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Preparation, evaluation and application of new pseudo-affinity chromatographic supports for penicillin acylase purification

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Abstract

New pseudo-affinity chromatographic supports for penicillin acylase were prepared and evaluated with three different samples: pure penicillin acylase, industrial clarified feedstock and crude extract.The different gels were studied for their purification fold (three to six) and their recovery power (80–100%). The best support was characterized by its dynamic capacity, (20 mg/ml) and its recovery power was tested at five flow-rates (30, 150, 300 and 750 cm/h) to determine the optimal flow-rate (300 cm/h). In addition we used cleaning in place to test the resistance to hard conditions of sanitization by 1 *M* NaOH (90% of recovery for 12 h of contact). These gels may therefore be used on an industrial scale.

Keywords: Pseudo-affinity; Enzymes; Penicillin acylase

1. Introduction

The production of semi-synthetic penicillins requires important intermediates such as 6-aminopenicillanic acid (6-APA). The immobilized penicillin acylase for 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 the enzymatic production. Therefore, a cost reduction would be of great interest.

Despite recent improvements [2], the industrial multi-step purification process is expensive and has low recovery [3,4].

The present work proposes a one-step purification process by pseudo-affinity chromatography.

Such chromatography is considered as the best selective technique for protein purification and has long been used for enzyme purification [5]. The ligand should exhibit specific and reversible high affinity for the substance to be purified and it should have chemically modifiable groups to allow its binding to the matrix.

Few authors have described the evaluation of affinity and pseudo-affinity adsorption processes for penicillin acylase purification [6–11]. For affinity adsorption, these authors used low salt conditions.

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The interaction is based only on the biological function or individual chemical structure, while in pseudo-affinity adsorption the interaction must be promoted with addition of high concentrations of salts. Although antibiotic ligands close to penicillin (ampicillin, amoxycillin, cephalexin, 6-aminopenicillanic acid) have been used, most authors have performed a hydrophobic interaction (because penicillin acylase possesses an hydrophobic region near the catalytic site) classified as a pseudo-affinity interaction [12] between the ligand and the substrate. In this paper we use the same terminology.

The drawbacks of ligands such as ampicillin, amoxycillin, cephalexin and penicillin are that they are hydrolyzable (56 to 18%) [6] and are expensive.

Mahajan and Borkar [6] demonstrated that a hydrophobic support (phenyl, octyl) was not appropriate for a full activity recovery due to the strong hydrophobic interaction. Our objective was to find a cheaper alternative to this support. We chose several ligands with a hydrophobic area allowing chromatography with a medium strength hydrophobic interaction.

In this paper, 10 new pseudo-affinity chromatographic supports were prepared and evaluated. These ligands were attached by epoxy function with spacer arms. The use of a spacer arm was necessary for low molecular mass ligands to increase their accessibility towards the interacting molecule [13]. The epoxy-activated gel allowed the introduction of the spacer arm at the same time as the coupling [14].The size and global structure of the ligands were selection criteria.

Moreover, the presence of an appropriate chemical reactive group (amine or hydroxyl) necessary for its immobilization and solubility in the appropriate buffer with a low percentage of dimethylformamide and stability at high pH were taken into account for this selection. Stability at high pH was important for the sanitization process. The ligands used were: 4-aminoantipyrin, 2-amino-3-benzyloxypyridine, 3-amino-5-phenylpyrazole, 3-amino-1-phenyl-2-pyrazolin-5-on, 1-benzyl 4-hydroxypiperdine, ethyl-4-hydroxybenzimidate, helicin, 4-hydroxybenzaldehyde, 4-hydroxybenzylalcool, salicin (Fig. 1).

Characterization, evaluation and application were performed by measuring selectivity, dynamic adsorption capacity and protein recovery with a pure penicillin acylase and with clarified feedstocks kindly supplied by an industrial company. Crude extracts were from our laboratory.

All the experiments were done on fast protein liquid chromatography (FPLC) and Biopilot workstations.

After a progressive selection, the best support was used to test the dynamic adsorption capacity with breakthrough curves. This capacity was determined for 20 mg/ml of gel. In addition, we determined optimal flow-rate (300 cm/h) and tested hard conditions by cleaning in place with 1 *M* NaOH at 15 min, 30 min, 1 h, 4 h and 12 h. The activity recovery remained stable during sanitization.

2. Experimental

2.1. Instruments

The chromatographic systems used throughout this study were the FPLC and Biopilot workstations from Amersham Pharmacia Biotech (Saclay, France). The data were collected and evaluated using the FPLC director and Unicorn 1-12 data system.

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

2.2. Chemicals

Pure penicillin acylase and clarified feedstock were kindly supplied by Gist-Brocades (Seclin, France). Epoxy-activated Sepharose 6B, XK16/20 and XK16/70 columns were purchased from Amersham Pharmacia Biotech. All ligands, salts and NIPAB (6-nitro-3-phenylacetamidobenzoic acid) were from Sigma (l'Isle d'Abeau Chesnes, France). All 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 were used to

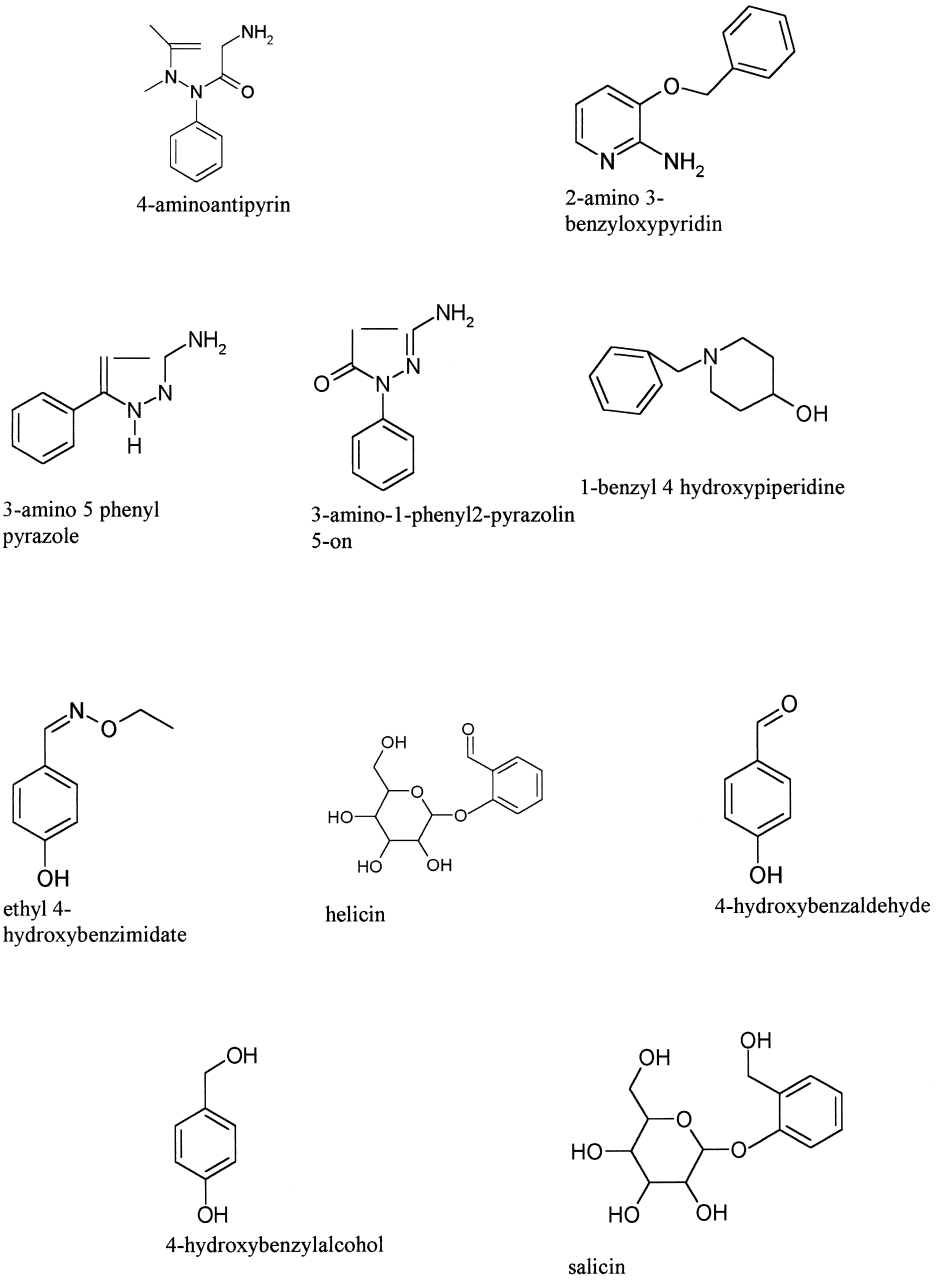

Fig. 1. Chemical structure of ligands immobilized on epoxy-activated Sepharose 6B.

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 two 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, diluted 1/4 in 2 *M* $(NH_4)_2SO_4$ and injected onto the column.

2.4. Preparation of supports

Epoxy-activated Sepharose is a pre-activated gel for immobilization of various ligands via stable ether linkages to hydroxyl, amino or thiol groups.

The gel has a long hydrophilic spacer arm which makes it particular suitable for immobilization of small molecules.

Preparation of gel and coupling the ligand were performed according to the manufacturer's instruction. First, 220 μmol of ligand were added per ml gel. The ligand was dissolved in coupling buffer, 50 m*M* sodium carbonate, pH 11.

Some ligands needed dimethylformamide for dissolution.

2.4.1. Ligand concentration assay

The ligand concentration linked to the support was calculated as the difference between the concentration of the ligand in the initial reaction mixture and that in the final wash mixture after filtration of the gel on the sintered glass. All ligands were chosen for their quality to absorb ultraviolet or visible light. This facilitated the spectrophotometric determination of the concentration.

2.4.2. Packing the gel

The gels were packed in an XK16/20 column according to the manufacturer's instructions.

2.5. Ligand selection

The selection of such chromatographic supports was performed in an XK16/20 column with 2 ml of gel. The dynamic adsorption was tested at 5 ml/min (150 cm/h). Two types of adsorption conditions were tested at room temperature.

(i) Affinity conditions: the enzymatic samples were adjusted to an appropriate ionic strength and pH with MES [2-(*N*-morpholino)ethanesulfonic acid] at pH 7, Tris–HCl at pH 8 and sodium acetate at pH 5.5 for pH adsorption experiments. The chromatographic supports were equilibrated with the same buffer and the desorption was studied by step gradient to 1 *M* NaCl in the same equilibration buffer and by pH variation with the equilibration buffer of two other experiments.

(ii) Pseudo-affinity conditions: the enzymatic samples were previously adjusted to an ammonium sulfate concentration of 2 *M*, 1.5 *M* and 1 *M* with 2 *M* ammonium sulfate–50 m*M* sodium phosphate, pH 7 buffer.

The chromatographic support was equilibrated with the same buffer and the desorption of penicillin acylase was promoted by decreasing the gradient with a 50 m*M* sodium phosphate, pH 7 buffer. Each gel was tested for these three starting concentrations.

After selecting the best starting conditions, pure penicillin acylase was injected at a fixed concentration (400 μl, 3.23 U) onto each column to calculate the different activity recovery and to select the best columns. 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 the selected resin for penicillin acylase was based on breakthrough curves with calculations made at 10% and 50% breakthrough and by the peak collection method.

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 (400 μl, 3.23 U) and protein elution was performed with a linear gradient with 50 m*M* sodium phosphate, pH 7.

2.8. Sanitization effect of recovery

The sanitization protocol is important for chromatographic media. We used sodium hydroxide, the most popular sanitization agent. Three columns were packed with the best support and tested with 1 *M* NaOH for 15 min, 30 min, 1 h, 4 h and 12 h. The recovery was studied before and after the treatment.

2.9. Analytical procedures

2.9.1. Enzyme assay

Penicillin acylase activity (U/ml) was assayed according to Refs. [15–17], using 250 μ*M* 6-nitro-3-phenylacetamidobenzoic acid (NIPAB, Sigma) as a substrate, in 10 m*M* potassium phosphate, pH 7.2 at 37°C and by monitoring the production of 6-nitro-3-aminobenzoic acid at 405 nm (extinction coefficient is 8980 1/mol cm).

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

2.9.2. Protein concentration

The protein concentration was estimated by the Bradford method [18] using bovine serum albumin as standard.

2.9.3. Size-exclusion chromatography

Size-exclusion chromatography was performed with Superdex 200 packed in an XK16/70 with 0.15 *M* NaCl–50 m*M* sodium phosphate, pH 7 as buffer at 1 ml/min.

3. Results and discussion

3.1. Characterization of supports

The amount of ligand attached to the supports was calculated by mass balance between the ligand concentration in the initial reaction mixture and its concentration in the final wash mixture. Table 1 presents the values of each ligand coupled with the epoxy-activated Sepharose 6B.

The amount of ligand attached to the supports was dependent on the chemical reactive group of the ligand. The amine was more reactive than hydroxyl (15–20 μmol/ml instead of 10–15 μmol/ml of gel).

Table 1
Ligand concentrations attached to epoxy-activated Sepharose 6B

Ligand	μmol of ligand/ml of gel
4-Aminoantipyrin	20
2-Amino-3-benzyloxypyridin	18
3-Amino-5-phenylpyrazole	18
3-Amino-1-phenyl-2-pyrazolin-5-on	20
1-Benzyl-4-hydroxypiperidine	10
Ethyl-4-hydroxybenzimidate	16
Helicin	15
4-Hydroxybenzaldehyde	16
4-Hydroxybenzylalcohol	16
Salicin	15

3.2. Ligand selection

Selectivity of the different gels was tested by a scouting experiment under affinity and pseudo-affinity conditions.

Under above mentioned affinity conditions performed at different pH (5.5, 7, 8), no penicillin acylase was retained on the different supports synthesized for equilibration and binding.

Under above mentioned pseudo-affinity conditions a starting condition scouting experiment was performed. All 10 supports retained penicillin acylase. We then tested three starting conditions with varying ammonium sulfate concentrations (2 *M*, 1.5 *M*, 1 *M*). This allowed us to choose appropriate starting conditions.

At 2 *M* all the supports retained penicillin acylase while at 1 *M* most did not.

At 1.5 *M*, most retained penicillin acylase well. Therefore, we used 1.5 *M* for the following experiments. This concentration of ammonium sulfate was slightly lower than that used in the literature (1.6 *M*) for antibiotic family ligands [6–11].

After choosing the starting conditions, activity recovery scouting was performed. We injected a fixed concentration of penicillin acylase (3.23 U) onto every column and studied the recovery after elution by decreasing ionic strength with 50 m*M* phosphate buffer, pH 7.

The results of these experiments are presented in Table 2.

Table 2
Tests of capture of pure penicillin acylase by different ligands[a]

Ligand	% of enzyme in through flow	% of eluted enzyme
4-Aminoantipyrin	0	100
2-Amino-3-benzyloxypyridin	29	25
3-Amino-5-phenylpyrazole	0.3	85
3-Amino-1-phenyl-2-pyrazolin-5-on	0.2	95
1-Benzyl-4-hydroxypiperidine	24	62
Ethyl-4-hydroxybenzimidate	0.2	64
Helicin	33	21
4-Hydroxybenzaldehyde	0.9	72
4-Hydroxybenzylalcohol	0.3	62
Salicin	34	13

[a] Pure penicillin acylase has a specific activity of 12 U/mg.

The choice of 4-aminoantipyrin, 3-amino-1-phenyl 2-pyrazolin-5-on and 3-amino-5-phenylpyrazole was made by the use of immobilized penicillin acylase for the synthesis of 2-acetyl-1-pyrroline and 2-propionyl-1-pyrroline [19]. We postulated that this type of molecule (pyrrole, pyrazole) was a good candidate for interaction with penicillin acylase. Table 2 shows that these three supports with the immobilized ligands constituted the best chromatography column for recovery (90–100%).

4-Hydroxybenzaldehyde and 4-hydroxybenzylalcohol differ from the classical phenyl hydrophobic ligand by their more polar properties. These molecules gave 62–72% recovery, indicating that this type of molecule was a possible candidate for the following experiments.

We then tested helicin and salicin which possess a hydrophilic molecule as an intermediate between the spacer arm and the attenuated hydrophobic ligand. These two molecules gave poor results probably because of their hydrophilicity.

2-Amino-3-benzyloxypyridin, 1-benzyl-4-hydroxypiperidine and ethyl 4-hydroxybenzimidate were then tested as alternative molecules. Their hydrophobicity was in between that described above and the strict hydrophobic ligand. Since 24% of the enzyme was in the through flow, we considered that 1-benzyl-4-hydroxypiperidine was not appropriate. Instead, we speculated that the lower hydrophobicity of this molecule compared to the other two reduced the capture of penicillin acylase. 2-Amino-3-benzyloxypyridin also contained a high percentage of enzyme in the through flow (24%) and a low recovery (25%). The large size of this molecule and its high hydrophobicity might explain these results.

Ethyl-4-hydroxybenzimidate behaved like 4-hydroxylbenzaldehyde and 4-hydroxybenzylalcohol. In fact, their structures and hydrophobicities are similar.

Seven supports allowed the recovery of penicillin acylase beyond 50%, two had a recovery of 95 and 100% and three others around 60–70%. We selected six of the seven supports to study an industrial clarified feedstock. For this, we used an industrial clarified, pre-concentrated and diafiltrated feedstock of recombinant penicillin acylase (50% purity by HPLC).

After injection of a fixed concentration (confidential values) of the feedstock onto the six supports and elution by decreasing the ammonium sulfate concentration, the activity recovery was found to be around 80% ($\pm$10%) for all (Table 3). The close similarities of these results indicated that the contaminants could modify the interaction between penicillin acylase and ligand. Two mechanisms are possible: (i) interaction between penicillin acylase and contaminants. In this case, the contaminants would carry the enzyme in the through flow; however, there was only 1 to 2% of enzyme in the through flow. The enzyme–contaminant complex gave a similar behavior in the different chromatographic supports. The contaminants were taken up by the enzyme and eluted with it. Consequently, only the hydrophilic contaminants passed through the column while the hydrophobic contaminants com-

Table 3
Purification of *E. coli* penicillin acylase from an industrial clarified feedstock by different ligands

Sample	Activity (U/ml)	Protein (mg/ml)	Sp activity[a] (U/mg)	Purification-fold	Recovery (%)
Clarified feedstock	*[b]	*[b]	5.84	–	–
4-Aminoantipyrin	0.52	0.059	8.81	1.5	85
3-Amino-5-phenylpyrazole	0.55	0.072	7.64	1.3	80
3-Amino-1-phenyl-2-pyrazolin-5-on	0.73	0.068	10.73	1.8	81
Ethyl-4-hydroxybenzimidate	0.64	0.065	9.85	1.7	68
4-Hydroxybenzaldehyde	0.83	0.065	12.77	2.2	80
4-Hydroxybenzylalcohol	0.85	0.09	9.44	1.6	81

[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.
[b] *: Confidential values.

plexed with the enzyme. Hence, since the hydrophobic contaminants could interact with the hydrophobic area near the catalytic site of the enzyme during the elution step, this complex might be modified and the catalytic site masked. (ii) Interaction between ligand and contaminants. In this scenario, the contaminants would block the interaction between the ligand and the enzyme. Moreover, we observed a good recovery and a low concentration of enzyme in the through flow. The hydrophobic contaminant would fix to the support, and during elution its concentration would increase. Hence, the interaction with the enzyme would be possible, thus masking the catalytic site.

Table 3 shows that the purification fold varied only from 1.31 to 2.18. This result was expected for a sample of 50% of purity. The contaminant was not eliminated by ion-exchange chromatography (data not shown). Fig. 2 shows the size-exclusion chromatography of feedstock before (A) and after purification (B). Note that the principal contaminant has been eliminated. This type of gel is a good alternative to a strict hydrophobic support for the treatment of an industrial clarified feedstock. It could be used at the capture, intermediate purification or polishing steps.

We tested all these gels with crude extract prepared in our laboratory. The samples were not pretreated, only centrifuged and diluted. One ml of crude extract (0.018 U/ml) was injected onto the column.

The results are given in Table 4.

A good recovery was obtained for most gels (87 to 100%) and these results are compatible with those obtained in the literature [11] for amoxycillin, ampicillin, 1-aminopenicillanic acid and 4-phenylbutylamine with the same level of purification fold (three to six). These authors worked in batch conditions (2 h of contact) with penicillin acylase from mutant cells of *Escherichia coli* ATTC9637. They used two different activated gels with a short spacer or without. It seems that to fix the same level of enzyme in dynamic conditions as in batch conditions, a long spacer arm is necessary. The ligands used in our work cost only 6% of the cost of ligands like ampicillin, amoxycillin, 14% of 4-phenylbutylamine and 30% of 1-aminopenicillanic acid. On an industrial scale, the saving would be great.

3.3. Determination of sorption capacity

To measure the dynamic capacities of the support, the frontal analysis method was used (Fig. 3). Fifty and 10% breakthrough points were taken to calculate the capacity. Moreover, we also used a peak collection method after step gradient to 50 m*M* sodium phosphate pH 7. Breakthrough curves were measured at 0.5 ml/min (Fig. 3). Under these experimental conditions, the capacity was 20 mg/ml at 10% breakthrough point and peak collection and 40 mg at 50% breakthrough point. This capacity is comparable to that of pseudo-affinity chromatographic supports.

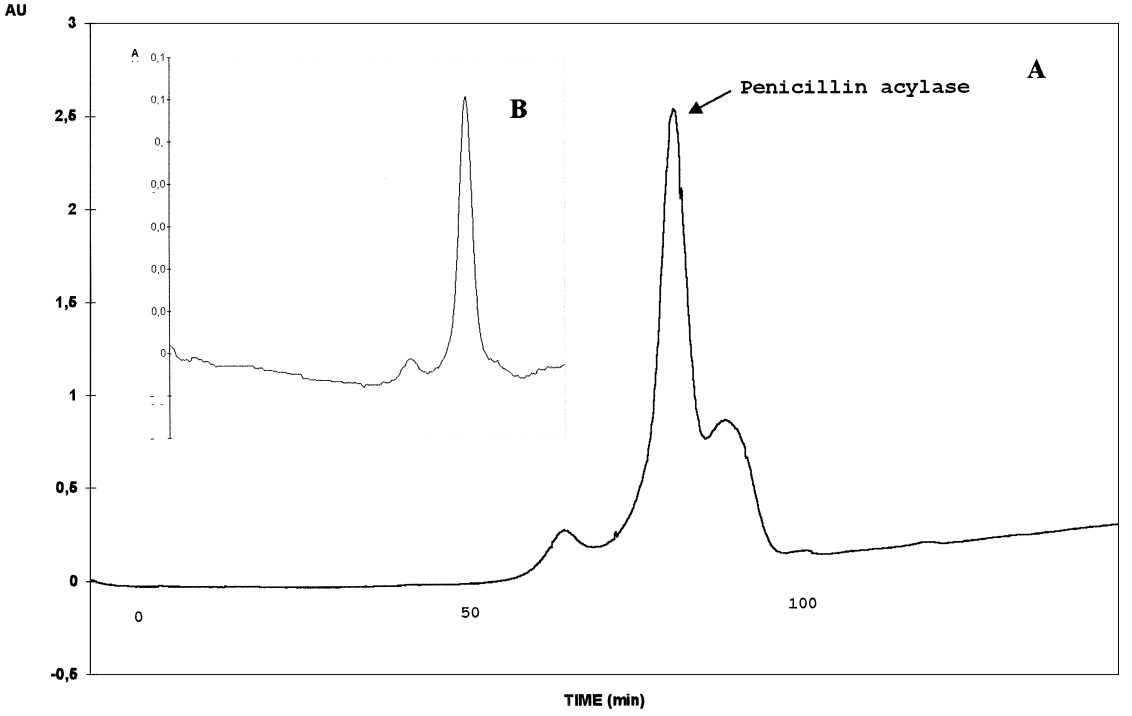

Fig. 2. Size-exclusion chromatography of clarified feedstock before (A) and after purification (B). Sample volume: 100 μl; equilibration buffer: 0.15 *M* NaCl–50 m*M* sodium phosphate, pH 7; flow-rate: 1 ml/min; detection at 280 nm.

3.4. Recovery versus flow-rate

Fig. 4 shows that recovery remained stable (95%) until 300 cm/h (10 ml/min) and decreased to 70% at 750 cm/h. 300 cm/h is an excellent flow-rate for pseudo-affinity chromatographic support.

3.5. Sanitization effect of recovery

Five contact times with 1 *M* NaOH were used to study the recovery (15 min, 30 min, 1 h, 4 h, 12 h).

Three gels were tested: 4-aminoantipyrin, 3-amino-1-phenyl-2-pyrazolin-5-on and 3-amino-5-

Table 4
Purification of *E. coli* penicillin acylase from a crude extract by different ligands

Sample	Activity (U/ml)	Protein (mg/ml)	Sp activity[a] (U/mg)	Purification-fold	Recovery (%)
Crude extract	0.018	0.52	0.035	–	–
4-Aminoantipyrin	0.0054	0.052	0.104	3	100
3-Amino-5-phenylpyrazole	0.003	0.015	0.200	5.7	100
3-Amino-1-phenyl-2-pyrazolin-5-on	0.0026	0.038	0.068	1.9	74
Ethyl-4-hydroxybenzimidate	0.0040	0.023	0.174	5	100
4-Hydroxybenzaldehyde	0.0033	0.030	0.110	3.1	73
4-Hydroxybenzylalcohol	0.0032	0.049	0.066	1.9	87

[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.

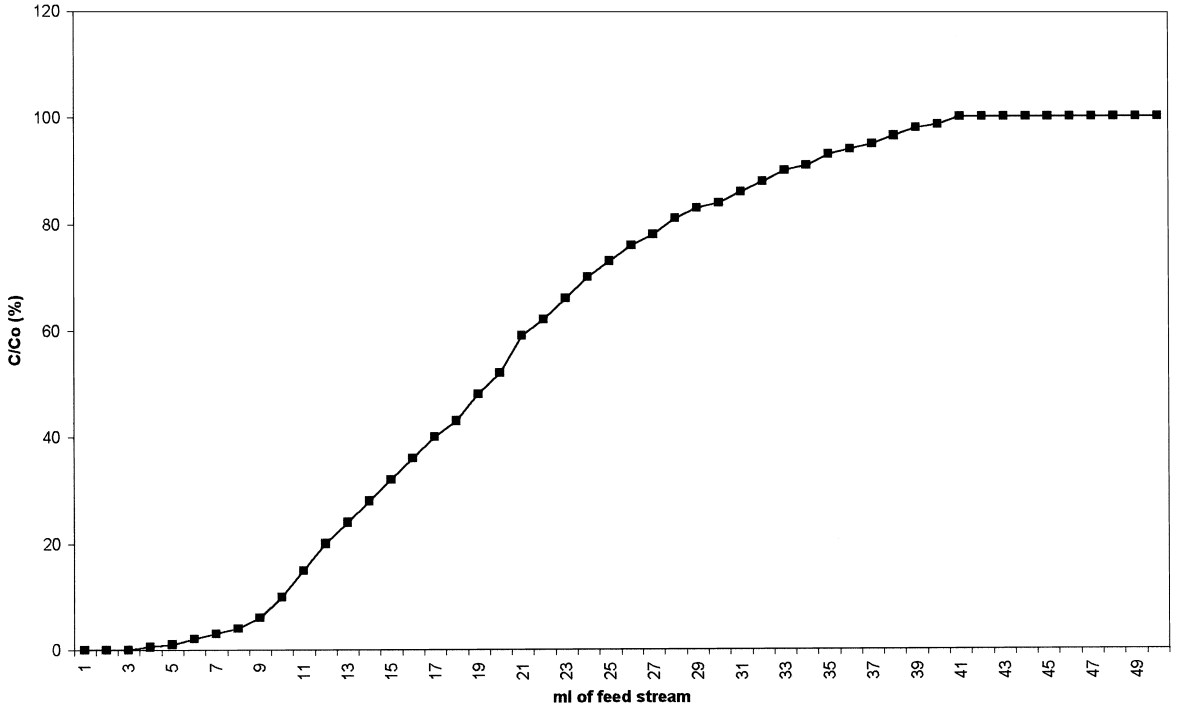

Fig. 3. Solute breakthrough curve with 4-aminoantipyrin Sepharose 6B (0.5 ml); 1 mg/ml penicillin acylase dissolved in 1.5 *M* $(NH_4)_2SO_4$–50 m*M* sodium phosphate, pH 7; detection at 280 nm; flow-rate: 1 ml/min.

phenylpyrazole Sepharose 6B. Recovery remained stable (100% of recovery) until 1 h of contact and was 90% between hours 4 and 12.

These results indicate that our pseudo-affinity chromatographic supports are not sensitive to cleaning in place by 1 *M* NaOH, which is classically used to remove pyrogen and to destroy microorganisms. Media compatible with sodium hydroxide are the most suitable for production purposes.

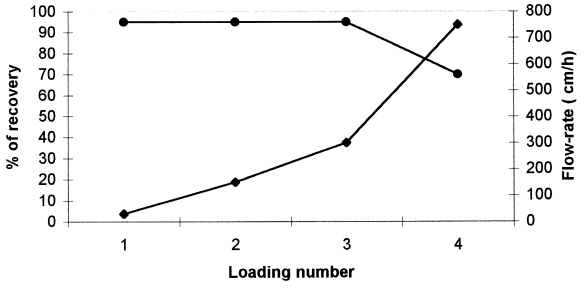

Fig. 4. Effect of flow-rate with 4-aminoantipyrin Sepharose on activity recovery in linear gradient elution; sample: 1 ml of 8 U/ml penicillin acylase in 1.5 *M* $(NH_4)_2SO_4$–50 m*M* sodium phosphate, pH 7; buffer A: 1.5 *M* $(NH_4)_2SO_4$–50 m*M* sodium phosphate, pH 7; buffer B: 50 m*M* sodium phosphate, pH 7.

4. Conclusion

New pseudo-affinity chromatographic supports for penicillin acylase purification were prepared and evaluated.

Six supports were selected after the first scouting experiment with penicillin acylase as the sample. These gels showed 70 to 100% of activity recovery. They were tested with an industrial clarified feedstock and with crude extract prepared in our laboratory.

The six gels presented a high recovery of around 80% at 1.5- to 2-fold purification with industrial clarified and diafiltrated feedstock, and 70 to 100% of recovery with crude extract at two- to six-fold

purification. Therefore, penicillin acylase was substantially purified in one step using this pseudo-affinity adsorption technique

This type of gel has a good sorption capacity (20 mg/ml) and an excellent flow-rate (300 cm/h).

Moreover, such gels can withstand the sanitization process. Therefore, they are suitable for use on an industrial scale.

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