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ACTION PATTERNS OF IMMOBILIZED DEXTRANASE

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ABSTRACT

Dextranase, isolated from *Penicillium funiculosum* and *P. lilacinum*, was immobilized on porous, silanized-silica beads and a phenol–formaldehyde resin. A commercial dextran of relatively low molecular weight ($\sim 2 \times 10^6$) was degraded by immobilized dextranase, with the formation of reducing sugars, but with little decrease in viscosity. In contrast, soluble dextranase caused rapid loss of viscosity, but only a slight increase in reducing sugar. Native dextran of high molecular weight, from *Leuconostoc mesenteroides* NRRL B-512 (F), was attacked very slowly by immobilized dextranase, with the release of oligosaccharides of low molecular weight.

INTRODUCTION

The action pattern of immobilized enzymes that degrade biopolymers may differ from those of their corresponding soluble forms. Earlier work from our laboratory[1,2] showed that, when immobilized, an endo-acting, alpha amylase degraded the amylose portion of corn starch in an exo-enzymic pattern, but left the amylopectin molecule only slightly degraded.

The present study is concerned with the action of an immobilized endo-dextranase on *Leuconostoc mesenteroides* NRRL B-512 (F) dextran. This dextran differs from corn starch, in that it consists of only a single species having 95% of α-(1→6) linkages with 5% of α-(1→3) branch-points. Most of the side chains are stubs of one or two D-glucose units, but a few side chains may have as many as 30 to 50 D-glucose units[3]. Although not a strictly linear molecule, the dextran more nearly resembles essentially unbranched amylose than it does amylopectin, which is highly branched with side chains that, in turn, are also branched. It therefore seemed possible that an immobilized endo-dextranase might react with the dextran to give some unusual products.

Dextranase was immobilized on Enzacryl Polyacetal by Epton *et al.*[4], who made no attempt to compare the mode of action of the immobilized enzyme with that of the soluble form. Cheetham and Richards[5] and Sugiura and Ito[6] immobilized dextranase on a variety of cellulosic supports, and claimed that there was no difference in activity between the immobilized and the soluble form. In our experience, cellulose

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was not a suitable carrier because dextrans, especially those of high mol. wt., displaced the dextranase from the carrier. Consequently, any unusual action-pattern exhibited by the immobilized enzyme would be masked by subsequent action of the soluble enzyme displaced. We found it necessary to wash the dextranase–carrier complex exhaustively with dextran solution until prolonged incubation of the washings showed no further dextranase activity.

Barker *et al.*[7] prepared a conjugate of dextranase and concanavalin A that showed strikingly different behavior on dextran and isomalto-oligosaccharides in comparison to that of soluble dextranase. The conjugate itself was soluble, and so its action pattern would not be expected to be comparable to that of insoluble dextranase.

EXPERIMENTAL

*Materials**. — Cultures of *Penicillium funiculosum* NRRL 1768 and *P. lilacinum* NRRL 396 were obtained from the Agricultural Research Service Culture Collection. Phenol–formaldehyde resin, Duolite S-761, was obtained from Diamond Shamrock Co., Cleveland, Ohio. Silanized, porous silica was furnished by Corning Glass Works, Corning, New York. Low-molecular-weight dextrans (T-2000, T-250, and T-150) were purchased from Pharmacia Fine Chemicals Co., Piscataway, New Jersey. Native *Leuconostoc mesenteroides* NRRL B-512 (F) dextran was kindly provided by Dr. Morey E. Slodki of this Center.

Preparation of dextranase. — Crude dextranase was prepared by culturing *P. funiculosum* and *P. lilacinum* on a medium consisting of 2% of B-512 (F) dextran and 3% of corn steep-liquor[8]. The impure dextranase was partially purified by precipitation in 50%-saturated $(NH_4)_2SO_4$. An aqueous solution of the precipitate was dialyzed against distilled water, the retentate was treated with lead acetate, and the soluble portion was dialyzed to remove the excess of lead acetate. The resulting dextranase solution was used as the source of enzyme for immobilization purposes.

Immobilization of dextranase. — Duolite S-761 resin (100 mg) was added to dextranase solution (2 mL) having 2.65 viscosity units/mL and an optical absorbance of 0.713 at 280 nm. The mixture was gently agitated by use of a tube roller; after 30 min, the absorbance had diminished to 0.316, and it remained constant thereafter. The resin–enzyme complex was thoroughly washed with water, followed by 1% dextran T-2000 solution until no residual enzyme could be detected in the wash solutions.

Dextranase was immobilized to silanized, porous silica by adding 4 mL of enzyme solution to 250 mg of the silica that had been pretreated with glutaraldehyde. The silica–enzyme complex was thoroughly washed with water and 1% dextran T-2000 to remove unattached enzyme.

Assay of dextranase. — The viscometric assay was conducted by adding 9.0 mL

*The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

of 1% T-2000 dextran at pH 5.1 to a No. 100 Oswald–Fenske viscometer tube, and warming to 40° in a water bath. Before addition of enzyme, the initial viscosity was determined. Up to 0.1 mL of enzyme solution was then added, and mixed by allowing a gentle stream of air to enter through the capillary side of the tube. The course of the enzyme reaction was monitored by measuring the viscosity every 5.0 min. Usually, a total time of 20 min was sufficient.

A unit of activity is that amount of enzyme needed to lower the specific viscosity (η_{sp}) by 0.1 min, and it is calculated according to the following equation:

$$\text{units/mg or mL} = (t_1 - t_2)/(t_0 \times te \times \text{mg or mL} \times 0.1),$$

where t_1 = seconds required for the substrate to pass through the viscometer tube, t_2 = seconds for reacted substrate, t_0 = seconds for water or buffer, and te = elapsed reaction-time (in minutes).

The potency of dextranase was also determined by measuring the increase in the reducing-sugar value obtained by incubating the enzyme with T-2000 dextran at 40°. Reducing sugar was measured in a Technicon Autoanalyzer by use of a $K_3Fe(CN)_6$ reagent. Results were calculated as mmol of isomaltose produced/mL, or mg/min.

Measurement of the activity of immobilized dextranase. — Dextran substrate (12 mL), warmed to 40°, was added to the immobilized dextranase, and the mixture was gently shaken in a water bath at 40°. At appropriate intervals, a 9-mL aliquot of the mixture, withdrawn with a syringe fitted with a porous-plastic tip, was added to a viscometer tube. After determination of the viscosity, the substrate was returned to the enzyme–carrier complex for further reaction. Results are expressed as change in specific viscosity with time, where the specific viscosity (η_{sp}) = $t/(t_0 - 1)$, t is the time (in s) for the reaction mixture to flow through the viscometer tube, and t_0 is the time required for water or buffer. When comparing soluble against immobilized dextranase, an attempt was made to carry each reaction to approximately the same specific viscosity.

Intrinsic viscosity was determined by Berlin's method[9], by converting η_{sp} to η_{rel}, and using the table provided in ref. 9.

L.c. analysis of isomalto-oligosaccharides. — Isomalto-oligosaccharides produced by the action of soluble and of immobilized dextranase were identified by l.c. in a μBondapak carbohydrate column supplied by Waters Associates. Elution of the oligosaccharides was accomplished with ratios of acetonitrile:H_2O of 7:3 or 3:1. The digests were prepared for l.c. analysis by addition of four parts of MeOH, to precipitate dextran fragments of high mol. wt. After centrifuging, the MeOH was removed from the supernatant liquors by evaporation.

RESULTS AND DISCUSSION

In Table I are given results showing the relationship of diminution of viscosity to formation of reducing sugar from T-2000 dextran by dextranase from both *P. funiculosum* and *P. lilacinum*. Dextranase, immobilized on Duolite resin, from both

TABLE I

RELATIONSHIP OF DIMINUTION OF VISCOSITY TO FORMATION OF REDUCING SUGARS BY FREE AND IMMOBILIZED DEXTRANASE FROM *Penicillium funiculosum* AND *P. lilacinum* ACTING ON DEXTRAN T-2000

Enzyme source	*Enzyme form*	*Δ (reciprocal viscosity)*	*Reducing sugar (mmol)*	*Reducing sugar/unit change in viscosity*	*Reducing sugar (immobilized)/reducing sugar (soluble)/unit change in viscosity*
P. funiculosum	soluble	0.282	0.15	0.53	—
NRRL-1768	resin[a]	0.067	0.31	4.6	8.7
	porous silica	0.145	0.81	5.6	10.6
P. lilacinum	soluble	0.204	0.108	0.53	—
NRRL-896	resin	0.352	1.58	4.5	8.5
	porous silica	0.232	2.75	11.8	22.2

[a]Resin as described in text.

TABLE II

INFLUENCE OF MOLECULAR WEIGHT OF DEXTRAN ON THE PATTERN OF ACTION OF IMMOBILIZED *Penicillium funiculosum* DEXTRANASE

Mol. wt. of dextran $\times 10^{-3}$	*Enzyme form*	*mM isomaltose/unit change in viscosity*	*Immobilized/soluble*
150	soluble	0.571	
	porous silica	0.658	1.15
250	soluble	0.277	
	porous silica	0.732	2.64
	resin	1.61	5.81
2000	soluble	0.53	
	porous silica	5.6	10.6
	resin	4.6	8.7

organisms yielded over eight times as much reducing sugar per unit change of viscosity as did the soluble form of the enzyme. Comparable results were obtained when the *P. funiculosum* enzyme was immobilized on porous silica. The *P. lilacinum* dextranase immobilized on porous silica consistently produced about twice as much reducing sugar per unit change in viscosity compared to the resin-attached enzyme, and about twenty times as much as the soluble dextranase.

Similar experiments were performed on dextrans T-150 and T-250 that have nominal mol. wts. of 150×10^3 and 250×10^3, respectively. In Table II are given the ratios of formation of reducing sugar by immobilized dextranase to that of the soluble enzyme. At the lowest molecular weight, there is little difference in the action pattern of either the bound or the free enzyme, but, as the molecular weight increases, the

TABLE III

DISTRIBUTION OF MOLECULAR WEIGHT OF DEXTRAN HYDROLYZATE PREPARED BY IMMOBILIZED AND BY SOLUBLE *P. funiculosum* DEXTRANASE

Dextran	*Enzyme*	[η]	*Molecular weight distribution*[a]	
			>11,000 (%)	*<11,000 (%)*
T-150	none	0.323	100	
	silica	0.232	68	32
	soluble	0.262	74	26
T-250	none	0.395	100	
	silica	0.248	59	41
	Duolite	0.343	81	19
	soluble	0.304	92	8
T-2000	none	0.629	100	
	silica	0.530	96.7	3.3
	Duolite	0.587	97.1	2.9
	soluble	0.555	100	0

[a]Distribution of mol. wt., determined by l.c. in a Waters μBondagel ε-linear column.

ratio increases, indicating that the bound dextranase converts from an endo to an exo type of action.

The contrasting patterns of action between the soluble and the immobilized dextranase were confirmed by gel-permeation chromatography. Table III records the proportion of dextran hydrolyzate whose mol. wt. is >11,000 to that <11,000. For T-150 dextran, the similarity in the size distribution confirms the results obtained by reducing-sugar analysis. As the size of the substrate increases, there is relatively more product of mol. wt. <11,000 in the immobilized dextranase digests than is found in the products produced by the soluble enzyme. The very high value of 41% below mol. wt. 11,000 in the silica-complex digest is probably due to greater enzyme action, as indicated by the low intrinsic viscosity. The Duolite–enzyme-treated material, however, has a higher intrinsic viscosity than the soluble-enzyme digest, but it still shows 2.5 times as much of components of low molecular weight than the soluble-enzyme digest.

The T-2000 digests showed little difference in molecular size of the material of mol. wt. >11,000, but only those containing the immobilized dextranases had appreciable amounts of product having mol. wt. <11,000. It seems quite evident that the immobilized dextranases exhibit a pronounced, exo-enzyme type of action on the dextrans of high molecular weight.

Native B-512 (F) dextran, having a mol. wt. of >10 million, was attacked only slightly by immobilized *P. funiculosum* dextranase. The enzyme attached to silica lowered the η_{sp} value from 1.02 to only 0.932 in 4.5 h, whereas the soluble enzyme caused η_{sp} to drop to 0.535 within 10 min, and yet, the reducing sugar amounted to 0.26mM isomaltose for the silica-dextranase, but only 0.15 for the soluble enzyme.

Consequently, the pattern for action of immobilized dextranase on native dextran is the same as that found with T-2000 dextran, even though the degree of hydrolysis was limited in the former case.

L.c. analyses revealed only oligosaccharides of low molecular weight (d.p. 2 to 5) in digests of native dextran by both the Duolite– and silica–dextranase complexes, with isomaltose preponderant. These products were also found in corresponding digests of both T-150 and T-250 dextran. Soluble *P. funiculosum* dextranase gave only traces of the di- and tri-saccharides.

This study reveals that immobilized endo-dextranases have decidedly different action patterns on B-512 (F) dextrans of various mol. wts. The immobilized enzymes rupture relatively few internal bonds of the dextrans of higher mol. wt., and yet they produce quite large proportions of reducing sugar. The inference is that the immobilized dextranases probably strip the backbone of side chains, especially the longer ones. The result would be a much more linear dextran molecule, presumably having properties different from those of the original, native polymer.

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