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Rapid protein purification using phenylbutylamine–Eupergit: a novel method for large-scale procedures

V. KASCHE*
AB Biotechnologie II, Technical University of Hamburg–Harburg, Postfach 901403, D-2100 Hamburg 90 (F.R.G.)
F. LÖFFLER
Röhm GmbH, Research Laboratories, Postfach 4242, D-6100 Darmstadt (F.R.G.)
T. SCHOLZEN
AB Biotechnologie II, Technical University of Hamburg–Harburg, Postfach 901403, D-2100 Hamburg 90 (F.R.G.)
and
D. M. KRÄMER and Th. BOLLER
Röhm GmbH, Research Laboratories, Postfach 4242, D-6100 Darmstadt (F.R.G.)

ABSTRACT

Electrophoretic desorption was used to compare the protein binding capacities of some hydrophobic adsorbents [the phenylbutylamine (PBA) derivatives of Eupergit C and agarose and Phenyl-Sepharose] for low-pressure chromatography. The highest capacity was observed for the bifunctional adsorbent PBA–Eupergit. The hydrophobically adsorbed proteins can be selectively desorbed by decreasing the pH of the eluent due to electrostatic repulsion between positive charges on the adsorbed proteins and positively charged secondary amines on the adsorbent. This was used to purify 1500 U penicillin amidase from *E.coli* homogenates per gram wet weight of PBA–Eupergit in 50 adsorption–desorption cycles without organic solvents (>90% yield, purification factor = 5.3).

INTRODUCTION

Hydrophobic adsorbents can be used for the selective adsorption of extracellular proteins from fermentation media or intracelular proteins from cell homogenates[1–4]. The adsorbed proteins can then be selectively desorbed by either decreasing the ionic strength or increasing the content of hydrophobic organic solvents in the buffer. In the former instance complete desorption of very hydrophobic proteins is difficult, and in the latter the rate of protein denaturation is generally increased. The hydrophobic solvents must therefore be removed from the protein in subsequent purification steps and recycled.

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Bifunctional adsorbents where the adsorption is governed by hydrophobic interactions and the desorption is caused by electrostatic repulsion between the support and the protein offer an alternative[1,5]. Such adsorbents can be formed when a secondary amine is produced during the covalent immobilization of the hydrophobic ligate to the support. With decreasing pH the density of positively charged groups on the support increases. The same applies for proteins. Hence hydrophobically adsorbed proteins could be desorbed by increasing the electrostatic repulsion simply by changing the pH of the aqueous eluent below the isoelectric point. When this repulsion is stronger than the hydrophobic adsorption, the use of organic solvents or denaturing agents[5] can be avoided in the desorption step.

The aim of this study was to investigate whether such bifunctional adsorbents can be formed by covalent immobilization of phenylbutylamine to Eupergit C and other supports. Their application to the preparative isolation of penicillin amidase from *E. coli* homogenates, without organic solvents, was also investigated.

EXPERIMENTAL

Materials

α-Chymotrypsin (CT, EC 3.4.21.1; Worthington CDI, Freehold, NJ, U.S.A.), trypsin (TRY, EC 3.4.21.4; Merck 8350, Darmstadt, F.R.G.), N-acetyl-L-tyrosine ethyl ester (ATEE; Sigma, St. Louis, MO, U.S.A.), phenylbutylamine (PBA; Aldrich, Milwaukee, WI, U.S.A.), N-benzoyl-L-arginine ethyl ester (BAEE; Merck), 2-nitro-5-(phenylacetamido)benzoic acid (NIPAB; Sigma), penicillin G (Welding, Hamburg, F.R.G.), Phenyl-Sepharose (Pharmacia), phenylbutylamine–agarose (Sigma) and Eupergit C (Röhm, Darmstadt, F.R.G.) were used as purchased. Alkaline protease was provided by Röhm. Penicillin amidase (PA) from *E. coli* (EC 3.5.1.11) was purified from crude enzyme preparations as described[6]. All other chemicals were of analytical-reagent grade.

Preparation of phenylbutylamine–Eupergit C

Eupergit C was added to a 1 *M* solution of PBA in ethanol and boiled for 4 h. The support was washed with ethanol and water until the UV spectrum of the eluate was negligible, then boiled for 30 min in 0.5 *M* sulphuric acid and washed with water. The amount of PBA immobilized to the support was calculated from the mass balance determined from the UV spectra and volumes of the stock and washing solutions used. PBA–Eupergit was stored at 4°C in water.

Enzyme activities

The CT, TRY and PA activities were determined using ATEE, BAEE and NIPAB as substrates as described[6,7]. The PA activity was also determined using 2% (w/v) penicillin G as a substrate [phosphate buffer (pH 7.8), 37°C]; 1 U is the amount of enzyme that hydrolyses 1 μmol/min of substrate. The reaction was monitored by high-performance liquid chromatography or a pH-stat[6].

Charge density of PBA–Eupergit as a function of pH

A known amount of support was suspended in 0.1 *M* potassium chloride solution and the pH adjusted to 10.5. The suspension was titrated with hydrochloric acid to pH 2.0. A blank without support was also titrated.

Binding curves

Solutions with different enzyme contents were added to the same amount of support equilibrated with the same buffer as used to prepare the enzyme solutions. The suspensions were incubated using a rotary mixer at 25°C for 60 min. Then the free enzyme concentration, C, was determined in the filtrate. From the mass balance, the concentration of adsorbed enzyme, X, was determined.

Comparison of the protein binding capacities of different supports with isoelectric focusing

To 50 μl (wet volume) of different supports equilibrated with pH 7.5 buffer (phosphate, $I = 0.2$ M), 1 ml of a crude enzyme solution was added. The suspension was incubated for 60 min using a rotary mixer and filtered. The support and 20 μl of the supernatant were then placed near the anode of an isoelectric focusing plate (LKB, pH 3.5–9.5) and analysed as described[7].

Preparative purification of penicillin amidase from E. coli homogenates

A sample of an *E. coli* homogenate (17 ml with 19 U/ml penicillin amidase) was applied to a column (volume 8 ml, diameter 1 cm, flow-rate 1 ml/min) packed with PBA–Eupergit, equilibrated with buffer A (0.05 M potassium phosphate–1 M sodium chloride, pH 7.5). After elution with 30 ml of buffer A, desorption with 35 ml of buffer B (0.1 M sodium formate, pH 3.8) was performed. After washing with 16 ml of buffer A, 40 ml of regeneration buffer (1% bacterial alkaline protease in 0.02 M glycine–sodium hydroxide, pH 10) and finally 60 ml of buffer A were pumped through the column before a new sample was applied. The protein contents in the sample and the eluates were determined as described[8].

RESULTS AND DISCUSSION

Protein binding capacities of different hydrophobic support with immobilized phenyl groups

The results are shown in Fig. 1. From the ratio of the intensity of the protein bands desorbed from the gel and the corresponding supernatant, the differences in the protein binding capacities of the adsorbents can be determined. The protein binding capacity was found to increase in the order Phenyl-Sepharose < PBA–agarose < PBA–Eupergit. The last adsorbent was therefore used in the subsequent experiments.

Charge density of PBA–Eupergit as function of pH

This was determined from the titration curve for a known amount of PBA–Eupergit corrected for the amount of titrant used to titrate the blank. The results are given in Fig. 2. It follows that the density of positive charges on the support increases with decreasing pH. The apparent pK values of the secondary amine groups that are protonated lie in the range 6–7. This value is lower than expected for free secondary amines owing to charge–charge interactions on the support[9].

Binding curves

Scatchard plots for the binding of CT, TRY and PA to PBA–Eupergit are given in Fig. 3. The non-linear binding curves show that the adsorbent sites differ with

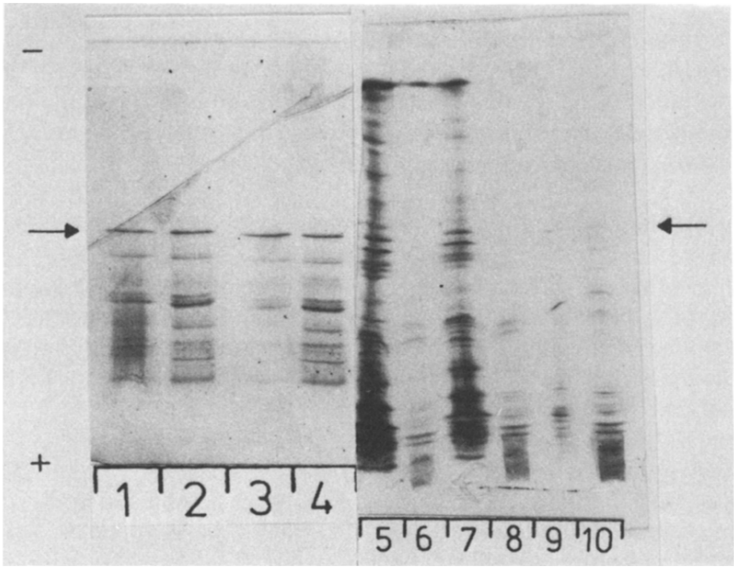

Fig. 1. Protein binding capacities of different hydrophobic adsorbents from a crude PA solution (lanes 1–4) and an *E. coli* homogenate (lanes 5–10). To 50 μl of the adsorbent (wet volume), 1 ml of crude enzyme or homogenate in phosphate buffer (pH 7.0, $I = 0.2$ *M*) was added. After equilibration for 60 min at 25°C, the adsorbent and 20 μl of the supernatant were placed near the anode (bottom) of an isoelectric focusing plate and separated. PBA–agarose (lanes 1 and 9, adsorbent; lanes 2 and 10, supernatant); Phenyl-Sepharose (lane 3, adsorbent; lane 4, supernatant); PBA–Eupergit (lanes 5 and 7, adsorbent; lanes 6 and 8, supernatant). Different preparations of this adsorbent were used in the two experiments. Arrows indicate the location of the main band with active PA (IP = isoelectric point = 7.0[6]).

respect to the dissociation constants, as is usually observed for affinity supports[10]. The average dissociation constants in buffer without added salt are 1, 4 and 5 μM for PA, TRY and CT, respectively. The binding capacity was found to increase in the presence of 1 *M* sodium chloride. For proteins of similar size, CT and TRY, the capacity decreases with increasing isoelectric point. This indicates that hydrophobic interac-

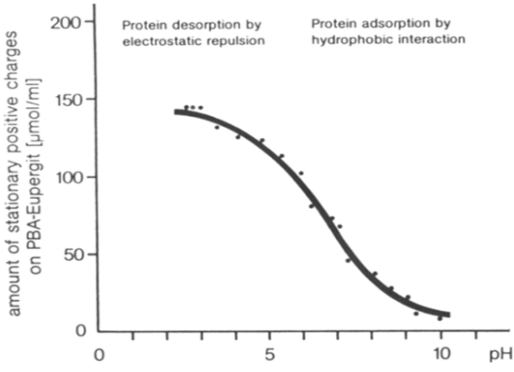

Fig. 2. pH dependence of the concentration of positive charges in PBA–Eupergit.

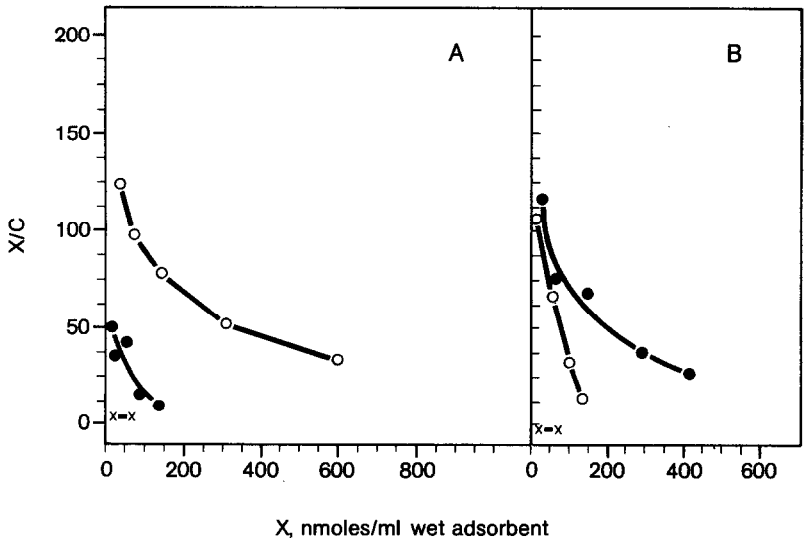

Fig. 3. Scatchard plots for the binding of different proteins to PBA–Eupergit at pH 7.5 (potassium phosphate–sodium phosphate buffer, $I = 0.2$ M) and 25°C. (A) CT, (×) with and (○) without 50% ethylene glycol; (●) TRY. (B) (○) PA from *E. coli*, (●) with 1 M NaCl and (×) with 50% ethylene glycol.

tions cause the adsorption, and that charge–charge repulsions on the support limit the adsorption capacity. From Fig. 2 it follows that these repulsive interactions should increase with decreasing pH. Hence it should be possible to desorb the adsorbed proteins simply by reducing the pH in the elution buffer. The negligible adsorption in the presence of ethylene glycol also demonstrates that hydrophobic interactions are responsible for the binding. Proteins adsorbed in the absence of this organic solvent could not be desorbed by the buffer with 50% ethylene glycol, with which no adsorption was observed. This indicates that an adsorbed protein is bound to the support by multiple-point interactions[3,11].

Preparative isolation of penicillin amidase from E. coli homogenates

The results for one adsorption–desorption–regeneration cycle are given in Fig. 4. The high salt content in buffer A was used to minimize the adsorption of negatively charged nuleic acids to the positively charged support. Their adsorption reduces the protein binding capacity of the column. The first peak in Fig. 4 has an absorption maximum at 260 nm, as expected for nucleic acids. The second peak with PA activity has an absorption maximum at 280 nm, indicating the selective adsorption of protein to the bifunctional support. The overall recovery of PA was found to be *ca.* 90% over 50 consecutive cycles. Hence at least 1500 U of PA per gram of PBA–Eupergit (wet weight) could be prepared by this method. The specific activity of the enzyme was increased from 2.0 U/mg protein in the sample to 10.6 U/mg protein in the eluate. The enzyme thus prepared can be used for immobilization without further purification in order to obtain a catalyst suitable for the hydrolysis of penicillin G or cephalosporin-G to prepare 6-aminopenicillanic acid and 7-aminodeacetoxycephalosporinic acid, respectively.

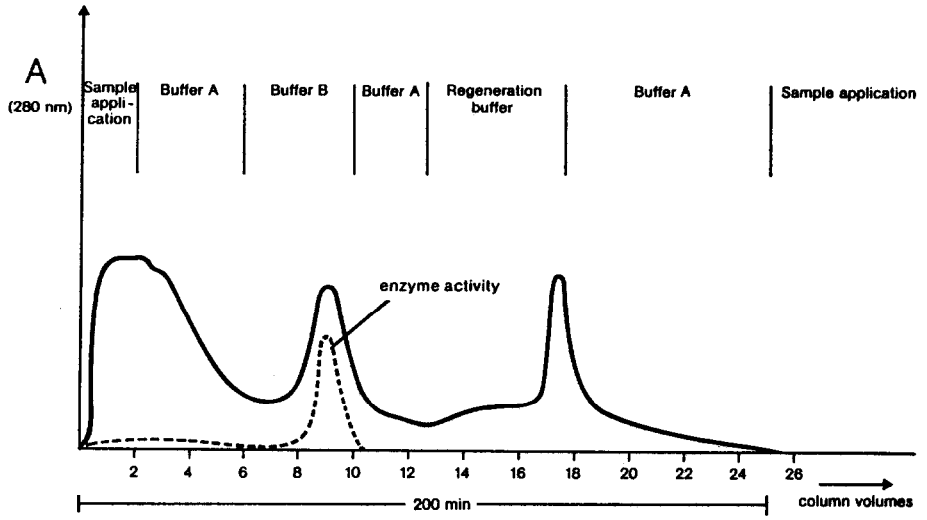

Fig. 4. One adsorption–desorption–regeneration cycle for the purification of penicillin amidase from an *E. coli* homogenate with a PBA–Eupergit column (8 ml, diameter = 1 cm) at a flow-rate of 1 ml/min at room temperature.

The results in Figs. 2–4 show that PBA–Eupergit is a bifunctional adsorbent that can be used for the isolation of enzymes on a preparative scale where the adsorbed proteins can be desorbed without the use of organic solvents.

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