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STUDIES ON DEXTRANASE

II. NEW EXO-DEXTRANASE FROM *BREVIBACTERIUM FUSCUM* VAR. *DEXTRANLYTICUM*

MAMORU SUGIURA[a], AKIRA ITO[a] and TSUTOMU YAMAGUCHI[b]

[a]*Department of Pharmacy, Tokyo College of Pharmacy, Ueno-sakuragi, 1-Chome, Taito-ku, Tokyo 110 and* [b]*Research Laboratory, Toyo Jozo Co. Ltd, Ohito-cho, Shizuoka 410-23 (Japan)*

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SUMMARY

Dextranase (EC 3.2.1.11) from *Brevibacterium fuscum* var. *dextranlyticum* was purified by ammonium sulfate fractionation, gel filtration of Bio Gel P-30 and DEAE-cellulose column chromatography. This preparation gave a single protein band with disc electrophoretic analysis.

The dextranase was most active over a pH range from 7.0–7.5 in a 30 min reaction and was stable over a wide pH range from 5.0–11.0 at 37 °C for 12 h. The enzyme was activated approximately 2 fold by cysteine and EDTA, and inactivated by iodine, $HgCl_2$, *N*-bromosuccinimide and $CuSO_4$.

In the dextranase hydrolysis reaction, isomaltotriose was the only digestion product with reducing power from dextran and reduced isomaltodextrins. Enzymatic hydrolysis also lowered the specific viscosity of the reaction mixture slowly. These results suggest that the enzyme is the new exo-type dextranase.

INTRODUCTION

It is widely reported that dextranase is produced by molds or bacteria. The purification and properties of dextranases from the mold *Penicillium funiculosum* have been reported [1]. However, there are few reports concerning bacterial dextranases, except for examples of *Lactobacillus bifidus* [2–4], two species of *Bacillus* [5] and intestinal bacteria [6]. Recently, Richards and co-workers have purified and obtained the properties of extracellular [7] and intracellular dextranases [8] from a bacterium. We have already reported the production of extracellular dextranase from *B. fuscum* var. *dextranlyticum* [9].

In this paper, the purification and properties of a new extracellular dextranase are described. The results suggest that with optimum pH and substrate specificity, this dextranase is different from those previously reported.

MATERIALS AND METHODS

Enzyme

The crude extracellular enzyme was the acetone precipitate of a culture filtrate of *B. fuscum* var. *dextranlyticum* [9].

Substrate and assay of enzyme activity

Dextran was produced by *Leuconostoc mesenteroides* N-4, and was partially hydrolyzed and fractionated. One fraction (mol. wt. $5.3 \cdot 10^4$) was used for the assay of enzyme activity and another (mol. wt. approx. $10 \cdot 10^6$) was used as the substrate in the hydrolysis experiments. These dextran fractions were supplied by Meito Sangyo Co. Ltd, Nagoya, Japan.

Dextranase activity was determined as follows. 2 ml of 2% dextran solution (in 100 mM phosphate buffer, pH 7.5) was preincubated for 5 min at 37 °C and then incubated with 1 ml of the enzyme solution. After 30 min the reducing sugar liberated in 1 ml of the reaction mixture was determined using Sumner's reagent [10]. One unit of dextranase activity was defined as the amount of enzyme which under the above conditions will produce reducing sugars in an amount equivalent to 1 μmole glucose per min.

Determination of protein concentration and disc electrophoresis

Protein concentration was determined spectrophotometrically by measuring the absorbance at 280 nm with a Hitachi 101 spectrophotometer. Disc electrophoresis was performed according to the procedure of Davis [11] with apparatus from M. S. Instrument Co., Osaka, Japan.

Paper chromatography

The enzymatic digestion products from dextran and isomaltodextrins were detected by paper chromatography on Toyo filter paper No. 50 with nitromethane–absolute ethanol–water (35:40:25, v/v/v). For the localization of sugars, $AgNO_3$–NaOH [12] or aniline hydrogen phthalate [13] was used.

Preparation of isomaltodextrins and reduction of isomaltodextrins

Isomaltodextrins were prepared by the partial digestion of dextran (mol. wt $5.3 \cdot 10^4$) with purified dextranase II from *P. funiculosum* [1] and fractionated by active carbon column chromatography as described by French [14]. The homogeneity of each sugar prepared was examined by paper chromatography and the degree of polymerization was determined by the method of French and Wild [15], as well as the ratio of reducing sugar to total sugar content. Reduction of isomaltodextrins was performed with sodium borohydride according to the procedure of Frush and Isbell [16].

Reagents used

Bio Gel P-30 was a product of Bio Rad Laboratories, Calif., U.S.A. Sodium borohydride was purchased from E. Merck A.G. DEAE-cellulose was a product of Pharmacia Fine Chemicals Co. Uppsala, Sweden. Other chemicals used were of special reagent grade.

RESULTS

Purification of dextranase

The crude enzyme powder (3.5 g) was extracted in 10 vols of water for 1 h and the solution centrifuged at 9000 rev./min for 10 min. The supernatant was fractionated by the addition of solid ammonium sulfate and the most active fraction (0.3–0.7 satn) was collected by centrifugation as above. The preparation obtained was dissolved in a small amount of water and loaded onto a Bio Gel P-30 column equilibrated with 25 mM phosphate buffer (pH 7.5) and eluted with the same buffer. A representative elution profile is shown in Fig. 1. The active fractions were combined and dialyzed

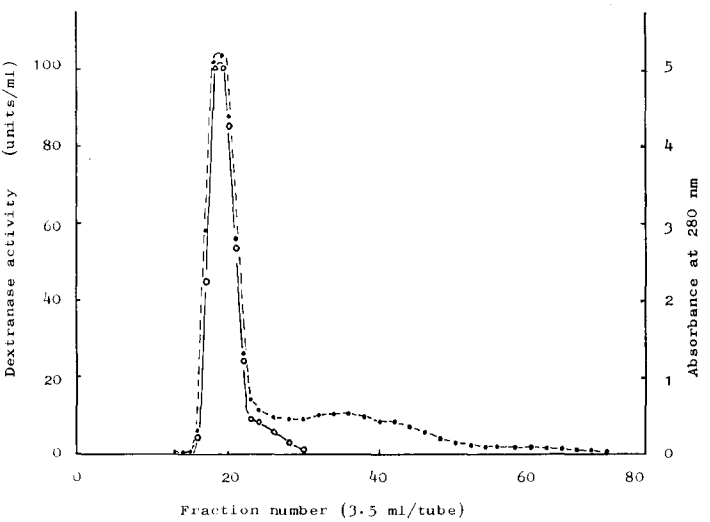

Fig. 1. Gel filtration of $(NH_4)_2SO_4$-treated preparation on Bio Gel P-30. The preparation dissolved in 4 ml water was applied on a column (2 cm × 56 cm) of Bio Gel P-30 equilibrated with 25 mM phosphate buffer (pH 7.5) and eluted with the same buffer at 4 °C. The flow rate was 15 ml/h. ○—○, dextranase activity; ●---●, absorbance at 280 nm.

against water for 2 days at 4 °C. The dialyzed enzyme preparation was adjusted to pH 7.5 with 0.05 M NaOH and adsorbed onto a column of DEAE-cellulose equilibrated with 10 mM phosphate buffer (pH 7.5). The adsorbed enzyme was eluted by a linear gradient system of NaCl (0–1.0 M) in the same buffer. The elution pattern of the enzyme is presented in Fig. 2. The active fractions were combined and dialyzed against 10 mM phosphate buffer (pH 7.5), and subjected to chromatography once again under the same conditions. A symmetrical protein peak with a constant specific activity of dextranase was obtained. The preparation was electrophoretically homogeneous as shown in Fig. 3. By these procedures, the enzyme was purified about 7.5 fold from the crude enzyme and the total recovery of activity was about 30%. The purification procedures are summarized in Table I.

Optimum pH and dextranase stability

The enzyme was incubated with 2% of substrate at various pH values, the

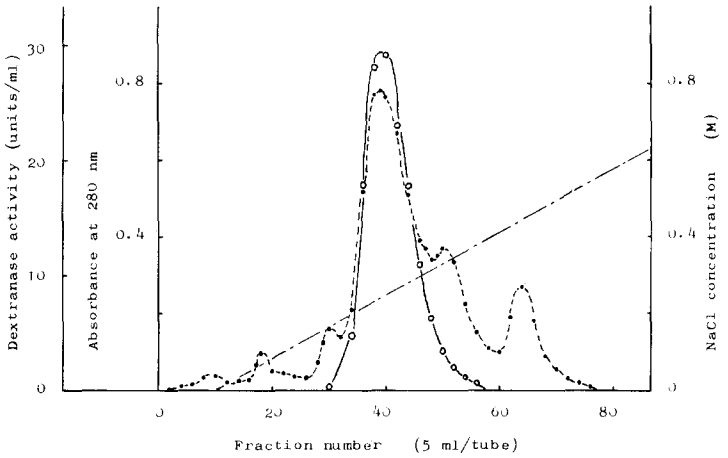

Fig. 2. Elution pattern of DEAE-cellulose column chromatography. The dextranase preparation was adsorbed on a DEAE-cellulose column (1.5 cm × 18 cm) equilibrated with 10 mM phosphate buffer (pH 7.5). The enzyme adsorbed was eluted by a linear gradient of NaCl (0–1.0 M) at a flow rate of 20 ml/h. ○—○, dextranase activity; ●---●, absorbance at 280 nm; ——, NaCl concentration.

other conditions being as described above. The optimum pH was found to be 7.0–7.5 and at pH 6.5 and 8.0, 85% of the maximum activity was observed.

For the examination of pH stability, the dextranase was incubated at various pH values at 37 °C for 12 h, and the residual activity was determined. The enzyme retained its maximum activity over a pH range from 6.0–9.0, and at pH 5.0, 10.0 and

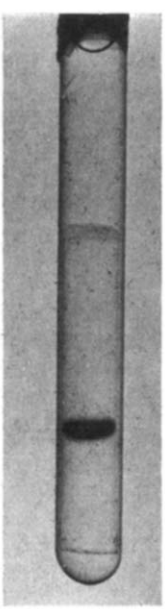

Fig. 3. Disc electrophoretic pattern of purified dextranase at pH 9.4, 4 mA/tube for 80 min at 4 °C. Staining was done by amidoschwarz 10 B.

TABLE I

PURIFICATION PROCEDURE OF *B. FUSCUM* DEXTRANASE

Specific activity was calculated as the activity per absorbance unit at 280 nm.

Procedure	Total activity (units)	Specific activity	Recovery (%)
Original powder	2880	4.5	100
↓ (extracted in water, fractionated with ammonium sulfate (0.3–0.7 satn) and dissolved in water)			
Solution	2175	20	82.4
↓ (fractionated with Bio Gel P-30)			
Effluent	1730	23	60.0
↓ (dialyzed against water and chromatographed on DEAE-cellulose with a linear gradient system of NaCl (0–1.0 M))			
Effluent	1166	34	40.4
↓ (dialyzed against phosphate buffer and rechromatographed on DEAE-cellulose)			
Purified dextranase	898	34.3	31.3

11.0, lost less than 10% of the activity. The thermal stability was also investigated. The dextranase was treated at pH 7.5 for 30 min and the residual activity was determined using standard procedures. The enzyme was found to be thermostable. Up to 50 °C, the enzyme lost less than 1% of its maximum activity and retained 70% of the maximum activity at 60 °C.

Effect of some metal salts and chemicals on enzyme activity

The effects of metal salts and chemicals on the enzyme were investigated as described in Table II. The dextranase was found to be activated 2 fold by cysteine and EDTA, and was activated weakly by $MnCl_2$. On the other hand, *N*-bromosuccinimide, iodine and $HgCl_2$ inactivated the enzyme completely and partial inactivation was observed with *p*-chlormercuribenzoic acid and $CuSO_4$.

Substrate specificity of the dextranase

Polysaccharides and oligosaccharides with various glucosidic linkages were subjected to digestion with the dextranase and the degree of hydrolysis of each substrate was determined. The results are presented in Table III, indicating that the enzyme hydrolyzed dextran and its derivatives with α-1,6-glucosidic linkages except CM-Sephadex and DEAE-Sephadex. Polysaccharides with other linkages were not attacked.

Action of the dextranase on dextran

Upon digestion of the dextran (mol. wt approx. $10 \cdot 10^6$) with the dextranase the specific viscosity of the reaction mixture, degree of hydrolysis and the digestion products were investigated simultaneously. In Fig. 4, the time course of hydrolysis and fall in specific viscosity are illustrated. In a similar experiment, *P. funiculosum* dextranase II, an endo-enzyme, was used as a comparative enzyme. The dextranase from *P. funiculosum* reduced the specific viscosity markedly in the initial stages of the

TABLE II

EFFECT OF SOME METAL SALTS AND REAGENTS ON *B. FUSCUM* DEXTRANASE

The enzyme was incubated with an equal volume of metal salt or reagent solution (2 mM, pH 7.5) at 37 °C for 30 min. Then the mixture was diluted 10 fold with deionized water and the residual activity was determined under standard conditions.

Reagent	Residual activity (%)
Cystein–HCl	203
Sodium dodecylbenzene sulfonate	104
Iodoacetic acid	108
N-Bromosuccinimide	0
p-Chloromercuribenzoic acid	69
Iodine	0
Urea	96
EDTA–Na_2	179
$AgNO_3$	3
$HgCl_2$	0
$MgSO_4$	94
$CaCl_2$	91
$FeCl_3$	94
$CuSO_4$	24
$CoCl_2$	118
$MnCl_2$	147
—	100

TABLE III

SUBSTRATE SPECIFICITY OF *B. FUSCUM* DEXTRANASE

Equal volumes of purified dextranase and 1% substrate solutions were incubated at pH 7.5 and 37 °C. After 30 min, the reducing sugar liberated was determined and the relative activity was calculated.

Substrate	Main linkage	Relative activity (%)
Dextran (mol. wt $5.3 \cdot 10^4$)	α-1,6	100
Isomaltose	α-1,6	0
Isomaltotriose	α-1,6	0
Sephadex G-100	α-1,6	24
Sephadex G-200	α-1,6	46
CM-Sephadex	α-1,6	0
DEAE-Sephadex	α-1,6	0
Xylan	α-1,3	0
Amylose	α-1,4	0
Glycogen	α-1,4	0
Starch	α-1,4	0
Cellulose	β-1,4	0

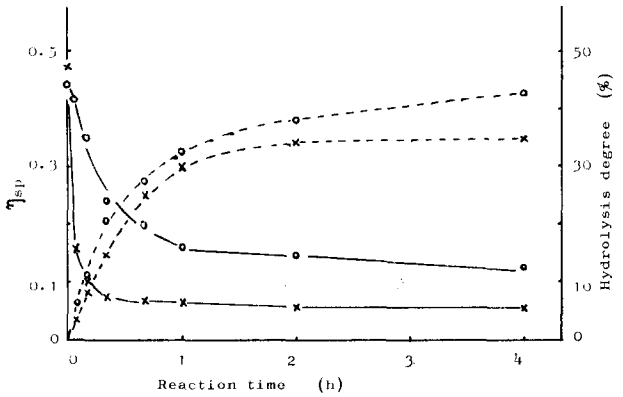

Fig. 4. Time course of dextran–dextranase reaction. Digest, 80 mg substrate and 8 units of the enzyme in 8 ml of 50 mM phosphate buffer (pH 7.5) for *B. fuscum* dextranase and pH 6.0 for *P. funiculosum* dextranase. 1 ml was used to determine the reducing sugar content by the Sumner method and 5 ml to determine the viscosity in an Ostwald viscometer. The degree of hydrolysis was calculated as glucose for *P. funiculosum* dextranase and as isomaltotriose for *B. fuscum* dextranase. ×—×, viscosity for *P. funiculosum* dextranase; ○—○, viscosity for *B. fuscum* dextranase; ×---×, hydrolysis degree for *P. funiculosum* dextranase; ○– –○, hydrolysis degree for *B. fuscum* dextranase.

reaction. On the other hand, the dextranase from *B. fuscum* var. *dextranlyticum* lower ed the specific viscosity relatively for a similar increase in reducing power. In the same reaction mixture the digestion products from the dextran were investigated by paper chromatography (Fig. 5). The dextranase produced isomaltotriose as the only reducing oligosaccharide, in progressively increasing amounts.

Action patterns of the dextranase on isomaltodextrins and their reduced derivatives

For an investigation of the action patterns of the dextranase, isomaltodextrins and their reduced derivatives (isomaltodextrinols) were digested by the enzyme and the digestion products determined by paper chromatography. The results are summarized in Table IV. Initially (5 min reaction), isomaltotriose and the residual chain of the isomaltodextrin were found as the digestion products with each substrate. After 120 min, the residual chain of the isomaltodextrin decomposed into isomaltotriose and a shorter residue (G_2 and G_1). The enzyme hydrolyzed isomaltotetraose more slowly than the other isomaltodextrins.

To determine whether the dextranase acts from the reducing end or the non-reducing end of the substrate, reduced isomaltodextrins were used as substrate and only digestion products with reducing power were detected by paper chromatography (Table IV). Isomaltotriose was the only reducing sugar detected in each case. In Fig. 6, paper chromatograms of the digestion products from isomaltoheptaitol and isomaltooctaitol are presented. The rate of hydrolysis seemed to be decreased markedly by the reduction of the endogroup. Sugar alcohols derived from isomaltotetraose and isomaltopentaose were not hydrolyzed under the conditions described in Table IV, but were very slowly hydrolyzed by a 10 fold increase in the enzyme activity and a long reaction time. These results suggest that the dextranase removes the isomaltotriosyl unit from the non-reducing endo of the isomaltodextrins. The proposed action patterns are shown in Fig. 7. The dextranase seems to be a new exo-dextranase.

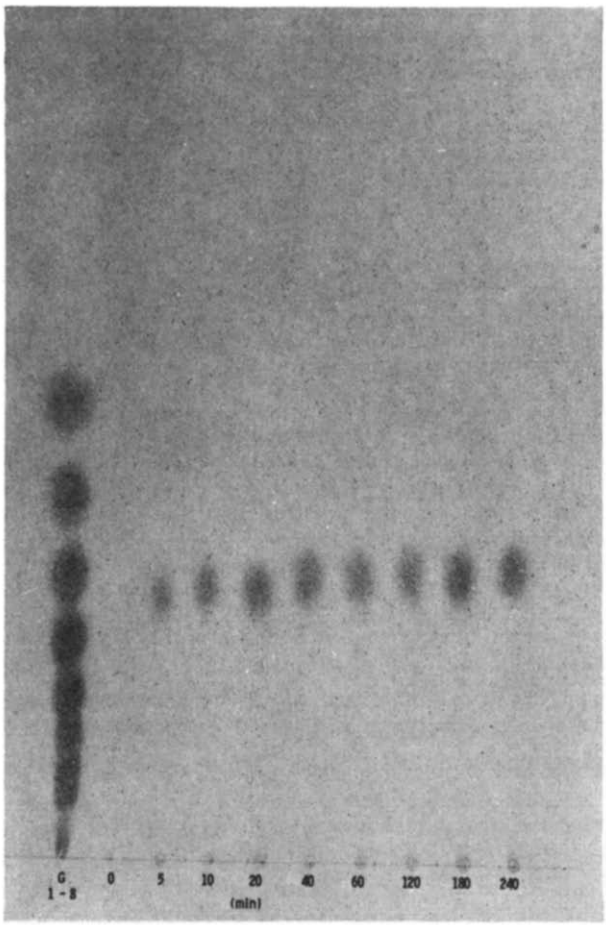

Fig. 5. Paper chromatography of digestion products from dextran by *B. fuscum* dextranase. Reaction mixture was the same as described in Fig. 4. Detection was done by $AgNO_3$–NaOH.

DISCUSSION

Richards and Streamer have reported an extracellular dextranase from a bacterium which showed maximum activity at pH 4.5–7.5 [7]. The dextranase from *B. fuscum* var. *dextranlyticum* shows maximum activity at a rather higher pH (7.0–7.5) and is stable over a wide pH range from 5.0–11.0.

Dextranase from bacteria, in general, have been endo-enzymes [17]. Only two species of *Bacillus* dextranase [5], intestinal dextranase [6] and intracellular dextranase from a bacterium [8] were described as exo-dextranase, but these also acted as endo-enzymes on dextran. However, the dextranase from *B. fuscum* var. *dextranlyticum* acts only as an exo-enzyme on the dextran as well as isomaltodextrins. Furthermore, the above enzymes produced glucose as the final digestion product whereas our dextranase yielded only isomaltotriose. These properties suggest that the enzyme is a new exo-type dextranase.

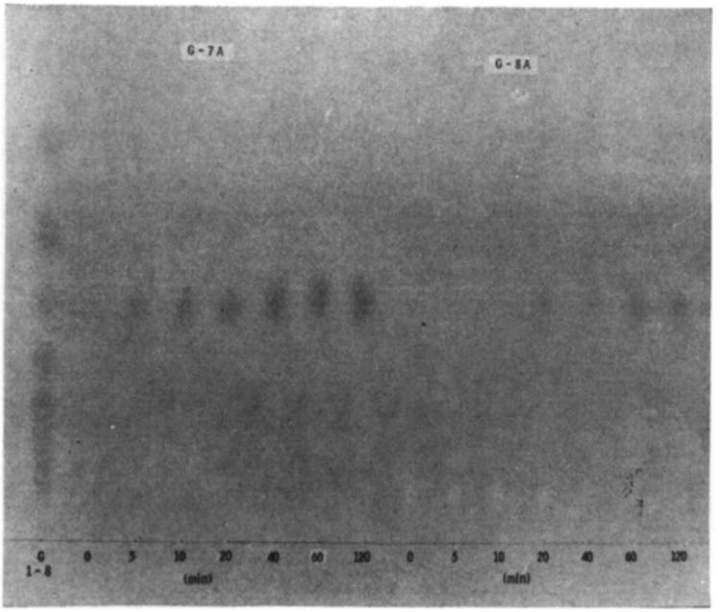

Fig. 6. Paper chromatography of digestion products from isomaltoheptaitol and isomaltooctaitol by *B. fuscum* dextranase. The experimental conditions were the same as described in Table IV. Detection was done by aniline hydrogen phthalate reagent.

TABLE IV

ACTION OF *B. FUSCUM* DEXTRANASE ON VARIOUS ISOMALTODEXTRINS AND THEIR REDUCED DERIVATIVES

Digest, 15 mg substrate and 1.0 unit of the enzyme in 1 ml of 50 mM phosphate buffer (pH 7.5). Reducing sugar products were detected by $AgNO_3$–NaOH with isomaltodextrins or aniline hydrogen phthalate with reduced isomaltodextrins. The products in parentheses were not detected under the above conditions, but were confirmed by use of 10-fold amounts of the enzyme after 360 min or more. G_1, glucose; G_2, isomaltose; $G_3 \cdots\cdots G_8$, isomaltotriose$\cdots\cdots$isomaltooctaose.

Substrate	Reducing sugar products	
	After 5 min	After 120 min
Isomaltose	–	–
Isomaltotriose	–	–
Isomaltotetraose	–	(G_3, G_1)
Isomaltopentaose	G_3, G_2	G_3, G_2
Isomaltohexaose	G_3	G_3
Isomaltoheptaose	G_3, G_4	G_3, G_4, G_1
Isomaltooctaose	G_3, G_5	G_3, G_2
Isomaltotetraitol	–	(G_3)
Isomaltopentaitol	–	(G_3)
Isomaltohexaitol	G_3	G_3
Isomaltoheptaitol	G_3	G_3
Isomaltooctaitol	G_3	G_3

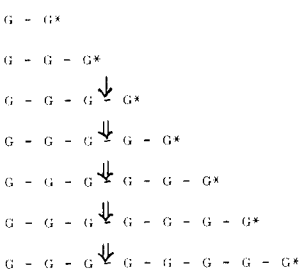

Fig. 7. Action patterns of *B. fuscum* dextranase toward isomaltodextrins. G, glucose; G*, reducing end; -,α-1,6 glucosidic linkage; ⇓, hydrolyzed very rapidly; ↓, hydrolyzed slightly.

P. funiculosum dextranases were not activated by cysteine and EDTA [1], but the new enzyme was markedly activated by these reagents. On the other hand, the enzyme was inactivated by $CuSO_4$ whereas the dextranases from *P. funiculosum* were activated by it.

The mechanisms of activation of the dextranase by cysteine and EDTA are to be investigated in the future.

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