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Novel Approaches to the Purification of Penicillin Acylase

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ABSTRACT

Penicillin acylase of *E. coli* NCIM 2400 has been purified to homogeneity using a combination of hydrophobic interaction chromatography and DEAE-cellulose treatment. A variety of substituted matrices were synthesized using D- or DL-phenylglycine, norleucine, ampicillin, or amoxycillin as ligands, all of which retained penicillin acylase at high concentrations of ammonium sulfate or sodium sulfate. The enzyme could be eluted nonbiospecifically by buffer of lower ionic strength with over 95% recovery of the activity. Ammonium chloride, ammonium nitrate, sodium chloride, sodium nitrate, and potassium chloride were ineffective in either adsorption or elution of the enzyme on these columns. Further purification of this partially pure enzyme with DEAE-cellulose at pH 7.0–7.2 yielded an enzyme preparation of very high purity according to electrophoretic and ultracentrifugal analyses, its specific activity being as high as 37 U/mg protein. The purified enzyme has a molecular weight of 67,000 a sedimentation coefficient of 4.0*S*, and resolves into two forms upon isoelectric focusing. Overall recoveries ranged between 75 and 85%. Ease of operation, high recoveries, high purity of the enzyme and prolonged reuse of the conjugates make the process economically feasible and possibly of great commercial importance.

Index Entries: Penicillin acylase, purification of; hydrophobic interaction chromatography, of penicillin acylase; 6-aminopenicillanic

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acid production; acylase, purification of penicillin; chromatography, of penicillin acylase;

INTRODUCTION

Penicillin acylase (penicillin amidohydrolase; EC 3.5.1.11) catalyzes the hydrolysis of penicillins to 6-aminopenicillanic acid (6-APA) and the side-chain organic acids (*1*). This property of penicillin acylase (PA) has been exploited commercially for the large scale production of 6-APA, which is an important intermediate in the manufacture of semisynthetic penicillins (*2*). Most of these processes utilize the enzyme in the immobilized form. Purity of the enzyme plays an important role in obtaining an immobilized enzyme preparation of high specific activity and thus in the efficiency and economics of the entire process. Various attempts have been made to purify PA from a large variety of sources (*3–10*). However, all these methods have suffered from one or many of the following drawbacks: (i) 7–8 steps are required and processes are time consuming, (ii) a low degree of purity, and (iii) very low yields.

Thus far, only limited attempts have been made to use affinity chromatographic methods for purifying this enzyme (*11–13*). Although the yields obtained in these cases were comparatively higher, the final products were very poorly defined. To overcome these difficulties, we have attempted to attack this problem in a more specific manner. We have successfully developed a process to purify PA from *E. coli* NCIM 2400 that involves only two chromatographic steps and yields a homogeneous enzyme preparation with a specific activity as high as 37 U/mg protein and overall recoveries betwen 75 and 85%. A preliminary report of our studies has been published earlier (*14*).

MATERIALS AND METHODS

Ampicillin was a product of Hindustan Antibiotics, Ltd. Amoxycillin was procured from Cipan-Companhia Industrial Produtora De Antibioticos, Lisbon. D- or DL-phenylglycine and norleucine were from BDH, England, and CNBr was from Fluka Chemicals. DEAE-cellulose was a product of Renal, Budapest, and alpha cellulose no. 125 was from Schleicher and Schuell. Sepharose-2B/4B/6B, as well as phenyl-Sepharose and octyl-Sepharose, were from Pharmacia Fine Chemicals Co., Upsala.

All other chemicals used were of technical grade and were purchased from Sarabhai Merck, India.

Preparation of Substituted Matrices

Coupling of the different ligands, viz., ampicillin, amoxycillin, phenylglycine, etc., to the agarose or cellulose matrices was carried out

essentially following the CNBr-activation method (*15*), and these details have been outlined elsewhere (*16*). The amount of phenylglycine and norleucine coupled to the matrices was determined by estimating the amount of these ligands recovered in the washings using the ninhydrin reaction (*17*). Similarly, the amount of ampicillin and amoxycillin recovered in the washings after 24 h of coupling reaction was determined by the method of Smith et al. (*18*).

Analytical Methods

Protein was estimated by the Lowry method (*19*) using bovine serum albumin as standard. Electrophoresis of the enzyme under native conditions was carried out at pH 8.3 and at pH 4.5 according to Gabriel (*20*). Electrophoresis was also carried out in the presence of sodium dodecyl sulfate (*21*).

Isoelectric Focusing

Isoelectric focusing of the purified enzyme was performed using an LKB flat bed apparatus following Vesterberg (*22*). The pH gradient was generated using ampholites of pH 2.5–9.8. After electrofocusing, the actual pH values were determined by cutting the gel pieces as described by Vesterberg (*22*).

Ultracentrifugation

Sedimentation analysis of the pure enzyme was carried out in a Spinco model E analytical ultracentrifuge using the An D rotor. The centrifuge was equipped with an automatic rotor temperature control device and Schlieren optics. The sedimentation coefficient value was calculated following the procedure given in the instruction manual.

Amino Acid Analysis

The purified enzyme was dialysed extensively against several volumes of distilled water at 4°C and lyophilized. The protein (1 mg/mL) was hydrolyzed using constant boiling 6*N* HCl in evacuated tubes at 110°C for 24 h, and the amino acid analysis was carried out on a Dionex D-300 instrument with a single column.

Assays

All enzymatic assays were performed using Na or K salts of benzylpenicillin as substrate in 0.1*M* phosphate buffer, pH 7.5. Typically, 100 μmol of the substrate was incubated with 10–200 μg of protein in a final volume of 2 mL of 0.1*M* phosphate buffer, pH 7.5, at 40°C and for 30 min. The reaction was terminated by pipetting 0.2 mL of the reaction mixture into 3.8 mL of citrate–phosphate buffer, pH 2.5. The enzy-

matic activity was estimated by determining the amount of 6-APA formed using *p*-dimethylaminobenzaldehyde reagent (*23*). One unit of the enzymic activity is defined as the amount of enzyme needed to catalyze the formation of 1 μmol of 6-APA per minute at 40°C and at pH 7.5.

Organism and Enzyme Production

E. coli NCIM 2400 cells were grown on nutrient broth containing 0.5% peptone, 0.3% beef extract and 1% NaCl for 24 h, and the acetone dried cells were prepared as described earlier (*14*).

Enzyme Purification

All the procedures were carried out at room temperature, unless mentioned otherwise.

Extraction

Acetone-dried cells of *E. coli* NCIM 2400 were suspended in distilled water (5% w/v) in 500 mL Erlenmeyer flasks. The suspension was adjusted to pH 7.0–7.2 with 0.5*N* NaOH and agitated on a rotary shaker for 2 h. The cell debris was then removed by centrifugation of the lysate at 10,000 rpm for 30 min in the presence of cell-flocculating agents. The crude supernatant thus obtained was used for further purification. If the protein concentration in this extract exceeded 5 mg/mL, it was adjusted to about 4 mg/mL by dilution of the extract with distilled water.

Chromatography on Substituted Matrices

The substituted matrices mentioned above were separately used for purifying PA from crude extracts. The conjugates were thoroughly washed with distilled water and equilibrated in 0.1*M* phosphate buffer, pH 7.5, containing ammonium sulfate and poured into glass columns (2.0 × 25 cm). The crude extract was also adjusted to the appropriate ammonium sulfate concentration by adding the salt slowly with constant stirring. The mixture was allowed to stand at room temperature for 3 h. At the end of this period, a slight turbidity appeared which was filtered off using Whatman No. 1 filter paper and the clear filtrate loaded on the column.

The extract was passed through the columns at a flow rate of 35–40 mL/h. The columns were thoroughly washed with the equilibration buffer until no protein could be detected by determination of the A_{280} of the effluent. Elution of the enzyme from the columns was achieved either by a stepwise or a linearly decreasing gradient of $(NH_4)_2SO_4$ in 0.1*M* phosphate buffer, pH 7.5. Protein was monitored by A_{280} and fractions were assayed for acylase activity. All fractions showing acylase activity over 0.5 U/mL were pooled and dialyzed in the cold against 0.05*M* phosphate buffer, pH 7.0, to remove salt. Alternatively, the salt concen-

tration was reduced by ultrafiltration using a Sartorius membrane filter system when larger volumes were involved.

Ion Exchange Chromatography

The dialyzed enzyme was further prufied on a DEAE-cellulose column. The ion-exchanger was regenerated using 0.5*N* NaOH/0.5*M* NaCl and 0.5*N* HCl followed by washing with distilled water and finally equilibrated in 0.05*M* phosphate buffer, pH 7.0. The dialyzed enzyme was passed through the column at a flow rate of 30 mL /h, and the column was further developed in the equilibration buffer. The enzyme activity appeared unadsorbed. All the fractions were monitored for protein and activity, pooled, and stored frozen at −20°C in the presence of 0.02% Na azide, or were dialyzed against distilled water and lyophilized.

RESULTS

Synthesis of Substituted Matrices

Conjugates of ampicillin, amoxycillin, norleucine, and phenylglycine with agaroses or cellulose were synthesized using the CNBr activation reaction. Under the conditions described (*15*), approximately 8–12 μmol of each ligand could be coupled per milliliter of settled agaroses. When mercerized cellulose was used as a matrix, however, the extent of coupling was less than half of that observed with Sepharose-4B. No leaking or bleaching of the ligand was observed over a long period of time, and even with repeated use of the matrices the capacity of the matrix to bind the enzyme (measured in terms of units of enzyme adsorbed per milliliter matrix) remained unaffected. However, the capacity of cellulose conjugates was much lower, as were the flow rates, when compared to Sepharose-4B. Therefore, all the subsequent experiments were carried out using the Sepharose-4B conjugate.

Purification of Enzyme

Purification of PA using a phenylglycyl-Sepharose column is summarized in Table 1. Lysis of acetone-dried cells of *E. coli* by shaking them in distilled water at pH 7.0–7.2 was used as an efficient method for extracting the enzyme. When the crude extract was made to 22% with ammonium sulfate and allowed to stand at room temperature for 3 h, no detectable loss in the enzyme activity was observed, if the protein concentration in the extract was 4.0–4.5 mg/mL. This extract was loaded onto the phenylglycine–Sepharose column (or other substituted matrices) preequilibrated with the buffer containing 22% ammonium sulfate. The enzyme activity was desorbed from the columns by decreasing ammonium sulfate concentration in the elution buffer either using a linearly decreasing gradient or a stepwise gradient of the salt concentrations. Figure

TABLE 1
Purification of *E. coli* Penicillin Acylase by Hydrophobic Interaction Chromatography

Step	Volume, mL	Activity, U/mL	Protein, mg/mL	Sp. activity,[a] U/mg	Purification, -fold	Recovery, %
Crude extract	700	3	4	0.75	—	100
Phenylglycine–Sepharose-4B	90	13.8	0.45	29.7	40	60
DEAE-cellulose	123	9.6	0.26	37	49	56

[a]One unit of enzyme is defined as the amount of enzyme needed for formation of 1 μmol of 6-APA per min at pH 7.5 and 40°C.

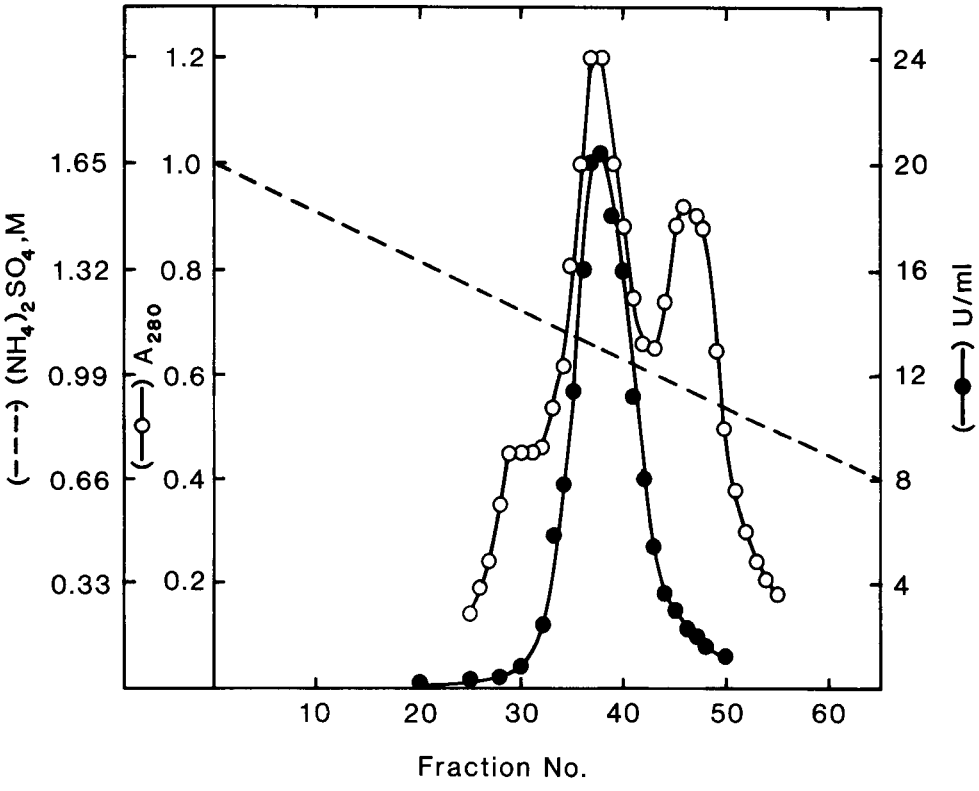

Fig. 1. Elution profile of penicillin acylase on phenylglycine–Sepharose column. About 700 mL of crude enzyme made 22% with $(NH_4)_2SO_4$ was loaded on the column (2.0 × 25 cm), and the column was washed extensively with equilibration buffer. Elution of the enzyme was achieved by a linearly decreasing gradient of $(NH_4)_2SO_4$ that was generated by mixing 400 mL of 0.1*M* phosphate buffer, pH 7.5, containing 22% $(NH_4)_2SO_4$ in the mixer and 400 mL of 0.1*M* phosphate buffer, pH 7.5, but devoid of $(NH_4)_2SO_4$ in the reservoir. Fractions of about 8 mL were collected and assayed for protein (○) and activity (●). Dashed line indicates nature of the salt gradient.

1 shows the elution profile of PA on the phenylglycine–Sepharose column. As is seen from the figure, the enzyme elutes out as a single peak of activity. The specific activity of this preparation (pooled sample) was 30 U/mg protein, thus giving about fortyfold purification. However, the recovery of the activity was only 60%.

Figure 2 represents the chromatographic profile of PA on columns of Sepharose-4B conjugated to ampicillin, amoxycillin, phenylglycine, or norleucine. In this case, the elution of enzyme was achieved by applying a stepwise gradient of ammonium sulfate using buffer devoid of salt. The enzyme was specifically retained on all these columns in the presence of 22% ammonium sulfate and could be desorbed by deleting the salt in the elution buffer. The specific activity of the enzyme thus eluted was between 18 and 24 U/mg protein. However, the recovery of enzyme activity across this step (which was always over 95%), in all these columns, was

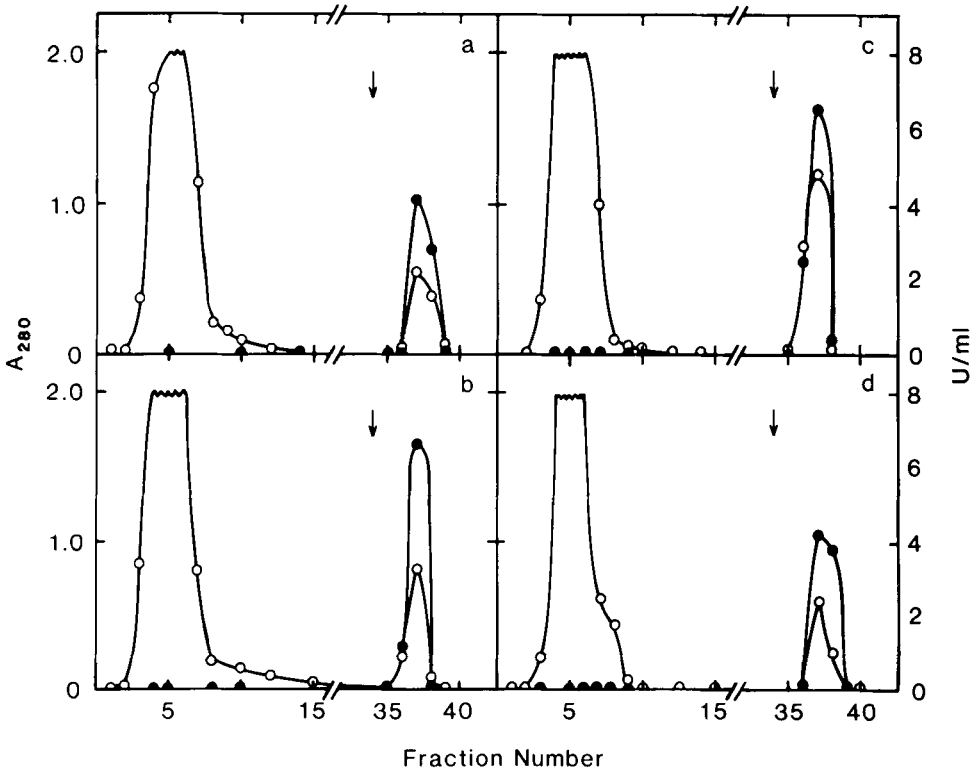

Fig. 2. Chromatography of penicillin acylase on substituted matrices. Crude PA was made 22% in $(NH_4)_2SO_4$ and loaded separately (about 50–55 U) on each column. The columns were washed exhaustively with equilibration buffer until no protein was detected in the effluent, and the enzyme activity was desorbed from the columns by applying a stepwise gradient of $(NH_4)_2SO_4$ from 22% to zero percent (indicated by arrow). Fractions were assayed for protein by reading absorbance at 280 nm (○) and for PA activity (●) as described in the text: (a) ampicillin–Sepharose; (b) amoxycillin–Sepharose; (c) phenylglycine–Sepharose; (d) norleucine–Sepharose.

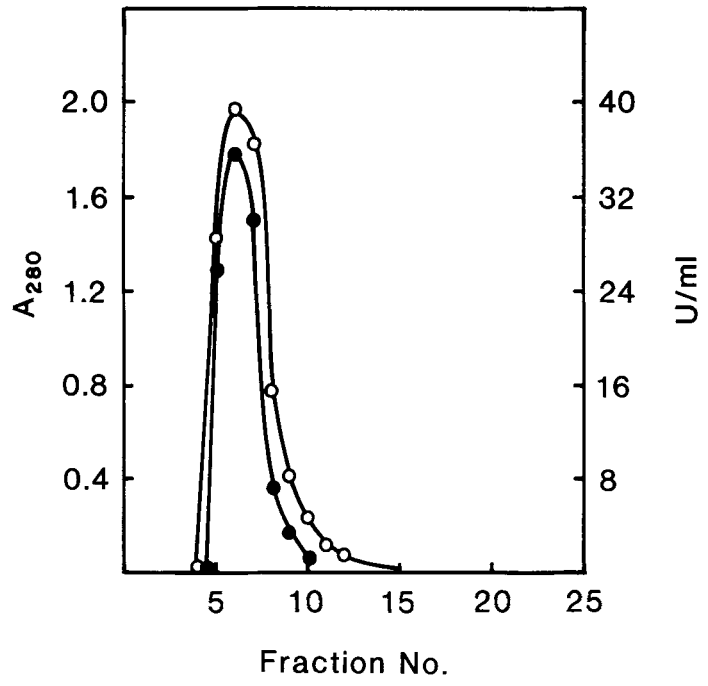

Fig. 3. Elution profile of penicillin acylase on DEAE-cellulose. Partially pure enzyme, pooled from the hydrophobic columns, was dialyzed against 0.05*M* phosphate buffer, pH 7.0, and loaded on a DEAE-cellulose column pre-equilibrated in the same buffer. The column was further washed with the equilibration buffer. Fractions were assayed for protein (○) and activity (●) as described in the text.

similar. A phenylglycine–Sepharose column was routinely used for subsequent studies.

The partially pure enzyme was further purified using DEAE-cellulose column chromatography (Fig. 3). At pH 7.0–7.1, PA does not adsorb to this anion exchanger, but all the impurities do. This enzyme preparation had a specific activity as high as 37 U/mg protein, and the overall recoveries were 55–60%.

Criteria of Purity

Purity of the enzyme thus obtained was determined using a variety of techniques. Polyacrylamide gel electrophoresis of PA was performed under native as well as denaturing conditions. The enzyme migrates as a single protein band in the absence as well as in the presence of sodium dodecyl sulfate (Fig. 4). The homogeneity of the preparation was further confirmed using ultracentrifugation during which the protein sediments as a single symmetrical peak (Fig. 5). Isoelectric focusing of the enzyme, however, shows two distinct protein bands (Fig. 6).

Molecular Properties

The enzyme thus purified behaves as a single molecular species on sodium dodecyl sulfate gel electrohporesis and has a molecular weight of 67,000 ± 1000. Similar results have been obtained by gel filtration on a Sepharose CL-6B column also. However, upon isoelectric focusing, the same preparation resolves into two bands of p*I* values between 6.5 and 6.7, indicating a charge heterogeneity. The purified enzyme has a sedimentation coefficient of 4.0*S*. The amino acid composition of PA is presented in Table 2. The enzyme has quite a high content of proline and other hydrophobic amino acids such as threonine, serine, tyrosine, as well as leucine and isoleucine. The purified enzyme is very active on Na

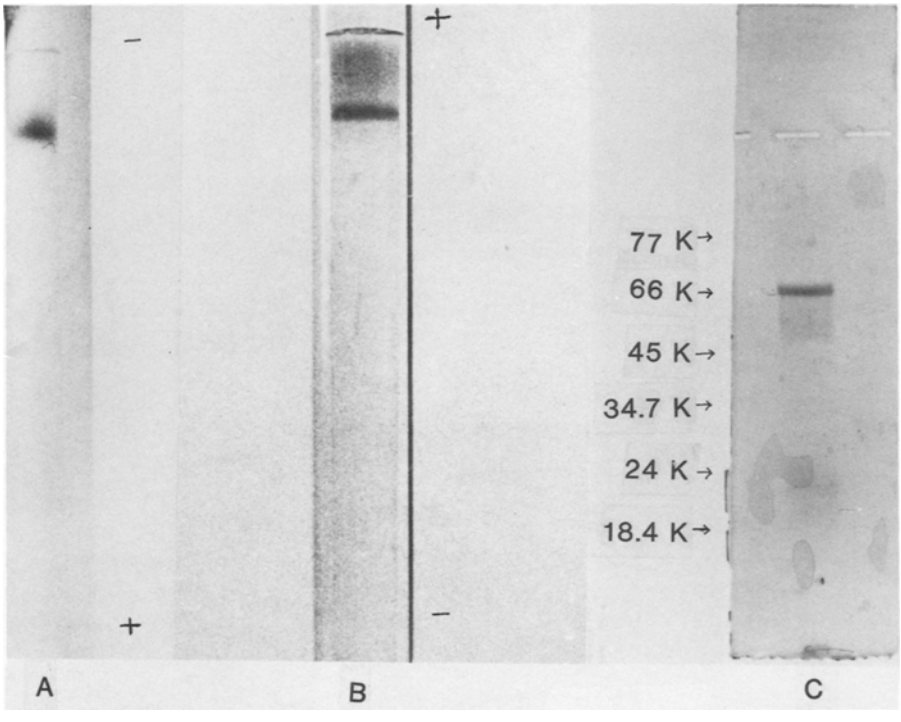

Fig. 4. Electrophoretic profile of penicillin acylase. Polyacrylamide gel electrophoresis of PA was carried out using 7.5% gels under nondenaturing conditions of pH 8.3 (A) as well as at pH 4.5(B). About 15–20 μg of the purified enzyme was electrophoresed in either experiment for 2 h, and the enzyme was located by staining gels with Coomassie blue. For electrophoresis under denaturing conditions (C), the enzyme was preteated with 1% sodium dodecyl sulfate and 1% β-mercaptoethanol. About 25–30 μg of protein was electrophoresed on 7.5% gels containing 0.1% sodium dodecyl sulfate for 6 h. Protein was made visible by staining the gel with Coomassie blue. The molecular weight markers used were transferrin (77,000), BSA (66,000), ovalbumin (45,000), pepsin (34,700), trypsinogen A (24,000), and β-lactoglobulin (18,400).

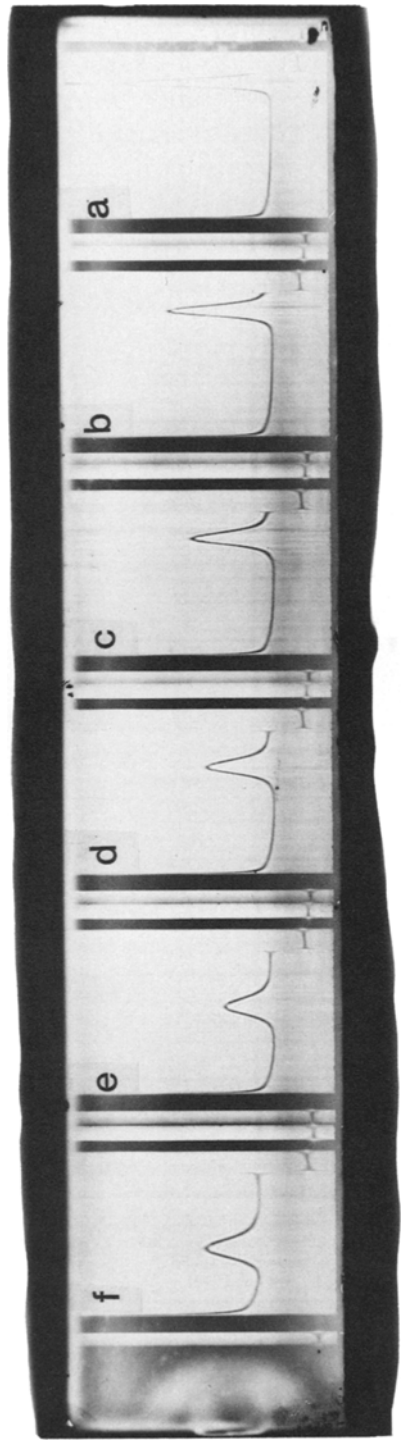

Fig. 5. Ultracentrifugal pattern of penicillin acylase. Sedimentation analysis of the purified enzyme (0.07% in 0.05*M* sodium phosphate, pH 7.2, specific activity 36.6 U/mg) was carried out at 4°C and 59,780 rpm using A D rotor in a Spinco model E analytical ultracentrifuge. Photographs were taken at an angle of 60° and after 20 min (a), 36 min (b), 52 min (c), 68 min (d), 100 min (e), and 132 min (f). The last photograph was taken at 50°. All exposures were for 15 s.

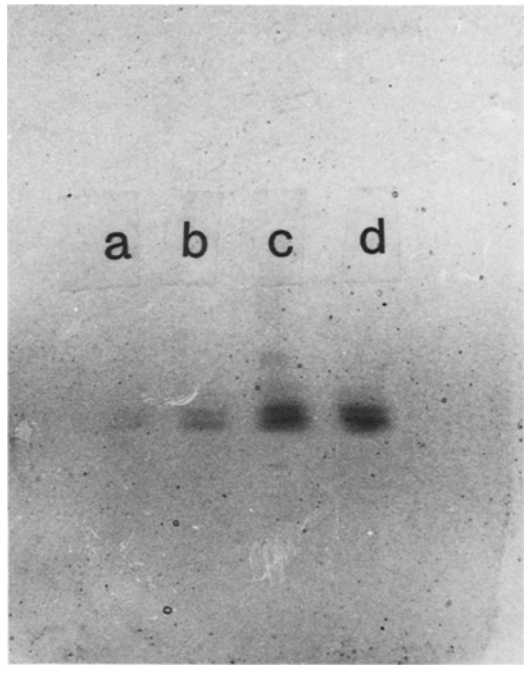

Fig. 6. Isoelectric focusing of penicillin acylase. Isoelectric focusing of PA was performed using a flat-bed apparatus with 1*N* phosphoric acid as anode and 1*N* NaOH as cathode. The ampholine gradient used was pH 2.5–9.8. Between 5 and 20 μg of sample was applied and electrofocussed for about 10 h. Protein was detected by staining with Coomassie blue, and pH was determiend by cutting the gel pieces as descibed by Vesterberg (ref. 22): (a) 5 μg; (b) 10 μg; (c) 15 μg; (d) 20 μg.

or K salts of benzylpenicillin. Using this substrate, the purified enzyme exhibits a specific activity of 37 U/mg protein, the highest value reported so far for *E. coli* enzyme. Cephalexin or penicillin V are hydrolyzed only insignificantly, but the enzyme hydrolyzes ampicillin and amoxycillin at about 50% the efficiency of penicillin G hydrolysis (Table 3).

Effects of Different Agents on Adsorption and Elution of PA on Substituted Matrices

Different salts were tested for their ability to either adsorb or elute the enzyme on phenylglycine–Sepharose column. Only sodium sulfate and ammonium sulfate were effective in retaining the enzyme on the column. None of the other salts mentioned in Table 4 could retain the enzyme on phenylglycine–Sepharose. The enzyme did not bind to the column when operated in 0.1*M* phosphate buffer between pH 5.5 and 8.5. Similarly, the effect of these salts (up to 2*M* concentrations) on the elution of enzyme adsorbed on phenylglycine–Sepharose in the presence of 22% ammonium sulfate was studied. None of the salts desorbed the en-

TABLE 2
Amino Acid Composition of Penicillin Acylase From *E. coli* NCIM 2400

Amino acid	Mol/mol enzyme[a]
Asx	32
Thr	20
Glx	30
Ser	15
Prl	16
Gly	21
Ala	25
Val	17
Met[b]	6
Cys[c]	8
Ileu	10
Leu	21
Tyr	11
Phe	11
His	5
Lys	15
Arg	12

[a]Calculated for a mol wt of 67,000.
[b]8% Methionine was detected as methionine sulfoxide.
[c]Obtained as cysteic acid. Tryptophan not determined. Values represent an average of three experiments.

zyme from the column when ammonium sulfate was present in the elution buffer. Moreover, penicillin G, penicillin V, phenylacetic acid, and 6-APA could not elute the enzyme from the column. When phenyl-Sepharose or octyl-Sepharose columns were used in these studies, the bound enzyme could be eluted only after inclusion of 50–80% ethylene glycol in buffer devoid of ammonium sulfate. However, the recovery of enzyme activity was as low as 20–25% in these cases.

TABLE 3
Substrate Specificity of *E. coli* Penicillin Acylase

	Substrate[a]	% Hydrolysis
1.	Penicillin G	100
2.	Ampicillin	56
3.	Amoxycillin	46
4.	Penicillin V	10
5.	Cephalexin	18

[a]All substrates were dissolved in 0.1*M* PO_4 buffer, pH 7.5. Assay conditions were as described in the text.

TABLE 4
Effect of Different Salts on Retention of Pencillin Acylase on Phenylglycine–Speharose[a]

	Salt	Maximum concentration, *M*	Adsorption
1.	NaCl	3.7	−
2.	NH_4 Cl	4.0	−
3.	KCl	2.9	−
4.	Na_2SO_4	1.5	+
5.	$(NH_4)_2SO_4$	1.6	+
6.	$NaNO_3$	2.6	−
7.	NH_4NO_3	2.7	−

[a]The column was equilibrated in 0.1*M* phosphate buffer, pH 7.5, and containing the individual salt. About 20 units of the crude enzyme were applied on the column in presence of the salt and washed with the same buffer. +, activity retained on column; −, activity not retained on column.

DISCUSSION

The Rationale

In this paper, we have described a novel approach for purifying PA that is based on the use of hydrophobic interaction chromatography. Earlier reports in the literature documented the use of affinity chroma-

TABLE 5
Comparison of the Capacity of Different Hydrophobic Matrices to Retain Penicillin Acylase[a]

Matrix	Ligand	Capacity[b]	Retention[c]
Sepharose-4B	Ampicillin	250	++
	Amoxycillin	266	++
	Phenylglycine	200	++
	Norleucine	134	++
	Octylamine	240	+++
	Phenyl-	210	+++
Cellulose	Ampicillin	83	++
	Amoxycillin	98	++

[a]Columns of the respective ligands coupled to Sepharose-4B or cellulose were equilibrated in 0.1*M* phosphate buffer, pH 7.5, containing 22% ammonium sulfate, and independently tested for retention of PA in presence of the salt.

[b]Capacity was determined as units of PA adsorbed per g dry matrix.

[c]+++ indicates very tight retention of the enzyme, which could be desorbed by inclusion of 50% ethylene glycol in the elution buffer.

tography for purifying PA. However, the degree of purity obtained in all these cases was very low, indicating the limitations of the methods used. Especially, the contributions of the spacers used for coupling ligands to the matrix have not been carefully examined (*12*). The rationale for using hydrophobic interaction chromatography in the present studies stems from the observations made by Klyosov et al. (*24*). These workers observed that the *E. coli* PA was inhibited by a series of aliphatic alcohols and that the type and extent of inhibition was a function of the carbon chain length of the alcohol used, indicating the involvement of hydrophobic interactions betwen the enzyme and the competitive inhibitors. This prompted us to visualize the presence of a hydrophobic "pocket" at or near the active site of the enzyme and the possibility of using this pocket as an anchorage for adsorbing the enzyme onto a hydrophobic column.

The Approach

A series of substituted matrices was synthesized using different ligands coupled to the agarose or cellulose without any spacers (*16*). When the enzyme was passed through the ampicillin–Sepharose or amoxycillin–Sepharose column equilibrated in 0.1*M* phosphate buffer, pH 7.5, it did not adsorb to the matrix. However, inclusion of ammonium sulfate in the equilibration buffer and enzyme solution dramatically changed the adsorption properties of these matrices, thereby effectively adsorbing all the enzyme activity onto the column. Deletion of ammonium sulfate in the washing buffer resulted in a rapid and total desorption of the enzyme from the column, indicating the involvement of hydrophobic interactions between the enzyme and the ligand. It was found that 22% ammonium sulfate was necessary and sufficient for adsorption of the enzyme onto these columns. We also observed that when the crude extract was made to 22% with ammonium sulfate, the loss of enzyme activity caused by precipitation was undetectable if the protein concentration in the crude extract was about 4 mg/mL. Therefore, the crude extract was adjusted to about 4 mg/mL by adding distilled water if the protein concentration was more than 4.5 mg/mL. However, at 22% ammonium sulfate, the adsorption of the enzyme was very tight, and washing the column with the buffer containing 22% salt removed a large number of impurities (Fig. 2). Of the various other salts tested, only sodium sulfate was effective in retaining the enzyme on the column. Both ammonium and sodium sulfate are known to increase the hydrophobic interactions (*25*).

Experiments were also carried out to evaluate whether the affinity of the enzyme for its substrates (i.e., ampicillin or amoxycillin) contributed to the adsorption. Our observation that pencillin G, penicillin V, phenylacetic acid, or 6-APA failed to desorb the enzyme from the column indicates that there are few or no "affinity" or "biospecific interactions"

in retaining the enzyme on these columns. A comparison of the adsorption and elution behavior of PA on the ampicillin–Sepharose, amoxycillin–Sepharose or phenyl-glycyl-Sepharose on one hand with that on the norleucine–Sepharose, phenyl-Sepharose or octyl-Sepharose on the other hand also supports this conclusion. Involvement of any ionic interactions is ruled out by the fact that the adsorption–elution phenomenon is totally insensitive to the presence or absence of NaCl or KCl. That the adsorption and elution event is solely a function of increased or decreased hydrophobic interactions was further confirmed when we observed that an increase in temperature increased these interactions and vice versa (data not shown), and that chaotropic agents such as ethylene glycol disrupted these interactions (*25*).

Elution of the enzyme from phenylglycine–Sepharose column using a lineraly decreasing gradient of ammonium sulfate resulted in about 40-fold purification of the enzyme as against 18- to 20-fold purification obtained by stepwise elution. However, the recovery of the enzyme activity was considerably enhanced by the stepwise elution, in addition to reducing the time and dilution of enzyme activity resulting from the linear gradient elution method. The enzyme preparation thus obtained was only partially pure. Further purification was achieved on a DEAE-cellulose column by taking advantage of the fact that the enzyme does not adsorb to the anion exchanger at neutral pH. Thus, the overall recovery of enzyme activity was between 75 and 85% when stepwise elution was used and between 50 and 60% when linear gradient was used for the hydrophobic interaction chromatography.

The Properties

The molecular properties of the enzyme thus purified are comparable to the crystalline PA of *E. coli* ATCC 11105 obtained by Kutzbach and Rauenbusch (*4*) in its behavior on isoelectric focusing, molecular weight, substrate specificity, and specific activity measurements using benzylpenicillin as a substrate. The major difference is observed in the electrophoretic behavior of the enzyme in the presence of sodium dodecyl sulfate. Whereas the enzyme from *E. coli* ATCC 11105 showed two protein bands corresponding to molecular weights of 71,000 and 20,500, we have obtained a single band of molecular weight of 67,000 for the *E. coli* NCIM 2400 enzyme. We had observed earlier that lyophilization causes a denaturation of the enzyme. Longer storage might also denature the enzyme and thus give rise to a low molecular weight species of the enzyme that may not be active. It is also possible that the low molecular weight species is absent in the strain used for present studies. Nevertheless, our data do not suggest the presence of any subunits for PA of *E. coli* NCIM 2400. Our results on the isoelectric focusing of the purified enzyme are in agreement with the earlier observations (*4*), but the significance of this charge heterogeneity is not clear at this time. The

ultracentrifugal (Fig. 5) as well as immunological analyses (our unpublished results) confirm the high purity of the enzyme. The apparent sedimentation coefficient value is different from those reported for *B. megaterium* (*26*). Amino acid analysis of the purified enzyme indicates the presence of hydrophobic amino acids to a large extent.

The Merits

Several advantages that make our method unique must be emphasized. Our method employs only two chromatographic steps, both of which can be performed in a batchwise manner with almost the same efficiency as in the columns. All the operations (except dialysis) can be performed at room temperature. Use of ammonium sulfate contributes to the improved stability of the enzyme during these operations. The enzyme obtained after the DEAE-cellulose step can be directly used for immobilization experiments without any further treatment. The high degree of purity of the enzyme thus purified enables one to obtain an immobilized enzyme preparation of very high specific activity. Use of sophisticated matrices such as agaroses might initially appear to be costly and hence a prohibitive factor as compared to the conventional "industrial matrices" such as celite. However, a careful analysis of the time factor involved, purity of the enzyme obtained and the prolonged reuse of the substituted matrices without any significant loss in the adsorption properties (unpublished results) makes this method of great commercial importance. We have successfully employed this method for purifying PA from *Kluyvera citrophila* (*27*) and studies are in progress for using this method for purifying PA from other microbial sources. This may also be important for gaining an insight into the complex active site topography of the enzyme molecule without chemically perturbing the structure of the enzyme. The possibility of using the hydrophobic matrices for immobilizing PA adds another interesting dimension to this area of research. Further studies are in progress in our laboratory.

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