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Studies on Dextranase. VII. The Kinetic Parameters of *Brevibacterium fuscum* Dextranase and Molecular Properties of the Digestion Products[1a,b]

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Hydrolysis actions of dextranase (EC 3.2.1.11) from *Brevibacterium fuscum* var. *dextranlyticum* on dextrans with various molecular weights (5.0×10^3-approx. 1.0×10^7) were investigated comparing with those of *Penicillium funiculosum* IAM 7013 dextranase. The specific activities were measured as an increase of reducing sugar per min per mg protein at the substrate concentration 1.34%. The activities of *B. fuscum* dextranase were 110—190 μmoles as glucose for various dextrans. Those of *P. funiculosum* dextranase were, however, almost constant and approximately 400 μmoles for dextrans examined.

The K_m values (%) and V_{max} values (μmoles/min per mg protein) of *B. fuscum* dextranase decreased with an increase in molecular weight of dextrans. K_m values were 0.041—0.065 and V_{max} values were 76—128. However, both values of *P. funiculosum* dextranase were constant, and K_m remained at about 0.2 and V_{max} was approximately 800.

Molecular properties of digestion products by *B. fuscum* dextranase were investigated. The polymerization degree of the products in the oligosaccharide fraction was constantly 3 in spite of an increase of reducing sugar content in the reaction mixture. Molecular size of the products was also investigated by the gel filtration on Bio Gel P-60. The fractionation patterns were markedly different from those of *P. funiculosum* dextranase as a typical endo-enzyme. These results indicated that the enzyme acted as an exo-dextranase at early reaction stage, and finally the enzyme might be able to hydrolyze the residual part of the substrate; *i. e.* core dextran having branching points.

In a previous paper,[3] the authors have reported some enzymatic properties of dextranase from *B. fuscum* var. *dextranlyticum*. In particular, it was noteworthy that the enzyme produced mainly isomaltotriose as the digestion product and was a new exo-type dextranase. The authors investigated the hydrolysis actions of *B. fuscum* dextranase on various dextrans comparing those of *Penicillium funiculosum* IAM 7013 dextranase II as an endo-type enzyme.[4,5] In this paper, kinetic parameters and molecular properties of the digestion products are described.

Materials and Methods

Enzyme——Purified *B. fuscum* dextranase was prepared by the procedures described in a previous paper.[3] Dextranase from *P. funiculosum* IAM 7013 was also purified as previous report[4] and the purified dextranase II was used in this series of investigations. These enzymes were homogeneous in disc electrophoretic analyses at pH 9.4.

Substrate——Dextran was a product of *Leuconostoc mesenteroides* N-4, and was partially hydrolyzed and fractionated. Dextrans as the substrate in this series of experiments having various molecular weights such as 5.0×10^3, 1.4×10^4, 5.3×10^4, 1.04×10^5, 2.0×10^5, approx. 6.0×10^5, approx. 1.0×10^6 and approx. 1.0×10^7 were used. These dextran fractions were supplied by Meito Sangyo Co., Ltd., Nagoya, Japan.

1) *a*) Part VI: M. Sugiura and A. Ito, *Chem. Pharm. Bull.* (Tokyo), **23**, 1304 (1975); *b*) This forms part C of "Studies on Enzymes" by M. Sugiura.
2) Location: *Ueno-Sakuragi, 1-chome, Taito-ku, Tokyo, 110, Japan.*
3) M. Sugiura, A. Ito, and T. Yamaguchi, *Biochim. Biophys. Acta* **350**, 61 (1974).
4) M. Sugiura, A. Ito, T. Ogiso, K. Kato, and H. Asano, *Biochim. Biophys. Acta* **309**, 357 (1973).
5) M. Sugiura and A. Ito, *Chem. Pharm. Bull.* (Tokyo), **22**, 1593 (1974).

Analytical Methods and Reagents used——Dextranase activity was determined by the same procedures described previously[3,4] except pH for incubation, and the reaction was done at pH 7.5 for *B. fuscum* dextranase and pH 6.0 for *P. funiculosum* dextranase, respectively. Reducing sugar liberated was measured using dinitrosalicylate reagent (Sumner's reagent[6]). One unit of dextranase was defined as the activity which liberated reducing sugars in an amount equivalent to 1 μmole of glucose per min. Total carbohydrate content was also estimated by phenol-H_2SO_4 method.[7] Protein concentration was determined according to the method of Lowry, *et. al.*[8] using bovine albumin as the standard protein. Bio Gel P-60 was a product of Bio Rad Laboratories, Calif., U. S. A. Other reagents used were of special reagent grade.

Results and Discussion

Specific Activities of Dextranases on Dextrans with Various Molecular Weights

In Table I, specific activities of each dextranase on various dextrans are presented.

TABLE I. Specific Activities of Dextranase from *B. fuscum* and *P. funiculosum* on Various Dextrans

Substrate (mol. wt.)	Specific activity (μmoles as glucose/min per mg protein	
	B. fuscum dextranase	*P. funiculosum* dextranase
5.0 x10³	186	385
1.4 x10⁴	176	380
5.3 x10⁴	144	414
1.04x10⁵	135	414
2.0 x10⁵	124	423
approx. 6.0 x10⁵	130	486
approx. 1.0 x10⁶	114	423
approx. 1.0 x10⁷	121	422

Reaction mixture contained 2 ml of substrate solution in 100 mM phosphate buffer (pH 7.5 for *B. fuscum* dextranase and pH 6.0 for *P. funiculosum* dextranase) and 1 ml of each enzyme solution containing 1.54 μg of *B. fuscum* or 1.095 μg of *P. funiculosum* dextranase. The final concentration of substrate was 1.34%. After 30 min incubation at 37°, 3 ml of Sumner's reagent was added to the reaction mixture and the reducing sugar was determined.

Dextranase activity was determined by measuring of the liberated reducing sugar. Under the conditions used for the assay, the initial reaction rate was in proportion to the incubation time. Dextranase from *P. funiculosum* had a constant specific activity on the various dextrans and the value was approximately 400 μmoles as glucose/min per mg protein. On the isomaltodextrins, we have already reported that the activity of the endo-type dextranase decreased relatively for a lowering in molecular weight of the substrate.[5] Fukumoto, *et al.* have also described similar observations on *P. luteum*[9] and *Aspergillus carneus* dextranases.[10] Results in Table I, however, indicate that specific activity of the endo-dextranase was approximately constant on the various substrates.

On the other hand, the specific activity of *B. fuscum* dextranase was lowered with an increase in molecular weight of the substrate examined, and 1 mg of the enzyme had about 110—190 units. These phenomena are just like those of β-amylase on amyloses with various molecular weights.[11] In particular, specific activity on the dextran with molecular weight approx. 1.0×10^7 was about 65% of the activity on the dextran with molecular weight 5.0×10^3 and the activity of *B. fuscum* dextranase was only observed from 1/4 to 1/2 that of *P. funiculo-*

6) J.B. Sumner, *J. Biol. Chem.* **65**, 393 (1925).
7) M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Roberts, and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
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9) N. Hiraoka, H. Tsuji, J. Fukumoto, T. Yamamoto, and D. Tsuru, *Int. J. Peptide Protein Res.*, **5**, 161 (1973).
10) D. Tsuru, N. Hiraoka, and J. Fukumoto, *J. Biochem. Tokyo*, **71**, 653 (1972).
11) E. Husemann and B. Pfamnemuller, *Makromol. Chem.* **87**, 139 (1965).

sum dextranase. In Fig. 1, progress curves of dextran hydrolysis by *B. fuscum* dextranase are shown. These hydrolysis patterns are similar to the results described in Table I. Libera-

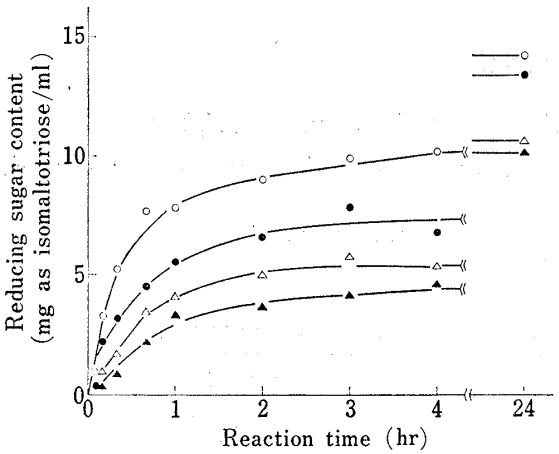

Fig. 1. Progressive Curves of Dextran Hydrolysis by *B. fuscum* Dextranase

The reaction mixture contained 80 mg substrate, 4 units of the enzyme in 4 ml of 50mM phosphate buffer (pH 7.5), and was incubated at 37°. Aliquots of the mixture were periodically sampled and the amount of reducing sugars liberated were determined by Sumner's reagent. ○; molecular weight 5.0×10^3, ●; molecular weight 1.4×10^4, △; molecular weight 5.3×10^4, ▲; molecular weight approx. 1.0×10^6

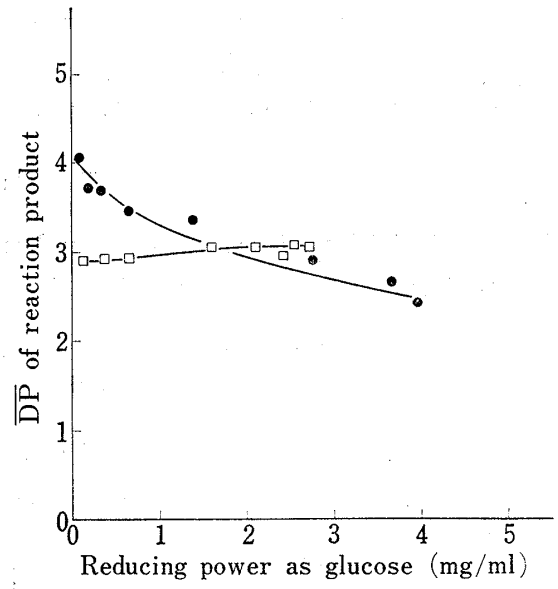

Fig. 2. Polymerization Degree ($\overline{DP}$) of Digestion Products from Dextran

Dextran (molecular wgieht approx. 1.0×10^7) was incubated with from 0.06 to 10 units of dextranase at 37° in a total volume of 2ml. After 30min, the reaction mixture was treated with 4 ml of MeOH and the $\overline{DP}$ of the digestion products in the supernatant was calculated as the ratio of their phenol-H_2SO_4 value to their reducing power as glucose. In this case, reducing sugar was determined by the method of Somogyi-Nelson.[a]

□; *B. fuscum* dextranase
●; *P. funiculosum* dextranase
a) M. Somogyi, *J. Biol. Chem.* **195**, 19 (1452).

TABLE II. Michaelis Constants and V_{max} Values of Dextranase from *B. fuscum* and *P. funiculosum* on Various Dextrans

Substrate (mol. wt.)	*B. fuscum* dextranase		*P. funiculosum* dextranase	
	K_m (%)	V_{max} (μmoles/min per mg protein)	K_m (%)	V_{max} (μmoles/min per mg protein)
1.4×10^4	0.0665	128	0.200	833
5.3×10^4	0.0625	102	0.204	825
1.04×10^5	0.0575	89	0.244	833
approx. 1.0×10^6	0.0435	78	0.232	800
approx. 1.0×10^7	0.0405	76	0.227	769

Reaction mixture contained 1 ml of various concentration of substrate in 100 mM phosphate buffer (pH 7.5 for *B. fuscum* dextranase and pH 6.0 for *P. funiculosum* dextranase) and 1 ml of enzyme solution containing 1.54 μg of *B. fuscum* or 1.095 μg of *P. funiculosum* dextranase. After 20 min incubation at 37°, 2 ml of Sumner's reagent was added to the reaction mixture and reducing powder was determined.

tion of reducing sugar on small molecular weight dextran was larger than that on large molecular weight dextran. These results and our previous observation that the enzyme removed isomaltotriose from the nonreducing end of the substrate,[3] suggested that the specific activity of exo-dextranase was influenced by the number of non-reducing ends in the reaction system.

Michaelis Constant (K_m) and Maximum Velocity (V_{max})

Michaelis constants (K_m) and maximum velocities (V_{max}) of dextranases calculated from Lineweaver-Burk plots are presented in Table II. The double-reciprocal plots were linear with all the substrates tested. With *B. fuscum* dextranase, K_m and V_{max} decreased with an increase in molecular weight of dextrans tested and K_m values (%) were from 0.067 to 0.041, and V_{max} values (μmoles/min per mgprotein) were from 76 to 128. However, both values of *P. funiculosum* dextranase were constant on various dextrans examined and K_m remained at about 0.2 and V_{max} approximately 800. The above results were similar to the phenomena described in Table I.

Polymerization Degree and Gel Filtration Patterns of Digestion Products by *B. fuscum* Dextranase

In a previous paper,[3] it has been reported that *B. fuscum* dextranase produced only isomaltotriose as the digestion product. In the present experiments, the degree of polymerization of the hydrolyzed products in the oligosaccharide fraction (after precipitation of remaining polysaccharides with 2-volume of methanol) was also investigated. As shown in Fig. 2, the average degree of polymerization ($\overline{DP}$) of the products by *B. fuscum* dextranase had a constant value of 3 in spite of an increase of reducing sugar content in the reaction mixture. This profile resembles the pattern obtained by an exo-dextranase from *Achromobacter* sp.[12] and by β-amylase,[13] respectively. On the other hand, $\overline{DP}$ of the products from *P. funiculosum* dextranase, an endo-type, became smaller as the reaction proceeded, corresponding to isomaltose and isomaltotriose as the final digestion products.[5] In Fig. 3, gel filtration patterns of the digestion products obtained by *B. fuscum* and *P. funiculosum* dextranases on Bio Gel P-60 are presented. By *P. funiculosum* dextranase, the substrate was roughly digested at the initial reaction stage and the molecular weight of digestion product was distributed over a wide range. After that, the digestion products were secondarily hydrolyzed to smaller products and the peak of products moved completely to small molecular weight fraction, from the initial stage, however, *B. fuscum* dextranase gave a low molecular weight peak being away from the original substrate peak, and the substrate peak disappeared as digestion proceeded. The patterns were obviously different from these of *P. funiculosum* dextranase. *B. fuscum* dextranase, however, did not remain the macromolecular residues, so called as limit dextrin for β-amylase, but the residue which could be precipitated by the addition of 2-volume of MeOH slightly remained.

When isomaltotriose was used as the standard, the maximum hydrolysis degree of *B. fuscum* was observed about 80% and the value was larger than 43% reported by Sawai, *et al.*[12] for *Achromobacter* sp. dextranase. After dialysis of the reaction mixture, 80% of the total sugar was dialyzed. These observations are strongly supported by the patterns indicated in Fig. 3. At the stage, the digestion products were also investigated by paper chromatography. Results are presented in Fig. 4 indicating that isomaltotriose and some isomaltodextrins larger than isomaltotriose are also detected, but it is not clear whether these isomaltodextrins are linear or branching. These results suggested that at early reaction stage, *B. fuscum* dextranase acted as an exo-type enzyme and removed isomaltotriose from the non-reducing end of the molecule as described previously,[3] and the enzyme, finally, might be able to hydrolyze the residual part of substrate; *i.e.* core dextran having branching points. To confirm the above possibility, the actions of *B. fuscum* dextranase on the various isomaltodextrins having branching will be investigated.

The above results strongly confirm the description in a previous paper[3] that *B. fuscum* dextranase is an exo-enzyme.

12) T. Sawai, K. Toriyama, and K. Yano, *J. Biochem. Tokyo* **75**, 105 (1974).
13) K.K. Tung and J.H. Nordin, *Anal. Biochem.*, **29**, 84 (1969).

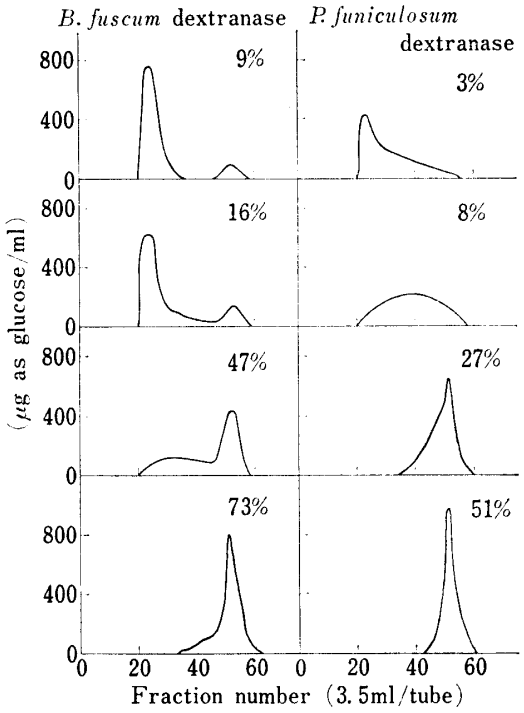

Fig. 3. Fractionation of Dextran on a Bio Gel P-60 Column after Treatment with Dextranases

1 g of dextran (molecular weight approx. 1.0×10^7) in 50 mM phosphate buffer was treated with 25 units of (A) *B. fuscum* dextranase, pH 7.5 (B) *P. funiculosum* dextranase, pH 6.0, in a total volume of 100 ml. With both enzymes, samples of 2 ml of the reaction mixture were taken at indicated hydrolysis degree. The degree of hydrolysis was calculated as isomaltotriose for *B. fuscum* dextranase and as glucose for *P. funiculosum* dextranase. The samples were fractionated on a Bio Gel P-60 column (2 cm×70 cm) equilibrated with 100 mM NaCl. The total sugars in 3.5 ml fraction of the elute were determined by phenol-H_2SO_4 method.

Fig. 4. Paper Chromatograms of Digestion Products from *B. fuscum* Dextranase Reaction after Long Incubation

Reaction conditions were the same as described in Fig. 3. 3.5 μl of the reaction mixture was withdrawn and spotted on a TOYO filter paper No. 50 (40 cm×40 cm) and developed by ascending method with the solvent of nitromethane: absolute EtOH: H_2O (30: 45: 25, v/v/v). Detection was done by NaOH-silver nitrate.[a] G_{1-6}; a series of isomaltodextrins (glucose-isomaltohexaose) Hydrolysis degree I; 9%, II; 47% and III; 73%

a) W.E. Trevelyan, D.P. Procter and J.S. Harrison *Nature* **166**, 444 (1950).

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