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PURIFICATION OF INTRACELLULAR DEXTRANASES AND D-GLUCOSIDASES FROM *Pseudomonas* UQM 733*

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ABSTRACT

The intracellular enzymes of *Pseudomonas* UQM 733 which act on dextran have been re-investigated mainly by isoelectric focusing. At least three dextranases are present, and one of them (D_4) has been purified and shown to be very similar to one of the extracellular endo-dextranases (D_1). Three different α-D-glucosidases have also been purified.

INTRODUCTION

Previous papers in this series have been concerned with the study of the extracellular dextranases [(1→6)-α-D-glucan 6-glucanohydrolases EC 3.2.1.11] of the bacterium *Pseudomonas* UQM 733[1,2]. A preliminary report has also been made of the intracellular enzymes of this bacterium[3]. We have now re-examined the intracellular enzymes of this bacterium.

EXPERIMENTAL

Materials. — B-512 native dextran was kindly supplied by Pharmacia-AB, Uppsala, Sweden. Isomaltose was a gift from Dr. Hubert Schiweck, Suddeutschen-zucker-AG, and isomaltotriose was prepared in this laboratory by M. Streamer[4]. Bovine serum albumin, D-glucose oxidase type V (*Aspergillus niger*), and peroxidase (horseradish) were obtained from the Sigma Chemical Co. The ampholytes, isoelectric-focusing columns, and gel-chromatography media were obtained from Pharmacia South Pacific. The mixed ion-exchangers were Amberlite IR-45(HO^-) and IR-120(H^+) resins.

General methods. — All enzyme solutions were stored and processed at 1–4° unless otherwise stated. Dextranase activity was assayed in 0.02M citrate buffer

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(pH 5.5, 0.5 ml) containing 0.5% of dextran. The solutions were incubated at 33°, usually for 1 h. The reducing sugars found during hydrolysis were assayed by the Nelson–Somogyi method[5], with anhydrous D-glucose as the standard. One unit of dextranase activity is defined as the amount of enzyme that produces 1 μmol equivalent of D-glucose under the above conditions, and specific activity as the units of activity per mg of total protein.

The D-glucose oxidase–peroxidase–guaiacol method[6] was used to determine D-glucose. Glucosidase activity was inhibited by the use of Tris–glycerol buffer[7]. Guaiacol was used as a chromogen instead of *o*-dianisidine, because it is non-carcinogenic. The colour developed with guaiacol is not as stable as that from *o*-dianisidine, and the absorbances at 436 nm were determined as rapidly as possible. The glucosidase activity was determined by incubating 0.5 mg of isomaltose in 0.02M citrate buffer (pH 5.5) with enzyme preparation in a final volume of 0.15 ml. The solutions were incubated for 1 h at 33°, and then adjusted to 1.0 ml with water, and the D-glucose released in the reaction was measured as described above. The glucosidase activity is defined as the amount of enzyme that produces 1 μmol of D-glucose per min under the above conditions.

Protein was determined either by measuring the absorbance at 280 nm, or by the modified Folin–Ciocalteau method[8] with bovine serum albumin as the standard. Carbohydrate was determined by the phenol–sulphuric acid method[9] with anhydrous

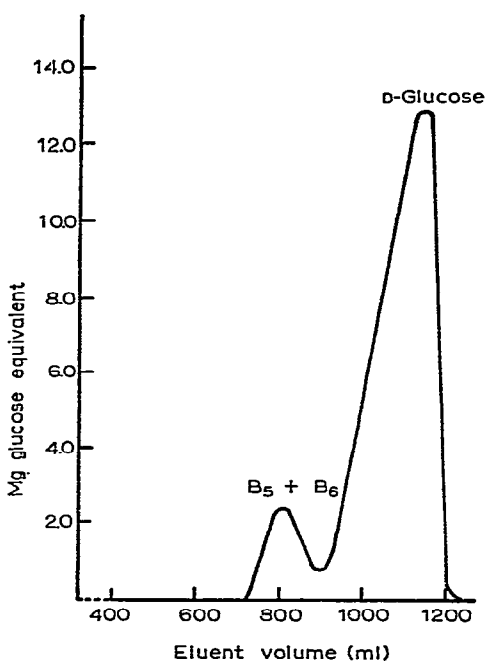


Fig. 1. Gel-permeation chromatography of branched isomaltopentaose (B_5), branched isomaltohexaose (B_6), and D-glucose on Sephadex G-15 (5.0 × 100 cm column, water eluent).

D-glucose as the standard. Thin-layer chromatography (t.l.c.) was carried out as described earlier[10].

Preparation of branched isomaltosaccharides. — Branched isomaltopentaose and branched isomaltohexaose were prepared by incubating a solution of 4 g of B-512 native dextran in 800 ml of 0.02M citrate buffer (pH 5.5) with 3.2 units each of dextranases D_1 and D_2 and 2 g of amyloglucosidase at 33° for 8 days. The hydrolysis was monitored by t.l.c. of deionised fractions. The dextranases D_1 and D_2 were prepared as described by Richards and Streamer[1]. The enzymes were denatured by heating the solution at ~100° for 15 min, and the solution was concentrated to 20 ml and then divided into 4 fractions of ~5 ml each. These fractions were then chromatographed separately on a column (5.0 × 100 cm) of Sephadex G-15 with deionised water as eluent (Fig. 1).

Extraction and purification of enzymes. — The bacterium was grown and harvested as described previously[1,3]. The cells were disrupted by sonication for a total of 20 min in an MSE 100-watt ultrasonicator at <5° in 0.02M citrate buffer (pH 5.5), followed by centrifugation. The intracellular enzymes were isolated from the supernatant solution by precipitation with ammonium sulphate followed by centrifugation. The precipitate (designated crude enzyme) was redissolved in 0.02M citrate buffer (pH 5.5) and dialysed against 1% glycine solution for 24 h.

Hydrolysis of dextran with crude enzyme. — The dextran solution [5 mg in 0.9 ml of 0.02M citrate buffer (pH 5.5)] was incubated at 33° with 0.1 ml (0.24 unit) of the crude enzyme preparation for 10 h. A 0.02-ml sample of the solution was examined by t.l.c. after deionisation with mixed ion-exchange resins.

Purification of intracellular enzymes by isoelectric focusing. — The isoelectric-focusing experiments were conducted at 4° with either the LKB 8101, 110-ml analytical column, or the LKB 8102, 440-ml preparative column. D-Glucitol (AR grade) was used as the density gradient, because the enzymes were inhibited by ethylene glycol, and invertase activity in the enzyme preparation precluded the use of sucrose. The focusing was conducted with 1% ampholytes in the pH ranges 3.0–10.0, 7.0–9.0, 6.0–8.0, and 4.0–6.0.

Effect of pH on dextranase D_4 and glucosidase activity. — The respective dextranase and glucosidase activities of the purified intracellular enzymes were examined at various pH values by using 0.02M citrate buffer (pH 4.0–6.0) and 0.02M phosphate buffer (pH 6.0–8.0).

Effect of temperature on activities of dextranase D_4 and glucosidases G-1, G-2, and G-3, and thermal stability of D_4. — Dextranase D_4 (0.01 unit) was incubated with 5 mg of dextran in 0.5 ml of 0.02M citrate buffer (pH 5.5), and glucosidases G-1, G-2, and G-3 (0.0016, 0.0013, 0.0015 unit, respectively) were incubated with 0.5 mg of isomaltose in 0.1 ml of 0.02M citrate buffer (pH 5.5) for 1 h at different temperatures. The dextranase and glucosidase activities were then determined as previously described.

The effect of temperature on the stability of the enzymes was measured by incubating D_4 and glucosidases G-1 and G-3 (0.01, 0.0016, and 0.0015 unit) in 0.1 ml (D_4 and G-1) or 0.2 ml (G-3) of 0.02M citrate buffer (pH 5.5) for 1 h at different

temperatures. The enzymes were then incubated with the appropriate substrates to determine the activities as described previously for D_4, G-1, and G-3; G-3 was also incubated with maltose under the conditions described for isomaltose.

Activity of purified enzymes against carbohydrates other than dextran and isomaltosaccharides. — To determine the activity of D_4, and glucosidases G-1, G-2, and G-3 towards carbohydrates other than dextran and isomaltosaccharides, 0.1-ml fractions (D_4 0.01, G-1 0.0016, G-2 0.0013, and G-3 0.0015 unit) of each of the enzyme solutions were incubated with solutions of ~5 mg of the carbohydrate in 0.5 ml of 0.02M citrate buffer (pH 5.5) for 20 h at 33°.

Products of hydrolysis of B-512 native dextran with dextranase D_4. — The dextran (5 mg) was incubated at 33° with 0.01 unit of D_4 in 0.50 ml of 0.02M citrate buffer (pH 5.5). The hydrolysis was followed by withdrawing 0.05-ml samples for reducing-power assay, and a further 0.01 unit of D_4 was added after hydrolysis for 29 h and 52 h. When the reaction had reached completion, the solution was deionised with mixed resin and examined by t.l.c.

RESULTS AND DISCUSSION

Purification of intracellular enzymes

The ultimate product of hydrolysis of B-512 native dextran with the crude enzyme preparation was shown by t.l.c. to be glucose only. Thus, the crude, intracellular enzyme preparation contained enzyme(s) capable of hydrolysing the α-(1→3) as well as the α-(1→6) linkages of this dextran. In earlier studies[3], the same enzyme mixture was fractionated by gel-permeation chromatography with Bio Gel P-200

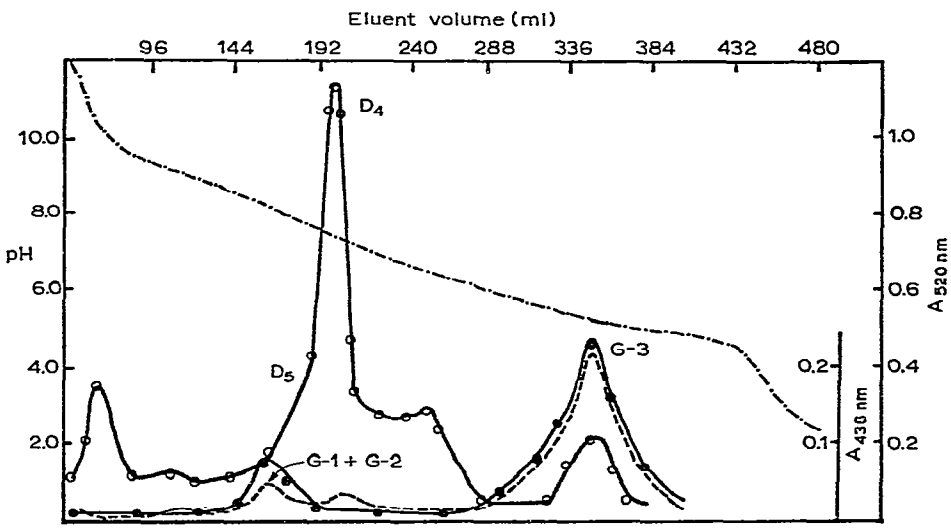


Fig. 2. Isoelectric focusing of crude intracellular enzymes in 1% pH 3–10 ampholyte and 0–60% of D-glucitol; 500 V for 64 h: —○—, dextranase activity; —●—, glucosidase activity (isomaltose); – – –, glucosidase activity (branched isomaltopentaose, branched isomaltohexaose); – · –, pH.

followed by chromatography on Hydroxylapatite. These purified extracts appeared to contain enzyme that attacked dextran in a random manner, initially liberating isomaltosaccharides, which then appeared to be attacked in an exo-manner, to give glucose and two oligosaccharides tentatively identified as branched isomaltopentaose and branched isomaltohexaose. The enzyme isolated had behaved as a single compound in gel chromatography and disc electrophoresis, and appeared to possess both endo- and exo-activity. The degradation of dextran completely to glucose by the crude enzyme preparations had not been noted previously.

The application of isoelectric focusing to the separation of the intracellular enzymes was first attempted by using pH 3–10 ampholytes. Dextranase activity was detected at pH 5.4, 6.6, 7.3, and 10.0 (Fig. 2). Glucosidase activity was detected at pH 5.4, 7.3, and 8.2 when the branched isomaltopentaose/branched isomaltohexaose mixture was used as the substrate, and at pH 5.4 and 8.2 when isomaltose was used as substrate. The dextranase activity peak at pH 7.3 showed "shoulders" of dextranase activity on either side of the major peak. The fractions containing the various dextranase and glucosidase activities were combined and designated D_4 (pI 7.3), glucosidase G-1 (pI 7.8), and glucosidase G-3 (pI 4.7). The glucitol was removed by dialysis for 24 h against 0.02M citrate buffer (pH 5.5) before gel-permeation chromatography. Attempts to purify the G-3 fractions further, using both Bio-Gel P-200 and Hydroxylapatite, and G-1 and G-2, using Bio Gel P-200, were unsuccessful, and resulted in complete loss of activity. The loss of activity on Bio-Gel P-200 is unusual, since such gels are generally regarded as relatively inert, chromatography media; for example, Janson[11] has used such gels to purify α-D-glucosidases with good recovery of activities. This loss of activity of glucosidases G-1, G-2, and G-3 during gel-permeation chromatography and absorption chromatography is an indication of the instability of these enzymes.

Janson[11] used preparative isoelectric focusing to purify α-D-glucosidases from large quantities of extraneous protein (7.3 g). In an attempt to scale up the preparation, the pH 3–10 focusing experiment was repeated with a 5-fold quantity of crude enzyme. The polarity of the column was reversed in order to reduce interference by precipitated protein, but the large-scale experiment gave less-effective resolution of the enzymes and loss of the glucosidase G-3 into the electrode solution (Fig. 3). Also, there appeared to be some selective loss of dextranase D_4 (pI 6.6), but this may be due to the change in glucitol concentration in the dextranase fractions which is associated with the reversal of polarity (glucitol slightly inhibits dextranase activity). The pH 5.5–9.0 fractions were combined, dialysed against glycine solution, and refocused at pH 7–9 (Fig. 4). This gave an improved resolution of the two dextranase peaks, separating one (D_4 pI 6.9) away from the glucosidases G-1 and G-2. The second dextranase activity (D_5) at pH 7.7 was associated, but not exactly coincident, with the G-2 activity. The glucosidase fraction eluted between 245–274 ml, which contained mainly glucosidase G-1 plus a minor amount of glucosidase G-2, with possibly a trace of D_5, was resolved into two separate glucosidase activities, namely,

G-1 (pI 7.5) and G-2 (pI 7.7), by further focusing with pH 6–8 ampholytes on an analytical (110-ml) column (Fig. 5).

The D_4 enzyme was further purified by gel-permeation chromatography with Bio-Gel P-200 (Fig. 6). The protein profile indicated the presence of protein at the void volume of the column in approximately the same amount as the protein associated with the enzyme activity, which was eluted in the fractionation range of the gel.

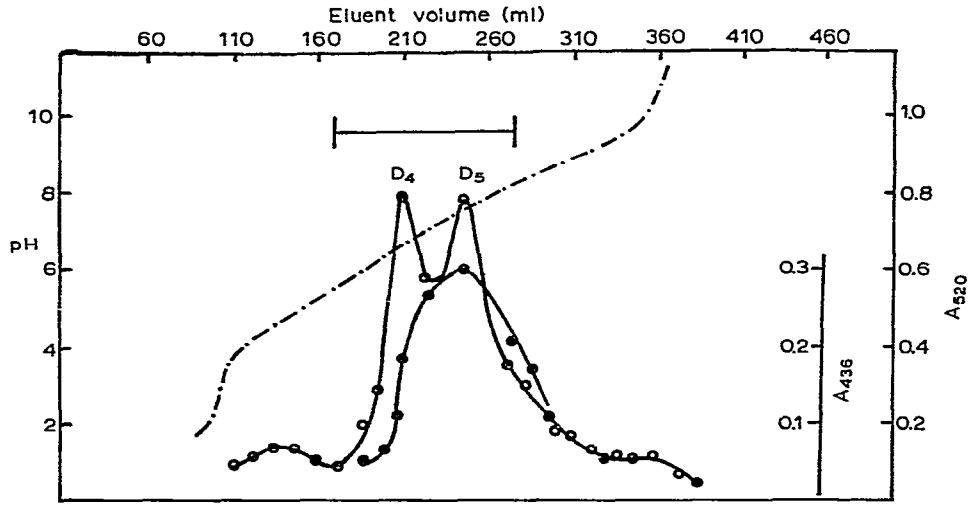


Fig. 3. Isoelectric focusing of crude enzymes in 1% pH 3–10 ampholyte and 0–60% of D-glucitol; 400 V for 62 h (large scale): —○—, dextranase activity; —●—, glucosidase activity (isomaltose); – –, pH.

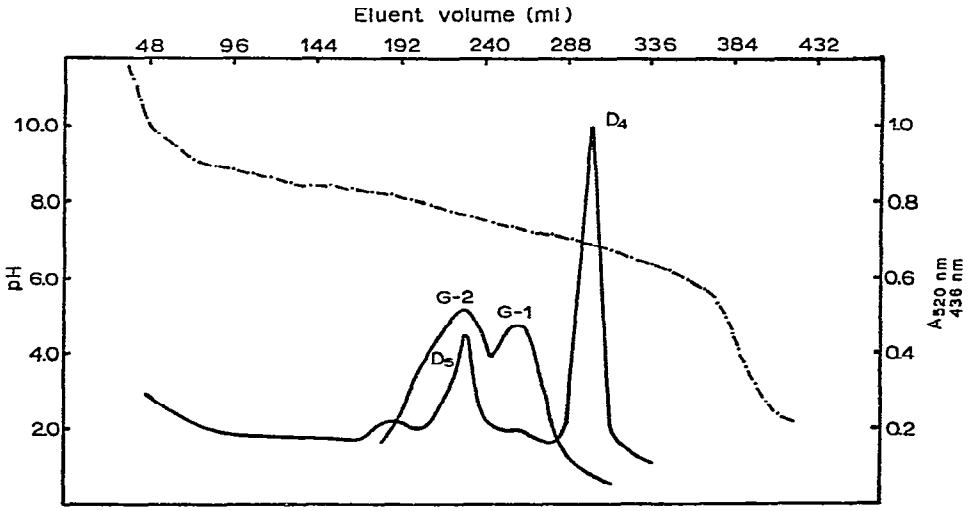


Fig. 4. Isoelectric focusing of crude intracellular enzymes in 1% pH 7–9 ampholyte and 0–60% of D-glucitol; 560 V for 108 h: curve including peaks D_4 and D_5, dextranase activity; curve including peaks G_1 and G_2, glucosidase activity (isomaltose); – –, pH.

The large amount of apparent protein at the inclusion volume of the column is due to ampholyte that had not been completely removed by dialysis prior to gel-permeation chromatography.

The group of enzymes that focus at pH 4.7 were separated from the enzymes that focus at pH $\geqslant$ 6.9 by isoelectric focusing of the crude enzyme preparation in pH 4–6 ampholyte (Fig. 7). In this experiment, there was again interference by denatured and/or precipitated protein which eluted in the same fractions as the

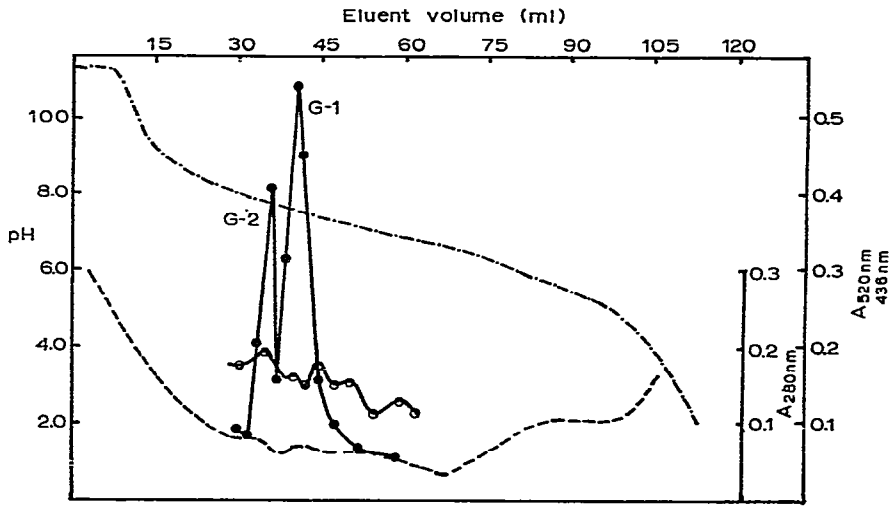


Fig. 5. Isoelectric focusing of glucosidases G-1 and G-2 in 1% pH 6–8 ampholyte and 0–60% of D-glucitol; 570 V for 50 h: —○—, dextranase activity; —●—, glucosidase activity (isomaltotriose); – –, pH; – – –, absorbance at 280 nm.

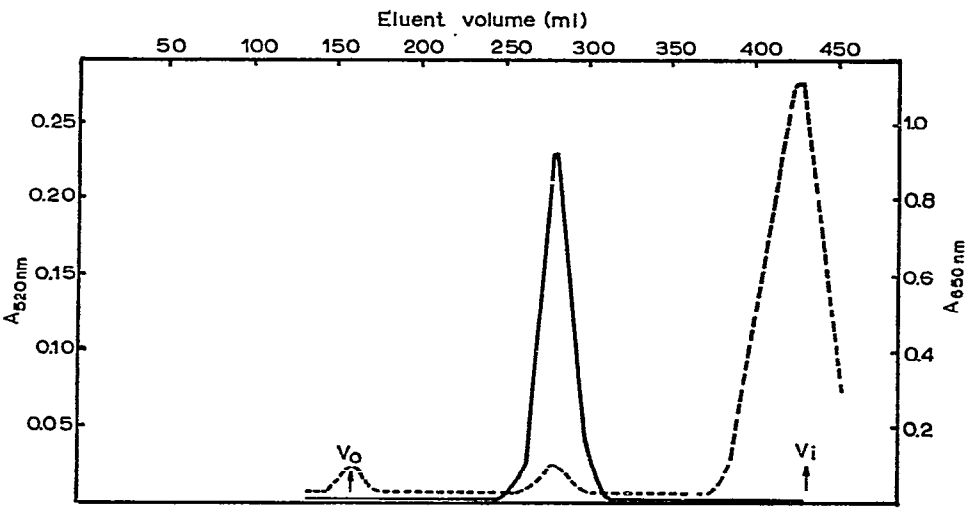


Fig. 6. Gel-permeation chromatography of enzyme D_4 on Bio-Gel P-200 in 0.02M citrate (pH 5.5): ——, dextranase activity; – – –, absorbance at 650 nm.

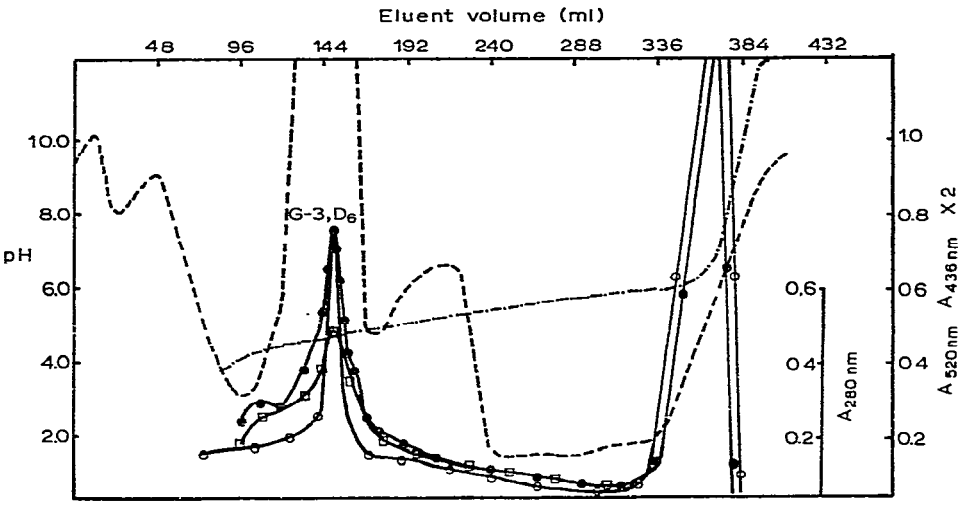


Fig. 7. Isoelectric focusing of crude enzyme preparation in 1% pH 4–6 ampholyte and 0–60% of D-glucitol; 420 V for 48 h: —○—, dextranase activity; —●—, glucosidase activity (isomaltose); —□—, glucosidase activity (branched isomaltopentaose, branched isomaltohexaose); –·–, pH; ———, absorbance at 280 nm.

focused enzymes. The enzymes that focused at pH 4.7 were obtained as cloudy solutions which showed glucosidase and dextranase activities apparently associated with two different enzymes. Thus, the ratio of glucosidase activity in two fractions taken at 144 ml and 150 ml was 0.75 : 1.0, whilst the ratio of dextranase activity in the same tubes was 0.3 : 1.0. Further evidence to support the existence of two separate enzymes with pI 4.7 was obtained when this combined fraction was centrifuged at 9,000 *g* for 15 min. This resulted in 25% loss of glucosidase activity and 97% loss of dextranase activity. Therefore, it was concluded that, in these fractions, the dextranase activity, and perhaps some of the glucosidase activity, was associated with precipitated protein.

The yields of glucosidases G-1, G-2, and G-3 were low, but similar to the yields obtained by Janson[11] when he purified α-D-glucosidases from the cytoplasm of *Cytophaga johnsonii* by similar methods. The yield decreased by a factor of approximately five with each isoelectric focusing experiment conducted (Tables I and II). The yield of G-2 (0.1%) was also diminished, because much of this enzyme was discarded as mixed fractions with enzyme D_5 (see Fig. 4).

General properties of dextranase D_4 and Glucosidases G-1, G-2, and G-3

The effect of pH on the activity of dextranase D_4 and the glucosidases G-1, G-2, and G-3 is shown in Fig. 8. The dextranase D_4 had a pH optimum of 5.5, similar to the extracellular dextranases D_1 and D_2 of this bacterium[1]. Glucosidases G-1 and G-2 showed a higher pH optimum of 6.0–6.5 and 6.0, respectively, whilst G-3, which was inhibited by phosphate buffer, had a pH optimum of 7.0. Subsequent experiments were conducted in 0.02M citrate buffer (pH 5.5).

TABLE I

PURIFICATION OF INTRACELLULAR ENZYMES[a] BY ISOELECTRIC FOCUSING AND GEL-PERMEATION CHROMATOGRAPHY

Procedure	*Specific activity*		*Yield (%)*		*Purification (−fold)*	
	D	*G*	*D*	*G*	*D*	*G*
Sonication	0.09	0.25	100	100		
30–50% Ammonium sulphate	0.11	0.45	39	31	1.2	1.8
Isoelectric focusing						
pH 3–10	4.5	0.59	35	7.0	50	3
pH 7–9	16.5[b]	1.2	8.3	2.3	183	5
pH 6–8		2.0[c]		0.25		8
		1.0[d]		0.10		4
Bio-Gel P-200	33.0		8.2		366	

[a]D, refers to dextranase activity; G, refers to glucosidase activity. [b]D_4 only. [c]Glucosidase G-1. [d]Glucosidase G-2 only.

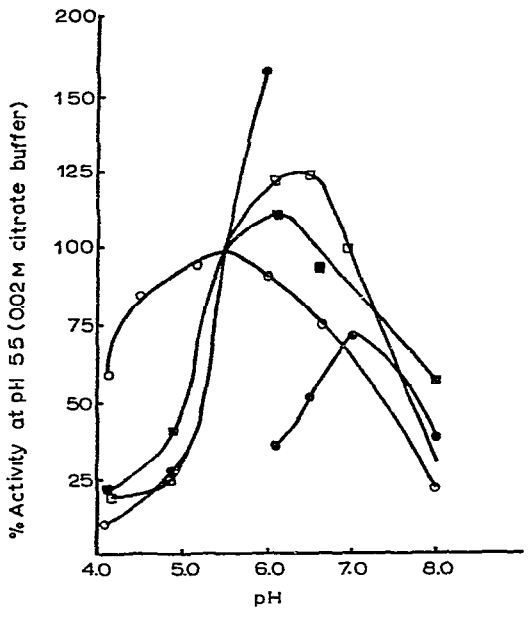
TABLE II

PURIFICATION OF GLUCOSIDASE G-3 BY ISOELECTRIC FOCUSING

Procedure	*Specific activity*	*Yield (%)*	*Purification (−fold)*
Sonication	0.3	100	
30–50% Ammonium sulphate	0.67	31	2.2
Isoelectric focusing (pH 4–6)	0.75	1.1	2.5

The effect of temperature on the activity of dextranase D_4 and the glucosidases G-1, G-2, and G-3 is shown in Fig. 9. The D_4 enzyme had a temperature optimum (~58°) similar to that of the extracellular dextranase[1] D_1. The glucosidases G-1 and G-2 had temperature optima of 37° and 33°, whilst G-3 had a higher temperature optimum of ~50°. The glucosidases G-1 and G-2 thus have low temperature optima, and are similar in this respect to the cytoplasmic glucosidases from *Cytophaga johnsonii* studied by Janson[11]. The effect of temperature on the stability of dextranase D_4 and glucosidases G-1 and G-3 is shown in Fig. 10. The increase in activity of G-3 towards isomaltose and maltose when heated at 38° is surprising and may indicate a change in conformation of enzyme between 33° (assay temperature) and 38°, which has a favourable effect on the rate of hydrolysis of these substrates.

The dextranase D_4 exhibited no activity towards soluble starch, sucrose, or hemicellulose "B" fraction. The glucosidases G-1 and G-2 also exhibited no activity towards these substrates, as well as exhibiting no activity towards maltose or cellobiose. Thus, these glucosidases differed from the α-D-glucosidases studied by Janson[11] and



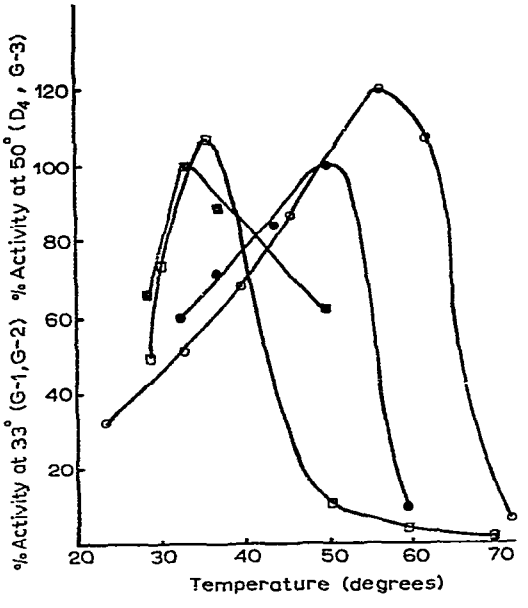


Fig. 8 (left). Effect of pH on activity of dextranase D_4 (—○—), and glucosidases G-1 (—□—), G-2 (—■—), and G-3 (—●—).

Fig. 9(right). Effect of temperature on activity of dextranase D_4 (—○—), and glucosidases G-1 (—□—), G-2 (—■—), and G-3 (—●—).

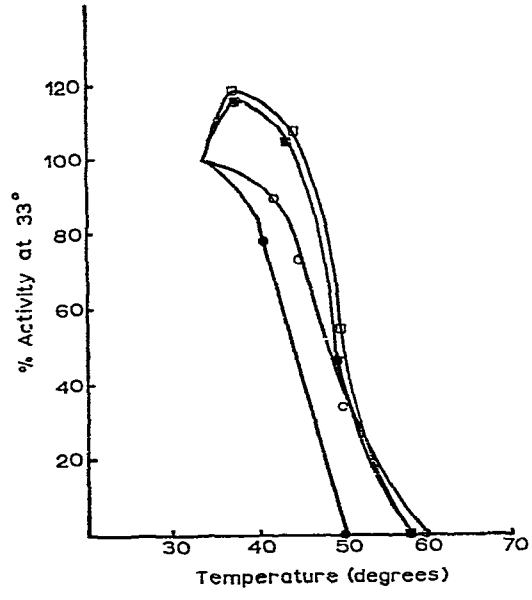


Fig. 10. Effect of temperature on stability of dextranase D_4 (—○—), and glucosidases G-1 (—●—), G-3 (IM_2) (—□—), and G-3 (M_2) (—■—).

by Jeffrey *et al.*[12]. The glucosidase G-3, however, possessed an affinity for maltose (M_2) similar to that for isomaltose (IM_2). The G-3 enzyme had no activity towards soluble starch, sucrose, or hemicellulose "B" fraction. However, the G-3 enzyme-preparation was not pure and, in order to ascertain whether the activity with M_2 was due to the presence of another glucosidase, the effect of temperature on the stability of the G-3 enzyme(s) was examined. The results shown in Fig. 10 show that G-3 retained the same relative activity towards M_2 and IM_2 at all temperatures examined, which would indicate the presence of one enzyme. This result is regarded as tentative evidence only, and further research is required to show whether two glucosidases having separate α-(1→4) and α-(1→6) activities are present.

The time course of hydrolysis of B-512 native dextran with dextranase D_4 followed a typical hyperbolic plot. The conversion into apparent glucose after 70-h hydrolysis was 34.5%, which was higher than that obtained for the hydrolysis of this dextran with the extracellular dextranase D_2. T.l.c. of the enzyme hydrolysate at the longest period of hydrolysis studied revealed the presence of glucose (trace amounts), and two series of oligosaccharides (isomaltose–isomaltotetraose and branched isomaltotetraose–branched isomaltononaose). Thus, the intracellular dextranase D_4 hydrolysed B-512 native dextran in an endo-type cleavage, giving rise to a series of linear and branched isomaltosaccharides similar to that obtained[1] from the extracellular enzyme D_1. The presence of branched isomaltotetraose indicated that dextranase D_4 was capable of hydrolysing α-(1→6) linkages close to, or even adjacent to, the branch point.

T.l.c. of the reaction mixtures of each of the glucosidases G-1, G-2, and G-3 with B-512 native dextran, after incubation for 1 week at 33°, revealed the products listed in Table III. They suggest that the G-2 enzyme contains a trace of endo-dextranase, whereas G-1 is free of endo-dextranase activity. The glucose in the case of G-1 probably arises from slow hydrolysis of dextran by G-1 itself. Glucosidase G-3 contains a small amount of endo-dextranase activity, as would be expected from the activity profile obtained after isoelectric focusing.

The dextranase D_4, when purified by gel-permeation chromatography using Bio-Gel P-200, had an elution volume similar to that of the extracellular enzyme[1] D_1. This indicates that D_4 and D_1 have similar molecular weights. The pH and tempera-

TABLE III

PRODUCTS OF HYDROLYSIS OF B-512 NATIVE DEXTRAN WITH GLUCOSIDASES G-1, G-2, AND G-3

Glucosidase	*Products*
G-1	Glucose and dextran
G-2	Glucose, isomaltose, branched isomaltopentaose, branched isomaltohexaose, and dextran
G-3	Glucose, branched isomaltopentaose, traces of oligosaccharides of higher molecular weight, and dextran

ture optima and mode of action of D_4 towards the oligosaccharides isomaltohexaose and isomaltoheptaose were also similar to those found for D_1. The intracellular dextranase D_4 is therefore very similar to the enzyme D_1 produced extracellularly by the *Pseudomonas* bacterium. These similarities between D_1 and D_4 in molecular weight, pH and temperature optima, and action pattern suggest that D_1 and D_4 are the same enzyme. However, it is usually assumed that extracellular enzymes are not found inside cells (*cf.* Ref. 14), and further research is required to establish the relationship (if any) between D_1 and D_4.

The low yield of the glucosidases is believed to be partly due to the purification technique used (isoelectric focusing), and partly due to the inherent instability of α-D-glucosidases when purified. The yields of α-D-glucosidases obtained by other research workers are usually low[11,12,14]. It is possible that the yield of glucosidases could be improved by using either ion-exchange chromatography or affinity chromatography. The mode of action of these enzymes will be described elsewhere.

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