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DEGRADATION OF DEXTRANS BY AN α-1,6-GLUCAN GLUCOHYDROLASE FROM *Streptococcus mitis*

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ABSTRACT

A dextran-glucosidase (α-1,6-glucan glucohydrolase) found in cell extracts of several strains of *Streptococcus mitis* has been purified, and some of its properties have been investigated. Dextran-glucosidase hydrolysed isomaltose and isomaltose saccharides at comparable rates, and a comparison of K_m values for isomaltose, isomaltopentaose and dextran showed that the enzyme had equal affinity for these substrates despite their different chain-length. The action pattern of the enzyme was not completely characteristic of an exo-glucanase or of a glucosidase. Its ability to act on polymers, and its complete specificity for α-(1→6)-D-glucosidic linkages were properties associated with exo-glucanases, whereas its transferring ability, the retention of configuration, and inhibition by nojirimycin supported its classification as a glucosidase. Dextrans were incompletely degraded by the enzyme, the extent of hydrolysis being related to the proportion of α-(1→6)-D-glucosidic linkages. It was concluded that the α-(1→4)- α-(1→3)-, and α-(1→2)-D-glucosidic linkages that occur in bacterial dextrans were resistant to the action of dextran-glucosidase, and arrested the further enzymic degradation of dextran. The use of the enzyme in investigations of the fine structure of dextrans is discussed.

INTRODUCTION

Certain dextrans can aid the deposition of plaque on the surface of teeth[1]. It has been proposed that the ability to synthesize an adhesive, insoluble dextran may be one of the determinants of the cariogenic potential of oral micro-organisms[2]. Oral streptococci produce a variety of dextrans with chemical and physical properties that distinguish them from one another[3,4]. The nature of the glycosidic linkages may be determined by periodate oxidation[5] and methylation analysis[6]. Additional information about the fine structure of polysaccharides can be obtained by examination of the fragments produced by enzymic degradation.

The endo-dextranases of *Penicillium funiculosum* and *P. lilacinum*, because of their requirements for a substrate that contains several consecutive α-(1→6)-D-glucosidic linkages[7], have only a limited activity towards oral glucans, many of which

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contain less than 50% of α-(1→6)-D linkages[8]. Glucanases having a different specificity are required to break down these polysaccharides. As part of a search for glucanases that could prove useful for dextran degradation, we have re-investigated the properties of an enzyme found in intracellular extracts of *Streptococcus mitis*. The enzyme, an α-(1→6)-D-glucosidase, was reported by Walker and Builder[9] to hydrolyse isomaltose and isomaltose saccharides, and the α-(1→6) linkages in panose and 6^3-α-D-glucosylmaltotriose were also cleaved despite the adjacent α-(1→4) linkages.

The present paper describes the action of this enzyme on dextrans, and, since the enzyme had equal affinity for α-(1→6)-D-glucans irrespective of chain length, the systematic name has been changed to α-1,6-glucan glucohydrolase. The trivial name for this enzyme is dextran-glucosidase.

MATERIALS AND METHODS

Carbohydrates. — A chemically synthesised, highly stereoregular dextran[10] was a generous gift from Professor C. Schuerch. The polymerization of a purely synthetic 1,6-anhydro-2,3,4-tri-*O*-benzyl-β-D-glucopyranose was catalysed by phosphorus pentafluoride, and subsequent debenzylation yielded dextran with $[\alpha]_D^{25}+199°$ (water). The glucan was synthesised 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), B-1415, B-742, and B-1355 were kindly provided by Dr. A. Jeanes, and dextran B-512 was from Dr. S. Svensson. Dextran was also prepared from *Streptococcus bovis* PC112 provided by Dr. R. T. J. Clarke, and from *S. bovis* NCDO 1253. The micro-organisms were grown under the conditions described by Bailey and Oxford[11], and the dextrans were isolated as described by Bailey[12].

Isomaltose and isomaltose saccharides* were isolated by paper chromatography from a partial, acid hydrolysate of dextran (Pharmachem Corp., Bethlehem, Pa., U.S.A.). Nigerose was a gift from Dr. E. T. Reese, and kojibiose was recovered by charcoal-column chromatography of a partial, acid hydrolysate of the teichoic acid[13] of *S. faecalis* strain 39. Maltose hydrate, supplied by T. Kerfoot Ltd., Vale of Bardsley, Lancs., U.K., was freed from small amounts of D-glucose and maltotriose by paper chromatography. Nojirimycin bisulphite[14,15] was provided by Dr. Reese.

Total carbohydrate was estimated with a cysteine–sulphuric acid reagent[16]. Reducing sugars were determined by the method of Nelson[17]. D-Glucose was assayed with D-glucose oxidase reagent[18] as modified by Dahlqvist[19]. Separations of isomaltose, isomaltose saccharides, and branched oligosaccharides were made by paper chromatography on Whatman No. 3MM paper in ethyl acetate–pyridine–water (10:4:3); detection was effected with alkaline silver nitrate[20]. In this solvent, branched oligosaccharides were easily separated from isomaltose saccharides of the same degree of polymerization.

*In this paper, in compliance with editorial policy, the term "isomaltose saccharides" has been used in place of "isomaltodextrins".

Preparation of cell extract. — The organisms were grown anaerobically (N_2+CO_2, 95:5) for 16 h at 37° in a medium[21] containing 2% of trypticase, 0.4% of dipotassium hydrogen phosphate, 0.1% of potassium dihydrogen phosphate, 0.2% of sodium chloride, and 0.5% of D-glucose. The cells were centrifuged at 2° for 10 min at 4,000 *g*, washed twice with 67mM sodium phosphate buffer (pH 7.1), and resuspended in 34mM sodium phosphate–25mM sodium citrate buffer (pH 6.8; 13 ml per 2 litres of medium). The suspension was placed in a cooling device[22] and disrupted for 6 min with a Branson Sonifier S-75 at full power. The cell debris was removed by centrifuging at 27,000 *g* for 20 min at 2°, and the supernatant solution was dialysed for 16 h against 50mM sodium citrate buffer (pH 6.5).

Purification of dextran-glucosidase. — All operations were carried out at 1–4°.

Step 1. A saturated solution of ammonium sulphate (pH 7.0) was added dropwise to the dialysed cell extract to give 45% saturation. The precipitate was removed by centrifugation for 10 min at 10,000 *g*, and ammonium sulphate was added to the supernatant solution to give 60% saturation. The precipitate was collected by centrifugation and redissolved in 50mM sodium citrate (pH 6.5, on one-tenth of the original volume of cell extract), and the solution was dialysed for 2 h against the same buffer.

Step 2. The dialysed solution (5.5 ml) was applied to a Pharmacia column (83 × 1.5 cm) packed with Bio-Gel P-150 (10 g) and pre-washed with 50mM sodium citrate buffer (pH 6.5). The column was eluted with the same buffer at a flow rate of 12 ml/h. Fractions (2 ml) were collected and those (tubes 14–26) containing dextran-glucosidase were combined and dialysed against 0.1M sodium chloride in 10mM sodium β-glycerophosphate–mM ethylenediaminetetra-acetic acid (pH 6.7). All transglucosylase[23] activity was removed in this step.

Step 3. The dialysed enzyme was applied to a DEAE-Sephadex (A-50) column (1 × 25 cm) that had been washed with the same buffer. The column was eluted with a linear gradient of sodium chloride at 10 ml/h. Dextran-glucosidase activity was eluted between 0.375 and 0.395M sodium chloride, the peak fraction being in 0.38M sodium chloride. Sucrase activity was eluted ahead of dextran-glucosidase, with the peak

TABLE I

PURIFICATION OF DEXTRAN-GLUCOSIDASE

Purification step	*Enzyme recovery*		*Specific activity (Units/mg of protein)*	*Purification*
	(Units)	(%)		
Cell extract	122	100	0.022	
Ammonium sulphate fractionation	92	75	0.11	5
Bio-Gel P-150 chromatography	65	53	0.35	16
DEAE-Sephadex chromatography	48	39	4.6	210

fraction in 0.36M sodium chloride. Fractions with high dextran-glucosidase activity, also containing slight sucrase activity, were combined and dialysed for 16 h against 50 mM sodium citrate buffer (pH 6.0). A summary of the purification is shown in Table I.

The enzyme retained 65% of its activity after being stored in the refrigerator for 12 months. It was not stable to freezing and thawing.

Determination of dextran-glucosidase activity. — The activity digest (0.5 ml) contained isomaltose (1 mg), 50 mM citrate buffer (pH 6.0, 0.2 ml), and enzyme (0.05 ml). After incubation for 15 min at 35°, a sample (0.1 ml) was withdrawn into 0.5M Tris–HCl buffer (pH 7.0, 0.15 ml) to stop the reaction. D-Glucose oxidase reagent (0.75 ml) was added, and the mixture was incubated for 1 h at 35°. The amount of D-glucose released was obtained by comparison with a standard curve. One unit of enzyme was defined as the amount that liberated 1 μmole of D-glucose per min in the activity digest.

RESULTS

Location of dextran-glucosidase. — The experiments described below were carried out with purified enzyme, isolated from an extract of *Streptococcus mitis* 439, obtained by complete disruption of the cells. Part of the enzymic activity could be released by a sonication so mild (30 sec at low power) that the cells appeared not to be broken. This cell-bound fraction amounted to 30% of the total activity. A further 30% was obtained on complete disruption, and the remaining 40% was found in the broth. Thus, the cell extract described under Methods comprised 60% of the total dextran-glucosidase activity, since it contained cell-bound as well as intracellular enzyme.

Action of dextran-glucosidase on isomaltose saccharides and on α-(1→6)-D-glucan. — The action of the enzyme on isomaltose saccharides up to isomaltopentaose was determined by Walker and Builder[9]. Isomaltotriose was hydrolysed at a similar rate to isomaltose, and the tetra- and penta-saccharides were hydrolysed at two-thirds of the

TABLE II

RELATIVE ACTIVITY OF DEXTRAN-GLUCOSIDASE ON ISOMALTOSE SACCHARIDES[a]

D.p.	*D-Glucose release (μg/h)*	*Relative rate of hydrolysis*
2	62.5	100
3	31.3	100
4	21.9	70
5	20.0	64
6	21.0	67
7	19.4	62
13	23.2	74

[a]The digests (0.5 ml) contained isomaltose (12 μmoles) or isomaltose saccharides (6 μmoles), and were incubated for 1 h with dextran-glucosidase (0.006 unit).

rate of isomaltose. In the present work, the enzyme was isolated by a different procedure, which yielded a more highly purified product. The action on isomaltose saccharides was repeated, using substrates of degree of polymerization (d.p.) up to seven, and the results obtained (Table II) were closely similar to those published previously. An isomaltose saccharide of d.p. 13, prepared by partial, acid hydrolysis of dextran, followed by dialysis to remove short-chain products, was hydrolysed at a rate similar to that for isomaltotetraose.

Since there was little fall in activity of dextran-glucosidase with increasing chain-length of isomaltose saccharide substrates, the ability of the enzyme to degrade α-(1→6)-D-glucans was investigated. Two dextrans that have an exceptionally high content of α-(1→6)-D-glucosidic linkages were the most suitable substrates for this test. These were (*i*) a dextran of d.p. 100–150, which was synthesised chemically[10], and contained only 2% of linkages other than α-(1→6)[25] and (*ii*) *Streptococcus bovis* dextran, which was reported to contain α-(1→6) linkages exclusively[12]. A third dextran, from *L. mesenteroides* B-512(F), was included as an example of a bacterial dex-

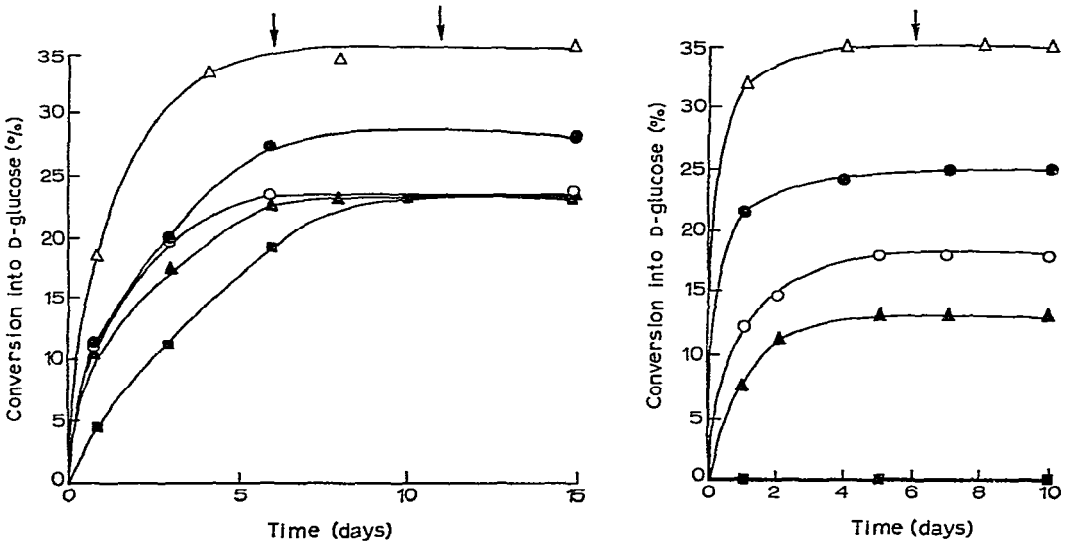

Fig. 1. Action of dextran-glucosidase on dextrans that contain a high proportion of α-(1→6)-D-glucosidic linkages. The digests (0.5 ml) contained 20mM sodium citrate buffer (pH 6), dextran-glucosidase (0.125 unit), and dextran (0.6 mg) from *S. bovis* PC112 (●), *S. bovis* NCDO 1253 (○), *L. mesenteroides* NRRL B-512 (▲), and synthetic dextran (△). The inhibitory effect of D-glucose (0.18 mg) on the hydrolysis of *S. bovis* NCDO 1253 dextran (■) is also shown. More enzyme (0.05 unit) was added at 6 and 11 days (↓).

Fig. 2. Action of dextran-glucosidase on dextrans with different proportions of anomalous linkages. The digests (0.5 ml) contained 20mM sodium citrate buffer (pH 6), dextran-glucosidase (0.3 unit), and dextran (0.6 mg) from *L. mesenteroides* NRRL B-512(F) [●, 5% of α-(1→3) linkages], B-1415 [○, 14% of α-(1→4) linkages], B-742 [▲, 21% of α-(1→4) linkages], and B-1355 [■, 35% of α-(1→3) linkages]. Synthetic dextran (△, 2% of unknown, anomalous linkages is included for comparison). More enzyme (0.1 unit) was added at 6 days (↓).

tran that contains 5% of α-(1→3)-D-glucosidic branch-linkages. The degradation of these dextrans was not complete (Fig. 1), but the results indicated the ability of the enzyme to act on polysaccharides.

The value for the percentage conversion of dextran B-512(F) into D-glucose obtained by reducing-power determination was the same as that found with D-glucose oxidase, and D-glucose was the only sugar found in samples withdrawn at intervals throughout the reaction for analysis by paper chromatography. Addition of more enzyme on completion of the reaction produced no more D-glucose, and removal of D-glucose by dialysis, followed by addition of more enzyme, had no effect, showing that a true limit of conversion had been attained.

The presence of linkages other than α-(1→6) could explain the incomplete hydrolyses of the dextrans. The occurrence of such linkages can also be revealed by hydrolysis of the dextrans with endo-dextranase, when they appear among the end products of higher molecular weight than isomaltotriose[25]. Similar amounts of these products were isolated, by paper chromatography, from digests containing *S. bovis* and B-512(F) dextrans that had been degraded to the limit by *Penicillium funiculosum* QM474 endo-dextranase (Table III). The proportion of anomalous linkages in these two dextrans must therefore be of the same order, and their incomplete hydrolysis by dextran-glucosidase can thus be explained.

TABLE III

PRODUCTS OF ENDO-DEXTRANASE ACTION ON DEXTRAN

Product[a]	*Synthetic dextran*	S. bovis *dextran*	L. mesenteroides *B-512(F) dextran*
D-Glucose	25	10	10
Isomaltose	58	62	58
Isomaltotriose	8	8	9
Higher	9	20	23

[a]The results are expressed as percentage yield of end products.

The limit of conversion of dextran into D-glucose was reached after several days of incubation, whereas the hydrolysis of isomaltose saccharides was complete within hours. The small number of chain ends in dextran digests could account for this. To obtain an accurate comparison of the affinity of the enzyme for isomaltose, isomaltopentaose, and dextran, the enzyme was incubated at various substrate concentrations. Lineweaver–Burk plots of the results gave K_m values of 5mM for isomaltose, 1.5mM for isomaltopentaose, and mM for Pharmachem dextran (average mol. wt. 40,000). These values indicated that the affinity of the enzyme for the substrates was virtually independent of their d.p.

Action of dextran-glucosidase on branched dextrans.—The incomplete hydrolysis of synthetic dextran, and of bacterial dextrans that contain 95% or more of α-(1→6)-D-glucosidic linkages, indicated that dextran-glucosidase was unable to hydrolyse or

by-pass the small percentage of anomalous linkages that these dextrans contained. The action of the enzyme on dextrans with a lower percentage of α-(1→6) linkages was then tested, and the extent of hydrolysis was found to be dependent on the percentage of α-(1→6) linkages. Both α-(1→3)- and α-(1→4)-D-glucosidic linkages arrested the action of dextran-glucosidase (Fig. 2).

Action of dextran-glucosidase on disaccharides. — Dextran-glucosidase (0.033 unit) was incubated in four digests (0.5 ml) containing kojibiose, maltose, nigerose, and isomaltose (1.2 mg), respectively. When isomaltose was completely converted into D-glucose, the hydrolysis of the other disaccharides was <0.25%. No further release of D-glucose occurred during incubation for 3 days.

Effect of dextran-glucosidase on the viscosity of dextran. — The presence of traces of endo-dextranase in the dextran-glucosidase preparation would cause a rapid reduction in the viscosity of dextran at a stage when the release of D-glucose was low. A digest (2.75 ml) containing dextran B-512(F) (44 mg), 50mM citrate buffer (pH 6), and purified enzyme (1.5 units) was incubated at 35° in an Ostwald viscometer. Measurements of viscosity were carried out during the initial stages of the reaction, and at intervals thereafter. The results (Fig. 3) showed the absence of an initial, rapid fall in viscosity.

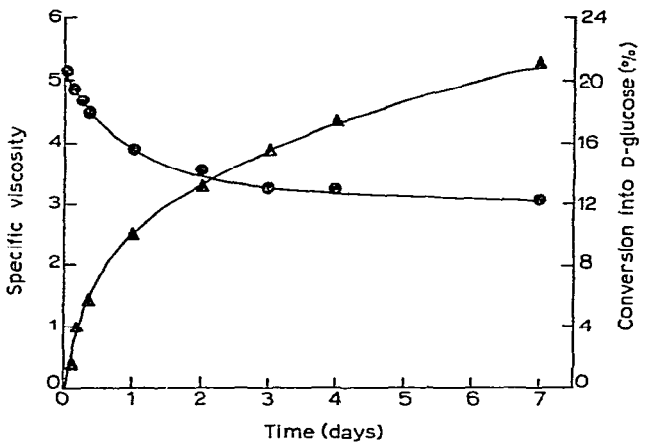

Fig. 3. Reduction in the viscosity (●) and release of D-glucose (▲) from dextran B-512(F) during incubation with dextran-glucosidase.

Limit of enzymic action on periodate-oxidized dextran. — It has been shown[26] that random oxidation of 5% of the D-glucose residues in polysaccharides eliminates virtually all exo-glucanase action, while having little effect on endo-glucanase activity. Synthetic dextran and dextran B-512(F) were oxidized to the extent of 5% with periodate, and then, after removal of iodate by dialysis, samples of the dextrans and the untreated controls were incubated with dextran-glucosidase and with *Penicillium funiculosum* endo-dextranase[24]. Degradation of the oxidized dextrans with dextran-glucosidase ceased at 10% conversion into D-glucose, whereas the endo-dextranase

caused an apparent conversion into isomaltose of 89%, a value not greatly different from the controls (95%).

Properties of dextran-glucosidase limit-dextran. — Dextran B-512(F) was degraded to the limit (25%) with dextran-glucosidase, and a methylation analysis[6] was carried out by Dr. S. Svensson on both the native and enzymically degraded dextran. The increase in the proportion of 2,4-di-*O*-methyl-D-glucose obtained from the branch points, and in that of 2,3,4,6-tetra-*O*-methyl-D-glucose from the non-reducing ends of the limit dextran, relative to native dextran (Table IV), indicated that 30% of the dextran had been eliminated, assuming the branch points had been left intact. The difference between the results obtained, and those expected for 25% degradation, were probably within the experimental errors.

TABLE IV

METHYLATION ANALYSIS OF DEXTRAN B-512(F) BEFORE AND AFTER HYDROLYSIS WITH DEXTRAN-GLUCOSIDASE

Product	*Yield of methylated sugar* (%)	
	Native dextran	*Limit dextran*
2,3,4,6-Tetra-*O*Me-Glc	3.7	5.3 (4.8)[a]
2,3,4-Tri-*O*Me-Glc	92.4	89.4 (91.4)
2,4-Di-*O*Me-Glc	3.8	5.3 (4.8)

[a]Calculated values for 25% degradation of dextran are shown in brackets.

When samples of native dextran B-512(F) and its limit dextran were passed through a column of Sepharose 4B, most of the material was eluted with the void volume, but the portion of the samples that was included demonstrated that the enzymic treatment had caused a small decrease in molecular weight (Table V). No fragments of low molecular weight, such as would be produced by traces of endo-glucanase activity, were found.

TABLE V

EFFECT OF DEXTRAN-GLUCOSIDASE ON THE AVERAGE MOLECULAR WEIGHT[a] OF *L. mesenteroides* DEXTRAN B-512(F)

Dextran	M_w	M_n
Native dextran	22×10^6	9.5×10^6
Limit dextran	20×10^6	4.5×10^6

[a]The molecular weight distribution refers to the portion of the dextrans that was included on Sepharose 4B.

Determination of the configuration of D-*glucose released from isomaltopentaose and dextran.* — Hydrolysis by exo-glucanase generally proceeds with inversion of con-

figuration, whereas glucosidase action is characterized by retention of configuration. The configuration of D-glucose released by dextran-glucosidase was determined with D-glucose oxidase, an enzyme that is highly specific for β-D-glucose. The rate of oxidation of D-glucose in a digest sample was determined before and after mutarotation. Mutarotation of α-D-glucose produces an increase in the rate of oxidation, whereas the reverse obtains for β-D-glucose. Dextran (20 mg) was incubated at room temperature with the enzyme (0.02 unit) in duplicate digests (0.25 ml). After 15 min, 0.5M Tris buffer (pH 7, 0.05 ml) was added to one digest to stop the reaction; the second digest was treated with 4% aqueous sodium carbonate (0.05 ml) to raise the pH to 10, thereby stopping the reaction and causing immediate mutarotation of D-glucose. D-Glucose oxidase reagent (0.75 ml) was added to both digests, and the rate of oxidation at room temperature was followed on the spectrophotometer. The mutarotated sample was the better substrate for D-glucose oxidase (Fig. 4*c*), thus proving that dextran-glucosidase had released α-D-glucose. The same result was obtained when a digest in which isomaltopentaose replaced dextran was treated similarly.

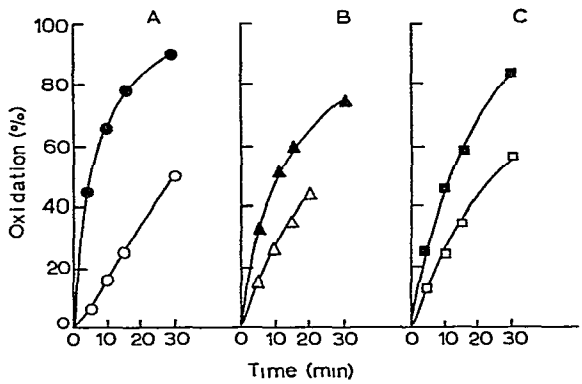

Fig. 4. Determination of the configuration of D-glucose released by dextran-glucosidase by comparing the rate of oxidation of α-D-, β-D-, and D-glucose with D-glucose oxidase. (*A*) Portions (0.25 ml) of freshly prepared solutions (0.005%) of α-D-glucose (○) and β-D-glucose (●) were incubated at room temperature with D-glucose oxidase (0.75 ml). (*B*) Effect of mutarotation; as (*A*), but a solution of D-glucose (▲) was compared with α-D-glucose (△) that had stood at room temperature for 15 min. (*C*) Dextran digests (0.25 ml) were incubated at room temperature for 15 min, then (■) mutarotated, and (□) untreated, before addition of D-glucose oxidase.

Effect of various inhibitors on dextran-glucosidase activity. — (*a*) *Nojirimycin.* The structure of nojirimycin differs from D-glucose only in the substitution of an NH group for oxygen in the ring. Nojirimycin is a potent, competitive inhibitor of glucosidases[14], and may be used to differentiate between glucosidases and exo-glucanases[15].

Dextran-glucosidase and glucoamylase (an exo-glucanase of *Aspergillus niger*) were incubated with isomaltotriose (mM) in the presence of various concentrations of nojirimycin bisulphite. Enzymic activity was determined with the usual reagent, since D-glucose oxidase was not affected by the concentrations of inhibitor used in these

experiments. The concentration of nojirimycin required to give 50% inhibition was 2 μM for dextran-glucosidase and 70 μM for glucoamylase. The ratio of the number of molecules of inhibitor per molecule of substrate required to give 50% inhibition was thus 0.002 for dextran-glucosidase, and 0.07 for glucoamylase. This ratio placed dextran-glucosidase in a class with the α-D-glucosidases.

(*b*) D-*Glucose*. Since D-glucose is a competitive inhibitor of many α-D-glucosidases, it was necessary to ensure that dextran-glucosidase could completely hydrolyse linear α-(1→6)-D-glucans despite the increase in D-glucose concentration that occurred during the reaction.

Complete conversion of isomaltose saccharides into D-glucose was obtained in digests where the substrate concentration was 0.5mM. Addition of D-glucose (168 μg) to a digest (0.5 ml) containing isomaltopentaose (210 μg) caused 57% inhibition during the first hour of incubation, but complete hydrolysis of isomaltopentaose was not prevented.

When D-glucose (180 μg) was added to a digest (0.5 ml) containing *S. bovis* NCDO 1253 dextran (600 μg), inhibition of dextran-glucosidase activity was 60% when hydrolysis of dextran in a control digest was 11%. The digest containing added D-glucose required 50% more time than the control to reach completion (Fig. 1).

Because of the inhibitory effect of D-glucose on dextran-glucosidase activity, dextran digests were incubated for several days (Figs. 1 and 3), and more enzyme was added at intervals so that the hydrolysis could reach the true limit.

(*c*) *Tris buffer*. The inhibitory effect of various concentrations of Tris on dextran-glucosidase activity in isomaltose digests was reported previously[9]. The reaction with dextran was also completely inhibited in 50mM Tris buffer (pH 7).

DISCUSSION

The exo-enzyme beta-amylase has played an important role in the determination of the fine structure of starch and glycogen. Similar enzymes are required for the elucidation of dextran structure, and with this objective, an investigation of the properties of dextran-glucosidase was begun.

It was first necessary to establish the complete absence of any trace of endo-dextranase activity from the purified enzyme preparation. On the basis of several criteria, we have concluded that the enzyme possesses exo-dextranase activity only. The results were as follows: (1) D-Glucose was the sole product of hydrolysis of dextran. Endo-dextranase activity would have resulted in the production of branched oligosaccharides resistant to the action of dextran-glucosidase. None was seen on paper chromatograms, and none was eluted from Sepharose 4B. (2) Enzymes capable of hydrolysing internal linkages cause a rapid fall in viscosity without concomitant release of reducing sugar. This sensitive method for detecting endo-dextranase failed to reveal any such activity, for no sharp fall in viscosity occurred when the purified enzyme was incubated with dextran. The observed fall in viscosity could be attributed to two factors: (*a*) the decrease in molecular weight as a result of exo-dextranase action, and

(*b*) the increase in flexibility of the molecule due to the changes in conformation of the substrate resulting from the removal of the side-chains. (3) A true limit of conversion of dextran into D-glucose was obtained in all cases, and subsequent additions of enzyme did not further raise the limit once the reaction was complete. If the enzyme were contaminated with endo-dextranase, each addition of enzyme would cause further degradation because of the production of new chain-ends susceptible to exo-dextranase action. (4) Oxidation of 5% of the D-glucose residues with periodate lowered the limit of hydrolysis of synthetic dextran by dextran-glucosidase from 35% to 10%, whereas the apparent conversion of the oxidized polysaccharide into isomaltose by the endo-dextranase of *Penicillium funiculosum* was virtually no different from that of untreated dextran. Traces of endo-dextranase activity in the dextran-glucosidase preparation would therefore have counteracted this effect of blocking exo-type degradation.

Secondly, it was necessary to ensure that the dextran-glucosidase was specific for α-(1→6)-D-glucosidic linkages. Dextrans may contain various proportions of α-(1→3)-, α-(1→4)-, or α-(1→2)-D-glucosidic linkages, or combinations of these linkages. These may occur exclusively as branch linkages, or may be present in the main polymeric chain. It was unlikely that the dextran-glucosidase preparation could hydrolyse a branch linkage attaching a long side-chain to the main chain, or act on any internal main-chain α-(1→3)-D-glucosidic linkages, if such occur, in dextran B-512(F), for this would have resulted in an initial, sharp fall in viscosity. Further, the results of methylation analysis indicated that enzymically degraded dextran B-512(F) retained all of the original branch-linkages. Dextran-glucosidase was also unable to hydrolyse the α-(1→3)-D-glucosidic linkage in branched oligosaccharides derived from dextran B-512(F) or the α-(1→4)-D-glucosidic linkage in those from dextran B-1415; the branched tetra- and penta-saccharide products of endo-dextranase action on these dextrans were stable in the presence of dextran-glucosidase (Walker and Pulkownik, unpublished results). Finally, the extent of degradation of dextrans decreased rapidly as the proportion of linkages other than α-(1→6) increased, showing that the enzyme could neither by-pass nor hydrolyse α-(1→3)- or α-(1→4)-D-glucosidic linkages.

Dextran-glucosidase therefore had a different specificity from the cell-bound exo-dextranase of *Bacillus* species[27]. Cell extracts of *B. megatherium* and *B. subtilis* hydrolysed commercial dextrans, containing 93–96% of α-(1→6)-D-glucosidic linkages, completely into D-glucose.

The investigation of a polysaccharide with an exo-enzyme provides a sensitive method for the detection of anomalous linkages. The presence of a single, anomalous linkage in an α-(1→6)-D-glucan would obstruct further degradation by dextran-glucosidase. A value for the percentage of anomalous linkages in synthetic dextran was calculated by substituting figures for its molecular weight and extent of hydrolysis into a theoretical expression. This was derived to account for the effect of unfavorable amino acids in poly-L-proline on the yield of free proline by the exo-peptidase, imino-peptidase[28]. The results agreed closely with those predicted from statistical considerations. Use of this expression indicated that the percentage of anomalous linkages in

synthetic dextran was 2%, a value in complete agreement with that calculated previously[25] from the amount and d.p. of the fraction resistant to endo-dextranase action.

The incomplete degradation of *S. bovis* dextran by dextran-glucosidase showed that this dextran, in common with all bacterial dextrans, contained linkages other than α-(1→6). The recent isolation of nigerose from the products of acetolysis of *S. bovis* dextran[29] proved that the anomalous linkages were α-(1→3).

In view of the apparent inability of dextran-glucosidase to hydrolyse any linkage other than α-(1→6), it is difficult to explain the extensive conversion of dextran B-512 into D-glucose. Recent studies[30] of the length of the side chains showed that ~40% contain one D-glucose residue, at least 45% are two D-glucose residues long, and the remaining 15% are longer than two. Essentially all of the side chains in dextran B-1415 consist of single D-glucose residues[31]. If the side chains are distributed at random, the hydrolysis of dextran B-512, which has 5% of branch linkages, should have ceased after only ~20 D-glucose residues had been released from a chain of d.p. 10,000. The conversion into D-glucose would then be less than 1%. Evidence provided from considerations of intrinsic viscosities, sedimentation constants, molecular weights, and molecular radii of dextran fractions indicate that a proportion of the side chains must be longer than 50 D-glucose residues[32–34]. In order to explain the 22% degradation of dextran B-512 into D-glucose, the proportion of side chains that are longer than 2 units (15%) would need to have an average chain-length of 33 D-glucose residues. But there seems no reason why such long side-chains should not also be branched, since, as Senti *et al.*[32] pointed out, long side-chains should be indistinguishable from the main chain to synthesizing enzymes. Nevertheless, the presence of long side-chains, whether or not they bear short side-chains, could largely explain the high conversion of dextran B-512 into D-glucose.

Another possible explanation for the extent of enzymic degradation of the dextrans might be the existence of several populations of molecules, in which the degree of branching ranged from nil to a highly branched structure. Formation of such a mixture of polysaccharides could result from the presence of more than one type of polymerizing and branching enzyme-system. Several D-glucosyl-transferases capable of synthesizing glucans of different appearance from sucrose have been isolated from *Streptococcus mutans*[4]. Similarly, many strains of *Leuconostoc mesenteroides* produce dextrans which have been separated by alcohol precipitation into fractions having not only different extents but also different types of branching[35]. Clearly, there is a need to purify dextran-sucrase and branching-enzyme systems so that they synthesize homogeneous populations of dextran, before chemical and enzymic studies of dextran structure can produce information of real value.

We have described dextran-glucosidase as an exo-dextranase to specify attack exclusively at the non-reducing end of the dextran chain, and so to distinguish it from the endo-dextranase type of activity, in which many internal linkages are susceptible to hydrolysis. The possibility remained, however, that the enzyme might be an α-D-glucosidase rather than an exo-dextranase, since exo-glucanases and glucosidases have substrates in common. Reese *et al.*[36] established criteria that may be used to differen-

tiate between the two types of enzymes. Their simplest test was to determine the relative activity of the enzyme on a dimer and on the corresponding tetramer or polymer. An exo-glucanase would be more active on the tetramer, a glucosidase on the dimer. The *S. mitis* enzyme could not be classified with certainty in this way, for its rate of action did not increase as the d.p. increased from 2 to 4, as would that of an exo-glucanase; neither did the rate of hydrolysis drop sharply, as would that of a typical glucosidase. There was no doubt that, weight for weight, isomaltose was a far superior substrate to dextran, yet at equimolar substrate concentration, the enzyme released D-glucose from dextran more rapidly than from isomaltose. This could be attributed to the presence in dextran of long side-chains which provide additional non-reducing ends that are susceptible to the action of the enzyme.

Exo-enzymes show greater specificity for dimer linkage than do glucosidases[36]. By this criterion, the *S. mitis* enzyme behaved as an exo-glucanase, for isomaltose was the only susceptible disaccharide. However, the enzyme was shown to catalyse transfer reactions[9] when acting on concentrated solutions of isomaltose and isomaltose saccharides. The ability to produce an oligosaccharide of d.p. one D-glucose residue higher than the substrate is a characteristic of glucosidases rather than exo-glucanases.

Hydrolysis by exo-glucanases proceeds by inversion of configuration[36], and according to this criterion, the *S. mitis* enzyme, which acted with retention of configuration, was clearly a glucosidase. Inhibition studies with nojirimycin also supported this view.

If the enzyme consisted of a mixture of an exo-glucanase with an α-D-glucosidase, some of the deviations from the pattern established by Reese *et al.* might be explained. This possibility is not considered likely, for the relative activity of the enzyme towards dextran and isomaltose did not change throughout its purification, nor after loss of activity due to storage, nor on inhibition with Tris buffer.

It is concluded that the enzyme is a glucosidase that releases D-glucose from the non-reducing end of α-(1→6)-D-glucans, and that the rate of hydrolysis is virtually independent of the degree of polymerization of the substrate.

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