.075 h^{-1} and B, D 0.45 h^{-1}. The proteins were separated by chromatography on hydroxyapatite. ——, phosphate gradient.

the column to which extracellular proteins released at high growth rate had been applied (Figure 2b), and none of the fractions displayed free dextranase inhibitor activity. Apparently only one type of activity, either that of the enzyme or the inhibitor, was free to be determined under a particular condition of growth, and this result indicated that results obtained previously with unfractionated culture filtrates were reliable measures of free dextranase and free inhibitor.

The rapidity with which *S. sobrinus* adapted to an alteration in growth conditions was illustrated when a batch culture of strain 6715-13WT in the chemostat vessel was converted into a glucose-limited continuous culture regulated at a low growth rate at pH 6.5. Initially, the dextranase activity of the batch culture was 16.8 Ug^{-1}, and inhibitor activity could not be detected. Medium was then pumped through the vessel at a dilution rate of 0.05 h^{-1}, and after only one culture volume had been collected, a sample was already devoid of dextranase, whereas dextranase inhibitor activity was 13.5 Ug^{-1}. After the flow of two culture volumes, inhibitor activity (30 Ug^{-1}) was close to the steady state value obtained after 5 culture volumes.

The influence of glucose concentration on dextranase inhibitor production was examined with a culture of mutant 201 in a steady state at D 0.05 h^{-1} under glucose limitation at pH 6.5. The medium reservoir was replaced with one containing an excess of glucose (4%), and samples were collected at intervals during the transition to the new steady state (N-limited). The culture responded almost immediately by releasing increased amounts of dextranase inhibitor (Figure 3). After approximately one culture volume had been replaced with the new medium, the accumulated inhibitor began to wash out, and after five volumes a steady state was achieved. The transient overproduction of inhibitor was accompanied by a similar increase in cell density, and inhibitor productivity gradually fell from 17.4 $Ug^{-1}\,h^{-1}$ under glucose limitation to 3.5 $Ug^{-1}\,h^{-1}$ under N limitation. Production of dextranase inhibitor by *S. sobrinus* strains K1-R and OMZ176 was also lower in glucose-sufficient than in glucose-limited cultures.[13]

The ability of dextranase inhibitor to inhibit *S. sobrinus* dextranase activity completely was tested with samples taken from a glucose-limited continuous culture of *S. sobrinus* OMZ176 operating at D 0.05 h^{-1} and pH 6.0. As little as 20 μl of cell-free filtrate had a significant effect on dextranase activity (7 mU), and 150 μl was sufficient to inhibit the enzyme completely (Figure 4). In this way it was possible to determine

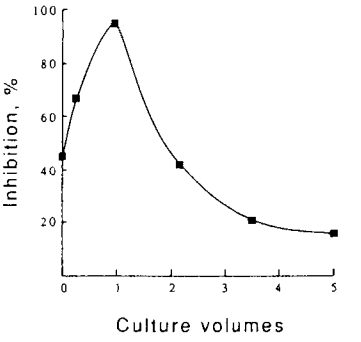

FIGURE 3 Inhibition of dextranase by increasing volumes of cell-free filtrates from a glucose-limited continuous culture of *S. sobrinus* OMZ176 grown at D 0.05 h^{-1}.

how much inhibitor was required to achieve complete inhibition of a sample of dextranase, and thus obtain a mixture that contained neither free inhibitor nor free dextranase activity.

Culture filtrates from other oral streptococci were tested for their ability to inhibit the activity of *S. sobrinus* K1-R dextranase. Although batch cultures of streptococci representing all serotypes of the mutans group were reported to produce dextranase inhibitor,[8] we found that only *S. downei* MFe28 (serotype *h*), and *S. cricetus* AHT and HS6 (serotype *a*) released inhibitor that was active against *S. sobrinus* dextranase. The results for *S. cricetus* AHT are shown in Table III. No inhibitor activity was found in culture filtrates of *S. rattus* BHT (serotype *b*), *S. salivarius* ATCC 13149 or *S. mitis* 53. *S. mutans* Ingbritt (serotype *c*) produced an inhibitor that was specific for *S. mutans* dextranase.

Qualitative tests carried out by the gel electrophoresis method (see Methods) revealed that concentrated cell-free filtrate from *S. sobrinus* K1-R, OMZ176 and mutant 201, and from *S. cricetus* AHT and HS6, contained inhibitors with slight activity against *Bacillus coagulans* endo-dextranase; these were well separated from the bands with activity against *S. sobrinus* dextranase. The gel method confirmed that none of the oral streptococci produced an inhibitor for *Penicillium funiculosum* dextranase.

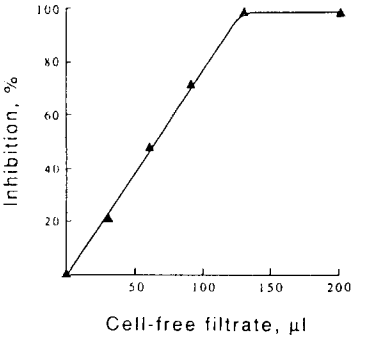

FIGURE 4 Inhibition of dextranase by dialysed cell-free filtrates (20 µl) of *S. sobrinus* 6715-13-201 during transition stages between two steady states at D 0.05 h^{-1}. The initial value was for a glucose-limited culture, then a glucose-sufficient medium was pumped in.

TABLE III
Productivity of a dextranse inhibitor, active against *S. sobrinus* K1-R dextranase, in continuous cultures of *S. cricetus* AHT grown at pH 6.5 under glucose limitation

Dilution rate (h^{-1})	Dry wt (gl^{-1})	Inhibitor activity (Ul^{-1})	Inhibitor activity (Ug^{-1})	Productivity ($Ug^{-1}\,h^{-1}$)
0.075	1.14	33	29	2.2
0.15	1.20	56	47	7.0
0.30	1.24	37	30	9.0
0.45	1.23	24	19	8.8

Effect of Various Conditions on the Activity of Dextranase Inhibitor Prepared from Mutant 201

The effect of preincubation of the inhibitor preparation with dextranase prior to the addition of dextran was investigated. Preincubation for up to 30 min gave a marked increase in the percentage inhibition of dextranase, while further preincubation resulted in a more gradual effect over 90 min (Figure 5). It was therefore convenient to set a preincubation time of 30 min for all determinations of inhibitor activity. Progress curves for dextran hydrolysis showed that the same rate of product formation was reached with or without preincubation of the inhibitor with dextranase.

The effect of variation in the pH of preincubation and digestion of dextran was examined between pH 4.6 and 6.8. This resulted in up to 7-fold variation in activity (Figure 6), but the percent inhibition of dextranase remained close to the average value of 49% over the pH range.

Preincubation of inhibitor with dextranase, and the subsequent incubation with dextran, was carried out at pH 6.0 over a range of temperatures from 15° to 45°C. Although this produced a 10-fold variation in activity, the inhibition of dextranase remained remarkably constant at *ca* 47% (Figure 7).

Specificity of the Inhibitor

The effect of purified inhibitor on dextranases from other sources was determined by substituting the enzymes for the *S. sobrinus* K1-R dextranase normally added to the

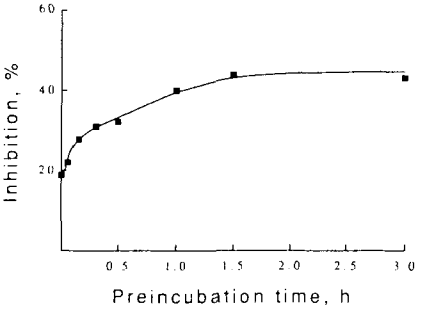

FIGURE 5 The effect on enzyme inhibition of preincubating the inhibitor (2.5 mU) with dextranase (7 mU) for various times before the addition of dextran. The values shown are the mean results of four experiments.

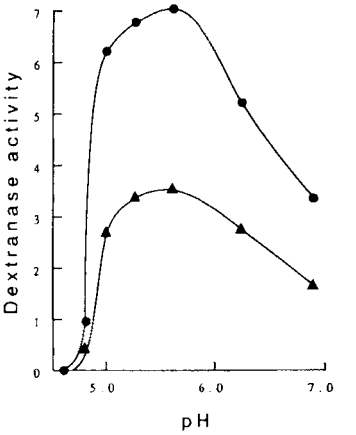

FIGURE 6 The effect of pH on the activity of dextranase (7 mU) in the presence (▲) and absence (●) of dextranase inhibitor (3.5 mU).

activity digests. The results indicated that the purified inhibitor was equally active towards dextranases from other strains of *S. sobrinus* (B13, OMZ176 and 6715-13WT). There was no inhibition of *S. mutans* Ingbritt endodextranase, nor of *S. mitis* exodextranase; dextranases from other bacteria (*B. coagulans*, *Arthrobacter globiformis* and *Flavobacterium sp.* M-73) and fungi (*P. funiculosum*, *Spicaria violaceae*, *Fusarium moniliforme* and *Chaetomium gracile*) were also totally resistant to inhibition.

Inhibition Characteristics

The type of inhibition of dextranase was studied in digests containing different concentrations of enzyme and substrate. The effect of variation in substrate concentration was investigated in a series of digests containing dextranase (7 mU), inhibitor

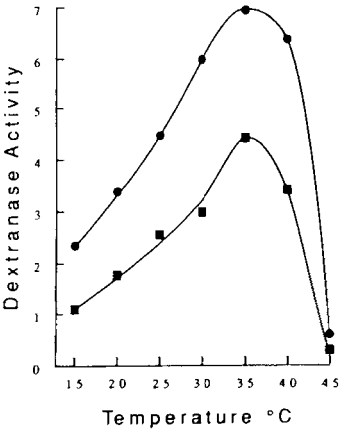

FIGURE 7 The effect of temperature on the activity of dextranase (7 mU) in the presence (■) and absence (●) of dextranase inhibitor (3.5 mU). The mixtures were buffered to pH 6.0

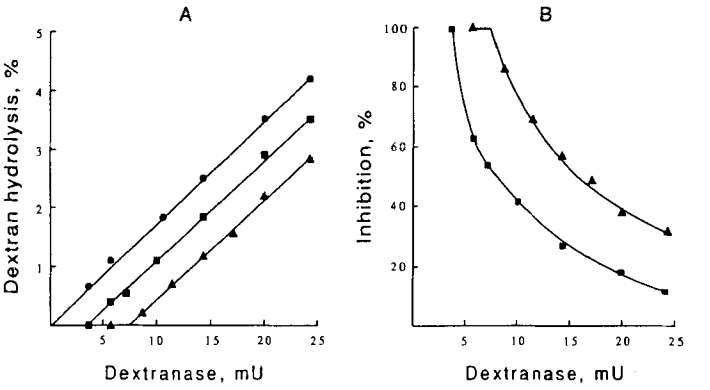

FIGURE 8 The effect of increasing dextranase concentration on dextran degradation (A) and on the percentage inhibition (B) in the absence (●) and presence of 3.5 mU (■) and 7.5 mU (▲) of dextranase inhibitor.

(3.5 mU) and from 0.4% to 2.0% dextran. This change in substrate concentration from the normal (1.5%) had no effect on the percentage inhibition, indicating that the inhibitor was binding to the enzyme at a different locus from the substrate.

The velocity of dextran hydrolysis as a function of total enzyme concentration was determined in the absence and presence of two concentrations of dextranase inhibitor. The incubation mixtures contained 0, 3.5 mU and 7 mU of inhibitor and from 3.5 mU to 28 mU of dextranase. A plot of the results (Figure 8a) gave two lines that were parallel to the line for the uninhibited reaction. Addition of the inhibitor had completely bound a portion of the enzyme, displacing the starting point of the reaction. The variation in percentage inhibition with enzyme concentration (Figure 8b) is indicative of a tight-binding inhibitor[10,11] capable of binding so firmly to dextranase that the complex would be difficult to dissociate into its components.

Evidence that the complex was in fact practically irreversible under natural conditions has been obtained. First, it was established that enzyme activity could not be recovered following dilution (up to 32-fold) of a mixture of enzyme and inhibitor. Secondly, a similar mixture failed to separate on a column of Sephadex G-50, and the yield of inhibitor was only 3% of that expected. Thirdly, when a mixture of dextranase and inhibitor was applied to a hydroxyapatite column, neither free inhibitor nor free enzyme was eluted from the column, whereas applied samples that contained either the inhibitor alone, or the enzyme alone, were eluted either with 0.01 M or with 0.05 M-potassium phosphate, respectively (*cf* also Figure 2). Finally, the mixture of enzyme and inhibitor did not dissociate into its individual components when treated with SDS at 25°C. Dissociation by boiling in SDS was required before the enzyme and dextranase inhibitor could subsequently be separated by SDS-PAGE.

Inhibition of the Hydrolysis of S. sobrinus *Soluble Dextran and Isomaltooctaose*

The standard assays of inhibitor activity determine the ability of *S. sobrinus* dextranase inhibitor to inhibit the hydrolysis of a commercial dextran with molecular weight 2×10^6 kDa derived from *Leuconostoc mesenteroides* NRRL B-512 (F). The dextran contains 5% of side chains attached with α-(1 → 3)-linkages. The effect of the inhibitor on the hydrolysis of substrates of lower molecular weight was examined with

a *S. sobrinus* linear dextran (d.p. 25–30) and an oligosaccharide of the isomaltose series (d.p. 8). In these assays the concentration of substrate was lowered to 0.3% to avoid high initial values for reducing power. Comparison of the results with those obtained with 0.3% of dextran 2000 revealed no significant difference between the percentage inhibition of hydrolysis of the three substrates.

DISCUSSION

The ability of *S. sobrinus* to produce extracellular inhibitor is not easy to observe when the organism is grown in batch culture. No free inhibitor is detected in cell-free filtrates during the exponential stage of growth, and it is only when the stationary stage is approached and the bacteria are growing very slowly, that some evidence of dextranase inhibition has been observed.[10] The precise effect of growth rate on the release of extracellular proteins can be determined in continuous culture, where microbial activity is measured in a steady state system. The dilution rate (D) is equal to the specific growth rate in the chemostat, and this is related to the mean generation time (t_d) by $D = (\log_e 2)/t_d$. Thus t_d can be controlled by adjusting D, which is the rate of input of fresh medium per volume of culture.

Determinations of free dextranase inhibitor activity in strains of *S. sobrinus*, which were grown in the chemostat under a variety of defined conditions, have shown that maximum release of dextranase inhibitor occurred at low growth rate (D 0.05 h^{-1}) in glucose-limited medium at pH 6.5.[13] This suggests that in dental plaque, where streptococci grow slowly under nutrient limitation, the conditions may be conducive to the production of dextranase inhibitor. By contrast, inhibitor activity was low or absent at high growth rate (D 0.45 h^{-1}), high glucose concentration and low pH. These conditions are identical to those obtained in batch cultures, where both the growth rate and the concentration of nutrients are high, and where, in the case of streptococci, the pH will fall, without control, to 4.6. Therefore, our results have clearly demonstrated the advantages of studying dextranase inhibitor production in continuous culture rather than in uncontrolled batch culture.

Quantitative assays of dextranase inhibitor in cell-free filtrates from continuous cultures of *S. sobrinus* have shown that all strains can produce inhibitor, and the present study has revealed that the mutant strain 6715-13-201 is an outstanding secretor compared with the wild types e.g. 6715-13WT (Table II). Qualitative SDS-gel electrophoresis studies with samples from various strains (Figure 1) have also demonstrated the exceptional abundance of inhibitor released by the mutant. Although fresh samples taken from the chemostat were always processed without delay, the electrophoretic separations gave multiple bands corresponding to inhibitors with molecular weights of 49, 40 and 25 kDa, respectively. Under some conditions, for instance growth at pH 7, the band at 49 kDa was predominant. A sample from *S. cricetus* AHT (serotype *a*) also gave a major band at 49 kDa and a minor band at 94 kDa. *S. cricetus* dextranase inhibitor was active against *S. sobrinus* (serotypes *d* and *g*) dextranases, in accord with the close relationships that exist between serotypes *a*, *d* and *g* of the mutans streptococci.

During storage at 4°C the inhibitors were gradually degraded to give inhibitory proteins of molecular weight 25 kDa. Even the partially purified inhibitor from mutant 201 was largely degraded to the lowest size, indicating that proteolysis had occurred during its preparation.

Kinetic Studies

The relationship between the concentration of inhibitor and the extent of inhibition of dextranase was linear up to an inhibition of 85% in mixtures containing dextranase (7 mU) and partially purified inhibitor (2–10 mU). With a classical type of reversible inhibitor, plots of product formation (in this case reducing oligosaccharides from dextran), against dextranase concentration should also be linear, going through the origin, but this was not the case with dextranase inhibitor (Figure 8a). As a result, the percentage inhibition depended on the enzyme concentration (Figure 8b), and this relationship is characteristic of tight-binding inhibition.[10,11] In practice, the reaction was always initiated by the addition of dextran following preincubation of the enzyme with inhibitor. Determinations of inhibitor activity were always carried out at a constant initial concentration of dextranase (7 mU) per assay.

Tight-binding inhibitors cause inhibition of enzymes at concentrations that are comparable to that of the enzyme. A considerable reduction in the concentration of inhibitor could therefore result from the formation of the enzyme-inhibitor (EI) complex,[10] and it cannot be assumed that the activity of free inhibitor in cell-free filtrates is equal to the total amount of inhibitor released by *S. sobrinus*.

Because a significant proportion of dextranase and inhibitor may be bound into an EI complex, it is clear that the productivities of dextranase and dextranase inhibitor that have been determined for *S. sobrinus* under different conditions of growth, refer only to the proportions that are free. At low growth rates, although the activity of inhibitor was high, more inhibitor could be bound in the EI complex. In that case, dextranase, hitherto detected only at high growth rates, would also have been produced, but none of it would be free. At high growth rates, when productivity of free dextranase was maximal, if more dextranase were bound into the EI complex, again it would follow that inhibitor had been produced, but none would be free. It is noteworthy that with *S. cricetus* AHT, a strain that produces no dextranase, the productivity of inhibitor did not fall as the growth rate was increased (Table III). It is probable that in this example the inhibitor could remain free because there was no dextranase to bind to it. A different explanation could be advanced for the so-called 'dextranase-deficient' mutant 201, where the excessive production of inhibitor released under all conditions (Table II) would be more than sufficient (e.g. 182 Ug^{-1} at D 0.45 h^{-1}) to complex with the amounts of dextranase normally released in continuous culture by *S. sobrinus* (up to 12 Ug^{-1} at D 0.50 h^{-1}).

To test whether significant amounts of enzyme and inhibitor are bound together in culture filtrates of *S. sobrinus* isolates, the EI complex should be dissociated into its components. This can be difficult to accomplish with tight-binding inhibitors. Reversibility has been demonstrated in other systems by diluting the solution or by separating the components by molecular sieve chromatography. Neither of these methods was successful with the dextranase-inhibitor complex. With some tight-binding inhibitors it has been mandatory to compete the inhibitor off with another ligand.[15] This would not be easy to do with a specific protein inhibitor. Despite these difficulties, the complex must be dissociated before the total (free + combined) activity of the enzyme and inhibitor can be determined.

The most obvious role for *S. sobrinus* dextranase is to hydrolyse the α-(1 $\rightarrow$ 6)-linked sequences in the mixed-linkage glucans that are synthesized from sucrose by the concerted action of up to four different extracellular α-D-glucosyltransferases.[3] When the dextran-like chains are removed, the resulting limit glucan is water-insoluble

and adhesive, having become a predominantly α-(1 → 3)-linked glucan. This type of polysaccharide is considered to be an important factor in the adherence and cariogenicity of *S. sobrinus*. Our quantitative results show that, due to the production of dextranase inhibitor, *S. sobrinus* would release little or no free dextranase when growing slowly in dental plaque. Thus the noncariogenic soluble dextran which is the product synthesized by the major glucosyltransferase (GTF-S) released at low growth rate, should be stable in resting plaque. However, in response to a sudden increase in the supply of nutrients (*cf* high growth rate in the chemostat), this organism can promptly realise its potential to produce dextranase[12] and also to produce more[3] of the glucosyltransferase (GFT-I) that catalyzes the synthesis of α-(1 → 3)-glucan. The strains of *S. sobrinus* that we have examined do not produce enough dextranase inhibitor at high growth rate to bind all the free dextranase, and therefore the glucan produced when the streptococci are growing rapidly, for instance at meal times, is mainly the adhesive type that promotes the adherence of *S. sobrinus*.

The properties of dextranase inhibitor, namely its stability, pH- and temperature-independence, specificity and inhibition characteristics, make it a suitable candidate for a therapeutic agent. Addition of dextranase inhibitor in adequate quantity to achieve the complete inhibition of dextranase even at high growth rate, should result in the glucans synthesized by GFT-S and GFT-I having a high enough proportion of dextran chains to be rendered soluble or non adhesive. This could be a sufficient strategy to disturb the adherence mechanism of *S. sobrinus* and thus to reduce its colonisation on teeth.

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