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UNEXPECTED AFFINITY OF POLYSACCHARIDES AND ITS APPLICATION IN SEPARATION OF ENZYMES ON FLUIDIZED BEDS

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ABSTRACT

Fluidized bed chromatography is emerging as a powerful tool in bioseparation of proteins/enzymes. In this work, alginate and chitosan beads have been used as affinity media in fluidized mode to selectively capture α-amylases and *Aspergillus niger* cellulase, respectively. Fairly high yields (>80%), with homogeneity at the level of single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, were achieved in all these cases. The main novel features are unexpected affinity of the polysaccharides towards the target enzymes; such macroaffinity ligands avoid any costly chemical steps for affinity ligands; and possibility of easy scale up in fluidized bed mode and single step purification protocols.

Key Words: Alginate; α-Amylase; Cellulase; Chitosan; Expanded bed affinity chromatography; Macroaffinity ligand

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INTRODUCTION

There are two major objectives, which generally have to be achieved in the beginning of protein purification at the industrial level.

1. The target protein is often present at fairly low concentration. Hence, it is necessary to start with larger volumes. Thus, reduction of volume or concentration of the protein(s) is invariably the primary step in any protein purification process. This is especially relevant in the context of purification of recombinant proteins as large amount of biomass is present in addition to the target protein (1).
2. Removal of particulate matter including cell and/or cell debris.

As pointed out by Thommes (2), only three approaches in bioseparation can achieve both of these objectives simultaneously:

- two-phase aqueous extractions;
- use of protein-binding ligands in crossflow microfiltration with membranes; and
- fluidized matrix chromatography.

The first two are not within the scope of this work and will not be discussed further. In fact, currently, the most commonly used method of primary protein recovery from whole broth is that of adsorption on fluidized beds (2–6).

Perhaps the earliest work on use of fluidized bed for whole broth processing in biotechnology was that of streptomycin production (2). As the clarification step was leading to large product losses, fluidized bed of a cation exchange resin was used and led to a 12% increase in streptomycin yield.

The application of fluidized beds in the area of protein bioseparation is of more recent origin. The development in this area has been critically dependent upon the availability of suitable fluidization media. The early pioneering work of Chase and Draeger was done with the commercially available Sepharose™ Fast Flow (7,8). Subsequently, some other matrices, such as those based upon controlled pore glass (9), crosslinked agarose with a quartz core (Streamline™, Pharmacia Biotech, Sweden) or steel core (5), zirconia (10), etc., have been described. Thommés (2) has tabulated the fluidization characteristics of different matrices.

In the fluidized bed, the adsorbent particles are freely suspended in the flowing liquid, the gravity being balanced by buoyancy. The minimum liquid flow velocity at which fluidization of the bed takes place is called minimum fluidization velocity (u_{mf}). The terminal velocity for the bed particle (u_t) is the velocity at which the particles will move out of the column if not restrained by the upper adapter. Operation of a fluidized bed for protein separation limits the range

of useful flow rates. One frequently used equation for defining this range is the Richardson–Zaki correlation (11):

$$u = u_t \varepsilon^n$$

This equation correlates the bed voidage, ε with the linear liquid velocity, u. The Richardson–Zaki parameter, n can be determined by plotting $\log u$ vs. $\log \varepsilon$. For useful behavior, the plot should be a straight line and n should be close to 4.8, which reflects laminar flow regime (7).

The second critical parameter for column behavior is residence time distribution (RTD), which evaluates dispersion of the loaded sample due to axial mixing in the mobile phase. Qualitative picture of RTD can be obtained easily by applying a sample of any tracer molecule, which is not absorbed by the column and by looking at the dilution of the sample as the tracer molecule exits the column. For quantitative analysis of the data, some good reviews are available (2,12,13).

Some of the advantages of expanded bed chromatography over other protein separation techniques are briefly discussed below.

1. The main motivation behind development of this technique has been the need to deal directly with crude suspensions. Such crude feedstock may include plant or animal tissue homogenates, fermentation products, blood serum products, or whey. Conventional chromatography with packed beds cannot deal with crude suspensions as the bed will ultimately get clogged up with the suspended particles.
2. Capital investments and labor costs associated with centrifugation/ultrafiltration are eliminated. Used properly, the chromatographic media (even if expensive) can be used several times. In this respect, cleaning-in-place procedures are especially important in fluidized bed chromatography since the bed interacts with crude feeds. Various cleaning-in-place procedures have been suggested and proved adequate in case of various purification processes based on the fluidized bed chromatography (1,2).
3. Like conventional chromatography, fluidized bed chromatography also reduces the initial volume and concentrates the sample. The main difference is that in the former case, a clarified feed is required so invariably the crude feeds are subjected to salt precipitation, etc. Precipitation reduces the processing volumes as well. Hence packed bed chromatography, coming as the second or third unit process in the protocol normally deals with samples, which are concentrated already. Fluidized bed chromatography thus combines the concentration step and retains the selectivity of packed bed chromatography as well.
4. As a selective process introduced early in the purification protocol, fluidized bed chromatography can often separate the desired protein

from hostile environments. The latter include proteases and glycosidases present in the crude extracts (2).

5. In many cases, fluidized bed chromatography has cut down the number of purification steps and hence the processing time.

The term "expanded bed" was introduced by Chase and Draeger to describe fluidized beds exhibiting reduced back mixing in the column and having an increased number of mass transfer units for achieving better utilization of the binding capacity of the adsorbent (7). Thus, these ideal fluidized beds for protein separation combine clarification, concentration, and capture in a single unit process. If an affinity adsorbent is used, the capture of the protein becomes selective. Thus, expanded bed affinity chromatography (EBAC) has emerged as a fairly powerful technique for protein purification (4,14–16).

The present works addresses the following two important issues:

need to develop a larger variety of chromatographic media for use in EBAC and

issue of cost involved in coupling affinity ligands to the matrix and having leak-proof affinity media

This work explores the possibility that naturally occurring polysaccharides can show unexpected affinity for some industrially important enzymes/proteins. The beads prepared from such polysaccharides as such constitute two-in-one affinity matrix as well as macroaffinity ligand.

MATERIALS AND METHODS

Sodium alginate (Cat. No. A-2158, composed predominantly of mannuronic acid residues) (abbreviated as HM alginate), Con A-agarose, wheat germ powder, and α-amylase (porcine pancreas) were purchased from Sigma Chemical Co. (St. Louis, MO). Protanal LF 10/60 (from brown seaweed, having high guluronic acid content of 65–75%) (abbreviated as HG alginate) was a gift from Prof. Bo Mattiasson, Lund University, Sweden. BAN 240L (*Bacillus amyloliquefaciens*) was generously supplied by NOVO Nordisk A/S (Bangalore, India). Fiberzyme (*Aspergillus fumigatus*) was obtained from Jayson Agritech Ltd., Mysore, India. Cellulase (from *Aspergillus niger*) was obtained from Central Drug House, Bombay, India and carboxymethylcellulose from Loba Chémie, Mumbai, India. The crude fungal extract (of *Scytalidium thermophilum*) was a generous gift from Prof. B. N. Johri (Department of Microbiology, GBPUAT, Pantnagar). All other chemicals were of analytical grade.

METHODS

Estimation of Enzyme Activities and Amount of Protein

Activity of α-amylase was estimated using starch (17) as the substrate. In the case of porcine pancreas, the assay was carried out at pH 7.0 (17) whereas in all other cases, the assay was done at pH 5.6 [Product sheet on BAN, NOVO Nordisk A/S (1990)]. The reducing sugar generated was measured using dinitrosalicyclic acid (18). One enzyme unit liberates 1 μmol of reducing sugar (calculated as maltose) per minute at 45°C (for the fungal enzyme) and at 25°C (for all other enzymes). In the case of the fungal amylase, the enzyme and substrate were pre-incubated at 45°C for 3–4 min. An amount of the enzyme (0.5 mL) was incubated with 0.5 mL of the substrate (1%, wt vol^{-1}) at a particular pH at 45°C for 5 min. Activity of cellulase was estimated using carboxymethyl-cellulose as the substrate (19). One enzyme unit liberates 1 μmol of reducing sugar (calculated as glucose) per minute at 50°C. Protein content was estimated by the dye binding method (20) using bovine serum albumin as the standard protein.

Polyacrylamide Gel Electrophoresis

SDS–PAGE of the samples using 12% gel was performed according to Hames (21) using a Genei electrophoresis unit (Bangalore Genei Pvt. Ltd., Bangalore, India) and standard molecular weight markers (Pharmacia Biotech, Hong Kong) for the purified cellulase from *Aspergillus niger* and purified fungal amylase and 15% gel for the purified α-amylase from wheat germ extract.

Preparation of Alginate Beads

Alginate beads were prepared from two different kinds of sodium alginate by a procedure outlined by Somers et al. (22). Beads were formed by dropping 50 mL of 2% alginate solution through a syringe into a 100 mL 0.1 *M* $CaCl_2$ solution. The beads obtained were kept for 2 hr in 0.1 *M* $CaCl_2$ solution and stored in a 0.006 *M* $CaCl_2$ solution at 4°C.

Extraction of Wheat Germ Amylase from Wheat Germ Powder

The amylase preparation was obtained by stirring 20 g of wheat germ powder with 20 mL of chilled acetone in cold for 2 hr. The dry powder, so

obtained, was used for extraction of amylase activity. The extraction was done with 40 mL of 0.05 *M* acetate buffer containing 0.006 *M* $CaCl_2$, pH 5.6. The mixture was stirred in cold for 4 hr and centrifuged at 12,000 *g* for 15 min at 4°C. The clear supernatant obtained was used as crude wheat germ amylase preparation.

Preparation of Broken, Heat-Killed Yeast Cells

Yeast cells were broken mechanically in a mortar and pestle. The broken yeast cells were suspended in 0.05 *M* acetate buffer containing 0.006 *M* $CaCl_2$, pH 5.2, heated at 80°C for 60 min and centrifuged at 12,000 *g* for 20 min. The suspension and centrifugation steps were repeated till the absorbance of the supernatant at 280 nm matched that of the suspension buffer. The concentrated cell suspension (obtained as residue) was suspended in the buffer and used.

Amylase Adsorption Isotherms

Adsorption isotherms of alpha amylases (from BAN 240L, a commercial preparation, porcine pancreas, wheat germ crude extract, and the fungal extract) were determined by equilibrating different concentrations of the enzyme with 2 mL alginate beads (in 0.05 *M* acetate buffer + 0.006 *M* $CaCl_2$, pH 5.2) overnight at 25°C. After equilibration, aliquots were removed and the amylase activity in the supernatant was measured to calculate bound amylase activity per milliliter of the adsorbent beads. The adsorption isotherms were determined with beads prepared from alginate with high guluronic acid content as well as from high mannuronic acid content.

To test the effect of presence of cells in the culture broth on the adsorption of wheat germ α-amylase on alginate beads, heat-killed yeast cells were added to flasks containing different concentrations of amylase activity (to a final concentration of 5 mg mL^{-1}) and treated as above.

Optimization of Binding of Fungal Enzyme to Alginate Beads in the Batch Mode

Three milliliters of the (appropriately diluted) crude fungal extract each (containing 46 U of amylase activity) was equilibrated with 3 mL of both types of alginate beads (high mannuronic acid and high guluronic acid content) at three different pH values (0.05 *M* acetate buffer containing 0.006 *M* $CaCl_2$, pH 5.2; 0.05 *M* acetate buffer containing 0.006 *M* $CaCl_2$, pH 6.0; and 0.05 *M* Tris acetate

buffer containing 0.006 *M* $CaCl_2$, pH 7.0) for 1 hr at 25°C. The bound activity was calculated by subtracting the activity remaining in the supernatant after incubation for 1 hr from the initially added enzyme activity.

Fluidization of the Settled Bed

The equilibration buffer (0.05 *M* acetate buffer containing 0.006 *M* $CaCl_2$, pH 5.2) was pumped through a bed of adsorbent alginate beads (bed volume 8.5 mL) contained in a 1.0 cm × 20.0 cm glass column (Supelco). The column volume could be adjusted with the help of a polytetrafluoroethylene (PTFE) flow adapter fitted with 20 mm polyethylene bed support. The flow to the bottom of the column was controlled using a peristaltic pump (Alitea AB, Sweden, model U4-MIDI). The sedimented adsorbent bed had a height of 11.0 cm. The liquid flow rate was first adjusted to calibrate the pump readings. The bed expansion was then studied as a function of the liquid flow rate. Once the maximum bed height (as allowed by the column length) had been attained, the flow rate was decreased in steps and the bed height was measured at each flow rate.

Separation in the Fluidized Bed

The equilibration buffer [0.05 *M* Tris acetate buffer containing 0.006 *M* $CaCl_2$, pH 7.0 for the fungal amylase and 0.05 *M* acetate buffer containing 0.006 *M* $CaCl_2$, pH 5.2 for all other amylases] was pumped through a bed of adsorbent HG alginate beads (for BAN 240L and porcine pancreatic amylase) and of HM alginate beads (for wheat germ and fungal amylases). The height of the sedimented adsorbent bed was 11.0 cm and the fluidized bed height was 16 cm at a flow rate of 2.0 mL min^{-1} for HM alginate beads and 2.4 mL min^{-1} for the beads made from HG alginate. 20 mL of the equilibration buffer containing different amounts of enzyme activities was loaded in the column. Elution was carried out in the packed bed mode by loading 10 mL of 1 *M* maltose (in 0.05 *M* acetate buffer containing 0.006 *M* $CaCl_2$, pH 5.2 and 0.05 *M* Tris acetate buffer containing 0.006 *M* $CaCl_2$, pH 7.0) into the column and keeping it for 4 hr in the cold room. The eluate was dialyzed against the same buffer to remove maltose and amylase activity determined. The eluate from the fungal amylase extract was abbreviated as E I.

To test the effect of presence of particulate matter in the culture broth on the shapes of breakthrough curves of wheat germ α-amylase on alginate beads under fluidized bed conditions, crude extract mixed with broken heat-killed yeast cells was pumped in under identical conditions as above.

Further Purification of Fungal Amylase on Con A-Agarose Column

The eluate from the above column was loaded onto a Con A-agarose column (0.5 × 3 cm). The column was washed with 0.01 *M* phosphate buffer, pH 6.9 containing 0.001 *M* $MgCl_2$, 0.001 *M* $MnCl_2$ and 0.001 *M* $CaCl_2$. The activity in the unbound fraction is abbreviated as E II. Elution of the bound activity was done with 0.01 *M* α-methyl-D-mannoside (abbreviated as E III).

PURIFICATION OF CELLULASE ON CHITOSAN BEADS

Preparation of Chitosan Beads

Chitosan beads were prepared (23) for use in fluidized bed chromatography of cellulase. Chitosan solution (1.5%) was prepared by dissolving 0.75 g chitosan in 50 mL of 0.1 *M* acetate buffer, pH 4.0 and centrifuging the solution to remove insoluble material. The supernatant was added to a mixture of water: methanol:NaOH (4:5:1) dropwise using a syringe and kept overnight at room temperature. The beads were washed thoroughly with distilled water and then stored in 0.05 *M* acetate buffer, pH 6.0.

Cellulase Adsorption Isotherms

Adsorption isotherms of cellulases (from *Aspergillus niger* and *Aspergillus fumigatus*) were determined by equilibrating different concentrations of the enzyme with 2 mL chitosan beads overnight at 25°C. After equilibration, aliquots were removed and the cellulase activity in the supernatant was measured to calculate bound cellulase activity per mL of the adsorbent beads. Centrifugation of the samples was not found to be necessary since the beads settle down on standing.

Bed Expansion Characteristics

The equilibration buffer (0.05 *M* acetate buffer, pH 6.0) was pumped through a bed of adsorbent chitosan beads (bed volume 8.5 mL) contained in a 1.0 cm × 20.0 cm glass column (Supelco). The column volume could be adjusted with the help of a PTFE flow adapter fitted with 20 mm polyethylene bed support. The flow to the bottom of the column was controlled using a peristaltic pump (Alitea AB, Sweden, model U4-MIDI). The sedimented adsorbent bed had a height of 11.0 cm. The liquid flow rate was first adjusted to calibrate the pump readings. The bed expansion was then studied as a function of the liquid flow rate. Once the

maximum bed height (as allowed by the column length) had been attained, the flow rate was decreased in steps and the bed height was measured at each flow rate.

Separation in the Fluidized Bed Mode

The equilibration buffer (0.05 *M* acetate buffer, pH 6.0) was pumped through a bed of adsorbent chitosan beads (bed volume 8.5 mL) contained in a 1.0 cm × 20.0 cm glass column (Supelco). The height of the sedimented adsorbent bed was 11.0 cm and the fluidized bed height was 14.5 cm at a flow rate of 3 mL min^{-1}. An amount of the crude (20 mL) containing cellulase activity was loaded in the column. Elution was carried out in the packed bed mode by loading 10 mL of 1 *M* phosphate buffer, pH 7.0 containing 20% ethylene glycol into the column and keeping it for 3 hr in the cold room. The eluate was then checked for cellulase activity.

RESULTS AND DISCUSSION

Separation of α-Amylases on Alginate Beads

In our laboratory, it was observed earlier that calcium alginate beads bind to α-amylases and the bound enzyme activities can be eluted selectively with maltose solution (24).

Table 1 shows the expansion behavior of these beads in the fluidized bed. In both cases, results were found to fit with Richardson–Zaki relation (11). The values of *n* for the alginate beads (with high and low mannuronic acid content) are in the laminar flow region.

Table 1. Richardson–Zaki Parameters for the Various Adsorbents Used. The Table Lists the Fluidizing Solutions Used to Calculate Richardson–Zaki Exponent and the Terminal Velocity by Studying the Variation of Bed Height as a Function of Liquid Velocity

Adsorbent	Fluidizing Solution	Richardson–Zaki Exponent	Terminal Velocity (cm hr^{-1})
HM[a] alginate beads	0.05 *M* acetate buffer + 0.006 *M* $CaCl_2$, pH 5.2	4.8	1636
HG[b] alginate beads	0.05 *M* acetate buffer + 0.006 *M* $CaCl_2$, pH 5.2	5.5	2807
Chitosan beads	0.05 *M* acetate buffer, pH 6.0	10.3	9214

[a] HM High mannuronate.
[b] HG High guluronate.

It was found that α-amylase from *Bacillus amyloliquefaciens* and porcine pancreas bind equally well with both kinds of alginate beads whereas the enzyme from wheat germ shows preference for alginate beads (of high mannuronic acid content) (Table 2). These adsorption isotherms also reveal some interesting features. The binding of porcine pancreatic amylase to alginate beads (with low mannuronic acid content) shows BET model of adsorption (25) whereas binding of amylases from wheat germ and porcine pancreas to alginate beads (with high mannuronic acid content) follows Langmuir adsorption pattern (26). The maximum binding capacity (q_m) for the wheat germ is around $50\,U\,mL^{-1}$ and the dissociation constant (K_d) is $325\,U\,mL^{-1}$. The corresponding values in the presence of heat-killed yeast cells are 37 and $252\,U\,mL^{-1}$, respectively. The values for the porcine enzyme are $q_m = 181\,U\,mL^{-1}$ and $K_d = 35\,U\,mL^{-1}$. The adsorption isotherms of the bacterial enzyme to both types of alginate beads show linear portion of the Langmuir isotherm. The equilibrium binding capacity as well as the dynamic binding capacity of the alginate beads (with high mannuronic acid content) for wheat germ amylase were found to decrease slightly in the presence of broken, heat-killed yeast cells (as model crude extract) (data not shown).

In the case of *Scytalidium thermophilum*, the enzyme binds more efficiently at pH 7.0 and with alginate beads of high mannuronic acid content (Fig. 1). All further data with this enzyme thus relate to these alginate beads.

The adsorption isotherm of the fungal enzyme on alginate beads was found to fit the Langmuir adsorption pattern (26). The maximum capacity of the alginate beads for the fungal enzyme was found to be $14.7\,U\,mL^{-1}$ and the dissociation constant equaled $0.66\,U\,mL^{-1}$. Thus, the maximum capacity of alginate beads for amylases from different sources is in the same range; however, the fungal amylase shows stronger affinity towards the alginate beads.

Table 2. Dependence of Affinity of α-Amylases on the Composition of Alginate Used for Preparing Beads

	Composition of Alginate[a]	
Source of α-Amylase	High Mannuronate	Low Mannuronate
Bacillus amyloliquefaciens	++	++
Porcine pancreas	++	++
Wheat germ	++	−
Scytalidium thermophilum	++	+

[a] −, 0–10% binding of enzyme activity to the matrix; +, 45–60% binding of enzyme activity to the matrix; ++, 85–100% binding of enzyme activity to the matrix.

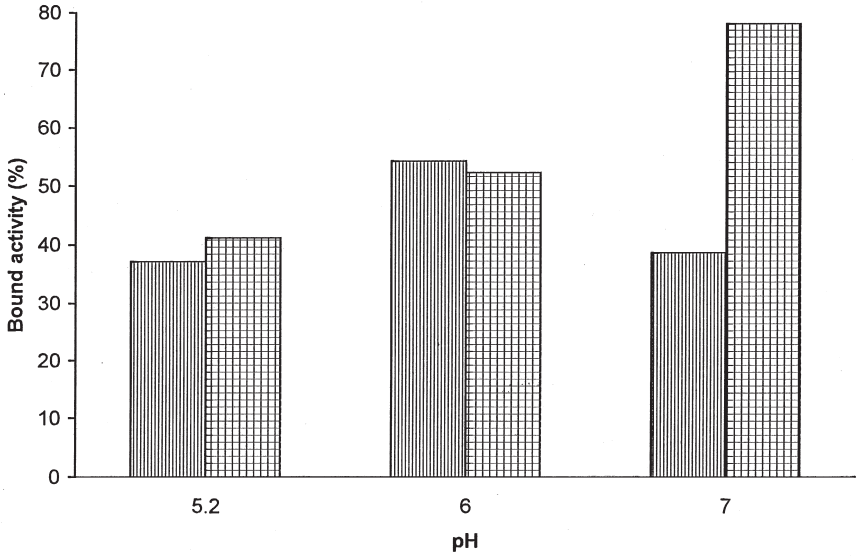


Figure 1. Optimization of binding of fungal α-amylase to two different types of alginate at different pH values. One milliliter (containing 46 U mL^{-1}) of the enzyme was incubated with high (cross-hatched) and low (vertically hatched) mannuronic acid-containing alginate beads at three different pH values. Details of the experiment are given in the text.

Table 3 shows the purification table for α-amylases from different sources. In all the cases, the recovery of the enzyme activity was 80% or above. The chromatography profiles on the fluidized beds for purification of the wheat germ enzyme and the fungal enzyme are shown in Fig. 2. These were the two cases where we started at the crude extract level. In both cases, the purified enzyme preparation showed a single band on SDS–PAGE (Fig. 3). On further analysis, the purified fungal amylase was found to consist of at least two isoenzymes, which could be separated on Con A-agarose column and had different thermal stabilities at 80°C.

Separation of Cellulase on Chitosan Beads

The values of Richardson–Zaki parameters describing the fluidization behavior of chitosan beads are given in Table 1. The slightly high value of n shows that the laminar flow may not be observed with fluidized beds of these beads. However, adsorption isotherms of cellulases from *Aspergillus niger* and *Aspergillus fumigatus* show Langmuir behavior (Fig. 4) and affinity for both cellulases. The maximum capacity, $q_m = 50$ and 1.8 U mL^{-1} for the enzymes

Table 3. Purification of α-Amylases from Various Sources on Fluidized Beds of Alginate Beads. The Conditions for Binding and Elution of the Enzyme Activities Are Described in the "Materials and Methods" Section. The Type of Alginate Beads Used for the Purification of the Various Amylases Is also Described in the Text

Steps	Source	Volume (mL)	Activity (U)	Protein (mg)	Specific Activity ($U\,mg^{-1}$)	Yield (%)	Fold Purification
Crude	(1) *Bacillus amyloliquefaciens*	20	20.0	0.2	102.4	100	1
	(2) Porcine pancreas	20	67.3	0.4	108.1	100	1
	(3) Wheat germ	20	1440.0	45.1	31.9	100	1
	(4) *Scytalidium thermophilum*	15	345.6	1.2	260.5	100	1
Wash	(1) *Bacillus amyloliquefaciens*	30	0	0	—	—	—
	(2) Porcine pancreas	30	0	0	—	—	—
	(3) Wheat germ	30	53	1.43	—	—	—
	(4) *Scytalidium thermophilum*	80	45.8	0.6	—	—	—
Eluate	(1) *Bacillus amyloliquefaciens*	10	18.5	0.01	1424.6	92.5	14
	(2) Porcine pancreas	10	67.1	0.03	1895.9	99.7	11
	(3) Wheat germ	10	1293.0	0.7	1841.8	89.8	58
	(4) *Scytalidium thermophilum*	10	245.0	0.1	2450.3	80.2	9.4

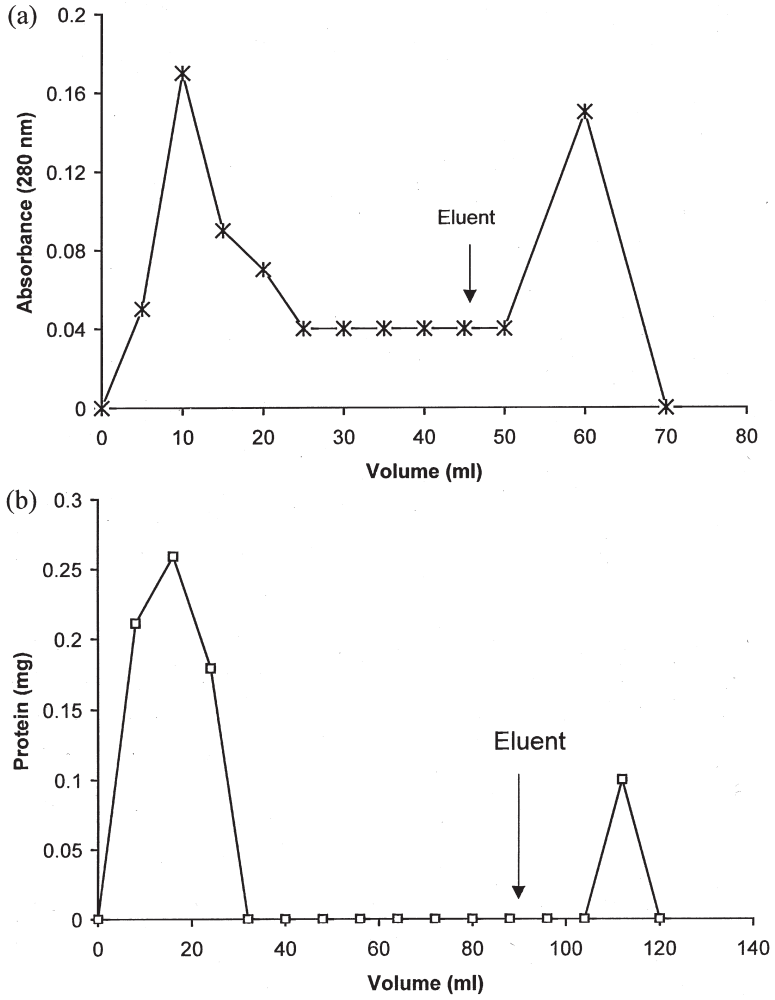


Figure 2. (a) Purification of α-amylase from wheat germ extract on a fluidized bed of alginate beads. The settled bed height was 11 cm and the column had an internal diameter of 1 cm. The degree of expansion was 1.6 at a linear flow rate of 2 mL min^{-1}. Twenty milliliters of the extract (in 0.05 *M* acetate buffer containing 0.006 *M* $CaCl_2$, pH 5.2) containing 1440 U of enzyme activity were introduced in the column after the bed had expanded to the desired height. (b) Purification of α-amylase from *Scytalidium thermophilum* on a fluidized bed of alginate beads. The settled bed height was 11 cm and the column had an internal diameter of 1 cm. The degree of expansion was 1.6 at a linear flow rate of 2.4 mL min^{-1}. Twenty milliliters of the extract (in 0.05 *M* acetate buffer containing 0.006 *M* $CaCl_2$, pH 5.2) containing 346 U of enzyme activity were introduced in the column after the bed had expanded to the desired height.

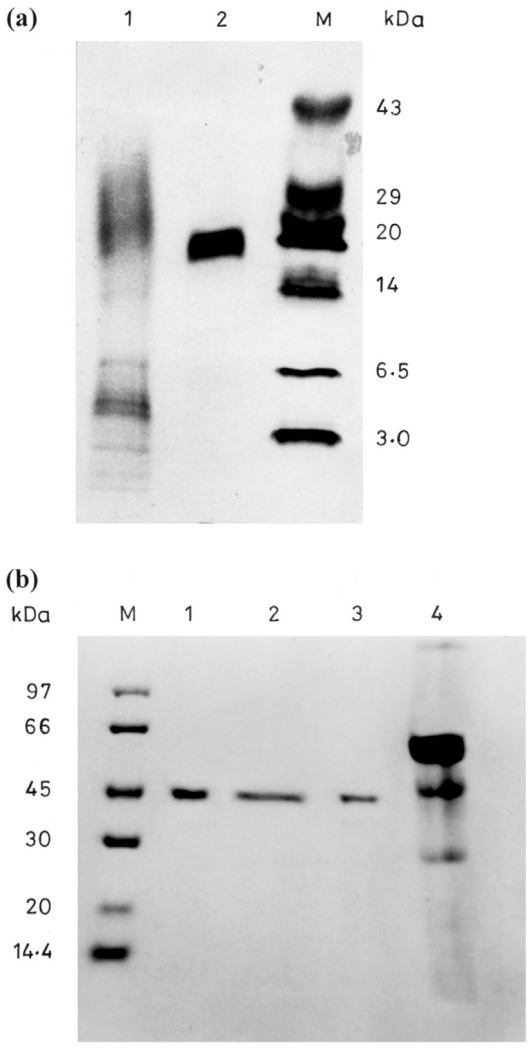


Figure 3. (a) SDS-PAGE pattern of wheat germ amylase: Lane 1. Crude wheat germ amylase. Lane 2. Purified preparation. Lane M. Standard molecular weight marker proteins. (b) SDS-PAGE pattern of fungal amylase: Lane 1. Fraction E I (5 μg) (Eluate from the column of alginate beads). Lane 2. Fraction E II (5 μg) (Part of E I, which did not bind to Con A-agarose). Lane 3. Fraction E III (5 μg) (Part of E I, which bound to Con A-agarose and was eluted with α-methyl mannoside). Lane 4. Crude extract (10 μg). Lane 5. Standard molecular weight marker proteins.

from *Aspergillus niger* and *Aspergillus fumigatus*, respectively. The corresponding values for dissociation constant, K_d are 5 and 28 U mL^{-1}, respectively. In view of the low capacity for the *Aspergillus fumigatus* enzyme, further work on purification was done with *Aspergillus niger* enzyme only. Conditions for elution of cellulase from chitosan beads were optimized in the batch mode. Elution with 1 *M* phosphate buffer, pH 7.0 containing 20% ethylene glycol gave nearly quantitative recovery of the bound enzyme activity (data not shown).

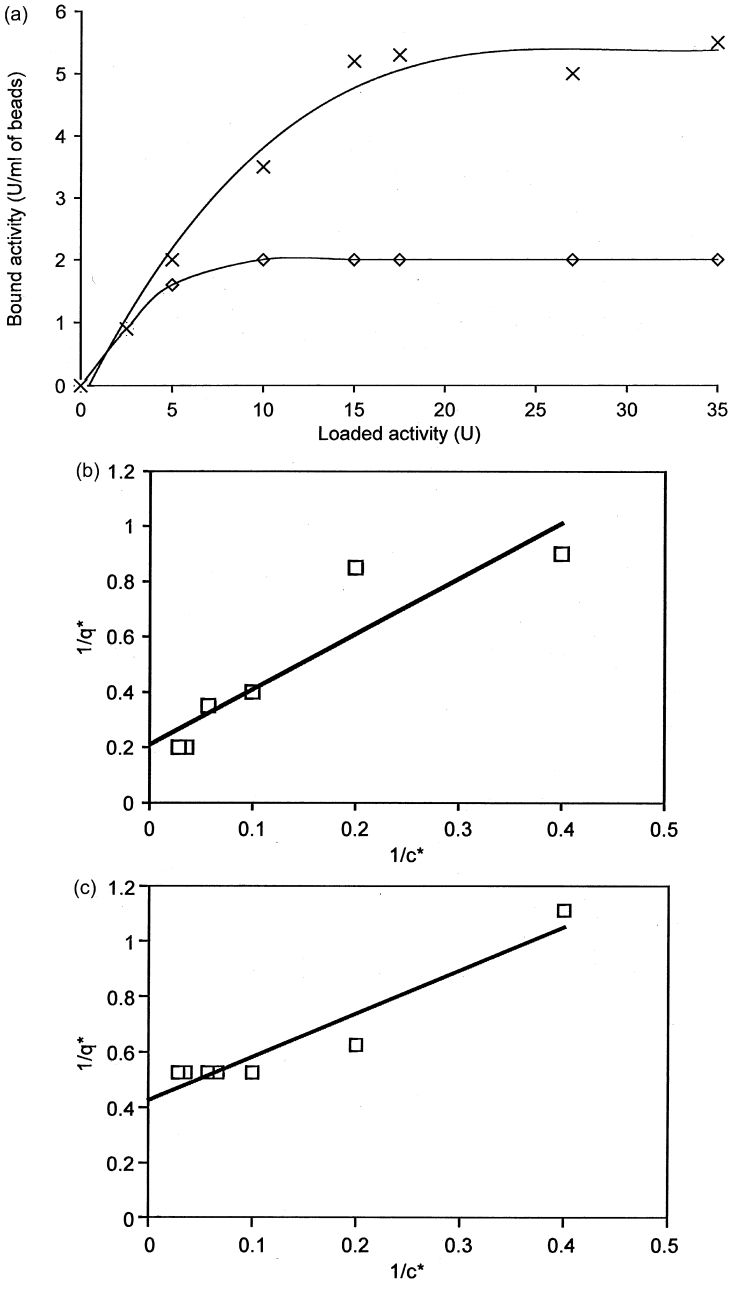
The data concerning purification of *Aspergillus niger* cellulase on fluidized bed of chitosan beads are shown in Table 4 and Fig. 5. About 80% recovery with 30-fold purification in a single step shows this to be a powerful approach for purification of cellulases. The SDS–PAGE of the purified enzyme shows a single band (Fig. 6). The purified enzyme was found to be free of β-glucosidase activity.

CONCLUSION

To summarize the results presented here, calcium alginate beads were found to affinity capture α-amylases from various sources. Similarly, chitosan beads were found to bind to cellulases. In all the cases, a single unit process consisting of EBAC yielded a purified product which, when analyzed on SDS–PAGE, showed a single band. It is noteworthy that the existing purification protocols in the case of most of these enzymes are multi-step procedures (27,28). Thus, these EBAC-based protocols are fast and economic alternatives. The significant finding of this work, however, has been that the polysaccharides having no "biological relationship" with the target enzyme seem to act as macroaffinity ligands. In fact, in both the cases, even chemical relationship between the macroaffinity ligand and the substrate is quite remote. Alginate is chemically quite different from starch and chitosan is chemically quite dissimilar from cellulose, the corresponding natural substrate.

Table 4. Purification of Cellulase from a Commercial Preparation Using a Fluidized Bed on Chitosan Beads. The Conditions of Binding and Elution of the Enzyme Are Described in the "Materials and Methods" Section

Steps	Volume (mL)	Activity (U)	Protein (mg)	Specific Activity (U mg^{-1})	Yield (%)	Fold Purification
Crude	20	18.0	1.9	9.7	100	1
Wash	30	0.0	1.1	—	0	—
Eluate	10	14.4	0.05	290.3	80	30



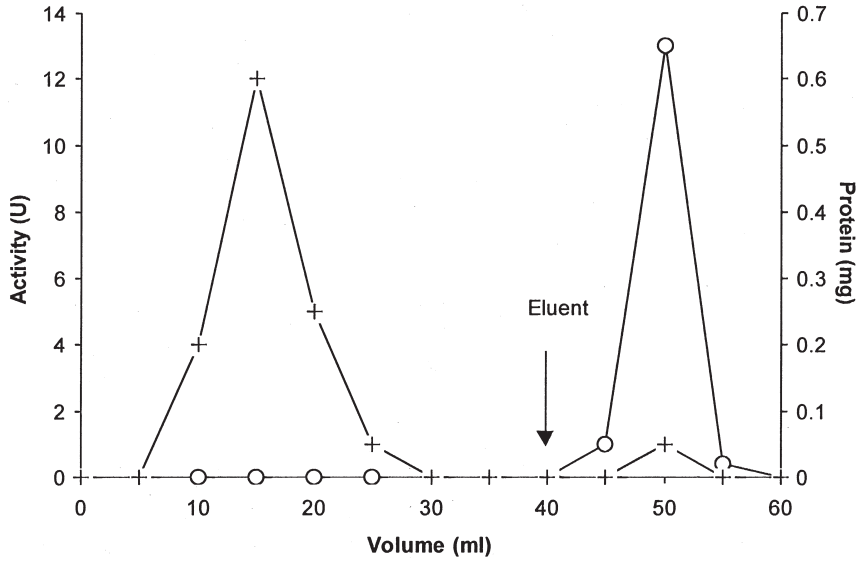


Figure 5. Fluidized bed purification of cellulase (*Aspergillus niger*) on chitosan beads. The settled bed height was 11 cm and the column had an internal diameter of 1 cm. The degree of expansion was 1.45 at a linear flow rate of 3 mL min^{-1}. Twenty milliliters of the extract (in 0.05 *M* acetate buffer, pH 6.0) containing 18 U of enzyme activity were introduced in the column after the bed had expanded to the desired height.

Figure 4. Equilibrium adsorption isotherms (a) for adsorption of cellulases [× , from *Aspergillus niger* (stock solution of 0.815 U mL^{-1}) and ◇, from *Aspergillus fumigatus* (stock solution of 0.6 U mL^{-1})] in 0.05 *M* acetate buffer, pH 6.0, to chitosan beads at 25°C. The stock solution of cellulases was made in 0.05 *M* acetate buffer, pH 6.0. Different volumes of the stock solution were made up to a total volume of 2.5 mL with the buffer and added to the chitosan beads (3 mL after decanting the excess liquid). The systems were incubated overnight on a shaker at 25°C. After this, a 0.5 mL aliquot was removed from each tube and the cellulase activity was determined in the supernatant to calculate the amount of cellulase activity bound to the chitosan beads. Centrifugation of the samples was not found necessary since the beads settle down completely on standing. Insets show the Langmuir model fit for the cellulases from *Aspergillus niger* (b) and *Aspergillus fumigatus* (c). Each set was run in triplicate and the difference in individual readings in each set was less than 5%.

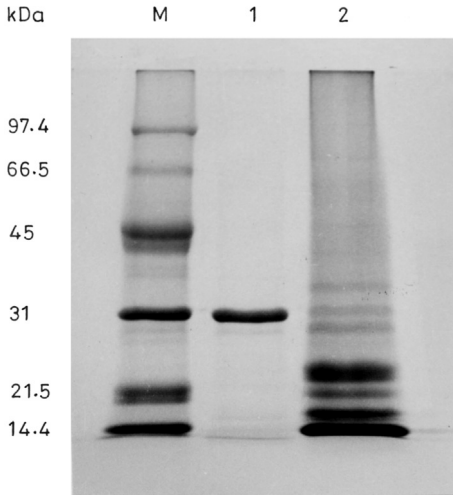


Figure 6. SDS-PAGE pattern of cellulase: Lane 1. Commercial cellulase. Lane 2. Purified preparation. Lane M. Standard molecular weight marker proteins.

The results described here also show that this unexpected molecular recognition is not random or nonselective in nature. It is not as if alginate or chitosan will bind to any (or many) enzyme(s). Even if they do, some selectivity is possible at the elution stage, e.g., elution of amylases from alginate with maltose. This is proved by the fold purification achieved in all these cases.

Hence, the approach outlined here demonstrates that it may be worthwhile to look for biological affinity, even in unexpected quarters, at least in the case of carbohydrate-degrading enzymes. A large number of polysaccharides occur in nature. These constitute a reservoir of economical and often nontoxic (compatible with regulatory requirements in food industries) affinity ligands. This should, in turn, make affinity chromatography, particularly in fluidized bed mode, a still more attractive technique for tomorrow.

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