

# Characterization of an immobilized protein matrix for use in an affinity method for $\beta$ -lactam antibiotics<sup>1</sup>

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Current methods for the isolation and analysis of  $\beta$ -lactam antibiotics in animal tissues and fluids are inadequate, primarily due to lack of multiresidue capabilities, limited selectivity, length of procedure, and the use of copious amounts of organic solvents. By employing immobilized affinity receptor techniques we have prepared an affinity matrix containing an enzyme with  $\beta$ -lactam-specific binding properties. Immobilization of DD-peptidase to an activated agarose gel results in substantial changes in the enzyme's tripeptide hydrolytic and penicillin-binding properties compared with the soluble enzyme. The efficiency and selectivity for binding different penicillins, the effect of pH, as well as the fate of the antibiotic molecule after prolonged interaction with the immobilized enzyme, are altered in comparison with their interaction with free DD-peptidase. Protein conformational changes occurring upon enzyme immobilization may explain the enzyme's modified reactivity with substrate and antibiotic. The utilization of immobilized DD-peptidase for isolation of penicillins from spiked animal tissues and fluids and the use of this technique for residue detection are discussed.

## Introduction

$\beta$ -Lactam antibiotics are used in the prevention and treatment of bacterial infections in food-producing animals. This class of antibiotics encompasses the penicillins and cephalosporins.

Because trace levels of these antibiotics may cause allergic reactions in humans, adequate methods for monitoring the residues in fluids and tissues of treated animals are needed. The existing microbial methods for monitoring  $\beta$ -lactam residues are non-specific, non-quantitative and time-consuming [1]. Many of the immunoassays developed as test kits are highly sensitive, but are generally more expensive and are more commonly used for milk samples rather than for tissues [1-3]. Spectrophotometric and chemical assays involving the use of iodometry, hydro-

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<sup>3</sup>Abbreviations: 6-APA, 6-aminopenicillanic acid; DTNB, 5,5'-dithiobis-(2'-nitrobenzoic acid); TNB<sup>-</sup>, thiobis-(2'-nitrobenzoate) anion; DLAA, *NN*-diacetyl-Lys-Ala-Ala; RP-, reversed-phase.

xylamine and a pH-stat, are limited by their low detection sensitivity and their requirement for relatively clean samples [4]. Many of the above methods are also limited by their inability to detect multiple residues of  $\beta$ -lactams. Extensive research has been dedicated to developing confirmatory methods for  $\beta$ -lactams, such as GLC, TLC and MS, but these techniques also require a high level of purity in the sample being measured. Presently,  $\beta$ -lactam drug residues analysed by liquid-chromatographic techniques are extracted from animal tissues and fluids by organic solvents or acid precipitation [5–8]. However, environmental concerns over the use of organic solvents and the deleterious effect that acid pH can have on some  $\beta$ -lactams present major drawbacks to these methods. In addition, acid precipitation may leave many interfering substances which may not be easily removed by ultrafiltration. Thus improved methods for multiresidue isolation and concentration, which would be rapid and simple to use, be specific and minimize solvent use, are needed to expand the present monitoring capability for  $\beta$ -lactams.

The bacteriostatic effect of  $\beta$ -lactam antibiotics occurs by their inactivation of a carboxypeptidase which catalyses one of the final steps of bacterial cell-wall biosynthesis, thereby inhibiting cellular growth [9–13]. The amide bond in the  $\beta$ -lactam ring acts as a structural analogue of the normal enzyme substrate. The  $\beta$ -lactams are able to form stable adducts with the DD-peptidases in the form of acyl-enzyme intermediates. The complex formed between the enzyme and benzylpenicillin is stoichiometric and eventually undergoes spontaneous breakdown ( $t_{1/2} = 9\text{h}$  at  $25^\circ\text{C}$ ) during which the active enzyme is regenerated and the benzylpenicillin is cleaved, forming two products, phenylacetyl-glycine and *N*-formyl-D-penicillamine [14,15]. From kinetic studies, the dissociation constant ( $K$ ) for the reaction between benzylpenicillin and DD-peptidase at  $25^\circ\text{C}$  was determined to be 13 mM, while for the reaction between ampicillin and DD-peptidase at  $37^\circ\text{C}$ ,  $K$  was determined to be 7.2 mM [16].

Immobilized-affinity-receptor techniques are useful and powerful for the isolation and purification of target macromolecules, as well as for the capture and detection of macromolecules for analytical purposes [17]. This non-immunoaffinity approach has the further advantage of being as inherently selective as antibodies without the cost and time associated with producing the antibody. In order to evaluate the use of the immobilized-affinity-receptor method as a tool for the isolation of  $\beta$ -lactam antibiotic residues, we have immobilized DD-peptidase on to a solid support and characterized its catalytic activity and  $\beta$ -lactam-binding properties.

## Materials and methods

### *Materials*

**Antibiotics** Benzylpenicillin sodium salt, ampicillin, amoxicillin, cloxacillin sodium salt, hetacillin potassium salt, 6-aminopenicillanic acid (6-APA),

cephapirin sodium salt, penicillamine and penicillamine disulphide were obtained from Sigma.

**Enzymes** DD-peptidase obtained from Dr. J.-M. Frère, Université de Liège, Liège, Belgium, was buffer-exchanged with 10 mM sodium phosphate, pH 7.0, prior to use.  $\beta$ -Lactamase I was purchased from Sigma.

**Chemicals and reagents** Hydroxylamine hydrochloride, *o*-dianisidine and D-alanine were from ICN Biochemicals. FAD, peroxidase, and D-amino acid oxidase were purchased from Boehringer Mannheim. Potassium pyrophosphate and ethanolamine hydrochloride were obtained from Aldrich. *NN*-diacetyl-Lys-Ala-Alal (DLAA) and 5,5'-dithiobis-(2'-nitrobenzoic acid) (DTNB) were from Sigma, and Na<sub>2</sub>HPO<sub>4</sub> and NaH<sub>2</sub>PO<sub>4</sub> were from Mallinckrodt. Phenylacetylglycine was purchased from Bachem. Sodium acetate was obtained from Fisher Scientific, and NaN<sub>3</sub> was obtained from J. T. Baker. Acetonitrile was from Burdick and Jackson. [<sup>14</sup>C]Benzylpenicillin (potassium salt; specific radioactivity 58 mCi/mmol) was obtained from Amersham. EcoScint scintillation solution was from National Diagnostics.

Swine serum and bovine kidney tissues were obtained from the local slaughterhouse.

### Methods

**Determination of enzyme concentration** DD-Peptidase concentration was determined either by active-site titration with benzylpenicillin [18] or by its absorption coefficient at 280 nm [9]. The preparation was devoid of  $\beta$ -lactamase activity as determined by the iodine/starch assay [9].

**Immobilization of DD-peptidase** The enzyme was immobilized via primary amino groups on to Affi-gel 10 (Bio-Rad), an agarose support gel containing a neutral 10-atom spacer arm and activated with *N*-hydroxysuccinimide ester functional groups. Affi-Gel 10 (0.5 ml) was transferred to a 5 ml disposable micro-column (Isolab Quik-Sep). The gel was washed and equilibrated in 0.1 M sodium acetate, pH 4.6. A 5 mg portion of DD-peptidase in the same buffer was added to the gel in a 1.5 ml volume. Up to 3 mg of DD-peptidase was immobilized/0.5 ml gel after a 16 h incubation at 4 °C with gentle rotation. Uncoupled protein concentration was determined by the bicinchoninic acid (BCA) protein assay (Pierce) or by its  $A_{280}$  ( $A_{1\text{ cm}, 280\text{ nm}}^{1\%} = 10.0$ ) [9]. Unchanged *N*-hydroxylsuccinimide groups on the Affi-gel were blocked with 1 M ethanolamine, pH 8.0, to prevent non-specific binding. The affinity column was stored at 4 °C in 0.1 M sodium phosphate, pH 7.0, with 0.02% NaN<sub>3</sub> added as a preservative. The catalytic activity of immobilized DD-peptidase was determined by the D-amino acid oxidase assay for liberated D-alanine after enzymic hydrolysis of the substrate DLAA [16].

**Binding reactions of  $\beta$ -lactam antibiotics with immobilized DD-peptidase** Binding studies were performed at 25 or 37 °C. The DD-peptidase affinity column was equilibrated to the desired temperature in 10 mM sodium phosphate, pH 7.0. All antibiotics were dissolved in the same buffer and also equilibrated at the proper temperature. The

antibiotic (0.5 ml) was applied on to the affinity gel and allowed to incubate for either 15 min or 1 h. Unbound antibiotic was washed off after the incubation time with sodium phosphate buffer and collected in 2 ml fractions for further analysis by reverse-phase (RP)-HPLC. Flow rates through the affinity column were typically 0.7 ml/min (by gravity). Neutral hydroxylamine (0.8 M) in 10 mM sodium phosphate buffer was used to elute the penicillins from the DD-peptidase column [19]. Thus the column was regenerated by stripping any bound drug with neutral hydroxylamine. Alternatively, since benzylpenicillin was observed to spontaneously dissociate from the affinity column with time (see the Results section), column regeneration after studies with benzylpenicillin was further carried out by allowing the column to sit for more than 2 h at 25 °C in phosphate buffer to permit complete dissociation and subsequently washed with more phosphate buffer.

**pH studies** The effect of pH on the binding of benzylpenicillin to immobilized DD-peptidase was also examined. The following pH buffers were used (10 mM): sodium citrate, pH 5; sodium phosphate, pH 7 and pH 8; boric acid, pH 9; and Na<sub>3</sub>PO<sub>4</sub>, pH 10. Benzylpenicillin (0.127 mM) was allowed to incubate with the DD-peptidase gel (0.127 mM enzyme) for 15 min at 25 °C at the desired pH in a microcolumn. After the reaction period, unbound drug was washed off the gel with buffer, filtered, and loaded on to the HPLC column. A parallel experiment was run in which a solution of benzylpenicillin was allowed to incubate in the same pH buffer under identical conditions (15 min, 25 °C) to determine if pH-induced hydrolysis had occurred during the time span of the binding experiment. In order to examine the formation of deacylation (acyl-enzyme breakdown) products at various pH values, benzylpenicillin was allowed to react with the immobilized enzyme at concentrations described above in a microcolumn for 15 min in the pH 7 buffer (25 °C). Unbound penicillin was washed off the gel with the same buffer. Buffers at various pH values were then each added to the penicillin-complexed enzyme gel and left for 1 h at room temperature. Filtrate containing deacylation products were collected, filtered and assayed by RP-HPLC as before.

**HPLC analysis** Determination of  $\beta$ -lactam binding to immobilized DD-peptidase was done by UV detection after isolation by RP-HPLC (Rainin HPXL) using a polymeric column [PLRP-S; 10 nm (100 Å); 5  $\mu$ m; 150 mm  $\times$  4.6 mm; Polymer Labs.] equilibrated in 10% acetonitrile/10 mM sodium phosphate, pH 7.0. The  $\beta$ -lactam drugs were eluted from the RP-column by a linear gradient of acetonitrile/10 mM sodium phosphate, pH 7.0, as described by Moats [20]. Wavelengths for UV detection (Gilson 115 Spectrophotometer) were 220 nm for the penicillins and 262 nm for cephalosporins. Samples were passed through Millex HV<sub>4</sub> filters (0.45  $\mu$ m; Millipore) and 0.4 ml was loaded on to the column. All mobile phases were filtered through a Kontes filtration device using Magna nylon 0.22  $\mu$ m membranes (MSI) and degassed with helium. The flow rate was 1 ml/min. Calibration curves of peak area or height versus concentration were

linear up to 10  $\mu\text{g}$  of penicillin on the basis of 200  $\mu\text{l}$  injections of each standard solution prepared in 10 mM phosphate buffer. Standard solutions of phenylacetyl-glycine dissolved in the same phosphate buffer were used to confirm the formation of degradation products resulting from the interaction between benzylpenicillin and the immobilized enzyme. Benzylpenicilloic acid was confirmed on the HPLC column by the reaction of  $\beta$ -lactamase I with benzylpenicillin.

**Thiol determination** Free SH resulting from the reaction of the  $\beta$ -lactam drug with free or immobilized peptidase was determined by reaction with DTNB [21]. Exchange of the disulphide in DTNB with free SH yields quantitative amounts of the thionitrobenzoic acid anion ( $\text{TNB}^-$ ), which exhibits strong visible absorption. The assay was carried out by adding a final concentration of 0.3 mM DTNB (stock prepared in 80% ethanol) to 0.2 ml of the reaction sample in a cuvette. The solution was immediately mixed and the absorbance was monitored for 20 min at 412 nm on a Beckman DU-70 spectrophotometer. For control samples, the drug was passed through Affi-gel containing no immobilized peptidase. The difference in absorbance between the enzyme-reacted drug and the control drug solutions was used to determine the thiol content of the reacted drug sample assuming an  $\Delta\epsilon_{412} = 13,600 \text{ M}^{-1}\cdot\text{cm}^{-1}$ .

**Binding of benzylpenicillin to immobilized DD-peptidase in fortified tissue and serum** Swine serum. A 1 ml portion of serum was fortified with 5 p.p.m. (in 200  $\mu\text{l}$ ) of a 10 mM sodium phosphate-buffered solution of benzylpenicillin and left for 5 min at 25 °C. Two general methods were followed to prepare the fortified samples prior to application on to the immobilized DD-peptidase column. In each case, recovery of the drug after this initial extraction step was determined either by HPLC or by using [ $^{14}\text{C}$ ]benzylpenicillin. Quantification of recovered [ $^{14}\text{C}$ ]benzylpenicillin was performed on a Beckman LS8000 liquid-scintillation counter in which 40  $\mu\text{l}$  of the labelled sample was mixed with 4 ml of scintillation fluid. In method 1 (ultrafiltration), the spiked serum was diluted 1:1 with phosphate buffer, pH 7, and then passed through a Centricon-10 ultrafiltration unit, resulting in a clear colourless filtrate. In method 2 (solvent extraction), a two-step extraction procedure using acetonitrile and methylene chloride was followed [22]. The resulting aqueous layer containing the penicillin was then ultrafiltered.

Calf kidney. A 1 g portion of minced tissue was fortified with 5 p.p.m. benzylpenicillin or [ $^{14}\text{C}$ ]benzylpenicillin and allowed to incubate for 30 min at 25 °C. The fortified tissue was homogenized with 2 ml of 10 mM sodium phosphate, pH 7, on a Janke and Kunkel Ultra-Turrax T25 homogenizer (IKA Labor Technik, Staufen, Germany) using the lowest power for 20 s in a 15 ml polypropylene centrifuge tube. An additional 2 ml of buffer was used to rinse the probe, this volume then being combined with the tissue homogenate. Drug extractions were performed as follows. In method 1 (heat extraction), a simple heat extraction was performed as follows. The spiked homogenate, contained in a 15 ml centrifuge tube, was heated in boiling water for 3 min. The extract was

centrifuged at 3000 *g* for 30 min. The supernatant was depigmented either by passing through a basic aluminium oxide column or through a 10 kDa-molecular-mass-cut-off ultrafiltration tube. The clarified solution was then analysed for recovered drug. In method 2 (solvent extraction), a five-step partitioning procedure [5] involving (1) deproteination by acetonitrile, (2) neutralization of acidic benzylpenicillin by addition of H<sub>3</sub>PO<sub>4</sub>, (3) extraction into methylene chloride, (4) treatment with an acetonitrile/hexane mixture and (5) partition into 10 mM buffer, pH 7, was followed.

The resulting filtrates/extracts from the above procedures containing recovered penicillin were applied to a column containing gel-immobilized DD-peptidase equilibrated in 10 mM phosphate buffer, pH 7, and allowed to incubate for 30 min at 25 °C. The molar ratio of penicillin to immobilized enzyme was approx. 1:1 in all cases. Unbound drug was collected and its concentration determined by HPLC or radioactivity counting.

## Results

### *Enzyme activity of immobilized DD-peptidase*

Bacterial cell-wall expansion occurs by the action of a set of DD-peptidases which utilizes a D-Ala-D-Ala dipeptide (synthesized by a cytoplasmic ADP ligase) as a carbonyl donor for transpeptidation and carboxypeptidation, with the liberation of D-alanine in each case [9,10]. Determination of the catalytic activity of immobilized DD-peptidase was made with the substrate DLAA, with freed D-alanine determined by the D-amino acid oxidase assay. As shown in Table 1, immobilization of DD-peptidase on the agarose support results in loss of >90% of its catalytic activity compared with that of free DD-peptidase. It is unclear whether the marked decrease in tripeptide hydrolysis is due to the unproductive binding of

**Table 1 Enzyme activity of free and immobilized DD-peptidase**

Unit values are defined as the amount of DD-peptidase catalyzing the hydrolysis of 1 μmol of DLAA into D-alanine and *NN*-diacetyl-Lys-Ala/min at 25 °C. The values shown represent the average of three determinations. Immobilized DD-peptidase concentration was 90.3 μM. DLAA was added in a 100-fold molar excess over the immobilized enzyme. Incubation of immobilized or free enzyme with the substrate was at 25 °C for 5 min. An additional 1 min incubation at 100 °C was made for the free DD-peptidase reaction to ensure termination of hydrolysis. Estimation of liberated D-alanine was made by the D-amino acid oxidase assay. D-Alanine was used as standard. In all cases, <10% of the initial DLAA was hydrolysed by the DD-peptidase.

	Activity	
	Units	Residual (%)
Immobilized enzyme	2.877 ± 0.009	3
Free enzyme	95.902 ± 0.058	100

the immobilized enzyme-substrate complex or to the inability of the immobilized enzyme to recognize and hence bind the substrate.

### *Binding of penicillins to immobilized DD-peptidase*

Seven  $\beta$ -lactam antibiotics were examined for their ability to bind to immobilized DD-peptidase: benzylpenicillin, ampicillin, cloxacillin, amoxicillin, hetacillin, 6-APA and cephalosporin. Table 2 displays the binding efficiency of the drugs when incubated with immobilized DD-peptidase for 15 min at 25 °C. As a comparison, Table 2 also presents the rate constants for the acylation (complex formation) and deacylation (complex breakdown) reactions of soluble DD-peptidase with the various drugs as reported by Frère and co-workers [9,10]. Benzylpenicillin and ampicillin binds the enzyme with similar efficiencies. This behaviour is in contrast with the soluble enzyme, which displays a catalytic efficiency ( $k_2/K$ ) for the acylation reaction with benzylpenicillin >100-fold higher than that for ampicillin (Table 2). The immobilized enzyme was also observed to bind to amoxicillin, cloxacillin, hetacillin and cephalosporin, albeit with lower efficiency. 6-APA, with its total lack of an acyl side chain, did not bind the immobilized enzyme. Extension of the incubation time between benzylpenicillin and the immobilized DD-peptidase to >1 h at 25 °C resulted in the recovery of all the bound benzylpenicillin in soluble form, as detected by HPLC (results not shown). The other drug tested, ampicillin, did not dissociate from the enzyme after the 1 h incubation. Variations in temperature and pH were also examined for their effect on the reaction between benzylpenicillin and the affinity matrix. No effect on binding was observed when the reaction was carried out at either 25 °C or 37 °C. Binding of benzylpenicillin, however, did

**Table 2 Binding of penicillins to immobilized DD-peptidase**

Each drug (67.9 nmol in 0.5 ml) was added to 0.5 ml of the immobilization gel containing 67.9 nmol of DD-peptidase. The drugs were allowed to react with the enzyme for 15 min at 25 °C. The incubation buffer was 10 mM sodium phosphate, pH 7.0. Unbound drug was washed off the gel with phosphate buffer and collected in 2 ml fractions at 1 ml/min. Fractions were passed through a 0.45  $\mu$ m-pore-size filter and 0.4 ml loaded on to a RP-HPLC column.

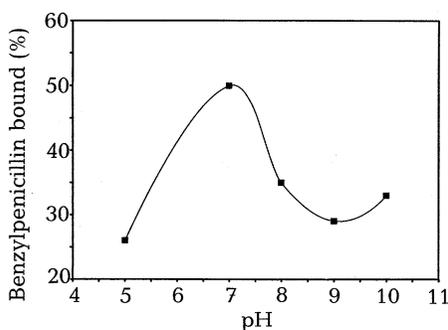
<i>Penicillin</i>	<i>Binding at 15 min (%)</i>	<i><math>k_2/K</math> (<math>M^{-1} \cdot s^{-1}</math>)<sup>a</sup> (complex formation)</i>	<i><math>k_3</math> (<math>s^{-1}</math>)<sup>a</sup> (complex breakdown)</i>
Benzylpenicillin	59	$1.2 \times 10^4$	$0.21 \times 10^{-4}$
Ampicillin	52	110	$1.4 \times 10^{-4}$
Cloxacillin	32	30	ND
Amoxicillin	32	ND	ND
Hetacillin	12	ND	ND
Cephalexin	12	ND	ND
6-APA	0	0.25	$<6 \times 10^{-5}$

<sup>a</sup> From [9,10], all values were determined at 37 °C, except for benzylpenicillin which was determined at 25 °C.

show a pH optimum of 7 (Figure 1). At higher pH values the efficiency of binding decreased by ~25%, but then levelled off.

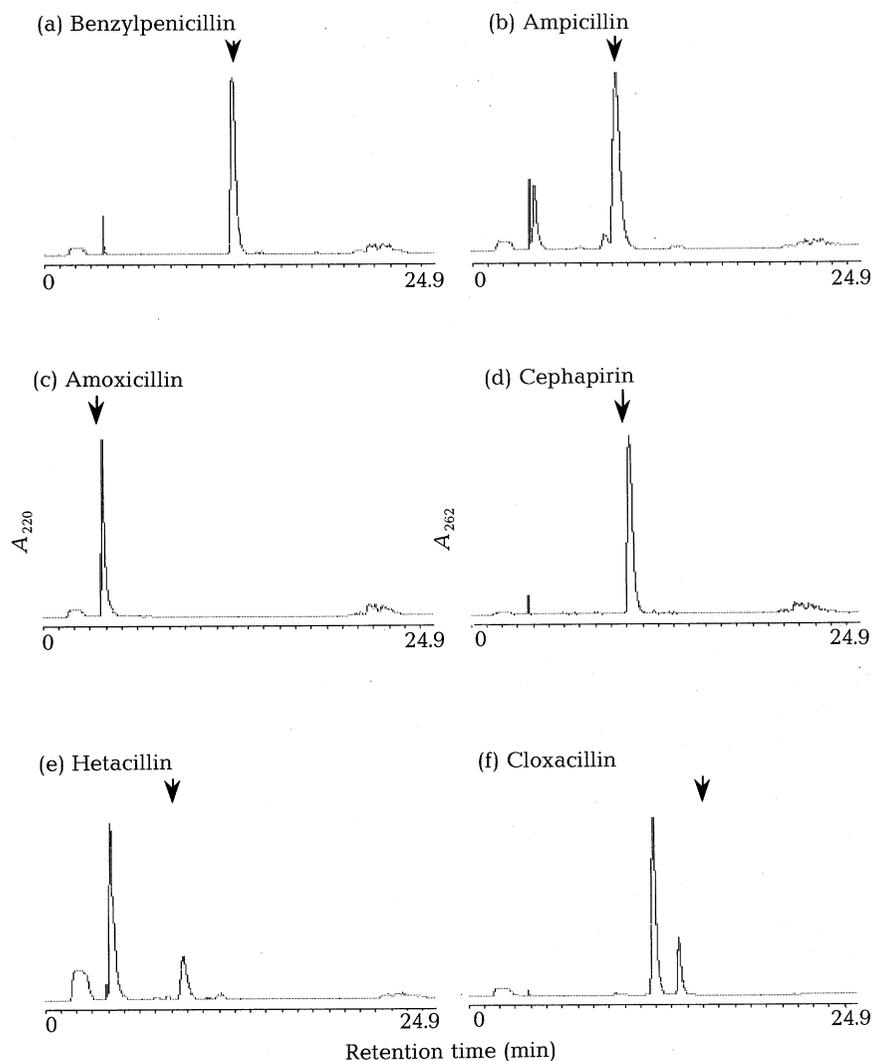
#### *Degradation of the drug-enzyme complex*

A 15 min incubation of an equimolar level of benzylpenicillin with immobilized DD-peptidase resulted in the appearance of an early-eluting peak due to phenylacetyl-glycine (Figure 2a). Assay of the same reaction sample by DTNB showed the presence of thiols (results not shown). Their formation indicated the presence of D-penicillamine, although the SH concentration determined accounted for <5% of the expected D-penicillamine formed, assuming complete conversion of the added antibiotic to equimolar amounts of phenylacetyl-glycine and penicillamine. No further increase in peak intensity was observed after reaction of benzylpenicillin with immobilized enzyme for >1 h at room temperature. However, the SH concentration was reduced to baseline levels after the extended reaction time, presumably due to the oxidation of the D-penicillamine initially released to the corresponding di-(D-penicillamine) disulphide. DD-Peptidase is believed to be involved in this oxidation process [15]. As with benzylpenicillin, interaction of ampicillin, amoxicillin, cephalixin or hetacillin with immobilized DD-peptidase also yields similar breakdown products, as determined by HPLC (Figure 2b-2e). DTNB analysis of the reaction of ampicillin and the immobilized enzyme again accounted for <5% of the total penicillamine expected to form as a result of reaction with stoichiometric amounts of the drug with immobilized DD-peptidase. No further HPLC or DTNB changes were observed upon prolonged reaction (>1 h) of ampicillin with the immobilized enzyme at room temperature. Similar observations were made for the reaction of these antibiotics with soluble DD-peptidase (results not shown). In contrast, reaction with cloxacillin yielded a chromatogram which was different from that which was observed when this drug was allowed to react with the soluble enzyme, reflecting a different mechanism of degradation of



**Figure 1**

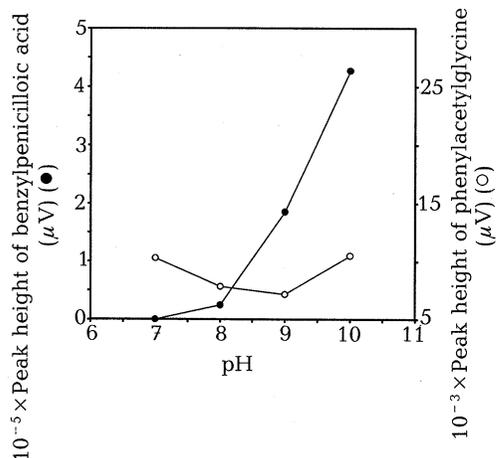
Dependence of benzylpenicillin-immobilized DD-peptidase complex formation on pH. A mol. equiv. amount of benzylpenicillin dissolved in the appropriate pH buffer (0.5 ml) was loaded on the DD-peptidase affinity gel, previously equilibrated in the pH buffer, and allowed to incubate with the gel for 15 min at 25°C. Collection and detection of unbound drug and composition of pH buffers are described in the text. The concentration of the immobilized peptidase was 127  $\mu$ M. In order to rule out the possibility of alkaline hydrolysis of benzylpenicillin during the time span of the binding experiment, a control sample of benzylpenicillin dissolved in the appropriate pH buffer was run in parallel.



**Figure 2**

Determination of deacylation products from the reaction between  $\beta$ -lactam drug and immobilized DD-peptidase at pH 7. Conditions were the same as in Figure 1. All HPLC procedures were as described in the Materials and methods section. Unbound drug was washed off the affinity column with the phosphate buffer, pH 7, filtered, and applied to a polymeric-based HPLC column (PLRP-S). UV detection was used to resolve the peaks attributable to degradation products. The peaks of the parent antibiotic are indicated by arrows. (a) benzylpenicillin; (b) ampicillin; (c) amoxicillin; (d) cephapirin; (e) hetacillin; (f) cloxacillin.

the immobilized protein with this penicillin (Figure 2f). Figure 3 shows the peak heights of benzylpenicilloic acid and phenylacetyl-glycine under various pH conditions. In all cases, breakdown of the acyl-enzyme was examined 1 h after the addition of the buffer to the penicillin-immobilized enzyme complex at 25 °C.



**Figure 3**

Effect of pH on the peak heights of deacylation products resulting from the reaction between benzylpenicillin and immobilized DD-peptidase. Enzyme and benzylpenicillin concentrations were the same as in Figure 1. The antibiotic was allowed to react with the immobilized enzyme for 15 min at 25°C and at pH 7. Unbound drug was washed off with pH 7 buffer. The penicillin-complexed enzyme gel was then incubated in various pH buffers for 1 h at 25°C. Deacylation products were washed off the gel with the pH buffer, collected, filtered and assayed using HPLC. The benzylpenicilloic acid peak was confirmed by reaction of  $\beta$ -lactamase I on benzylpenicillin. Phenylacetyl glycine was confirmed by running a standard solution in phosphate buffer. Retention times were 2 min (benzylpenicilloic acid) and 4 min (phenylacetyl glycine).

#### *Binding of benzylpenicillin to immobilized DD-peptidase in fortified tissue and serum*

We evaluated the effectiveness of immobilized DD-peptidase in capturing penicillin from more complex matrices, swine serum and calf kidney, with the results shown in Table 3. Two types of sample preparation methods were performed prior to application to the affinity column. In one, organic solvents were first used to extract the drug. In the other, solvents were omitted completely and fortified samples were either simply heated or ultrafiltered to separate the drug from the matrix. The goal was to determine the level of drug sample purity required for effective interaction with the immobilized peptidase.

It appears from the data that, for either serum or kidney, solvent deproteination is required for effective binding of the extracted drug to the immobilized enzyme. In fact, binding efficiencies were similar to that obtained for pure solutions of penicillin. In samples not treated with organic solvents, the crude drug extracts are still recognized by the immobilized enzyme, although the degree of binding is much lower. In addition, in swine serum, lower drug recoveries are obtained, which may be due to the binding of penicillin to the serum proteins that are concomitantly removed in the ultrafiltration step. Protein precipitation by acetonitrile not only reduces the level of protein available for complexation to the drug, but may also aid in the liberation of any protein-bound penicillins, thus allowing reaction with the affinity gel. Despite the fact that organic solvents can overcome these drug recovery and purity problems to some extent, it is still encouraging to see that the cruder drug solutions obtained by aqueous extraction methods can still be recognized by the immobilized enzyme. An interesting result with respect to

**Table 3 Recoveries and immobilized-DD-peptidase-binding efficiencies of benzylpenicillin extracted by various methods**

A 1 ml portion of swine plasma or 1 g of minced calf kidney was fortified with 5 p.p.m. of benzylpenicillin or [<sup>14</sup>C]benzylpenicillin. Fortified samples were either treated with organic solvents as described in methods adapted from [5,22], or not, prior to application to the affinity column (see the Materials and methods section). Extracted penicillin solutions were divided into portions for assay by HPLC or by radioactive counting to determine recovered drug. The solutions were then applied to a column of immobilized DD-peptidase (equilibrated in 10 mM sodium phosphate, pH 7.0) in equimolar concentrations relative to the immobilized protein. The penicillin-immobilized enzyme reaction was allowed to proceed for 30 min at 25 °C, and material was subsequently washed off in the same equilibrating buffer for analysis by HPLC or radioactive counting.

Fortified sample	Method	Extraction recovery (%)	Binding efficiency with immobilized DD-peptidase (%)
Swine plasma	Ultrafiltration	20	13
	Acetonitrile	81	41
Bovine kidney	Heat	50	17
	Acetonitrile	38	50

the recovery levels of drug from calf kidney is that a higher percentage recovery was obtained with the simple heat-extraction step (50%) compared with the solvent-partitioning method (38%). This is probably a consequence of the greater chance of drug loss during the multiple steps of the labour-intensive partitioning method.

## Discussion

Techniques using immobilized receptor molecules, such as proteins, provide an effective way of isolating biomolecules for analytical determinations. However, alteration of structural and behavioural properties of proteins often occur upon immobilization on solid supports [17,24,25]. Thus we have examined the hydrolytic activity and  $\beta$ -lactam-binding characteristics of DD-peptidase coupled to a solid support.

The soluble DD-peptidases from *Streptomyces* have been well characterized by Frère and co-workers with respect to their binding and inhibition properties by  $\beta$ -lactam drugs [9,10]. Penicillins and cephalosporins form equimolar complexes with DD-peptidase, rendering the enzyme inactive. The reaction is believed to occur by the following mechanism:



where E is the active enzyme, A is the antibiotic molecule, EA is the inactive antibiotic-enzyme complex, EA\* is the acyl-enzyme and P is the product(s). The half-life of the benzylpenicillin-acyl-enzyme complex at 25 °C is ~9 h. Breakdown of the complex regenerates the active enzyme plus phenylacetylglycine and N-formyl-D-penicillamine [14,15].

Numerous site-directed-mutagenesis studies performed on invariant residues located near or at the catalytic cavity, including the active-site residue serine-62, indicate that the carbonyl donor substrate and penicillin bind to the same serine residue in the active site or to residues located close to each other along the polypeptide backbone [26–30].

Immobilization of DD-peptidase resulted in complete loss of hydrolytic activity of the tripeptide substrate DLAA when compared with the soluble enzyme. In spite of the loss in activity, the enzyme was observed to bind readily to a number of  $\beta$ -lactam antibiotics. This is not entirely surprising, given the fact that, in site-directed mutations of DD-peptidase, the hydrolytic activity is always more sensitive to the mutation than penicillin-binding properties [26–30]. Apparently, the consequence of immobilization of DD-peptidase on a solid support is the substantial modification of its behaviour toward binding ligands, which may reflect significant changes in protein conformation.

Our work shows that the immobilized enzyme appeared to exhibit some selectivity of binding for different penicillins. The order of specificity for the drugs tested are: benzylpenicillin  $\approx$  ampicillin  $>$  cloxacillin  $\approx$  amoxicillin  $>$  hetacillin  $\approx$  cephalirin  $>$  6-APA. Benzylpenicillin and ampicillin were complexed most readily by the enzyme at room temperature and neutral pH. Total prevention of recognition and binding occurs in the absence of an acyl substituent, as in the case of 6-APA.

It is surprising that both ampicillin and benzylpenicillin bind to immobilized DD-peptidase with similar efficiency considering that Frère and co-workers [9,10] determined the bimolecular rate constant for the acylation reaction between benzylpenicillin and the soluble enzyme to be at least 100-fold higher than for ampicillin (also shown in Table 2). Interestingly, we observed that complexed benzylpenicillin dissociated reversibly from the matrix-bound enzyme after an extended incubation time at room temperature, whereas ampicillin did not. We also found that changes in temperature (25 °C and 37 °C) did not appear to affect the binding efficiency of the affinity gel with benzylpenicillin.

The effect of pH on the binding of benzylpenicillin to the solid-phase enzyme is shown in Figure 1. At lower pH, a 2-fold increase in the amount of drug bound occurred in going from pH 5 to pH 7. This behaviour is in contrast with that observed for the reaction between soluble peptidase with benzylpenicillin [31]. In the latter case, no changes in the acylation rate constants were detected between pH 5 and 8. Instead, the drug-binding behaviour of the immobilized enzyme more reflects the binding (based on  $K_m$  values) of substrate (DLAA) by the soluble peptidase. Our studies suggest that an enzyme group having a  $pK$  of  $\sim 6$  may be involved in the binding of antibiotic, a value that is not much different from that (5.3) of a group implicated in the interaction of soluble enzyme with substrate in previous studies [31]. Thus it appeared that immobilization increased the accessibility of this group for binding to penicillin. At higher pH values ( $> 8$ ), a reversal of events occurred. The immobilized enzyme lost some penicillin binding ability in going from pH

7 to pH 8, but then binding remained steady without further loss even up to pH 10. With the soluble enzyme [32], a dramatic loss in penicillin binding was observed at pH values  $>8$ . The  $pK$  of 9.5 in the experiments with soluble enzyme suggests that a highly conserved lysine located three residues away from the active-site serine may play a crucial role in the initial binding of the antibiotic. Upon immobilization, as shown in the present studies, this lysine probably no longer exerted the same influential effect on penicillin binding by the enzyme. The slight decrease in binding occurring at pH values  $>7$  is characterized by a group with a  $pK$  of  $\sim 7.5$ , which may be a putative imidazole ( $pK \sim 7$ ) also occurring in DD-peptidase, chymotrypsin and other related peptidases [28].

Studies by Frère et al. [9] show that DD-peptidases reacts with benzylpenicillin at room temperature and neutral pH to form stoichiometric and inactive complexes. Their studies showed that prolonged incubation of the penicillin-DD-peptidase complex at  $25^{\circ}\text{C}$  resulted in the spontaneous breakdown of the enzyme-drug complex, exhibiting a half-life of 9h. The fate of benzylpenicillin molecule on its interaction with the DD-peptidase is its cleavage into two fragments, phenylacetyl-glycine and D-penicillamine [14,15]. Despite the differences in substrate and penicillin reactivity between soluble and immobilized DD-peptidase, the degradation behaviour did not appear to be significantly altered for most of the antibiotics studied at neutral pH. Although some breakdown products (phenylacetyl-glycine and penicillamine) were rapidly formed,  $>95\%$  of the complex remained stable throughout the time period (15 min) studied. The exception was cloxacillin, which yielded different degradation products when allowed to react with the immobilized enzyme than with soluble enzyme. Virtually all of the parent cloxacillin molecule was converted into degradation products, as shown by the disappearance of the native peak in Figure 2f. Thus immobilizing DD-peptidase caused the enzyme to undergo a different degradation mechanism with cloxacillin. The nature of these degradation products needs to be identified in future studies. Studies were also performed to investigate the effect of pH on the deacylation process undergone by the benzylpenicilloyl-enzyme complex. Detection of possible product formation was resolved using RP-HPLC with UV detection. There was no effect of pH on phenylacetyl-glycine formation, although increasing pH resulted in an increase in the formation of benzylpenicilloic acid. This latter reaction is due to the attack of  $\text{OH}^-$  ions on the benzylpenicilloyl-enzyme. These results are in agreement with the observations made for soluble DD-peptidase [31].

Finally, the use of the immobilized peptidase in the selective extraction of penicillins from animal samples was examined. Because the immobilized enzyme was still able to bind the drug in crude extracts from animal tissue and serum, this immobilized-ligand technique may provide a simple alternative method for purifying and detecting these residues in animal samples. It is possible that the binding efficiency may be enhanced or selectivity modified with changes in ionic strength or when different immobilization supports are used. When employed as a pre-

analytical clean-up step for  $\beta$ -lactams, efficient elution of the drug from the affinity matrix would allow drug concentration, thereby increasing the level of sensitivity of the subsequent detection method. These are factors which need to be considered in future studies for optimization of the affinity method for drug residues.

Because an immobilization process often leads to alterations in the biophysical properties of a biomolecule, it was necessary in the present studies to carefully characterize the behaviour of immobilized DD-peptidase toward peptide substrate and its various  $\beta$ -lactam inhibitors. We found differences in catalytic activity, analyte binding and analyte degradation between immobilized and soluble DD-peptidases. The solid-phase enzyme demonstrates a clear ability to recognize multiple residues of penicillin, as well as having selectivity for the residues. Several studies have been reported describing the use of immobilized penicillinase as a 'bioreactor' for penicillin analysis in a flow-injection-analysis configuration [33–36]. To our knowledge, however, the studies presented here demonstrate the first application of a 'bioaffinity' method for  $\beta$ -lactam drug detection. Moreover, we show that the use of DD-peptidase as the receptor molecule in an affinity method may be promising for the purification or scavenge of  $\beta$ -lactam antibiotics from animal tissue and serum.

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