STUDIES ON DEXTRANASES

PART II*. AN INTRACELLULAR BACTERIAL DEXTRANASE

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ABSTRACT

An intracellular dextranase preparation has been isolated from the bacterium described in the preceding paper. The enzyme degrades dextrans to D-glucose principally, but apparently cannot attack certain non-(1→6)-linkages. The initial attack on dextran appears to be *endo*, but attack on linear isomaltodextrins seems to be *exo*, one D-glucose unit at a time being liberated from the non-reducing end of the substrate.

INTRODUCTION

Dextranases (E.C. 3.2.1.11) are enzymes capable of hydrolysing α-D-(1→6) linkages of dextrans. They can be produced adaptively by certain bacteria when cultured on dextran-containing media, and have been found in the cell-free culture fluids[1,2], or associated with the cells[3–5]. Recent studies in this laboratory on dextranases produced by a soil bacterium dealt with two extracellular enzymes[6]. The present work describes the purification and properties of an intracellular enzyme from the same organism.

EXPERIMENTAL AND RESULTS

Materials and methods. — Dextran Type 100-C (Sigma) was used as substrate for assay of dextranase activity. D-Glucose oxidase, Type II from *Aspergillus niger*, and peroxidase (from horseradish) were obtained from the Sigma Chemical Co. D-Glucose was determined enzymically by the D-glucose oxidase–peroxidase–*o*-dianisidine system[8,9]. Methods for disc electrophoresis at pH 5.0, paper chromatography, and dextranase assay are described in the preceding paper[6]. Hydroxylapatite (Bio-Gel H.T.P.) was obtained from Bio-Rad Laboratories, Richmond, California.

Extraction and purification of the enzyme. — Cells from the culture medium[6] were harvested at the end of the log phase of growth by centrifugation at 20,000 *g* and

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4°, given two washings with 0.02M citrate buffer (pH 5.5), and suspended in an equal volume of the same buffer. Dextranase activity of the washed cells was virtually zero. Two methods of cell disruption were attempted.

(*a*) French pressing at 0–4° and approximately 8 tons/in.^{2}, followed by centrifugation at 20,000 *g*. Four successive pressings gave an optimal yield of dextranase activity.

(*b*) Sonication for 20 min in an M.S.E. 100-watt ultrasonicator, while being cooled in ice, followed by centrifugation.

Method (*a*) released ~40% of the total activity into solution (total activity being measured by assaying the disrupted-cell suspension prior to centrifugation), whereas method (*b*), as well as being more convenient, released some 50% of the total activity into solution. Attempts to remove more enzyme from the cell debris, using the detergents Triton-100 (Calbiochem, B grade) and sodium lauryl sulphate, were unsuccessful.

The purification procedure, which was carred out on sonicated cells from 6 l of culture medium, is summarised in Table I, for which the following points require comment.

TABLE I

PURIFICATION OF INTRACELLULAR DEXTRANASE

Procedure	*Specific activity*	*Yield* (%)	*Purification*
Extract after sonication	0.022	100	
Ammonium sulphate (40–47% saturation)	0.091	78	4
Bio-Gel P-200 column	0.99	48	45
Hydroxylapatite column	3.29	37	150

(*a*) The number of units of enzyme activity/mg of protein (specific activity) was determined at each stage of the purification. One unit is defined as that amount of enzyme which gives rise to 1 μg equivalent of D-glucose/min.

(*b*) Solid ammonium sulphate was added in small portions to the stirred solution of crude enzyme at 4°.

(*c*) Chromatography on a column (90 × 2.5 cm) of Bio-Gel P-200 was carried out at 4°, by upward elution with 0.02M citrate buffer (pH 5.5). A sample (4 ml) containing ~12 mg of protein was applied to the column. The dextranase activity was eluted in a single, symmetrical peak between 235 and 265 ml (Fig. 1). Disc electrophoresis of the material from this peak showed two sharp bands of protein, and a small amount of protein which remained at the point of application.

(*d*) Attempts to purify the material from the Bio-Gel P-200 column by chromatography on DEAE- and CM-cellulose were unsuccessful.

(*e*) The hydroxylapatite (Bio-Gel H.T.P.) column (0.8 × 3.0 cm, 1.5 g dry wt.) was prepared in 0.01M phosphate buffer (pH 7.0). The enzyme solution (4 ml, con-

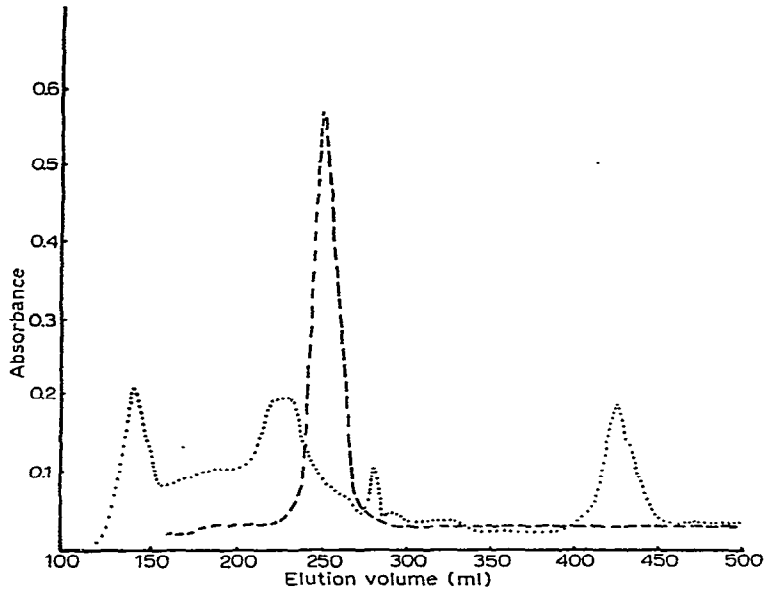

Fig. 1. Elution pattern of intracellular dextranase from Biogel P-200. ·····, Absorbance at 280 nm (protein); ——, absorbance at 520 nm (Nelson reducing assay).

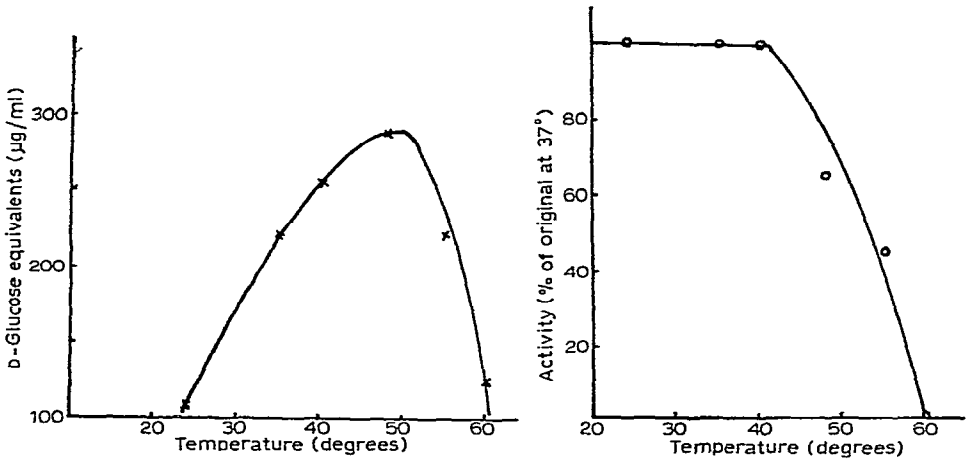

Fig. 2. Temperature optimum curve.

Fig. 3. Temperature stability curve.

taining ~90 μg of protein) was added to the column, followed by 6 ml of buffer. All of the enzymic activity was adsorbed. Stepwise elution with phosphate buffer (pH 7.0) of increasing molarity (6 ml each sample) removed successively 15 (0.02M buffer), 78 (0.05M buffer), and 1.8% (0.1M buffer) of the added activity. The material removed by

TABLE II

ACTION OF INTRACELLULAR DEXTRANASE ON DIFFERENT SUBSTRATES

Substrate	*1 h*	*3 h*	*19 h*	*72 h*
Dextran 100-C	G,[a] IM2 to IM7 and higher	G, IM2 to IM7 and higher	G, IM2 to IM7	G, B_3, B_6[b]
Isomaltose (IM2)	G, IM2[c]	G, IM2	G, IM2	G
Isomaltotriose (IM3)	G, IM2, IM3	G, IM2, IM3	G, IM2	G
Isomaltotetraose (IM4)	G, IM2, IM3, IM4	G, IM2, IM3, IM4	G, IM2, IM3	G
Isomaltopentaose (IM5)	G, IM2 to IM5	G, IM2 to IM5	G, IM2 to IM5	G
Isomaltohexaose (IM6)	G, IM2 to IM6	G, IM2 to IM6	G, IM2 to IM4	G

[a] G = D-glucose, IMx = linear isomaltodextrins of d.p. x. [b] B_3 and B_6 are tentatively identified as branched isomaltose oligosaccharides of five and six units, respectively. [c] The degree of underlining reflects the relative intensity of the spots.

the 0.05M buffer was used in subsequent experiments. Disc electrophoresis of a concentrated aliquot of this fraction, run at the same time as a sample of material from the Bio-Gel P-200 column, showed a single protein-band which moved the same distance as the faster-moving band in the cruder sample.

Properties of the enzyme preparation. — The enzyme solution in citrate buffer (pH 5.5, 0.02M) lost <10% activity when stored for up to 6 weeks at 4°. Freezing and thawing of such a solution caused ~10% loss in activity for each cycle.

The temperature optimum (Fig. 2) was obtained by incubating the enzyme solution (0.1 ml), citrate buffer (pH 5.5, 0.02M, 0.7 ml), and dextran solution (1%, 0.5 ml) for 1 h at various temperatures, followed by an assay of reducing power.

Temperature stability (Fig. 3) was determined by incubating the enzyme solution (0.1 ml) and buffer (0.7 ml) for 1 h at each temperature and then at 37° for 1 h with added dextran solution (1%, 0.5 ml), followed by assay of reducing power.

The pH optimum (Fig. 4) was found by incubating enzyme solution (0.1 ml) and dextran solution (1%, 0.5 ml) in phosphate–citrate buffers (0.02M, 0.7 ml) of various pH values for 1 h at 37°, followed by assay of reducing power.

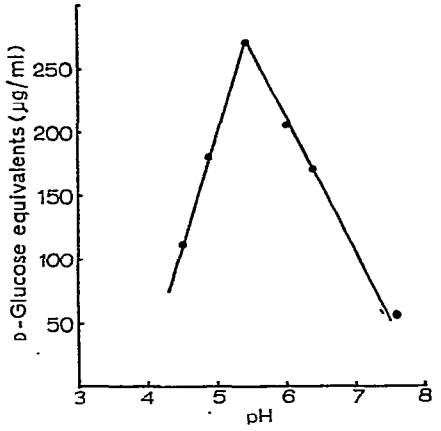

Fig. 4. pH optimum curve.

Substrate studies on the purified dextranase preparation. — Unless otherwise specified, substrate studies were carried out by incubating the various substrate solutions (1%, 0.5 ml), citrate buffer (0.02M, pH 5.5, 0.5 ml), and enzyme solution (0.1 or 0.2 ml) at 37° for the times indicated. The resultant solutions were deionized by shaking for 1 h with Amberlite IR-120(H^+) and IR-45(HO^-) resins, filtered, and examined by paper chromatography. The results are shown in Table II.

The enzymic hydrolysis of dextran 100-C was monitored by measuring (*a*) the increase in reducing power and (*b*) the D-glucose liberated, the results being shown in Fig. 5. After 3 days, paper chromatography showed only glucose and two oligosaccharides (B_5 and B_6) having R_I 0.23 and 0.14, where R_I indicates migration

relative to isomaltose. These values did not correspond to any of the isomaltodextrins (IM5 and IM6 have R_I 0.17 and 0.10).

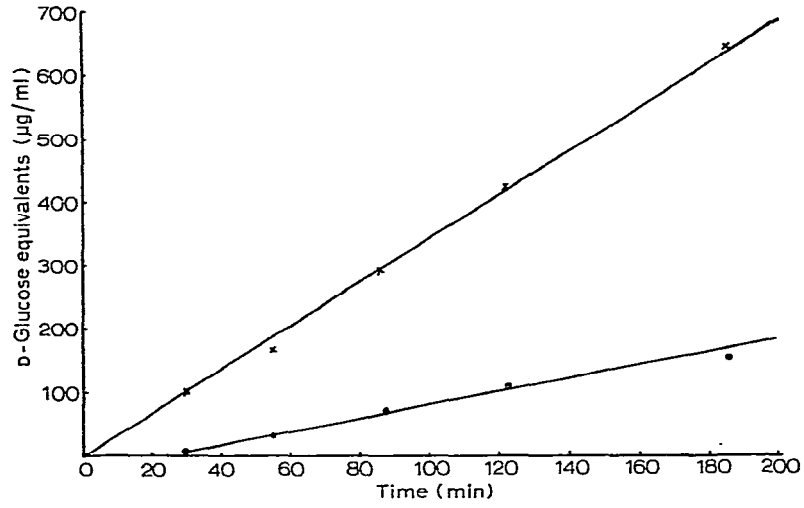

Fig. 5. Attack of intracellular dextranase 100-C, as followed by reducing-power increase (–×–) and D-glucose assay (–O–).

Study of the chromatograms of the oligosaccharide digests seemed to indicate that single D-glucose units were progressively hydrolysed from these substrates. To confirm this, the progress of the enzymic attack on borohydride-reduced isomaltotetraose was followed by (*a*) assay for increase in reducing power (Fig. 6); (*b*) assay for D-glucose liberation by the enzymic system described above (Fig. 6); (*c*) paper chromatography, which showed glucose and a non-reducing trisaccharide to be released initially, followed after 4 h by a small proportion of non-reducing disaccharide.

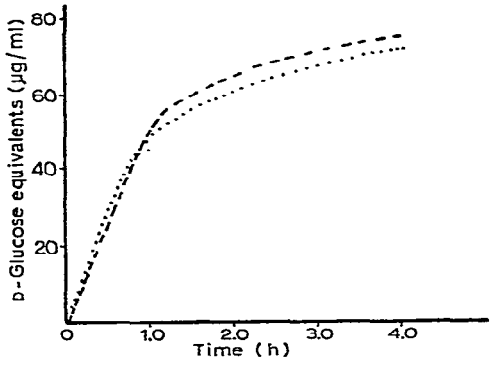

Fig. 6. Attack of intracellular dextranase on reduced isomaltotetraose, as followed by reducing-power increase (——) and D-glucose assay (····).

DISCUSSION

Initial attack of the enzyme preparation on dextran liberated oligosaccharides, but D-glucose was not liberated before 30 min (Fig. 5). The mean d.p. of the liberated oligosaccharides progressively decreased with time of hydrolysis, until after 3 days only D-glucose and the two supposedly branched oligosaccharides, B_5 and B_6, remained. Similar studies of linear isomaltodextrins of d.p. 2 to 6 indicated that these were degraded entirely to D-glucose, probably by liberation of one D-glucose unit at a time from the non-reducing end. This mechanism was confirmed for the case of reduced isomaltotetraose (IM4). Fig. 6 shows that D-glucose was the only reducing sugar released, and paper chromatograms confirmed that D-glucose and a non-reducing trisaccharide were released initially.

Thus, apparently, the initial attack on the dextran molecule is *endo*, whereas the attack on the lower oligosaccharides studied is *exo*. The results could be interpreted as the action of (*a*) a mixture of endo- and exo-enzymes, the later detection of D-glucose (Fig. 5) being due to the few end-groups originally available for exo-attack, or (*b*) a single enzyme, similar in action to some of the alpha-amylases, which appear to attack amylose in a random fashion initially, but degrade lower oligosaccharides in a non-random fashion[7]. Points in favour of (*b*) include the single band obtained on disc electrophoresis and the single, smooth peaks in the pH and temperature activity curves.

On the basis of the above evidence, we conclude that the enzyme is probably pure and is different from the extracellular dextranases produced by the same bacterium. Since we have designated the latter as D_1 and D_2, we designate this intracellular enzyme as D_3. A probable *in vivo* function of such an intracellular enzyme is to degrade to D-glucose the oligosaccharides formed by the action of the extracellular enzymes D_1 and D_2 on dextran.

Accumulation of the supposedly branched oligosaccharides B_5 and B_6 from dextran degradation would appear to indicate that the enzyme is incapable of attack on non-(1→6)-linkages. No evidence of transglycosylation was obtained from any of the chromatograms.

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