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STUDIES OF THE DEXTRANASE ACTIVITY OF PIG-SPLEEN ACID α-D-GLUCOSIDASE

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ABSTRACT

Pig-spleen acid α-D-glucosidase, when purified over 2000-fold, has a molecular weight of ~106,000 and is homogeneous by disc-gel electrophoresis. The enzyme splits reducing α-D-glucosyl disaccharides and almost completely degrades dextrans that contain (1→3)- and (1→6)-linkages. Dextrans containing (1→2)-linkages are only partially hydrolysed. The kinetic parameters for the acid α-D-glucosidase were obtained by using oligo- and poly-saccharide substrates. Variation of pH, temperature, and inhibitors caused changes in the activity of the acid α-D-glucosidase towards oligo- and poly-saccharide substrates. These results support the earlier suggestion that the enzyme has multiple substrate-binding sites.

INTRODUCTION

Enzymes that degrade dextran are found in bacteria[1], moulds[2], plants[3], and in animal and human tissues[4]. Enzymic degradation of dextrans by animal tissues occurs with the formation of D-glucose under *in vitro*[5] and *in vivo*[6] conditions. The enzymic system in liver, kidneys, spleen, and other tissues, which hydrolyses dextrans, probably differs in properties from that present in the small intestine[7]. The mammalian acid α-D-glucosidase (also called γ-amylase or glucoamylase, E.C. 3.2.1.20) possessing dextranase activity[8] was shown to be an exoglucosidase with broad substrate specificity localized in the lysosomes[9,10]. This enzyme seems to be responsible for the utilization of clinical dextran used as a plasma expander. We now report on the dextranase (earlier called dextranglucosidase [11,12]) activity of acid α-D-glucosidase, and on its degradation of clinical and native dextrans having different molecular structures.

RESULTS AND DISCUSSION

Isolation of acid α-D-glucosidase. — The purification procedure adopted was essentially that described by Bruni *et al.*[8]. The method takes advantage of the pronounced retardation of acid α-D-glucosidase on Sephadex G-100 which, apparently,

is a result of an enzyme–substrate type interaction. The adsorbed enzyme was thereby separated from inactive proteins, and was eluted by methyl α-D-glucopyranoside[13] which is a competitive inhibitor of α-D-glucosidase[14] (Fig. 1). Acid α-D-glucosidase activity was determined by measuring the rate of formation of D-glucose from dextran (dextranase activity). Dextranase activity was almost absent from the major peak of protein and was eluted as one symmetrical peak having a low content of protein. A typical purification of pig-spleen acid α-D-glucosidase is summarized in Table I. On

TABLE I

PURIFICATION OF ACID α-D-GLUCOSIDASE FROM PIG SPLEEN[a]

Step	*Volume (ml)*	*Total activity[b] (units × 10³)*	*Total protein (mg)*	*Specific activity (units/mg of protein)[b]*	*Yield (%)*
Crude extract	3,200	4.2	29.5×10^3	0.142	100
Autolysis supernatant	3,000	3.8	8.2×10^3	0.462	89
70% Satd. $(NH_4)_2SO_4$	500	3.2	6.1×10^3	0.525	76
Sephadex G-100 pool	95	1.85	6.5	285	43

[a]The crude extract was the supernatant from 1500 g of pig spleen. [b] Dextranase activity of acid α-D-glucosidase.

disc-gel electrophoresis, the enzyme migrated as a single protein band at low (Reisfeld system[15]) and high pH (Davis system[16]). Determination of maltase and dextranase activities of α-D-glucosidase in the gel demonstrated that the band of each activity coincided with the protein band.

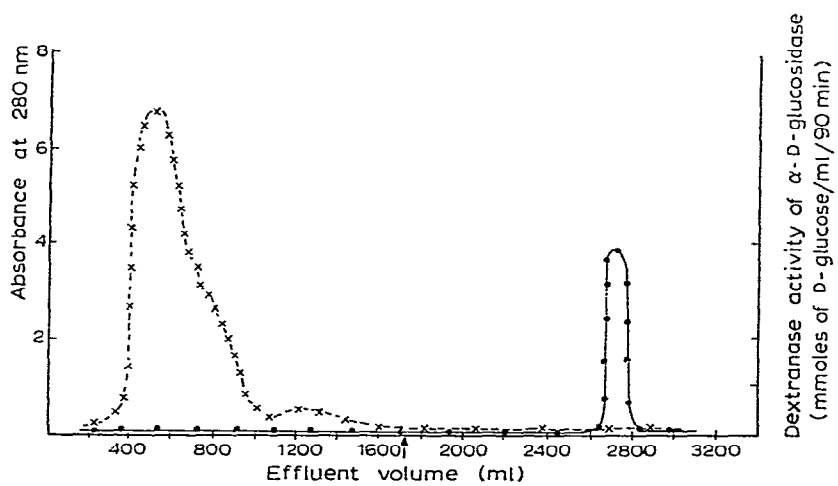

Fig. 1. Chromatography of acid α-D-glucosidase on Sephadex G-100. Absorbance at 280 nm ×—×; dextranase activity ●—●. The arrow indicates addition of methyl α-D-glucopyranoside.

Action of acid α-D-glucosidase on oligosaccharides having different types of linkages. — As demonstrated by Torres and Olavarria[9], crude preparations of dog-liver α-D-glucosidase catalyzed the hydrolysis of oligosaccharides having different types of linkages. The electrophoretically homogeneous, acid α-D-glucosidase had the same activity as the crude preparation[12]. The extrapolated values for K_m and V_{max} for the action of the enzyme on different oligosaccharides are shown in Table II.

TABLE II

KINETIC PARAMETERS FOR THE ACTION OF ACID α-D-GLUCOSIDASE ON OLIGOSACCHARIDES

Oligosaccharide	*Linkages*	K_m (*mM*)	V_{max}/V_{max} (*maltose*)
Maltose	1→4	5.8	1.00
Nigerose	1→3	7.3	0.73
Kojibiose	1→2	2.7	0.16
Isomaltose	1→6	29.0	0.07
Isomaltotriose	1→6	53.0	0.13
Isomaltotetraose	1→6	47.0	0.12

The relatively low K_m value for kojibiose might be due to greater substrate-binding affinity of α-D-glucosidase for a (1→2)-linkage, but V_{max} is also low. The trisaccharide *O*-α-D-glucopyranosyl-(1→2)-*O*-[α-D-glucopyranosyl-(1→6)]-D-glucose is split by acid α-D-glucosidase to give isomaltose, the rate of hydrolysis of the (1→2)-linkages being equal to that of kojibiose. The K_m value for the action of the pig-spleen enzyme

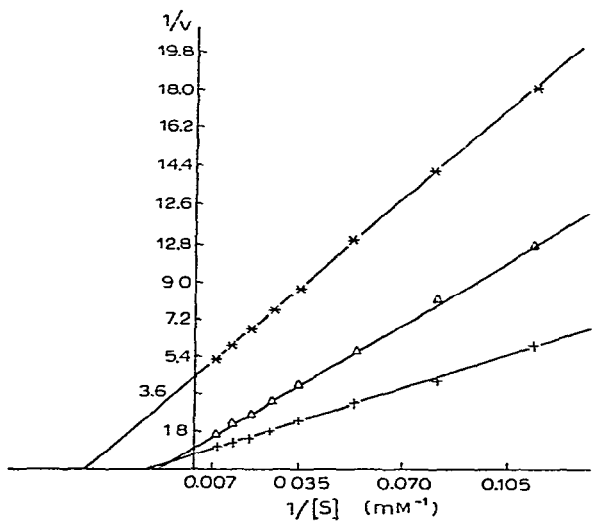

Fig. 2. Double reciprocal plot of the dependence on substrate concentration of the rate of hydrolysis of maltose (+—+), kojibiose (*—*), and nigerose (△—△) by acid α-D-glucosidase. Incubation was for 15 min at 37° in 50mM sodium acetate buffer (pH 5.0) with 1.5 units of the enzyme for maltose and nigerose, and 3 units for kojibiose.

on maltose is similar to that of the enzyme from other sources (rabbit liver[14] 4.9mM, rat liver[17] 4.7mM, dog liver[9] 4–5mM). In contrast to the α-D-glucosidases described earlier[10,18], pig-spleen α-D-glucosidase activity was not inhibited by oligosaccharide substrates (Fig. 2).

Action of acid α-D-glucosidase on dextrans. — Pig-spleen acid α-D-glucosidase hydrolyses polysaccharides[12] (glycogen, amylose, amylopectin, nigeran, pseudonigeran, and different dextrans) at rates depending on their structures. Table III shows the kinetic parameters for the action of acid α-D-glucosidase on three groups

TABLE III

KINETIC PARAMETERS FOR THE ACTION OF ACID α-D-GLUCOSIDASE ON GLYCOGEN AND DIFFERENT DEXTRANS

Substrates	*Linkages (%)*			K_m (*mg/ml*)	V_{max} (*relative*)
	1→6	*1→3*	*1→2*		
Dextrans					
Group 1					
Polyglukin (M_w 60,000)	97	2	1	25	1.00
Dextran T 500 (M_w 500,000)	90	—	—	25	1.00
Dextran T 2000 (M_w 2,000,000)	90	—	—	25	1.00
SF-4 (native)	95	3	2	25	1.00
Group 2					
63-1 (native)	65	2	29	55	0.43
62-6 (native)	66	1	33	50	0.53
LU-122 (native)	68	3	29	40	0.53
Group 3					
44m-2 (native)	87	13	0	10	0.30
44b-2 (native)	70	29	1	10	0.66
44p (native)	70	30	0	11	0.66
Rabbit-liver glycogen				2.6	9.60

of dextrans containing (1) >90% of (1→6)-linkages, (2) ~30% of (1→2)-linkages, and (3) 10–30% of (1→3)-linkages. The values of K_m and V_{max} for the action of the enzyme on the dextrans of different groups varied considerably, but they were very similar within a single group. These values did not depend on the strains and molecular weights of the dextrans. The substrate affinity of α-D-glucosidase for the dextrans from the second group is lower than those of groups 1 and 3. However, it is unlikely that this lower affinity is due to the presence of (1→2)-linkages, since the affinity for kojibiose is higher than that for nigerose and isomaltose (see Table II).

According to Palmer's data[18], rabbit-muscle acid α-D-glucosidase has a greater affinity for glycogen having short outer-chains (from shell fish) than for glycogen having longer outer-chains (from rabbit liver). There may be differences in the

lengths of the outer chains in the dextrans of groups 1–3. There is also direct correlation between the content of (1→3)-linkages and the V_{max} values for the dextran in group 3 (Table III). A similar correlation was also observed for the initial rates of hydrolysis of these dextrans (Table IV).

TABLE IV

RELATIVE, INITIAL RATES OF HYDROLYSIS OF DEXTRANS BY ACID α-D-GLUCOSIDASE

Dextrans	*(1→3)-Linkages* (%)	*Initial rates* (%)
44b-2	29	100
44m-1	23	80
54-2	18	69
44m-2	13	54
SF-4	2	29
63-1	2	29
62-6	2	29

TABLE V

PERIODATE OXIDATION OF DEXTRAN 44b-2 BEFORE AND AFTER DEGRADATION BY α-D-GLUCOSIDASE

	Linkages (%)		
	1→6	*1→3*	*1→2*
Dextran 44b-2			
Control	70	29	1
After 14% degradation	80	20	0

The periodate-oxidation data obtained before and after degradation of dextran 44b-2 by α-D-glucosidase show a decrease in the content of (1→3)-linkages (Table V). Thus, the acid α-D-glucosidase preferentially attacks (1→3)-linkages which are probably located at the non-reducing ends of the molecules. The dextrans of group 3 probably have a different number of side chains containing only one D-glucose residue linked to position 3 of a backbone unit[19]. Dextrans containing (1→2)-linkages may have quite a different type of structure, which results in a low affinity for α-D-glucosidase.

As acid α-D-glucosidase splits all types of α-D-glucosidic linkages in disaccharides (Table II), it was of interest to determine the extent of degradation of dextrans. Using low concentrations of dextrans and long incubation times, the results given in Table VI were obtained. Thus, the clinical dextran polyglukin [97% of (1→6)-linkages] and its native precursor, dextran SF-4, were almost totally degraded, as was dextran 44b-2 [30% of (1→6)-linkages]. Dextrans containing (1→2)-linkages were only 20% degraded, and the resulting dextrin was resistant to fresh enzyme. Since the initial rates of hydrolysis of dextrans SF-4 and 63-1 are almost equal (Table IV), the

TABLE VI

EXTENT OF HYDROLYSIS OF DEXTRANS BY ACID α-D-GLUCOSIDASE

Dextran[a]	*Degree of conversion into* D-*glucose* (%)
Polyglukin	97
SF 4	95
44b-2	92
63-1	21
62-6	13

[a]Types of linkage are given in Table III.

dextran 63-1 molecule may contain some site(s) readily split by α-D-glucosidase and a backbone which is resistant to the enzyme.

Some properties of pig-spleen acid α-D-glucosidase. — As shown above, acid α-D-glucosidase catalyzed the hydrolysis of oligo- and poly-saccharides having different types of linkages. Most of the studies of acid α-D-glucosidase[8,14,18] have involved maltose and glycogen as substrates.

The molecular weight of pig-spleen acid α-D-glucosidase, as determined[20] by gel filtration with Bio-Gel P-200 and comparison with proteins having known molecular weights, was 106,000 (Fig. 3).

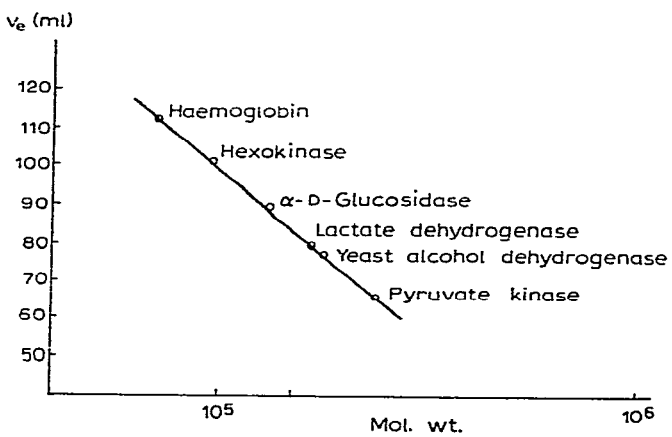

Fig. 3. Determination of the molecular weight of acid α-D-glucosidase. See Materials and Methods.

The pH dependence of α-D-glucosidase action on dextran (dextranase activity) and maltose (maltase activity) is shown in Fig. 4. The pH optimum of dextranase activity was 4.8–5.0, and that of maltase activity 4.0–4.5. Dextranase, but not maltase, activity was significantly stimulated by 200mM KCl in the range pH 3.5–5.0. Similar enhancement of the α-D-glucosidase action on glycogen (glucoamylase activity) has been shown for the enzyme obtained from other sources[10,18].

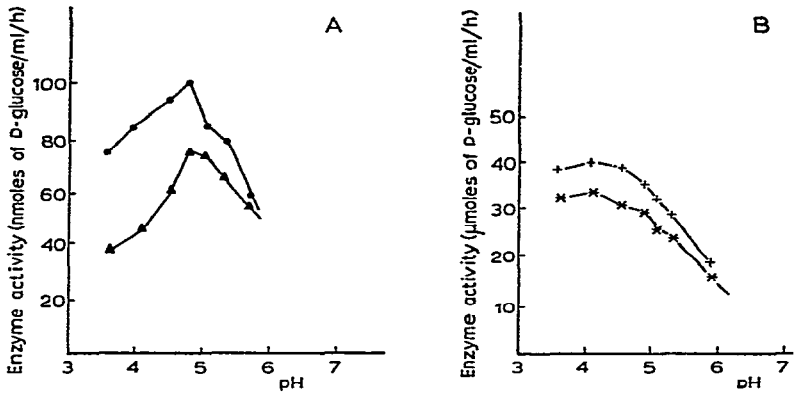

Fig. 4. Dependence of acid α-D-glucosidase activity on pH and salt concentration. A. Dextran: the reaction mixtures (0.3 ml) contained 3 mg of polyglukin, 50mM sodium acetate buffer, and 6 units of the enzyme, and were incubated at 37° for 90 min with (●—●) and without (▲—▲) the addition of 200mM KCl. B. Maltose: the reaction mixtures (0.3 ml) contained 600 μg of maltose, 50mM sodium acetate buffer, and 1.5 units of the enzyme, and were incubated at 37° for 15 min with (+—+) and without (*—*) the addition of 200mM KCl.

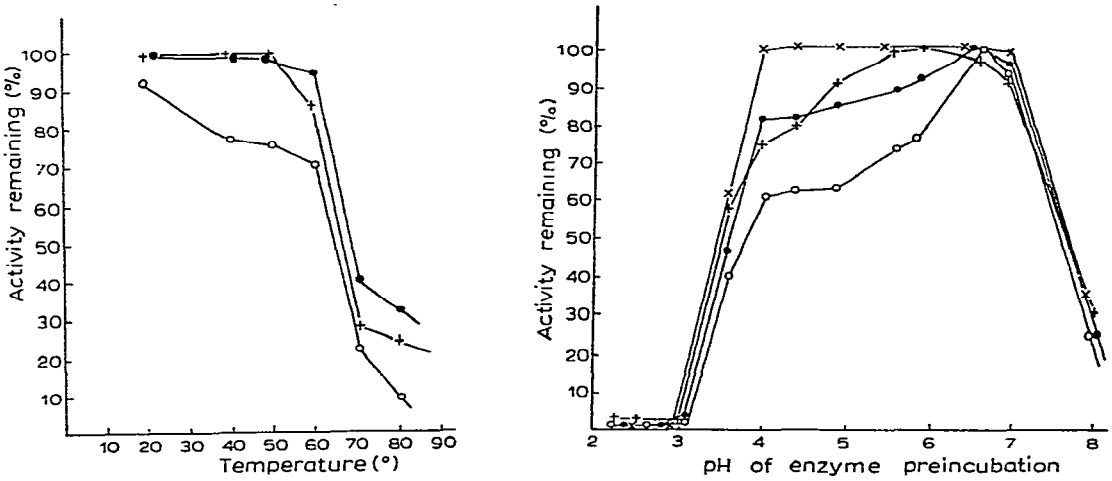

Fig. 5. Thermal stability of dextranase (●—●), maltase (+—+), and glucoamylase (○—○) activities of acid α-D-glucosidase: 0.3 ml of the enzyme was added to 0.3 ml of 50mM sodium acetate buffer (pH 5.0) and incubated for 60 min at various temperatures (see Materials and Methods).

Fig. 6. Effect of pH on stability of dextranase (●—●), maltase (+—+), glucoamylase (○—○), and isomaltase (×—×) activities of α-D-glucosidase: 0.3 ml of the enzyme was added to 0.6 ml of 0.1M citrate–0.2M Na_2HPO_4 of pH value indicated, and incubated at 37° for 60 min (see Materials and Methods).

Purified preparations of acid α-D-glucosidase could be stored at 4° for several months without loss of activity. At pH 5.0, incubation of the enzyme at 60° for 1 h had no effect on dextranase activity and an insignificant influence on maltase

activity, whereas glucoamylase activity was noticeably decreased (Fig. 5). The different thermostabilities of maltase and glucoamylase activities of bovine spleen α-D-glucosidase have been demonstrated[21].

When the α-D-glucosidase was incubated for 60 min at 37° in acid media, its activity declined (Fig. 6). The effect on the activity towards glycogen was greater than for maltose, and that for dextran was more pronounced than that for isomaltose. The dextranase activity of the enzyme was a little more stable on incubation at acid pH values than glucoamylase activity.

Urea inhibited both dextranase and glucoamylase activities. The inhibition of maltose activity was less pronounced (Fig. 7). A difference in the action of urea on the glucoamylase and maltase activities of rabbit-liver α-D-glucosidase has been reported[22].

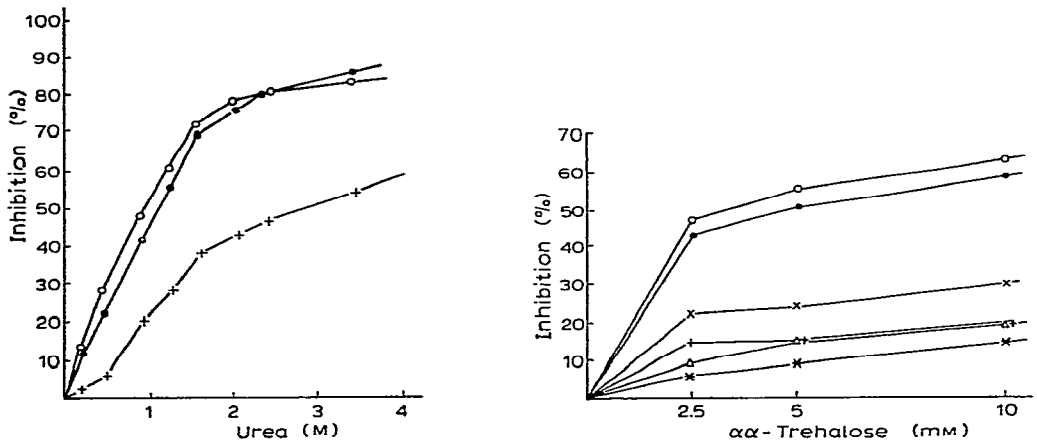

Fig. 7. Inhibition of dextranase (●—●), maltase (+—+), and glucoamylase (○—○) activities of α-D-glucosidase by urea (see Materials and Methods).

Fig. 8. Inhibition of α-D-glucosidase by αα-trehalose. The incubation mixtures (0.2 ml) contained various concentrations of αα-trehalose (as indicated), 50mM sodium acetate buffer, and, severally, 2 mg of polyglukin (●—●) or glycogen (○—○), 0.4 mg of maltose (+—+), or isomaltose (×—×), or nigerose (△—△), or kojibiose (*—*). When glucoamylase, maltase, and nigerase activities were tested, 1.5 units of the enzyme were added to each incubation mixture; when other activities were tested, 30 units of the enzyme were added. The incubation time was 15 min, except for dextranase activity (90 min).

αα-Trehalose is a competitive inhibitor of α-D-glucosidase[14,18], and this disaccharide inhibited both the dextranase and the glucoamylase activity of the enzyme to the same extent (Fig. 8). The inhibition of disaccharidase activities is rather low and is virtually independent of the linkage type. Inhibition was competitive and incomplete for all the substrates. An increase in αα-trehalose concentration up to 20mM did not markedly change the degree of inhibition.

The data presented in this paper support earlier suggestions[10,14,18] about the multiplicity of substrate binding-sites of acid α-D-glucosidase, in that there are

different binding-subsites in acid α-D-glucosidase molecules for oligo- and polysaccharides having different types of α-D-glucosidic linkages.

MATERIALS AND METHODS

Oligosaccharides. — Maltose and αα-trehalose were products of Chemapol (Czechoslovakia) and were purified by paper chromatography. Isomaltosaccharides were prepared by partial hydrolysis of polyglukin[23]. *O*-α-D-Glucopyranosyl-(1→2)-*O*-[α-D-glucopyranosyl-(1→6)]-D-glucose was a gift from Dr. M. Torii (Osaka University, Japan). Nigerose and kojibiose were prepared by partial acetolysis[24] of dextrans 44m-2 and 63-1, respectively.

Polysaccharides. — Dextrans T-2000, T-500, blue dextran, and Sephadex G-100 were products of Pharmacia Fine Chemicals Co. (Sweden). Polyglukin, a clinical dextran, was repeatedly precipitated by ethanol from a 6% aqueous solution. Glycogen was isolated from rabbit liver by TCA extraction and repeatedly precipitated from aqueous solution by ethanol. Most of the native dextrans were produced by *Leuconostoc mesenteroides* strains, and dextrans 62-6 and LU-122 by *Leuconostoc dextranicum* strains[25,26]. Isolation of the dextrans and determination of types of linkages were described earlier[24–27].

Isolation of α-D-glucosidase. — Unless stated otherwise, the steps in the purification procedure were performed at 4°. Pig spleen was homogenized with 2 vol. of 0.1M sodium acetate buffer (pH 5.0) containing mM EDTA and centrifuged for 30 min at 6000 r.p.m. after overnight extraction. The crude extract was heated and then stored overnight at 37°. The precipitate was removed by centrifugation, and dry $(NH_4)_2SO_4$ was added to 70% saturation. The precipitate was collected by centrifugation, dissolved in a small volume of 25mM NaCl–mM EDTA (pH 6.7) (medium *A*) and extensively dialyzed against medium *A*. The solution of non-diffusible material was clarified by centrifugation and concentrated under diminished pressure at 20°. A portion (~100 ml) of the solution of partially purified enzyme was applied to a column (5 × 95 cm) of Sephadex G-100, equilibrated with medium *A* and then eluted by the same medium. Fractions (12 ml) were collected at a flow rate of 25 ml/h, and assayed for absorption at 280 nm and dextranase activity. All inactive protein was eluted in the total volume of the column, but dextranase activity was retarded on the dextran gel. The dextranase activity was displaced from the column with a 1% solution of methyl α-D-glucopyranoside in medium *A* (Fig. 1). The fractions containing dextranase activity were combined, dialyzed against medium *A* to remove D-glucose and methyl α-D-glucopyranoside, and used in subsequent experiments.

Enzyme assays. — The dextranase activity of α-D-glucosidase was determined by addition of the enzyme solution (0.1 ml) to polyglukin dissolved in 200mM sodium acetate buffer (pH 5.0, 0.1 ml, 20 mg/ml). After storage at 37° for 90 min, the reaction was stopped by heating in a boiling water-bath for 2 min, or, when the enzyme assays were carried out on crude material, by the addition of 5% $ZnSO_4$ (0.1 ml) and 0.3M NaOH (0.1 ml). The concentration of D-glucose in the samples was determined

directly or after centrifugation (for crude perparations) by the specific D-glucose oxidase method[28]. Protein was determined by the Lowry method[29] with dried, bovine serum albumin as standard. For purified enzyme preparations, a micro-modification of this method was used. One unit of dextranase activity is defined as the amount of enzyme that catalyzes the liberation from polyglukin of 1 nmole of D-glucose/min. For the determination of maltase, isomaltase, and glucoamylase activities of α-D-glucosidase, the incubation mixtures contained 0.1 ml of the enzyme solution added to 0.1 ml of solutions of maltose (0.4 mg), isomaltose (0.4 mg), or glycogen (20 mg) in 200mM sodium acetate buffer (pH 5.0). After 15 min at 37°, the reactions were stopped as described above.

Polyacrylamide disc-gel electrophoresis. — Disc-gel electrophoresis was performed at low pH as described by Reisfeld[15], and at high pH in the Davis system[16]. Runs were performed for 90 min with 50 mamp per tube. The gel was stained for protein with 0.25% Coomassie Brilliant Blue (Serva, West Germany) in 7% acetic acid. To demonstrate dextranase and maltase activities of α-D-glucosidase in the gel, the method of Dahlqvist and Brun, as modified by Belenki[22], was used. Dextran (polyglukin) was incorporated into the gel (final concentration, 10 mg/ml) to demonstrate dextranase activity.

Determination of molecular weight. — The molecular weight of α-D-glucosidase was determined by gel filtration[20], using Bio-Gel P-200. The Bio-Gel (Bio Rad, U.S.A.) was equilibrated in medium *A* and packed to give a final column dimension of 23 × 500 mm. The column was calibrated with blue dextran and proteins of known molecular weight: ovalbumin (45,000), bovine serum albumin (67,000), haemoglobin (63–70,000), hexokinase (96,000), lactate dehydrogenase (135,000), alcohol dehydrogenase (150,000), pyruvate kinase (235,000), and urease (480,000). 4 mg of each standard protein and 100 μg of α-D-glucosidase were applied to the column, which was eluted with medium *A* at 4 ml/h; 2-ml fractions were collected. Protein peaks were determined by measurement of absorbancy at 280 nm. The activities of the enzymes were determined by generally adopted methods. The peak of α-D-glucosidase was detected by measuring maltase and dextranase activities. The elution volume was determined by the apexes of peaks of these activities, which coincided.

Hydrolysis of dextrans by α-D-glucosidase. — (*a*) Total hydrolysis was performed as described earlier[12].

(*b*) Lyophilized dextran was dissolved in 0.1M sodium acetate buffer (pH 4.8, 20 mg/ml), added to an equal volume of the enzyme solution, and the mixture was incubated at 37°. The D-glucose liberation was monitored, and when the degree of breakdown reached 10–15%, the reaction was stopped by heating in a boiling water-bath for 2–3 min. The dextran was precipitated with 3 vol. of 96% ethanol, collected by centrifugation, redissolved, deproteinized[30] with chloroform, purified by repeated reprecipitation, and freeze-dried. A control sample of the dextran was treated under the same conditions except that the buffer was used instead of the enzyme.

Periodate oxidation of dextrans was performed as described by Rankin and Jeanes[31]. The periodate consumption was measured by the method of Lichosherstov

and Brossar[32], and formic acid was titrated with 5mM $Ba(OH)_2$ in an atmosphere of nitrogen. The percentage of (1→3)-linkages was taken as the calculated amount of unoxidized D-glucose in polyaldehydes isolated after periodate oxidation of the dextrans[33]. For the preparation of polyaldehydes, each sample of dextran was oxidized by periodate, as described above. The remaining periodate was reduced with ethylene glycol, and the mixture was dialyzed against distilled water at 4° (until it gave a negative test for iodate) and then freeze-dried. The yield of polyaldehyde was close to theoretical. The preparation was hydrolyzed with 2M H_2SO_4 (90 min, boiling water-bath) and neutralized, and D-glucose was assayed by means of D-glucose oxidase[28].

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