ELSEVIER

Journal of Chromatography B, 753 (2001) 45–50

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Efficient two-step chromatographic purification of penicillin acylase from clarified *Escherichia coli* ultrasonic homogenate

J. Sanchez, N. Verdoni, V. Fitton, X. Santarelli*

Ecole Supérieure de Technologie des Biomolécules de Bordeaux, CNRS-UMR 5544, Université Victor Segalen Bordeaux 2, 146 Rue Leo Saignat, 33076 Bordeaux Cedex, France

Abstract

A two-step chromatographic purification procedure from clarified *Escherichia coli* ultrasonic homogenate was evaluated. The capture step included immobilized metal affinity chromatography with Cu^{2+} as metal ion. Two elution methods were performed: 1 *M* NH_4Cl and 0.01 *M* imidazole. Respectively, we obtained a different purification fold (16.5 to 3.15) and a similar result for the recovery of activity (90–99%). The best elution method was chosen for the procedure. The second step, hydrophobic interaction chromatography, gave a 3.8-fold purification with 77.7% of activity. The total procedure gave a 66-fold purification in relation to the initial crude extract with 70% for the recovery of activity and was performed without any conditioning step and at the same pH value.

Keywords: Enzymes; Penicillin acylase

1. Introduction

Semi-synthetic penicillins are produced from intermediates such as 6-aminopenicillanic acid (6-APA). The immobilized penicillin acylase for routine production of 6-APA is well known in biotechnology [1], so the production cost of 6-APA is partially dependent on enzymatic production. Therefore, the ultimate aim is to be able to produce large quantities of penicillin acylase at a low cost. However, despite recent improvements, the purification process has long proved expensive with a low recovery [2–10].

Recently, the partial purification of penicillin acylase by aqueous two-phase systems was reported [11] with 85% recovery and 5.7 purification fold. In a previous article [12] in which we evaluated different ligands which are structural analogs to antibiotics, we also found good results for recovery (73 to 100%) and purification fold (1.9 to 5).

Therefore, we tested another method to purify penicillin acylase by immobilized metal affinity chromatography [13] and obtained 95% of recovery and 12-fold purification. In this technique, proteins are separated on the basis of their affinity for a metal ion [14–20].

Recently, some authors [21] reported the continuous recovery and purification of the penicillin acylase under pseudo-affinity conditions using phenyl Sepharose gel with 74% of recovery but a low purification factor (2.4).

The most recent paper with an efficient multi-step purification procedure [2] presented ammonium sulfate precipitation (70 and 40%), dialysis and a two-step chromatographic procedure.

In the present paper, we present an efficient and fast procedure for the purification of penicillin

*Corresponding author.

E-mail address: xavier.santarelli@estbb.u-bordeaux2.fr (X. Santarelli).

0378-4347/01/$ – see front matter
PII: S0378-4347(00)00529-6

acylase with only two chromatographies (66-fold purification, 70% recovery of activity) and without any conditioning step.

2. Experimental

2.1. Instruments

The chromatographic system used throughout this study was the fast protein liquid chromatography (FPLC) workstation from Amersham Pharmacia Biotech (Saclay, France). The data were collected and evaluated using the FPLC director.

For recovery studies, we used a Uvikon 930 spectrophotometer (Kontron, Montigny Lebretonneux, France) to measure absorbance at 280 nm, 405 nm and 595 nm.

The ultrasonic homogenizer Vibracell 72412 was purchased from Fisher Biobloc Scientific (Illkirch, France).

2.2. Chemicals

Chelating Sepharose fast flow, XK16/20 column were purchased from Amersham Pharmacia Biotech.

The 3-amino-5-phenylpyrazole Sepharose was prepared in our laboratory [12].

All metals, salts and NIPAB (6-nitro-3-phenylacetamidobenzoic acid) were from Sigma (l'Isle d'Abeau Chesnes, France).

Buffer with NH_4Cl was treated with activated charcoal to avoid undesirable absorption of UV. It was then filtered.

All other salts were of HLPC grade, and the buffers were filtered through a 0.22-μm membrane filter.

2.3. Cell culture and preparation of crude extract

The strain was maintained in 20% glycerol at −80°C. *Escherichia coli* ATCC 9637 was grown at 37°C and 120 rpm in nutrient broth containing per liter: 5 g tryptone, 5 g yeast extract, 5 g NaCl, and 1 g glucose, pH 7. Briefly 2.5 ml of this culture was used to inoculate 250 ml of the following medium: 3 g KH_2PO_4, 7 g K_2HPO_4, 1 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 7H_2O$, 2 g phenylacetic acid, 7 g tryptone and 0.1 g yeast extract per liter, pH 7. The organisms were grown and enzyme production initiated at 24°C and 120 rpm for 2 days.

The cells were harvested by centrifugation at 8000 *g* for 20 min and the pellet was resuspended in the lysis buffer (0.1 *M* KH_2PO_4, 1 m*M* EDTA, pH 7.8) and kept at −20°C. After thawing, the sample was placed in an ice-water bath and sonicated (100 W) in three short pulses of 30 s. The sample was centrifuged in an Imac CS 100 Hitachi microfuge at 25 000 *g* for 30 min and the supernatant was collected and injected onto the column.

2.4. Preparation of supports

The gels were packed in an XK16/20 column. Slurry was prepared with binding buffer in a ratio of settled gel–buffer (75:25) and was de-gassed.

The column was filled through the outlet with a few centimeters of binding buffer and was closed. The slurry was poured into the column in one continuous motion. The remainder of the column was filled with buffer and the top mounted and connected to a pump. The bottom outlet of the column was opened and the pump set at 133% of the flow-rate to be used during chromatography. The packing flow-rate was maintained for three bed volumes after a constant bed height was reached.

2.5. Chromatographic procedure

2.5.1. Immobilized metal affinity chromatography

Immobilized metal affinity chromatography (IMAC) was performed with a crude extract without any conditioning step. The crude extract was injected at 2 ml/min onto the column (3.5 ml). The flow-rate during the chromatography was maintained at the same value. The column was then washed with 0.3 *M* NH_4Cl. Two types of elution were performed; the first type used 1 *M* NH_4Cl and the second type used 0.01 *M* imidazole.

2.5.2. Hydrophobic interaction chromatography

The hydrophobic interaction chromatography (HIC) support, 3-amino-5-phenylpyrazole Sepharose was packed in an XK16/20 column (2.5 ml). For chromatography, the column was equilibrated at 3.5 ml/min. The equilibration buffer was 2 *M*

$(NH_4)_2SO_4$, 0.05 *M* sodium phosphate, pH 7.2 and the elution was performed by step gradient with 0.05 *M* sodium phosphate, pH 7.2.

2.6. Analytical procedures

2.6.1. Enzyme assay

Penicillin acylase activity (U/ml) was assayed according to previously reported techniques [22–24]. Briefly, 250 μ*M* NIPAB was used as a substrate, in 10 m*M* potassium phosphate, pH 7.2 at 37°C and the production of 6-nitro-3-aminobenzoic acid was monitored at 405 nm (extinction coefficient is 8980 l/mol cm).

One unit of enzyme activity (U) was defined as the amount of enzyme which catalyzes the formation of 1 μmol of 6-nitro-3-aminobenzoic acid/min.

2.6.2. Protein concentration

The protein concentration was estimated with Coomassie blue [25] using bovine serum albumin as standard.

2.6.3. Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using a Mini-PROTEAN II apparatus and a Tris–glycine buffer system according to the previously reported method [26] were used to monitor the purification during the chromatographic steps. Electrophoresis was performed for 45 min at 200 V using 16% polyacrylamide gels. Detection was done by Coomassie brilliant blue R250.

3. Results and discussion

3.1. Optimization of elution conditions of immobilized metal chelate affinity chromatography

IMAC was performed with chelating Sepharose fast flow (4 ml) and Cu^{2+} as metal. At this stage, the gel was equilibrated with 0.5 *M* NaCl, 0.05 *M* sodium phosphate, pH 7.2. The crude extract was injected onto the column without any dilution or conditioning step and washing was performed with equilibration buffer until the UV absorbance returned to baseline. The first step at 0.3 *M* NH_4Cl made it possible to eliminate contaminants. For the elution step, two methods were compared. The first was with 1 *M* NH_4Cl (Fig. 1) and the second with 0.01 *M* imidazole (Fig. 2).

The different fractions were analyzed for their total protein concentration and activity, and the results are presented in Table 1. The two methods of elution gave different results for purification fold (16.5 to 3.15) but similar results for recovery (90–99%). The difference about purification fold is due to

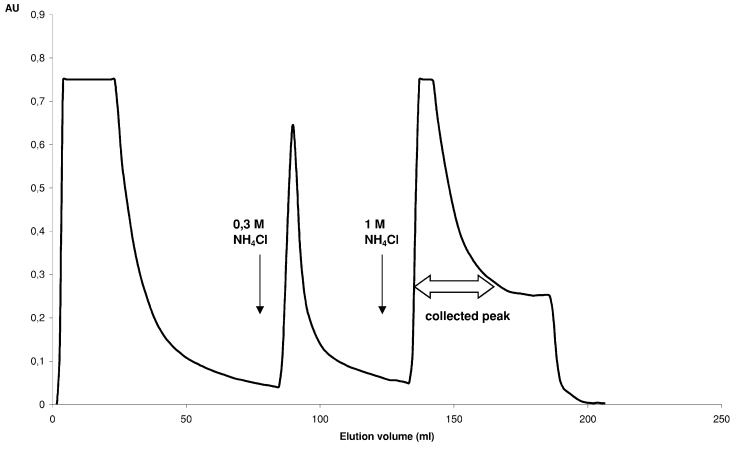

Fig. 1. Immobilized metal chelate affinity chromatography (IMAC). Column: XK16/20 (4 ml of chelating Sepharose fast flow). Sample: crude extract (10 ml), buffer A: 0.5 *M* NaCl, 0.05 *M* sodium phosphate, pH 7, buffer B: 1 *M* NH_4Cl in buffer A. Detection at 280 nm; flow-rate: 2 ml/min.

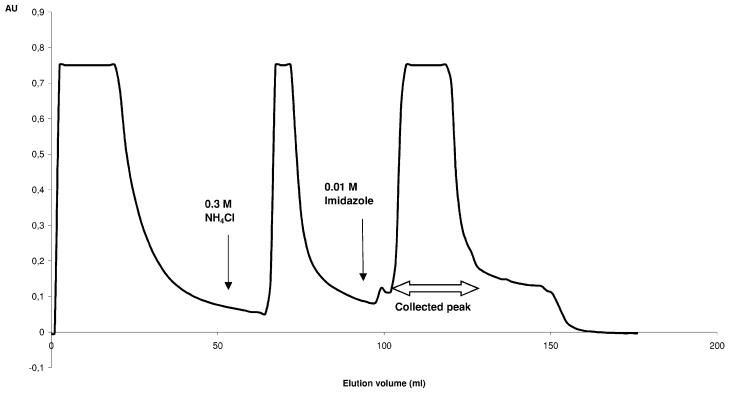

Fig. 2. Immobilized metal chelate affinity chromatography (IMAC). Column: XK16/20 (4 ml of chelating Sepharose fast flow). Sample: crude extract (10 ml), buffer A: 0.5 *M* NaCl, 0.05 *M* sodium phosphate, pH 7, buffer B: 0.01 *M* imidazole in buffer A. Detection at 280 nm; flow-rate: 2 ml/min.

the fact that 0.01 *M* imidazole elutes the penicillin acylase and a small part of the contaminants. Lower concentrations of imidazole were tested without penicillin acylase elution (data not shown). At 0.01 *M* imidazole, the totality of activity was recovered. Therefore, the elution with imidazole seems to be less selective than with 1 *M* NH_4Cl, the protein concentration for the two experiments (Table 1) shows that with imidazole the elution fraction was more contaminated than with 1 *M* NH_4Cl. This was also observed with the two chromatograms.

Elution with 1 *M* NH_4Cl gave the best results in terms of purification fold, so we used a hydrophobic chromatography as second step because the high salt concentration allowed this chromatographic sequence.

After these optimizations, a new preparation of crude extract was used to performed the IMAC–HIC sequence.

3.2. Hydrophobic interaction chromatography

The fraction eluted with 1 *M* NH_4Cl from IMAC was injected onto the 3-amino-5-phenylpyrazole Sepharose without any conditioning step (Fig. 3). The recovery of activity and purification fold are represented in Table 2. In fact, this two-step procedure gave a 66-fold purification in relation to the initial crude extract with 70% of activity recovery.

Electrophoretic analysis (Fig. 4) showed the purified fraction (lane 3) with the two bands corresponding to the two subunits of the penicillin acylase. This demonstrated the efficiency of the purification.

Table 1
Purification of *E. coli* penicillin acylase from a crude extract and elution by different eluents

Sample	Activity (U/ml)	Protein (mg/ml)	Specific activity[a] (U/mg)	Purification (-fold)	Recovery (%)
Crude extract	0.008	1.992	0.004		
Elution with 1 *M* NH_4Cl	0.00231	0.035	0.066	16.5	90
Elution with 10 m*M* imidazole	0.0032	0.254	0.0126	3.15	99

[a] One unit of enzyme is defined as the amount of enzyme needed to form 1 μmol of 6-nitro-3-aminobenzoic acid per min at pH 7.2 and 37°C.

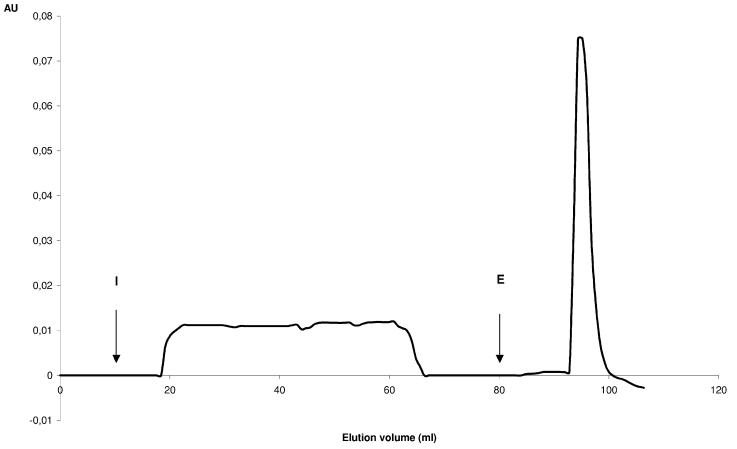

Fig. 3. Hydrophobic interaction chromatography of eluate of IMAC Fig. 1. Column: XK16/20 (4 ml of 3-amino-5-phenylpyrazole Sepharose). Sample: eluate of Fig. 1A (I); buffer A: 2 *M* $(NH_4)_2SO_4$, 0.05 *M* sodium phosphate, pH 7, (E) buffer B: 0.05 *M* sodium phosphate, pH 7. Detection at 280 nm; flow-rate: 2 ml/min.

4. Conclusion

In this work, IMAC was performed on an *E. coli* ultrasonic homogenate crude extract without any conditioning step. After removing the contaminants, we compared elution with NH_4Cl and with imidazole. Respectively, we obtained different results for purification fold (16.5 and 3.15) and similar results for recovery of activity (90 and 99%). After elution with NH_4Cl, HIC was performed without any conditioning step. Therefore, the final purification fold of 66 and 70% of activity recovery demonstrates that this two-step procedure is very suitable for the purification of penicillin acylase.

During the purification process, the penicillin acylase remained at an environmental pH near 7, which is compatible with its activity and allows enzymatic assaying without any conditioning step.

Since the elution of penicillin acylase at the end of the gradient was at low ionic strength, the protein in the final sample may be used under physiological conditions.

Acknowledgements

We thank R. Cooke for linguistic help. This work was supported by the Université V. Segalen Bordeaux 2 and the Conseil Régional d'Aquitaine.

Table 2
Purification of *E. coli* penicillin acylase from a crude extract by a two-step procedure

Sample	Activity (U/ml)	Protein (mg/ml)	Specific activity[a] (U/mg)	Purification (-fold)	Recovery (%)
Crude extract	0.0026	0.3	0.0086	–	–
First step: IMAC	0.0139	0.095	0.1463	17	90
Second step: hydrophobic interaction	0.0346	0.061	0.5672	3.88	77.7
Total				66	70

[a] One unit of enzyme is defined as the amount of enzyme needed to form 1 μmol of 6-nitro-3-aminobenzoic acid per min at pH 7.2 and 37°C.

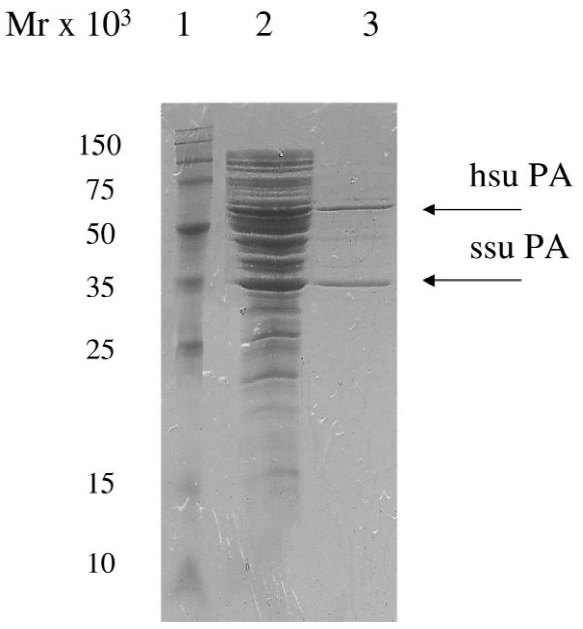

Fig. 4. Electrophoretic analysis of the purification of penicillin acylase. A 15-μg amount of soluble protein was loaded on a 16% SDS–PAGE system and Coomassie-stained. Lanes: 1=molecular mass marker, 2=crude extract, 3=purified fraction. hsu PA: high subunit of penicillin acylase, ssu PA: small subunit of Penicillin acylase.

References

[1] S.S. Ospina, A. Lopez-Munguia, R.L. Gonsalez, R. Quintero, J. Chem. Tech. Biotechnol. 53 (1992) 205.
[2] C. Fargues, S. Chanel, G. Grévillot, Bioseparation 6 (1997) 343.
[3] T.A. Sadvige, J. Vandamm (Eds.), Biotechnology of Industrial Antibiotic Drugs and the Pharmaceutical Sciences, Vol. 22, Marcel Dekker, New York, 1984, p. 172.
[4] A. Erarslan, I. Terzi, A. Guray, E. Bermek, J. Chem. Tech. Biotechnol. 51 (1991) 27.
[5] P.B. Mahajan, P.S. Borkar, Appl. Biochem. Biotechnol. 9 (1984) 421.
[6] V.K. Sudhakaran, J.G. Shewale, Biotechnol. Lett. 9 (1987) 539.
[7] S.K. Karyekar, M.V. Hegde, Biotechnol. Tech. 3 (1989) 145.
[8] V. Kasche, F. Loffer, T. Scholzen, D.M. Kramer, Boller, J. Chromatogr. 510 (1990) 149.
[9] E. Boccu, T. Gianferra, L. Gardossi, F.M. Veronese, Farmaco 45 (1990) 203.
[10] L.P. Fonseca, J.M.S. Cabral, Bioseparation 6 (1996) 292.
[11] J.C. Marcos, L.P. Fonseca, M.T. Ramalho, J.M.S. Cabral, J. Chromatogr. B 734 (1999) 15.
[12] X. Santarelli, V. Fitton, N. Verdoni, C. Cassagne, J. Chromatogr. B 739 (2000) 63.
[13] V. Fitton, X. Santarelli, J. Chromatogr. B, submitted for publication.
[14] J. Porath, J. Carlsson, I. Olsson, G. Belfrage, Nature 258 (1975) 598.
[15] J. Porath, M. Belew, in: I.M. Chaiken, M. Wilchek, I. Parikh (Eds.), Affinity Chromatography and Biological Recognition, Academic Press, San Diego, CA, 1983, p. 173.
[16] J. Porath, B. Olin, B. Granstrand, Arch. Biochem. Biophys. 225 (1983) 543.
[17] B. Lönnerdal, C.L. Keen, J. Appl. Biochem. 4 (1982) 203.
[18] E. Sulkowski, Trends Biotechnol. 3 (1985) 1.
[19] J. Porath, B. Olin, Biochemistry 2 (1983) 1621.
[20] J. Porath, Protein Expr. Purif. 3 (1992) 263.
[21] L.J. Fonseca, I.M. Patricio, J.M. Cabral, J. Mol. Recognit. 11 (1–6) (1998) 252.
[22] C. Kutzbach, E. Ravenbush, Hoppe-Seyler's Physiol. Chem. 354 (1974) 45.
[23] N. Robas, H. Zouheiry, G. Branlant, C. Branlant, Biotechnol. Bioeng. 41 (1993) 14.
[24] C. Fargues, Doctorate, INPL-ENSIC, Nancy, 1993.
[25] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[26] U.K. Laemmli, Nature 277 (1970) 680.