Carbohydrate Research, 59 (1977) 553–561

THE USE OF TITANIUM(IV) OXIDE COATED WITH DIAZOTISED 1,3-DIAMINOBENZENE FOR THE IMMOBILISATION OF CARBOHYDRATE-DIRECTED ENZYMES

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(Received December 22nd, 1976; accepted for publication, March 28th, 1977)

ABSTRACT

The utility of porous titanium(IV) oxide particles as a matrix for the immobilisation of enzymes on column packings has been extended. On coating the particles with diazotised 1,3-diaminobenzene, their capacity for binding dextranase was increased two-fold. The stability of the enzyme–matrix bridge was enhanced by the covalent bond so formed. Excess diazonium groups were reacted with 2-naphthol. Investigations of the effects of change of dextran concentration, pH, temperature, and flow rate upon a continuously operated column of the immobilised dextranase permitted assessment of the kinetic aspects of the enzyme *via* Lineweaver–Burk plots. The change of reaction rate with temperature showed, according to Arrhenius plots, an abrupt change at 28°. Possible sources of the kinetic characteristics of the immobilised dextranase are discussed.

INTRODUCTION

Whereas hydrous forms of titanium and zirconium oxides are effective matrices for the immobilisation of polysaccharides and proteinaceous macromolecules with retention of biological activity[1–5], the column-packing properties of the titanium matrix can be improved considerably by use of a porous titanium(IV) oxide in particulate or bead form[6]. However, the latter matrix is fired during its production, and therefore contains a very low degree of chelating ability compared with that of hydrous titanium(IV) oxide. Consequently, the attachment of enzyme occurs to a lower degree and is reversible. Whereas reversibility may sometimes be advantageous, we have sought to retain the excellent column-packing characteristics of porous titanium(IV) oxide and protective nature of its pores concomitant with irreversible enzyme attachment. Coating with diazotised 1,3-diaminobenzene[7] was considered suitable for this purpose and we now report thereon.

EXPERIMENTAL AND RESULTS

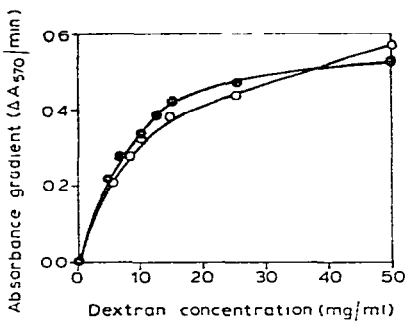
Determination of reducing groups, protein, and dextranase activity. — Reducing groups were determined by an automated process[6,8] based on reaction with 3,5-dinitrosalicylic acid, enzyme protein by an automated ninhydrin technique[9] after hydrolysis[6], and dextranase activity manually[6] by using a modified reaction with 3,5-dinitrosalicylic acid[10] of the products liberated from dextran.

The preparation of an insoluble dextranase derivative of porous titanium(IV) oxide coated with diazotised 1,3-diaminobenzene. — Porous titanium(IV) oxide (type M27, 0.3-mm particle diameter, large pore size) (kindly supplied by Dr. A. R. Thompson, AERE, Harwell) was washed copiously with water and oven-dried, and a portion (2 g) was added to a solution of 1,3-diaminobenzene (25 mg/ml) in M hydrochloric acid (40 ml) maintained at 0°. 6% Aqueous sodium nitrite (30 ml) at 0° was added very slowly with continuous shaking; the temperature was kept below 4° and freezing was avoided. The mixture was then shaken at 0° for 30 min and decanted, and the solid was washed rapidly with 0.2M sodium acetate buffer (pH 5.0, 3 × 100 ml) at 0°. A solution of dextranase (1,6-α-D-glucan 6-glucanohydrolase, EC 3.2.1.11, Koch-Light Ltd., 5 mg/ml) in the same buffer (20 ml) at 4° was added and the mixture was shaken at 4° for 2 h. The solid was then shaken with a saturated solution of 2-naphthol in saturated, aqueous sodium acetate (200 ml) at 4° for 4.5 h; after decantation, the solid was washed with 0.2M sodium acetate buffer (pH 5.0, 5 × 100 ml) at 4°. The immobilised enzyme derivative was stored at 4° in the same buffer. The material had 17 units/g of dextranase activity, 4.08 mg/g of bound protein, and values for the specific activity and retention of activity of bound enzyme of 4.16 units/mg (original, 35.6 units/mg) and 11.7%, respectively.

Determination of the Michaelis constant for immobilised dextranase. — The foregoing, insoluble dextranase derivative was assayed for dextranase activity as described above but using various concentrations (0–10%) of dextran. The shaking rate was adjusted to the minimum necessary for adequate agitation of the granules. The experiment was repeated firstly with dextrans of different molecular weights [17.0×10^4 (Sigma), 50.0×10^4, 15.8×10^4, 7.0×10^4, and 4.4×10^4 (Pharmacia)] and secondly for soluble dextranase.

The Michaelis constants were determined from Lineweaver–Burk plots in which the reciprocal of the initial absorbance gradient was plotted against the reciprocal of the dextran concentration (Figs. 1 and 2, Table I).

Use of a column of immobilised dextranase. — The immobilised dextranase was packed under gravity in a jacketed column (9.0 × 0.4 cm) and eluted for 24 h with 0.1M sodium acetate buffer (pH 5.0) at 0.53 ml/min. Solutions of dextran (mol. wt., 17.0×10^4) were passed down the column continuously as described below in (*a*)–(*d*), and the eluted reducing-power was monitored and expressed as the D-glucose equivalent. These conditions were used between experiments to monitor loss of dextranase activity.



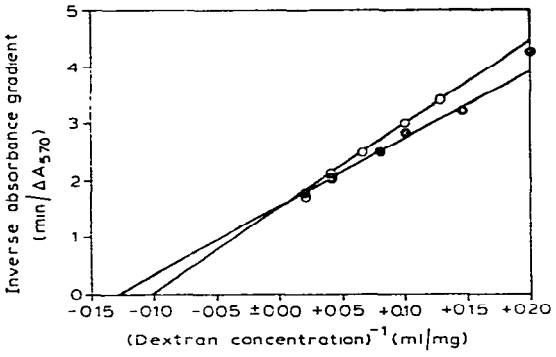


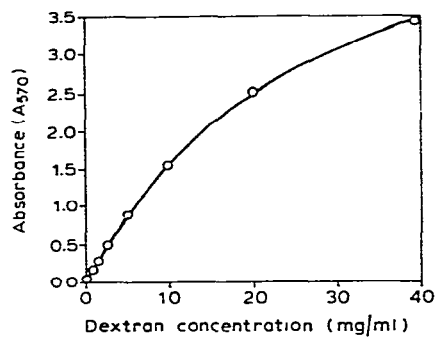
Fig. 1 (left). Typical curves for the dependence of activity of free dextranase (—●—) and immobilised dextranase (—○—) upon concentration of dextran substrate (mol. wt., 4.4×10^4); —●— is × 10.

Fig. 2 (right). Lineweaver–Burk plots for dextranases described in the legend to Fig. 1; —●— is ×0.1.

TABLE I

MICHAELIS CONSTANTS FOR DEXTRANASE FREE AND IMMOBILISED ON POROUS TITANIUM(IV) OXIDE COATED WITH DIAZOTISED 1,3-DIAMINOBENZENE

Mol. wt. of dextran	K_m *for soluble enzyme* (%)	K_m^{app} *for immobilised enzyme in stirred suspension* (%)	K_m^{app} *in column* (%)
50.0×10^4	0.92	1.10	1.08
17.0×10^4	1.32	3.01	3.57
15.8×10^4	0.70	0.78	0.80
7.0×10^4	0.73	1.07	0.99
4.4×10^4	0.81	0.91	0.85



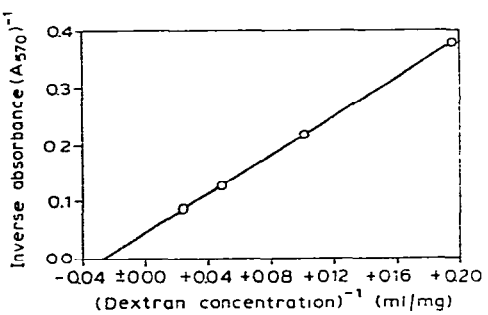


Fig. 3 (left). Relationship between substrate and product concentrations for a packed column of immobilised dextranase.

Fig. 4 (right). Lineweaver–Burk plot for dextranase described in the legend to Fig. 3.

(*a*) *Variation of dextran concentration.* Dextran solutions of various molecular weights (15.8×10^4, 7.0×10^4, and 4.4×10^4) and concentrations (0.025–4.0% in 0.1M sodium acetate buffer, pH 5.0) were passed continuously down the column at 0.53 ml/min for a minimum of 30 min to ensure stabilisation of the recorder trace. The concentration of D-glucose equivalents in the eluate was plotted against substrate concentrations (Fig. 3) for the different substrates, and the Michaelis constants were found by Lineweaver–Burk plots (Fig. 4, Table I).

(*b*) *Variation of flow rate.* A 0.25% solution of dextran (mol. wt., 17.0×10^4) in 0.1M sodium acetate buffer (pH 5.0) was passed down the column at 25° at various flow-rates (0.36–2.7 ml/min). The analysis rate was kept constant at 0.204 ml/min. The concentration of D-glucose equivalents in the eluate was plotted against the flow rate (Fig. 5).

(*c*) *Variation of pH.* Solutions (0.25 and 1.0%) of dextran in 0.1M sodium acetate buffer of various pH values (3.5–5.0) were passed down the column at 25° at 0.53 ml/min. The column was equilibrated with the appropriate buffer between runs, and the dextran solution was not applied until the pH of the eluate was the same as that of the buffer entering the column. Expression of the results as a percentage of the product concentration at pH 5.0, and comparison with the values obtained for soluble dextranase show that in each case the activity value was 100% (within experimental limits; range, 98–103%).

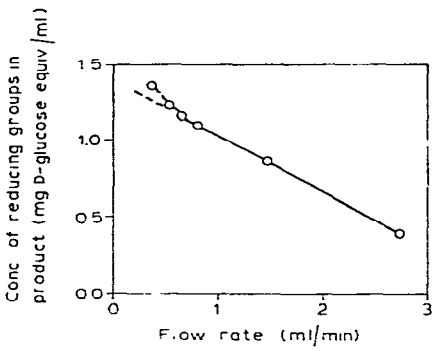


Fig. 5 (left). Dependence upon column flow-rate of the generation of reducing groups from a dextran substrate by a packed column of immobilised dextranase.

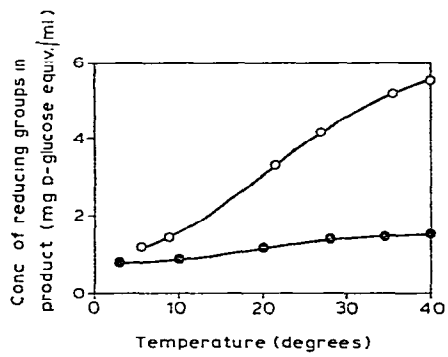


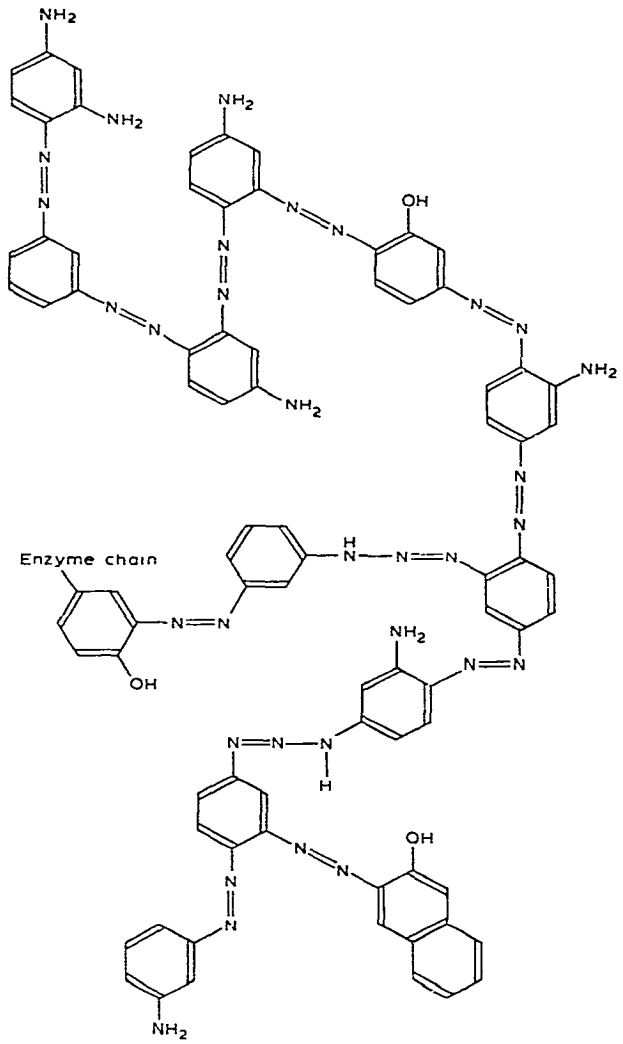
Fig. 6 (right). Dependence upon temperature of the generation of reducing groups from a dextran substrate by a packed column of immobilised dextranase.

(*d*) *Variation of temperature.* Solutions (0.25 and 1.0%) of dextran in 0.1M sodium acetate buffer (pH 5.0) were passed down the column at 0.53 ml/min at various temperatures (3–35°). The concentration of D-glucose equivalents in the eluate was plotted against temperature (Fig. 6).

DISCUSSION

The support for enzyme immobilisation is first coated with diazotised 1,3-diaminobenzene and subsequently coupled conventionally with the enzyme *via*, for example, a tyrosyl residue.

Polymerisation of the diazotised 1,3-diaminobenzene molecules occurs prior to coupling, but some amino groups remain unchanged. Subsequent reaction with 2-naphthol was employed to remove any unreacted diazonium groups. That this matrix (Scheme A) was effective for enzyme immobilisation was evident from the



Scheme A Schematic representation of enzyme coupled to support coating

activity and stability of the immobilised dextranase. Comparison of the method with that of immobilisation of dextranase directly on porous titanium(IV) oxide[6] showed that the use of the 1,3-diaminobenzene coating increased the amount of activity bound more than two-fold. The percentage retention of specific activity was also improved to a certain extent.

The Michaelis constants determined for the free enzyme and for stirred suspensions of the insolubilised enzyme with substrates having various molecular weights (Figs. 1 and 2, Table I) showed little change in the K_m with molecular weight for either form of the enzyme. The high values obtained with the dextran of molecular weight 17.0×10^4 are due to the fact that this dextran was branched whereas the others were unbranched.

The higher K_m^{app} values for the immobilised enzyme may be a result of a number of effects. The value of K_m^{app} at low ionic strengths decreases by more than one order of magnitude when substrates of opposite charge to the carrier matrix were used[11]. The electrostatic potential was calculated by insertion of the Maxwell–Boltzmann distribution into the Michaelis–Menten equation using the changes in Michaelis constant, and good agreement was obtained with the value for the electrostatic potential calculated from the pH-activity shifts. The diffusion of substrate from the solution to the microenvironment of an immobilised enzyme can also be a major factor in the rate of the enzyme reaction. A diffusion film which covers the surface of an insoluble particle has been postulated[12], and within which the substrate concentration is lower than in the solution. The rate at which substrate passes over the insoluble particle affects the thickness of the diffusion film, which in turn determines the concentration of substrate in the vicinity of the enzyme and hence the rate of reaction. For an enzyme immobilised on Sepharose[13], the values of K_m^{app} were reversibly ~10-fold higher than that obtained for the native enzyme. A further reason for diffusion may be the association of dextran with the coating of diazotised 1,3-diaminobenzene. Dextrans are adsorbed by Celite that has been coated with diazotised 1,3-diaminobenzene[14]. Linear dextrans, however, were adsorbed to a lesser degree (1.2 g/g of matrix) than branched dextrans (7.9 g/g of matrix).

The effect of the molecular weight of the substrate upon activity can be very pronounced. Diffusion of large molecules will be limited by steric interactions with the matrix, and this effect is reflected in the fact that the relative activity of bound enzyme towards substrates of high molecular weight is generally lower than towards those of low molecular weight. However, this property may sometimes be an advantage, as the immobilised enzyme may be protected from attack by large inhibitor molecules. The increase of K_m on immobilisation was slightly greater for substrates of higher molecular weight, indicating that limitations of reaction rate due to diffusion may also be playing a role.

The immobilised dextranase was also examined, when packed in the form of a column, in relation to stability, reuseability, and flow characteristics. Lineweaver–Burk[15] plots (Fig. 4) of reciprocal substrate concentration versus reciprocal product concentration were used to find the K_m^{app} values (Table I). The small residence times

used (~3 min) ensured that initial rates were being measured. The K_m values for the column vary with flow rate, as this is the main factor affecting the thickness of the diffusion layer around the particles[15], and this is the reason for referring to them as "apparent" Michaelis constants. The K_m^{app} values obtained for the column of immobilised dextranase agree well with those for the stirred reactor situation. The latter was subject to greater error due to the difficulty of achieving adequate mixing without causing fragmentation of the granules, a problem which did not exist in the column because a constant flow-rate was used.

A form of the integrated rate equation[14]

$$P.S_0 = K_m^{app}.\ln(1-P)+c/Q,$$

where P is the fraction of substrate reacted within the column, S_0 is the initial concentration of substrate, c is the reaction capacity of the column, and Q is the rate of flow through the column, was also used to assess the column characteristic of the immobilised enzyme. If values of P are measured when various initial concentrations of substrate are passed through the same column at identical flow-rates (*i.e.*, Q is constant), then a plot of $P.S_0$ versus $\ln(1-P)$ should give a straight line if K_m^{app} and c are constant at this flow rate, and the slope of the line will equal K_m^{app}. The fact that the expected straight line was not obtained (Fig. 7) is probably due to deviation from the plug-flow conditions which the integrated rate equation requires. It is noteworthy that no activity was lost from the column during these tests (Table II).

TABLE II

RETENTION UNDER VARIOUS CONDITIONS OF ACTIVITY OF A COLUMN OF DEXTRANASE IMMOBILISED ON POROUS TITANIUM(IV) OXIDE COATED WITH DIAZOTISED 1,3-DIAMINOBENZENE

Progressive treatment	*Activity remaining after treatment*[a] (%)
0.25% dextran solution for 66 h at pH 5.0, 25°, 0.53 ml/min	88.5
Various concentrations of dextran (0.025–4%) for a total of 4 h at pH 5.0, 25°, 0.53 ml/min	88.5
0.25% dextran at various flow-rates (0.36–2.70 ml/min) for a total of 3 h at pH 5.0, 25°	88.5
0.25% dextran at various pH values (5–3.5), repeated with 1% dextran, for a total of 8 h at 25°, 0.53 ml/min	82.8
0.25% dextran at various temperatures (3–40°), repeated with 1% dextran, for a total of 12 h at pH 5.0, 0.53 ml/min	82.8

[a]Calculated from original activity measured at pH 5.0, 25°, 0.53 ml/min with 0.25% dextran.

With increase of flow rate of substrate through the column of immobilised dextranase, the percentage conversion decreased due to the inverse proportionality of residence time with flow rate (Fig. 5). The rate of production of D-glucose equivalents increased with increase in flow rate (Fig. 8), because more substrate passed over the

enzyme in a given time. At flow rates less than ~ 1.8 ml/min, this effect was predominant, but, at higher flow-rates, the residence time was so low that the percentage conversion decreased rapidly enough for the overall effect to be one of decreasing rate of reaction with increasing flow-rate (Fig. 8).

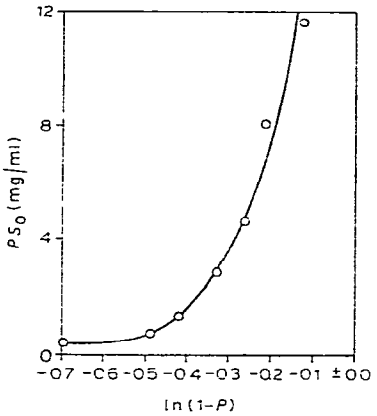


Fig. 7 (left). Variation of $P.S_0$ with $\ln(1-P)$ for continuous operation of a column of immobilised dextranase (dextran substrate mol. wt., 17.0×10^4) (see Text).

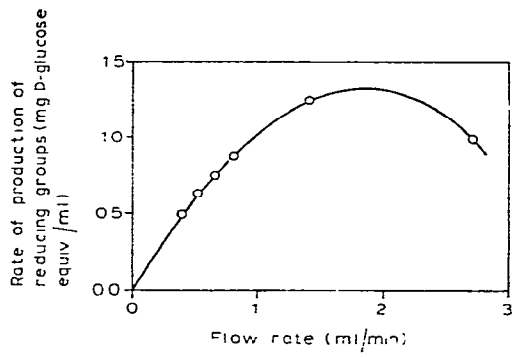


Fig. 8 (right). Effect of flow rate upon rate of generation of reducing groups from dextran by a column of immobilised dextranase (see text).

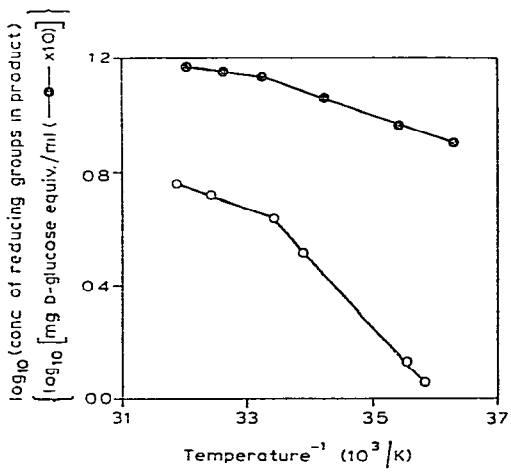


Fig. 9. Arrhenius plots for a column of immobilised dextranase (1% dextran substrate, —○—; 0.25% dextran substrate, —●—) (see text).

That no change in reaction rate was observed over the pH range 5.0–3.5 is in contrast to the pH profile of free dextranase (pH optimum, 5.0–5.5), in which the reactivity falls off rapidly with decreasing pH over the same range. This effect may be due to the fact that the microenvironment in the vicinity of the enzyme remains at pH ~5.0 irrespective of the pH in the solution. Such effects have been observed frequently with polyelectrolytes. An almost identical pH-activity profile was obtained with a dextranase derivative of diazotised 1,3-diaminobenzene-coated cellulose[7]. It is suggested that increase of the acidity of the phenolic hydroxyl group on the tyrosine residue on reaction with the diazo group on the matrix changed the overall charge of the protein, causing a shift in pH optimum to the alkaline side. The variation of pH caused a loss of activity in the column of 6% of the former activity (Table II).

The reaction rate increased with temperature (Fig. 6), levelling off somewhat above ~25°. This increase was more pronounced at a higher concentration of substrate, there being a greater excess of substrate available for reaction. The change of reaction rate can be seen more clearly on Arrhenius plots (Fig. 9), which are both straight lines, the gradients of which change abruptly at 28°. The cause of this phenomenon may be due to such factors as reversible configurational changes of the enzyme arising from temperature changes, or changes in the degree of ionisation of residue near the active site. However, it is evident that it is not due to thermal deactivation, as no activity was lost during these tests (Table II).

ACKNOWLEDGMENTS

The authors thank Professor S. A. Barker for his advice and interest, and the S.R.C. for a research scholarship (to I.M.K.).

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