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Phenylboronate-Chitosan Resins for Adsorption of β-Amylase from Soybean Extracts

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Abstract

Isolation and purification of bioproducts from crude extracts can be obtained by affinity methods based on reversible binding of a specific molecule to ligand immobilized in a porous matrix. In the present work, nicrospheres based on chitosan matrix, which incorporated aminophenylboronic acid as a derivative, were prepared and characterized, aimed at developing a β-amylase adsorption process. Kinetic curves and adsorption isotheriru of the crude extracts as well as the breakthrough curves for a frontal chromatographic separation method of a commercial sample of β-amylase from soybean are presented. These results were compared to similar data obtained with a comercial microspheres gel based-on agarose.

Index Entries: Purification; β-amylase; soybean; phenylboronate; chitosan.

Introduction

Interest in bioproducts has been increasing as a result of biotechnological development. Industrial demands for new applications, specificity, and renewability of products have also increased dramatically. Because of the expansion of downstream processing in biotechnology, the methods aimed at concentration and purification of enzymes and biopolymers after their production from a variety of sources require new advances *(1–3)*. β-Amylase (β-1,4-glucanmaltohydrolase, EC 3.2.1.2) is an exoenzyrne that removes units of nonreduced maltose terminals from polysaccharide chains, producing β-maltose and β-limit dextrins. There is a considerable industrial interest in this enzyme for the production of syrups rich in mal-

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tose. Separation and purification of β-amylase still require new methods because of the expansion market.

The phenylboronate ligand, which is used in affinity separation of some diol-containing analytes, presents the following main characteristics: high connection capacity, good flexibility under conditions of adsorption and elution (usually sorbitol), chemical and biologic stability, and lower cost than that of natural lectins *(4)*.

Chitosan, poly(D-glucosainine), is obtained by deacetylization of chitin, poly(*N*-acetyl-D-glucosamme). Chitosan dissolves in acids of medium strength and its molecule contains an anine group used extensively in chemical modifications *(5)* for several purposes. Compared with other immobilization matrices, the chitosan matrix has wider pores and a larger surface area, which allows new preparation conditions to be devised *(6)*, and larger amounts of enzyme to be immobiized under the same reaction conditions. Compared to the other tested matrices, chitosan showed the best performance, owing to lower resistance to diffusion and higher fiow rates *(7)*. Chitosan is similar to amylose in its structure, and it was chosen for its potential in imparting specificity to the separation *(8)*.

Batista-Viera et al. *(9)* described reversibility for adsorption and elution as well as covalent immobilization of soybean amylase on agarose-phenylboronate in their discussion of the potential recovery possibility for purification of β-amylase from soybean extracts by affinity methods. The present article describes our study of two ways to extract β-amylase from soybean to optimize the extraction mode. We have also synthetised an affinity phenylboronate-chitosan (PBC) adsorbent and studied several aspects of the interaction mechanism involved in the recognition of β-amylase on the PBC sorption material. Aiming at a possible scale-up of the process, we studied the adsorption kinetics and isotherm and the breakthrough curves in a frontal mode. The prepurification, adsorption, and elution steps were dialyzed by quantification of the specific activity during the various steps, aiming at the purification of soybean β-amylase.

Materials and Methods

Chitosan–crab shells of a practical grade and sodium borohydride were obtained from Sigma (St. Louis, MO), 3-aminophenylboronic acid and dinitrosalicylic acid (DNS) from Aldrich (Milwaukee, WI), phenylboronate-agarose (PBA-30) from Amicon (Denver, CO), and Coomassie blue reagent from Bio-Rad (Richmond, CA). All other chemicals used were of reagent grade.

Preparation of Chitosan Microspheies and Adsorbent

The required volumes (usually 250 mL) of an aqueous solution containing chitosan-free base were prepared: Dissolving chitosan-free base in acetic acid produced chitosan acetate salt. The spray method was used with a standard 0.5 mm SSBR-JBC nozzle (Spraying Systems,

Campinas, Brazil). When the liquid was fed into the nozzle with a peristaltic pump, atomization occurred owing to the force of the compressed air, separating the liquid into small droplets. The product was then collected. Under standard conditions, the temperature, spray flow, and compressed spray airflow (represented as the volume of air input per unit of time) were set at 25°C, 6 mL/min, and 10 L/min, respectively *(10)*. Noncrosslinked chitosan atmospheres were prepared using a spray method by adding 2.5% (w/w) chitosan solution in 0.75 *N* acetic acid to 1 *N* NaOH and were left to stand for 15 min. The microspheres prepared in this way were highly spherical and had a smooth but distorted surface morphology. Preparation conditions also had some influence on particle size. Porosity is a very important property of chitosan matrices and can be changed using different experimental conditions during preparation (stirring, ratio of chitosan solution in AcOH/NaOH, temperature, stabilizer, and crosslinking).

Synthesis was carried out in three stages: preparation of the matrix, activation, and derivatization (with reduction). Preparation of the matrix was a process of obtaining porous particles from commercial chitosan. Crosslinking and activation of the matrix involved reaction with a dialdehyde agent. The derivatization was a reaction between aldehyde groups in the matrix and amine groups in the ligand, 3-aninophenylboronic acid, under mild conditions and the final reduction to eliminate unsaturated sites. The resulting microparticulate gel (beaded noncrosslinked chitosan) was conditioned overnight in 50 m*M* phosphate buffer at pH 8.0 and crosslinked and activated for 6 h at 4°C, using a ratio 0.45 mol/mol aldehyde/amino groups ratio. The activated matrix was washed with phosphate buffer. Then a solution containing 16 μmol of boronic acid/mL was added, and the ligand was immobilized for 72 h at 4°C under stirring (175 rpm). The immobilization density of the ligand in the chitosan matrix was evaluated using ultraviolet (UV) absorption at 280 nm through the determination of ligand concentration in solution before and after immobilization *(11)*. The PBC resin obtained was washed with distilled water and reduced with sodium borohydride at 4°C.

Characterization of Adsorbent and Batch Experiments

Particle size distribution of the PBC adsorbent was obtained through the light-scattering method using Malvern Mastersize equipment. The total micropore and mesopore volumes of the dried and crosslinked chitosan matrix were determined by nitrogen adsorption (Brunauer, Emmet, and Teller [BET] method) and desorption methods. Batch adsorption experiments were carried out in Eppendorf tubes to characterize the effects of pH and ionic strength on adsorption capacity.

Extraction of β-Amylase from Soybean

To verify the extraction efficiency and the content of β-amylase in soybean (PL-1/IAC, Brazil), extraction procedures developed by Smith et al. *(12)* and Rai et al. *(13,14)* were used. Extraction was done with 1 g of defatted

soybean flour, and diluted with buffer to obtain a protein solution used in capillary electrophoresis.

Capillary Electrophoresis

The equipment used was Beckman P/ACE model 5010 in CGE mode with an uncoated, fused silica capillary tube (47 cm × 75 μm). Samples were dissolved in 120 m*M* Tris-HC1 buffer, pH 6.6, containing 1% (w/w) sodium dodecyl sulfate. Beckman provided the run gel buffer. Voltage was 14.1 kV at 20°C. The extracts were lyophilized for further use.

Determination of Protein

The total protein content was determined by the Bradford *(15)* method, using bovine serum albumin as a reference.

Determination of β-Amylase Activity

β-amylase activity in the extracts was determined by the Bernfeld *(16)* method, in 125 m*M* acetate buffer, pH 5.0. One unit of β-amy1ase (activity unit [AU]) was defined as the amount of enzyme that catalyzed formation of 1 μmol of maltose per minute using 1 mL of enzyme solution under given conditions. In a typical batch determination, 400 μL of 2.5% (w/w) starch solution was added to 400 μL of 125 m*M* acetate buffer, pH 5, with 200 μL of the enzyme solution and the solution was immediately incubated at 60 °C for 30 min. After 4 mL of reagent DNS was added, the reagent was mixed vigorously and boiled for 15 min in a bath, and the resulting sample was collected in an ice bath. The UV absorbance was measured at 640 nm against a blank with the same component mixture without the enzymatic solution.

The calibration curve of maltose (0.2–2 mg/mL) was prepared for conversion of spectrophotometiic readings of the enzyme activity. The specific activity was expressed as the activity of β-amylase/mg of total protein.

Kinetic Curves and Adsorption Isotherms

The experimental procedures for determination of both the kinetic and isotherm curves were similar. Setups containing syringes and disposable pistons with retention were used. Samples with the adsorbent and the enzyme solution were placed in the syringes and conditioned in 25 m*M* phosphate buffer, pH 6.8, containing 0. 1 *M* NaCl (adsorption buffer) for 1 h with temperature control. In those experiments, I mL of the enzyme solution mixed with the adsorbent and 15 mg of the lyophilized extract diluted in 50 mL of phosphate buffer were added and placed on a rotation device (10 rpm). The revolution simulates an agitated tank and allows dispersion and homogenization of the suspension. Samples were withdrawn at a few minutes' intervals for determination of the adsorption kinetics and after 180 min for acquisition of the adsorption equilibrium data. The supernatant was evaluated in tern of total protein and activity as the amount of total protein or activity. For each concentration, the capacity was determined as the amount of total protein or activity (protein mg, or AU) con-

tained in 1 g of the adsorbent. The equilibrium data were fitted with the Langmuir model *(17)*.

Chromatographic Experiments

Isocratic Elution

The PBC and phenylboronate-agarose (Matrix gel PBA-30; Amicon) adsorbents were conditioned in a column and tested. A Pharmacia HR 5/5 column (0.5-cm diameter) was used and coupled to a Shimadzu high-performance liquid chromatography system and a data acquisition system. The tests were carried out with 0.85 g of each adsorbent (ie., 6 cm of the adsorbent bed in the column) at a flow rate of 0.25 mL/min. A sample of a prepurifled and dialyzed soybean β-amylase was injected into the column and preequilibrated with 25 m*M* phosphate buffer (pH 6.8). The same buffer was used for isocratic elution with 0.1 *M* sorbitol and 0.1 *M* NaC1, or 0.5 *M* NaCl for PBA-30 and PBC, respectively, at a flow rate of 0.25 mL/min. The eluted phase was collected in 1-mL fractions and samples were analyzed for protein concentration and activity.

Frontal Analysis

In the fixed-bed adsorption experiments, soybean extract was continuously fed into the column containing the solid phase, formed by beads of the PBC adsorbent. The feed was maintained until the desired product (β-amylase) appeared in the effluent at the feed concentration. The column was packed with 0.5 g of the adsorbent beads and had a diameter of 0.5 cm, a length of 2 cm, and a constant operational flow rate of 0.5 mL/min.

Results and Discussion

Physicochemical Characterization of Adsorbent

The particle size distribution of the prepared adsorbent was measured through a Malvern 3100 Light Scattering Instrument. The PBC adsorbent contained particles in the range of 10–300 μm with a wide size distribution profile and an average Sauter diameter of 98 μm. The particle size distribution of the coninercial boronate-agarose adsorbent showed a bimodal distribution with a predominance of particles in the range of 40–200 μm, with an average Sauter diameter of 110 μm. The micropore and mesopore size distribution characteristics of the various reticulated matrices are presented in Figs. 1 and 2, for several molar ratios of glutaraldehyde (-CHO)/chitosan matrix ($-NH_2$). PBC adsorbent with a molar ratio of 0.45 was prepared. Cumulative pore volume increased with molar ratio, and pore sizes between 50 and 100 nm were responsible for a great part of the total pore volume.

During the drying of the swollen adsorbent containing water, the pore structure collapses and data obtained do not describe porosity of the original adsorbent. To study the contribution of the matrix-spacer arm and ligand in the adsorption, the influences of pH and of ionic strength were

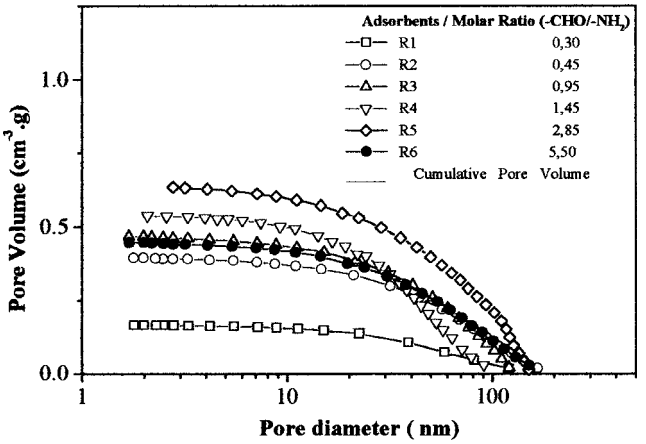

Fig. 1. Cumulative pore volume of chitosan matrix crosslinked with glutaraldehyde.

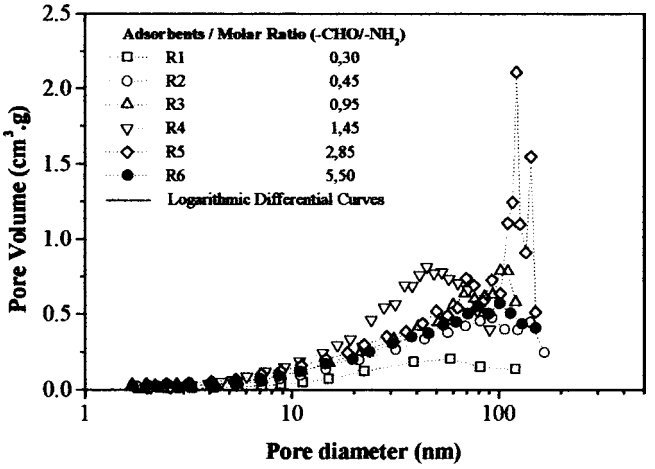

Fig. 2. Logarithmic differential pore volume of chitosan crosslinked with glutaraldehyde.

evaluated (Figs. 3 and 4), Figure 3 shows the effect of ionic strength on total protein adsorption capacity and activity in terms of NaCl content at a fixed pH 6.8. A similar behavior was observed for PBC, with a minimum capacity at an NaCl concentration of about 0.5 *M*, in terms of both total protein and activity. For PBA-30 there was a continuous increase in both capacities with increasing ionic strength. Evaluation of the adsorbents in relation to pH showed a typical behavior for the adsorption of β-amylase at the isoelectric point of the molecule and in its vicinity. As can be seen in Fig. 4, the PBC adsorbent had an optimum pH value for adsorption for both total protein and activity of about 5.5, which is equivalent to the isoelectric point of soybean β-amylase. For the PBA-30 adsorbent, minimum adsorption capacity of total protein occurred at a pH of 7.0, while a capacity in terms of activity decreased continually with an increase in pH. The differ-

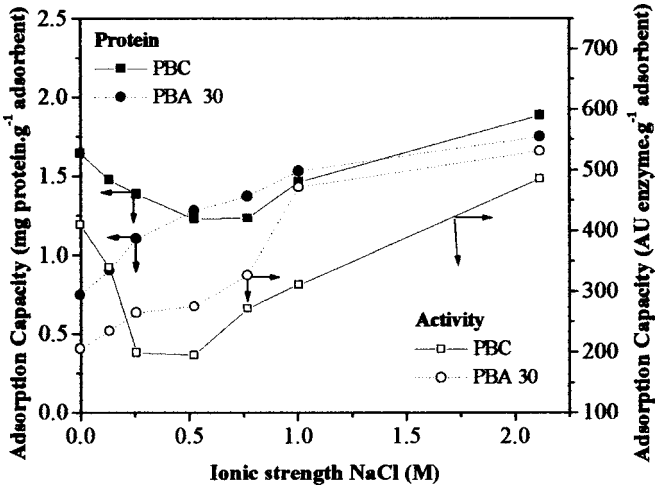

Fig. 3. Effect of ionic strength on adsorption capacity of adsorbents (pH 6.8).

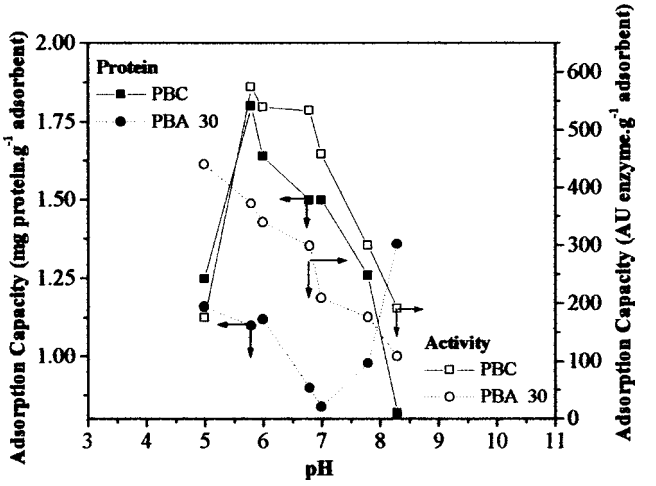

Fig. 4. Effect of pH on adsorption capacity of adsorbents (ionic strength of 0. 1 *M* NaCl).

ences in ionization of the adsorbents have an influence on the amount of β-amylase adsorbed in the studied pH range.

Interestingly, because of so-called secondary interactions (ionic, charge transfer, hydrophobic, and hydrogen bound), phenylboronate ligand can be used for purification of nonglycosylated proteins, such as in the case of soybean β-amylase. Both adsorbents retained β-amylase at low concentrations of buffer and salt. This reflected a recognition mechanism based on the formation of an ion pair between the ligand and β-amylase (ionic interactions). However, secondary interactions, such as hydrogen bridges, and also hydrophobic interactions cannot be excluded. In fact, Figs. 3 and 4 show

a maximum adsorption efficiency for PBC with no NaCl in the buffer; this decreased to a minimum adsorption in the NaCl concentration range of 0.25–I *M*, and again increased for an NaCl concentration higher than 1 *M*. By contrast, for PBA-30 it was necessary to use at least 0.1 *M* NaCl to reach the same yield. The hydrophobic contribution was more pronounced in PBA-30, and an increasing amount was adsorbed as ionic strength increased. The behavior of the total protein adsorbed is identical to the activity, which shows that the adsorbed chemical species maintain a close relationship. A similar behavior was reported *(19)* for the system immunoglobulin G on agarose and histidine.

It is worth pointing out that nitrogen groups can be protonated, depending on the pH in both cases reported (chitosanglutaraldehyde-phenylboronate and agarose-carbodiimide-histidine), and, therefore, this is a way to modify the intensity and the type of interaction of the matrix and ligand (i.e., ion exchange).

Characterization of the Soybean Extract

Figure 5 displays an electrophoretogram for two soybean protein extracts obtained with the two extraction methods described. The migration times of β-amylase (58.8 kDa) and trypsin inhibitor (20.7 kDa) molecules were 23.34 and 19.67 min, respectively. The composition of Ren et al.'s *(13)* extracts was approx 77% trypsin inhibitor and 21.5% β-amylase. The extraction methods for the fractions of soybean β-amylase, described earlier, showed differences in selectivity. The method proposed by Smith et al. *(12)* showed less selectivity, extracting the same number of molecular species of low as well as of high molecular mass (3–300 kDa). In addition to selectivity, the extraction method proposed by Ran et al. *(13,14)* restricted the present molecular species to a quite narrow range of molecular masses (60 kDa).

Adsorption Kinetics and Isotherms

Kinetic curves were obtained for both adsorbents for total protein and activity. Figure 6 shows typical results. A fast decrease in the protein concentration and initial activity—70 and 50% for PBA-30 and 90 and 70% for PBC, respectively—can be observed during the first 25 min. This demonstrates a higher capacity and a faster kinetics of adsorption for PEC, probably caused by a higher density of the ligand and a smaller resistance to diffusion because of wider pores. Experimental data on equilibrium adsorption in the batch procedure are shown in the isotherms depicted in Fig. 7. The adsorption capacity of the adsorbents in terms of activity were adjusted as a function of the enzyme activity by the model proposed by Langmuir *(17)*, described by Eq. 1 as

$$q^* = \frac{q_m c^*}{(k_d + c^*)} \tag{1}$$

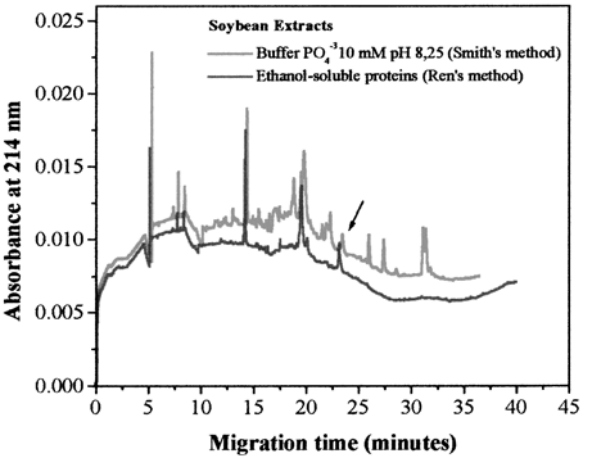

Fig. 5. Electrophoretogram for two soybean extracts obtained with extraction methods described.

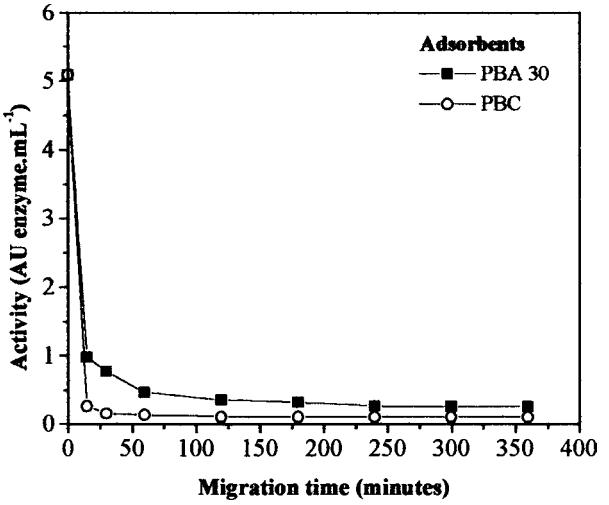

Fig. 6. Kinetic curves for PBA-30 and PBC adsorbents measured in batch experiments.

in which q^* (mg/g) is the total protein adsorbed per g of the adsorbent, c^* (mg/mL) is the concentration of the protein in the solution, q_m (mg/g) is the maximum capacity of the adsorbent, and k_d (mg/mL) is the dissociation constant. A fitting to experimental data was obtained by nonlinear regression for the PBA-30 and PBC adsorbents for both the crude and dialyzed extracts. Table 1 gives the parameters calculated for the isotherms obtained with the crude extract, and Table 2 displays the results for the dialyzed soybean extract. The values of the parameters obtained indicate that the adsorbents have similar adsorption capacities and PBC has greater affinity characteristics than PBA-30.

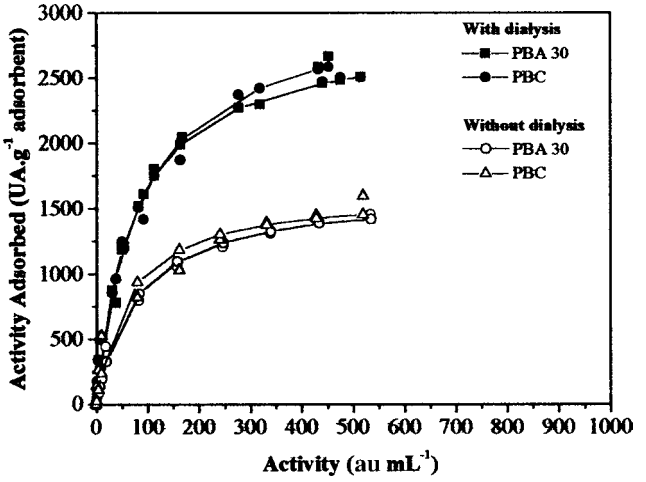

Fig. 7. Activity isotherms at 25°C, pH 6.8, and ionic strength of 0. 1 *M* NaCl carried out in batch. Langmuir parameters for crude extracts for PBA-30 are: q_m = 1617 AU/g, k_d = 75.92 AU/mL; and for PBC are q_m = 1626 AU/g, k_d = 59.32 AU/mL. For dialyzed extracts for PBA-30 they are: q_m = 2865 AU/g, k_d = 86.23 AU/mL; and for PBC are q_m = 3082 AU/g, k_d = 72.22 AU/mL.

Table 1
Langmuir Parameters of Adsorption Isotherms with Crude Extract

	Total protein			Total activity		
Adsorbent	q_m (mg/g)	k_d (mg/mL)	R^2	q_m (AU/g)	k_d (AU/mL)	R^2
PBA-30	9.83	0.61	0.96	1.617	75.92	0.98
PBC	12.62	0.43	0.95	1.625	59.32	0.97

Table 2
Langmuir Parameters of Adsorption Isothern with Dialysed Extract

	Total protein			Total activity		
Adsorbent	q_m (mg/g)	k_d (mg/mL)	R^2	q_m (AU/g)	k_d (AU/mL)	R^2
PBA-30	39.18	1.54	0.95	2.865	86.23	0.99
PBC	39.02	0.68	0.97	3.083	72.22	0.97

Breakthrough Curves

Figures 8 and 9 display the breakthrough curves for PBA-30 and PBC adsorbents, determined in frontal chromatographic experiments. Curves of total protein concentration and β-amylase activities are shown for crude and prepurified systems to demonstrate the effect of contaminants on com-

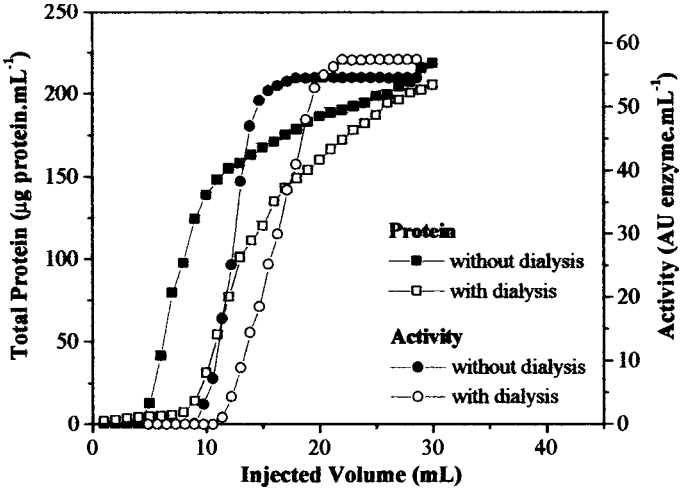

Fig. 8. Breakthrough curves for crude soybean extracts flowing through PBA30. Column height is 2 cm, and column diameter is 0.5 cm. Mass of adsorbent is 0.5 g and flow rate is 0.5 mL/min.

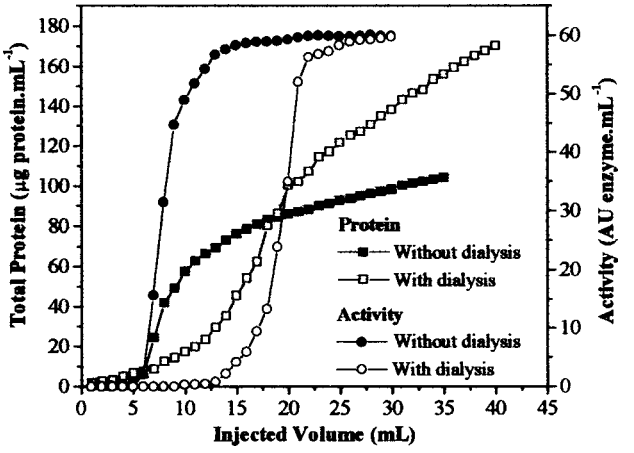

Fig. 9. Breakthrough curves for PBC. Column height is 2 cm, and column diameter is 0.5 cm. Mass of absorbent is 0.5 g and flow rate is 0.5 mL/min..

petition at the adsorption sites. The behavior of the activity breakthrough curves is typical of one-component ideal adsorption.

Enzyme Punfication Protocol

In a typical experiment with the aim of evaluating the quality of the adsorbent (PBC) and comparing it with that of phenylboronate-agarose (PBA-30), 200 μL with 180 U of the enzyme and 0.93 mg of total protein was

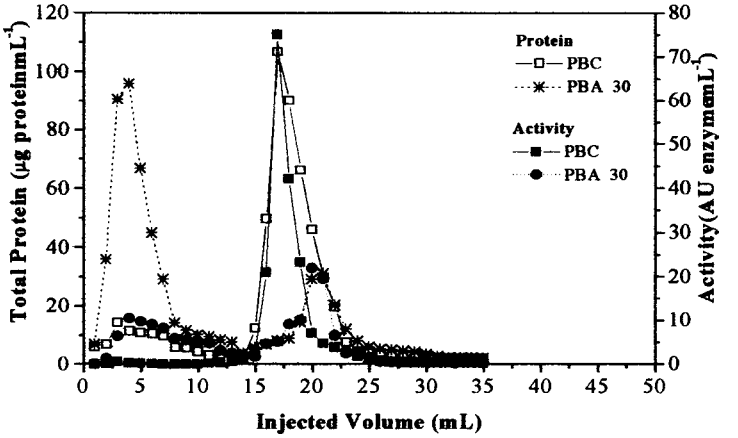

Fig. 10. Comparison of elution curves of prepurified soybean extracts for PBA-30 and PBC. Isocratic elution was done with 0.1 *M* sorbitol and 0.1 *M* of 0.5 *M* NaCl, respectively, and a flow rate of 0.25 mL/min.

injected into the columns filled with PBA-30 and PBC, respectively, both the same adsorbent and bed height described in the procedures above. As shown in Fig. 10, after elution, fractions of the eluted peak had a purification factor at a specific activity of approx 5 for both PBA-30 and PBC. For the pool of fractions eluted, the enrichment in specific activity was 4 (12.2%) and 4 (41.7%), respectively. The isocratic elution of the enzyme in the column of PBA-30 showed a well-defined peak. The different behavior of PBA-30 in the process of adsorption of the soybean proteins cannot be explained only by the difference in density of the ligands of the adsorbents, because the matrix and spacer are different. For the same matrix and spacer the specificity of the adsorbent with pheny1boronate depends first on the quality of the ligand, and second on ligand density *(20)*.

To verify the contribution of each step in the whole purification process, total protein content and activity were observed dialyzed extract all the prepurification (concentration) and purification steps. Figure 11 displays a flow sheet of the complete purification scheme, including the prepurification and adsorption steps. Table 3 shows the evolution of those variables in the prepurification steps. The purification factor in these preliminary schemes reached a value of 25. The results of the adsorption experiments applied to the prepurification steps, presented in Table 4, indicate a further 4-fold enrichment of the enzyme's specific activity, leading to a 97-fold enrichment in the overall enzyme purification process.

Conclusion

The affinity PEC adsorbent, which was synthesized with up to 100 μmol/mL, was compared with the commercial phenylboronate-agarose adsorbent (PBA-30), containing 51 μmol/mL with precipitated extract of commercial soy and dialysate with β-amylase. Both adsorbents showed

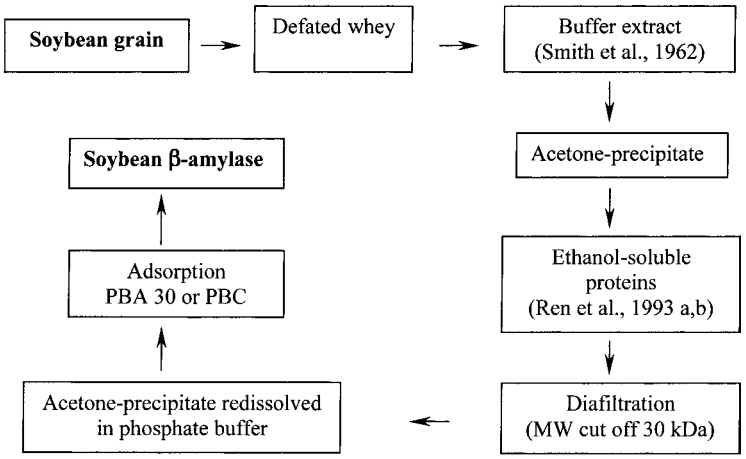

Fig. 11. Flow sheet of prepurification and absorption steps.

Table 3
Prepurification Steps Starting from Deffated Soybean Flour

Fraction	Volume (mL)	Protein (mg/mL)	Activity (AU/mL)	Total activity (AU)	Specific activity (AU/mg)	Yield (%)	Purification (fold)
Buffer extract	12.0	15.70	134.4	1,61	8.6	100	1
Ethanol-soluble proteins	12.0	1.08	105.0	1,26	96.7	78.1	11
Diafiltration (mol wt cutoff of 30 kDa)	8.6	0.86	139.0	1,19	161.6	74.1	19
Acetone precipitate redissolved[a]	1.0	4.67	1,00	1,00	215.2	62.3	25

[a] Acetone precipitate = prepurified soybean extract.

Table 4
Adsorption Steps Applied to Prepurified Extract

Fraction	Volume (mL)	Protein (mg/mL)	Activity (AU/mL)	Total activity (AU)	Specific activity (AU/mg)	Yield (%)	Purification (fold)
Acetone precipitation	0.20	4.67	900	180	192	100	1
PEA-30	1	0.029	22	22	759	12.2	3.9
PBC	1	0.100	75.1	75.1	752	41.7	3.9

viability for the enzyme purification procedures. The extraction of buffered alcoholic solutions was shown to be advantageous for obtaining a narrower range of molecular masses (60 kDa). The interaction was shown to be complex and competitive as a result of formation of an ionic pair

between ligand and matrix. Otherwise, interactions such as hydrophobic and hydrogen bridges, should not be disregarded. The kinetic experiments indicated the greater affinity of PBC for the extract and less restriction of diffusion in PBC because of wider pores. The isotherms were fitted to the Langmuir model over the whole range of concentrations tested and showed high values for the dissociation constants, which suggests that competition with another molecular species for the ligand in the adsorption of soybean β-amylase does exist. The breakthrough curves for total protein suggest a competitive adsorption mechanism with a possible multilayer modeling attack. The adsorption steps, performed in columns and coupled to prepuriflcation steps, resulted in a total purification factor of 97 for the specific activity of soybean β-amylase.

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