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Studies on Dextranase. VIII. Some Enzymatic Properties of Immobilized Dextranase from *Brevibacterium fuscum* var. *dextranlyticum*[1a,b]

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Dextranase (EC 3.2.1.11) of *Brevibacterium fuscum* var. *dextranlyticum* 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 stability, although thermal stability, optimum pH and temperature were similar to that of the native enzyme, respectively. The immobilized dextranase was very stable on storage at 4° and retained more 90% of the initial activity in the 9th weeks whereas the native enzyme lost about 50% of it after 6 days later. Relative activity of immobilized dextranase on dextrans with various molecular weights decreased relatively for an increase in molecular weight. Michaelis constant (K_m) of the immobilized enzyme was larger 2—5 times than that of the native enzyme on each molecular weight of dextran. Action pattern of dextranase was not effected by the immobilization. Immobilized dextranase was very stable in a column reaction and suitable for continuous enzyme reaction, and also found to keep steady activity by the repeated use. These properties of immobilized dextranase of *B. fuscum* were very similar to those of the immobilized enzyme of *P. funiculosum*.

The authors have reported the immobilization of dextranase from *Penicillium funiculosum* IAM 7013 and its enzymatic properties to develop the application of the enzyme.[3] We also prepared the immobilized dextranase from *B. fuscum* which had a wider optimum pH range and pH stability range than those of the *P. funiculosum* enzyme and examined its enzymatic properties. In this paper, some enzymatic properties of the immobilized enzyme are described.

Materials and Methods

Enzyme and Reagents used——Dextranase from *B. fuscum* was purified by the procedure described in the previous paper[4] and was used as the native enzyme. The specific activity of the enzyme was 167 units per mg protein by the standard enzyme assay.[4]

Dextran was produced by *Leuconostoc mesenteroides* N-4, and was partially hydrolyzed and fractionated. One fraction (mol. wt 5.3×10^4) was used for the assay of the enzyme activity. Various dextran fractions were gifts of Meito Sangyo Co. Ltd. Nagoya, Japan. Sepharose 4B was obtained from Pharmacia Fine Chemicals Co. Uppsala Sweden. Other chemicals used were of reagent grade.

Immobilization of Dextranase and Assay of Enzyme Activity——Immobilization of dextranase was carried out as described in a previous paper[3] and Sepharose 4B activated with cyanogen bromide was used as a supporting material. The specific activity of the immobilized dextranase prepared as above was 50—60 units per mg protein bound and was about 30% of the native enzyme. Native dextranase activity was determined as described in a previous paper[4] and the activity of the immobilized enzyme was estimated as follows. To 2 ml of 2% dextran solution (in 100 mM phosphate buffer pH 7.5), 1 ml of immobilized dextranase suspended in water was added and the mixture was incubated at 37° with mechanical shaking. After 30 min, 3 ml of dinitrosalicylate reagent (Sumner's reagent[5]) was added to the mixture and the liberated reduc-

1) *a*) Part VII: M. Sugiura and A. Ito, *chem. Pharm. Bull.* (Tokyo) **23**, 1532 (1975); *b*) This form is part CXIII of "Studies on Enzymes" by M. Sugiura.
2) Location: *Ueno-sakuragi, 1-chome, Taito-ku, Tokyo, 110, Japan.*
3) M. Sugiura and A. Ito *Chem. Pharm. Bull.* (Tokyo) **22**, 2941 (1974).
4) M. Sugiura, A. Ito and T. Yamaguchi, *Biochim. Biophys. Acta*, **350**, 61 (1974).
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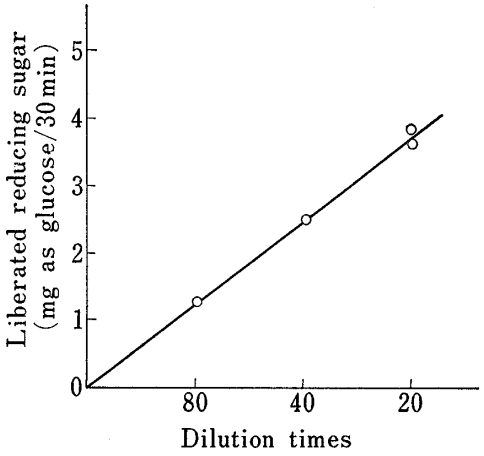

Fig. 1. Effect of Concentration of Immobilized Dextranase on the Reaction Rate

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

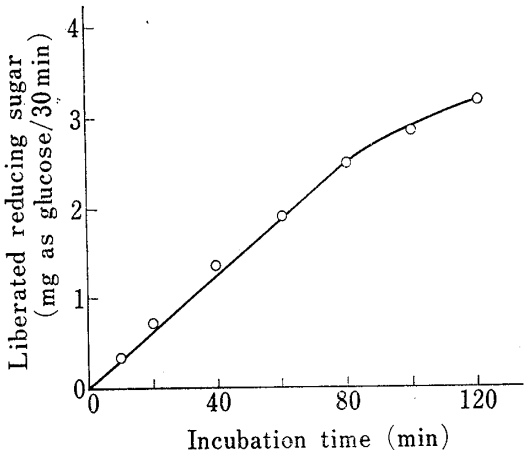

Fig. 2. Effect of Incubation Period on the Reaction Rate

0.2 unit of immobilized dextranase was used and the enzyme assay was carried out under the standard conditions with varied incubation period.

ing sugar was determined as described previously. Under these assay systems, the effect of amount of immobilized dextranase and incubation period on the reaction rate were investigated. Results are presented in Figs. 1 and 2, respectively. Reducing sugar liberated was increased in proportion to the amount of the enzyme and incubation period, respectively.

Results

Effect of pH and Temperature on the Reaction Rate

In Fig. 3, the effect of pH on the reaction rate is presented. Optimum pH was found to be 7.0—7.5 for both native and immobilized dextranases, thus the shift of optimum pH caused by the immobilization was not observed. Optimum temperature of both enzymes was also investigated under the standard enzyme assay. The results suggested that the influence of the immobilization on the optimum temperature was not observed and the maximum activity was shown at 55° with native and immobilized dextranases.

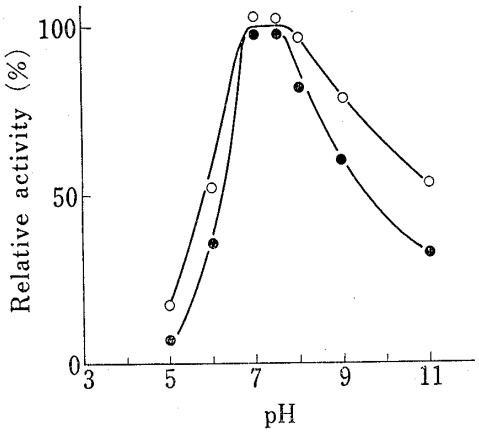

Fig. 3. Effect of pH on Dextranase Activity

The enzyme assay was carried out under standard conditions except for buffers employed. buffers; pH 3—8: McIlvaine buffer, pH 9—11: 100 mM carbonate buffer ● :native dextranase, ○ : immobilized dextranase

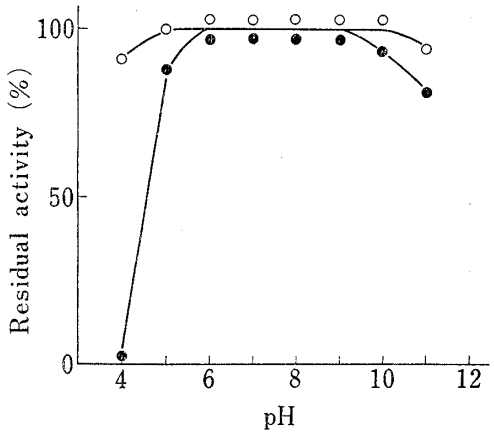

Fig. 4. pH Stability of the Enzymes

The enzyme was treated at various pH values and 37° for 12 hr, and then the residual activity was determined under the standard conditions. buffers; pH 4—8: McIlvaine buffer, pH 9—11: 100 mM carbonate buffer ●:native dextranase, ○:immobilized dextranase

pH and Thermal Stability of Immobilized Dextranase

For the investigation of pH stability of immobilized dextranase, the enzyme suspended in buffer with various pH values was treated at 37° for 12 hr and the residual activity was determined under the standard procedure. Results are shown in Fig. 4. *B. fuscum* dextranase was originally stable over a wide pH range,[4] and the immobilization led the increase of its pH stability; *e.g.* at pH 4.0, the residual activity of the immobilized enzyme was about 90%, whereas that of the native enzyme was less than 1%. Thermal stability was also examined as follows. The enzyme suspended in 100 mM phosphate buffer (pH 7.5) was treated at various temperatures for 30 min and the remaining activity was determined. It was found that the both enzymes were stable up to 50° and the loss of activity was less than 1%, but the immobilization did not lead the significant increase of thermal stability at higher temperatures.

Stability of Immobilized Dextranase on Storage

To investigate the stability of immobilized dextranase on storage, the enzyme suspended in 100 mM phosphate buffer (pH 7.5) or deionized water was stored at 4° and the residual activity was determined periodically under the standard conditions. As shown in Fig. 5, native dextranase in deionized water was very labile and lost about 50% of the initial activity in the 6 th, but the immobilized enzyme was very stable and less than 10% of it was only lost in the 9 th week. Both native and immobilized dextranases in buffer, however, were found to be very stable under the conditions.

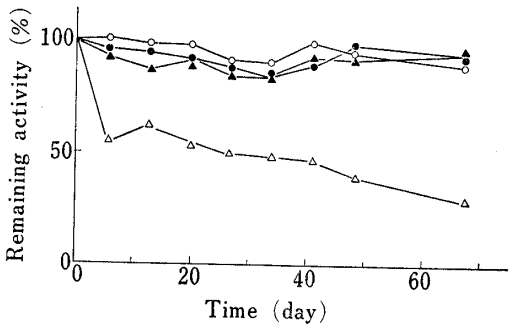

Fig. 5. Stability of Immobilized Dextranase on Storage

The enzyme was stored at 4° in deionized water or 100 mM phosphate buffer (pH 7.5) and the activity was assayed after the time intervals. Remaining activities are expressed as percentages of the original activity.
native dextranase; △ : in deionized water, ○ : in buffer immobilized dextranase; ▲ : in deionized water, ● : in buffer

Action Patterns of Immobilized Dextranase

For an investigation of the change of action patterns by the immobilization, dextran (mol. wt 5.3×10^4) was hydrolyzed by the immobilized enzyme and the digestion products were detected by paper chromatography. The results suggested that action patterns of native and immobilized dextranases were identical and isomaltotriose was the only digestion product detected by the procedure.

Effects of the immobilization on the activity and Michaelis constant (K_m) were examined. Results are presented in Table I. The authors have reported that on *B. fuscum* dextranase, the activity was decreased in relation to the increase of dextran molecular weight.[6] By the immobilization, this tendency was also found remarkable and the enzyme showed about 50% activity towards the dextran with mol. wt 10×10^6 as compared with that of the mol. wt 5×10^3 dextran. These phenomena were found more clear on the Michaelis constants. The immobilization led also to increase the K_m values at 2—5 times that of the native enzyme, *e.g.* on the dextran with mol. wt 5.3×10^4, the immobilization resulted in increase of K_m values from 0.070 to 0.278.

Stability of Immobilized Dextranase on a Continuous Enzyme Reaction in a Column

The immobilized dextranase mixed with cellulose powder to obtain faster flow rate was packed into a jacketed column and maintained at 37°. Effect of flow rate of the substrate on the reaction rate was investigated in the column and the results are presented in Fig. 6. Up to 200 sec/ml of flow rate, the reaction rate was found to increase in proportion to flow rate and the results indicated that variation of the flow rate of the substrate was able to control the

6) M. Sugiura and A. Ito, *Chem. Pharm. Bull.* (Tokyo), **23**, 1532 (1975).

reaction rate in the column. During the continuous enzyme reaction, the stability of the immobilized enzyme in the column was also examined. 0.5% of the substrate solution (in 100 mM phosphate buffer pH 7.5) was continuously passed through the column at a flow rate of 56 ml/hr at which the reaction rate was increased in proportion to the flow rate of substrate. As shown in Fig. 7, the immobilized enzyme was very stable and after 160 hr, the enzyme retained almost equal activity to that of which was observed at the beginning. At the same time, the digestion products produced in a column reaction were periodically examined by paper chromatography. It was found that the action pattern of immobilized dextranase was identical to that of the native enzyme.

Table I. Dextranase Activity for Dextrans with Various Molecular Weights

Dextran (mol. wt)	Relative activity: (%)		Michaelis constant (K_m): (%)	
	Native dextranase	Immobilized dextranase	Native dextranase	Immobilized dextranase
5×10^3	100	100	0.108	0.286
5.3×10^4	90.9	81.8	0.070	0.278
10.4×10^4	87.6	70.5	0.068	0.272
Approx. 10×10^5	83.8	54.4	0.047	0.223
Approx. 10×10^6	82.1	49.8	0.044	0.225

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

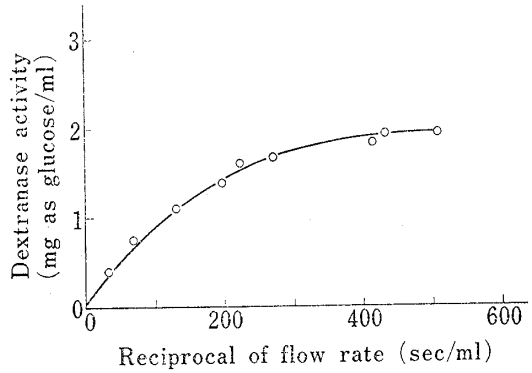

Fig. 6. Relation between Flow Rate and the Dextranase Activity

Immobilized dextranase was packed into a jacketed column (1.5×1 cm). The column was maintained at 37° and 0.5% of dextran solution (in 100 mM phosphate buffer, pH 7.5) was passed through at indicated flow rate.

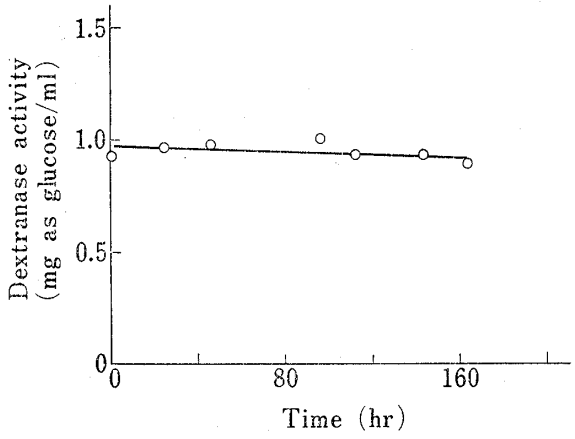

Fig. 7. Continuous Dextranase Reaction in a Column

Substrate was passed through the immobilized dextranase column (1.5×1 cm) at flow rate of 56 ml/hr. Other conditions were the same as described in Fig. 6.

Table II. Stability of Immobilized-dextranase after Repeated Use

Number of times used	Remaining activity (%)
1	100
2	91
3	94
4	91
5	85
6	83
7	85

A mixture of 50 mM phosphate buffer (pH 7.5), 200 mg of dextran and the enzyme in a total volume 15 ml, was incubated for 20 min at 37° with shaking and then filtered. The residual immobilized enzyme was washed with water and again incubated under the same conditions as above.

On the immobilized dextranase, the enzyme reaction was considered to be carried in a batch system and removed from the reaction system for repeated use. Therefore, the stability of the enzyme after repeated use was investigated. After reaction in a flask with mechanical shaking, the immobilized enzyme was collected by filtration and washed with water repeatedly and used for the reaction in the same system. The stability of the enzyme was monitored by the determination of the reducing sugar in a filtrate. Results are presented in Table II indicating that after 6 times of repeated use, the immobilized enzyme retained about 85% of the initial activity. From the results, the immobilized enzyme was found to be very stable, to be recovered and to be suitable for repeated use.

Discussion

For an immobilization of enzyme, the enzyme prepared was possibly retained a high specific activity compared with that of the native enzyme and the same substrate specificity were very important point of views. An immobilized enzyme fixed by ionic linkage, in general, was well known that changing pH or ionic strength caused in the liberation of the enzyme from the supporting material during the enzyme reaction. In a case of microcapsulated enzyme, it was also known that large substrate did not enter into a microcapsule and caused a decrease of the remarkable specific activity.

In consideration of above phenomena, the authors attempted to produce an immobilized dextranase with a high specific activity and obtained the immobilized enzyme by the procedure described above. The liberation of the enzyme from Sepharose 4B was examined on our immobilized dextranase. After dextranase reaction under the standard conditions, the enzyme activity in the filtrate was estimated and the liberation was not detected at all.

On the immobilized dextranase, some enzymatic properties were examined and the excellent properties such as an increase of stability of pH and of storage, production of the same hydrolyzed product as that of the native enzyme, ability of continuous enzyme reaction and repeated use were confirmed. Amount of the enzyme bound to Sepharose 4B and specific activity of the immobilized enzyme were very similar to those of immobilized dextranase of *P. funiculosum* of which properties have been reported.[3] These properties were considered due to similarity of molecular weight, isoelectric point and amino acid composition of both dextranases.[7] This is also explained by the description of Axén and Ernback[8] that fixation of a enzyme to Sepharose 4B activated with BrCN was carried out by binding of free amino groups on the enzyme to it and also the fact that both dextranases were the acidic protein,[7] and free amino groups contained were therefore very low.

It has been reported that enzymatic properties of *B. fuscum* dextranase were better than those of *P. funiculosum* enzyme[4] and the immobilization of *B. fuscum* enzyme resulted in increase of those excellent properties. Therefore, the authors expect that utilization and application of the enzyme are developed in a future.

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7) M. Sugiura and A. Ito, *Chem. Pharm. Bull.* (Tokyo), **23**, 1304 (1975).
8) R. Axén and S. Ernback, *Eur. J. Biochem.*, **18**, 351 (1971).