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MODIFIED GENERAL AFFINITY ADSORBENT FOR LARGE-SCALE PURIFICATION OF PENICILLINASES

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SUMMARY

N-Acetyl-D-(−)-penicillamine as a stable second-generation biospecific affinity ligand has previously been suggested for purification of *Bacillus cereus* 569/H β-lactamase I. A complex spacer arm is coupled with the matrix by using epichlorohydrin and phloroglucinol doubly activated with divinyl sulphone in the *meta* position. Coupling of D-(−)-penicillamine ligand resulted in an active affigel. However, we found that two affinity ligands in close proximity prevents simultaneous binding of two penicillinase molecules, therefore one ligand is superfluous. Our results show that: (1) shortening the spacer arm by direct activation of the matrix with divinyl sulphone is satisfactory to produce the affinity material with N-acetyl-D-(−)-penicillamine; (2) incorporation of 15 μmol of N-acetyl-D-(−)-penicillamine per ml of wet Sepharose 4B satisfies the maximum binding capacity requirements of the affigel (about half of the originally incorporated amount of ligand); (3) our simplified affinity adsorbent is generally applicable for large-scale purification of penicillinases to homogeneity from various bacterial sources by the convenient batch method without prior concentration of these enzymes; (4) reacetylation for four/five times can regenerate the original binding capacity of the affigel.

INTRODUCTION

In view of the worldwide and considerable β-lactamase resistance of Gram-positive and Gram-negative bacteria, much attention has been paid to the purification of these enzymes, mainly by classical column chromatographic methods but several affinity chromatography procedures have also been reported[1–9]. The inherent instability of the early first generation ligands (specific β-lactams) rendered the absorbents unsuitable for repeated use[2–7].

The present report describes the application of N-acetyl-D-(−)-penicillamine in a simplified manner[8,9] for the biospecific affinity purification of penicillinases to homogeneity from various bacterial strains. This stable affinity ligand was originally

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introduced into laboratory practice by Clarke *et al.*[1]. Quantitation of the optimization of the penicillinase binding capacity of N-acetyl-D-(−)-penicillamine–Sepharose 4B, however, convinced us that there is no need to attach approximately 24.5 μmol of D-(−)-penicillamine per ml of matrix because less than 1% of the coupled ligand can bind penicillinases on a mol per mol basis. On the other hand, only the N-acetylated ligand is an active binder, therefore special care must be taken to regenerate the affigel from time to time to maintain its performance.

EXPERIMENTAL

Materials

Divinyl sulphone (DVS) was obtained from Aldrich (Aldrich Europe, c/o Jansen Pharmaceuticals, Belgium). D-(−)-Penicillamine (DPA) was a product of Biogal (Debrecen, Hungary). Sepharose 4B was obtained from Pharmacia (Uppsala, Sweden). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was from Sigma (Dorset, U.K.).

Bacterial strains

Bacillus cereus 569/H and *B. cereus* 569/H/9 (a hyper magno mutant) constitutive β-lactamase producing (β-lactamase I as exopenicillinase and β-lactamase II as exocephalosporinase) and *B. licheniformis* 749 C strains were kindly provided by Dr. S. Fleming, Department of Molecular Biology, University of Edinburgh, U.K. *Staphylococcus aureus* NCTC 9789 samples were supplied by Dr. R. H. Pain, Department of Biochemistry, University of Newcastle upon Tyne, U.K. or by Dr. I. N. Simpson and Dr. G. W. Ross, Chemotherapy Department, Microbiology Division, Glaxo Group Research, Greenford, U.K. and also by Dr. R. P. Ambler, Department of Molecular Biology, King's Building, University of Edinburgh, U.K. *S. aureus* PC 1 1711 E was a gift from Dr. I. N. Simpson, or from Dr. G. W. Ross and from Dr. R. P. Ambler (*S. aureus* PC 1 11195 NCIB). In order to control the penicillinase specificity of our affigel we used caphalosporinases of different origins: *Escherichia coli* J5-3 R46$^+$ (OXA-2) and *E. coli* J6-2 RTEM-1$^+$ strains from Dr. J. T. Smith, Microbiology Section, School of Pharmacy, University of London, U.K.; *Pseudomonas aeruginosa* MAR, a TEM-1-like β-lactamase producing strain from Dr. A. M. Philippon, Service de Biologie, C. H. U. Cochin, Paris, France; *E. cloacae* 53 strain from Dr. J. T. Smith.

Enzyme assay, protein determination

β-Lactamase activity was assayed by measuring the absorbance of nitrocefin at 486 nm in a cell of pathlength 1 cm as described by O'Callaghan *et al.*[10]. One unit is that amount of enzyme which is able to hydrolyze 1 μmol of nitrocefin in 1 min at 37°C.

The protein content of the enzymes was estimated by measuring their absorbances at 280 nm or as described by Lowry *et al.*[11].

In vitro susceptibility studies

Minimum inhibitory concentration (MIC) values were measured by the broth dilution method with two-fold serial dilutions. Inocula of 10^8 c.f.u. (colony forming unit) ml^{-1} were used for induction in preparative scale studies. All MIC values were determined in Brain-Heart Infusion (BHI) medium (Code CM 225; Oxoid, Basingstoke, U.K.).

Monitoring of exopenicillinase induction

BHI was used for excess enzyme production by induction. Overnight inocula of the *S. aureus* strains were diluted in the same broth (200 ml in 500-ml conical flasks) containing inducers and grown at 37°C with shaking at 120 rpm. In order to reduce the lag phase, we selected an initial c.f.u. value of 10^8 for preparative exopenicillinase production. Changes of about two orders of magnitude in c.f.u. were monitored in 10 h with *S. aureus* NCTC 9789 strains. An activity of 0.78 U ml^{-1} enzyme was found in the non-induced control culture, 6 U ml^{-1} in the culture induced by 1 $\mu g\ ml^{-1}$ Methicillin (MET) (MIC/3). *S. aureus* PC 1 1911 E produced maximum enzyme level of 30 U ml^{-1} in 8 h without induction, however induction with 0.1 $\mu g\ ml^{-1}$ MET (MIC/30) in 12 h resulted in 479.5 U ml^{-1} enzyme or with 1 $\mu g\ ml^{-1}$ MET (MIC/3), 1800 U ml^{-1} exopenicillinase were found in 12 h. *S. aureus* PC 1 11195 NCIB showed a basic enzyme level of 223.1 U ml^{-1} without induction in 8 h, while the use of 0.39 $\mu g\ ml^{-1}$ of Cefotaxim (MIC/4) gave 377.6 U ml^{-1} enzyme in 8 h and 0.19 $\mu g\ ml^{-1}$ Cefoxitine (MIC/4) produced a 353.6 U ml^{-1} increase by induction in 8 h.

Preparation of affinity absorbent

N-Acetyl-D-(−)-penicillamine–Sepharose 4B was prepared either according to Clarke *et al.*[1] or by simplification and modification of the original method as follows.

A 10-ml volume of Sepharose 4B was suspended in 10 ml of 1 *M* sodium hydroxide–sodium bicarbonate buffer, pH 11.0, and directly treated with 1.5 ml of DVS at 40°C, keeping the pH constant. The time dependence of the DVS activation was investigated: 4-ml samples were removed from the reaction mixture at 20-, 30-, 40- and 60-min intervals. The gel samples were then filtered in small sintered glass funnels and washed extensively with distilled water to neutrality. The wet activated gel samples were treated separately and uniformly with 40 mg of DPA in 2 ml of 0.05 *M* phosphate buffer, pH 7.0, with shaking for 18 h at room temperature to achieve maximum incorporation of the ligand.

Coupling of DPA to the activated gel was assessed by monitoring the absorbance at 412 nm, $A^{1\ cm}_{412\ nm}$, produced upon treatment of a suitable aliquot of the reaction mixture with DTNB[12]. Quantitation of the results revealed 7 μmol of DPA bound in the 20-min, 15 μmol in the 30-min, 25 μmol in the 40-min and 30 μmol in the 60-min DVS-activated gel samples per ml. The excess of DPA was then removed by filtration in sintered glas funnels and the gel samples were resuspended in 2 ml of distilled water and treated with 1 ml of acetic anhydride continuously added over a

TABLE I

TIME DEPENDENCE OF DIRECT DVS ACTIVATION OF SEPHAROSE 4B AS MEASURED BY CHANGES IN THE BINDING CAPACITY OF *B. CEREUS* 569/H β-LACTAMASE I

DVS activation time (min)	*Ligand bound (μmol)**	*Binding capacity (mg enzyme)**
20	7	0.09
30	15	0.58
40	25	0.6
60	30	0.61

* Per ml affigel.

TABLE II

TIME DEPENDENCE OF N-ACETYL-DPA LIGAND INCORPORATION AS MEASURED BY CHANGES IN THE BINDING CAPACITY OF *S. AUREUS* PC 1 1711 E EXOPENICILLINASE

DPA incorporation time (h)	*Ligand bound (μmol)**	*Binding capacity (mg enzyme)**
4	7.5	0.06
8	15	0.38
13	25	0.4
18	30	0.4

* Per mg affigel.

period of 80 min at pH 8.0. The mixtures were then tested separately for maximum *B. cereus* 569/H β-lactamase I binding capacity in proportionally reduced volumes of a crude extract as described below. The results are shown in Table I.

Unfortunately, direct assessment of the extent of DVS activation is not possible, therefore, based on the above findings, we activated 10 ml of Sepharose 4B with 1.5 ml of DVS for 60 min and investigated the time dependence of the incorporation of DPA into this activated matrix to determine the stoichiometry of the N-acetyl-DPA for penicillinase binding on a mol per mol basis. Simple calculation shows that in principle much less than 1% of the ligand incorporated by Clarke *et al.*[1] is sufficient to achieve maximum enzyme binding capacity to their affigel.

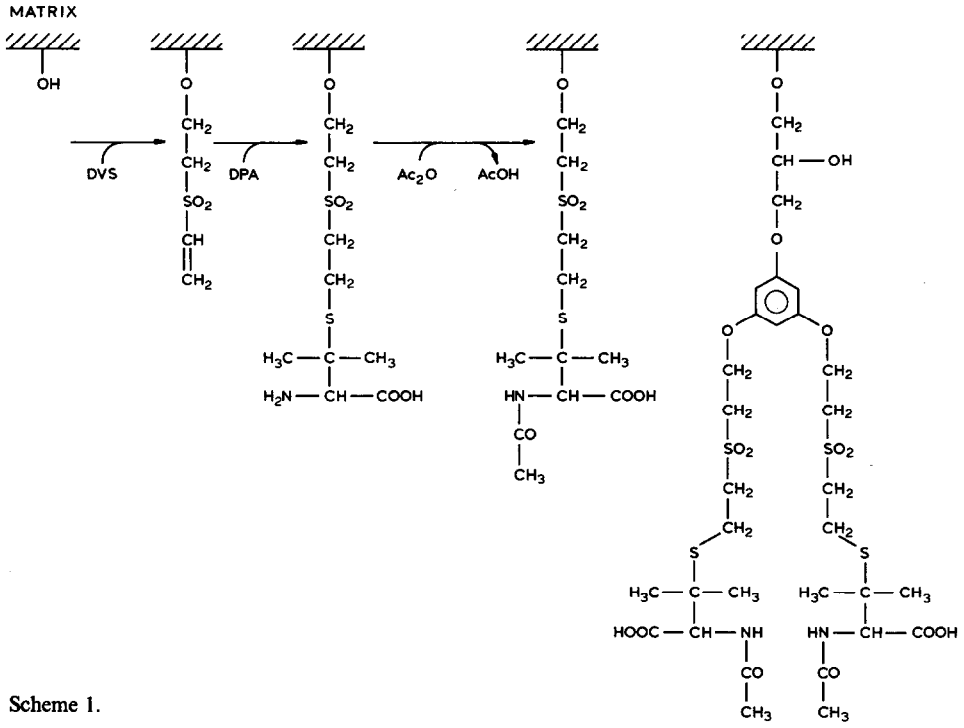
Under the above experimental conditions, the wet DVS-activated gel was treated with 100 mg of DPA in 10 ml of 0.05 *M* phosphate buffer, pH 7.0, and 4-ml samples were withdrawn at 4-, 8-, 13- and 18-h intervals. Assessment of DPA with the DTNB method[12] revealed about 25% ligand incorporation (7.5 μmol per ml gel) in 4 h, 50% (15 μmol per ml gel) in 8 h, 83% (25 μmol per ml) in 13 h and 100% (30 μmol per ml) in 18 h. Incorporation of greater than 30 μmol of DPA cannot occur under these experimental conditions. Acetylation was performed as described above. The binding capacity with *S. aureus* PC 1 1711 E exopenicillinase is shown in Table II.

As by 8 h of incorporation the maximum enzyme binding capacity was achieved for *B. cereus* 569/H or *B. cereus* 569/H/9 β-lactamase I (0.6 mg pure enzyme per ml affigel), we concluded that the incorporation of 15 μmol of DPA per ml gel is sufficient. An 100-ml volume of Sepharose 4B was converted into N-acetyl-D-(−)-penicillamine–Sepharose 4B affinity material using the above reaction conditions. Further proportional increases in the amounts of Sepharose 4B and the reactants are possible.

The chemical reactions involved in the preparation of the affigel are illustrated in Scheme 1.

Preparation of crude enzymes

In the case of Gram-positive bacteria, the cell-free BHI media of induced cultures were considered as crude enzyme preparation only; however, Gram-negative bacteria were grown overnight without induction in Nutrient Broth media, followed by centrifugation of the cells. The sediments were treated by sonication in 0.05 *M* phos-



Scheme 1.

phate buffer, pH 7.0, and after nuclease treatment, supernatants were prepared by ultracentrifugation at 105000 *g* for 1 h and extensive dialysis against the above buffer.

Adsorption to the affigel and elution

A typical medium-scale experiment was as follows. A 2-l volume of crude cell-free Gram-positive bacterium culture supernatant was stirred with 40 ml of affinity adsorbent at 4°C for 15 min at a low speed which just prevented sedimentation of the gel. The gel was then allowed to sediment, transferred quantitatively to a suitable sintered glass filter and washed free from protein with 0.05 *M* phosphate

TABLE III

PURIFICATION OF *B. CEREUS* 569/H/9 EXOPENICILLINASE BY N-ACETYL-D-(−)-PENICILLAMINE SEPHAROSE 4B AFFINITY CHROMATOGRAPHY

Parameter	*Cell-free supernatant*	*Purified enzyme*
Volume (ml)	2000	100
Protein (mg ml^{-1})	2.5	0.0225
Total activity, nitrocefin (U · 10^{-3})	3600	3312
Specific activity		
Nitrocefin (U mg^{-1} · 10^{-3})	0.72	1472
Benzylpenicillin (U mg^{-1} · 10^{-3})	–	1100
Purification	1	2044.4
Recovery (% total activity)	100	92

TABLE IV

PURIFICATION OF EXOPENICILLINASES OF *S. AUREUS*- AND *B. LICHENIFORMIS* 749 C STRAINS BY N-ACETYL-D-(−)-PENICILLAMINE SEPHAROSE 4B AFFINITY CHROMATOGRAPHY

Source and inductor	*Cell-free supernatant*			*Purified enzyme*			*Purification fold*	*Capacity (mg ml^{-1})*
	Protein (μg ml^{-1})	*U ml^{-1} · 10^{-3}*	*Specific activity (U mg^{-1} · 10^{-3})*	*Protein (μg ml^{-1})*	*U ml^{-1} · 10^{-3}*	*Specific activity (U mg^{-1} · 10^{-6})*		
S. aureus PC 1 1711 E 1 μg ml^{-1} MET	610	1.8	2.95	3.4	17.82	5.241	1777	0.4
S. aureus NCTC 9789 1 μg ml^{-1} MET	600	6	10	4.6	58	12.609	1261	0.3
B. licheniformis 749 C No inductor	308	1.2	3.9	2.9	26.85	9.259	2374	0.3

buffer, pH 7.0. The removal of unbound protein was monitored spectrophotometrically at 280 nm, $A^{1\,cm}_{280\,nm}$. The bound enzyme was eluted separately with two 50-ml portions of 1 *M* sodium chloride containing 0.05 *M* phosphate buffer, pH 7.0. The pooled filtrates were desalted by ultrafiltration at 4°C. After sterile filtration and freeze-drying in sterile vials, the enzyme was distributed as about 1000 U per vial. The purification parameters are shown in Tables III and IV. The products are homogeneous according to sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis[13].

RESULTS AND DISCUSSION

The use of N-acetyl-D-(−)-penicillamine–Sepharose 4B as an affinity material by Clarke *et al.*[1] for β-lactamase purification was an excellent improvement because gradual hydrolysis of earlier β-lactam ligand-based affigels[2–7] prevented their use more than once or twice for efficient β-lactamase preparation and purification.

The above authors successfully used their affinity adsorbent for the purification of *B. cereus* 569/H β-lactamase I in two ways. In columns, however, direct chromatography of the crude cell-free culture medium is hampered by the large volumes, therefore they advised preconcentration of the enzyme on Celite. In this manner in relatively large amount of penicillinase was successfully purified.

We suggest treatment of large volumes of cell-free culture media by the batch technique, which makes possible convenient handling of penicillinases of different bacterial origins.

During optimization experiments we found that adsorption of the enzymes onto the affigel was best achieved at 4°C and not more than 15 min of slow and continuous stirring was sufficient for maximum binding of the enzyme. The stirring speed was just enough to prevent spontaneous sedimentation of the affigel. Handling of the gel was easy when, following adsorption of the enzyme, the suspension was allowed to settle.

As adsorption is an equilibrium process, a minimum amount of active principle should remain in the supernatant above the affigel, but repeated batch treatment of two pooled affigel supernatants with a calculated amount of affigel (according to its capacity for the respective penicillinase) proved to be satisfactory. It follows that the maximum binding capacity of the affigel should be assessed for each penicillinase prior to its use. Enzymes of different bacterial sources differ from each other significantly as shown in Tables III and IV, however the affigel can be used generally for penicillinases. The biospecificities of both affigels, that of Clarke *et al.*[1] and ours is shown also by the fact that β-lactamase II, an exocephalosporinase of the *B. cereus* strains used in these studies, is not bound to the affigel, although it is coproduced in about 10% and excreted into the culture medium together with the exopenicillinase β-lactamase I.

When cephalosporinases of Gram-negative bacteria were tried in small-scale experiments (examples listed in Experimental, *Bacterial strains*) we found no adsorption at all. This clearly shows the biospecific binding capability of the N-acetyl-D-(−)-penicillamine–Sepharose 4B affinity material.

We also should like to draw attention to the slow deacetylation of the affigel upon repeated use to an appreciable extent. Deacetylation also takes place during

storage of the affigel at 4°C for a relatively long period (several weeks) if it is not used frequently, but reacetylation four/five times may lead to the recovery of full capacity and good performance. The affigel, however, cannot be used indefinitely probably because of disadvantageous changes in the gel structure.

As mentioned above the stoichiometry of maximum binding capacity theoretically can be considered on a mol ligand per mol enzyme basis. However this is not true in practice. Several factors play a rôle in enzyme protein binding, among which are the following:

(a) Only ligands that are sterically free for access are active; ligands inside the gel beads require diffusion which may or may not be possible during adsorption, *i.e.*, "buried" ligands may not act as binders even if they are linked to the matrix with relative long spacer arms. The enzymes should also avoid the diffusion barrier of ordered water molecules surrounding the matrix backbone. Reduction of the amount of ligand molecules to about half of the original without decreasing the maximum binding capacity shows that most of the coupled ligands in both affigels are sterically handicapped; possibly they are inside the open pore structure of Sepharose 4B (Tables I and II).

(b) Steric hindrance may be caused by gel aging.

(c) As the applicability of the immobilized ligand depends on its specific adsorption, the matrix must be devoid of non-specific absorptive effects because these could obscure the biospecificity and interfere with the action of the bound ligand.

(d) The length of the spacer group between DPA and the matrix. Since shortening of the original spacer arm by omitting epichlorohydrin and phloroglucinol did not reduce the maximum binding capacity of our affigel, we feel that simplification of its preparation is justified.

Finally we should like to mention that both N-acetyl-D-(−)-penicillamine-based affigels show biospecificity for penicillinases, as revealed by their definite inability to bind β-lactamases of cephalosporinase character. This, however, may prompt the suggestion that biospecific cephalosporinase-binding affigels can be constructed with one of the possible cephalosporamine optical isomer derivatives, on a similar theoretical basis.

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