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Studies on Dextranase. IV. Immobilization of Dextranase from *Penicillium funiculosum* IAM 7013[1a,b]

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Dextranase (EC 3.2.1.11) from *Penicillium funiculosum* was immobilized by the fixation to Sepharose 4B activated with cyanogen bromide. The specific activity of the preparation was about 30%, compared with that of native dextranase.

The immobilization led to increase pH and thermal stabilities, although optimum pH and temperature were the same as that of the native enzyme, respectively. Relative activity of Sepharose-dextranase on various dextrans decreased relatively for an increase in the molecular weight. Michaelis-constant (K_m) of the immobilized enzyme was higher 2—5 times than that of the native enzyme on each molecular weight of dextran examined. Digestion products by the immobilized dextranase were almost the same as those by the native enzyme. Sepharose-dextranase was very stable in a column reaction and useful for continuous enzyme reaction, and also found to keep steady activity by the repeated use.

Levin, *et al.* have reported that immobilization led to increase in thermal and pH stability of trypsin.[3] Takami and Ando have also reported that the resistance to the inhibotors and metal ions were gained.[4] Recently, numerous enzymes were immobilized by various methods because continuous enzyme reaction in a column, repeated use and elimination of the immobilized enzyme from the reaction system became possible.[5]

Recently, it was considered that dextranase (EC 3.2.1.11) might be useful to remove the dextran which disturbed the milling and refining processes from the sugar-cane juices. Really, Cheetham and Richards have immobilized a bacterial dextranase for this purpose.[6] Pharmacological actions of dextran sulfate and partially hydrolyzed dextran sulfate were investigated.[7] The authors prepared the immobilized dextranase which was considered to be useful in obtaining low molecular dextran to derive the sulfate, and in the removal of dextran from sugar-cane juices.

For an immobilization, dextranase II from *Penicillium funiculosum* of which enzymatic properties were reported[8,9] was used to obtain the immobilized dextranase. In this paper, some properties, action patterns and continuous enzyme reaction of the immobilized enzyme are described.

Materials and Methods

Enzyme and Reagents used——Dextranase II from *P. funiculosum* purified by the methods described in the previous paper[8] was used as the native enzyme and its activity was 428 units per mg of protein by the standard enzyme assay.

1) *a*) Part III: M. Sugiura and A. Ito, *Chem. Pharm. Bull.* (Tokyo), **22**, 1593 (1974). *b*) This form is part XCVII of "Studies on Enzymes" by M. Sugiura.
2) Location: *Ueno-sakuragi, 1-Chome, Taito-ku, Tokyo, 110, Japan.*
3) Y. Levin, M. Pecht, L. Goldstein, and E. Katchalski, *Biochemistry* 3, 1905 (1964).
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Dextran was produced by *Leuconostoc mescenteroides* N-4, and was partially hydrolyzed and fractionated. One fraction (mol. wt. 5.3×10^4) was used for the assay of enzyme activity. Sepharose 4B was obtained from Pharmacia Fine Chemicals Co. Uppsala, Sweden. Other chemicals used were of special reagent grade.

Immobilization of Dextranase——Sepharose 4B was activated with cyanogen bromide (BrCN) by the procedure of Axén and Ernback[10] as follows. To the mixture of Sepharose 4B (5 ml) and water (2.5 ml), 10% of BrCN water solution (2.5 ml) was added, and the mixture was adjusted at pH 11 by the addition of 2 M NaOH and allowed to stand for 10 min. The activated Sepharose 4B gel was rapidly washed on a glass filter with cold H_2O (100 ml) and HCl–borate buffer (Sörensen buffer, pH 8.0, ionic strength 0.5 and 100 ml) under suction, and suspended in 10 ml of the same buffer. To the suspension was then added native dextranase, and the mixture was stirred very gently for 24 hr at 4°. Sepharose-dextranase obtained was washed on a glass filter with 100 ml of 100 mM acetate buffer (pH 4.0) in 1 M NaCl, and with 100 mM borate buffer (pH 8.5) in 1 M NaCl under suction. The dextranase bound to Sepharose 4B was calculated as the difference between the protein originally added in the reaction mixture and that in the washings. Protein was determined by the method of Lowry, *et al.*[11]

Assay of Enzyme Activity——Native dextranase activity was estimated by the method described previously.[8] Sepharose-dextranase activity was determined as follows. To 2 ml of 2% dextran solution in 100 mM phosphate buffer (pH 6.0) was added 1 ml of Sepharose-dextranase suspended in H_2O and the mixture was incubated at 37° with mechanical shaking. After 30 min, 3 ml of Sumner's reagent[12] was added to the mixture and liberated reducing sugar was determined as described previously. In this determination, the presence of Sepharose-dextranase did not effect on the colorization. The effect of amount of Sepharose-dextranase and incubation time on the reaction rate are shown in Fig. 1 and 2, respectively.

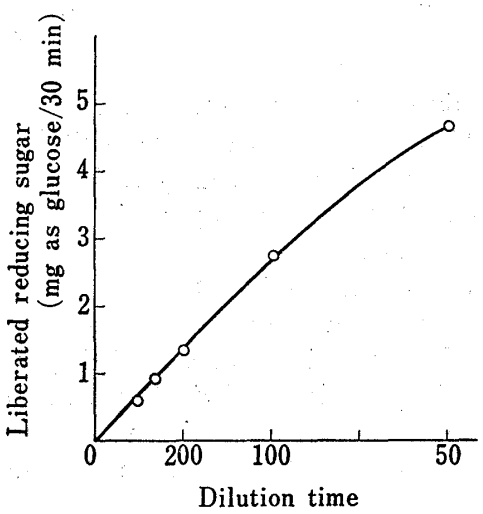

Fig. 1. Effect of Concentration of Sepharose-dextranase on the Reaction Rate

The enzyme reaction was carried out under the standard conditions except for the concentration of Sepharose-dextranase.

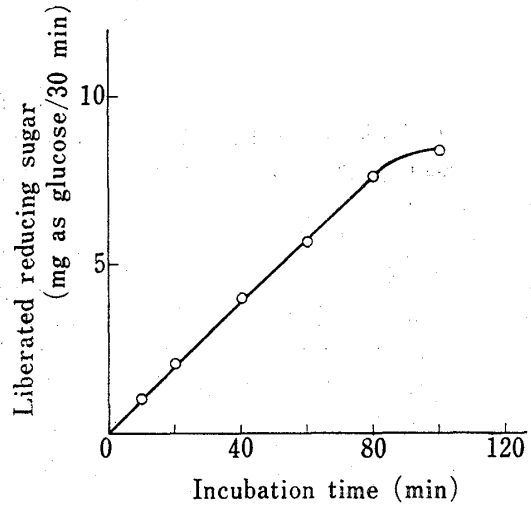

Fig. 2. Effect of Incubation Period on the Reaction Rate

0.5 unit of Sepharose-dextranase was used and the enzyme assay was carried out under the standard conditions with varied incubation time.

In the case of immobilized enzyme, since the enzyme without shaking falls to the bottom of reaction vessel, association between enzyme and substrate will become the limiting step of the enzyme reaction. By the shaking, the reaction rate was increased with increase in amount of the enzyme and reaction time, these confirmed that in this assay system, effect of association rate on the reaction rate was negligible.

Results

Factors which Effect on the Amount of Protein Bound

On immobilization of the enzyme, some factors effecting on the amount of protein bound to the matrix were considered. We regarded some factors and investigated the follow ex-

10) R. Axén and S. Ernback, *Eur. J. Biochem.*, **18**, 351 (1971).
11) O.H. Lowry, H.J. Rosegrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
12) J.B. Sumner, *J. Biol. Chem.*, **65**, 393 (1925).

Table I. Activity of Sepharose-dextranase Preparations

Experiment No.	Dextranase added (mg. protein)	Uptake of dextranase (mg. protein)	Specific activity (units/mg. protein)
1	2.5	1.4	33.9
2	6.6	2.1	145.8
3	6.6	2.3	135.2
4	8.4	2.8	125.0
5	12.6	3.1	143.9
6	16.8	5.5	108.0

Various amounts of purified dextranase were added to the 5 ml of activated Sepharose 4B suspended in 10 ml of HCl-borate buffer (pH 8.0, ionic strength 0.5). Other procedures were the same described in the text.

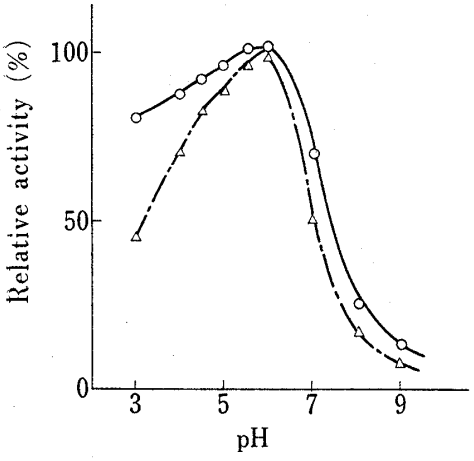

Fig. 3. Effect of pH on Dextranase Activity

The enzyme assay was carried out under standard conditions except for buffers employed. At pH 3—8 and 9, McIlvaine buffer and 100 mM carbonate buffer were employed, respectively.
---△---: native dextranase
—○—: sepharose-dextranase

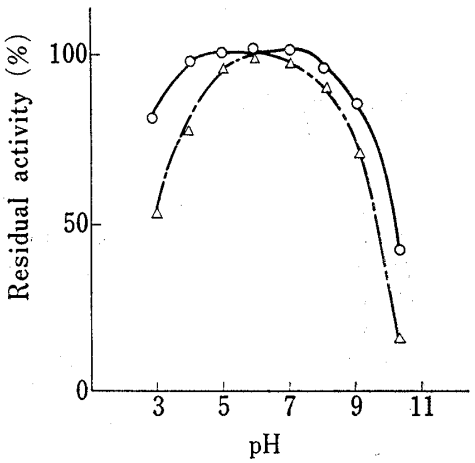

Fig. 4. pH Stability of the Enzymes

The enzyme was treated at various pH values and 37° for 60 min, and then the residual activity was determined. Buffers; pH 3—8: McIlvaine buffer, pH 9—11: 100 mM carbonate buffer, ---△---: native dextranase, —○—: sepharose-dextranase

periments. Effect of dextranase concentration on the amount of enzyme bound is presented in Table I. The amount of protein bound to the Sepharose 4B was increased according to the increase of the enzyme added and the specific activity of the preparation obtained was about 140 units per mg of protein and about 30% that of the native enzyme. At various protein concentartions, the specific activity was also constant, and effect of the enzyme concentration of the amount of the protein bound was not observed in these cases. The effect of activation period of Sepharose 4B with BrCN on the protein bound was also examined, but the periods longer than 10 min were found to be insignificant.

Effect of pH and Temperature on the Reaction Rate

In Fig. 3, the effect of pH on the reaction rate is presented. Both for the native and Sepharose-dextranase, the optimum pH value was 6.0, thus shift of optimum pH caused by the immobilization was not observed. On the Sepharose-dextranase, whereas at acidic pH range the relative activity was higher than that of the native enzyme. The effect of temperature on the reaction rate was also investigated under the standard method. The results suggested that influence of the immobilization on the optimum temperature was not observed and that maximum activity was shown at 50° with native- and Sepharose-dextranase, respectively.

pH and Thermal Stability of Sepharose-dextranase

The effect of pH on the stability of Sepharose-dextranase is shown in Fig. 4. The figure indicated that Sepharose-dextranase was stable within a wider pH range, pH 4 to 8, and that loss of activity was less than 2% of the maximum activity. Thermal stability was also examined. The enzyme was incubated at pH 6.0 for 20 min, and the residual activity was determined. Under these conditions, both native enzyme and Sepharose-dextranase were stable at temperatures up to 40°, and at 55° the former showed only 17% of residual activity, whereas on the later, 37% of it was noted. These results suggested that the immobilization led to increase in thermal stability.

Effects of Metal Ions and Reagents on Sepharose-dextranase

The effects of metal ions and reagents on Sepharose-dextranase were investigated. Results are presented in Table II. On each compound, significant difference was not observed between

Table II. Effect of Metal Ions and Reagents on the Enzyme Activity

Metal ions and reagents	Concentration (M)	Residual activity (%)	
		Native-dextranase	Sepharose-dextranase
None	—	100	100
$AgNO_3$	1×10^{-6}	52	69
$HgCl_2$	1×10^{-6}	71	89
$CoCl_2$	1×10^{-5}	113	133
$CuSO_4$	1×10^{-4}	128	137
NBS[a)]	1×10^{-4}	0	0
Iodine	1×10^{-5}	11	20
Iodoacetic acid	1×10^{-4}	88	98
Urea	1	53	73
Guanidine·HCl	1	43	47

The enzyme was incubated with indicated metal ion or reagent at 37° and pH 6.0 for 30 min, and then the residual activity was determined under the standard conditions.
a) *N*-bromosuccinimide

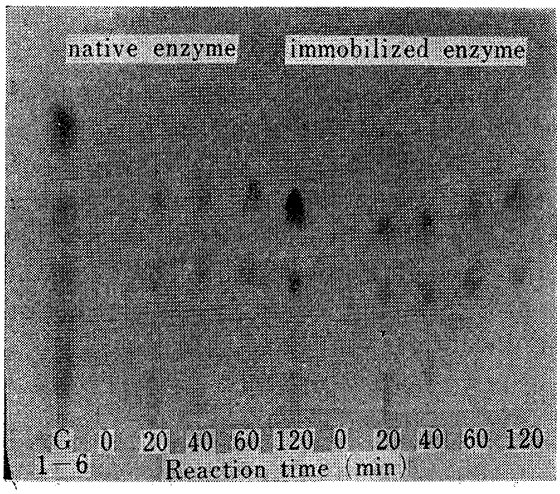

Fig. 5. Paperchromatograms of Digestion Products from Dextran by the Enzymes

The reaction mixture contained 20 mg of dextran (mol. wt., 5.3×10^4) and 2 units of the enzyme in 1 ml of 50 mM phosphate buffer (pH 6.0), and was incubated at 37° with shaking. solvent; nitromethane:ethanol:water (35:40:25 v/v), development: siliver nitrate-sodium hydroxide, G1—6; standard isomaltodextrins (glucose-isomaltohexaose)

Sepharose-dextranase and the native enzyme. It is known that the protection from these compounds is, in general, increased by immobilization, whereas in the case of dertranase, the protection was not gained significantly.

Actions of Sepharose-dextranase on Dextrans with Various Molecular Weights

In Fig. 5, paperchromatograms of digestion products from dextran (mol. wt., 5.3×10^4) by Sepharose-dextranase and the native enzyme are shown. Almost equal amount of isomaltodextrins as the digestion products were observed from the both reaction mixture, and their products were not different significantly. Relative activities on the various dextrans were also examined. As shown in Table III, relative activities and Michaelis-constants (K_m) of native dextranase were found to be constant, but the activity of

Table III. Dextranase Activity for Dextrans with Various Molecular Weights

Dextran (mol. wt)	Relative activity (%)		Michaelis constant (K_m): (%)	
	Native-dextranase	Sepharose-dextranase	Native-dextranase	Sepharose-dextranase
5×10^3	100	100	0.220	0.500
5.3×10^4	96.2	80.3	0.204	0.612
10.4×10^4	95.8	71.8	0.208	0.972
ca. 10×10^5	99.0	54.9	0.217	1.120
ca. 10×10^6	103.3	45.4	—	—

Each molecular weight of dextran was used as the substrate and the other conditions were the same as standard methods. Michaelis constant was calculated from Lineweaver-Burk plots.

Sepharose-dextranase was decreased relatively for an increase in molecular weight. On the dextran (mol. wt., *ca.* 10×10^6), about 50% of the activity was only observed. On the contrary, K_m values were increased. In the action on the same dextran, the immobilization led also to increase the K_m values at 2—5 times that of native dextranase, respectively.

Continuous Enzyme Reaction in a Column

A continuous enzyme reaction which was one of the usefulness of immobilized enzyme was examined in Sepharose-dextranase. The preparation was packed into a jacketed column and maintained at 37° and substrate solution was passed through the column at constant flow rate. The stability of Sepharose-dextranase packed was observed by the determination of reducing sugar contents in the effluent. Results are presented in Fig. 6, indicating that Sepharose-dextranase was very stable and after 170 hr, the activity observed at beginning was almost retained. At various flow rate of substrate, the reaction rate was also investigated. Results are shown in Fig. 7. It was found that varying of the flow rate of substrate was able to control the reaction rate in the column.

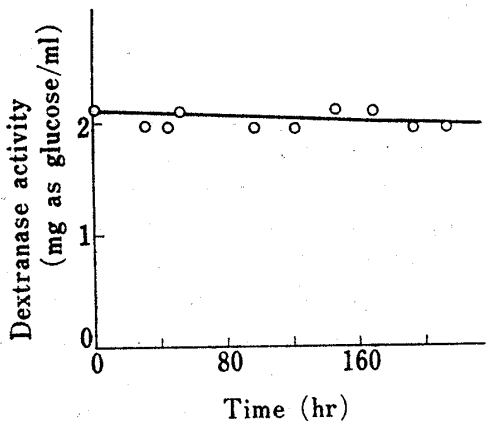

Fig. 6. Continuous Dextranase Reaction in a Column

Sepharose-dextranase was packed in a jacketed column (1.5 cm×2 cm). The column was maintained at 37° and 0.5% of dextran solution (in 100 mM phosphate buffer, pH 6.0) was passed through at a flow rate of 9 ml/hr. Reducing sugar in 0.5 ml of effluent was determined by Sumner's reagent.

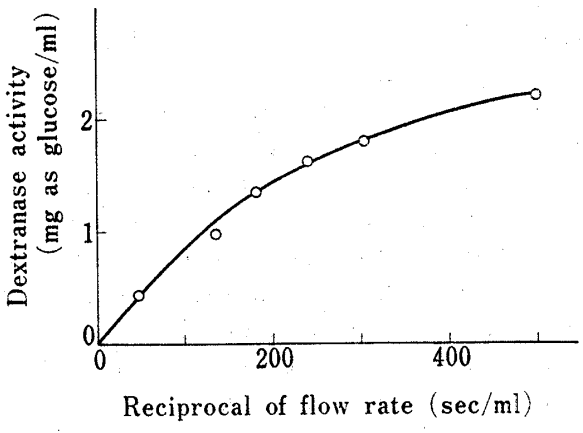

Fig. 7. Relation between Flow Rate and Dextranase Activity

Substrate was passed through the Sepharose-dextranase column (1.5 cm×1 cm) at indicated flow rate. Other conditions were the same as described in Fig. 6.

Stability of Sepharose-dextranase after Repeated Use

In consideration of repeated use of Sepharose-dextranase in a batch system, the stability of it was examined. Results are presented in Table IV, indicating that Sepharose-dextranase was found to be very stable, to be recovered and to be used repeatedly.

Table IV. Stability of Sepharose-dextranase after Repeated Use

Number of times used	Remaining activity (%)	Number of times used	Remaining activity (%)
1	100	5	93
2	100	6	93
3	96	7	93
4	98		

A mixture of 50 mM phosphate buffer (pH 6.0), 200 mg of dextran and Sepharose-dextranase, in a total volume of 15 ml, was incubated for 20 min at 37° with shaking and then filtered. The residual Sepharose-dextranase was washed with water, and again incubated under the same conditions as described above. The interval between each use was 20 min.

Discussion

Immobilization of enzymes are known to carried out by various linkages such as ionic and covalent linkages and microencapsulation, and many immobilized enzymes produced by the above methods have been reported.[4,6,13,14]

In order to prepare an immobilized dextranase with high specific activity, we have attempted to produce an immobilized dextranase by various methods. At first, it was attempted to fix dextranase to diethylaminoethyl (DEAE)-cellulose and the fixed enzyme was obtained. However, we found the enzyme to liberate from DEAE-cellulose to the reaction system during the proceeding of the enzyme reaction and the liberation was observed at substrate concentration less than 0.1%. Thus, the preparation was not attained our purposes. Cheetham and Richards have reported an immobilized bacterial dextranase fixed to carboxymethyl (CM)-cellulose azide, although its specific activity was very low.[6] But, we failed to fix *P. funiculosum* dextranase II to CM-cellulose azide. After all, the fixation of dextranase to Sepharose 4B activated with BrCN was found to be the only successful procedure.

It is known, in general, that an immobilization of enzyme lead to change the optimum pH or pH stability, but these changes were not observed significantly on the dextranase. This is due to the considerations that, in the dextranase reaction system, both dextran and the digestion products are uncharged.

Mitz and Summaria have described that immobilized chymotrypsin hydrolyzed easily the low molecular synthesized substrates, but not the high molecular weight casein.[15] Similar results were shown on our immobilized dextranase (Table III). These observations are, in general, considered that by the ionic or steric effect of supporting material, the diffusion rate of substrate or products from the reaction system was disturbed. Since on Sepharose-dextranase, the reaction rate was decreased relatively in increasing molecular weight of the substrate, the main effect was considered that steric effect of Sepharose 4B disturbed the diffusion of substrate to the active site on the enzyme.

With Sepharose-dextranase, the excellent properties such as an increase of pH and thermal stability, production of same digestion products as those of native enzyme, ability of continuous reaction and repeated use were confirmed and our first purposes to produce the immobilized dextranase were achieved.

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13) T. Tosa, T. Mori, and I. Chibata, *Agr. Biol. Chem.* (Tokyo), **33**, 1053 (1969).
14) T. Mori, T. Tosa, and I. Chibata, *Biochim. Biophys. Acta*, **321**, 653 (1973).
15) M.A. Mitz and L.J.S. Summaria, *Nature*, **189**, 576 (1961).