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Penicillin acylase purification with the aid of hydrophobic charge induction chromatography

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Abstract

The aim of this work was to test a chromatographic support, 4-mercaptoethyl pyridine (4-MEP) Hypercel, for penicillin acylase purification by using pure penicillin acylase and crude extract. Two equilibration buffers with various salt concentrations and different flow rates were tested. The relationships between electrostatic and hydrophobic interactions and proteins are demonstrated. $(NH_4)_2SO_4$ proved preferable because no salting-in occurred, contrary to NaCl. The recovery and purification fold were similar to those obtained in pseudo-affinity chromatography with a three-fold reduction of the $(NH_4)_2SO_4$ concentration.
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1. Introduction

The production of semi-synthetic penicillins requires important intermediates such as 6-aminopenicillanic acid (6-APA), which is produced industrially by using immobilized penicillin acylase [1]. The high cost of producing 6-APA is mainly due to the enzyme purification process and, despite improvements over the years, the process has low recovery [2–4]. Affinity and pseudo-affinity adsorption processes for penicillin acylase purification using antibiotic ligands similar to penicillin (ampicillin, amoxycillin, cephalexin) were investigated in the past [5–10]. Most led to hydrophobic interactions but the drawbacks of these ligands are that they are hydrolysable (18–56%) [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 interactions.

Different ligands which are structural analogs to antibiotics have also been studied [11]. Although the hydrophobic interaction process was used, good recovery (73–100%) and purification fold (1.9–5) results were found. Recently, immobilized metal affinity chromatography (IMAC) [12–18] was investigated [19] but the presence in the process of heavy metals which are toxic did not facilitate the validation for biopharmaceutical uses.

In the search for the ideal ligand for penicillin acylase purification [20], aromatic rings substituted by amino, hydroxyl or another group to modulate the hydrophobic interactions have been tested. The presence of a sulphur group in the ligand structure was not investigated, although it is present in this substrate.

For ligand adsorption chromatography, the importance of sulphur atoms is now well known [21–23] thanks to investigation of the phenomenon of aromatic adsorption. The synergistic adsorption effect of sulphur was evidenced when it was close to the aromatic ring. By using heterocycles in thiophilic chromatography [24,25], it has been demonstrated that aromatic or heterocyclic compounds linked by a thioether bond on the matrix are able to adsorb some proteins. The specific influence of sulphur atom was induced by the π-electron system of the pyridine residue.

In literature [20], specific ligands for penicillin acylase purification were listed and the best ligand selected contained a heterocycle. In addition, as the penicillin acylase amino acid composition revealed an abundance of tryptophan, phenylalanine and tyrosine, which are considered to be electron donors, we assumed that the interaction of penicillin acylase via the heterocycle and thiophilic system was

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possible. This was reinforced by the presence of a sulphur atom in the enzyme substrate.

Therefore, we investigated a new chromatographic support, MEP Hypercel [26–29], which includes 4-mercaptoethyl pyridine (4-MEP), an ionizable head group with a hydrophobic tail and which is designed for antibody purification through hydrophobic charge induction chromatography (HCIC). The pK_a of 4-MEP is 4.8 and at physiological pH, the aromatic pyridine ring is uncharged. For antibody purification, the adsorption is only based on a mild hydrophobic interactions whereas desorption is based on charge repulsion and by reducing the pH to 4.

The aim of this paper is to evaluate 4-MEP Hypercel for penicillin acylase purification in order to find a new method to avoid working with IMAC and heavy metals as in our current process. The use of hydrophobic charge induction chromatography with its thiophilic effect, hydrophobicity and its ionizable ring makes it possible to reduce the $(NH_4)_2SO_4$ concentration three-fold in comparison with pseudo-affinity methods [11].

2. Experimental

2.1. Instruments

The chromatographic system used throughout this study was the FPLC workstation from Amersham Pharmacia Biotech (Saclay, France). The data were collected and evaluated using the FPLC director data system. For recovery studies, we used an Uvikon 930 spectrophotometer (Kontron, Montigny Lebretonneux, France) to measure absorbance at 280, 405 and 595 nm.

2.2. Chemical

Pure penicillin was kindly supplied by DSM-Food specialties France (Seclin, France). MEP Hypercel was kindly supplied by BioSepra (Cergy-Saint-Christophe, France). All salts and 6-nitro-3-phenylacetamidobenzoic acid (NIPAB) were from Sigma (l'Isle d'Abeau Chesnes, France). Buffer with $(NH_4)_2SO_4$ was treated with activated charcoal to avoid undesirable absorption of UV. It was then filtered. All other salts were of HLPC grade, and all 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 were 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 mM 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 then diluted 1/4 in equilibration buffer: 0.5 M NaCl, 0.02 M sodium phosphate pH 7 before injection onto the column.

2.4. Preparation of supports

The MEP Hypercel chromatographic support (2 ml) was packed in a XK16/20 column according the manufacturer's procedure.

2.5. Stability of penicillin acylase activity in different buffers at different times

Stability of penicillin acylase activity was studied in the different buffers used in the chromatographic studies (50 mM sodium phosphate, pH 7; 50 mM sodium phosphate, 2 M NaCl, pH 7; 50 mM sodium phosphate, 2 M $(NH_4)_2SO_4$, pH 7; 50 mM sodium acetate pH 4) at different times (1, 2, 5, 8 and 24 h) at 25 °C.

2.6. Study of recovery versus flow rate and equilibration buffer

Four flow rates were evaluated to study recovery versus flow rate: 1, 2.5, 5 and 10 ml/min. Two equilibration buffers were tested: 50 mM sodium phosphate pH 7 with various concentrations of $(NH_4)_2SO_4$ (0, 0.5, 1 and 2 M); and 50 mM sodium phosphate pH 7 with various NaCl concentrations (0, 0.5, 1 and 2 M). A fixed concentration of pure penicillin acylase was injected (0.95 U) and protein elution was performed with a linear gradient with 50 mM sodium acetate pH 4.

2.7. Evaluation of the method with a crude extract

The ultrasonic homogenate was diluted 1/4 in equilibration buffer: 0.5 M $(NH_4)_2SO_4$, 0.02 M sodium phosphate pH 7 and injected onto the column. The experiment was performed at 2.5 ml/min. The fractions were collected and analyzed.

2.8. Analytical procedures

2.8.1. Enzyme assay

Penicillin acylase activity (U/ml) was assayed according to [30–32]. Briefly, 250 μM 6-nitro 3-phenylacetamidobenzoic acid (Sigma) were used as a substrate in 10 mM 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 M/cm).

One unit of enzyme activity (U) was defined as the amount of enzyme which catalyzes the formation of 1 μmole of 6-nitro 3-amino benzoic acid per minute at 37 °C.

2.8.2. Protein concentration

The protein concentration was estimated with the Bradford protein assay [33]. Bovine serum albumin was used as standard.

3. Results and discussion

3.1. Stability of penicillin acylase activity in different buffers at different times at 25°C

Before evaluating the efficiency of 4-MEP Hypercel for the recovery of penicillin acylase, we verified that the different buffers used in the different experiments did not modify the penicillin acylase activity even after several hours.

Table 1 show that the stability of penicillin acylase activity in the different buffers at 25 °C was high until 24 h. Even at pH 4 or with high salt concentration, the activity remained higher than 90% after 24 h. Therefore, the evaluation of the 1-MEP Hypercel matrix was performed.

3.2. Recovery versus flow rate and concentration of ammonium sulfate in 50 mM sodium phosphate pH 7 equilibration buffer

Fig. 1 shows that recovery remained stable (100–95%) until 2.5 ml/min and decreased to 83% at 10 ml/min for 2 M in $(NH_4)_2SO_4$. With 1 and 0.5 M $(NH_4)_2SO_4$, the decrease was greater and reached 70% at 10 ml/min.

At a flow rate of 1 ml/min the recovery was similar for the three equilibration buffers. This indicated that at low flow rate, the hydrophobic interactions occurred without the salting-out effect or with only a slight effect. When the flow rate increased, the hydrophobic interactions compensated the inherent decrease in interaction.

3.3. Recovery versus flow rate and concentration of NaCl in 50 mM Tris–HCl pH 8 as equilibration buffer

Fig. 2 shows that without NaCl, the recovery at 1 ml/min was only 70% and decreased quickly to 20% at 10 ml/min.

Table 1
Stability of penicillin acylase activity in different buffers at different times at 25 °C

	1 h	2 h	5 h	8 h	24 h
50 mM sodium phosphate, pH 7	100	100	100	100	99.5
50 mM sodium acetate, pH 4	100	98.6	98	96.8	92.7
50 mM sodium phosphate, 2 M NaCl, pH 7	100	100	99.3	98.7	95.5
50 mM sodium phosphate, 2 M $(NH_4)_2SO_4$, pH 7	100	100	100	100	100

Activity is expressed as % of activity recovery.

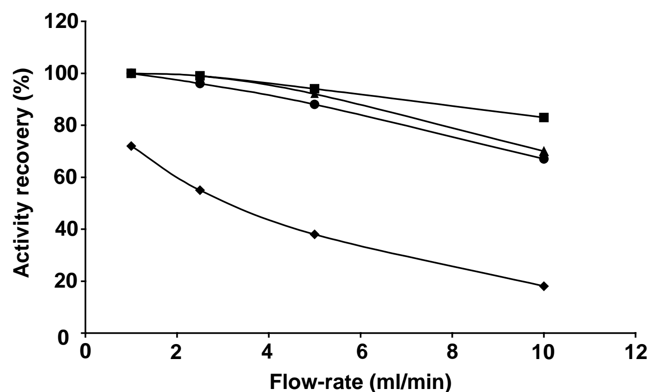

Fig. 1. Activity recovery vs. flow rate and concentration of $(NH_4)_2SO_4$. A fixed concentration of pure penicillin acylase was injected (0.95 U) in the different equilibration buffers: 2 M $(NH_4)_2SO_4$ in 50 mM sodium phosphate pH 7 (■), 1 M $(NH_4)_2SO_4$ in 50 mM sodium phosphate pH 7 (▲), 0.5 M $(NH_4)_2SO_4$ in 50 mM sodium phosphate pH 7 (●), 50 mM sodium phosphate pH 7 (◆) and protein elution was performed with a linear gradient with 50 mM sodium acetate pH 4.

With 0.5 M NaCl, the recovery at 1 ml/min was 95% and decreased to 30%. With 1 M NaCl, the recovery was 95% at 1 ml/min and decreased to 40% at 10 ml/min. However, at 2 M NaCl, the recovery was very low and was below 10% at 1 and 2.5 ml/min. When the flow rate reached 5 ml/min, the recovery was 45% and reached 35 at 10 ml/min. Beyond 5 ml/min, the experiment at 2 M NaCl gave similar results to those at 0.5 and 1 M NaCl.

In hydrophobic interaction chromatography, both the electrostatic and hydrophobic interactions with the protein affect the efficiency of the separation [34]. The hydrophobic interaction can be predicted by the molal surface tension increment, which depends on the nature of the salt, whereas the electrostatic interplay is correlated with the salt concentration. The behavior of penicillin acylase with NaCl versus $(NH_4)_2SO_4$ can be explained by the difference of

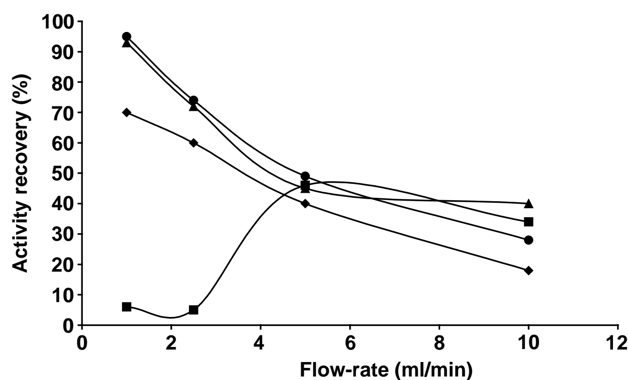

Fig. 2. Activity recovery vs. flow rate and concentration of NaCl. A fixed concentration of pure penicillin acylase was injected (0.95 U) in the different equilibration buffers: 2 M sodium chloride in 50 mM sodium phosphate pH 7 (■), 1 M sodium chloride in 50 mM sodium phosphate pH 7 (▲), 0.5 M sodium chloride in 50 mM sodium phosphate pH 7 (●), 50 mM sodium phosphate pH 7 (◆) and protein elution was performed with a linear gradient with 50 mM sodium acetate pH 4.

Table 2
Purification of *E. coli* penicillin acylase from a crude extract

Buffer	Fraction	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)	Purification fold	Recovery (%)
$(NH_4)_2SO_4$ (1 M)	Injection	0.1522	0.062	2.455		
	Through flow	0.0041	0.008	0.512	0.208	5.79
Elution (pH 4)	Elution	0.067	0.006	11.167	4.548	94.21
$(NH_4)_2SO_4$ (0.5 M)	Injection	0.1592	0.088	1.809		
	Through flow	0.0028	0.006	0.467	0.258	7.06
Elution (pH 4)	Elution	0.061	0.008	7.625	4.215	92.94
$(NH_4)_2SO_4$ (0.0 M)	Injection	0.173	0.138	1.253		
	Through flow	0.0146	0.016	0.912	0.727	29.32
Elution (pH 4)	Elution	0.0304	0.007	4.342	3.465	70.67

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

molal surface tension increment between the two salts. The molal surface tension increment of NaCl ($\sigma_{NaCl} = 1.64 \times 10^{-4}$ N/m) was close to the limit molal surface tension increment between the salting-in and salting-out effect ($\sigma = 1.5 \times 10^{-4}$ N/m), whereas the molal surface tension increment of $(NH_4)_2SO_4$ was higher ($\sigma = 2.16 \times 10^{-4}$ N/m). This phenomenon was observed also with the cell surface hydrophobicity behaviour [35].

For low NaCl concentrations, the electrostatic effect was negligible compared to the hydrophobic interactions, leading to interactions between proteins and the chromatographic support. On the contrary, at high NaCl concentrations (2 M), as σ_{NaCl} was low, the electrostatic interaction became dominant, thus promoting the salting-in effect and then a poor efficiency of the purification. In addition, the salting-in effect decreased with the increase of the flow rate. The NaCl had no effect on the capture of the protein from 5 ml/min.

Similar results have been mentioned in the literature for the purification of *Trichoderma reeisei* cellulases [36].

3.4. Hydrophobic charge induction chromatography with crude feedstock

The results are presented in Table 2 and Fig. 3. The results are similar for 1 and 0.5 M $(NH_4)_2SO_4$ in the equilibration buffer with more than 90% of recovery and 4.5 purification fold. Without $(NH_4)_2SO_4$, recovery and purification fold were low. Around 30% of the enzyme was found in the through flow. This reinforces what we had previously observed regarding the importance of $(NH_4)_2SO_4$ in pseudo-affinity chromatography and consequently in enzyme binding to the ligand in hydrophobic charge induction chromatography with the advantage of reducing the $(NH_4)_2SO_4$ concentration by 3 and decrease the cost of the experiments.

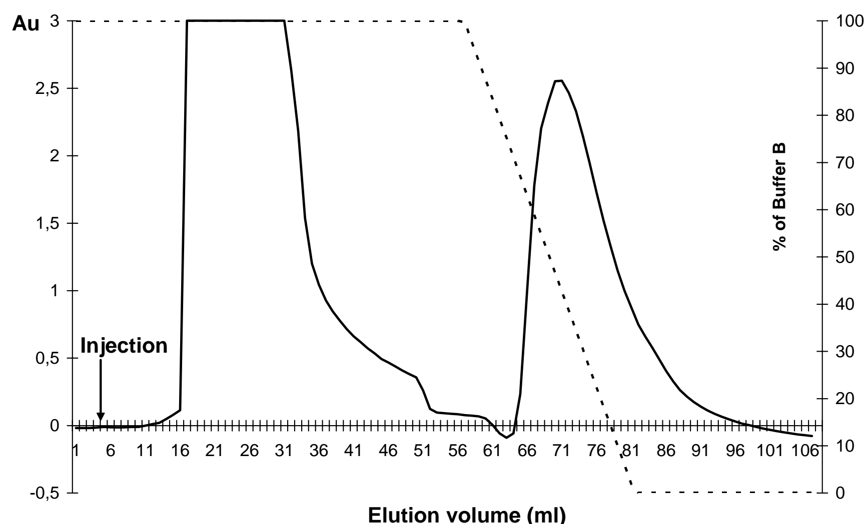

Fig. 3. Hydrophobic charge induction chromatography (HCIC). Column: XK 16/20 (2 ml of MEP Hypercel). Sample: crude extract (10 ml); buffer A: 0.5 M $(NH_4)_2SO_4$; 0.02 M sodium phosphate pH 7; buffer B: 50 mM sodium acetate pH 4. Detection at 280 nm; flow-rate: 2 ml/min.

4. Conclusion

In this paper, we show that hydrophobic charge induction chromatography with 4-MEP Hypercel as ligand is suitable for the capture of penicillin acylase according to the ligand design for penicillin acylase purification described in literature [20]. The recovery and purification fold values are similar as those obtained in pseudo-affinity chromatography [11] with the advantage of reducing the $(NH_4)_2SO_4$ concentration by 3. The use of HCIC with this type of ligand makes it possible to combine thiophilic, hydrophibicity and charge induction effects for the satisfactory purification of penicillin acylase for biopharmaceutical purposes. The use of 4-MEP Hypercel design for antibodies purification allowed us to avoid IMAC and therefore the use of heavy metals which are toxic.

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