Purification and Some Properties of *Arthrobacter globiformis* Exo-1,6-α-glucosidase

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A gram-positive and pleomorphic bacterium (strain I-42) isolated from soil as a producer of exo-1,6-α-glucosidase [EC 3.2.1.70] was identified as *Arthrobacter globiformis*. This *Arthrobacter* enzyme, inducible by dextran extracellularly, was partially purified from a cell-free culture supernatant. It was found most active at pH around 6.0 and most stable at pH 6.0–6.5. The enzyme was proved, by several experiments, to attack dextran in the exo-wise fashion to release only glucose leaving a macromolecular limit dextran dextrin. Transglucosylation from dextran to accumulating or added glucose was not observed.

As to the dextranase that releases glucose from dextran in the exo-wise fashion, several examples have so far been reported. In 1956, Sery and Hehre\(^1\) first observed an enzyme system that did so in the culture broth of human intestinal bacteria belonging to *Bacteroides*. Recently Walker *et al.*\(^2\)\(^,\)\(^3\) described many features of a glucose-releasing dextranase (α-1,6-glucosidase,*\(^1\) dextranglucosidase*\(^1\)) of *Streptococcus mitis* isolated from human dental plaque. A similar dextranase (exodextranase*\(^1\)) of *Bacillus* species isolated from soil was briefly characterized by Zevenhuizen.\(^4\) These two enzymes were intracellular. For the enzymes of this type [EC 3.2.1.70, 1,6-α-D-glucan glucohydrolase] a name, exo-1,6-α-glucosidase, was recommended in 1972 as it was added to the classified enzyme system in Enzyme Nomenclature 1972.\(^5\) Enzymes having a similar activity occur in a variety of mammalian tissues.\(^6\)

By screening soil bacteria we were able to isolate several bacteria that produced enzymes of this type extracellularly. This paper deals with identification of one of the bacteria, together with purification and some basic properties of its glucodextranase.*\(^2\)

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\(^1\) Names actually used in their papers for glucose-releasing dextranase.

\(^2\) Abbreviated name used in this paper for exo-1,6-α-glucosidase.

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MATERIALS AND METHODS

**Dextrans.** Dextran T2000 derived from dextran B-512 was a product of Pharmacia Fine Chemicals AB, Uppsala, Sweden. Native dextran B-512 was a gift from Dr. Allene Jeanes of the Northern Regional Research Laboratories, Peoria, Ill., U.S.A. Dextrans N-4 were supplied by Meito Sangyo Co., Nagoya. Dextran B-512 and N-4 are known to contain about 95% and 93–96% α-1,6-glucosidic linkage, respectively.\(^7\)\(^,\)\(^8\)

**Cultures.** The bacterium, strain I-42, was one of those isolated from soil as glucodextranase producers and used throughout. It was identified as *Arthrobacter globiformis* as described in RESULTS AND DISCUSSION. The identification was carried out in accordance with the system of Yamada and Komagata\(^9\) and with the 8th edition of Bergey's Manual of Determinative Bacteriology.\(^10\)

The bacterium was grown for dextranase production in a medium containing 0.2% NH\(_4\)NO\(_3\), 0.1% KH\(_2\)PO\(_4\), 0.05% MgSO\(_4\)-7H\(_2\)O, 0.02% CaCl\(_2\)-2H\(_2\)O, 0.3% peptone, 0.3% meat extract and 1.5% dextran in deionized water at pH 7.4 (adjusted with NaOH). Glucodextranase was induced only in the presence of dextran. The dextran used for this purpose was a product (M\(_w\), 36,000–39,000) of Meito Sangyo Co., a derivative from native dextran N-4. For systematic purification of glucodextranase, 20-liter culture was incubated at 30°C for 2 days in a jar fermentor with aeration at the rate of 15 liters per min (by courtesy of Shinnihon Chemical Co., Anjo, Aichi). The stock culture was maintained on slants of the same medium in 2% agar.

A culture of *Penicillium luteum* ATCC 9644 (IFO 6345) was purchased from the Institute for Fermentation, Osaka. Its dextranase [EC 3.2.1.11] was prepared...
as before\textsuperscript{11}) and used in an experiment for comparison.

**Dextranase assay.** Five ml of the reaction mixture for glucodextranase assay contained dextran T2000 (1\%), acetate buffer (0.08 M), calcium acetate (0.005 M) and enzyme solution (1 ml) at pH 6.0. After incubation at 37°C for 20 to 60 min, the reaction was stopped by adding 2 ml of 1\% Na\textsubscript{2}CO\textsubscript{3}, and the increase of reducing sugar was measured. One unit was defined as the amount of enzyme required to release reducing sugar equivalent to 1 μmole glucose per min under the above-stated conditions.

Unless otherwise specified, miscellaneous reaction mixtures were composed as assay mixtures irrespective of kinds of dextrans, kinds of buffers, pH or potency of enzymes, and were always incubated at 37°C for reaction.

**Analytical methods.** Reducing sugars were measured by Somogyi's reductometry.\textsuperscript{12} In order to determine only glucose in reaction mixtures, Glucostat (glucose oxidase reagent) of Worthington Biochemical Corp., Freehold, N.J., U.S.A., was employed. The extents of enzymatic hydrolysis of dextrans were calculated on the basis of substrate concentration values obtained by the phenol-sulfuric acid method\textsuperscript{13}) using glucose as standard.

Protein was measured by absorbance at 280 nm with egg albumin as standard. For measurement of specific fluidity (reciprocal of specific viscosity) of dextran digests an Ostwald viscometer was used.

Paper chromatograms (Toyo filter paper, No. 50) were developed with aqueous 65\% 1-propanol\textsuperscript{14}) ascendingly and stained by the AgNO\textsubscript{3}–NaOH dipping technique.\textsuperscript{15}

**RESULTS AND DISCUSSION**

**Identification of strain I-42**

The bacterium, strain I-42, was identified as *Arthrobacter globiformis*. Its description follows:

Rods, 0.4 to 0.6 by 1.0 to 3.0 μ in young cultures (Fig. 1B). Coccioid or slightly ellipsoidal cells appear as it ages (Fig. 1A). Curved and irregularly formed cells are observed. Develop in a bending pattern of cell division.\textsuperscript{16}) Pleomorphic. Non-motile. Spore not formed. Gram positive. Not acid-fast.

Nutrient agar colonies: Circular, smooth, entire, raised, glistening, grey.

Peptone-dextran agar colonies: Circular, raised, entire, glistening, creamy.

Nutrient agar slant: Smooth, glistening, medium unchanged.

Nutrient broth: Turbid and sediment.

Potato: Raised, smooth, brownish grey.

Nutrient gelatin stab: Liquefaction.

P.C.B. milk: Alkaline and peptonization.

Nitrate not reduced.

Indole not produced.

Hydrogen sulfide not produced (Kligler's agar).

Starch weakly hydrolyzed.

No acid formed from L-arabinose, xylose, rhamnose, glucose, fructose, mannose, galactose, sorbose, sucrose, lactose, maltose, trehalose, cellobiose, dextrin, starch, dextran, inulin, glycerol, mannitol, sorbitol, inositol, arbutin,
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esculin, salicin or methyl α-glucoside in peptone medium. \(^{17}\)

Acetate, pyruvate, lactate, malate, succinate, fumarate, α-keto-glutarate, citrate, formate, propionate, butyrate, malonate, gluconate, glyoxylate, glycolate, hippurate and urate are assimilated, but oxalate, glutarate, adipate and pimelate are not. \(^{17}\)

Ammonium sulfate is utilized as a sole nitrogen source.

No vitamin is required.

Tyrosine is dissolved with browning.

Casein is hydrolyzed.

DNase: Positive.

Urease: Positive.

Oxidase: Negative.

Catalase: Positive.

Effect of pH: Grows between pH 5.0 to 9.0.

Effect of sodium chloride: No growth at 10% sodium chloride.

Effect of temperature: Optimum temperature, 25~32°C. No growth at 39°C.

Aerobic.

Principal amino acid in cell wall: Lysine. \(^{18}\)

%GC: 64.5 \(^{19}\)

Source: Road soil in Shuzenji, Shizuoka, 1971.

**Purification of glucodextranase**

The starting material for purification of glucodextranase was a jar-grown culture possessing 0.3-unit/ml dextranase activity. \(^{*}\) Every purification procedure was carried out at 5°C. The enzyme was first collected by adsorption on TEAE-cellulose (Serva Feinbiochemica GmbH & Co., Germany) by the batch-wise method from the cell-free culture supernatant diluted about twice with deionized water at pH 7.0. The TEAE-cellulose (ca. 120 g) used had been equilibrated to an acetate mixture (sodium acetate in 0.025 M and calcium acetate in 0.005 M). From the cellulose once washed with acetate mixture, glucodextranase was eluted with acetate mixture containing 0.7 M sodium chloride. The eluate (820 ml), after well dialyzed against acetate mixture, was subjected to Duolite A2 (Chemical Process Co., Calif.) column chromatography (four 1.1 x 36 cm columns). Glucodextranase was eluted as a single peak by 0 to 0.6 M sodium chloride gradient in acetate mixture. The combined dextranase-rich fractions (ca. 300 ml) were dialyzed against a less dense acetate mixture (sodium acetate in 0.01 M and calcium acetate in 0.005 M), concentrated to 84 ml by ultrafiltration (Diaflo membrane PM10, Amicon Corp., Mass.) and subjected to two runs of electofocusing with a 110 ml column (LKB Produkter AB, Sweden).

Glucodextranase focused at pH about 3.9 as a single peak as shown in Fig. 2. The

![Fig. 2. Profile of Electofocusing of Glucodextranase.](image)

enzyme was electofocused once more under the same conditions as the first runs. The combined glucodextranase fractions thus obtained were dialyzed against acetate mixture, concentrated to 7.5 ml by ultrafiltration as described above and finally chromatographed on a column (2.5 x 82 cm) of Bio-Gel P-150 (Bio-Rad Laboratories, Calif.) in acetate mixture. Glucodextranase fractions eluted as a single peak and combined were the final product of purification. The purification procedures are summarized in Table I. It was

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\(^*\) The glucodextranase activity of jar-grown cultures was variable and lower than that of flask-grown cultures (0.7 to 1.0 unit/ml). The bacterium produced a similar dextranase intracellularly, too, but its amount was much smaller than that in the culture supernatants.
known through the enzyme purification that Arthrobacter I-42 produces only a single glucodextranase extracellularly.

The glucodextranase thus purified was not completely pure as known from a disc electrophoresis pattern (pH 8.3 Tris buffer), where at least one minor protein band having no dextranase activity was visible on the cationic side of the main one that was glucodextranase. The purified glucodextranase solution hydrolyzed not only various dextrans but also isomaltodextrins to release only glucose. Kojibiose, nigerose, sucrose or cellobiose was not attacked by this purified glucodextranase solution. Maltose and soluble starch were hydrolyzed by the enzyme solution very slightly compared with dextran with liberation of only glucose. Whether or not glucodextranase itself has a glucoamylase activity is still under investigation.

**pH-dependency of glucodextranase**

The activity of glucodextranase upon dextrans was most significant at around pH 6.0 as shown in Fig. 3. Glucodextranase seemed most stable at pH 6.0 to 6.5 as long as acetate or cacodylate buffer was used (Fig. 4). Glucodextranase was markedly inactivated in buffers containing citrate or phosphate even at pH 6.0 to 6.5 under conditions similar to those in Fig. 4.

**Mode of action**

Figure 5 shows the action of Arthrobacter glucodextranase on two dextrans of the commercialized type, dextrans T2000 and N-4. Both dextrans were similarly hydrolyzed by the dextranase and their hydrolysis limit was around 28%. Native dextran B-512 was also hydrolyzed to a comparable extent. The hydrolysis limit rose to 30–32% for these dextrans when their concentration in digests was lowered to 0.05% and the incubation time was lengthened to four days with a more potent enzyme (8 units/5 ml digest). From each of the 1% dextran digestes of the three dextrans incubated for a day as in Fig. 5, a carbohydrate residue was recovered by precipitation with 67% methanol. Its amount, as assayed by the phenol-sulfuric acid method, ranged from 70 to 75% of the dextran given as substrate. Therefore, it is evident that
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dextran T2000 by 42±43%.* The fairly wide difference in hydrolysis limit of the dextrans tested between the two Arthrobacter dextranases, gluco- and isomalto-dextranases, implies a dissimilarity in action specificity between these exo-type enzymes. Dextranglucosidase of Streptococcus mitis was reported to hydrolyze 23±25% of dextran B-512,3) while Bacillus exodextranase was claimed to fully hydrolyze a dextran having 93±96% α-1,6-glucosidic linkage.4)

Figure 6 is the paper chromatogram of a dextran T2000-glucodextranase digest similar to that of Fig. 5. The reaction product is only glucose and no oligosaccharide spot is visible. Temporal oligosaccharide formation by possible transglycosylation from dextran to accumulating or added glucose was not observed even in digests of higher dextran concentrations, such as 3.6%, with or without

* Unpublished recent experiments (substrate concentration, 0.05%) showed that the isomalto-dextranase hydrolyzed those dextrans referred to in this paper to an extent as great as about 60%.

the glucodextranase leaves a macromolecular limit dextrandextrin even after extensive attack on dextrans.

It was reported previously20) that an isomaltose-releasing dextranase produced by another strain of Arthrobacter (strain T6) hydrolyzed the glucodextranase leaves a macromolecular limit dextrandextrin even after extensive attack on dextrans.

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In order to know more about the mode of action of glucodextranase, dextran T2000 digestion was followed by two analytical methods simultaneously.

Figure 7 is a result where Somogyi's reductometry and glucose oxidase method (Glucostat) were used. Two curves showing the reaction processes obtained by the two methods actually overlap with each other. This result indicates that glucose is the only reducing sugar product from the dextran and that the release of any oligosaccharide product is least probable.

In the next experiment, dextran T2000 digestion was followed by viscosimetry and by reductometry using two identical digests. In this case, digestion of the same dextran by *P. luteum* dextranase was also followed in the same way for comparison. The result is illustrated in Fig. 8.

With mold dextranase, which splits dextran molecules at random to produce glucose and a mixture of oligosaccharides, specific fluidity of its digest rapidly increases from the beginning. With glucodextranase, on the other hand, specific fluidity of its digest rises very little in spite of a much greater reducing sugar release than with mold dextranase. The striking contrast between the two dextranases in fluidity increase vs reducing sugar increase resembles that observed by Moore and Stone\textsuperscript{21} between endo- and exo-types of $\beta$-1,3-glucan hydrolases. Accordingly, Fig. 8 indicates that the mode of action of glucodextranase does not involve endo-mechanism.

From the several experimental results described above, it can be concluded that exo-1,6-$\alpha$-glucosidase (glucodextranase) of *Arthrobacter globiformis* strain I-42 hydrolyzes dextran by successively splitting $\alpha$-1,6-glucosidic linkage from the chain ends, namely in the exo-wise fashion, to release only glucose, leaving a macromolecular limit dextrandext-
rin, perhaps without causing transglycosylation directly from the substrate.

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REFERENCES