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PURIFICATION AND SUBSTRATE SPECIFICITY OF AN ENDO-DEXTRANASE OF *Streptococcus mutans* K1-R

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ABSTRACT

An extracellular endo dextranase has been isolated from *Streptococcus mutans* K1-R Incubation of cell-free culture fluid with sucrose permitted the removal of a large proportion of the extracellular D-glucosyltransferases by irreversible adsorption onto the insoluble glucans that these enzymes synthesize from sucrose The remaining D-glucosyltransferases were separated from dextranase by precipitation with ammonium sulphate, chromatography on hydroxylapatite and DEAE-cellulose, followed by filtration on Ultrogel The major products of action of the purified dextranase on (1→6)-α-D-glucans were isomaltotriose (IM_3), isomaltotetraose (IM_4), and isomaltopentaose (IM_5) Further hydrolysis of IM_4 and IM_5 occurred after prolonged incubation with excess of enzyme, to give D-glucose, IM_2, and IM_3 The relative rate of hydrolysis of isomaltose saccharides fell sharply with decreasing chainlength from IM_{12} to IM_5 The hydrolysis of dextrans containing 96% or more of (1→6)-α-D-glucosidic linkages, expressed as apparent conversion into IM_3, was virtually complete, and substrates such as *Streptococcus sanguis* glucan, containing sequences of (1→6)-α-D-glucosidic linkages, were also effectively hydrolyzed Dextranase activity towards the soluble glucan of *Streptococcus mutans* was limited and there was no action on the insoluble glucan synthesized by *S mutans* sucrose 3-D-glucosyltransferase

INTRODUCTION

The significance of dextran as a factor in dental caries arises from its role in the adherence and colonisation of *Streptococcus mutans* on tooth surface[1] Certain reactions of *S mutans* are greatly influenced by extremely small amounts of dextran Addition of trace amounts of dextran to the growth medium promotes cell-associated D-glucosyltransferase activity of *S mutans*[2], and the dextran-binding capacity of the cells is increased A few nanograms of dextran of high molecular weight is sufficient to agglutinate the cells[3], and the same effect has been demonstrated between *S mutans* dextran and *Actinomyces viscosus* cells[4] Synthesis of glucans by the extracellular D-glucosyltransferases of *S. mutans* is greatly accelerated by a few micrograms

of dextran, which not only acts as a primer for the reaction[5], but also prevents adsorption of the enzyme to the newly synthesized, insoluble glucan[6]

In view of these different effects produced by dextran, a better understanding of the role of dextranases released from some micro-organisms found in dental plaque, particularly *S mutans*, is required Most, if not all, strains of *S mutans* produce extracellular dextranase[7–9], but the isolation of this enzyme free from D-glucosyltransferase has not been reported The pattern of glucan synthesis by streptococcal cells[10] and by their isolated D-glucosyltransferases[11] is altered both by added dextranase and by the reaction products of dextranase, furthermore, adherence of *S mutans* to various surfaces is strongly inhibited by dextranase[12] None of these experiments was carried out with the indigenous dextranase of *S mutans* The effect of variation in growth conditions on the relative proportion of dextranase and D-glucosyltransferases produced by *S mutans* is not known, but when dextranase is released, some modification of the sequelae of D-glucosyltransferase activity must follow In particular, the reactions that are mediated by trace amounts of dextran would be sensitive to low levels of dextranase

Guggenheim and Burckhardt[8] commented on the close association of a D-glucosyltransferase with *S mutans* dextranase, and on the difficulty of its removal from dextranase by absorption or gel chromatography Yet the separation of these two enzymes is essential, for D-glucosyltransferase cannot be assayed correctly in the presence of dextranase, nor can the effects of this dextranase on D-glucosyltransferases be studied unless the dextranase itself is free from D-glucosyltransferase activity

We now report a procedure for the isolation of endo-dextranase from the cell-free filtrate of *S mutans* K1-R, in which the complete removal of D-glucosyltransferase from the dextranase has been achieved

MATERIALS AND METHODS

Substrates — Dextran 2000 (mol wt 2×10^6) was purchased from Sigma Chemical Co, St Louis Mo, U S A A chemically synthesized, linear dextran (d p 100–150) was a gift from Professor C Schuerch[13] This dextran was synthesized at State University College of Forestry under research grant GM06168 from the Division of General Medical Sciences, National Institutes of Health Dextrans from *Leuconostoc mesenteroides* strains NRRL B-512(F) and NRRL B1355 (fraction L) were kindly provided by Dr A Jeanes The streptococcal glucans were synthesized by incubating sucrose (4%) for 24 h with (*a*) crude, culture fluid of *Streptococcus sanguis* 804, (*b*) a D-glucosyltransferase[14] isolated from the culture fluid of *S mutans* OMZ 176 by fractionation with ammonium sulphate followed by chromatography on hydroxylapatite; (*c*) combined fractions of *S mutans* K1-R D-glucosyltransferase eluted with potassium phosphate buffer (0 28M) from the hydroxylapatite column described in this paper (Fig 1) The three glucans were synthesized under dextranase-free conditions The insoluble glucans from (*a*) and (*c*) were washed with water (x 6), and then freeze-dried The soluble glucan from (*b*) was precipitated and washed with

60% ethanol, dissolved in water, then reprecipitated and washed with 75% ethanol, and a solution in water was freeze-dried

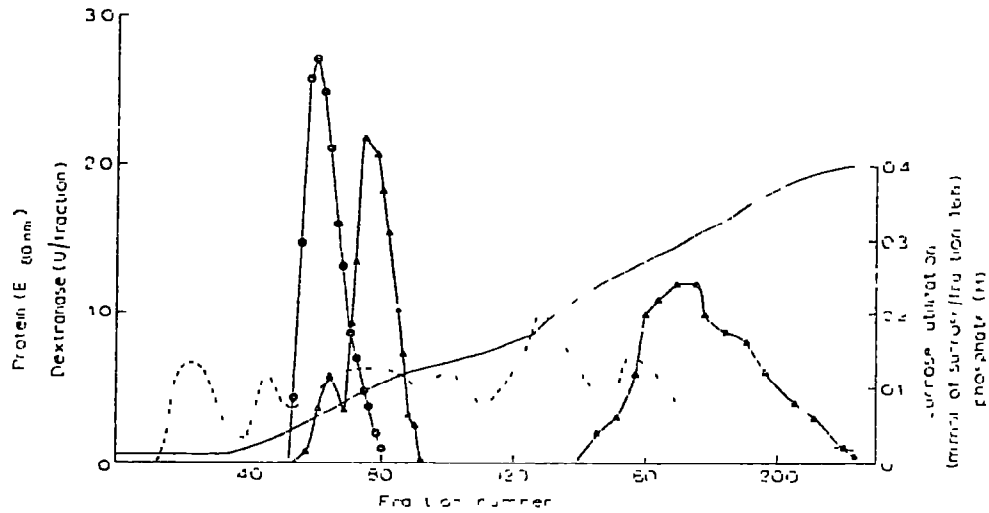


Fig 1 Chromatography of 20–80% $(NH_4)_2SO_4$ fraction on hydroxylapatite Fractions containing dextranase activity (55–72) were combined Fractions 160–180 were also combined and used to prepare K1-R insoluble glucan ———, phosphate gradient, —●— dextranase activity —▲— sucrose-utilising activity, – – – –, $E_{280\,nm}$

Oligosaccharides of the isomaltose series were isolated from a partial, acid hydrolysate of clinical dextran Isomaltose (IM_2) and higher oligosaccharides up to isomalto-octaose (IM_8) were separated by chromatography on charcoal–Celite[15], and IM_9–IM_{12} were separated by gel filtration[16] on Bio-Gel P2 at 50° The oligosaccharides were further purified by preparative paper chromatography[17]

Materials — The following chromatographic supports were used. Bio-Gel HTP (hydroxylapatite) and Bio-Gel P2, from Bio-Rad Corp, Calif; Whatman DEAE-cellulose, DE 52, Ultrogel AcA34 (polyacrylamide–agarose gel), from LKB Produkter, AB, Bromma Sweden The supports were prepared for use in accordance with the manufacturers' instructions Ammonium sulphate was a BDH laboratory reagent especially low in heavy metals for enzyme work All other chemicals were analytical reagent grade

Organisms and growth conditions — *S mutans* strain K1-R was obtained from Dr R G Fitzgerald, *S mutans* OMZ 176 from Professor B Guggenheim, and *S sanguis* 804 from Professor J Carlsson The organisms were grown in a medium containing 2% of Trypticase (BBL), 0 5% of yeast extract (Difco), 0 4% of dipotassium hydrogen phosphate, 0 1% of potassium dihydrogen phosphate, 0 2% of sodium chloride, and 2% of D-glucose The growth was performed under anaerobic conditions (N_2+CO_2, 95·5) for 16 h at 37° in a fermenter (MF-114, New Brunswick Scientific

Co. Inc., New Brunswick, N.J., U.S.A.) The pH of the final growth-medium was adjusted to pH 6.0 with lactic acid prior to inoculation and maintained at this value throughout the growth period by automatic addition of NaOH

Analytical methods — Total carbohydrate was determined by cysteine–sulphuric acid reagent[18]. Reducing sugars were determined by Nelson's method[19] on one-fifth the recommended scale, the extinction being read at 510 nm. In experiments with isomaltose oligosaccharide substrates, determinations were performed on one-tenth scale and extinction values were read at 570 nm. D-Glucose was determined by a D-glucose oxidase-peroxidase reagent[20].

Oligosaccharides were separated by descending chromatography on Whatman No. 3MM paper, with nitromethane–ethanol–water[17] (41:36.23) and detection by alkaline silver nitrate[21]. Where necessary, solutions were deionized with Amberlite MB-3 resin (carbonate form) prior to chromatography.

Protein was determined by Hartree's modification[22] of the Folin–Ciocalteu procedure, using bovine serum albumin (Fraction V, Sigma Chemical Co.) as a standard; protein in column fractions was monitored by measuring the extinction at 280 nm. Inorganic phosphate was determined by the method of Allen[23], and sodium chloride by titration with 0.1M silver nitrate, with potassium chromate as the indicator.

Enzyme assays — An incubation temperature of 35° was used throughout.

Dextranase activity was determined by the release of reducing sugars in a digest containing Sigma 2000 dextran (1.4 mg/ml), sodium citrate buffer (25 mM, pH 6.0), and an appropriate volume of enzyme. The digests were sampled at intervals, and the enzyme was inactivated by boiling (2 min, 100°) or by the addition of copper reagent. One unit (U) of activity is defined as the amount of enzyme that caused the release of 1 μmol of reducing power per min, under the conditions of assay.

Activities of the polysaccharide-synthesizing enzymes, D-glucosyltransferase and D-fructosyltransferase, were determined by measuring the rate of sucrose utilization in the presence of dextran which has been reported to stimulate D-glucosyltransferase activity[5,6]. The activity digest contained sucrose (0.6%), Sigma dextran 2000 (160 μg/ml), sodium citrate buffer (25mM, pH 6.5), and enzyme. Sucrose remaining in the digests was determined in aliquots (0.05 ml, withdrawn at various intervals) by a modification of the anthrone method[24]. When the contribution by the individual transferases was required, the polysaccharide in the digests was precipitated by the addition of ethanol (2 vol.) and washed with 75% aqueous ethanol. The polysaccharide was dissolved in 36% sulphuric acid, and its glucose and fructose contents were determined by the "hot"[25] and "cold"[26] anthrone methods.

Invertase activity was also detected by this assay. To establish whether the sucrose in the digest was hydrolysed by invertase to D-glucose and D-fructose, or used for polysaccharide synthesis by the transferase(s), the release of reducing sugars was compared on a molar basis with the disappearance of sucrose in the digest. The presence of invertase was indicated if the ratio of reducing sugars to sucrose consumed was greater than unity. A ratio of two, together with the absence of polysaccharide formation in the digest, implied that the sucrose hydrolysis was due solely to invertase.

Enzyme preparation — Culture fluid was obtained from each cell growth (5 l) by centrifugation (10 min, 12,000 *g*) All subsequent operations were carried out at 0–2° unless otherwise specified The culture fluid from *S mutans* K1-R was dialysed against distilled water (10 vol), then concentrated to 2% of the original volume by ultrafiltration on a PM-10 membrane (Amicon Corp , Lexington, Mass , U S A), and dialysed against 50mM sodium citrate buffer (pH 6 0) Solid sucrose was then added to give a final concentration of 10% (w/v), and the mixture was incubated at 35° for 2 h The insoluble material was removed by centrifugation (20 min, 1,800 *g*), and the supernatant solution was dialysed against 50mM sodium citrate buffer (pH 6 0) for 3 days to remove the remaining sucrose

The dialysed preparation was fractionated with solid $(NH_4)_2SO_4$, the fraction precipitating between 20 and 80% saturation of salt was dissolved in 50mM sodium citrate buffer (pH 6 0) to one-tenth of the original volume, and dialysed against the same buffer to remove the salt

Hydroxylapatite chromatography -- The material obtained by $(NH_4)_2SO_4$ fractionation was dialysed against 10mM potassium phosphate buffer (pH 6 5) and applied to a column (43 × 3 cm) of hydroxylapatite prepared in the same buffer The enzyme was washed on with 45 ml of the buffer, and the elution gradient was set up with 10mM potassium phosphate buffer (pH 6 5) in the mixing vessel (500 ml) and 0 2M potassium phosphate buffer (pH 6 5) in the reservoir Fractions (6 ml) were collected at the rate of 20 ml/h When the phosphate concentration of the eluate reached 0 11M, the reservoir buffer was changed to 0 5M potassium phosphate (pH 6 5) and the elution was continued

DEAE-cellulose chromatography — Combined fractions containing dextranase activity from the hydroxylapatite column were dialysed against 5mM sodium citrate buffer (pH 6 0) and applied to a column (30 × 1 5 cm) of DEAE-cellulose prepared in the same buffer The column was then washed with 5mM sodium citrate buffer (pH 6 0) until the extinction at 280 nm of the eluate was negligible (200 ml) Gradient elution with 5mM sodium citrate buffer (pH 6 0) in the mixing vessel (250 ml) and 0 15M NaCl in the same buffer in the reservoir was then begun Fractions (6 ml) were collected at the rate of 35 ml/h When the chloride concentration of the eluate reached 0 075M, the reservoir buffer was changed to 0 3M NaCl in 5mM sodium citrate buffer (pH 6 0) The elution was continued until the eluate buffer was 0 24M NaCl, when the reservoir buffer was changed to 0 8M NaCl in 5mM sodium citrate buffer (pH 6 0)

Ultrogel filtration — Fractions containing dextranase from the DEAE-cellulose column were pooled and dialysed against 50mM sodium citrate buffer (pH 6 0) The pool was then concentrated to one-fifth of the volume by ultrafiltration on UM 20-E membrane A portion (5 ml) of this enzyme was applied to a column of Ultrogel (70 × 2 5 cm) prepared in 50mM sodium citrate buffer (pH 6 0) The column was eluted with the same buffer at the rate of 27 ml/h, 3-ml fractions were collected Fractions containing dextranase were combined, and concentrated to 10 ml by ultrafiltration on UM 20-E membrane

RESULTS

Purification of dextranase — Several enzymic activities apart from the dextranase were detected in the crude, culture fluid These included invertase, dextran-glucosidase, and glucan- and fructan-synthetases Approximately 26% of the total cell dextran-glucosidase was present in the culture fluid. This enzyme lost activity rapidly unless the cell-free culture fluid was dialysed against citrate buffer soon after harvesting Because the culture fluid remained undialysed for a considerable period during concentration, the dextran-glucosidase activity was absent from the concentrated material

A loss of 50% in the sucrose-utilizing activity was also detected after the concentration step It was not established whether this was due to the loss of invertase or polysaccharide synthetase activities In contrast, the loss in dextranase activity was negligible (Table I) The apparent decrease in protein content after ultrafiltration was probably due to the removal of peptides of relatively low mol wt. present in the medium and not of the bacterial protein, since the cut-off of PM-10 membrane was at mol wt 10,000

TABLE I

PURIFICATION OF *S mutans* DEXTRANASE

Fraction	*Total units*	*Total protein (mg)*	*Specific activity (units/mg)*	*Purification (-fold)*	*Yield (%)*
1 Culture filtrate (5 l)	119 4	8250	0 014	—	100
2 Concentrated filtrate	116 3	1706	0 07	5	97
3 After sucrose incubation	70 3	883	0 08	6	59
4 20–80% $(NH_4)_2SO_4$	62 3	348	0 18	13	52
5 Hydroxylapatite fractions	50 6	27 6	1 83	131	42
6 DEAE-Cellulose fractions	34 6	3 5	9 88	706	29
7 Ultrogel fractions	23 4	0 9	26 0	1857	20

The next step in purification made use of the fact that D-glucosyltransferases are adsorbed easily on the polysaccharides they synthesize Preliminary, small-scale experiments showed that the proportion of synthetase removed by incubating culture supernatant-fluid with sucrose far exceeded the amount adsorbed when cell-free filtrate was treated directly with *S mutans* K1-R insolubie glucan D-Glucosyltransferases were therefore exposed to the copious quantities of insoluble polysaccharide that they synthesized from sucrose *in situ* The enzyme that synthesized soluble glucan, the D-glucosyltransferase that was most difficult to remove from dextranase by any other procedure, was preferentially adsorbed Provided the incubation time was short (2 h). losses in dextranase activity were negligible. However, when a more-purified preparation (containing less D-glucosyltransferase) was used, losses of up to 40% of the dextranase activity were encountered The high loss after

this step in the large-scale purification shown in Table I was therefore uncharacteristic The loss of sucrose-utilizing activity in this step was 80%

Ammonium sulphate fractionation removed 60% of the protein from the dextranase preparation, and resulted in 11% loss in dextranase activity, 10% of sucrose-utilizing activity was also removed

Partial purification of dextranase from the remaining sucrose-utilizing activity was achieved by hydroxylapatite chromatography (Fig 1) The dextranase peak, eluted with 55mM phosphate, overlapped the peak of sucrose-utilizing activity eluted with 0 1M phosphate A second peak of sucrose-utilizing activity (eluted with 0 28M phosphate) was a D-glucosyltransferase that synthesized insoluble glucan from sucrose, and this α-D-glucan was among the substrates tested for extent of hydrolysis by the purified dextranase This fractionation step also removed 80% of the sucrose-utilizing activity and resulted in 19% loss in the dextranase activity

Chromatography on DEAE-cellulose fractionated the sucrose-utilizing contaminant of dextranase into three overlapping peaks which were eluted ahead of dextranase activity (Fig 2) The first peak (I) was eluted with 0 10M NaCl, while the last one (III) partially overlapped the dextranase peak (eluted with 0 19M NaCl) In order to establish the identity of the enzymes in these peaks, determinations of reducing sugars and polysaccharide synthesis were carried out in conjunction with the sucrose-utilization assay of the peak fractions (Table II) The results indicated that the first two peaks contained little polysaccharide-synthesizing activity and that sucrose was hydrolysed by invertase On the other hand, the peak closest to the dextranase peak contained little invertase activity and consisted of a mixture of D-glucosyl- and

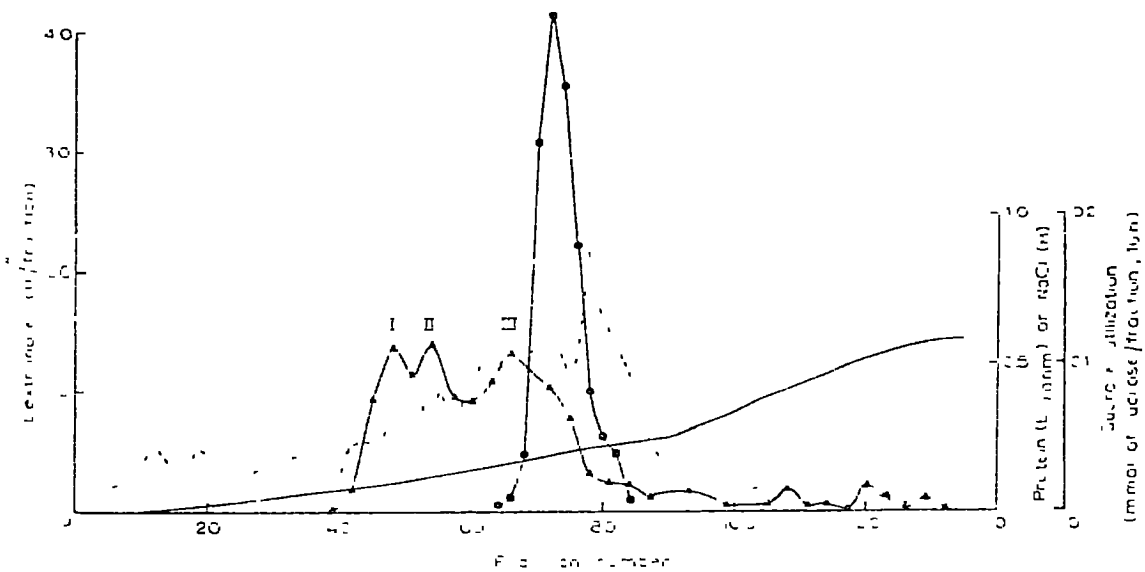


Fig 2 Chromatography of fractions 55–72 from hydroxylapatite column on DEAE-cellulose Fractions (70–79) containing dextranase activity were combined Symbols are as for Fig 1

TABLE II

PRODUCTS OF SUCROSE UTILIZATION ASSAYS[a] OF PEAK FRACTIONS FROM THE DEAE CELLULOSE COLUMN

Peak	*Sucrose used (μmol)*	*Products*			
		Glucose (μmol)	*Reducing sugars*	*Glucan (as μmol of glucose)*	*Fructan (as μmol of fructose)*
I	14 0	19 5	39 4	0.5	0 5
II	5 4	5 6	11 2	0 9	0 7
III	7 4	1 5	8 0	4 0	1 3

[a]Sucrose utilization assays were carried out as described in Enzyme assays The digests (1 ml), containing a sample (0 4 ml) from each fraction, were incubated at 35° for 16 h.

D-fructosyl-transferases The amount of synthetase removed from dextranase by this chromatography was difficult to determine because its activity could only be detected after incubation for 16 h

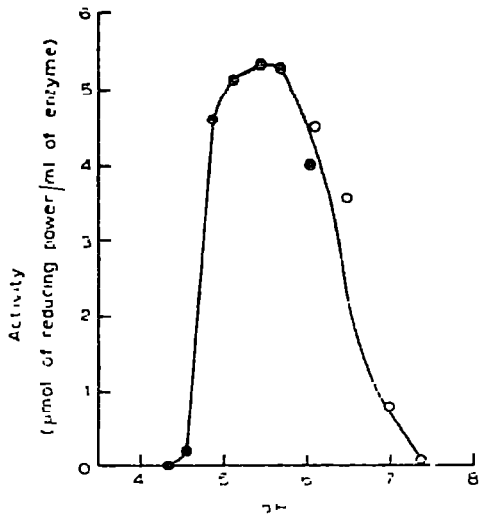
Gel filtration of the dextranase peak obtained from DEAE-cellulose column on a column of Ultrogel resulted in complete separation of the two activities The transferase peak was eluted in 0 4 bed-volume, while dextranase was eluted in 0 7 bed-volume This does not imply that the DEAE-cellulose step was unnecessary, since fractionation of hydroxylapatite fractions on Ultrogel resulted in elution of invertase together with dextranase

This purification scheme resulted in 1800-fold purification of dextranase activity and in the complete removal of contaminating enzymes, including D-glucosyl-transferase There was no release of reducing groups in activity digests when dextran was replaced with glycogen or fructan

Properties of dextranase — The conditions for optimal activity of the enzyme were at pH 5 5 and 35° (Figs 3 and 4, respectively) The enzyme was rapidly inactivated at temperatures above 40° (Fig. 4) The activity was linear with time up to 40-min incubation, and with the volume of enzyme, provided the digest contained less than 0 17 U/ml Addition of bovine serum albumin (80 or 200 μg/ml of digest) increased the activity by 17% Exhaustive dialysis of the enzyme against 10mM EDTA followed by dialysis in 50mM sodium citrate buffer (pH 6 0) did not alter the activity Treatment of the dextran solution in a similar way did not make it a more-suitable substrate The enzyme lost activity at the rate of 4% per week when stored at 2°

Substrate specificity — The Michaelis constant (K_m) of the dextranase for Sigma dextran determined from a Lineweaver–Burk plot (Fig 5), was 29 mg/ml or 1.4×10^{-5}M The K_m value for IM_8, determined from activities obtained with a range of substrate concentrations (0 2–1 5mM), was 18mM.

The action on dextran was also studied by observing the order of appearance of products over a period of reaction The initial action of the enzyme was to hydrolyse Sigma dextran to a series of higher isomaltose saccharides, the lowest member of



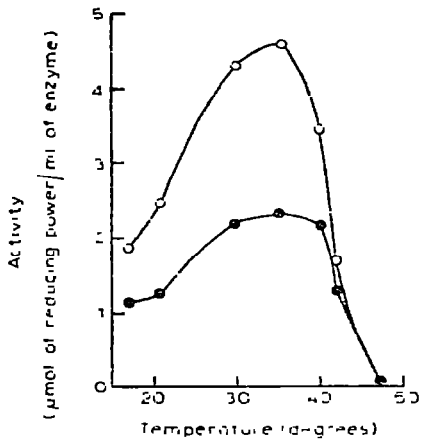


Fig 3. The effect of pH on the activity of dextranase Activity digests containing Sigma dextran (14 mg/ml), enzyme (16 mU/ml), and either 35mM sodium citrate buffer (●) or 30mM sodium phosphate–5mM sodium citrate buffer (○) were incubated for 30 min at 35

Fig 4 The effect of temperature on the activity of dextranase Activity digests containing Sigma dextran (14 mg/ml), enzyme (64 mU/ml), and 25mM sodium citrate buffer (pH 6 0) were incubated for 15 min (●) and 30 min (○)

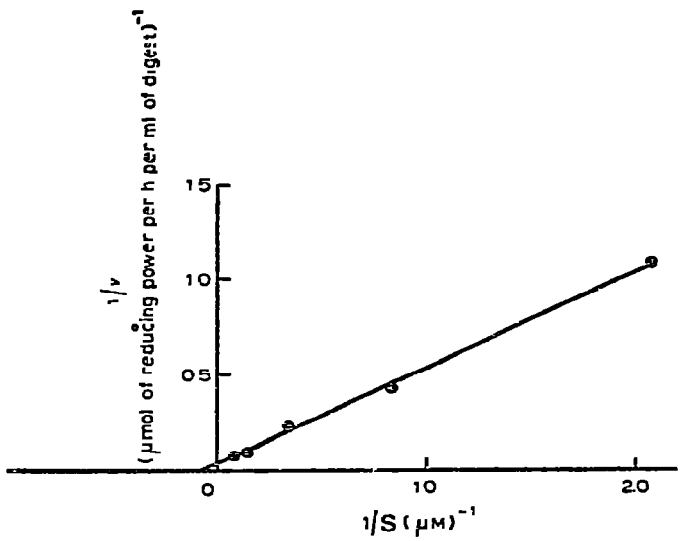


Fig 5 Lineweaver–Burk plot of the action of dextranase on Sigma dextran 2000 Standard digests contained 0 16 U of enzyme per ml

which was IM_5 This sugar remained prominent throughout the course of the reaction, while the higher saccharides were apparently fully converted into IM_4, IM_3, and, to a lesser extent, isomaltose and D-glucose The apparent conversion into IM_3 (determined from the release of reducing power in the digest) was 0 3 h, 23%, 0 7 h (36%) 1 h (39%), 1 5 h (46%), 2 h (49%) 4 h (64%), 6 h (70%), 24 h (95%) After reaction for 24 h, low amounts of sugars of molecular weight higher than IM_5 appeared to consist mostly of branched saccharides This was indicated by spots at positions in between those corresponding to isomaltose saccharides On addition of more enzyme to the dextran digest, IM_5 was further hydrolysed, D-glucose and IM_2 became more prominent, and the apparent conversion into IM_3 rose from 95% to 100% Various other glucans, when treated under the same conditions, were considered to be hydrolysed to the limit, the second incubation with dextranase giving similar, small increases in conversion values The hydrolysis of dextrans (Sigma 2000, B-512, and synthetic) that contain 96% or more of (1→6)-α-D-glucosidic linkages was largely complete, whereas dextran (fraction L) from *L mesenteroides* NRRL B-1355 with 38% of (1→6)-α-D-glucosidic linkages[27], was hydrolysed to the extent of 84% The products ranged from D-glucose to IM_5, while the dextrans that contain branch linkages also yielded branched saccharides of d p >4 The synthetic dextran, being essentially linear, was hydrolysed completely to isomaltose saccharides

The streptococcal glucans showed considerable differences in product distribution The action of the enzyme on the soluble glucan synthesized by a purified D-glucosyltransferase of *S mutans* OMZ 176 was limited (17% conversion into IM_3) as shown by the relative lack of products smaller than IM_5, by the presence of "branched products" of higher d p , and by the large quantities of saccharides close to the origin of the chromatogram. Another *S mutans* glucan, the insoluble polysaccharide synthesized by a purified D-glucosyltransferase of strain K1-R, was totally resistant to the dextranase In contrast to the *S mutans* glucans, the insoluble glucan from *S sanguis* 804 was solubilized and hydrolyzed to the extent of 85% and 72%, respectively

These results indicated that the rate of action of dextranase on isomaltose saccharides was dependent on the chain-length The members of the series were therefore compared as substrates for the enzyme at equimolar concentrations (Fig 6) Because the saccharides themselves had finite reducing-power, the rate of reaction was determined as the percentage increase in reducing power per hour A 100% increase per hour thus describes a situation where every molecule of substrate has been cleaved once. The rates were determined during a period when the release of reducing power was linear, the time interval ranged from 5 h for IM_5 to 10 min for IM_{12} It was observed that the rates of reaction rose sharply with increasing chain-length (Fig 6), there being a 40-fold difference between the rates for IM_5 and IM_{12} To compare the rate of reaction of Sigma dextran 2000 under the same conditions, the concentration of dextran in the digest would need to have been 1 4 g/ml As this was experimentally impossible, synthetic dextran was used instead Determinations of reducing power in each isomaltosaccharide digest were continued until there was no

further increase, and the products were then separated by paper chromatography. The main products from each substrate were IM_4, IM_3, IM_2, and D-glucose No evidence of transglucosylation was obtained from any of the chromatograms There was no activity towards IM_3 and IM_4 under the conditions used in this experiment However, when the concentration of the enzyme in the digest was increased ten-fold, the rate of action on IM_4 was 6% per h, *i e* , approximately the same as that for IM_5 in the presence of one-tenth the activity The major products from this reaction were shown by paper chromatography to be IM_3 and D-glucose, with lower amounts of isomaltose Isomaltotriose was not a substrate even with the higher concentration of enzyme

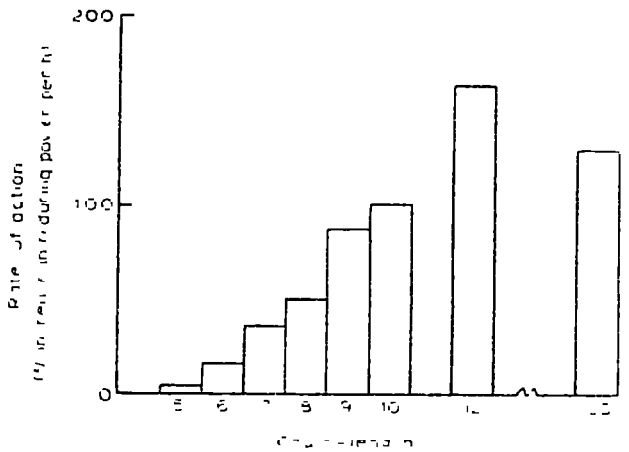


Fig 6 The effect of chain-length of isomaltose saccharides on their initial rate of hydrolysis The substrates (0 7mM) were incubated with dextranase (0 16 U/ml) at 35 and pH 6

DISCUSSION

Several studies have shown that *S mutans* produces dextranase[7–9], and Guggenheim and Burckhardt[6] concluded that almost all strains covering serological groups a–e release an endo-dextranase into the medium, with a proportion of the enzyme being cell-bound Some strains also produce an exo-dextranase[7], and this enzyme is found in cell extracts as well as in the medium Nevertheless, investigations into dextran metabolism by *S mutans* have centered mainly on synthesis, and reports of experiments with D-glucosyltransferases have not generally indicated the level of dextranase activity accompanying the synthesizing enzymes Provided that *S mutans* is grown under conditions suitable for the survival of extracellular enzymes, dextranases and D-glucosyltransferases will be present together in the medium, and a simple fractionation with ammonium sulphate does not separate these enzymes The procedure we have described for the purification of endo-dextranase allows the complete removal of all traces of D-glucosyltransferase activity The enzyme is also

free from exo-dextranase activity, as shown by its inability to hydrolyze isomaltose and isomaltotriose

The endo-dextranase of strain K1-R is characterized by its high affinity for dextran The value (1.4×10^{-5}M) for K_m is smaller by almost two orders of magnitude than that (0.97×10^{-3}M) of *S mutans* strain OMZ 176 dextranase[8] The major products of the K1-R enzyme on several dextrans were shown by paper chromatography to be IM_3, IM_4, and IM_5 Small amounts of D-glucose and IM_2, together with higher oligosaccharides containing one or more linkages other than (1→6)-α-D- were also observed Since both IM_4 and IM_5 were slowly further hydrolyzed to D-glucose, IM_2, and IM_3, the extent of hydrolysis of the dextrans was expressed as their conversion into apparent IM_3

The K_m value for IM_8, 1.8×10^{-2}M, being a thousand-fold higher than the value for dextran, illustrates the reduced affinity of the dextranase for oligosaccharides The rate of hydrolysis of isomaltose saccharides fell rapidly (Fig 6) when the chain length decreased from 7 to 5, and the hydrolysis of IM_4 was extremely slow These results may be compared with those reported[28] for a dextranase (D1) of *Pseudomonas* UQM733 The initial rates of degradation of isomaltosaccharides by enzyme D1 also decreased with d p from IM_8 to IM_5, and only traces of products were seen from IM_4 and IM_3 Although the two enzymes gave similar, early products of dextran hydrolysis, dextranase-D1 finally produced equivalent amounts of IM_2, IM_3, and IM_4, and traces of D-glucose were released from IM_3–IM_6, only after prolonged incubation. In this paper, we have not given the detailed account of the hydrolysis of various oligosaccharides that has been presented for dextranase D1, because the final distribution of products depends on the enzyme concentration and the duration of incubation, and the results do not give precise information on linkage hydrolysis A study of the action pattern of *S mutans* dextranase on two series of ^{14}C-isomaltosaccharides, labelled at opposite ends, is now in progress

S mutans dextranase hydrolyzed (1→6)-α-D-glucans containing 6% or less of anomalous linkages to give an apparent conversion into IM_3 of 99–100%, but the enzyme had little action on the glucans synthesized from sucrose by the isolated D-glucosyltransferases of *S mutans* This result is consistent with the known specificity of endo-dextranases Hydrolysis of *Leuconostoc* dextrans by *Penicillium* dextranases was markedly lowered with increasing degree of branching[29], and a bacterial endo-dextranase[30] CB (Calbiochem) would not act on *S mutans* (1→3)-α-D-glucan Furthermore, dextranase CB did not hydrolyze the crude glucans[30] synthesized from sucrose by the culture filtrates of *S mutans* strains K1-R and B13 The insoluble glucan synthesized by a purified D-glucosyltransferase of *S. mutans* K1-R (see Methods) is a (1→3)-linked α-D-glucan as shown by the extent of its hydrolysis (91%) by a specific (1→3)-α-D-glucanase[31], and thus it is completely resistant to dextranase Dextranase can inhibit[11] or alter the synthesis of insoluble glucan by *S mutans* D-glucosyltransferases only when the enzymes are present together Dextranase inhibition of (1→3)-α-D-glucan synthesis is partly due to the high primer activity[14] of small amounts of dextran for sucrose (1→3)-α-D-glucan 3-α-D-glucosyltransferase; the

soluble glucan produced by *S mutans* is also an efficient primer for sucrose 3-D-glucosyltransferase This glucan (OMZ 176) is not easily fragmented by endo-dextranase, thus assuring the availability of a primer for the synthesis of insoluble glucan. The insoluble glucan synthesized from sucrose by *S sanguis* 804 culture-filtrate was a good substrate for *S mutans* dextranase, and the apparent conversion into IM_3 (72%) was similar to that (67%) produced by dextranase CB[30] Both these bacterial endo-dextranases are without action on (1→3)-α-D-glucan The presence of (1→3)-α-D-glucosidic linkage sequences in *S sanguis* dextrans was indicated by the action of (1→3)-α-D-glucanase[7], which released D-glucose (15%) from strain-804 glucan The same enzyme also released D-glucose (11–17%) from glucans of *Streptococcus* spp that resemble *S sanguis*

The complete specificity of *S. mutans* dextranase for (1→6)-α-D-glucosidic linkages and its total inactivity towards (1→3)-α-D-glucan, indicates its suitability for studies on the fine structure of streptococcal dextrans The choice of dextranases that can be used for such studies is limited, because the enzymes isolated from micro-organisms grown in dextran media may contain endo-(1→3)-α-D-glucanase activity. This enzyme has proved difficult to remove from dextranases of fungal origin[32], and most other dextranases have not been rigorously tested on (1→3)-α-D-glucan

The degrees of hydrolysis of the various glucans investigated were considered to be true limits, because the digests were given a second addition of enzyme followed by further incubation The results for clinical dextran (Sigma 2000) differ from those published for the hydrolysis of a similar dextran (Fluka) by the dextranase[8] of *S mutans* OMZ 176 The specific activity of this enzyme (19 4 i u per mg) was close to that of strain K1-R dextranase (26 0), and the enzyme concentration in the dextran digest (0 13 i u per ml) was similar to that used here (0 18 i u per ml) However, Guggenheim and Burckhardt[8] used a 6-fold higher concentration of substrate, and relied on one addition of enzyme Their analysis by g l c of the products released from a number of soluble dextrans of *Streptococcus* spp revealed small amounts of D-glucose and IM_2, but no IM_3 or higher oligosaccharides Yet the mean d p of the products, estimated from reducing-power determinations, gave values ranging from 6 (Fluka), 14 (*sanguis*), to 57 (*mutans*) In a preliminary study of dextranases from *Streptococcus* spp , Dewar and Walker[7] found that the endo-dextranase of *S mutans* OMZ 176 hydrolysed clinical dextran to give isomaltosaccharides shown by paper chromatography to be mainly IM_3–IM_6 The apparent disagreement between these results is probably caused by the difficulty of analysing oligosaccharides by g l c , compared to the simplicity and accuracy of paper chromatography, and there is no reason to doubt that endo-dextranases from the two strains of *S mutans* have a similar substrate specificity Both enzymes released D-glucose and IM_2 from IM_4 and IM_5, whereas IM_2 and IM_3 were not attacked It was suggested[8] that the release of D-glucose from IM_4 and IM_5 by OMZ 176 dextranase was due to traces of exo-dextranase impurity, but there are two considerations that do not support this view Firstly, *S mutans* exo-dextranase, if present, would also have hydrolyzed IM_2 and

IM_3, secondly, *S mutans* endo-dextranase releases D-glucose only from the reducing end of IM_4 and IM_5[33], thus proving that exo-dextranase, which attacks from the non-reducing end, could not have been responsible for the appearance of D-glucose

Staat and Schachtele[9], who studied the dextranase activity of cell extracts of *S mutans* B2, could not detect the liberation of reducing groups from clinical dextran, but found some oligosaccharides by gel chromatography of the products from dextran-*t* The absence of D-glucose-*t* among the products from this end-labelled dextran was cited as evidence that D-glucose was not a product of the reaction in "agreement" with this result, the fact that *S mutans* did not produce acid from dextran under the conditions employed for carbohydrate-fermentation tests[34] was cited as indicating that this organism might not utilize dextran Although the conditions for optimal dextranase production by *S mutans* have not been defined, it is already known that the enzyme is not stable below pH 5, and accordingly may not survive unless the pH is suitably controlled Therefore, neither the apparent absence of D-glucose during the early stages of dextran degradation, nor the characteristics of growth displayed in uncontrolled batch-culture, nor even the result of a fermentation test proves that dextran cannot be utilized by *S mutans* We have consistently observed that washed suspensions of *S mutans* cells readily hydrolyse clinical dextran, and that cells of *S mutans* strains that produce exo-dextranase, as well as endo-dextranase, give D-glucose as the main end-product

It seems clear, therefore, that *S mutans* can realize its potential to utilize extracellular dextrans that are susceptible to hydrolysis by endo-dextranase The most-prevalent species of streptococcus in dental plaque, *S sanguis*, is also the best producer of dextran from sucrose The proportion and arrangement of the (1→6)-α-D-glucosidic linkages in *S sanguis* dextran and in some regions of certain *S mutans* glucans, account for their hydrolysis by endo-dextranases Degradation of the dextrans by the indigenous dextranases of plaque micro-organisms can explain the presence of oligosaccharides and D-glucose in dental plaque[35]

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