A Simple Purification Procedure for and Further Characterization of a Glucodextranase* from *Arthrobacter globiformis* I42†

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A highly purified glucodextranase (EC 3.2.1.70) was simply prepared from cell-free culture broth of *Arthrobacter globiformis* I42 by ammonium sulfate fractionation, affinity chromatography on Sephadex G-100 and column chromatography on DEAE-cellulose. The purified enzyme was judged to be essentially homogeneous on PAGE and SDS-PAGE as well as isoelectric focusing. $E_{1\text{cm}}^{1\%}$ at 280 nm was 18.6. The enzyme is an acidic protein with a pI of 4.31. The molecular weight of the enzyme was estimated to be about 120,000 by SDS-PAGE. The enzyme is rich in alanine and glycine. No carbohydrate moiety seemed to be associated with the enzyme protein.

The optimum pH and temperature of the enzyme are pH 6.0 and 45°C, respectively. The enzyme was completely stable over the pH range of 5.5~7.5 at 4°C for 24 hr, and retained about 40% of its original activity after heating at 60°C for 10 min. Inactivation of the enzyme was found to be partial with 5 mM Pb^{2+} or Zn^{2+} , and complete with 5 mM each of Cu^{2+} , Hg^{2+} , Fe^{3+} , $KMnO_4$ and NBS. The enzyme split dextran or isomaltose to produce β -glucose, indicating that the α -glycosidic linkages in these substrates are inverted. The K_m value of the enzyme for dextran is 0.014%. Phenyl α -D-glucoside is a potent competitive inhibitor ($K_i = 1.69$ mM).

In 1976, Sawai *et al.*¹⁾ first reported that an exo-1,6- α -glucosidase (1,6- α -D-glucan glucosylhydrolase, EC 3.2.1.70) was produced extracellularly by a Gram-positive and pleomorphic soil bacterium identified as *Arthrobacter globiformis* strain I42. They described the partial purification and some catalytic properties of this enzyme.^{1,2)} The enzyme was characterized as a glucodextranase, because it released only β -glucose from the non-reducing ends of dextran molecules.^{1,2)} Later on the enzyme was obtained in a crystalline state by Ohya *et al.*³⁾

This paper describes an improved purification procedure for and further characterization of the glucodextranase from *A. globiformis* I42.

MATERIALS AND METHODS

Chemicals. Dextran T2000 (MW: 2×10^6) and Sephadex G-100 were products of Pharmacia Fine Chemicals, Uppsala, Sweden. DEAE-Cellulose (0.85 meq/g) was a product of The Brown Co., West Germany, crystalline bovine serum albumin from Miles Laboratories Inc., U.S.A., methyl α -D-glucoside, used as an elution ligand, from Tokyo Kasei Kogyo Co., Ltd., Japan, methyl α -D-glucoside, used for investigating the inhibition kinetics, from Wako Pure Chemical Industries, Ltd., Japan, phenyl α -D-glucoside from Nakarai Chemicals, Ltd., Japan, isomaltose from Hayashibara Biochemical Laboratories, Inc., Japan, polypeptone from Daigo Eiyo Kagaku Co., Ltd., Japan, and carrier ampholyte (pH 4~6) from LKB Produkter AB, Stockholm, Sweden. Standard proteins for SDS-PAGE (SDS-6H) were products of Sigma Chemical Company, St. Louis, U.S.A. Clinical dextran (Meito 70) was kindly supplied by Meito Sangyo Co., Ltd., Japan. All other reagents were of analytical grade.

† Studies on a Glucodextranase from *Arthrobacter globiformis* I42. Part I.

* Abbreviation used in this paper for exo-1,6- α -glucosidase.

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Abbreviations: PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; NBS, *N*-bromosuccinimide; EDTA, disodium ethylenediaminetetraacetic acid; pCMB, *p*-chloromercuribenzoate.

Organism. The organism used for preparation of the glucodextranase was a Gram-positive soil bacterium, *Arthrobacter globiformis* I42 (NRRL B-4427, IAM 12102).

Cultures. The standard culture medium for enzyme production comprised 6l of a liquid medium containing 1.5% clinical dextran (Meito 70), 0.2% NH_4NO_3 , 0.1% KH_2PO_4 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.04% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.3% polypeptone, 0.3% meat extract and 0.1% asparagine. The medium was adjusted to pH 8.5 by the addition of 1N NaOH and then sterilized in a Tokyo Rikakikai 11.5-l jar-fermentor (model M-200) at 120°C for 40 min. The precultured broth (200 ml) of *A. globiformis* I42 grown in the same medium for 2 days was inoculated into the standard culture medium, and then the cultivation was performed at 20°C for 65 hr at an aeration rate of 3 l/min and a rotational speed of 50 rpm. The cell-free culture fluid was obtained using a Tomy Seiko continuous centrifuge (model RS-20 II, 10,000 $\times g$) and then used as the starting material for enzyme purification.

Substrate and enzyme assay. Dextran T2000 was used as the substrate for the enzyme assay. Dextran-saccharifying activity was examined for the standard assay. The reaction mixture for the glucodextranase assay contained 0.25 ml of 1% dextran T2000, 0.5 ml of 40 mM acetate buffer (pH 6.0), containing 10 mM calcium acetate, and 0.25 ml of the enzyme solution. The mixture was incubated at 30°C for an appropriate period. Glucose produced, in 0.5 ml of the reaction mixture, was measured by the glucostat method.^{4,5} One unit of enzyme activity was defined as the amount of enzyme that catalyzed the liberation of 1.0 μmol of D-glucose from the substrate per min under the above conditions.

Measurement of protein and carbohydrate. Protein concentrations were measured by the method of Lowry *et al.*⁶ with crystalline bovine serum albumin as a standard. The absorbance at 280 nm was used for monitoring protein in column effluents.

The total carbohydrate in the purified enzyme was determined by the phenol-sulfuric acid method⁷ with D-glucose as a standard.

Polyacrylamide gel electrophoresis. PAGE was performed in 7.5% polyacrylamide with Tris-diethylbarbituric acid buffer (pH 7.0) according to the method of Davis.⁸ SDS-PAGE was performed in 5% polyacrylamide containing 0.1% SDS by the method of Weber and Osborn.⁹ In both cases, the gels were stained in 0.25% Coomassie Brilliant Blue R-250–50% methanol–10% acetic acid.

Isoelectric focusing. The *pI* of the enzyme was measured, using an LKB Ampholine electrofocusing apparatus (110 ml), by the method of Vesterberg and Svensson.¹⁰

Amino acid analysis. Amino acid analysis of the purified enzyme was performed with a Nihondenshi amino acid analyzer (model JLC-200A) or a Waters HPLC apparatus (model QA-1), under the conditions given under Results.

Column chromatography. To purify the enzyme, column chromatographies on Sephadex G-100 and DEAE-cellulose were performed.

Polarimetric procedure. A 1.0 ml sample of the buffered enzyme solution, pH 6.0 (100 units), containing 5 mM calcium acetate was added to 4.0 ml of a 3% isomaltose solution, followed by thorough mixing. Then a part of the resultant mixture was quickly transferred to a 0.1-dm polarimeter cell and the changes in the rotation were measured at 30°C at intervals with a Union Gikken automatic digital polarimeter (model PM-101). After incubation for 15 min, one drop of concentrated ammonium hydroxide was added to the polarimeter cell and then the rotation was again measured after thorough mixing.

Measurement of *K_m* and *V* for dextran. A mixture of 0.5 ml of a 0.01–0.25% dextran T2000 solution and 1.0 ml of 40 mM acetate buffer, containing 10 mM calcium acetate (pH 6.0), with or without 10 mM phenyl α -D-glucoside or 20 mM methyl α -D-glucoside was equilibrated at 30°C. The reaction was started by the addition of 0.5 ml of the purified enzyme (0.044 units) warmed to the same temperature. After incubation for 10 min, 0.5-ml samples were removed and the glucose liberated was measured by the glucostat method. The *K_m* and *V* values were calculated from Lineweaver–Burk plots.¹¹

RESULTS

Time courses of cell growth and glucodextranase production during the cultivation

Samples of about 20 ml each of the culture medium in a jar-fermentor were taken at appropriate times, and the absorbance at 660 nm was immediately measured as cell growth. Each sample was subjected to centrifugation at 10,000 $\times g$ at 4°C to remove bacterial cells. The glucodextranase activity, protein concentration and pH of each culture broth were then measured. The time courses of growth of the bacterium and the enzyme production are summarized in Fig. 1.

Purification of glucodextranase

Unless otherwise stated, all purification procedures for the enzyme were performed in a cold room (4°C).

FIG. 1. Time Courses of Cell Growth and Glucodextranase Production.

---●---, protein concentration ($A_{280\text{nm}}$); —○—, glucodextranase activity; —●—, turbidity ($A_{660\text{nm}}$); ---■---, pH. The experimental details are given in the text.

FIG. 2. Elution Patterns of the Crude Enzyme after $(\text{NH}_4)_2\text{SO}_4$ Fractionation on Affinity Chromatography on Sephadex G-100.

5% Methyl α -D-glucoside was added to the elution buffer at the position indicated by the arrow: ---●---, protein concentration ($A_{280\text{nm}}$); —○—, glucodextranase activity. The active fractions (tube Nos. 145~165) were combined.

Step 1. Ammonium sulfate fractionation. Solid ammonium sulfate was added to the cell-free culture broth (ca. 6 l) to 90% saturation. After being kept overnight, the solution was subjected to continuous centrifugation at $10,000 \times g$. The precipitates were dissolved in 230 ml of 20 mM acetate buffer containing 5 mM calcium acetate.

Step 2. Affinity chromatography on Sephadex G-100. The precipitate solution was applied on a column of Sephadex G-100 (6.0×42 cm) preequilibrated with 20 mM acetate buffer containing 5 mM calcium acetate (pH 6.0). The glucodextranase protein was

completely adsorbed onto Sephadex G-100 under the above conditions. After washing the column with ca. 5.6 l of the buffer mentioned above, the enzyme was eluted with the same buffer containing 5% methyl α -D-glucoside at a flow rate of 45 ml/60 min/tube. Fraction Nos. 145 to 166, which contained most of the glucodextranase activity (Fig. 2), were collected and then concentrated to about 8 ml by ultrafiltration with a Diaflo YM-2 membrane (Amicon Corp., Lexington, Mass., U.S.A.) and a collodion bag (Sartorius GmbH). The concentrated solution was dialyzed against the same buffer overnight.

FIG. 3. DEAE-Cellulose Column Chromatography of the Enzyme after the Sephadex G-100 Column Chromatography.

---●---, protein concentration ($A_{280\text{nm}}$); —○—, glucodextranase activity. The active fractions (tube Nos. 131~140) were combined.

TABLE I. RECOVERIES AND ACTIVITIES OF THE ENZYME DURING PURIFICATION

Purification step	Total volume (ml)	Total protein ^a (mg)	Enzyme activity		Sp. activity (units/mg protein)
			Total units	Yield (%)	
1. $(\text{NH}_4)_2\text{SO}_4$ fraction (90% saturation)	252	3500	5430	100	1.55
2. Sephadex G-100	990	198	4400	81.0	22.2
3. DEAE-Cellulose	60	144	3680	67.8	25.6

^a Measured by the method of Lowry *et al.*⁽⁶⁾

Step 3. DEAE-Cellulose column chromatography. The dialyzed sample was applied on a column of DEAE-cellulose (2.2×45 cm) pre-equilibrated with 20 mM acetate buffer containing 5 mM calcium acetate (pH 6.0). After washing the column with *ca.* 600 ml of the buffer mentioned above, the enzyme was eluted with the same buffer containing 0.3 M NaCl at a flow rate of 6.0 ml/20 min/tube. A symmetrical protein peak containing only glucodextranase activity was obtained (Fig. 3). The active fractions (tube Nos. 131~140) were then combined.

Table I summarizes the overall purification steps. The purified enzyme had a specific activity of 25.6 units per mg of protein, the final yield being about 68%, and it was used for the subsequent characterization.

Purity and molecular weight of the purified enzyme

The purified enzyme gave a single protein band on PAGE and SDS-PAGE (Fig. 4). $E_{1\text{cm}}^{1\%}$ at 280 nm was determined to be 18.6 from the absorbance and dry weight of the purified enzyme. No carbohydrate moiety associated with the enzyme protein was detected.

The molecular weight of the purified enzyme was found to be about 120,000 on comparison of its relative mobility on SDS-PAGE with those of standard proteins (SDS-6H).

Isoelectric point

The purified enzyme was subjected to isoelectric focusing to determine its *pI* and also to check its homogeneity. A sharp and symmetrical protein peak containing only glucodextranase activity was obtained, as can be

FIG. 4. Polyacrylamide Gel Electrophoresis of the Purified Glucodextranase.

Samples of the purified enzyme, 10 μ g (a) and 40 μ g (b), were applied on gel columns of 7.5% polyacrylamide and then run at 4°C at a constant current of 2 mA per gel column. (c) A sample of the purified enzyme (10 μ g) was applied on a gel column of 5% polyacrylamide containing 0.1% SDS, followed by electrophoresis at room temperature for 3.5 hr at a constant current of 8 mA per gel column.

seen in Fig. 5. The enzyme was judged to be homogeneous, its *pI* being 4.31.

Amino acid composition

A 10 mg sample of the purified enzyme preparation, dried at 80°C under reduced pressure over phosphorus pentoxide, was hydrolyzed with 6N redistilled HCl in a sealed tube at 110°C for 24 hr. The hydrolyzate was evaporated to dryness to remove HCl and then analyzed with an automatic amino acid analyzer. Tryptophan was analyzed by HPLC. The data are summarized in Table II. The enzyme is rich in alanine and glycine, but poor in lysine and methionine. Both histidine and tryptophan were found in small amounts. Cystine (half) was not detected, even with performic

FIG. 5. Isoelectric Focusing of the Purified Glucodextranase.

The purified enzyme, 3.9 mg of protein and 99.8 units of enzyme activity, was applied to a 110 ml LKB column. After electrophoresis at 4°C for 50 hr at a constant voltage of 1,100 V, fractions of 2 ml were collected, and then the enzyme activity, protein concentration and pH of each fraction were assayed.

---●---, protein concentration ($A_{280\text{ nm}}$); —○—, glucodextranase activity; ■, pH.

TABLE II. AMINO ACID COMPOSITION OF THE PURIFIED ENZYME

Amino acid	Molar ratio (%)
Arginine	4.47
Lysine	2.88
Histidine	1.33
Phenylalanine	2.24
Tyrosine	4.28
Leucine	7.38
Isoleucine	2.32
Methionine	1.21
Valine	7.02
Alanine	14.56
Glycine	12.51
Proline	5.56
Glutamic acid	8.22
Serine	5.65
Threonine	8.77
Aspartic acid	9.42
Tryptophan	2.18
Cystine (half) ^a	0

^a Measured by the performic acid oxidation method.¹²⁾

acid oxidation.¹²⁾

Effect of pH on the activity of the enzyme

The effect of pH on the activity of the purified

enzyme was studied under the standard assay conditions at 30°C for 6 min using 50 mM β , β' -dimethylglutaric acid-NaOH buffer containing 5 mM calcium chloride, pH 4.0~7.0. The enzyme showed a single maximum at pH 6.0.

Stabilities of the enzyme toward pH and temperature

Two series of enzyme solutions were prepared, the solutions containing equal amounts (0.01 ml) of the purified enzyme (0.48 units) and individually adjusted to a pH from 3.5 to 7.5 by adding 0.59 ml of 50 mM β , β' -dimethylglutaric acid-NaOH buffer containing 5 mM

calcium chloride. After one set of solutions had been kept at 4°C for 24 hr and the other set at 45°C for 2 hr, both sets of buffered enzyme solutions were diluted 5-fold with the same buffer (pH 6.0) as described above. Each 0.25 ml sample (0.04 units) was examined for the remaining glucodextranase activity by means of the standard assay at 30°C for 10 min. The results are shown in Fig. 6. The enzyme was completely stable over the pH range of 5.5~7.5 at 4°C and that of 6.5~7.5 at 45°C, under the conditions used.

Purified enzyme solutions (5.40 units) in 0.2 ml of 20 mM acetate buffer (pH 6.0) containing 5 mM calcium acetate were heated at various temperatures for 10 min and then cooled immediately in an ice-bath. After heat treatment, the enzyme solutions were diluted 170-fold with the same buffer as mentioned above. The remaining glucodextranase activity was then measured with 0.25 ml of each enzyme solution (0.04 units) by means of the standard assay at 30°C for 10 min. The results (Fig. 7) indicate that the enzyme is completely stable at temperatures below 55°C. The enzyme retained about 40% of its original activity on heating at 60°C, but was completely inactivated on heating at 65°C under the conditions used.

FIG. 6. Stabilities of the Purified Glucodextranase with Respect to pH and Temperature.

—●—, held at 4°C for 24 hr before assaying for enzyme activity; —○—, held at 45°C for 2 hr before assaying for enzyme activity. The experimental details are given in the text.

FIG. 7. Effects of Temperature on the Stability (—●—) and Activity (—○—) of the Purified Glucodextranase.

The experimental details are given in the text.

Effect of temperature on the activity of the enzyme

Enzyme solutions (0.034 units) in 1.0 ml of 40 mM acetate buffer (pH 6.0) containing 10 mM calcium acetate were incubated with 1.0 ml of a 0.5% dextran T2000 solution at different reaction temperatures for 10 min. The glucodextranase activity per 1.0 ml of each reaction mixture was assayed. The optimum temperature of the activity of the enzyme was 45°C, as shown in Fig. 7.

Effects of various metal ions and several enzyme inhibitors on the activity of the enzyme

The enzyme was incubated with an equal volume of a metal ion or inhibitor solution (10 mM, pH 6.0) at 30°C for 30 min and then cooled immediately in an ice-bath. Then the

FIG. 8. Changes in Optical Rotation during the Hydrolysis of Isomaltose by the Purified Glucodextranase and after Base-catalyzed Mutarotation.

A drop of concentrated ammonium hydroxide was added to the digest at the time indicated by an arrow and then the optical rotation was followed until 19 min incubation. The experimental details are given in the text.

mixtures were diluted 20-fold with 20 mM acetate buffer (pH 6.0) and the remaining activity per ml of each reaction mixture containing 0.020 units of enzyme was examined by means of the standard assay at 30°C for 20 min. Inactivation of the enzyme was partial with 5 mM Pb^{2+} or Zn^{2+} , being about 20% inhibition in each case. On the other hand, 5 mM each of Cu^{2+} , Hg^{2+} , Fe^{3+} , $KMnO_4$ and NBS inactivated the enzyme completely. EDTA, pCMB and other metal ions tested had no inhibitory effect on the activity of the enzyme.

Anomeric configurations of hydrolysis products

The anomeric configurations of the reaction products formed by the enzyme were examined. The optical rotation of isomaltose decreased gradually in a negative direction after addition of the enzyme. However, an immediate upward mutarotation was observed upon the addition of ammonium hydroxide to the reaction mixture (Fig. 8). This result indicates, at least qualitatively, that the enzyme releases β -glucose, and that the α -glycosidic linkages of the substrates are inverted.

FIG. 9. Lineweaver-Burk Plots for the Purified Glucodextranase.

—○—, glucodextranase activity toward dextran in the absence of 5 mM phenyl α -D-glucoside or 10 mM methyl α -D-glucoside; —●—, glucodextranase activity toward dextran in the presence of 5 mM phenyl α -D-glucoside; —○—, glucodextranase activity toward dextran in the presence of 10 mM methyl α -D-glucoside. s , dextran concentration (%); v , initial velocity (μ mol glucose/min/mg protein). The experimental details are given in the text.

Inhibition kinetics

The Lineweaver-Burk plots of glucodextranase activity toward dextran T2000 in the presence or absence of 5 mM phenyl α -D-glucoside or 10 mM methyl α -D-glucoside are shown in Fig. 9. The K_m and k_0 values of the enzyme for dextran were 0.014% and 95.24 sec^{-1} , respectively. As can be seen in Fig. 9, both phenyl α -D-glucoside and methyl α -D-glucoside are typical competitive inhibitors for the enzyme. The K_i values of the enzyme for phenyl α -D-glucoside and methyl α -D-glucoside were calculated to be 1.69 and 8.28 mM, respectively.

DISCUSSION

The first aim of this study was to establish a simpler and more effective enzyme purification procedure than the previous one.¹⁾ A glucodextranase was simply purified from cell-free culture broth of *A. globiformis* I42 by ammonium sulfate fractionation, affinity chromatography on Sephadex G-100 and column chromatography on DEAE-cellulose. It is remarkable that the activity recovery of the enzyme at the final purification stage (67.8%, Table I) is about 7 times higher than the

previous one (10%).¹⁾ The purified enzyme showed a specific activity of 25.6 units/mg protein and was judged to be essentially homogeneous on PAGE and SDS-PAGE as well as isoelectric focusing. The specific activity of this enzyme preparation is considerably higher than that of the previous one¹⁾ (18.0 units/mg protein). Using the purified enzyme, we obtained new data as to the physicochemical and enzymatic properties of the enzyme, which were lacking in the previous reports.^{1~3)}

The molecular weight of the purified enzyme (MW 120,000) is close to those of both the purified dextran α -1,2 debranching enzyme (MW 125,000)¹³⁾ and isomaltotriose-producing dextranase II (MW 114,000)¹⁴⁾ from *Flavobacterium* sp. M-73, however, it is quite different from that of the purified isomaltodextranase (MW 69,000) from *A. globiformis* T6.¹⁵⁾ Both endodextranases CD-I and -II from *Chaetomium gracile* contain a sugar moiety (4.5% each).¹⁶⁾ However, no carbohydrate moiety seemed to be associated with the purified glucodextranase from *A. globiformis* I42, as is the case of the purified isomaltodextranase from *A. globiformis* T6.¹⁵⁾

The amino acid composition of the purified glucodextranase was somewhat different from those of the two endodextranases of *C. gracile*.¹⁶⁾ The most striking discrepancy is that both the *C. gracile* enzymes have cystine (half) residues, while the purified enzyme does not. Another interesting point is that the alanine content of the purified enzyme is much higher than those of the *C. gracile* enzymes. These results resemble well the case of the purified isomaltodextranase from *A. globiformis* T6.¹⁵⁾

On the basis of the observed mutarotation upon hydrolysis of isomaltose by the purified enzyme (Fig. 8), we concluded that the anomeric carbon atoms of the reaction products

were of the β -configuration. This supports the finding of Ohya *et al.*²⁾ Using a gas-liquid chromatographic method, we also confirmed that the purified enzyme released only β -glucose from substrates, inverting the original α -anomeric configuration.¹⁷⁾

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