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IMMOBILIZATION OF ENZYMES ON COLUMNS OF BRUSHITE

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SUMMARY

The use of brushite ($CaHPO_4 \cdot 2H_2O$) for enzyme immobilization is described and exemplified with β-galactosidase. Immobilization was carried out at 5°C in a 0.01 *M* Tris buffer, pH 7.6, containing 0.01 *M* phosphate. The immobilized enzyme showed high activity over a long period of time (after 90 days the activity was still 44% of the initial value). The leakage of enzyme from these matrices is very low, amounting to no more than 4% upon washing a column fully saturated with enzyme with 300 column volumes of buffer. A method is described in which the enzyme leakage is virtually negligible. One advantage of brushite as a matrix for enzyme immobilization is that the immobilization can be done in almost any buffer, even at high salt concentration, provided that high concentrations of phosphate are avoided. The immobilized enzymes can be desorbed easily by increasing the phosphate concentration. Since the enzyme is adsorbed at the top of the column, no preconcentration of the enzyme solution is required prior to immobilization.

INTRODUCTION

Artificially immobilized enzymes have been prepared by a variety of methods, including adsorption on ion-exchange resins[1], on charged[2] and neutral[3,4] amphiphilic gels, covalent binding to water-insoluble carriers[5,6] and covalent cross-linking of the proteins by bifunctional reagents[7]. The choice of carriers for immobilized enzymes involves consideration of such factors as ease of immobilization, ease of desorption, porosity of carrier, extent of leakage, capacity to withstand high or low ionic strength, mechanical and bacterial resistance and cost.

In this report, we describe the use of brushite ($CaHPO_4 \cdot 2H_2O$) for enzyme immobilization. We chose β-galactosidase as a model enzyme, partly because a coloured product is obtained with 2-nitrophenyl β-D-galactopyranoside as substrate, thereby allowing the movement of the product on the column to be followed visually. A great advantage of brushite as a matrix is that in most cases only phosphate ions will affect the adsorption of proteins[8,9]. Most enzymes can thus be immobilized in the

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presence of any concentrations of other salts, for instance potassium chloride[10]. (Immobilization at high salt concentrations is not feasible with ion-exchangers.) Therefore, when brushite is used as the enzyme carrier one can select the buffer that is most suitable for the enzyme, provided only that high concentrations of phosphate are avoided. When immobilization is based on adsorption, the enzyme is retained in the top section of the column, thereby eliminating the need for concentration of dilute enzyme solutions prior to immobilization. Since the adsorption is reversible the enzymes can be released from the column. An advantage with this is that the same column can be used for different enzymes and that the enzyme can be converted back to a free form, for instance, to attempt renaturation.

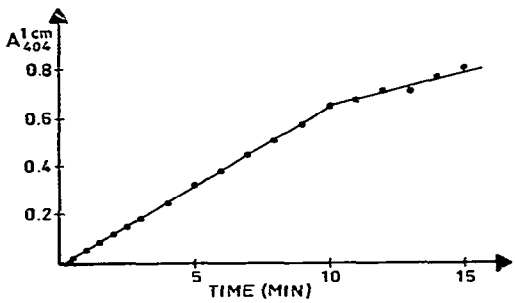
MATERIALS AND METHODS

The β-galactosidase used was a crude product obtained from *Escherichia coli*[11]. 2-Nitrophenyl β-D-galactopyranoside (E. Merck, Darmstadt, G.F.R.) was employed as substrate. Immobilization was done in Tris–HCl buffer containing 0.01 *M* magnesium chloride, 0.001 *M* disodium hydrogen phosphate and 0.02% sodium azide; adjustment to pH 7.6 was made with hydrochloric acid. This buffer is subsequently referred to as buffer A. In one experiment, magnesium chloride was not included in the buffer; the magnesium-free buffer is referred to as Tris buffer B. The enzyme stock solution, made in buffer A, had a protein concentration of 1.8 mg/ml. Brushite was prepared according to the method of Tiselius *et al.*[8]. All experiments were carried out at 5°C.

EXPERIMENTAL AND RESULTS

Determination of the activity of the immobilized enzyme

A glass column was packed with 50 ml of sedimented brushite. After equilibration of the bed with buffer A, a solution of β-galactosidase was pumped into the column until the enzyme began to appear in the effluent. The excess of non-adsorbed enzyme was then removed from the column by washing with buffer A. The column, thus saturated with enzyme, was then emptied into an erlenmeyer flask containing 60 ml of buffer A. About 10 ml of fresh brushite were added to the flask to adsorb any enzyme leakage from the saturated brushite. Ten aliquots, each consisting of 0.7 ml of sedimented brushite, were withdrawn from the erlenmeyer flask and transferred to ten test-tubes. Substrate solution (200 μl) was added to each of the test-tubes, which were then shaken to keep the brushite in suspension. The enzymatic reaction in tubes 1, 2, 3 ... 10 was stopped after 1, 2, 3 ... 10 min, respectively, by adding 0.7 ml of 1 *M* K_2CO_3 solution (the reaction was linear during the first 10 min under the experimental conditions used; see Fig. 1). The brushite was removed by centrifugation. The absorbances of the supernatants measured at 404 nm were plotted against time (see Fig. 1). Enzyme activity, defined as the change in absorbance per min, was calculated from the slope of the straight line obtained. By relative enzyme activity, determined *n* days after the immobilization, we mean the ratio of the enzyme activity measured after *n* days and the activity measured immediately after immobilization. The activity was measured over a period of 90 days. The results are shown in Fig. 2.



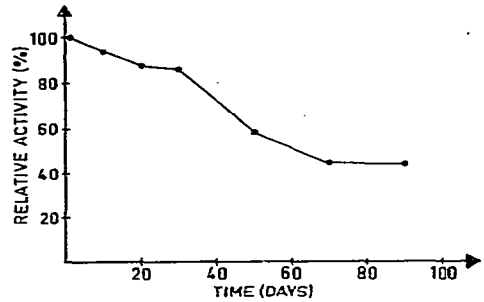


Fig. 1. Hydrolysis of 2-nitrophenyl β-D-galactopyranoside by β-galactosidase immobilized on brushite (batchwise operation). The slope of the straight line obtained during the first 10 min was used for the determination of the activity of the enzyme. The release of 2-nitrophenol was followed by monitoring the absorbance at 404 nm.

Fig. 2. The activity of immobilized β-galactosidase as a function of time.

Determination of an operational conversion factor

The activity measurements described above are cumbersome and time-consuming. For the experiments described in the following two sections, exact activity values were not required, and we therefore chose to measure an operational conversion factor by the following simple column procedure.

A brushite column (volume, 10 ml; length, 10 cm) was equilibrated with buffer A. An aliquot (1 ml) of the stock enzyme solution was applied to the column, which was then washed with buffer A to remove non-adsorbed and loosely bound enzyme. When the colourless substrate (0.2 ml) was filtered into the column bearing the immobilized enzyme it turned yellow immediately (absorption maximum at 404 nm). The product was eluted from the column with buffer A at a flow-rate of 10 ml/h. The effluent was collected in a fraction collector at intervals of 12 min. The absorbance of each fraction was measured at 404 nm ($A_{404}^{1\,cm}$). The amount of product formed, P_{immob}, was then expressed as Σ (fraction volume $\times$ $A_{404}^{1\,cm}$).

An aliquot (1 ml) of the stock enzyme solution was then mixed in a test-tube with 0.2 ml substrate. After 30 min $A_{404}^{1\,cm}$ was measured. The amount of product, P_{free}, was again estimated from the product of $A_{404}^{1\,cm}$ and the volume of the solution (= 1.2 ml). The ratio $(P_{immob}/P_{free}) \cdot 100$ is referred to as the relative conversion factor. This factor appeared to be reasonably constant during at least 3 months under the experimental conditions used (Fig. 3).

Effect of magnesium ions on the activity of the immobilized enzyme

The enzyme was immobilized on a brushite column (volume, 10 ml; length, 10 cm) as described under *Determination of the activity of the immobilized enzyme*. The relative conversion factor was determined at intervals of a few days. Nineteen days after immobilization, during which interval the relative conversion factor remained largely unchanged, the column was washed with a magnesium-free buffer (buffer B) and the relative conversion factor was again determined. As seen in Fig. 4 it decreased abruptly from 98% to 28%. When the column was washed with buffer A the relative

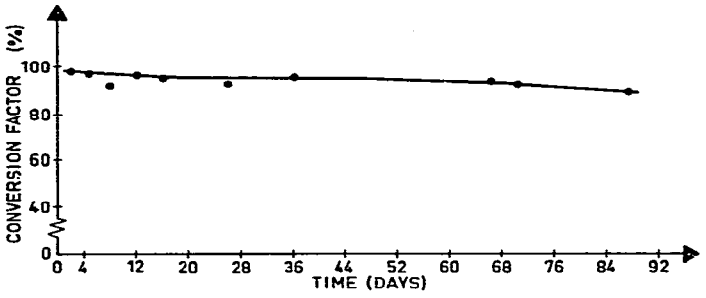


Fig. 3. The time dependence of the release of 2-nitrophenol from 2-nitrophenyl β-D-galactopyranoside by β-galactosidase immobilized on brushite (column operation).

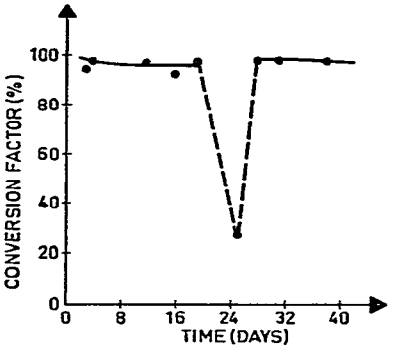
conversion factor increased to its previous value. (It is known that β-galactosidase can be reactivated with magnesium ions[12].)

Height of the column carrying immobilized enzyme

As stated in the Introduction, an attractive feature of brushite as a matrix for immobilized enzymes is that the enzymes are adsorbed at the top of the column. To determine the height of this top section, brushite was packed into a glass column (length, 10 cm; volume, 5 ml) and equilibrated with buffer A. A 1-ml volume of the enzyme solution was applied on the column which was then washed with buffer A. The relative conversion factor was determined. The top 1-cm portion of the column was removed and the conversion factor of the enzyme left on the column was determined. The next 1 cm of brushite was then removed and the conversion factor was again determined. This procedure was repeated until only negligible activity remained in the column. The results shown in Fig. 5 demonstrate that all of the enzyme was contained in the top 4-cm portion of the column. This corresponds to a volume of 2 ml.

Leakage: extent and prevention

Enzyme leakage is a common occurrence when enzymes are immobilized on carriers[13]. As stated above, the enzyme is immobilized near the top of the column (Fig. 5). Any enzyme desorbed during continuous washing is simply adsorbed again further down the column and very little, if any, appears in the effluent (provided that the column is not saturated with enzyme). Therefore, to determine the extent of leakage we completely saturated brushite packed in a plastic column (volume, 10 ml; length, 10 cm) with enzyme. The saturation point was determined by pumping enzyme into the column until 1 ml of the effluent from the column developed a yellow colour with 200 μl of the substrate within 1 min. A 55-ml volume (*i.e.*, about 100 mg protein) of the enzyme stock solution was found to be sufficient. The saturated column was then washed until the effluent showed negligible activity. The saturated column was connected to an identical column of brushite containing no enzyme. The function of this second column was to trap any enzyme released from the saturated column. This is subsequently referred to as the "trapping" column. A 0.73-l volume of buffer A (= 73 column volumes) was passed through the coupled columns. When the enzymes in the two columns were desorbed separately, 7.9 mg of the enzyme were



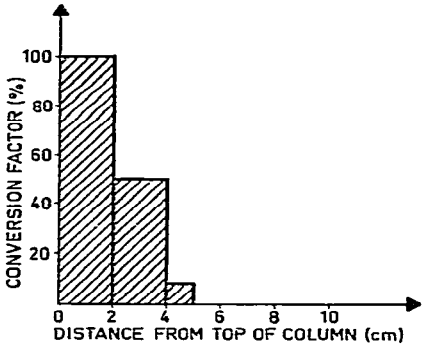


Fig. 4. Effect of magnesium ions on the activity of the immobilized enzyme. After 19 days buffer A (containing magnesium ions) was replaced by buffer B (devoid of magnesium ions), causing a loss in activity. The activity was restored upon replacement of Mg^{2+}.

Fig. 5. The localization of the immobilized enzyme along the column. Only the top part of the bed was covered with enzyme. Column: volume, 5 ml; length, 10 cm.

desorbed from the previously saturated column, while 0.3 mg were desorbed from the "trapping" column. This amounts to only 4% protein leakage.

DISCUSSION

Reversible immobilization of enzymes as described above has many advantages:

(1) The attachment procedure is very simple.

(2) No preconcentration of the enzyme solution is necessary, as is the case when the enzyme is immobilized by covalent linkages. (The enzyme will automatically be concentrated at the top of the column, see Fig. 5.)

(3) The enzyme can be desorbed easily by increasing the phosphate concentration of the buffer. This is of great importance, since the activities of many immobilized enzymes decrease with time but can often be restored if the enzyme is desorbed and kept in solution for some time and then readsorbed on the same bed[14-16]. The same enzyme preparation can then be used repeatedly, which is of particular interest when economy is important, as is the case when enzyme reactors are used in industry.

(4) The same bed can be used for different enzymes, which also is an economic advantage.

(5) The enzyme leakage is small if the column is not completely saturated with protein.

In a previous paper[16] we described the usefulness of amphiphilic agarose derivatives for reversible immobilization of enzymes; in this paper we have shown that brushite can be used for similar purposes. Since all proteins seem to be adsorbed to columns of brushite[8,9], this bed material may be a universal matrix for the immobilization of proteins.

Both brushite and hydroxyapatite (which is another form of calcium phosphate) are widely used as adsorbents for the fractionation of proteins. An advantage of brushite is that it is easier and cheaper to prepare and gives considerably higher flow-rates than hydroxyapatite. However, it is not completely stable at room tem-

perature[8], being slowly transformed to hydroxyapatite. Although this is a drawback in chromatographic purification procedures, it should not be a problem in immobilization experiments, since both brushite and hydroxyapatite adsorb proteins under similar conditions. For immobilization experiments the mentioned advantages of brushite are so important that we have only done a few experiments with hydroxyapatite. The results are, however, similar to those obtained with brushite columns.

As previously shown, the adsorption of most proteins to calcium phosphate is dependent on the concentration of phosphate alone[8,9]; *cf.*, ref. 17 which describes the adsorption of a protein on hydroxyapatite in the presence of 20% NaCl. Therefore it was not surprising that β-galactosidase was immobilized in buffer A containing 3 *M* NaCl (experiments not described in this paper).

A common feature of immobilized enzymes is that their activities diminish with time[14,15]. The activity of β-galactosidase attached to brushite also decreased with time, but relatively slowly. After 3 months the relative activity was still about 45%, as shown in Fig. 2 (the conversion factor was about 90%, see Fig. 3).

The slow rate of enzyme leakage is an other attractive feature of enzyme reactors based on brushite as matrix. (In our experiment the release was 0.3 mg protein per litre eluent from a *saturated* 10-ml column, corresponding to a leakage of 4% of the protein applied.) However, the leakage can virtually be neglected if the column containing the immobilized enzyme is coupled to a "trapping" column of brushite which has not been treated with enzyme solution. Alternatively, one can choose a ratio of column volume and amount of sample applied such that only the upper part of the column will be covered by enzyme.

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