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Purification of a bacterial pullulanase on a fluidized bed of calcium alginate beads

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Abstract

Pullulanase from *Bacillus acidopullulyticus* was purified on a packed bed and a fluidized bed of calcium alginate beads. The binding of enzyme activity to the medium was found to follow Langmuir isotherm pattern. The maximum binding capacity was 1476 U ml^{-1} matrix and the dissociation constant was 142 U ml^{-1}. The dynamic binding capacities at 5% breakthrough in the packed and fluidized beds were 472 U ml^{-1} and 644 U ml^{-1}, respectively. In the packed bed as well as the fluidized bed, an activity recovery of more than 95% with fold purification in the range of 46–59 was observed. The elution with a competitive inhibitor, viz. maltose, and high-fold purification indicate an affinity-based process. The purification process worked equally well with columns of bed volumes of 3.8 and 10 ml.

Keywords: Expanded bed chromatography; Calcium alginate beads; Affinity sorbents; Pullulanase; Enzymes

1. Introduction

It is now well-established that downstream processing costs account for about 50–80% of the total production cost of enzymes/proteins [1]. Hence development of efficient downstream processing strategies has become an area of major activity. Expanded bed chromatography [2–4] in general, and expanded bed affinity chromatography (EBAC) [5,6] in particular, are two such emerging approaches which have shown considerable promise as an efficient unit operation in downstream processing. Expanded bed chromatography is able to use crude suspensions directly, thus obviating the need for pre-clarification. One factor, which has limited the application of this approach in bioseparation, is the high cost of commercially available media. Thus, development of suitable alternative media is a desirable goal. The conventional approach of designing an affinity medium is to covalently couple a suitable affinity ligand to the chromatographic medium [7,8]. We have, in recent years, explored the possibility of developing media which have inherent affinity for the target protein. This, wherever possible, avoids the cost of covalent coupling. Moreover, one no longer has to worry about slow ''leaching'' of the affinity ligand during repeated use of the affinity media. Successful applications of this concept have been the purification of cellulases [9], α-amylases [6] and phospholipase D [10] by fluidized bed affinity chromatography.

We have recently observed that pullulanase also

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shows affinity towards calcium alginate [11]. In this work, we describe the successful purification of pullulanase activity from a crude commercial preparation of the enzyme from *Bacillus acidopullulyticus*. The effect of scale-up on purification has also been studied.

2. Materials and methods

Sodium alginate (catalogue No. A-2158, composed predominantly of mannuronic acid residues) was purchased from Sigma (St. Louis, MO, USA). Promozyme, a commercial preparation of pullulanase from *Bacillus acidopullulyticus*, was a generous gift from Novozymes South Asia (Bangalore, India). This was a viscous brown liquid. Wherever necessary, it was diluted three times with 0.05 *M* acetate buffer, pH 5.0 containing 6 m*M* $CaCl_2$. The chromatographic column (cat. No. C3919) was purchased from Sigma (St. Louis, MO, USA). The peristaltic pump (Model U4-MIDI) was from Alitea (Stockholm, Sweden). Yeast cells were purchased from the local market. All other chemicals were of analytical grade.

2.1. Estimation of enzyme activity and amount of protein

Activity of pullulanase was estimated using pullulan as the substrate [12]. One enzyme unit liberates 1 μmol of reducing sugar (calculated as glucose) per minute at 40 °C and pH 5.0. The amount of reducing sugar produced was estimated by the dinitrosalicylic acid method [13]. Protein content was estimated by the dye binding method [14] using bovine serum albumin as the standard protein.

2.2. Preparation of alginate beads

Alginate beads were prepared by a procedure outlined by Somers et al. [15]. Beads were formed by dropping 50 ml of 2% alginate solution through a syringe (fitted with a gauze 23 needle) into a 100 ml 0.1 *M* $CaCl_2$ solution. Care was taken not to disturb the liquid during bead preparation as any disturbance led to deformation of the beads. The beads obtained were kept for 2 h in 0.1 *M* $CaCl_2$ solution and stored in a 0.006 *M* $CaCl_2$ solution at 4 °C.

2.3. Preparation of broken, heat-killed yeast cells [6]

Yeast cells (20 mg, dry mass) were broken mechanically in a mortar and pestle. The broken yeast cells were suspended in 4 ml of 0.05 *M* acetate buffer containing 0.006 *M* $CaCl_2$, pH 5.0, heated at 80 °C for 60 min and centrifuged at 12 000 *g* for 20 min. The suspension and centrifugation steps were repeated until the absorbance of the supernatant at 280 nm matched that of the suspension buffer. The broken yeast cells, obtained as residue, were suspended in the buffer (to obtain a concentration of 5 mg ml^{-1}) for further use.

2.4. Adsorption isotherms

Adsorption isotherm of pullulanase was determined by equilibrating different concentrations of the enzyme with 1 ml alginate beads (in 0.05 *M* acetate buffer+0.006 *M* $CaCl_2$, pH 5.0) overnight at 25 °C. After equilibration, aliquots were removed and the pullulanase activity in the supernatant was measured to calculate bound enzyme activity per ml of the adsorbent beads. The volume of the beads throughout this work refers to the volume of the settled beads (the beads settle easily with gravity).

2.5. Separation in the column

The equilibration buffer (0.05 *M* acetate buffer containing 0.006 *M* $CaCl_2$, pH 5.0) was pumped through a bed of adsorbent alginate beads contained in a 20.0×1.0 cm glass column. The column volume could be adjusted with the help of a PTFE flow adapter fitted with a 20 mm polyethylene bed support. The flow to the bottom of the column was controlled using a peristaltic pump. Initially, the sedimented height was 3.0 cm (bed volume 3.8 ml). A 36-ml volume of the (appropriately diluted) enzyme was loaded into the column. In the case of the fluidized bed, the height of the expanded bed was 4.4 cm at a flow-rate of 2.0 ml min^{-1}. Elution, in both the cases, was carried out in the packed bed mode by loading 10 ml of 1 *M* maltose (in 0.05 *M* acetate

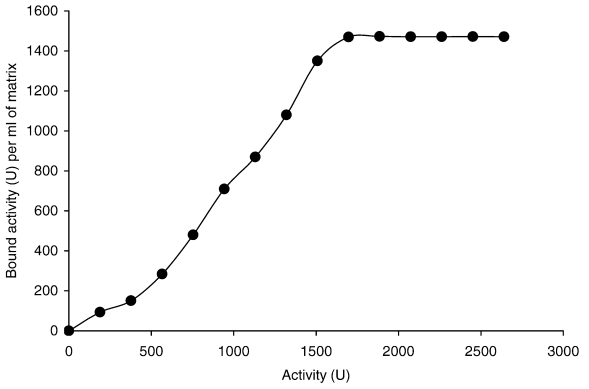

Fig. 1. Adsorption isotherm of pullulanase on alginate beads. Different concentrations of pullulanase were made up to 2 ml with 0.05 *M* sodium acetate buffer, pH 5.0 and added to 1 ml of alginate beads and equilibration carried out overnight at 25 °C. Aliquots were removed and checked for pullulanase activity to determine the amount of enzyme bound per ml of the beads. Each set was run in triplicate and the difference in individual readings in each set was less than 6%. Quantitative assessment of the results was done by fitting the data to the Langmuir isotherm of the form $q^*=q_m c^*/(K_d+c^*)$, where c^* is the equilibrium liquid concentration in U ml^{-1} and q^* is the capacity of the matrix at a particular enzyme load. The other symbols used have been described in the text.

buffer containing 0.006 *M* $CaCl_2$, pH 5.0) into the column and keeping it for 4 h in the cold room. The eluate was dialyzed against the same buffer to remove maltose and pullulanase activity was determined.

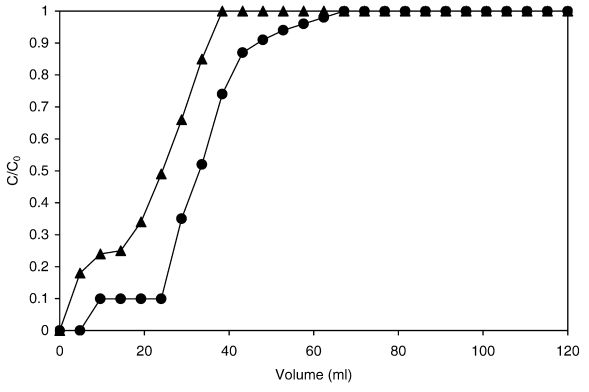

Fig. 2. Breakthrough curve of pullulanase activity on a packed bed of alginate beads. *C* refers to the concentration of enzyme activity (●, U ml^{-1}) and the concentration of protein (▲, mg ml^{-1}) in the effluent, C_0 refers to the same in the load.

Scale up of the process was also investigated with a sedimented bed of 10 cm. The fluidized bed height was 14.5 cm, under the flow-rate conditions used above. The volume of the (appropriately diluted) crude extract loaded on the column was 78 ml. Elution of the bound enzyme activity was carried out as described previously.

To test the effect of presence of particulate matter in the culture broth on the shape of breakthrough curve of pullulanase on alginate beads under fluidization conditions, the crude extract was mixed with broken heat-killed yeast cells and pumped in under identical conditions as above.

3. Results and discussion

Starch-degrading enzymes constitute a major class among biotechnologically useful industrial enzymes [16]. Hence, their production in a more economical way is a desirable goal. Pullulanase is an important starch-degrading enzyme which is useful in producing high-maltose syrups [17]. Thus, an efficient purification protocol using EBAC is worth developing.

3.1. Equilibrium adsorption

To estimate the enzyme binding capacity of the alginate beads, batch equilibrium adsorption experiment was performed with the crude extract. Fig. 1 shows adsorption isotherm of pullulanase on alginate beads. The binding of pullulanase to alginate beads was found to follow Langmuir isotherm pattern [18]. The maximum binding capacity (q_m) of the beads was found to be 1476 U ml^{-1} and the dissociation constant (K_d) was 142 U ml^{-1}. The capacity of the beads for this enzymes compares favourably with the binding capacities reported with other enzymes and their affinity matrices in the expanded bed format [5,6,19,20].

3.2. Frontal analyses in packed and fluidized beds

The crude extract (appropriately diluted with 0.05 *M* acetate buffer, pH 5.0 containing 6 m*M* $CaCl_2$) was loaded on a packed bed of alginate beads. The effluent was monitored for enzyme activity till the

enzyme activity (in U ml^{-1}) matched that of the input ($C=C_0$) [21,22] (Fig. 2). The dynamic capacity at 5% breakthrough (calculated from the volume corresponding to $C/C_0=0.05$ [21,22]), is found to be 473 U ml^{-1}. This corresponds to 32% of the maximum equilibrium binding capacity.

A similar experiment was performed with a fluidized bed of alginate beads (Fig. 3). The dilution of the crude extract with the irrigating buffer was found to be necessary since otherwise, the viscosity of the extract was sufficient to move all the beads towards the upper adapter. The dynamic binding capacity at 5% breakthrough in this case was determined to be 644 U ml^{-1} which corresponds to 42% of the maximum equilibrium binding capacity. Clemmitt and Chase have reported that in the case of histidine-tagged proteins purified on commercially available Streamline chelating column, the dynamic binding capacity at 5% breakthrough was 51% of the maximum equilibrium binding capacity which ''emphasizes the efficient loading of the adsorbent'' [22]. Thus, in the present case also, the dynamic binding capacity at 5% breakthrough reflects reasonably efficient loading of the adsorbent.

The purpose of using a fluidized/expanded bed is to be able to load crude broth/homogenate containing particulate matter directly on the column. To this end, broken, heat-killed yeast cells were introduced in the crude extract as a model mixture. As expected, the dynamic binding capacity of the beads decreased slightly in the presence of broken, heat-killed yeast cells (Fig. 3). It was however still higher than the dynamic binding capacity in the packed bed mode and the equilibrium binding capacity in the batch mode.

3.3. Chromatographic procedures

3.3.1. Packed bed chromatography

The elution pattern of pullulanase on a packed bed of alginate beads is shown in Fig. 4. The purification data are summarized in Table 1. The high-fold purification in a single step reflects the affinity-based process. Also, the elution of the enzyme with maltose, a competitive inhibitor of the enzyme, indicates that alginate is acting as an affinity media for pullulanases.

3.3.2. Fluidized bed chromatography

The chromatographic profile for the purification of pullulanase on a fluidized bed of alginate beads is shown in Fig. 5. The significant level of purification achieved can be judged from the specific activity of the purified fraction (Table 2), which is of the same

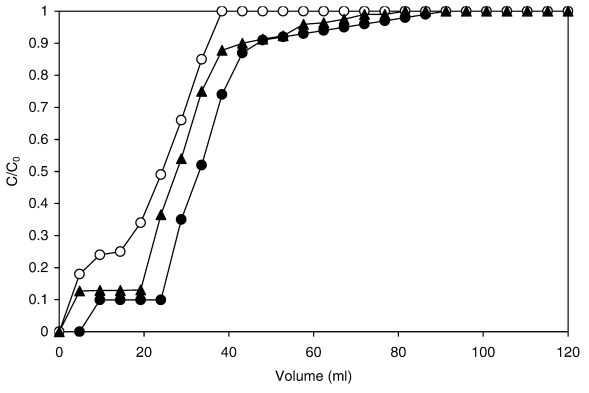

Fig. 3. Breakthrough curves of pullulanase in the presence (○) and absence of heat-killed yeast cells (●) on a fluidized bed of alginate beads. The height of the settled bed was 3 cm. The bed was expanded to a height of 4 cm at a flow-rate of 2.0 ml min^{-1}. The breakthrough curve for protein (▲) is also shown in the figure.

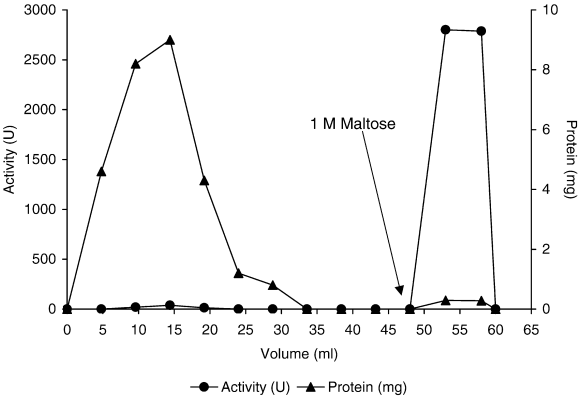

Fig. 4. Purification of pullulanase on a packed bed of alginate beads. The bed height was 3 cm. The enzyme was pumped in at a flow-rate of 2 ml min^{-1} and then washed with 0.05 *M* sodium acetate buffer containing 0.006 *M* $CaCl_2$, pH 5.0 until the absorbance at 280 nm had levelled off. Elution was carried out with 1 *M* maltose in the packed bed. Fractions were collected and assayed for enzyme activity (●) and protein (▲).

Table 1
Purification of pullulanase from *Bacillus acidopullulyticus* on a packed bed of alginate beads

Step	Activity (U)	Protein (mg)	Yield (%)	Specific activity (U mg^{-1})	Fold purification
Crude	5657	29	100	195	1
Wash	71	28	1	2	–
Eluate	5587	0.6	99	9312	48

Pullulanase activity (5657 U, from a commercial preparation) was passed through a packed bed column of alginate beads and eluted using 1 *M* maltose as described in the text.

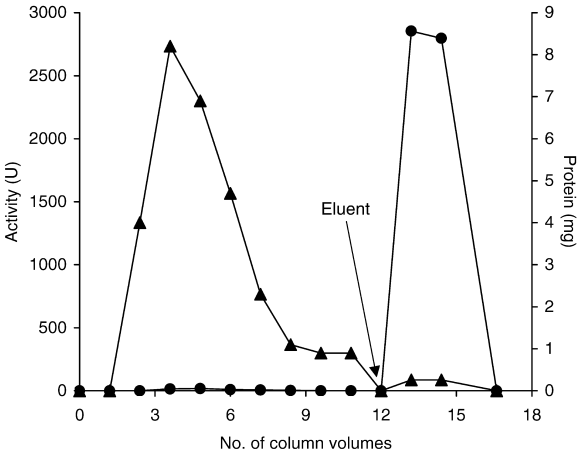

Fig. 5. Purification of pullulanase from *Bacillus acidopullulyticus* on a fluidized bed of alginate beads. The settled bed height was 3 cm and the column had an internal diameter of 1 cm. The degree of expansion was 1.46 at a linear flow-rate of 2 ml min^{-1}. 5700 U of the enzyme activity (in 0.05 *M* acetate buffer containing 0.006 *M* $CaCl_2$, pH 5.0) was introduced into the column after the bed had attained the desired height. The fraction size was 4.8 ml. Elution was carried out in the packed bed mode after switching off the pump and allowing the bed to settle down.

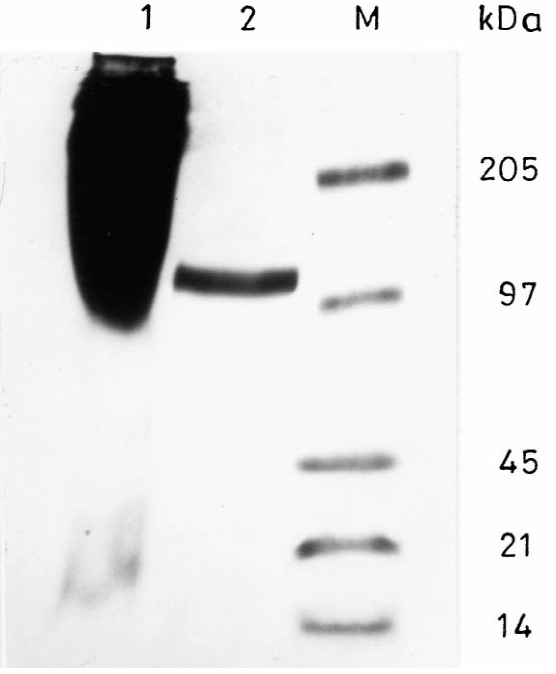

Fig. 6. SDS–PAGE of purified enzyme. Lanes: 1=crude enzyme; 2=purified enzyme; M=marker proteins. The gel was stained with Coomassie Brilliant Blue R-250 for 45 min and then destained in 40% methanol and 10% acetic acid. kDa=Kilodalton.

Table 2
Purification of pullulanase from *Bacillus acidopullulyticus* on a fluidized bed of alginate beads

Step	Activity (U)	Protein (mg)	Yield (%)	Specific activity (U mg^{-1})	Fold purification
Crude	5700	30	100	190	1
Wash	54	29	1	2	–
Eluate	5650	0.5	99	11 300	59

The enzyme was loaded in the column with settled bed height of 3 cm. The column was fluidized by pumping in 0.05 *M* sodium acetate buffer containing 0.006 *M* $CaCl_2$, pH 5.0 until the bed attained a height 1.46 times the settled bed height. Other experimental details are described in the text.

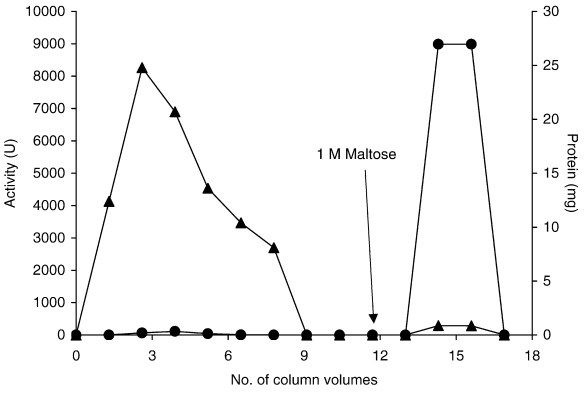

Fig. 7. Scale up of purification of pullulanase from *Bacillus acidopullulyticus* on a fluidized bed of alginate beads. The settled bed height was 10 cm and the column had an internal diameter of 1 cm. The degree of expansion was 1.46 at a linear flow-rate of 2 ml min^{-1}. 18 525 U of the enzyme activity (appropriately diluted in 0.05 *M* acetate buffer containing 0.006 *M* $CaCl_2$, pH 5.0) was introduced into the column after the bed had attained the desired height. The fraction size was 17 ml. Elution was carried out in the packed bed mode as described in the legend to Fig. 5.

order as that achieved for the enzyme using magnetite–alginate beads in the batch mode [11]. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of the purified preparation showed a single band corresponding to a minimum molecular mass of 100 000 (Fig. 6) which agrees well with the earlier reported molecular mass [11,23].

To test the potential of scale-up, the column with 10 ml of bed volume was used. A 78-ml volume of the crude extract (containing 18 525 U of enzyme activity) was passed through the fluidized column. The amount of pullulanase eluted as well as the purity of the effluent (Fig. 7 and Table 3) were comparable to the experiment carried out on a smaller scale. The recoveries (in the smaller scale and with 10 ml bed volume) were 99 and 97%, respectively. The fold purification in the fluidized bed mode, if anything, has been slightly better than the packed bed mode. This is especially interesting in this case since the data in Tables 1–3 show that the media shows very high selectivity for the target enzyme in the binding stage.

We have mentioned earlier that fluidized beds of these beads show greater dispersion than ideal expanded beds [6]. However, the results obtained here show that despite this deviation from the ideal fluidization behaviour, fairly good purification can be obtained.

Earlier we had described the purification of pullulanase using magnetite–alginate beads [11]. Magnetic methods of bioseparation, however, tend to be expensive [2] and not suitable for industrial enzymes like pullulanase. This work shows that an inexpensive media like alginate beads can be used in fluidized bed mode for the purification of pullulanases. The non-toxic nature of calcium–alginate beads also makes them suitable for purification of these enzymes in view of its application in food processing industries [17]. Alginate has been used in a variety of processed foods for a long time [24].

Acknowledgements

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Table 3
Scale up of purification of pullulanase from *Bacillus acidopullulyticus* on a fluidized bed of alginate beads

Step	Activity (U)	Protein (mg)	Yield (%)	Specific activity (U mg^{-1})	Fold purification
Crude	18 525	96	100	193	1
Wash	222	90	1	2	–
Eluate	17 970	2	97	8985	46

The enzyme was loaded in the column with settled bed height of 10 cm. The column was fluidized by pumping in 0.05 *M* sodium acetate buffer containing 0.006 *M* $CaCl_2$, pH 5.0 until the bed attained a height 1.46 times the settled bed height. Other experimental details are described in the text.

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