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STUDIES ON DEXTRANASES

PART III. INSOLUBILIZATION OF A BACTERIAL DEXTRANASE

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ABSTRACT

A bacterial dextranase has been insolubilized by three methods, *viz.*, by coupling to cellulose carbonate, to carboxymethylcellulose azide, and to cyanogen bromide-activated cellulose. Properties of the insolubilized forms of the enzyme are described and compared with those of the enzyme in solution. Increased temperature stability of the insolubilized forms was noted in each case, whilst pH optima and action patterns showed no significant changes.

INTRODUCTION

Insolubilized enzymes are now receiving a great deal of attention[1–5]. In applied research[6–11], the value of water-insoluble enzymes lies in their potential as specific, re-usable, non-contaminating catalysts, often of increased stability, for use in industrial processes. A commercial process for the resolution of acyl-DL-amino acids, using immobilized aminoacylase, has been developed[12].

Many enzymes are membrane-bound *in vivo*, and artificially immobilized enzymes can serve as model systems in studies to elucidate the mode of action of such enzymes[13]. Urease has been employed in semipermeable microcapsules to remove urea from blood in the artificial kidney[14]. Other medical[15] and pharmaceutical[16] uses have been proposed.

As an extension of previous work[17,18] on dextranases, it was considered that an insolubilized dextranase might prove useful for the removal of dextran from sugar-cane juices. The presence of dextran in cane juices causes problems in the milling and refining processes. The anticipated qualities most useful in this regard would be improved thermal stability and ease of recovery, both factors being of possible importance in the economics of such a scheme.

EXPERIMENTAL AND RESULTS

Materials and methods. — Dextran type 100-C (Sigma) was used as substrate for the assay of dextranase activity. Dextranase-CB (bacterial) from Calbiochem was

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supplied as lyophilized powder in sodium chloride and dextran. Cellulose powder (Whatman CC41) was used as starting material in the cellulose carbonate and cyanogen bromide procedures. Carboxymethylcellulose (Cellex-CM, 0.68 mequiv./g, Bio-Rad Labs.) was used as starting material for the preparation of carboxymethylcellulose azide.

The following buffer systems were used: (*a*) 20mM sodium citrate (pH 6.2), (*b*) 0.2M sodium citrate (pH 6.2), (*c*) 50mM sodium phosphate (pH 7.8), (*d*) 20mM sodium phosphate (pH 7.8), (*e*) 0.1M sodium hydrogen carbonate, (*f*) 50mM sodium phosphate (pH 7.0), (*g*) 0.2M sodium citrate (pH 6.0), M in sodium chloride and M in sucrose. Phosphate–citrate (McIlvaine) buffers of various pH values were used for studies of pH optima and pH stability.

The washing procedures to remove any excess of uncoupled enzyme or physically adsorbed enzyme were the same for each support used. Controls using the enzyme and the original (underivatised) cellulose and carboxymethylcellulose were utilized to show that the washing procedures were adequate (Table I). The washing was carried out on sintered-glass filters at 4°, the sequence, for 200 mg of solid support, being: (*i*) buffer (*e*), 50 ml; (*ii*) buffer (*b*), 50 ml; (*iii*) buffer (*g*), 50 ml; (*iv*) buffer (*c*) 50 ml; (*v*) buffer (*a*), 50 ml. Each buffer was added in 10-ml batches, and the slurry was stirred thoroughly on the filter. The washed solid was suspended in 1 ml of buffer (*a*), and 0.1 ml of this suspension (containing 20 mg of solid) was taken for assay of bound-enzyme activity. For assay, the enzyme slurry (0.1 ml), dextran (1% solution, 0.5 ml), and buffer (*a*) (0.5 ml) were shaken gently for 60 min at 40°. After centrifugation, 1-ml samples were removed for assay by the Nelson procedure adopted previously[17].

TABLE I

PROPERTIES OF PREPARATIONS OF INSOLUBILIZED DEXTRANASE

	Specific activity[a]	*Units of enzyme activity/g of matrix*[b]	*Activity (as % of unbound enzyme)*	*Protein (mg)/g of matrix*
CC-dextranase	6.5	104	7.9	16
ICC-dextranase	26.4	2166	32.2	82
CMC-dextranase	7.5	83.3	9.1	11
Underivatised dextranase	81.8		100	
Underivatised cellulose		9		
Underivatised CM-cellulose		7		

[a]As μg of D-glucose liberated/min/mg of protein. [b]As μg of D-glucose liberated/min.

Preparation of cellulose carbonate-bound dextranase (CC-dextranase). — Cellulose carbonate (5 g) was prepared, using the standard conditions described by Barker *et al.*[19], and stored *in vacuo* over silica gel at room temperature. A mixture of dextranase (4 mg), buffer (*f*) (1 ml), and cellulose carbonate (200 mg) was stirred

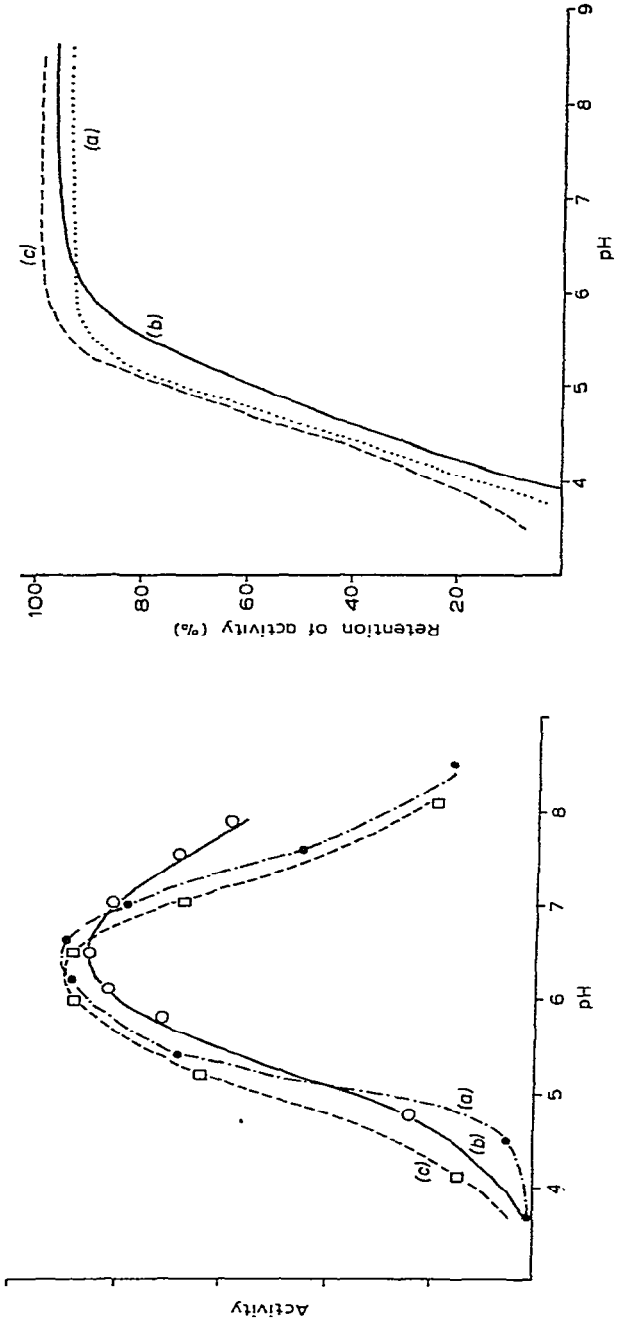

Fig. 1. pH Activity: (*a*) free dextranase; (*b*) CC-dextranase; (*c*) ICC-dextranase.

Fig. 2. pH Stability. See Experimental Section for details: (*a*) free dextranase; (*b*) ICC-dextranase; (*c*) CC-dextranase.

magnetically in a test tube for 24 h at 4° and then centrifuged, and a sample (10 μl) of the supernatant liquor was assayed before carrying out the washing procedure outlined above.

The original enzyme activity was determined by taking a 10-μl aliquot of the enzyme solution prior to the addition of the cellulose carbonate.

Other conditions for the insolubilization step were attempted, *e.g.*, different times, and different ratios of enzyme to cellulose carbonate carrier, but there was little increase in uptake of activity after ~6 h. The most active preparations were those having the higher ratios of enzyme to cellulose carbonate, the above quantities giving the preparation with maximum bound activity (104 μg of glucose/min/g of matrix). A pH of 7.8 for the coupling step gave a preparation having an activity similar to that of the product obtained at pH 7.0. It was considered that the stability[23] of the cyclic carbonate groups did not permit the use of a pH higher than 7.8.

In assessing the pH optimum (Fig. 1), pH stability (Fig. 2), and temperature stability (Fig. 3) of the CC-enzyme, great care had to be taken with control tubes,

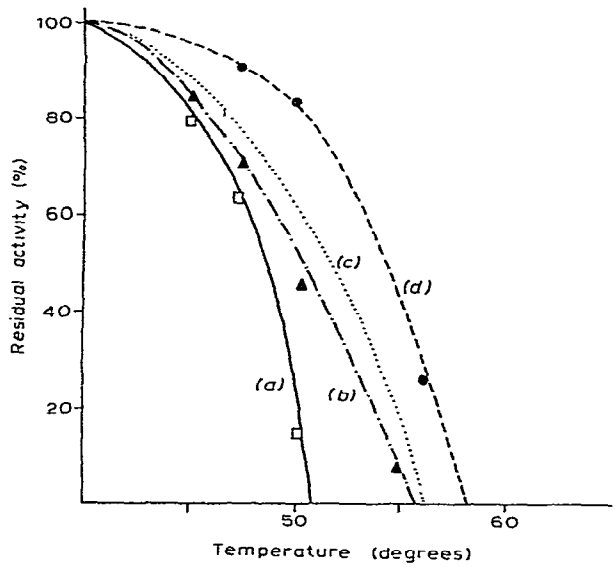

Fig. 3. Temperature stability at pH 6.2. See Experimental Section for details: (*a*) free dextranase; (*b*) CC-dextranase; (*c*) CMC-dextranase; (*d*) ICC-dextranase.

since, at the higher pH and temperature values, material which gave increased reducing-power readings was leached from the cellulose carbonate. Attempts, using buffers, to wash such materials from the cellulose carbonate preparation prior to enzyme coupling resulted in a marked lowering of the cyclic carbonate content, as determined[19] by the decreased intensity of infrared absorption at 1810 cm^{-1} relative to that at 1750 cm^{-1}.

The CC-enzyme was stable for several months when stored in buffer (*d*) at 4°

with little loss (~7%) of activity. After having been used six times for incubations of 1 h at 40°, the CC-enzyme retained ~53% of its original activity.

Preparation of dextranase bound to cyanogen bromide-activated cellulose (ICC-dextranase). — Activation of cellulose by cyanogen bromide to form the reactive cyclic imidocarbonate was carried out by the procedure of Axén and Ernbach[20]. Cellulose powder (200 mg) was treated with 2.5% aqueous cyanogen bromide (25 ml) at pH 11.0 for 6 min at 23°, the pH being kept constant by addition of sodium hydroxide from a Metrohm Herisau Combititrator. Coupling of the imidocarbonate cellulose (ICC) samples to the dextranase was carried out in buffer (*c*) as follows. Buffer (1 ml) was added, the flask was flushed with nitrogen and then stoppered, and the slurry was stirred magnetically for 16 h. Washing of the resulting ICC-enzyme was carried out as previously described. Stability and substrate studies were also carried out as described later, and the results are shown in Figs. 1–3, and in Table I. In one experiment, loss of enzyme activity from solution was determined after 2 h (23% loss), 4 h (24.5% loss), and 19 h (27.6% loss). Thus, most of the coupling reaction took place rapidly.

Other conditions of coupling and activation of the cellulose were investigated with a view to increasing the activity and temperature stability of the product. Cellulose samples (100 mg) were activated in cyanogen bromide solution at pH 11 and 11.5, and for periods of 5 and 10 min. The resulting samples of activated cellulose were coupled with enzyme at two concentrations to determine the effect on the temperature stability and the activity of the insolubilized preparation. The results are summarised in Table II.

TABLE II

ACTIVITIES OF ICC-DEXTRANASE PREPARED UNDER VARIOUS CONDITIONS

Sample	*Activation pH*	*Activation time (min)*	*Enzyme/ICC (mg/mg) × 10³*	*Units of enzyme activity/g of matrix*	*Loss of activity after heating (%)*
1	11.0	10	6.25	1249	61.3
2	11.0	5	6.25	2166	47.7
3	11.0	10	1.25	316	69.2
4	11.0	5	1.25	474	48.4
5	11.5	10	6.25	66.4	68.7
6	11.5	5	6.25	999	53.1
7	11.5	10	1.25	8.33	100
8	11.5	5	1.25	341	55.7

The ICC-enzyme was stable for several months at 4° in buffer (*a*), with only 5% loss of activity occurring in this time. It was used up to eight times in typical 1-h incubations at 40°, with less than 20% loss of activity. The products of action on dextran were the same as for the CC-enzyme and the free enzyme.

Preparation of carboxymethylcellulose azide-bound dextranase (CMC-dextranase). — Carboxymethylcellulose (20 g) was converted into the hydrazide by the

method of Crook *et al.*[21], and stored at 4°. The CMC-azide was prepared for immediate use as follows. Cold, 5% aqueous sodium nitrite (10 ml) was added during 30 min to a stirred suspension of CMC-hydrazide (2 g) in 0.6M hydrochloric acid (80 ml); the temperature was kept at 0°. The azide was washed at ~2° with water (2 × 100 ml), M sodium chloride (2 × 100 ml), and water (300 ml).

Coupling to Calbiochem dextranase. — CMC-azide (0.5 g) was added to a solution of dextranase (4 mg) in water (3 ml) immersed in an ice bath and stirred magnetically. The pH was rapidly adjusted to pH 8.7 with saturated, aqueous sodium tetraborate, and maintained thereat for 1 h by further additions of borate. Washing was carried out as before, and the CMC-enzyme was suspended in buffer (*a*) (2.5 ml). The most active preparation (Table I) had a much lower activity than that of the ICC-enzyme. Attempts to increase the activity by using other samples of freshly prepared CMC-azide, and by varying the pH and time of coupling, were unsuccessful. One explanation for the low activity could be the limitation imposed upon the number of azide groups/g by the content of carboxymethyl groups in the original material.

The temperature stability was the only factor studied in detail (Fig. 3). The action pattern on the dextran was the same as for the ICC-enzyme, the CC-enzyme, and the soluble enzyme.

Quantitative ninhydrin procedure. — Stock solutions were made up as follows for the ninhydrin determination of hydrolysed enzyme samples: *A*, methyl cellosolve (50 ml), water (50 ml), ninhydrin (0.6 g), and sodium acetate (3.78 g), adjusted to pH 5.5 with glacial acetic acid; *B*, 2mM hydrazine sulphate. Solution *A* (2 ml) was added to solution *B* (1 ml) just prior to use. Samples (1 ml) were added, and the solutions were heated for 15 min in a boiling water-bath, cooled to room temperature, and their absorbances read at 570 nm.

Determination of bound protein. — Samples of each insolubilized enzyme preparation and of the corresponding activated matrix were dried *in vacuo* for two days over phosphorus pentaoxide. Weighed quantities (20–30 mg) were heated under nitrogen with 6M hydrochloric acid (2 ml) for 48 h at 110°. Each hydrolysate was filtered through glass fibre (Whatman G.F. 81). The filtrates and washings were evaporated to dryness, and the residues were stored *in vacuo* over sodium hydroxide for 24 h and then reconstituted with water (0.5 ml). Hydrolysates of the free enzyme were treated similarly.

The hydrolysates were subjected to ascending paper chromatography on Whatman 3MM paper, using the organic phase of 1-butanol–acetic acid–water (4:1:5). Ninhydrin-positive material arising from protein was extracted from the paper into a mixture of ethanol–water (3:1), and determined quantitatively by the procedure described above.

The amount of enzyme bound in each sample was calculated by relating the absorbance at 570 nm to a standard curve obtained by hydrolysis and assay of known amounts of the free enzyme. The results are expressed in Table I.

The pH optima were determined in citrate–phosphate (McIlvaine) buffer as follows. Insoluble-enzyme slurry (0.1 ml), buffer (0.5 ml), and 1% aqueous dextran

(0.5 ml) were shaken for 4 h at 40°, and, after centrifugation, samples (1 ml) were withdrawn for assay of reducing power. The results are shown in Fig. 1. The pH stability studies were carried out in McIlvaine buffer (0.5 ml), in which enzyme slurry (0.1 ml) was shaken gently for 16 h at 4°. The tubes were centrifuged, and the buffer was decanted. The insolubilized enzyme was washed with buffer (*a*) (3 × 2 ml) and suspended in 0.5 ml of that buffer. Aqueous dextran (1%, 0.5 ml) was added, and the activity assayed as described above. The results are shown in Fig. 2.

Temperature stability studies. — Insolubilized-enzyme slurry (0.1 ml) was incubated with buffer (*a*) (0.5 ml) at various temperatures, with gentle shaking, for 1 h. The tubes were transferred to a water bath at 40°, aqueous dextran (1%, 0.5 ml) was added, and the activity was assayed as described previously. The results are shown in Fig. 3.

Effect of substrate on thermal stability. — Insolubilized-enzyme slurry (0.1 ml), buffer (*d*) (0.5 ml), and 1% aqueous dextran (0.5 ml) were shaken gently for 1 h at 55°. The slurries were then centrifuged, and the pellet was washed with buffers (*a*) (3 × 5 ml), (*c*) (1 × 5 ml), (*a*) (2 × 1 ml), and finally suspended in buffer (*a*) (0.5 ml). Aqueous dextran (1%, 0.5 ml) was added, and the enzyme activity was assayed. Control tubes, using 0.5 ml of water in place of the dextran solution, were used to give values for activity lost in the absence of substrate, during the heat treatment. The washing and assay procedures used were the same as those for the enzyme in the presence of substrate.

Action pattern of the insolubilized enzymes on dextran. — Enzyme slurries (0.4 ml), buffer (*a*) (0.5 ml), and aqueous dextran (1%, 0.5 ml) were stoppered and shaken at 40°. Samples were removed after 1.5, 6, and 23 h, and 3 days, deionized with Amberlite IR-120 (H^+) and IR-45 (HO^-) resins, and chromatographed on Whatman No. 1 paper with ethyl acetate–pyridine–water (10/4/3). Spots were visualized with the alkaline silver nitrate reagent[22]. In all cases, essentially the same oligosaccharide pattern was obtained as from the action of a sample of the free enzyme of comparable activity, *i.e.*, isomaltotriose (IM_3) and isomaltotetraose (IM_4) were the principal, final products, with some isomaltose (IM_2) and higher saccharides of the isomaltose series, together with small proportions of branched oligosaccharides having d.p. 3 and 4.

DISCUSSION

The most active, insoluble-enzyme preparations, both in terms of specific activity of the enzyme and enzyme activity per gram of matrix, were obtained from cyanogen bromide-activated cellulose (ICC-dextranase, Table I).

The number and type of covalent linkages formed between the enzyme and the matrix thus appear to affect the specific activity. In the case of cellulose carbonate, a urethane linkage is proposed between the carbonate group and the free amino groups of the enzyme, most probably the ε-amino of lysine[19,23]. Three types of linkage are proposed for the cyclic imidocarbonate, again involving only free amino groups of the

enzyme[20]: (*a*) an isourea derivative, (*b*) an *N*-substituted imidocarbonate, and (*c*) an *N*-substituted carbamate. In the case of the acid azide, the involvement of several different amino acid residues is proposed, *viz.*, lysine, tyrosine, cysteine, and serine[3,24,25]. The amount of protein bound per gram of matrix in each complex is quite low (*cf.* Table 3 in Ref. 3, and Tables 2–4 in Ref. 20). Thus, enzyme overcrowding, as a factor affecting the *relative* specific activities, is not likely to be significant. It is possible that the CMC-azide method, involving possible linkages between four different amino acid residues, produces an insolubilized derivative firmly bound in a conformation unfavourable for maximum activity. However, such an explanation would not apply in the case of cellulose carbonate, which has the lowest specific activity of all. Although no quantitative data are available for the cyclic carbonate content of the cellulose carbonate, a reactive derivative was undoubtedly used, as the amount of protein bound per gram of matrix is at least an order of magnitude greater than that estimated in Ref. 23, where an active preparation was obtained.

All of the insolubilized enzymes showed a significant increase in thermal stability (Fig. 3), the ICC-enzyme being the most stable, with 50% of its activity retained after storage for 1 hour at 55° and pH 6.2. The presence of the substrate (dextran) further increased the thermal stability. Thus, when ICC-dextranase was kept for 1 h at 55° and pH 7.8 in the presence and absence of substrate, the activity retained was 34 and 22%, respectively. The corresponding figures for CMC-dextranase were 38 and 17%. The study of this effect was facilitated for the insolubilized enzymes because of the ease of removal of substrate by simple washing after the heat treatment.

Because the preliminary experiments indicated that the cyanogen bromide-activated cellulose was the most effective agent for insolubilising the dextranase, further experiments were carried out with this derivative in attempts to optimise conditions (Table II). However, increases in both activation time and pH failed to increase the amount of insoluble enzyme. It is probable that there are more reactive groups present in the ICC prepared[20] at pH 11.5. It is also possible that, perhaps as a result of the foregoing, greater amounts of dextranase are coupled to ICC activated at pH 11.5, but that the enzyme molecules so coupled are more hindered sterically, and exhibit lower catalytic rates toward the large dextran substrate. Reduction of the ratio of total enzyme to cellulose derivative gave a less-active, insoluble-enzyme product, which also had a lower thermal stability.

The pH–activity profile of the free, ICC-, and CC-enzymes were very similar. The changes observed could be due partly to charged groups introduced into the cellulose carrier, and partly to changes caused by the chemical modification of the enzyme that occurs in the coupling reactions. Large shifts in pH would not be expected in the case of a dextranase, where both substrate and products are uncharged. This is in contrast, for example, to an esterase, where acid is likely to accumulate in the proximity of the insolubilized enzyme and affect its activity. This effect has been observed, *e.g.*, with agarose-bound chymotrypsin acting upon *N*-acetyl-L-tyrosine

ethyl ester[20], where the pH–activity profile is shifted to more alkaline pH-values (optimum 9.7) than the free enzyme (optimum 7.8). The pH-stabilities of the CC- and ICC-enzymes show little change from the free enzyme in the range studied.

The ease and economy of preparation of the ICC derivative, coupled with the higher activity and temperature stability of the ICC-enzyme conjugate, indicate that this is the best of the methods studied for attachment to a cellulose carrier.

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