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Purification of β-lactamases by affinity chromatography on phenylboronic acid–agarose

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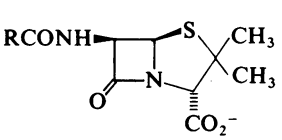
Several β-lactamases, enzymes that play an important part in antibiotic resistance, have been purified by affinity chromatography on boronic acid gels. The procedure is rapid, appears to be selective for β-lactamases, and allows a one-step purification of large amounts of enzyme from crude cell extracts. We have found the method useful for any β-lactamase that is inhibited by boronic acids. Two kinds of boronic acid column have been prepared, the more hydrophobic one being reserved for those β-lactamases that bind boronic acids relatively weakly. β-Lactamase I from *Bacillus cereus*, P99 β-lactamase and K1 β-lactamase from Gram-negative bacteria are among the better-known β-lactamases that have been purified by this method. The procedure has also been used to purify a novel β-lactamase from *Pseudomonas maltophilia* in high yield; the enzyme has an exceptionally broad substrate profile and hydrolyses monocyclic β-lactams such as azthreonam and desthiobenzylpenicillin.

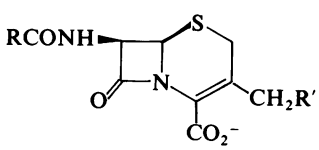
By catalysing the hydrolysis of the β-lactam ring in penicillins (I) and cephalosporins (II), the β-lactamases defend growing bacteria against the lethal effect of these antibiotics. The diversity and prevalence of these enzymes ensures continued attention to the study of inhibitors (Cartwright & Waley, 1983). Several irreversible inactivators of these enzymes are now known, and at least one is used clinically. Inert reversible inhibitors are rarer. To date, the only known inert inhibitors of β-lactamases are boric acid and phenylboronic acids (Kiener & Waley, 1978; Beesley *et al.*, 1983). The classification of β-lactamases based on their amino acid sequences recognizes three classes: A, B and C (Ambler, 1980; Jaurin & Grundström, 1981). Classes A and C are 'serine enzymes', i.e. they function by an acyl-enzyme mechanism, and have a serine residue in their active sites (Knott-Hunziker *et al.*, 1979, 1980, 1982*a*,*b*; Cohen & Pratt, 1980; Fisher *et al.*, 1980, 1981). The β-lactamases belonging to classes A and C are inhibited by boric acid and boronic acids. The class-B enzyme β-lactamase II from *Bacillus cereus* requires Zn(II) for activity, and there is no evidence that it is a serine enzyme, nor is it inhibited by boric acid or boronic acids. Thus, as far as we know, there is a perfect correlation between a β-lactamase being a serine enzyme and its being inhibited by boronic acids.

Two β-lactamases are now known to require metal ions for activity, β-lactamase II and β-lactamase L1 from *Pseudomonas maltophilia* (Kuwabara & Abraham, 1967; Saino *et al.*, 1982). Apart from these two, all the other β-lactamases that we have tested so far are inhibited by boronic acids, although the extent of inhibition varies widely. That β-lactamases are inhibited by boronic acids raised the question of whether such inhibitors could be immobilized and used to purify β-lactamases. Since β-lactamases vary so much in size, isoelectric point and stability, the purification of any newly discovered β-lactamase by conventional techniques is a time-consuming affair, and purification of relatively large amounts of enzyme for X-ray crystallography or peptide sequencing is not entered into lightly. There is a need, therefore, for a rapid, simple and mild method for purifying

Abbreviations used: SDS, sodium dodecyl sulphate; CH-Sepharose, carboxyhexyl-Sepharose; QAE-Sephadex, quaternary aminoethyl-Sephadex; DNAase, deoxyribonuclease.



(I)



(II)

large quantities of β-lactamases in high yield. We report here that the use of boronic acid–agarose columns fulfils this need.

The inhibition constant of *m*-aminophenylboronic acid for a β-lactamase in a cell extract may be measured to determine whether the enzyme will be bound to the affinity gel. If the result is positive, the enzyme can then be selectively bound and eluted pure in one step. As one example, 100mg of the β-lactamase from *Pseudomonas aeruginosa* 1822S/H (Berks *et al.*, 1982) have been purified in one stage from an extract of freeze–thawed cells in nearly 100% yield. This procedure has been used on several other β-lactamases, including a new β-lactamase from *Pseudomonas maltophilia* (Saino *et al.*, 1982).

Materials and methods

Materials

β-Lactams. The following compounds were gifts from the firms cited. Desthiobenzylpenicillin, nocardicin A and cefazolin (Fujisawa Pharmaceutical Co., Osaka, Japan); cefoxitin (Merck, Sharp and Dohme Research Laboratories, Rahway, NJ, U.S.A.); azthreonam (Squibb Institute for Medical Research, Princeton, NJ, U.S.A.); nitrocefin, cephalosporin C, penicillin G, cephaloridine and cefuroxime (Glaxo Research Laboratories, Greenford, Middx., U.K.); cloxacillin and carbenicillin (Beecham Pharmaceuticals, Brockham Park, Betchworth, Surrey, U.K.); and cefalexin (Eli Lilly and Co., Indianapolis, IN, U.S.A.).

Other chemicals. Affi-Gel 10 was obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A.; activated CH-Sepharose 4B from Sigma, Poole, Dorset, U.K., and *m*-aminophenylboronic acid hemisulphate from Aldrich Chemical Co., Gillingham, Dorset, U.K.

Enzymes. The *Pseudomonas* β-lactamase was from the constitutive mutant *Pseudomonas aeruginosa* 1822S/H (Flett *et al.*, 1976; Berks *et al.*, 1982). Crude enzyme preparation was obtained by freezing and thawing cells four times in 31.4mM-sodium phosphate/9.2mM-citric acid, pH6.3. DNAase (Worthington Biochemical Corp., Freehold, NJ, U.S.A.) was added to a concentration of 3μg/ml and the suspension stirred for 48h at 4°C. Cell debris was then centrifuged (at 24000*g* for 60min) and the supernatant dialysed against loading buffer (see below).

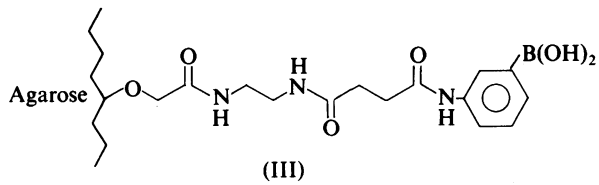
We are indebted to Dr. E. L. Emanuel (of this Laboratory) for extracts containing P99 β-lactamase (from *Enterobacter cloacae* P99) and K1 β-lactamase (from *Klebsiella aerogenes* 1082E), and to Dr. G. Ross (Glaxo Research Laboratories) for the organisms. The bacteria were grown as described by Marshall *et al.* (1972) and extracted with phenylethyl alcohol and EDTA (Choma & Yamazaki, 1981). The cell-free extracts were dialysed against loading buffer.

The constitutive mutant *Bacillus cereus* 569/H provides β-lactamase I, which is released into the culture medium along with β-lactamase II (Baldwin *et al.*, 1980). This organism also produces a third β-lactamase which is membrane-bound ('γ-penicillinase'; Pollock, 1956) (re-named β-lactamase III; Connolly & Waley, 1983). Cells freeze–thawed 6–8 times were centrifuged (at 13000*g* for 60min) and the supernatant, containing small amounts of β-lactamase I, was discarded. The disrupted cells were then stirred with 20mM-sodium citrate buffer, pH6, containing 0.5M-NaCl and 0.5% Triton X-100 for 36h at 4°C. Cell debris was centrifuged down (at 75000*g* for 90min), and nucleic acids in the supernatant precipitated by lowering the pH to 5.0 at 4°C overnight. After centrifugation (at 10000*g* for 30min) the pH was adjusted to 6.0 before loading.

β-Lactamase L2 was obtained from *Pseudomonas maltophilia* IID1275, kindly provided by Dr. Y. Saino, Gunma University, Gunma, Japan. The organism was grown as described previously (Saino *et al.*, 1982). Crude β-lactamase L2 was kindly prepared by Mr. R. Bicknell (this School) as follows. Cells were disrupted by freeze–thawing in 50mM-Tris/100μM-$ZnCl_2$, pH8.0. Nucleic acids were precipitated by the addition of protamine sulphate to 0.4% and the supernatant, after centrifugation (at 10000*g* for 40min) passed down a QAE-Sephadex A50 column to remove β-lactamase L1 (which was required for other studies) (Saino *et al.*, 1982). The eluate containing β-lactamase L2 was adjusted to pH7 before loading.

Methods

Boronic acid columns. A typical boronic acid column with a hydrophilic spacer arm (III) (called



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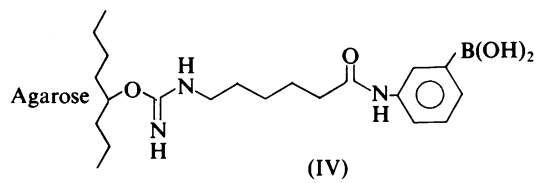


(IV)

a 'type-L column') was made as follows. A 20ml portion of Affi-Gel 10 was washed with propan-2-ol and then water at 4°C and transferred to 20ml of 1M-$KHCO_3$ containing 2g of *m*-aminophenylboronic acid hemisulphate and 2g of sorbitol. The suspension was agitated at room temperature for 1h, the pH being maintained at 8.0 with solid $KHCO_3$. The gel was then made into a column 4.1cm high × 2.5cm diameter and washed successively with 1M-NaCl/0.5M-sorbitol, pH7 (200ml), 0.5M-borate, pH7 (200ml), and finally 20mM-triethanolamine hydrochloride/0.5M-NaCl, pH7. The column was then ready for use. A column this size could bind at least 100mg of the *Pseudomonas* 1822/SH enzyme. Analysis of the gel showed an incorporation of 14 ± 3μmol of dihydroxyboryl groups/ml of gel (Weith *et al.*, 1970).

Boronic acid gels with a hydrophobic spacer arm (IV) (type-B columns) were made exactly as above, except that succinimide-activated CH-Sepharose 4B was substituted for Affi-Gel 10. In the structures shown (III and IV) the left-hand oxygen atom is derived from the carbohydrate moiety, and the boronic acid group will be largely unionized at pH7. The incorporation of boron in (IV) was 12 ± 3μmol of dihydroxyboryl groups/ml of gel (Weith *et al.*, 1970).

Except where stated otherwise, columns were run as follows. Crude enzyme was dialysed against 20mM-triethanolamine hydrochloride buffer/0.5M-NaCl, pH7 (loading buffer) and run through a column at a flow rate of 30ml/h. The column was then washed with loading buffer until the A_{280} of washings was zero. The β-lactamase was then eluted with 0.5M-borate/0.5M-NaCl, pH7. The columns were regenerated by washing with this borate buffer, and stored at 4°C. They have been re-used repeatedly, and no loss of binding capacity has been detected. The use of buffers of high ionic strength minimizes ionic interactions.

β-lactamase L2 was loaded on to columns in 50mM-Tris/100μM-$ZnCl_2$, pH7. The column was washed and the enzyme eluted as described above. β-Lactamase III was loaded in 20mM-sodium citrate/0.5M-NaCl/0.5% Triton X-100, pH6, at 5ml/h. The column was washed with 20mM-triethanolamine hydrochloride/0.5M-NaCl/0.5% Triton, pH6, and elution was effected with 0.5M-borate/0.5M-NaCl/0.5% Triton, pH6. β-Lactamase III is unstable in the absence of Triton or for long periods at pH values above 6.5 (Connolly & Waley, 1983). β-Lactamase I was loaded on to columns in culture supernatant adjusted to pH7 (Baldwin *et al.*, 1980).

Assays. β-Lactamase activity was measured in the pH-stat. The hydrolysis of a penicillin or cephalosporin [1ml of a 5mM solution in 0.5M-NaCl (0.5% with respect to Triton X-100 in the case of β-lactamase III)] in a water-jacketted vessel was followed by the uptake of 40mM-NaOH at 30°C. A unit of β-lactamase activity is the amount of enzyme that hydrolyses 1μmol of substrate/min at 30°C at the pH optimum of the enzyme. Under the conditions described, the rate is the limiting rate (Nomenclature Committee of the International Union of Biochemistry, 1982). Thus the term 'pH optimum' here means the pH optimum of $k_{cat.}$.

Inhibition constants. Inhibition constants were determined by a new, quick method (Waley, 1982). Progress curves for the hydrolysis of cephalosporin C, nitrocefin or penicillin G were performed in 20mM-triethanolamine hydrochloride/0.5M-NaCl, pH7, in the presence of *m*-aminophenylboronic acid or boric acid, and compared with those of controls lacking inhibitor. The value of K_i was a guide to the type of column to use.

Substrate profile. The substrate profile for β-lactamase L2 was determined by monitoring the hydrolysis of β-lactams either in the pH-stat or spectrophotometrically in a Cary 219 recording spectrophotometer, where the buffer employed was 50mM-phosphate/0.5M-NaCl, pH7. U.v. spectra before and after hydrolysis gave the molar absorption coefficient (ε). Apparent values of $V_{max.}$ and K_m were obtained from progress curves by a modification of the intersection method (Schonheyder, 1952; Cartwright, 1979). Spectral parameters for the difference spectrum (substrate − product) used in the present study were as follows [λ(nm), Δε (litre·mol^{-1}·cm^{-1})]: nocardicin A, 232, 1040; desthiobenzylpenicillin, 232, 785; benzylpenicillin, 232, 940; azthreonam, 318, 660; cephalosporin C, 260, 6000; cephaloridine, 280, 4100; cefazolin, 260, 2540; cefuroxime, 280, 6820; cephalexin, 260, 6800; cefoxitin, 261, 7775.

Determination of isoelectric points and M_r. Isoelectric focusing was performed on LKB Ampholine PAG plates, pH3.5–9.5, with markers from Pharmacia. Gels were stained to detect β-lactamase activity with filter paper dipped in nitrocefin and dried; proteins were detected by staining with Coomassie Brilliant Blue R250. Electrophoresis in 10% (w/v) polyacrylamide gels in the presence of SDS was used (Laemmli, 1970), with protein markers from Sigma.

Results

Table 1 shows the β-lactamases that we have examined to date on boronic acid–agarose columns with hydrophilic (type L) or hydrophobic (type B) spacer arms, together with the inhibition data from *m*-aminophenylboronic acid and boric acid. (The amino acid group of the former will not be ionized at pH7.)

Table 1. *Purification of β-lactamases on phenylboronic acid affinity columns*

The β-lactamase 'PA' marked with an asterisk below was from *Pseudomonas aeruginosa*; the sources of the other β-lactamases are given in the Materials and methods section, where their names are also given in full. The purification started from cell extracts, except for β-lactamase L2, where an eluate from QAE-Sephadex was used.

β-Lactamase	Class	Purification (fold)†	K_i‡ (μM)		β-Lactamase purified on:	
			m-apba	Borate	Type-L column**	Type B column††
'PA'*	C	220	5§	630§	Yes	Yes
P99	Not assigned	12	60§	5000§	Yes	Yes
L2	Not assigned	2000	200§	370§	Yes	Yes
βI	A	15	2000‖	1000‖	No (retarded)	Yes
βII	B	–	Not inhibited¶		No	No
βIII	Not assigned	60	1650‖	4400‖	No (retarded)	Yes
K1	Not assigned	40	770¶	600¶	No (retarded)	Yes
L1	Not assigned	–	Not inhibited¶		No	No

† (Units/mg of pure protein)/(units/mg of protein in starting material).

‡ Assays were performed in the presence and absence of inhibitor in 20mM-triethanolamine hydrochloride/0.5M-NaCl, pH7, at 30°C. K_i values were determined by the method of Waley (1982). Abbreviation used: *m*-apba, *m*-aminophenylboronic acid.

§ Cephalosporin C as reporter substrate.

‖ Nitrocefin as reporter substrate.

¶ Penicillin G as reporter substrate.

** Column matrix Affi-Gel 10.

†† Column matrix activated CH-Sepharose 4B.

Type-L affinity columns

β-Lactamases with K_i values for boronic acids $< 200\,\mu M$ are bound by these columns. The β-lactamase from *Pseudomonas aeruginosa* is particularly tightly bound. For instance, in a large-scale preparation of pure enzyme, 105000 units (against cephalosporin C, pH8) of crude enzyme in 300ml of loading buffer was loaded on to a column 4.1cm high × 2.5cm diameter. No detectable activity came through either on loading or with washing. The enzyme was eluted in a sharp band (25ml) (Fig. 1). A total of 104000 units were eluted: virtually 100% recovery. The enzyme gave a single band on isoelectric focusing (pI8.7) (Fig. 3 below). The specific activity was 840 units/mg (505 units·A_{280}^{-1}), rather higher than that of the conventionally (Berks *et al.*, 1982) purified enzyme (700units/mg).

Purification of the β-lactamase from *Enterobacter cloacae* P99 was achieved similarly. Isoelectric focusing of the eluted enzyme gave one major band and several minor bands, all of which had β-lactamase activity (Fig. 3). The main band had a pI of 8.25. Multiple bands of the P99 β-lactamase were also reported by Ross & Boulton (1973). The enzyme was homogeneous by SDS/polyacrylamide-gel electrophoresis. The specific activity of the pure enzyme was 540 units·A_{280}^{-1} (against cephalosporin C pH7) or 750 units/mg, compared with 250 units/mg reported by Marshall *et al.* (1972).

Fig. 1 shows the affinity chromatography of crude β-lactamase L2 from *Pseudomonas maltophilia*. The purified enzyme, on isoelectric focusing, gave one major band (and one close minor one), pI9.3, both of which had β-lactamase activity (Fig. 3). The enzyme was homogeneous by SDS/polyacrylamide-gel electrophoresis, the estimated M_r being 32400. It had a specific activity (against penicillin G at pH7) of 655 units·A_{280}^{-1}. The capacity of this type of column for β-lactamase L2 is rather low (1–2mg for a column of dimensions 2cm long × 1cm diameter), and it seems likely that, with a K_i of 200 μM for *m*-aminophenylboronic acid, binding to this type of column is rather weak.

The ability of the type-L column to effect a 2000-fold purification of β-lactamase L2 in one step is particularly noteworthy. Only 2–3mg of β-lactamase L2 are produced from a 15-litre growth of *Pseudomonas maltophilia*, so it would have been hard to purify this enzyme by conventional methods.

When crude K1 β-lactamase from *Klebsiella aerogenes* was loaded on to a type-L column, which was then washed, the β-lactamase activity was eluted behind the main protein band (Fig. 2). No further β-lactamase activity was eluted with borate buffer. Clearly retardation rather than binding occurs. K_i for *m*-aminophenylboronic acid is 700 μM (Table 1). Where affinity is this low, hydrophobic type-B columns are required.

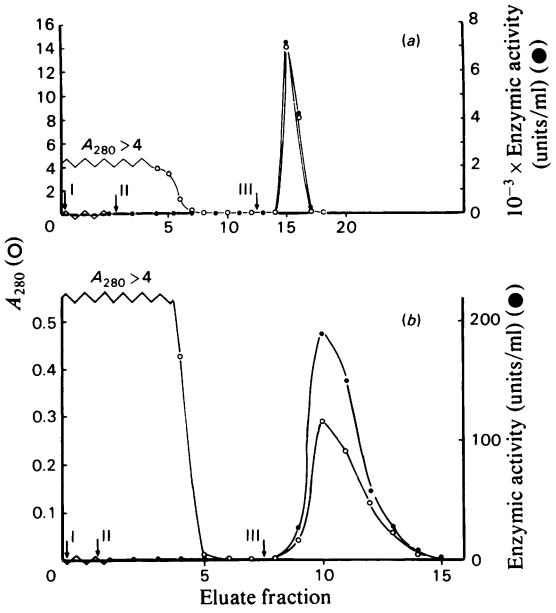


Fig. 1. *Affinity chromatography of β-lactamases on type L boronic acid columns*

(*a*) Chromatography of approx. 120mg of crude β-lactamase from *Pseudomonas aeruginosa* 1822S/H on a boronic acid column (2.5cm diam. × 4.1cm high). I, Crude cell extract (300ml) (dialysed against 20mM-triethanolamine hydrochloride/0.5M-NaCl, pH7); II, column washed with 20mM-triethanolamine hydrochloride/0.5M-NaCl, pH7; III, elution with 0.5M-borate/0.5M-NaCl, pH7. The volume of the fractions was 10ml. (*b*) Chromatography of the QAE-Sephadex eluate (see the Materials and methods section) of β-lactamase L2 from a 7.5-litre growth of *Pseudomonas maltophilia* IID1275 on a boronic acid column (1cm diam. × 2cm. high). I, Crude cell extract (1.5litres) in 50mM-Tris/100μM-$ZnCl_2$, pH7; II, column washed with 20mM-triethanolamine hydrochloride/0.5M-NaCl, pH7; III, elution with 0.5M-borate/0.5M-NaCl, pH7. Fraction volumes were 10ml for I and II; and 1ml for III.

Type-B affinity columns

The purification of K1 β-lactamase on these columns was achieved under the same conditions as those employed for the type-L columns. No activity was eluted from type-B columns during either loading or extensive washing. The K1 β-lactamase was eluted with the borate buffer with >95% yields (Fig. 2). Isoelectric focusing of purified K1 β-lactamase showed a major band of pI6.3 and two minor bands of pI5.8 and 5.3. All the bands had β-lactamase activity (Fig. 3). The enzyme was homogeneous by SDS/polyacrylamide-gel electrophoresis and had a specific activity of 4000 units·A_{280}^{-1} against penicillin G

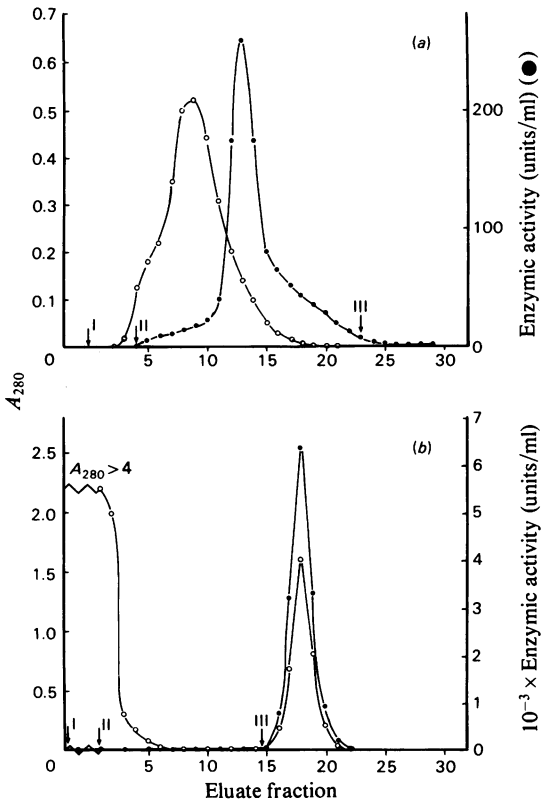


Fig. 2. *Affinity chromatography of β-lactamases on type-L and type-B boronic acid columns*

(*a*) Chromatography of a crude extract of β-lactamase K1 from *Klebsiella aerogenes* 1802E on a type-L boronic acid column (4.1cm high × 2.5cm diam.). I, Freeze-dried crude cell extract loaded on the column in 4ml of 20mM-triethanolamine hydrochloride/0.5M-NaCl, pH7; II, column washed with 20mM-triethanolamine hydrochloride/0.5M-NaCl, pH7; III, 0.5M-borate/0.5M-NaCl, pH7. Fractions were of volume 2.5ml. (*b*) Chromatography of a crude extract of β-lactamase K1 from *Klebsiella aerogenes* 1802E on a type-B boronic acid column (2cm high × 1cm diam.). I, Crude cell extract (dialysed against 20mM-triethanolamine hydrochloride/0.5M-NaCl, pH7; II, column washed with 20mM-triethanolamine hydrochloride/0.5M-NaCl, pH7; III, 0.5M-borate/0.5M-NaCl, pH7. Fractions were of volume 2.5ml.

at pH7. It seems likely that the minor bands observed on isoelectric focusing are variants possibly due to deamidation of asparagine or glutamine side chains.

β-Lactamase I and β-lactamase III from *B. cereus* (K_i for *m*-aminophenylboronic acid 2.0mM and 1.65mM respectively) were barely retarded by type-L columns. They were, however, bound, eluted from and purified by, type-B columns. A

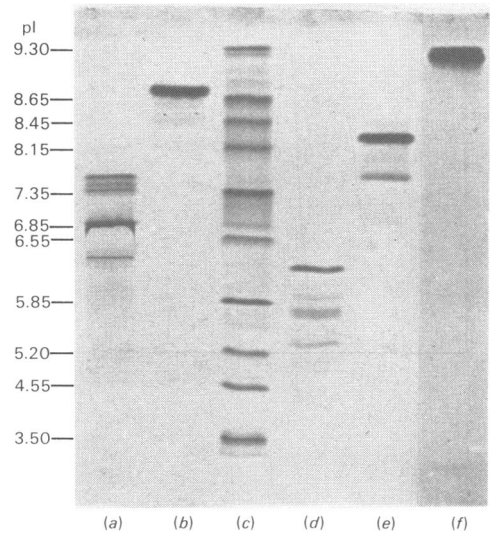


Fig. 3. *Isoelectric focusing of β-lactamases purified on phenylboronic acid–agarose*

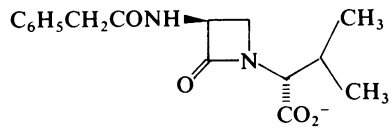
A 15μl portion of enzyme at a concentration of 1–2mg/ml was applied to each track and the gel subjected to 1500V and 30W for 1.5h. Protein was detected with Coomassie Brilliant Blue R250 (0.1%) in aq. 25% (v/v) ethanol containing acetic acid (8%, v/v). The anode is at the bottom of the gel. (*a*) β-lactamase III from *Bacillus cereus* 569/H; (*b*) *Pseudomonas aeruginosa* 1822S/H β-lactamase; (*c*) Marker mixture (LKB) (pI values given on the left); (*d*) β-lactamase K1 from *Klebsiella aerogenes* 1082E; (*e*) *Enterobacter cloacae* P99 β-lactamase; (*f*) β-lactamase L2 from *Pseudomonas maltophilia* IID1275.

type-B column (2cm high × 1cm diameter) bound 10mg of β-lactamase I, and the enzyme could be purified from crude supernatant. β-Lactamase II does not bind to the column (Table 1). The capacity of the same column for β-lactamase III is very much lower. About 1mg could be bound from crude cell extract on this column. Elution with borate buffer gave homogeneous enzyme as judged by SDS/polyacrylamide-gel electrophoresis, with M_r 31500, as found previously (Connolly & Waley, 1983). The specific activity was 2500units/mg (against penicillin G, pH7). Isoelectric focusing of the purified enzyme gave several bands, all having β-lactamase activity (Fig. 3). Neither β-lactamase II nor the L1 β-lactamase from *Pseudomonas maltophilia* (the metal-ion-requiring enzymes) are inhibited by boronic acids. As expected, they are not bound by either the L-type or the B-type affinity columns (Table 1). This provides an effective way of separating the two extracellular β-lactamases from *B. cereus* and the two enzymes from *Pseudomonas maltophilia*.

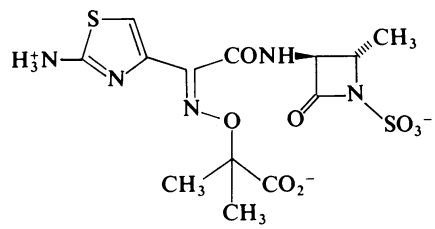
Table 2. *Substrate profile of β-lactamase L2 from Pseudomonas maltophilia*

Progress curves were constructed from measurements made in 50mM-sodium phosphate/0.5M-NaCl, pH7, at 30°C. Values for $V_{max.}$ are given relative to a value of 100 for penicillin G.

Substrate	$V_{max.}$(rel.)	Apparent K_m(μM)
Penicillin G	100	35
Cloxacillin	11	<10
Carbenicillin	8	<10
Cephalosporin C	24	51
Cephaloridine	196	375
Cefuroxime	19	28
Cefazolin	186	190
Cefalexin	1.0	20
Cefoxitin	0.0013	4
Nocardicin A	24	46
Azthreonam	9	7
Desthiobenzylpenicillin	1.1	24



(V)



(VI)

Properties of β-lactamase L2

Preliminary investigations on the purified β-lactamase L2 have centred on its substrate profile (Table 2). Of those β-lactams tested, only cefoxitin appears to be relatively resistant. Of special interest is the enzyme's unusual ability to hydrolyse monocyclic lactams, including desthiobenzylpenicillin (V) and azthreonam (VI) (Sykes *et al.*, 1982). The enzyme has a sharp pH optimum at pH7 with penicillin G as substrate. It does not show biphasic kinetics with cloxacillin, as reported for class-A β-lactamases (Kiener *et al.*, 1980). Its ability to hydrolyse many β-lactam antibiotics no doubt contributes to the organism's multiple resistance to these drugs (Saino *et al.*, 1982).

Discussion

The present results show that boronic acid–agarose gels provide an effective method for purifying certain β-lactamases. For those β-lactamases with a relatively high affinity for *m*-aminophenylboronic acid ($K_i < 200\,\mu M$), gels having a hydrophilic spacer arm (type L) provide adequate binding. About half the free energy change on binding in solution has been ascribed to non-polar forces (Beesley *et al.*, 1983). The capacity (about $5\,mg \cdot cm^{-3}$) is unusually high; Scopes (1982) quotes a capacity of $1–2\,mg \cdot cm^{-3}$ as 'good'. For those β-lactamases with low affinity for *m*-aminophenylboronic acid ($K_i > 200\,\mu M$), an additional binding force is required. A hydrophobic spacer arm would be expected to provide this additional binding. Neither the specific interaction nor the hydrophobic interaction alone is sufficient for binding when the K_i is in the millimolar region, but the two interactions are additive (Scopes, 1982). Triton X-100 binds to the type-B gels, but not to the type-L gels; if this binding were to the spacer arms, it would account for the low capacity of the type-B gels for β-lactamase III.

We have not observed any interference by nucleotides, or by glycoproteins, during chromatography of crude cell extracts; their adsorption needs different conditions, namely a very slow flow rate, and a pH near the pK_a of boronic acids, and the avoidance of buffers such as triethanolamine that interact with boronic acids (Rosenberg *et al.*, 1972; Middle *et al.*, 1983). Proteolytic enzymes that are serine enzymes interact with boronic acids (e.g. subtilisin; Philipp & Bender, 1971), but we have not detected proteins other than β-lactamases in our final preparations. Whether this method is applicable to penicillin-binding proteins (Waxman & Stromonger, 1983) we do not know.

We have found a perfect correlation between a β-lactamase being inhibited by boronic acids and its being retained on one of the boronic acid columns. This supplements the correlation (see the introduction) between a β-lactamase being a serine enzyme and its being inhibited by boronic acids. This suggests that a β-lactamase may be regarded as a serine enzyme if it is retained on a boronic acid column. Further work is necessary to see how widely this generalization holds.

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