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THE USE OF TITANIUM(IV) OXIDE FOR THE IMMOBILISATION OF CARBOHYDRATE-DIRECTED ENZYMES

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ABSTRACT

The use of particles of porous titanium(IV) oxide as a suitable matrix for enzyme immobilisation has been investigated with dextranase. Treatment of the particles with enzyme in the presence and absence of ammonium ions showed that the presence of ammonia induced a greater coupling of protein, whereas a greater retention of enzyme specific activity was achieved in the absence of ammonia. Properties of the immobilised enzyme include a pH-dependence and reversibility of the coupling between enzyme and matrix. The immobilised dextranase was most stable at pH 5.0. Automated analytical techniques for measuring the activity of dextranase and other polysaccharidases in soluble and insoluble forms are also reported.

INTRODUCTION

Most of the methods used[1–4] to derivatise enzymes to give active immobilised-forms are laborious and cumbersome, and the matrix in activated form may be unstable. We have attempted to circumvent such problems by using a chelation technique in which a matrix can be quickly activated for enzyme attachment by treatment with simple titanium compounds[5–8]. Enzymes could be attached to such derivatives as ligands by replacing water or chloride ligands on the titanium species chelated to the support. More recently, we have combined the matrix and the chelate bridge by devising methods of preparing hydrous oxides of titanium which, by an analogous ligand-replacement system, are effective matrices for enzyme immobilisation[9], and other immobilisation phenomena[10–12]. This method of immobilisation has also been extended to a hydrous oxide of zirconium[10–13], and these supports have the advantages that they can be simply and quickly produced *in situ*.

We now report an extension of this work, which involves porous titanium(IV) oxide (titanium dioxide).

EXPERIMENTAL AND RESULTS

Measurement of dextranase activity. — (*a*) *Manual.* Dextranase activity was measured in terms of the rate of release of reducing groups from dextran, by the

method of Bernfeld[14] modified as follows. The assay reagent consisted of 3,5-dinitrosalicylic acid (1 mg/ml) and sodium potassium tartrate (300 mg/ml), dissolved in 0.5M sodium hydroxide. Insoluble enzyme-derivative (25 mg, damp) or an aqueous solution of the soluble enzyme (1 mg/ml, 25 μl) was incubated with a 0.5% solution of dextran (Sigma Chemical Co.; mol. wt., 170,000) in 20mM sodium phosphate buffer (pH 5.0, 1 ml) at 37° for 30 min. The reaction was terminated by rapid cooling, and removal of the solid by centrifugation. An aliquot (500 μl) of the supernatant was immediately transferred to the assay reagent (2.5 ml), which was then heated in a boiling water-bath for 10 min, and cooled to room temperature. The absorbance at 570 nm was determined, and standard solutions of D-glucose were similarly treated to provide a linear calibration graph. The unit of dextranase activity was defined as that which leads to the release of reducing groups equivalent to one micromole of D-glucose under the conditions of the assay.

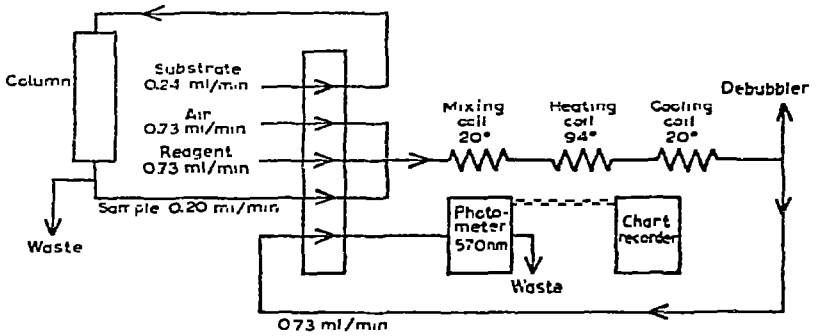

Fig. 1. Pumping system for automated 3,5-dinitrosalicylic acid assay.

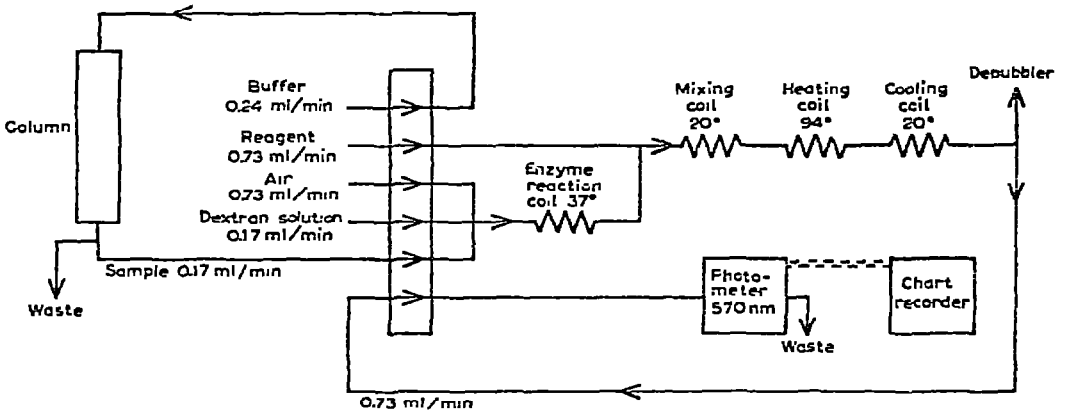

Fig. 2. Pumping system for automated dextranase assay.

(*b*) *Automated.* An automated assay for reducing groups[15] was modified as shown in Fig. 1, with a Carlo Erba peristaltic proportionating pump, a Fisons Vitatron Colorimeter, and a Leeds and Northrup recorder. The assay reagent consisted of 3,5-dinitrosalicylic acid (1 mg/ml) and sodium potassium tartrate (0.75 mg/ml) dissolved in 0.5M sodium hydroxide. For the automated determination of elution of dextranase activity, the system was modified to that shown in Fig. 2 with a 0.5% dextran solution. The system was tested by the passage of standard solutions of dextranase (0–0.01 mg/ml) in 0.02M sodium phosphate buffer (pH 5.0), and it was confirmed that the pH of the eluate did not affect the assay by use of a solution of dextranase (0.01 mg/ml) in 0.02M sodium phosphate buffer (pH 7.3).

Determination of protein contents of enzyme samples. — Rigorously dried, insoluble-enzyme derivatives (~50 mg) were heated in sealed Pyrex ampoules with 6M hydrochloric acid (AnalaR, 2 ml) and water (0.2 ml) at 110° for 18 h. Aqueous solutions of the original, soluble enzymes (25 mg/ml, 0.2 ml), untreated matrix blanks, and water blanks were hydrolysed in the same way. The amino acid contents of the hydrolysates were determined by ninhydrin assay.

The acid hydrolysates were transferred to volumetric flasks (5 ml), and the ampoules were washed with 8M sodium hydroxide (1.5 ml). The washings were added to the hydrolysates, the pH was adjusted to 5–8 by the addition of 0.8M sodium hydroxide and/or 0.6M hydrochloric acid, and the volume was adjusted to 5 ml with water. The solutions were diluted (1–10 times) with water, and the amino acid contents determined by an automated assay[16] with ninhydrin.

Preparation of an insoluble dextranase derivative of porous titanium(IV) oxide. — Porous titanium(IV) oxide spheres (kindly supplied by Dr. A. R. Thompson, AERE, Harwell; type M27, 0.3-mm particle diameter, large pore-size, 50 mg), which had been washed with an excess of water to remove the fines, and oven-dried at 50°, were added to a solution of dextranase (1,6-α-D-glucan 6-glucanohydrolase, EC 3.2.1.11, Koch-Light Labs. Ltd., 35.6 units/mg, 4 mg/ml) in 0.02M sodium phosphate buffers (pH 4.5–8.0, 1 ml), and shaken at a minimum rate for 18 h at 4°. Other conditions investigated were (*a*) dextranase concentration of 4 mg/ml, coupling at pH 5.0, shaking for 0.5–18 h; and (*b*) dextranase concentration of 0.5–8.0 mg/ml, coupling

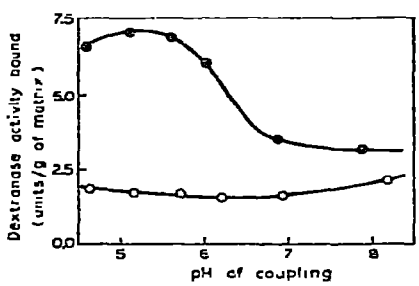

Fig. 3. Effect of variation of pH on the coupling, in the presence (—○—) and absence (—●—) of ammonia, of dextranase activity to porous titanium(IV) oxide.

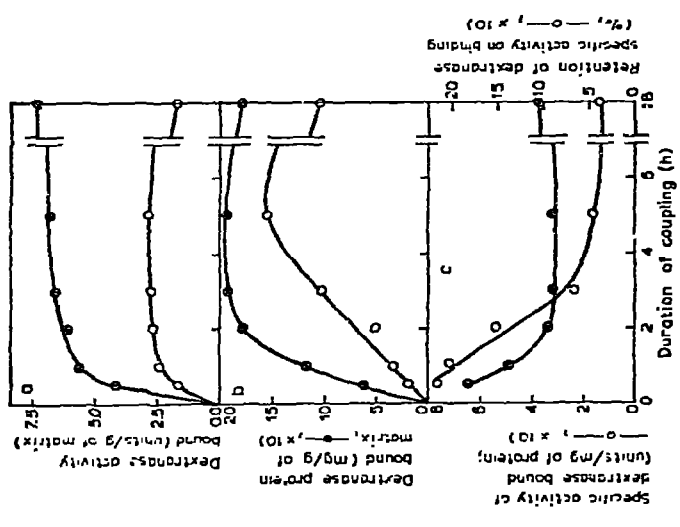

Fig. 4. Effect of variation of reaction time on the coupling, in the presence (—○—) and absence (—●—) of ammonia, of dextranase to porous titanium(IV) oxide: (*a*) activity bound, (*b*) protein bound, (*c*) specific activity of bound enzyme.

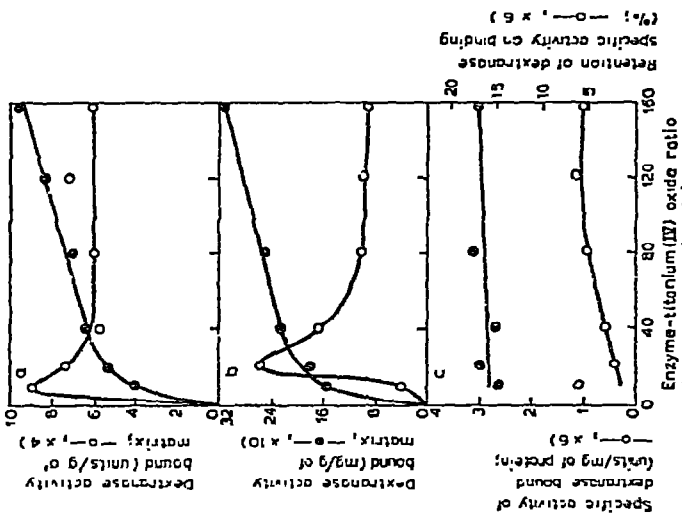

Fig. 5. Effect of variation of enzyme–matrix mass ratio on the coupling, in the presence (—○—) and absence (—●—) of ammonia, to porous titanium(IV) oxide: (*a*) activity bound, (*b*) protein bound, (*c*) specific activity of bound enzyme.

at pH 5.0, shaking for 18 h. The spheres were washed by shaking with 0.02M phosphate buffer (pH 5.0, 12 × 1 ml, 5 min each) and assayed manually for dextranase activity and protein content. Samples containing no enzyme were treated similarly to provide a blank in the enzyme assay, or to check, *via* oven-drying and weighing, that no titanium(IV) oxide had been lost by attrition. The results are shown in Figs. 3–5.

The above couplings of enzyme to porous titanium(IV) oxide were repeated, but replacing the buffer with 0.9M ammonium hydroxide which had been added slowly to 6M hydrochloric acid to give the desired pH values (4.5–8.0). Such solutions (0.9 ml) were then mixed with aqueous solutions (0.1 ml) of dextranase (5–80 mg/ml). The results are shown in Figs. 3–5.

Effect of variation of reaction conditions on the performance of a column of dextranase immobilised on porous titanium(IV) oxide. — Dextranase was immobilised on porous titanium(IV) oxide (4 × 1 g) in the absence of ammonia as described above, but on 20 times the scale with a pH of 5.6, a coupling time of 18 h, and a dextranase concentration of 4 mg/ml. The washed batches were combined, and packed under gravity in a column (8.5 × 0.4 cm). The column was eluted with 0.02M sodium phosphate buffer (pH 5.0) at 20°, at a flow rate of 0.2 ml/min; after 24 h, a 0.5% solution of dextran (Sigma Chemical Co.; mol. wt., 170,000) in the same buffer was passed continuously through the column. The eluate was analysed automatically for reducing groups. A constant value was obtained, and this remained unchanged when the dextran concentration was increased continuously and linearly (0.5 → 20%) and then brought back to 0.5%. Similarly, the level of eluted reducing-activity remained unchanged when the dextran concentration was kept at 0.5% and the molarity of the sodium phosphate buffer (pH 5.0) was increased (0.02 → 0.2M) continuously and linearly, and then returned to 0.02M.

The dextran concentration was kept at 0.5% in 0.02M sodium phosphate buffer, and the pH was increased continuously and linearly (5.0 → 7.3), at a low rate to permit close observation of the behaviour of the immobilised enzyme. The pH in the column

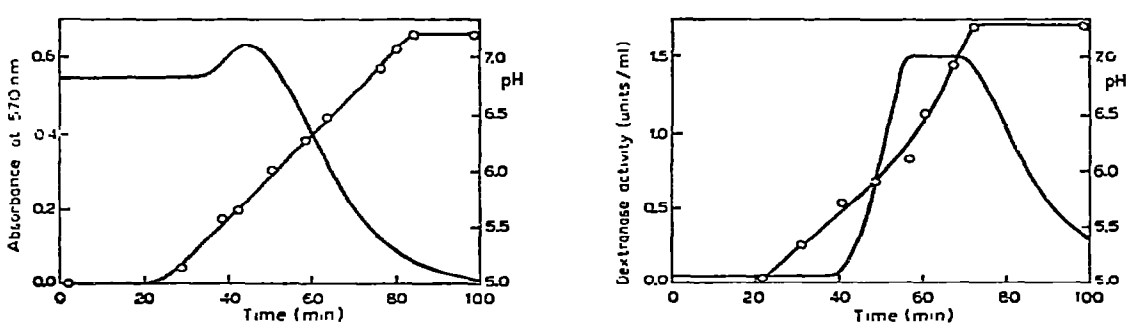

Fig. 6. Activity of dextranase (———) immobilised on porous titanium(IV) oxide as a function of environmental pH (—○—).

Fig. 7. Release of dextranase activity, (———) from dextranase immobilised on porous titanium(IV) oxide, as a function of environmental pH (—○—).

was monitored in terms of the pH in the mixing vessel for the column input and, from the residence time in the column and the delay time of the analytical system, the level of reducing sugar could be equated with the pH value (Fig. 6). On returning the pH to 5.0, the level of reducing sugar in the eluate was 73% of the original. The column of immobilised dextranase was eluted with 0.02M sodium phosphate buffer, the pH being increased continuously as described above. The enzyme activity eluted was determined (Fig. 7) by using the automated dextranase assay and the calibration graph obtained for standard solutions of dextranase.

DISCUSSION

A form of titanic oxide having excellent packing properties has been developed by AERE, Harwell; this material has a uniform particle size and pore dimension. Specialist treatment during its production renders it porous. Derivatisations were attempted in the presence of ammonia, which affects[9] the chelating ability of hydrous titanium(IV) oxide. These reactions therefore simulated those with hydrous titanium-(IV) oxide (equivalent to method 5 in Ref. 9). Derivatisations were also attempted in the absence of ammonia.

It was found that dextranase could be readily immobilised on the porous titanium(IV) oxide at pH 5, and products with bound activities of ~7.5 units/g were obtained. Optimisation of the coupling with respect to reaction pH, time, and enzyme-to-titanium(IV) oxide ratio showed the following effects. In the presence of ammonia, pH had little effect (Fig. 3). However, in the absence of ammonia, higher activity was obtained for coupling at pH values lower than 6, there being little variation in the range pH 4.5–6.0. Although the coupling reaction was largely complete within 2 h (Fig. 4), activity continued to be coupled in the absence of ammonia, whereas, in the presence of ammonia, there was a drop in the activity on prolonged stirring of the mixture. The fall of specific activity with prolonged reaction time reflects steric effects as the density of the coupled molecules is increased. Increases in the amount of enzyme offered to the substrate (Fig. 5) showed, for the absence of ammonia, gradual increases in the amounts of activity and protein bound and a nearly constant retention of specific activity. Different phenomena clearly operate in the presence of ammonia, where the overall degree of coupling is much lower, resulting in the initial coupling of a small amount of enzyme with high retention of activity (little crowding), followed by further coupling whereupon the specific activity remains relatively constant.

Thus, porous titanium(IV) oxide is a suitable matrix for enzyme immobilisation. Maximum amounts of dextranase attached, in the presence and absence of ammonia, were as follows. activity bound, 2.2 and 9.6 units/g of matrix; protein bound, 25.1 and 3.2 mg/g of matrix: specific activity of bound enzyme, 0.6 and 8.5 units/mg (original 35.6 units/mg), respectively. Whereas the presence of ammonia induced a greater coupling of protein, it was clear that greater activities could be bound in the absence of ammonia.

Immobilised dextranase (prepared in absence of ammonia) was effective when

used in column form, and acceptable flow-rates could be achieved. No loss of activity was detected even after elution with a 20% solution of dextran or with 0.2M buffer. As the pH of the medium was raised, the activity passed through a maximum (Fig. 6) corresponding to the pH optimum of the enzyme (pH ~5.5). Decrease in activity on further increase of the pH could reflect the activity–pH profile for the enzyme (activity at pH 6.0 and 7.0 is 50 and 12%, respectively, of that at pH 5.5) or be caused by loss of enzyme from the matrix. After reaching pH 7.3, the activity level continued to fall without further increase in pH; on returning the pH to 5.0, 27% of the original activity had been lost, but the pH value at which the activity loss had started could not be deduced from this experiment alone.

In order to separate the effects of pH on enzymic activity and on the elution of enzyme from the column an analysis system was used which detected dextranase coming off the column by automatic assay of its reaction with dextran (Fig. 2). At pH ~6, enzymic activity began to be eluted, but at higher pH values the release was more rapid until virtually all the enzyme had been eluted. It is therefore concluded that the immobilised enzyme is stable at lower pH values, but that the enzyme is eluted at higher pH values. This phenomenon may have application in the area of reversible enzyme-immobilisation; few matrices display this phenomenon, although it is one of practical use where changing the exhausted enzyme may be more convenient than changing the packing (see Ref. 17).

Comparison with data for immobilisation of carbohydrate-directed enzymes upon hydrous titanium(IV) oxide shows that lower activities are retained by the porous titanium(IV) oxide. However, the stability of the activity retained, which is at an acceptable level, and the fact that the material can be packed into a column for continuous use show that the oxide holds potential for enzyme immobilisation. Production of the matrix with various degrees of controlled porosity could therefore offer the basis for an immobilised-enzyme reactor separator. The matrix should be equally suitable for immobilisation of other molecules such as immunogens, immunoglobulins, and affinants.

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