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HIGH-PERFORMANCE LIQUID AFFINITY CHROMATOGRAPHY OF β-LACTAMASE

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SUMMARY

New silica and organic polymer-based, wide-pore, affinity chromatographic phases have been developed for β-lactamase purification. The resins have been evaluated in terms of binding capacity, ligand coverage, protein recovery and long-term stability of activated resins prior to ligand attachment. The columns have been applied to the isolation of β-lactamase from fermentation media. Protein purity following high-performance liquid affinity chromatography was assessed by polyacrylamide gel electrophoresis.

INTRODUCTION

Since it was realised that β-lactamases are involved in the cause and spread of microbial resistance to β-lactam antibiotics, these enzymes have been extensively investigated[1]. It is now known that a wide range of microorganisms are capable of β-lactamase production, and several distinct classes of this enzyme have been isolated and classified[2]. Purification has been performed by a combination of various classical methods. Of these, possibly the most successful has been affinity chromatography. A variety of ligands, including cephalosporin C[3,4], ampicillin[5], methicillin[6] and D-penicillamine[7] have been used as immobilized substrates, mainly on an agarose matrix.

Recently, high-performance liquid affinity chromatographic (HPLAC) techniques have been described, which combine the biological specificity with the inherent speed, resolving power, and sensitivity of high-performance liquid chromatography (HPLC). HPLAC has been used for isolation of carbohydrates[8], glycoproteins[9], isoenzymes[10], and other important biomolecules. The column matrix used has been almost exclusively limited to wide-pore silicas. Affinity stationary phases have been prepared by attaching spacer molecules to the surface hydroxyl groups by a silylation procedure, followed by covelantly linking the ligand to a suitable functional group on the spacer arm.

In recent years, resins, rather than silica-based matrices, have been developed for use in HPLC. They offer the major advantage of stability at high pH values. The separation of biopolymers by gel-filtration HPLC, has also been achieved on wide-

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pore, silica-based and hydrophilic, organic matrices as stationary phases[11,12]. TSK column packings have proved to be particularly successful for this type of HPLC. In this report, we investigate the possibility of using these types of matrices for the application of HPLAC to the isolation of penicillinase. Several ligand coupling procedures are examined, including activation with cyanogen bromide[13]. The affinity resins are investigated in terms of non-specific protein binding, enzyme elution, sample loading, and stability.

MATERIALS AND METHODS

Reagents

The packing materials, TSK 4000PW (pore size, *ca.* 500 nm; particle size, 17 μm) and TSK 3000SW (pore size, 25 nm; particle size, 10 μm) were kindly supplied by Toya Soda (Yamaguchi, Japan), through the auspices of Anachem (Luton, U.K.). 7-Pentanamido-cephalosporanic acid and nitrocefin were gifts from Glaxo (London, U.K.). β-Lacatamase type I, bovine serum albumin (BSA), myoglobin, cyanogen bromide, cephalosporin C (sodium salt), sodium periodate, 1,6-diaminohexane, sodium cyanoborohydride, sodium borohydride, 1,1'-carbonyldiimidazole and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide were purchased from Sigma (Poole, U.K.). L-[1-^{14}C]Valine (55 mCi mmol^{-1})was obtained from Amersham International (Amersham, U.K.) and the protein assay kit from Bio-Rad Labs. (Watford, U.K.). Stainless-steel tubes and fittings were supplied by H.E.T.P. (Macclesfield, U.K.). All other reagents, of the highest possible grade, were obtained from British Drug House (Poole, U.K.).

Preparation of resins

Based on the presence of free hydroxyl groups on the resin surface[14], affinity resins were prepared by using four ligand coupling procedures, as summarised in Fig. 1. Resin 1 was prepared in a similar way to that previously described for the attachment of cephalosporin C to agarose[15]. TSK 4000PW resin (4 g) was washed thoroughly with distilled water (2 l), resuspended, and further washed with 100 ml of 2 *M* phosphate buffer (pH 12.0) at 0–4°C. The resin was then suspended in 10 ml of the same buffer and, maintaining a low temperature, 1 ml of cyanogen bromide (0.9 g ml^{-1} in acetonitrile) was added dropwise over 2–3 min. The reaction was allowed to continue with gentle stirring for a further 7 min and the solid product was collected by vacuum filtration and washed rapidly with ice-cold distilled water (2 l). This activated product was then immediately suspended in 20 ml of chilled 0.1 *M* bicarbonate buffer (pH 10.3), containing cephalosporin C (2.3 m*M*). The coupling reaction was allowed to continue for 48 h at 4°C, after which time the liganded resin was thoroughly washed free of any unbound cephalosporin with distilled water (3 l).

The initial steps in the second coupling procedure to prepare resin 2, were identical to the cyanogen bromide activation previously described. Immediately following activation, the resin was suspended in 10 ml of 0.5 *M* bicarbonate buffer (pH 9.0), containing 1,6-diamino hexane (50 m*M*). The coupling reaction was allowed to proceed for 24 h at 4°C with continual, gentle stirring. After thorough washing with distilled water, the filtered resin was transferred to 10 ml of a solution containing 1ethyl-3-(3-dimethylaminopropyl)carbodiimide (1.0 m*M*) and 7-pentanamido-ce-

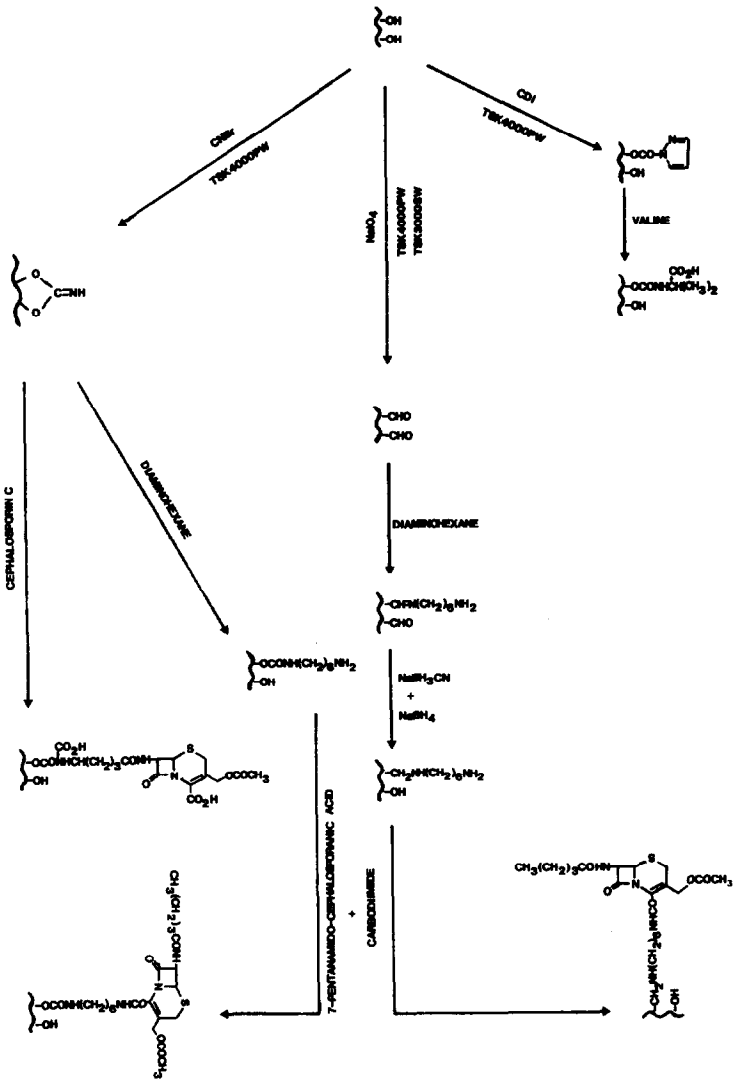

Fig. 1. Coupling of ligands to TSK 3000SW and TSK 4000PW.

phalosporanic acid (0.6 m*M*), adjusted to pH 4.7 with 2 *M* hydrochloric acid. This pH was maintained for 2 h by continual acid addition, and the reaction was allowed to continue for a further 48 h at 4°C. The affinity packing material was then washed free of any uncoupled material with distilled water, prior to column packing.

The third coupling procedure, used to produce affinity resins 3 and 4 was performed with the TSK 3000SW and TSK 4000PW materials, respectively. Because the former support is silica-based, this precluded the use of cyanogen bromide treatment which requires a high pH. After washing with distilled water, each resin (4 g) was added separately to a solution containing sodium periodate (0.25 *M*) in a tightly closed polyethylene container. This was gently shaken for 2 h at room temperature,

and the solid product was filtered and washed with distilled water (2 l). The oxidized resins were then each added separately to 10 ml of 0.5 *M* phosphate buffer (pH 6), containing sodium cyanoborohydride (0.5 m*M*) and 1,6-diaminohexane (50 m*M*). The suspensions were gently shaken for 3 days at room temperature and, after filtration, they were extensively washed with distilled water. Residual aldehyde groups on the resin surface were reduced by suspending the resins in 10 ml of a solution, containing 1 *M* sodium borohydride, and shaking the suspensions for 15 h at 4°C. After filtration and thorough washing, coupling of 7-pentanamido-cephalosporanic acid to each of the resins was performed as previously described for resin 2, using the water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

The final method of ligand coupling was based on resin activation with 1,1′-carbonyldiimidazole[16] and was used with the cyanogen bromide-activated resin in a comparative study on ligand binding and stability of the resin in an activated form. TSK 4000PW (4 g) was thoroughly washed with distilled water (2 l) and then with 100 ml of aqueous dioxane in the sequence: 25%, 50% and 75% (v/v). Final washing was carried out with pure, sodium-dried dioxane. The resin was then suspended in 20 ml of dioxane containing 1,1′-carbonyldiimidazole (5.5 m*M*). The reaction was allowed to proceed for 1 h at room temperature, and the activated resin was then thoroughly washed on a filter with dioxane (2 l), dried under vacuum and stored under dessication at 26°C. When required, the dried, activated product (0.1 g) was removed and suspended in 1 ml of sodium carbonate buffer (pH 10), containing 5 μCi of L-[1-^{14}C]valine and "cold" valine (0.9 μM). The coupling reaction was allowed to proceed for 24 h at 4°C, and the filtered resin was then washed with distilled water until no significant radioactivity was detected in the washings. The radiolabelled resin was thoroughly dried under vacuum. After suspension of each sample in 20 ml of scintillating fluid (Scintillator 299, United Technologies Packard, Downers Grove, IL, U.S.A.), the β-emission was measured for 10 min in a 2425 Tri-Carb scintillation counter (Packard). Once activated (as previously described for resin 1), the cyanogen bromide-activated resin was washed with solutions of dioxane and treated and stored in the same way as the 1,1′-carbonyldiimidazole-activated resin.

Chromatographic procedure

Prior to chromatography and column packing, all stainless-steel components were treated for protection against high salt concentrations by successive washings with distilled water, 30% (v/v) nitric acid, distilled water, 0.5 *M* ethylenediamine tetraacetic acid (pH 6), and finally, distilled water. Affinity and "control" resins (untreated) were packed into stainless-steel columns (100 × 4.6 mm I.D.) with a Milton Roy HPLC pump (Laboratory Data Control, U.K.). Resins were suspended in 15 ml of 0.1 *M* phosphate buffer (pH 7) and slurry-packed upwards via a stainless-steel bomb (300 × 8.0 mm I.D.) at 2 ml min^{-1}. Packing was continued with the same buffer for 45 min, and after column inversion the flow continued for a further 30 min. Affinity and control columns, following washing with distilled water, were stored at 4°C in 50% (v/v) aqueous methanol.

In the HPLAC procedure used, the mobile phases were delivered at 0.5 ml min^{-1} by two double-reciprocating pumps controlled by a high-pressure mixing unit (Applied Chromatography Systems, Luton, U.K.). Injections were made with a Model 7125 Rheodyne valve (Anachem, U.K.), and detection was performed by

recording UV absorption at 280 nm with a Spectromonitor III (Laboratory Data Control). Fractions were collected with a Frac100 fraction collector (Pharmacia, Uppsala, Sweden) and, prior to enzyme determination, were diluted tenfold with 0.1 *M* phosphate buffer (pH 7).

Enzyme assay

The enzyme assay was performed with the chromogenic cephalosporin, nitrocefin. The cephalosporin was dissolved in dimethylsulphoxide (10 mg ml^{-1}) and further diluted to 50 μg ml^{-1} with 0.1 *M* phosphate buffer (pH 7). Each sample was mixed with an equal volume of the nitrocefin reagent (50 μg ml^{-1}) and the rate of increase in absorbance was monitored at 482 nm in a double-beam SP800 spectrophotometer (Pye-Unicam, Cambridge, U.K.) at 37°C. β-Lactamase activity was determined from a calibration line, constructed from the initial rate of nitrocefin hydrolysis by a series of β-lactamase solutions of known concentrations.

Preparation of standards and fermentation media samples

Standard aqueous solutions (2 mg ml^{-1}) of β-lactamase type I, bovine serum albumin and myoglobin were prepared and stored at −20°C.

Samples in fermentation media were prepared by addition of the standard β-lactamase to *Aspergillus* complete media[17] to give a final penicillinase concentration of 100 μg ml^{-1} and, prior to chromatography were centrifuged (10000 *g* for 10 min) at 4°C. Samples collected from the eluent were dialysed overnight at 4°C against 5 l of 0.01 *M* phosphate buffer (pH 7). After lyophilization, the residue was dissolved in distilled water (40 μl) and analysed by electrophoresis.

Estimation of the β-lactamase binding capacity of the resins

Samples of each resin (0.05 g) were packed in 1-ml syringes containing filter discs to contain the resin. Each resin was equilibrated by gravity elution with 10 ml of 0.03 *M* phosphate buffer (pH 7). A 200-μl aliquot of β-lactamase (20 μg ml^{-1}), prepared in the equilibrating buffer, was added to each resin and allowed to pass through the resin bed under gravity. This was repeated with further 200-μl aliquots of the enzyme until β-lactamase break-through was detected in the eluent by assay with nitrocefin.

Protein determination and electrophoresis

Protein concentrations were estimated by the method described by Bradford[18]. Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis was performed at 4°C as previously described by Laemmli[19]. Gels were stained by the silver-staining technique of Morresey[20].

RESULTS AND DISCUSSION

Development of mobile phases for HPLAC

Initially, it was considered that binding and elution of β-lactamase from the affinity columns could be successfully achieved with acetate buffers, as had previously been described for agarose-based affinity columns[15]. However, use of these buffers produced severe, non-specific protein adsorption, even on untreated "control" resins.

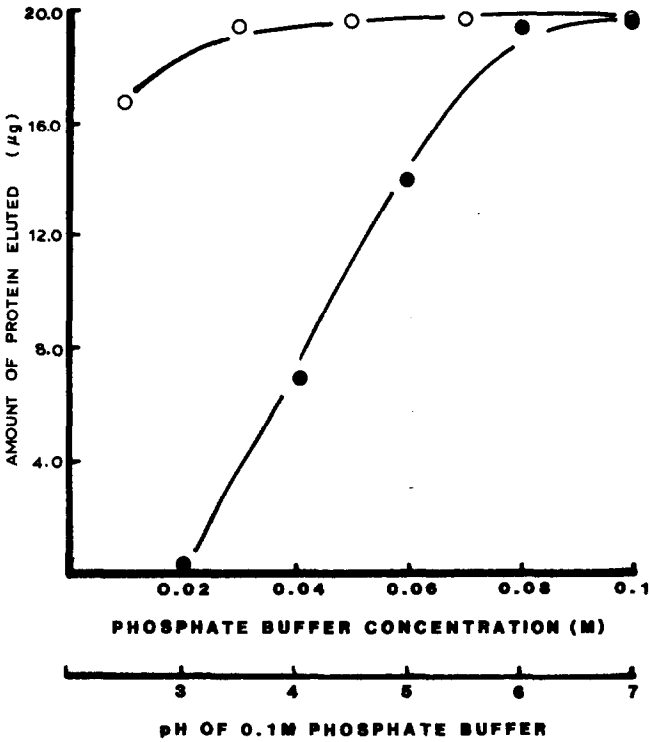

Fig. 2. Recovery of protein from a column of TSK 4000PW. Chromatography was performed at 0.5 ml min^{-1} with a mobile phase containing various phosphate buffer concentrations (○) and a 0.1 *M* phosphate buffer at various pH values (●). Folowing a 20-μl injection of myoglobin (1 mg ml^{-1}), protein elution was detected at 280 nm (0.1 a.u.f.s.), and concentration was estimated as described in Materials and methods.

Phosphate salts were investigated as possible mobile phase buffers and found to be more suitable on the basis of low, non-specific protein binding at pH 7. Using phosphate buffers, non-specific adsorption was markedly affected by the mobile phase pH, and to a much lesser extent by the buffer concentration. These effects are illustrated in Fig. 2 for myoglobin, applied to a control column of TSK 4000PW. Similar results were obtained when the affinity columns were used.

A mobile phase containing 0.03 *M* phosphate buffer (pH 7) was finally chosen to provide selective binding of the β-lactamase which could be subsequently eluted by increasing the ionic strength with 0.5 *M* potassium chloride. Further increases in salt concentration resulted in no additional elution of penicillinase.

β-Lactamase binding capacity of the resins

Studies on the capacity of each resin to bind β-lactamase indicated that 0.05 g of the affinity resins 1, 2, 3 and 4 were capable of binding 103, 3.3, 4.7, and 1.3 μg, respectively. These values were calculated from known amounts of enzyme, added to each resin before activity was detected in the eluate. No adsorption was demonstrated on the untreated TSK 4000PW or TSK 3000SW matrices used as controls. This wide range of binding capacities, notably between resin 1 and the others, may

be due to the choice of ligand and/or the methods used for ligand coupling. Cephalosporin C was chosen, as it had been previously shown to be an excellent ligand for the affinity chromatography of β-lactamase, possessing a high enzyme affinity but low hydrolysis rate[21]. 7-Pentanamido-cephalosporanic acid was used as an alternative ligand, because its structure was analogous to cephalosporin C, and the presence of only one carboxylic acid function allowed unambiguous coupling to the amino matrices by means of the carbodiimide reaction. However, the K_m value of 7-pentanamido-cephalosporanic acid is approximately half that of cephalosporin C for β-lactamase type I[22] and this may contribute to the differences in capacity of the resins for enzyme binding.

An alternative explanation may lie in the differences in the procedures employed for ligand coupling. Resin 1 was prepared by a two-step procedure. However, resins 2, 3 and 4 were obtained after a series of reactions. This may have resulted in a reduction of final ligand surface coverage. Coupling through the carboxylic acid group in the dihydrothiazine ring of the cephalosporin may also restrict binding with β-lactamase and thus reduce the resin capacity.

TABLE I

PROTEIN AND ENZYME RECOVERY FROM AFFINITY AND CONTROL RESINS

Of each protein 20 μl were injected in the mobile phase given. Protein concentration and enzyme activity (following elution from the column) were determined as described in Materials and methods.

Resin	*Protein*	*Composition of eluent*			
		0.03 M Phosphate (pH 7)		*0.03 M Phosphate (pH 7) + 0.5 M potassium chloride*	
		Protein recovery (% prot. applied)	*Enzyme recovery (% uts. applied)**	*Protein recovery (% prot. applied)*	*Enzyme recovery (% uts. applied)**
1	Myoglobin	101	–	72	–
	BSA	100	–	79	–
	β-Lactamase**	–	8.6	–	91.6
2	Myoglobin	100	–	101	–
	BSA	40	–	98.5	–
	β-Lactamase	–	5.3	–	87.8
3	Myoglobin	95	–	96.3	–
	BSA	13.7	–	98.1	–
	β-Lactamase	–	8.5	–	70.5
4	Myoglobin	101	–	101	–
	BSA	62.3	–	101	–
	β-Lactamase	–	24.8	–	51
TSK 4000PW	Myoglobin	95	–	100	–
Control	BSA	95	–	101	–
	β-Lactamase	–	80.0	–	95
TSK 3000SW	Myoglobin	93	–	75	–
Control	BSA	97	–	80	–
	β-Lactamase	–	83.0	–	90

* 1 uts. (Unit) hydrolyses 1 μg of nitrocefin min^{-1}.

** Amount of β-lactamase applied was 700 units. The protein concentration was too low to be determined by the method used in materials and methods.

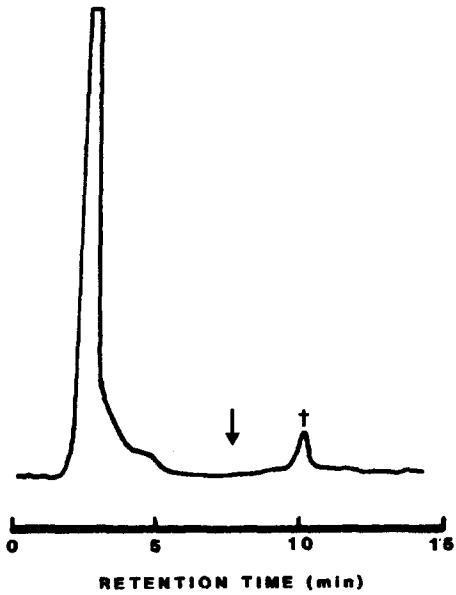

Fig. 3. Isolation of β-lactamase on resin 1 from fermentation media. Following a 20-μl sample injection, elution at 0.5 ml min^{-1} was performed with 0.03 *M* phosphate buffer (pH 7). After 7 min (↓), the mobile phase was switched to 0.03 *M* phosphate buffer (pH 7) containing 0.5 *M* potassium chloride, and flow continued until the active β-lactamase (†) was eluted. Detection was at 280 nm (0.1 a.u.f.s.).

Binding specificity of the resins

Further investigations were made concerning the binding specificity of the affinity columns and their ability to purify active β-lactamase. Myoglobin and bovine serum albumin were chosen as model proteins.

Table I shows protein and active enzyme recovery from the four affinity columns and controls. Resin 1 clearly provides superior performance with respect to enzyme recovery and low adsorption of myoglobin and BSA.

The purity of β-lactamase recovered from complex fermentation media was assessed by SDS polyacrylamide gel electrophoresis. With resin 1, it was possible to purify the enzyme to homogeneity. Fig. 3 illustrates binding and elution of the enzyme from this column by detection at 280 nm. When resins 2, 3 and 4 were used in the same HPLAC procedure, the protein fractions collected showed the presence of multiple protein bands (including β-lactamase) in the electrophoretic analysis. These contaminating proteins were particularly notable in samples obtained from resins 2 and 4.

This non-selective protein adsorption may be explained by ionic interactions between the protein and residual charged groups on the resin surface. In addition, hydrophobic interactions may arise between the protein and the aliphatic N-acyl side chain on the 7-pentanamido-cephalosporanic acid exposed at the resin surface.

Stability of the activated resins

The stability of the TSK 4000PW resin after cyanogen bromide activation was examined and compared with the same resin after activation with 1,1'-carbonyldi-

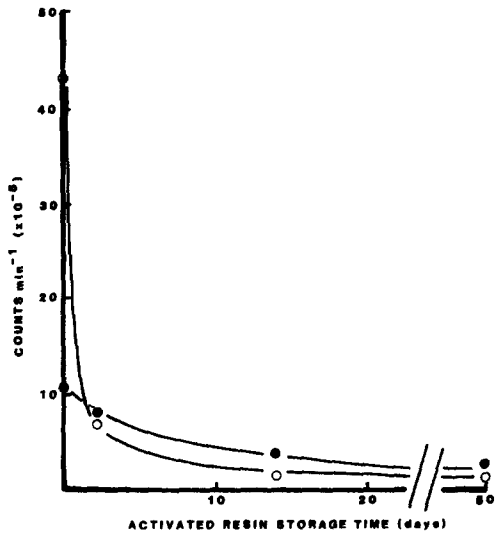

Fig. 4. Effect of storage time on the ligand-coupling ability of TSK 4000PW, activated with cyanogen bromide (○) and 1,1′-carbonyldiimidazole (●). 5 μCi of radiolabelled valine ($54 \cdot 10^5$ counts min^{-1}) were added to a sample of each activated resin and, after coupling and washing, the amount of radioactivity was measured in a liquid scintillation counter, as described in Materials and methods.

imidazole. Since it was not possible to obtain radiolabelled cephalosporin C or 7-pentanamido-cephalosporanic acid, L-[1-^{14}C]valine was chosen as a model ligand. Fig. 4 illustrates the effects of storage on the ability of the activated resins to couple with the amino acid. Although the amino acid loading on the cyanogen bromide-activated resin is initially significantly higher, the ability of this resin to couple the ligand is considerably reduced after a relatively short storage time. In contrast, the ligand binding capacity of the 1,1′-carbonyldiimidazole-activated resin decays slowly with time, although its initial ligand coverage is considerably lower. Clearly, the half-life of both types of activated resins would be significantly extended by storage at 0°C.

CONCLUSION

Different matrices, ligands, and mobile phases have been investigated for the isolation of β-lactamase by HPLAC. The optimum stationary phase was found to be TSK 4000PW, containing cephalosporing C, linked to the surface by means of cyanogen bromide activation. Specific enzyme binding was achieved by using a phosphate buffer at pH 7 and elution with the same buffer for which the ionic strength had been increased by salt addition. Elution was also possible by using the irreversible β-lactamase inhibitor, clavulanic acid, in the buffer. Thus, the mechanism of binding was proven to be a true affinity process.

The HPLAC technique proved to be a simple and very rapid method for homogeneous purification of active β-lactamase type I. Relatively small amounts of the column matrix are required, the specific binding capacity is high and the pH of the mobile phase is not restricted to below 8, as in the case of silica-based stationary

phases. The use of this type of activated, column material for the preparation of a wide variety of affinity matrices is possible, although storage of the material, once activated, is limited to short periods of time. In contrast, the liganded materials, stored in packed columns under aqueous methanol at 4°C, were used for several months without significant loss in performance.

An important application of this particular affinity material (resin 1) is in the detection of trace levels of β-lactamase in large sample volumes when the concentration is too low for conventional assay. The penicillinase may be concentrated from large-volume samples on the column and then eluted by a step gradient in the form of a sharp peak.[22]

Since the ligand is a β-lactam, the possibility exists for HPLAC analysis and isolation of penicillin-binding proteins[23]. There also exists the possibility of coupled-column chromatography as recently described[9]. It combines HPLAC with a further on-line purification technique, such as gel filtration or ion-exchange HPLC.

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