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Evaluation of immobilized metal affinity chromatography for purification of penicillin acylase

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Abstract

The aim of this work was to test immobilized metal affinity chromatography (IMAC) for the purification of penicillin acylase. After evaluation of different metals, Cu^{2+} was selected. Different samples were tested: pure penicillin acylase, industrial clarified feedstock and crude extract. After comparing two eluents, NH_4Cl and imidazole, it appeared that although both gave good results for recovery and activity, NH_4Cl was a more selective eluent with a higher fold purification than imidazole (4.64 versus 2.04). Moreover, we shown that a multistep gradient of NH_4Cl, greatly increased the degree of purification (12.36) compared with the one-step process as control (4.64). In addition, good recovery was obtained (97–100%).

Keywords: Enzymes; Penicillin acylase

1. Introduction

Important intermediates such as 6-aminopenicillanic acid (6-APA) are required to produce semi-synthetic penicillins. The immobilized penicillin acylase for the routine production of 6-APA is well-known in biotechnology [1]. Since penicillin acylase is obtained from enzymatic solutions of low purity, the production cost of 6-APA is partially dependent on enzymatic production. Moreover, it is necessary to purify large quantities of penicillin acylase at a low cost. Currently, and despite recent improvements, the purification process is expensive and has low recovery [2–4].

Few authors have described the evaluation of affinity and pseudo-affinity adsorption processes for penicillin acylase purification using antibiotic ligands close to penicillin (ampicillin, amoxycillin, cephalexin) [5–10]. Most have performed hydrophobic interactions but the drawbacks of ligands such as ampicillin, amoxycillin, cephalexin and penicillin are that they are hydrolyzable (56 to 18%) [5] and expensive. Moreover, some authors [5] have demonstrated that a hydrophobic support (phenyl, octyl) is not the best for a full activity recovery due to the strong hydrophobic interaction.

In a previous article [11], we evaluated different ligands which are structural analogs to antibiotics. Although we used a hydrophobic interaction process, we obtained good recovery (73 to 100%) and good degree of purification (1.9 to 5). Recently [12] a partial purification of penicillin acylase by aqueous

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two-phase systems was presented with similar results: 85% recovery and 5.7 purification factor.

In the present work, we evaluate another method to purify penicillin acylase by immobilized metal affinity chromatography (IMAC) [13–19]. In this case, proteins are separated on the basis of their affinity for a metal ion.

2. Experimental

2.1. Instruments

The chromatographic systems used throughout this study were 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, 405 and 595 nm.

2.2. Chemicals

Pure penicillin acylase and clarified feedstock were kindly supplied by Gist-Brocades (Seclin, France).

Chelating Sepharose fast flow and the XK 16/20 column were purchased from Amersham Pharmacia Biotech. 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 UV absorption. It was then filtered. All other salts were HLPC grade, and the buffers were filtered through a 0.22-μm membrane filter.

2.3. Cell culture and preparation of crude extract

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. 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 $MgSO_4.7\ H_2O$, 2 g phenylacetic acid, 7 g tryptone and 0.1 g yeast extract per liter. 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, then diluted 1:4 in equilibration buffer: 0.5 *M* NaCl, 0.02 *M* sodium phosphate, pH 7, and injected onto the column.

2.4. Preparation of supports

The gels were packed in an XK16/20 column. A slurry was prepared with binding buffer in a ratio of 75% settled gel to 25% buffer 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 (150 cm/h). The packing flow-rate was maintained for three bed volumes after a constant bed height was reached.

2.5. Metal affinity selection

The selection of immobilized metal chromatographic supports was performed in an XK16/20 column with 2 ml of gel. Four metals were tested: Ni^{2+}, Cu^{2+}, Fe^{3+}, Zn^{2+}. The adsorption of metal was obtained with 0.3 *M* metal solution in distilled water at 5 ml/min (150 cm/h). The columns were equilibrated with 0.5 *M* NaCl, 0.02 *M* sodium phosphate, pH 7 and elution performed with a linear gradient of NH_4Cl with 1 *M* NH_4Cl, 0.5 *M* NaCl, 0.02 *M* sodium phosphate, pH 7. Pure penicillin acylase was diluted (1:10) in the equilibration buffer and 1 ml at a fixed concentration (0.95 U) was injected onto the column and analyzed for penicillin acylase activity recovery. An industrial clarified feedstock and crude extract prepared in our laboratory were also used.

2.6. Determination of sorption capacity

Measurement of the sorption capacity of IMAC with the selected metal for penicillin acylase was based on breakthrough curves with calculations made at 10% breakthrough and by the peak collection method.

Pure penicillin acylase (0.5 mg/ml) in equilibration buffer was prepared and injected onto 1 ml of gel in a continuous manner at 1 ml/min.

2.7. Study of recovery versus flow-rate

Five flow-rates were used to study recovery versus flow-rate: 1 ml/min (30 cm/h), 5 ml/min (150 cm/h), 10 ml/min (300 cm/h), 25 ml/min (750 cm/h). A fixed concentration of pure penicillin acylase was injected (0.95 U) and protein elution was performed with a linear gradient with 50 m*M* sodium phosphate, pH 7.

2.8. Analytical procedures

2.8.1. Enzyme assay

Penicillin acylase activity (U/ml) was assayed according to Refs. [20–22]. Briefly, 250 μ*M* 6-nitro-3-phenylacetamidobenzoic acid (NIPAB, SIGMA) were 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 1/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.

2.8.2. Protein concentration

The protein concentration was estimated with Coomassie blue [23] using bovine serum albumin as standard.

3. Results and discussion

3.1. Metal selection

Under the conditions described above, a metal scouting experiment was performed (Table 1). Three IMAC supports retained penicillin acylase. Cu^{2+} gave the best recovery (>70%), while Ni^{2+} and Zn^{2+} retained only 22 and 15% of pure penicillin acylase, the majority being in the through flow. The IMAC support with Fe^{3+} did not retain penicillin acylase at all (100% in the through flow). For all experiments, elution was performed at the end of the gradient (1 *M* NH_4Cl). From these results, we selected Cu^{2+} as the metal for IMAC support.

Table 1
Scouting experiments for capture of pure penicillin acylase by different metals

Metal	Enzyme in through flow (%)	Eluted enzyme (%)
Ni^{2+}	44.33	22
Cu^{2+}	0.093	72
Zn^{2+}	69.7	15.3
Fe^{3+}	100	0

3.2. Determination of sorption capacity

To measure the dynamic capacity of IMAC for penicillin acylase, the frontal analysis method was used. A 10% breakthrough point was used to calculate the capacity. Moreover, we also used a peak collection method after a step gradient at 1 *M* NH_4Cl. Breakthrough curves were measured at 1 ml/min. Under these experimental conditions, the capacity was 45 mg/ml at 10% breakthrough point and peak collection.

3.3. Recovery versus flow-rate

After selecting the metal, we performed an experiment to test the binding capacity of IMAC at different flow-rates.

Five flow-rates were used (Fig. 1). Up to 10 ml/min (300 cm/h) the recovery of penicillin acylase was about 75%. When the flow-rate was twice as large, the recovery decreased by only 10% and when the flow-rate increased fourfold (1200 cm/h) the recovery decreased by 50%. This indicated that a high flow-rate (300 cm/h) could be used as a capture step of penicillin acylase and that up to 1200 cm/h the binding capacity was 50% of recovery.

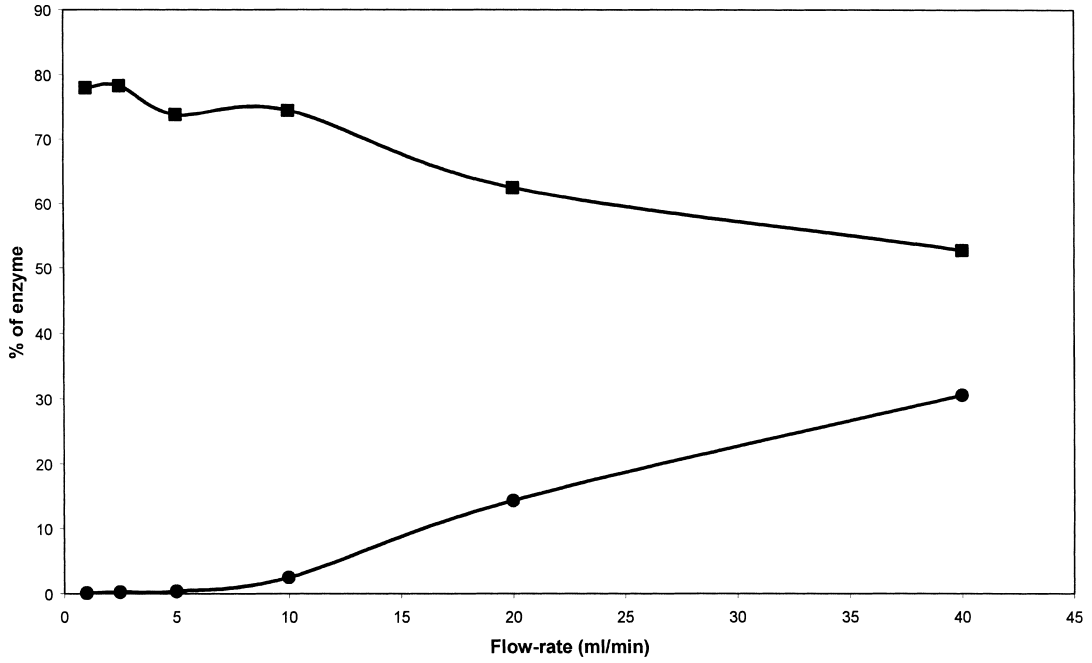

Fig. 1. Effect of flow-rate with chelating Sepharose fast flow on activity recovery in linear gradient elution. (■) % of activity recovery in the eluate fraction, (·) % of activity in the through flow; sample: 0.95 U penicillin acylase (pure penicillin acylase has a specific activity of 12 U/mg) suspended in equilibration buffer: 0.5 *M* NaCl, 0.02 *M* sodium phosphate, pH 7, elution buffer: 1 *M* NH_4Cl, 0.5 *M* NaCl, 50 m*M* sodium phosphate, pH 7.

3.4. Elution conditions

A multistep gradient elution of imidazole was tested with pure protein in addition to the elution method used above and the maximum elution was obtained with 5 m*M* imidazole (70% of recovery).

Contrary to the elution with NH_4Cl, the position of elution with imidazole was at the beginning of the gradient. This difference in elution was studied with a clarified feedstock and a crude extract.

In the next experiment, the two methods of elution were compared with an industrial clarified feedstock (Table 2). There was a slight advantage for the NH_4Cl elution with regard to purification factor (1.77 against 1.21). Therefore, we tested a three-step elution by NH_4Cl. In the case of a one-step elution

Table 2
Purification of *E. coli* penicillin acylase from an industrial clarified feedstock (specific activity: 4.32 U/mg) and elution by steps of NH_4Cl and imidazole

Sample	Activity (U/ml)	Protein (mg/ml)	Specific activity[a] (U/mg)	Purification (-fold)	Recovery (%)
Elution with 5 m*M* imidazole	1.17	0.22	5.25	1.21	80
Control (one step): 1 *M* NH_4Cl	0.92	0.12	7.67	1.77	80
First step: 100 m*M* NH_4Cl	$5.94 \cdot 10^{-3}$	0.103	0.058	0.013	0.10
Second step: 300 m*M* NH_4Cl	0.19	0.102	1.86	0.43	6
Third step: 1 *M* NH_4Cl	1.39	0.101	13.76	3.18	73

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

Table 3
Purification of *E. coli* penicillin acylase from a crude extract and elution by steps of NH_4Cl and imidazole

Sample	Activity (U/ml)	Protein (mg/ml)	Specific activity[a] (U/mg)	Purification (-fold)	Recovery (%)
Crude extract	0.014	0.64	0.022	-	–
Elution with 5 m*M* imidazole	$6.35 \cdot 10^{-3}$	0.14	0.045	2.04	100
Control (one step): 1 *M* NH_4Cl	$5.93 \cdot 10^{-3}$	0.058	0.102	4.64	100
First step: 100 m*M* NH_4Cl	$4.58 \cdot 10^{-5}$	$2.17 \cdot 10^{-3}$	0.021	0.97	0.55
Second step: 300 m*M* NH_4Cl	$2.46 \cdot 10^{-4}$	$5.06 \cdot 10^{-3}$	0.05	2.24	0.68
Third step: 1 *M* NH_4Cl	$4.84 \cdot 10^{-3}$	0.013	0.26	12.36	97

[a] One unit of enzyme as defined in Table 2.

with 1 *M* NH_4Cl as control, 80% recovery was obtained with a degree of purification of 1.77. With a multistep gradient at 100 m*M* and 300 m*M* of NH_4Cl, only 0.1% and 6% of penicillin acylase, respectively was recovered. With 1 *M* NH_4Cl as a final step, a similar result to the control value was found (73%), but interestingly the purification factor was greatly increased from 1.77 to 3.18.

After the study with an industrial clarified feedstock, we tested a crude extract produced in our laboratory. Table 3 shows that elution with 1 *M* NH_4Cl gave a degree of purification twice as large as that obtained with imidazole. For the two types of elution the recovery was 100%.

A three-step elution of NH_4Cl was done: for 100 m*M* and 300 m*M* of NH_4Cl the purification factor was only 0.97 and 2.24 with 0.55 and 0.68% recovery, respectively. At 1 *M* NH_4Cl, the degree of purification was 12.36 with 97% recovery. Therefore, the same result was obtained as for the clarified feedstock, i.e., a three-step elution of NH_4Cl gave a better purification factor about twice as large as the control value. Moreover, elution with NH_4Cl was more efficient than with imidazole as tested both with clarified feedstock and crude extract.

4. Conclusion

In this paper, we show that IMAC is suitable for capture of penicillin acylase. The results of recovery are similar as those obtained with His–Tag proteins for which IMAC is known to be a very adequate support [24]. The eluents: NH_4Cl and imidazole were compared and in both cases a good recovery was obtained, although NH_4Cl appeared to be more selective with a higher fold purification. By using NH_4Cl as eluent, we demonstrate that a multistep gradient of NH_4Cl increases the degree of purification by at least a factor of two compared with a one-step process as control. Therefore, by coupling IMAC and a multistep gradient of NH_4Cl, a very efficient and easy-to-use process for purification of penicillin acylase has been obtained, which can be readily used on an industrial scale.

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