Metabolism of the Polysaccharides of Human Dental Plaque: Release of Dextranase in Batch Cultures of *Streptococcus mutans*

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Dextranase activity was determined in cell extracts and cell-free filtrates of *Streptococcus mutans* strains which had been grown in batch culture. Exo-dextranase activity was located chiefly in cell extracts, whereas endodextranase was mainly extracellular. Release of endo-dextranase began early in the exponential phase of growth, and ended when the concentration of residual sugar was low. Thus, dextranase expression was associated with rapidly growing cells, and the yield of dextranase was increased several fold when the initial concentration of D-glucose in the medium was changed from 0.5% to 2%. The endo-dextranase was not stable at pH 5, and control of the pH of the culture was essential to preserve active dextranase during overnight growth. Strain Ingbritt (serotype c) and serotype d strains were the best dextranase producers; other strains (serotypes a, b, c, e and f) displayed much lower activity. The ability to produce endo-dextranase, and to synthesize α -D-glucans with a high proportion of (1→3)-linked sequences, appeared to be related properties. The possibility is discussed that the release of two enzymes, namely endo-dextranase and the D-glucosyltransferase (GTF-I) that synthesizes (1→3)- α -D-glucan, are factors that contribute to the cariogenicity of *S. mutans* serotype d.

INTRODUCTION

A significant proportion of bacteria in human dental plaque produce enzymes capable of hydrolysing (1→6)- α -D-glucosidic linkages in dextran (Staat *et al.*, 1973). An investigation into the occurrence of intracellular dextranases in batch cultures of human oral streptococci (Dewar & Walker, 1975) revealed that over 33% of the strains contained 1,6- α -D-glucan glucohydrolase, an exo-dextranase (EC 3.2.1.70) previously reported in *Streptococcus mitis* (Walker & Builder, 1967). The enzyme was capable of releasing D-glucose from dextrans (Walker & Pulkownik, 1973) and from oligosaccharides derived from dextrans (Walker & Pulkownik, 1974). Several strains, later classified as *Streptococcus mutans* (Hare, 1977) produced an endo-dextranase (EC 3.2.1.11), that released oligosaccharides of the isomaltose series from dextran. Some of the *S. mutans* strains produced both types of dextranase. Water-insoluble glucans produced from sucrose by *Streptococcus sanguis* and *S. mutans*, which contain a proportion of (1→6)-linked sequences of D-glucose residues, were also substrates for *S. mutans* endo-dextranase (Hare *et al.*, 1978).

Many reactions of *S. mutans* are greatly influenced by small amounts of dextran. These include the aggregation of the cells (Gibbons & Fitzgerald, 1969), their adherence to hard surfaces (Gibbons & Van Houte, 1973), the synthesis of extracellular glucans from sucrose (McCabe & Smith, 1973), the distribution of D-glucosyltransferases between cells and culture filtrate, and the dextran-binding capacity of the cells (Spinell & Gibbons, 1974). Such reactions would be sensitive to low levels of dextranase, and it is therefore important that

dextranase production by *S. mutans* be assessed correctly. It can be inferred from discrepant reports on *S. mutans* dextranase that one or more factors may make it difficult to determine its activity accurately. The existence of the enzyme has variously been acknowledged (Guggenheim & Burkhardt, 1974; Germaine *et al.*, 1977), denied or simply neglected. We have investigated the release of endo-dextranase by *S. mutans* grown in batch culture under conditions commonly employed in different laboratories. The results indicate that it is not feasible to expect consistent values for dextranase activity to emerge from batch growths.

METHODS

Organisms and growth conditions. *Streptococcus mutans* strains AHT and BHT were received from D. D. Zinner, FA-1 and K1-R from R. J. Fitzgerald, JC2 from J. Carlsson, GS-5 from A. Bleiweis, Ingbritt from B. Krasse, B13 from S. Edwardsson, OMZ176 from B. Guggenheim, LM7 from L. A. Thomson, and OMZ65 and OMZ175 from D. Bratthall.

The organisms were grown in a medium (Van Houte & Saxton, 1971) that contained (per l distilled water): 20 g Trypticase Peptone, 5 g yeast extract, 4 g K_2HPO_4 , 1 g KH_2PO_4 , 2 g NaCl, and usually 20 g D-glucose or D-fructose. Dialysed medium consisted of that fraction of the complete medium that passed through an Amicon hollow-fibre filter cartridge. Bacteria were grown under anaerobic conditions (N_2/CO_2 , 95:5) at 37 °C in 350 ml medium in a 1 l capacity New Brunswick Multigen model F-1000 fermenter; the pH of the culture was maintained at 6.0 ± 0.05 by automatic addition of NaOH.

Fractionation of cultures. Samples of the culture were cooled to 4 °C and centrifuged for 10 min at 12000 g. Portions (about 20 ml) of the cell-free filtrate were dialysed against 50 mM-sodium citrate buffer (pH 6), with two changes of buffer over a 20 h dialysis period. The cells were washed twice with the same buffer, resuspended in one-seventh of the original volume of the culture sample, and disintegrated by shaking with 0.1 mm diam. glass beads (Glasperlen; Braun, Melsungen, F.R.G.) in a cell homogenizer (MSK; Braun) for 3 min at 2 °C. The beads were filtered off, and the filtrate was centrifuged at 34800 g for 10 min to yield a soluble extract which was dialysed against 50 mM-sodium citrate buffer (pH 6.0).

Determination of dextranase activity. Endo-dextranase activity of dialysed cell-free filtrates and cell extracts was determined from the release of reducing sugars in digests (1 ml) that contained 15 mg dextran (Sigma, Type 2000) and an appropriate volume of enzyme, usually 0.5 ml, in 25 mM-sodium citrate buffer (pH 6.0). Samples were taken at intervals for up to 24 h for the determination of reducing power with neocuproine reagent (Dygart *et al.*, 1965). One unit of activity was defined as the amount of enzyme that released 1 μ mol reducing power min^{-1} under the conditions of the assay.

Exo-dextranase activity of cell-free filtrates and cell extracts was determined by measuring the release of D-glucose from isomaltose as described previously (Dewar & Walker, 1975). One unit of activity was defined as the amount of enzyme that released 1 μ mol D-glucose min^{-1} .

Determination of dry weight of cells. Triplicate portions of the culture (5 ml) were centrifuged and washed once with sodium citrate buffer as described above. The pellets were then washed with water and dried to constant weight at 90 °C. The activities of cell extracts and cell-free filtrates were converted from units l^{-1} to units per g dry wt of the cells with which they were originally associated.

Analysis of S. mutans α -D-glucans. Methylation analysis and determination of the limit of hydrolysis by endo-dextranase were as described by Hare *et al.* (1978).

RESULTS

Location of endo- and exo-dextranases

The distribution of the two dextranases between cells and cell-free filtrates of several strains of *S. mutans* was determined. The organisms were grown in complete medium supplemented with 2% (w/v) glucose with the pH controlled at 6.0, and harvested in the stationary phase of growth. The bulk of the exo-dextranase (74–79%) was present in the cell extracts, whereas the major portion of the endo-dextranase (60–90%) was extracellular (Table 1). No glucose was released during the incubation of the cell-free filtrates with dextran in the activity tests, so the initial stages of dextran degradation were mediated entirely by endo-dextranase. The results that follow therefore refer to the endo-dextranase activity of culture filtrates.

Table 1. *Distribution of endo-dextranase and exo-dextranase activities in batch cultures of Streptococcus mutans*

Activities are expressed as units (g dry wt cells)⁻¹. One unit of endo-dextranase releases 1 μ mol reducing power from dextran min⁻¹, and one unit of exo-dextranase releases 1 μ mol D-glucose from isomaltose min⁻¹.

Strain	Endo-dextranase		Exo-dextranase	
	Cell extract	Filtrate	Cell extract	Filtrate
K1-R	2.1	16.0	4.6	1.6
B13	4.2	5.9	6.4	1.6
Ingbritt	0.5	6.1	3.5	1.5
OMZ176	6.7	11.0	0.5	0.14

Fig. 1

Fig. 2

Fig. 1. Release of endo-dextranase activity (●) in batch culture of *S. mutans* strain OMZ176 in complete medium supplemented with D-glucose (2%, w/v). The pH of the culture (▲) was allowed to fall as a result of acid production by the bacteria. Samples were withdrawn at intervals for determination of culture density (A_{600}) (■), dry weight of the cells (not shown), and residual glucose (% w/v) (○).

Fig. 2. Release of endo-dextranase activity (●) in batch culture of *S. mutans* strain Ingbritt in complete medium supplemented with D-fructose (2%, w/v). The pH of the culture was controlled at 6.0 ± 0.1 . Samples were withdrawn at intervals for determination of culture density (A_{600}) (■), dry weight of the cells, and residual fructose (% w/v) (○).

Effect of growth at controlled pH on endo-dextranase activity of cell-free filtrates

Extracellular endo-dextranase activity of *S. mutans* strain OMZ176 was measured at intervals during growth in complete medium supplemented with 2% glucose, without pH control (Fig. 1). The dextranase activity increased during the early-exponential phase of growth but began to decline when the pH had fallen to 5 (about 4 h after inoculation). Thereafter the rate of glucose utilization was low, and the dextranase activity had fallen to zero after a further 4 h. Qualitatively similar results (not shown) were obtained with strain Ingbritt grown under the same conditions; with this strain the pH had fallen to 5 by about 6 h after inoculation and dextranase was undetectable 10 h after inoculation.

Table 2. *Extracellular endo-dextranase activity of different Streptococcus mutans serotypes grown at pH 6 in medium supplemented with D-glucose or D-fructose (2%)*

Strain	Serotype	Glucose medium			Fructose medium		
		Culture density (g dry wt l ⁻¹)	Dextranase activity*		Culture density (g dry wt l ⁻¹)	Dextranase activity*	
			Units l ⁻¹	Units (g dry wt) ⁻¹		Units l ⁻¹	Units (g dry wt) ⁻¹
AHT	<i>a</i>	1.72	0	0	0.90	0	0
FA1	<i>b</i>	2.74	0.15	0.06	2.65	1.4	0.53
BHT	<i>b</i>	2.50	0.23	0.09	2.63	0.72	0.27
JC2	<i>c</i>	1.41	0.57	0.40	1.38	1.2	0.87
GS5†	<i>c</i>	1.38	1.0	0.72	1.36	2.4	1.76
Ingbritt	<i>c</i>	1.34	10.3	7.7	1.26	23.5	18.7
B13	<i>d</i>	1.42	3.4	2.4	1.35	10.6	7.9
K1-R	<i>d</i>	1.55	25.0	16	—	—	—
OMZ176	<i>d</i>	2.02	22.2	11	—	—	—
LM7†	<i>e</i>	0.45	0.4	1	0.82	0.4	0.5
OMZ175†	<i>f</i>	1.48	0.8	0.5	1.23	0	0
OMZ65†	<i>g</i>	1.44	0	0	1.33	0	0

* One unit of activity is the amount of enzyme that releases 1 μ mol reducing power min⁻¹.

† Results for these strains are from a single culture which was not repeated. In all other cases, similar results were obtained from up to 10 replicate cultures.

When *S. mutans* strain Ingbritt was grown in batch culture controlled at pH 6, in complete medium supplemented with 2% fructose (Fig. 2), dextranase was released during exponential growth, and once the maximum activity had been reached [16.5 units l⁻¹; 10.2 units (g dry wt cells)⁻¹] there was no subsequent fall up to 24 h after inoculation. Similar results (not shown) were obtained in medium supplemented with 2% glucose [maximum activity 14.5 units l⁻¹; 8.2 units (g dry wt cells)⁻¹]. These results may be compared with the maximum, but transitory, values of 6.5 units l⁻¹ and 6.0 units (g dry wt cells)⁻¹ obtained with strain Ingbritt in glucose medium without pH control.

Growth in medium supplemented with sucrose

During growth in media containing sucrose, *S. mutans* extracellular D-glucosyltransferases synthesize α -D-glucans which bind to dextran receptors on the cell surface (McCabe, 1976). Under this condition it was possible that dextranase might also become cell-associated because of its affinity for its own substrate. However, the distribution of dextranase activity of Ingbritt cells and culture filtrate was not altered when sucrose replaced glucose in the medium. Dextranase activity increased throughout the exponential phase of growth, finally reaching a value of 11.5 units l⁻¹. Thus, growth conditions under which D-glucosyltransferases became cell-associated did not influence the release of dextranase, and 95% of the total dextranase activity was found in the cell-free filtrate.

Dextranase activity of S. mutans strains grown in medium supplemented with D-glucose or D-fructose

Strains chosen from the seven identified serotypes of *S. mutans* were grown in dialysed medium with the pH controlled at 6.0 and harvested early in the stationary phase. Extracellular dextranase activity was generally about twofold higher in fructose medium than in glucose medium. Representative strains of serotype *d* clearly displayed the highest dextranase activity, and only one strain (Ingbritt) from serotype *c* showed comparable activity (Table 2). Serotype *b* strains released less enzyme than those of serotype *c*, and the single representatives of serotypes *a*, *e*, *f* and *g* showed little or no activity under these conditions.

Table 3. *Effect of medium composition on the release of dextranase activity*

Strain	Medium	Glucose (%)	Dextranase activity [units (g dry wt cells) ⁻¹]
Ingbritt	Dialysed	2	7.7
Ingbritt	Complete	2	8.2, 8.5, 6.1
Ingbritt	Complete	0.5	0.70, 0.4, 1.6
K1-R	Complete	2	16
K1-R	Complete	0.5	4.9
OMZ176	Complete	2	15, 11, 8
OMZ176	Complete	0.5	2.2, 0

Effects of glucose concentration on dextranase release

A reduction in glucose concentration from 2% to 0.5% resulted in a fall of up to 90% in dextranase activity per g dry wt of cells (Table 3). At the lower concentration of glucose there was more variability in the activity measured in different batches, and on one occasion, strain OMZ176, one of the best dextranase producers, was devoid of activity. Replacement of complete medium with dialysed medium did not affect the yield of dextranase.

DISCUSSION

A detailed examination of the specificity and action pattern of *S. mutans* endo-dextranase proved that the main hydrolysis products from dextran were isomaltotriose, isomaltotetraose and isomaltopentaose (Pulkownik & Walker, 1977, Pulkownik *et al.*, 1978). D-Glucose was not a product of endo-dextranase action. In contrast, glucose is the only product of dextran hydrolysis by the exo-dextranase which is formed by some strains of *S. mutans*. This specificity clearly distinguished between the two types of dextranase. The exo-enzyme was mainly intracellular, and its activity in the culture filtrate was so low that its activity could only be determined by measuring the rate of release of glucose from isomaltose: no release of glucose was detected when isomaltose was replaced by dextran which is a poorer substrate for the enzyme (Dewar & Walker, 1975). Dextran was used as the substrate for the experiments described in this paper, so only the activity of the endo-enzyme has been studied. The endo-dextranase activity of intracellular extracts of several strains of *S. mutans* was reported earlier (Dewar & Walker, 1975), and we have now presented values for the extracellular endo-dextranase of selected strains.

Strains from serotypes *c* and *d* were the most active producers of dextranase. The enzyme was released early in the exponential phase of growth, and dextranase production was clearly a property associated with rapidly growing cells. Any conditions that were not conducive to rapid growth, such as a low concentration of glucose or a pH below 5, gave a significantly lower enzyme activity (Table 3; Fig. 1). Unless the pH of the culture was controlled, extracellular enzyme produced early in the exponential phase became completely inactivated by the adverse conditions so that the activity could not be detected when the culture reached the stationary phase. Dextranase was absent in cultures of *S. mutans* growing slowly under glucose limitation at a mean generation time of 14 h (Walker *et al.*, 1980). The relationship between growth rate and dextranase production should therefore be studied under defined conditions such as can be obtained in a chemostat, which is an ideal system for studying a growth rate-linked property. In the closed system of batch culture where the environmental conditions are essentially variable, it has not proved possible to obtain completely consistent results for dextranase activity between batches. Nevertheless, by controlling the pH, by providing sufficient glucose and by harvesting near the end of the exponential phase, we have shown that activities up to 25 units (1 cell-free filtrate)⁻¹ can be obtained. It has long been known that addition of dextranase to incubation mixtures

Table 4. *Relationship between the dextranase activity of oral streptococci, the methylation analysis of their α -D-glucans, and the hydrolysis of water-insoluble glucans by the endo-(1 $\rightarrow$ 6)- α -D-glucanase of Bacillus coagulans NRRL B-3949*

The methylation analysis and the determination of the limit of hydrolysis by endo-dextranase were as described by Hare *et al.* (1978).

Source of glucan	Linkage analysis			Hydrolysis (%)
	Methyl ether (mol %)		Linkage Ratio (1→6)/(1→3)	
	2,3,4-Tri-O-Me-D-Glc	2,4,6-Tri-O-Me-D-Glc		
<i>S. mutans</i> serotype <i>d</i> (high dextranase)				
K1 R	11	64	0.17	5
OMZ176	18	57	0.31	22
B13	11	64	0.17	3
<i>S. mutans</i> serotype <i>c</i> (moderate dextranase)				
Ingbritt	36	53	0.67	31
NSW1	32	51	0.63	38
NSW47	39	38	1.0	41
<i>S. sanguis</i> (no dextranase)				
804	49	15	2.3	62
ATCC 10558	47	17	2.8	53
NSW13	48	19	2.5	39

containing sucrose and D-glucosyltransferases will strongly inhibit the synthesis of D-glucans (Walker, 1972; Schachtele *et al.*, 1975). The possibility that indigenous dextranases of *S. mutans* will similarly interfere with glucan synthesis by D-glucosyltransferases is nevertheless often neglected (Ebisu *et al.*, 1974), and even when the presence of dextranase and its effect on glucose metabolism have been acknowledged, the actual amount of dextranase activity in the D-glucosyltransferase preparations is generally not quoted in defined units (Germaine *et al.*, 1977). Schachtele *et al.* (1975) have shown that 5 units of dextranase l⁻¹ reduced the adherence of *S. mutans* cells to glass by 80%, and as little dextranase as 2 units l⁻¹ inhibited by 60% the synthesis of D-glucans from sucrose by crude *S. mutans* glucosyltransferase. Hence under optimum conditions, *S. mutans* cells release more than enough dextranase to inhibit the synthesis of α -D-glucans by D-glucosyltransferases.

A comparison was made between the proportion of (1 $\rightarrow$ 6)- and (1 $\rightarrow$ 3)-linked glucose sequences in glucans synthesized from sucrose by cell-free filtrates from various strains of *Streptococcus* spp., and the dextranase activity of each culture filtrate (Table 4). This provided circumstantial evidence for the ability of dextranase to modify the structure of the glucans. Strains from *S. mutans* serotype *d*, which were the best dextranase producers, gave the lowest values for the ratio of (1 $\rightarrow$ 6)- to (1 $\rightarrow$ 3)-linked sequences, which ranged from 0.17 to 0.31. Strains from serotype *c*, which released less dextranase, gave values in the range 1.0 to 1.6, whereas *S. sanguis* strains, which produce little or no dextranase, gave the highest values (from 2.3 to 2.8). The glucans produced by serotype *d* organisms were the most resistant to further hydrolysis by exogenous endo-dextranase (Table 4), in accord with the probability that the glucans were modified by indigenous dextranase so that most of the (1 $\rightarrow$ 6)-linked sequences were already removed. In contrast, the glucans of *S. mutans* serotype *c* and *S. sanguis* were more susceptible to hydrolysis by added dextranase. The glucans of *S. sanguis*, which were synthesized in the absence of indigenous dextranase, contained the highest proportion of (1 $\rightarrow$ 6)-linked glucose sequences, and were thus subject to extensive hydrolysis by exogenous dextranase (up to 62%).

The cariogenic potential of oral streptococci is strongly correlated with the production of

water-insoluble glucans that contain a high proportion of (1→3)-linked sequences (Gibbons & Van Houte, 1975*a*), and this requires the participation of a specific D-glucosyltransferase designated GTF-I (Walker & Hare, 1977). However, since *S. mutans* strains produce a second glucosyltransferase (GTF-S) that is responsible for the synthesis of (1→6)-linked sequences (Fukui *et al.*, 1974; Kuramitsu, 1975; Walker & Hare, 1977), it is clear how the production of indigenous dextranase could also be a caries-conducive property. By decreasing the proportion of (1→6)-linked sequences, the dextranase contributes towards the production of a predominantly (1→3)-linked glucan. This would explain why dextranase-deficient mutant strains of *S. mutans* serotypes *c* and *d* produced virtually no smooth surface carious lesions in rats, compared with the severe effect of the wild-type strains (Tanzer *et al.*, 1977).

Streptococcus sanguis produces copious amounts of a predominantly (1→6)-linked glucan, and although it is far more abundant than *S. mutans* in dental plaque, it is apparently not capable of inducing caries in germ-free rats (Green *et al.*, 1974). It is the proportion of *S. mutans* on human teeth that correlates to a high degree with caries activity (Gibbons & Van Houte, 1975*b*). Although *S. mutans* serotype *c* strains have received the most attention because of their prevalence, two recent studies have indicated the importance of serotype *d* strains. Sidaway (1979), by accurate sampling of microscopically identified areas of tooth surface, found that serotype *d* strains appeared more frequently than serotype *c* in smooth-surface lesions of primary teeth. Huis in't Veld *et al.* (1979) in a study of the distribution of different serotypes in human dental plaque, found that serotype *d* was predominantly present in approximal plaque of adults with active caries. These results support our conclusion that the caries-conducive properties of *S. mutans* serotype *d* merit increased attention.

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