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Separation, Purification and Properties of β-Lactamase I and β-Lactamase II from *Bacillus cereus* 569/H/9

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1. A procedure was devised which is suitable for the isolation of β-lactamase I and β-lactamase II from *Bacillus cereus* 569/H/9 on a large scale. After adsorption on to Celite both enzymes were eluted in good yield and separated by chromatography on Sephadex CM-50. 2. β-Lactamase I was separated into three main components by isoelectric focusing and into two components by chromatography. 3. The Zn^{2+}-requiring β-lactamase II obtained by this procedure had a lower molecular weight (22000) than β-lactamase I (28000) and also differed from the latter in containing one cysteine residue. 4. The β-lactamase II contained no carbohydrate, but showed the thermostability of the enzyme isolated earlier as a protein–carbohydrate complex. 5. Amino acid analyses and tryptic-digest 'maps' indicate that some degree of homology between β-lactamase I and β-lactamase II is possible, but that β-lactamase I is not composed of the entire sequence of β-lactamase II together with an additional peptide fragment. 6. A 6-methylpenicillin and a 7-methylcephalosporin showed much lower affinities for both enzymes than did penicillins and cephalosporins themselves.

Two types of extracellular β-lactamases are produced by *Bacillus cereus* 569 and 569/H, in which they are inducible and constitutive respectively. β-Lactamase I, a penicillinase with relatively little activity against many cephalosporins, has been purified by methods involving, among others, adsorption on and elution from glass powder (Kogut *et al.*, 1956) or Celite (Miller *et al.*, 1965), and chromatography on Amberlite IRC-50 resin (Puetzer & Boschetti, 1961), cellulose phosphate (Imsande *et al.*, 1970) and DEAE-cellulose (Kuwabara, 1970). The enzyme has been reported to consist of three or more distinct species, separable by electrophoresis on agar or polyacrylamide gel (Pechère & Zanen, 1962; Arcos, 1968; Imsande *et al.*, 1970). β-Lactamase II, a Zn^{2+}-requiring enzyme with activity against many cephalosporins as well as penicillins (Sabath & Abraham, 1966; Kuwabara & Abraham, 1967), has been separated from β-lactamase I by fractional precipitation with $(NH_4)_2SO_4$ and purified by chromatography on DEAE-cellulose and precipitation of inactive material with acetone after removal of β-lactamase I by selective inactivation of the latter at 65°C (Kuwabara, 1970). The resulting preparation of crystalline β-lactamase II contained a carbohydrate moiety (Kuwabara *et al.*, 1970) which was later separated from the protein moiety by filtration through Sephadex G-100 (Kuwabara & Lloyd, 1971).

The purification of β-lactamase II has been complicated by the failure to recover it in eluates containing β-lactamase I after adsorption of the β-lactamase activity of the culture filtrate on powdered glass or Celite (Sabath & Abraham, 1965; Kuwabara, 1970), and by the failure to find it in the eluate from a column of cellulose phosphate (Sabath & Abraham, 1965). The present paper describes conditions under which adsorption on Celite can be used efficiently as the first step in the large-scale purification of β-lactamase II, as well as β-lactamase I. It also describes a simple procedure for the separation of the two enzymes from each other and includes a comparison of some of the properties of the purified enzymes obtained.

Materials and Methods

Materials

Reagents. Tris ('Trizma') was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Ethylenediamine was from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. and was distilled before use. All other general chemicals were from BDH, Poole, Dorset, U.K., and were of AristaR or AnalaR quality, except glycine (laboratory grade) and guanidinium chloride (especially low in heavy metals). Benzylpenicillin (Crystapen) and potassium cephalosporin C were from Glaxo Research Laboratories, Greenford, Middx., U.K. One batch of cephalosporin C was crystallized twice from 70% (v/v) ethanol

before use. 6-Aminopenicillanic acid was a gift from Beecham Research Laboratories, Brockham Park, Surrey, U.K. Cefazolin was from Fujisawa Pharmaceutical Co., Osaka, Japan. Other cephalosporins, 7-methoxycephalosporins and 7-aminocephalosporanic acid were from Eli Lilly and Co., Indianapolis, Ind., U.S.A.

Phenoxymethyl-6-methylpenicillin and a 7-methylcephalosporin were from the Squibb Institute for Medical Research, Princeton, N.J., U.S.A. Trypsin was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Celite 545 was from Johns Manville Products Corp., Lompol, Calif., U.S.A.; before use it was washed well with tap water and then with three portions of distilled water (10ml/g of Celite) and sucked damp-dry on a Pyrex glass sinter, porosity 1. Iodo[2-^{14}C]acetic acid was from The Radiochemical Centre, Amersham, Bucks., U.K. It was diluted with non-radioactive iodoacetic acid and the mixture neutralized with NaOH to give 0.2M-sodium iodoacetate (0.0465μCi/mmol).

Buffers. The pH of all buffers was determined at room temperature. For 'Tris' buffer, 0.1mol of Tris was dissolved in 900ml of water and the solution adjusted to pH7.0 with 12M-HCl and diluted to 1 litre. For 'Tris–Zn^{2+}' buffer, 1.0ml of 1.0M-$ZnSO_4$ was added before dilution. For 'Tris–Zn^{2+}–citrate' buffer, pH7.0, 0.1mol of Tris, 0.1mol of trisodium citrate and 1.5mol of NaCl were dissolved in 900ml of water. Adjustment of pH, addition of $ZnSO_4$ and dilution to 1 litre were then carried out as above For 'Tris–Zn^{2+}–salt' buffer, 0.1mol of Tris and 0.5mol of NaCl were dissolved in 900ml of water and the solution was then treated as above. Buffers were used between 0° and 4°C for all preparative work except the thermal precipitation of β-lactamase I and ultrafiltration of β-lactamases I and II.

General methods

Determination of protein. Protein was determined as a routine by the method of Lowry *et al.* (1951) with bovine serum albumin (Koch–Light Laboratories Ltd.) as standard, or (with purified enzyme preparations) by measurement of E_{280} at pH7.0; with solutions used for determinations of molecular weight, refractometry was used with a Brice–Phoenix differential refractometer as described by Lloyd & Peacocke (1970). The values for the weights of β-lactamases I and II obtained by the method of Lowry *et al.* (1951) were 112 and 80% respectively of those obtained by refractometry.

Determination of carbohydrate. The phenol–H_2SO_4 method of Dubois *et al.* (1956) for the detection of total hexose content was used, with galactose as standard.

Assay of β-lactamase activities. The unit of activity is the amount of enzyme required to hydrolyse 1μmol of substrate/min at pH7.0 and 30°C. Routine assays were carried out with a recording pH-stat, type PHM25/TTT 11a/ABU 13/SBR2c (Radiometer, Copenhagen, Denmark). The stock solution of substrate for the assay of β-lactamase I only consisted of: benzylpenicillin, 60mg; 1% (w/v) gelatin, 14.0ml; 50mM-EDTA (adjusted to pH7.0 with 0.1M-NaOH), 4.0ml; water to 40.0ml. For the assay of β-lactamase II only the stock solution consisted of: potassium cephalosporin C, 60mg; 1% (w/v) gelatin, 14.0ml; water to 40.0ml. In later assays gelatin was omitted from these solutions and the latter were made up in 0.5M-NaCl.

A sample (2.0ml) of the solution required was pipetted into the titration vessel maintained at 30°C. For the assay of β-lactamase II only, 5μl of 0.14M-$ZnSO_4$ was added at this point. The solution was adjusted to pH7.0 at 30°C by titration with 40mM-NaOH. With benzylpenicillin as substrate the very low rate of Zn^{2+}-catalysed hydrolysis was established. A small amount of the suitably diluted enzyme solution (usually 1–10μl) was then added and the assay solution was subsequently maintained at pH7.0 by the recorded addition of 40mM-NaOH. Dilutions of the enzymes were usually made in Tris–Zn^{2+}–salt buffer containing 1% (w/v) gelatin. β-Lactamase activities were determined from the initial rates of addition of NaOH. It was assumed that 1equiv. of acid was liberated on hydrolysis of 1mol of benzylpenicillin at pH7.0, but that 2equiv. were liberated from 1mol of cephalosporin C and from other cephalosporins with a leaving group attached to the exocyclic methylene (Sabath *et al.*, 1965). When the rate of hydrolysis of benzylpenicillin was measured at lower pH values allowance was made for incomplete ionization of the penicilloate (Woodward *et al.*, 1949).

Preparations of enzymes in which the β-lactamase II activity was at least 20% of that of β-lactamase I could be assayed conveniently in one operation with benzylpenicillin as substrate. The total activity of both β-lactamases was determined in the solution used for assay of β-lactamase I only, except that EDTA was replaced by water. After about 30% of the benzylpenicillin had been hydrolysed, EDTA (200μl of a 50mM solution adjusted to pH7.0 with 0.1M-NaOH) was added to the reaction vessel to inactivate β-lactamase II. The resulting decrease in the rate of reaction corresponded to the β-lactamase II activity and the new initial rate to the β-lactamase I activity.

Measurements of the rates of hydrolysis of cephalosporins in relatively low concentrations, for determinations of Michaelis constants, were made spectroscopically. Opening of the β-lactam ring is accompanied by loss of an absorption band with $\lambda_{max.}$ at 260nm (Hamilton-Miller *et al.*, 1970). Continuous recording of the extinction at 260nm after addition of the enzyme to the substrate was made in a double-

beam spectrophotometer (SP800; Pye Unicam, Cambridge, Cambs., U.K.). The extinction fell towards a final value which was 20% of the original value. Extrapolation of a line representing the slope of the resulting curve at the origin to the final value gave the time for complete hydrolysis of the substrate at the initial rate.

Ultrafiltration. Concentration of proteins by ultrafiltration was carried out at room temperature in either an ultrafiltration cell with a stirrer, model 50 (for volumes less than 100ml), or continuous-flow apparatus, model TC1S or TC5 (for larger volumes), both from Amicon Ltd., High Wycombe, Bucks., U.K. The membrane used for filtration was UM-2 (Amicon Ltd.) which retains molecules of mol.wt. greater than about 2000. With UM-10 membranes (which retain molecules of about 10000–20000mol.wt.) there was incomplete retention of β-lactamase II (70%) on some occasions, though in all cases essentially complete retention of β-lactamase I. In the cell with a stirrer (model 50), agitation was kept to a minimum by stirring intermittently for a few seconds at 120rev./min.

Paper electrophoresis and chromatography. Electrophoresis on Whatman no. 1 paper at 75V/cm was carried out in an apparatus similar to that of Katz *et al.* (1959) at pH3.5 in pyridine–acetic acid–water (1:10:135, by vol.). Electrophoresis at pH1.8 in acetic acid–formic acid–water (10:1:39, by vol.) was carried out similarly or in a Camag high-voltage electrophoresis apparatus at 105V/cm (Camag, Muttenz, Switzerland). The latter apparatus was also used for electrophoresis at pH4.5 in pyridine–acetic acid–water (2:3:95, by vol.) and at pH8.9 in 2% (w/v) $(NH_4)_2CO_3$. Chromatography on Whatman no. 1 paper was in butan-1-ol–acetic acid–water (upper phase from 4:1:4, by vol.) or, for sulphonic acids, in butan-1-ol–pyridine–water (1:1:1, by vol.). Amino acids and peptides were detected with the ninhydrin–cadmium acetate reagent (Heilmann *et al.*, 1957). Amino sugars were detected by the method of Partridge (1948). Radioautography and counting of spots on paper were carried out as described by Smith *et al.* (1967). In some cases spots were first located by use of a spark chamber from Birchover Instruments, Hitchin, Herts., U.K.

Gel electrophoresis. A solution obtained by mixing 1% (w/v) ammonium persulphate (10ml), 28.5% (w/v) acrylamide containing 1.5% (w/v) *NN'*-methylenebisacrylamide (10ml), *NNN'N'*-tetramethylethylenediamine (60μl) and 0.1M-Tris–HCl buffer, pH7.0, containing 8.3% glycerol (20ml), was poured rapidly into a former to give a gel 140mm × 50mm × 3.4mm with four sample slots (4mm × 1mm × 3mm) 63mm from one end. Paper wicks (Whatman no. 1, 10cm × 6cm) made contact between the buffer for the electrodes (0.1M-Tris–HCl, pH7.0, containing 8.3% glycerol) and the gel. The apparatus was kept at 4°C. An initial voltage of 30V/cm was applied to the gel for 15min. Samples of purified β-lactamase I and II (10–25μg) were then placed in the sample slots and a current was passed (15mA, 30V/cm) for about 3h. Protein bands were stained in 0.1% Amido Black 10B (Edward Gurr Ltd., London S.W.14, U.K.) in 3% (v/v) acetic acid followed by destaining in 7% (v/v) acetic acid.

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis was carried out by the method of Weber & Osborn (1969). Bovine serum albumin (mol.wt. 68000), ovalbumin (mol.wt. 43000) and lysozyme (mol.wt. 14300) were used as internal standards.

Isoelectric focusing. This was carried out in an Ampholine electrofocusing column, type 8101 (LKB-Producter A.B., 16125 Bromma 1, Sweden), essentially by the method of Haglund (1967). The columns were maintained at 0.3°±0.3°C. The electrode solutions were: cathode, 0.2ml of ethylenediamine (redistilled) in 10.0ml of water; anode, 0.2ml of conc. H_2SO_4 in 14.0ml of water, containing 12.0g of sucrose. For isoelectric focusing β-lactamase preparations were dialysed against two changes of 100vol. of 1% (w/v) glycine.

Preliminary experiments were carried out in a 110ml column with wide-range Ampholines, pH3–10 (LKB 8131, batch no. 11). At the end of the experiment the contents of the column were removed into a fraction collector (0°C) by attachment of a peristaltic pump. In later experiments an 85ml column and narrow-range Ampholines, pH8–10 (LKB 8156, batch no. 29), were used, with a proportional decrease in the volume of the density gradient. Elution was by displacement of the density gradient with water at a rate of 45.5ml/h. Then 60 2.5min fractions were collected and stored at 0°C. β-Lactamase activities, pH and E_{280} values of the fractions were determined, and the active fractions were dialysed against water (72h; two changes of 500vol.) and then freeze-dried. The residues were dissolved in 5mM-NH_4HCO_3 (0.5ml) and the E_{280} values of the resulting solutions determined after centrifugation (2000*g*; 15min). The material in these solutions was used for amino acid analyses.

Amino acid analysis. Protein solutions were dialysed against water or 5mM-NH_4HCO_3. A solution of about 50–200μg of protein in a 3ml hydrolysis tube was diluted 1:1 with 12M-HCl. The solution was then frozen and degassed (Moore & Stein, 1963). Hydrolysis was for 18, 48 or 96h at 108°C and the values for serine and threonine were extrapolated to zero time. The hydrolysates (of 200μg of protein) were run on a Biocal 200 analyser or (of 50μg of protein) on a Biocal Minichrom analyser with the discontinuous buffer system of Spackman *et al.* (1958).

Cysteine was determined as cysteic acid, and methionine (sometimes) as its sulphone after oxida-

tion of freeze-dried samples of protein by the method of Hirs (1956), or Moore (1963), and subsequent hydrolysis. The relative proportions of tyrosine and tryptophan were determined spectroscopically by the procedure of Bencze & Schmid (1957).

Reaction with iodoacetate. Tris (121.1mg), guanidinium chloride (4.77g) and dithiothreitol (7.7mg) were dissolved in water to give 10ml of solution and the pH of the latter was adjusted to 8.6 with 12M-HCl. A freeze-dried preparation of β-lactamase II (50mg) or a heat-denatured (60°C in solution for 1h) preparation of β-lactamase I (36mg) was dissolved in 2.5ml of this solution and N_2 passed slowly through the mixture for 2h. A solution (0.25ml) of iodo[2-^{14}C]acetate (50μmol) in fourfold molar excess over dithiothreitol was then added to the solution at room temperature and N_2 passed through the latter for 1h. The reaction was terminated by addition of formic acid (0.3ml) and the solution diluted to 10ml with 1% (v/v) formic acid.

The resulting solution was desalted on a column (84cm×2.5cm diam.) of Sephadex G-25 (medium grade) and the protein fraction freeze-dried.

Tryptic-digest 'maps'. Samples (5mg) of the β-lactamases were oxidized with performic acid (Hirs, 1956). A solution of 1% (w/v) trypsin in 1.2mM-HCl (AristaR) was kept at 27°C for 1h. Suspensions (0.5%, w/v) of the oxidized β-lactamases in 1% (w/v) NH_4HCO_3 were treated with 1% (v/v) of the trypsin solution and the mixtures kept at 37°C overnight, by which time all the suspended material had dissolved. Samples (from about 1mg of protein) of the peptides obtained by freeze-drying the digest were subjected to electrophoresis on paper at pH3.5 for 45min, followed by chromatography in a perpendicular direction overnight in butan-1-ol–acetic acid–water.

Production of β-lactamase I and β-lactamase II

Organisms. B. cereus 569/H/9 is a constitutive β-lactamase-producing strain kindly given by Professor M. R. Pollock, Department of Molecular Biology, University of Edinburgh. It is a mutant of strain 569/H selected by the method of Kogut *et al.* (1956) for its ability to produce high amounts of penicillinase. Spore suspensions of the organism were prepared as described by Kuwabara (1970).

Laboratory-scale production. Media for production of β-lactamases I and II and the preparation of inocula were as described by Kuwabara (1970). In some experiments the inocula were stored in ice overnight, after growth for 6h, and warmed to 37°C before transfer to Kuwabara's (1970) modified CH/C medium. This storage had no apparent effect on the final β-lactamase activities.

The modified CH/C medium was distributed in 5-litre long-necked flat-bottomed flasks lightly plugged with cottonwool and maintained at 35°C (1470ml/flask). The inoculum (30ml/flask) was added and the flasks were then rotated at 200rev./min in an apparatus similar to that of Mitchell (1949). In most experiments cultures were harvested after 9h. For the determination of β-lactamases produced at different times, growth was continued for 24h and samples (10ml) were removed from each of two flasks at hourly intervals. Measurements were made of the extinctions of these samples and their contents of β-lactamase I and β-lactamase II. Extinctions were measured in a Spekker Absorptiometer (Hilger–Watts, London N.W.1, U.K.) with matched tubes, 1.5cm diam., and a neutral grey filter, H508. The cultures used for isolation of the enzymes were harvested by centrifugation at 0°C (1875*g*, 30min) and the decanted supernatant was stored at 4°C until required.

Large-scale production. B. cereus 569/H/9 was also grown in the medium of Kuwabara (1970) in a 400-litre fermenter as described by Melling & Scott (1972). The pH was automatically maintained at pH7.0 by controlled addition of 3M-HCl and cultures were aerated with 300 litres of air/min and stirred at 250rev./min. The temperature of both inoculum and production cultures was maintained at 30°C. Cultures were harvested when maximum amounts of β-lactamase II had been attained. The 400 litres of culture were cooled to 10°C and centrifuged in a Westphalia type K08006 continuous-flow centrifuge at a flow rate of about 250 litres/h. The supernatant was stored in a stainless-steel vessel fitted with a stirrer.

Results

Growth of B. cereus 569/H/9 *and β-lactamase production*

During the phase of exponential growth in which measurements were made the β-lactamase I and II activities in the medium increased rapidly, but the rates of increase then declined as the culture approached its stationary phase. Subsequently the β-lactamase II activity of the culture fluid began to fall, whereas the β-lactamase I activity continued slowly to increase. The changes in activity with one culture are shown in Fig. 1. This behaviour is similar to that described by Crompton *et al.* (1962) with respect to β-lactamase production by *B. cereus* 569 and to that recorded by Kuwabara (1970) on the basis of data supplied by the Microbiological Research Establishment, Porton, from a 142-litre culture of *B. cereus* 569/H in a fermenter.

To obtain the maximum amounts of β-lactamase II mixed with the smallest possible amount of β-lactamase I from laboratory-scale cultures, the latter were harvested 9h after inoculation. At this time the proportion of β-lactamase I to β-lactamase II was effectively one-half that at 24h. In the supernatants from six different cultures the amount of β-lactamase I

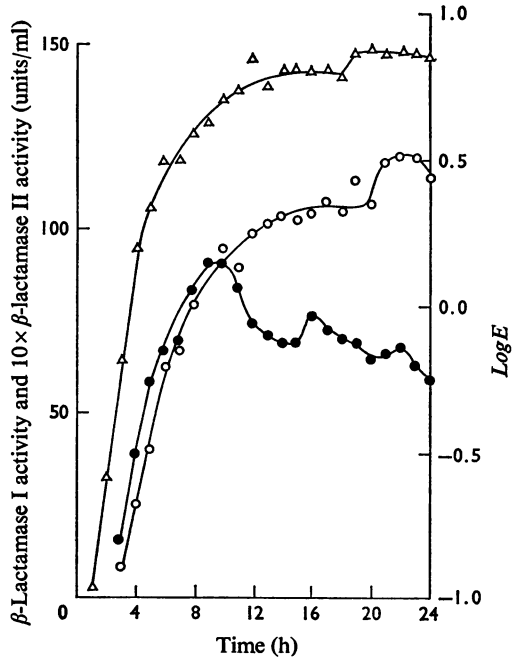

Fig. 1. *Growth of B. cereus 569/H/9 and β-lactamase production*

○, β-Lactamase I activity; ●, β-lactamase II activity; △, extinction of culture (see the text for details).

varied from 32 to 88 units/ml and that of β-lactamase II from 3.5 to 9.3 units/ml. With similar cultures harvested after 24h the amount of β-lactamase I varied from 82 to 150 units/ml and that of β-lactamase II from 2.5 to 6.5 units/ml.

Similar yields of both β-lactamases were obtained from large-scale cultures grown in fermenters at 35°C with appropriate scaling-up of the inoculum and production stage of the laboratory procedure, but low yields were obtained when the procedure was modified by growth of the inoculum in two stages. However, a decrease in the temperature of growth from 35° to 30°C resulted in high yields from the two-stage inoculum. It was subsequently shown by the method of Kogut *et al.* (1956) that an increasing proportion of cells which did not produce β-lactamase appeared in cultures at the higher temperature. Thus on plating-out a culture obtained by growth of *B. cereus* 569/H/9 from a spore suspension and five daily transfers on agar at 38°C, none of the cells gave β-lactamase-positive colonies. With corresponding cultures grown at 35° and 30°C 13 and 100% respectively of the colonies were β-lactamase-positive.

A typical large-scale culture grown at 30°C yielded 105 units of β-lactamase I/ml and 9.7 units of β-lactamase II/ml.

Purification and separation of β-lactamases I and II

Preliminary investigations. Re-examination of the procedure of Miller *et al.* (1965) showed that both β-lactamases I and II could be adsorbed rapidly and almost completely from a clarified culture supernatant at neutral pH by stirring the latter with Celite 545. Tris buffers at various pH values between 7.0 and 9.0 and containing NaCl in concentrations up to 3M eluted a large proportion (up to 85%) of β-lactamase II, but in no case more than 20% of the β-lactamase I. However, elution with a Tris buffer, pH7.0, containing 0.1M-trisodium citrate, 1.5M-NaCl and 1mM-$ZnSO_4$ removed both enzymes in excellent yield. The Tris–Zn^{2+}–citrate buffer could be replaced by a Tris–Zn^{2+}–maleate buffer, but the latter was not used as a routine because its strong absorption of u.v. light prevented spectroscopic analysis of the eluate.

Attempts to separate the two β-lactamases by elution from Celite with gradients of trisodium citrate in Tris buffers containing NaCl were only partly successful. It was thus decided to use adsorption on Celite primarily for the concentration and purification of both enzymes together.

Isoelectric focusing in a sucrose density gradient with a freeze-dried enzyme preparation from a dialysed Celite eluate suggested that the isoelectric points of both enzymes lay in the range pH8.4–9.5. Studies were then made of the use of Sephadex CM-50 for the resolution of β-lactamase I and β-lactamase II.

Adsorption on and elution from Celite. (*a*) Supernatants from six 1.5-litre cultures were pooled in a stainless-steel bucket and adjusted to pH7.0 at 4°C with 12M-HCl. Damp-dry Celite 545 (120g dry wt.) was added and the suspension stirred for 1h at 4°C with a stainless-steel stirrer at 50rev./min. The Celite was then removed by filtration on a glass sinter and rinsed three times on the sinter in water (10ml/g dry wt. of Celite) at room temperature. A slurry of the Celite in about 600ml of water was poured into a Pharmacia K25/100 column, maintained at 0°C, to give a column, after settling, of about 66cm×2.5cm diam. Excess of supernatant was removed from the top of the column and replaced with the Tris–Zn^{2+}–citrate eluent.

The pale-yellow effluent corresponding to the void volume of the column (about 200ml), containing suspended particles, was followed by a clear dark-yellow buffer front. The elution of β-lactamase I and II and the values for E_{280} of the fractions obtained in a typical experiment are shown in Fig. 2. The active fractions (25–100) were pooled and dialysed at 4°C against 10vol. of 1mM-$ZnSO_4$ (one change) for a total of 24h. At this stage (stage 2 of the representative

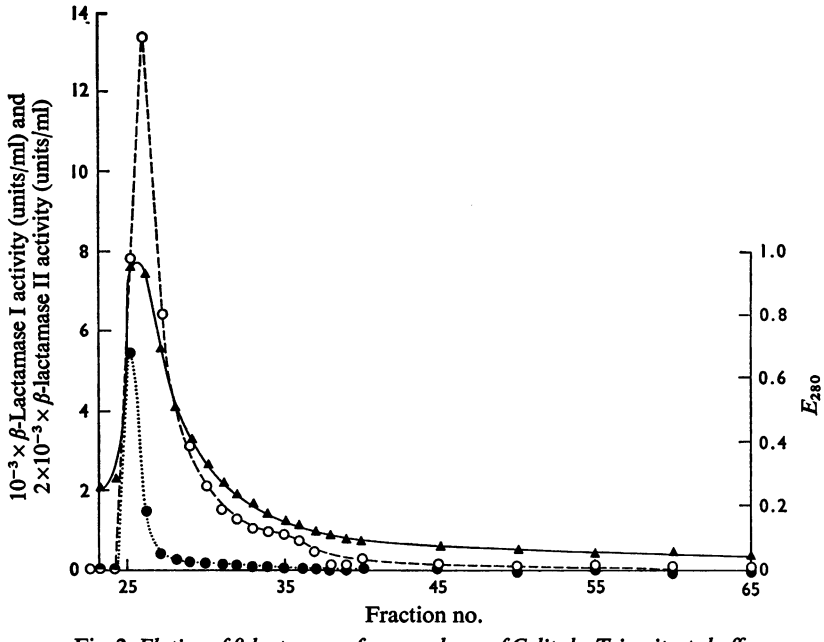

Fig. 2. *Elution of β-lactamases from a column of Celite by Tris–citrate buffer*

The column was 66cm×2.5cm diam. The flow rate was 175ml/h and fractions (9.91ml) were collected at 0°C. The eluate was monitored at 280nm in a Uvicord II (LKB-Producter AB, Stockholm, Sweden). ○, β-Lactamase I activity; ●, β-lactamase II activity; ▲, E_{280}.

purification shown in Table 1) the solution could be freeze-dried without loss of activity to a white powder containing a mixture of β-lactamase I and β-lactamase II.

(*b*) With 400-litre cultures Celite 545 [washed by suspension in 20vol. (v/w) of deionized water] was added to the stirred culture supernatant until all the β-lactamase II and most of the β-lactamase I had been adsorbed. About 15kg of washed Celite was required. The Celite was collected by centrifugation in a domestic spin dryer with a canvas bag and washed by resuspension and further centrifugation in 3×20vol. (v/w) of demineralized water. The washed Celite was packed into two sections of a KS370 column (Pharmacia Fine Chemicals A.B., Uppsala, Sweden), forming a column about 25cm×37cm diam. Elution was carried out with Tris–Zn^{2+}–citrate buffer at a flow rate of 1 litre/h, and 4-litre fractions were collected. The elution profile was similar to that observed with the laboratory-scale process.

An attempt to concentrate the β-lactamases in the eluate (21 litres) by ultrafiltration was abandoned when it was found that these enzymes showed anomalous behaviour in the presence of high salt concentrations and passed through membranes with nominal molecular-weight retention values as low as 10000. Similar behaviour was observed with the exopenicillinase from *Staphylococcus aureus* (Melling *et al.*, 1973). The eluate was therefore equilibrated by pressure dialysis with a buffer containing 0.1M-Tris and 1.0mM-$ZnSO_4$ adjusted to pH7.0 with 12M-HCl, and then equilibrated with a similar buffer containing 0.1mM-$ZnSO_4$. The dialysis was done by use of a twin-coil 90 Cron-a-coil with a Plexiglass unit (Travenol Laboratories, Thetford, Norfolk, U.K.) and buffer and enzyme solutions were circulated in closed loops at 12litres/h. The diluted buffer solution containing the β-lactamase (6.3 litres) was then concentrated in an Amicon TC5 ultrafiltration unit with PM-10 membranes to 1.4 litres. The concentrate was freeze-dried in 3lb (1.4kg) Kilner jars (200ml in each) and the product stored at −20°C. The overall yield of β-lactamase I was 77% and that of β-lactamase II was 98%.

Chromatography on Sephadex CM-50. The dialysed eluate from Celite (723ml for the batch referred to in Table 1) was pumped at a rate of 175ml/h through a column of Sephadex CM-50 (6.1cm×2.5cm diam.), kept at 0°C, which had been equilibrated in Tris–zinc buffer and washed with water. The material percolating through (collected at 0°C) contained a very small amount of β-lactamase I activity and no β-lactamase II activity. The loaded Sephadex CM-50 column was washed with 200ml of water and elution then carried out with a linear NaCl gradient. The results of a representative experiment are shown in Fig. 3.

Table 1. *Purification of β-lactamases I and II*

Stages 3A, 4A and 5A refer to fractions containing β-lactamase II after chromatography on Sephadex CM-50, and stages 3B and 4B to fractions containing β-lactamase I (for details see the text and Figs 1–3). Amounts (units) of β-lactamase I and β-lactamase II activities were determined with benzylpenicillin and cephalosporin C respectively as substrates. The values given in the Table were obtained during purification of the enzymes from a single batch of culture fluid, but Figs. 1–3 refer to separate experiments.

Stage	Material from	Total vol. (ml)	β-Lactamase activity (units/ml)		Amount (units) of β-lactamase activity recovered (%)		Sp. activity of β-lactamase (units/mg of protein)		β-Lactamase I activity (units) / β-Lactamase II activity (units)	E_{280}/E_{260}	Carbohydrate (μg/ml)
			I	II	I	II	I	II			
1	Culture supernatant	8438	32.7	4.0	100	100	270	33	8.25	0.86	27
1A	Supernatant after Celite	8418	0.23	0	0.7	0	—	—	—	—	24
2	Eluate from Celite	723	375	48	98.4	104	3535	453	7.81	—	6
3	Sephadex CM-50 percolate	920	2.0	0.9	0.7	2.6	—	—	—	—	<5
3A	Sephadex CM-50 eluate 1	314	4.7	87	0.6	86.0	127	2360	0.05	—	<5
4A	Heat-treated stage 3A	294	1.1	89	0.1	84.4	30	2317	0.013	—	<5
5A	Concentrate of stage 4A*†	6.0	0.08	880	0.002	83.4	0.17	2302	0.0001	‡1.73	<5
3B	Sephadex CM-50 eluate 2	377	657	0.3	94.2	0.4	7222	3.3	2190	—	<5
4B	Concentrate of stage 3B*	6.0	6683	4.4	95.7	0.5	7492	5.0	1507	‡1.79	<5

* Solutions from the preceding stage concentrated tenfold. † Subjected to further heat-treatment at 60°C for 30min.
‡ Mean of determinations on several runs.

Fractions 35–65 and 66–110, containing mainly β-lactamase II and almost exclusively β-lactamase I activity respectively, were pooled separately. The pooled fractions containing mainly β-lactamase II (see Table 1, stage 3A) were then heated to 60°C for 30min, during which time a slight precipitate formed. After centrifugation the resulting solution contained 95% of the β-lactamase II activity of the solution before heat-treatment and no measurable β-lactamase I activity. The yields and specific activities of the enzymes from one batch of culture fluid at successive stages of the purification procedure are given in Table 1. With some batches of the purified enzymes the final solutions were dialysed against Tris–Zn^{2+} buffer diluted 100-fold with 1mM-$ZnSO_4$ and then freeze-dried (without significant loss of activity) to yield white powders. These freeze-dried preparations were stable for at least several months when kept in sealed ampoules at 4°C. In other cases the final solutions of the enzymes were concentrated tenfold by ultrafiltration. The resulting concentrates retained 90% of their activity during 5 months at 4°C.

β-Lactamase II was eluted from columns of Sephadex CM-50 as a single symmetrical peak. The β-lactamase I obtained from laboratory-scale cultures, however, was eluted in a peak which showed asymmetry (Fig. 3). With material from 400-litre cultures the β-lactamase I peak was partially resolved into two components, the minor one being concentrated in the later fractions.

A solution of β-lactamase I (10mg/ml; stage 4B, Table 1), which had been dialysed against water and then concentrated threefold by dialysis against Carbowax (20M), yielded a crystalline mass on storage for 2 weeks at 4°C in open vessels. The crystals were about 10μm in length.

Properties of β-lactamases I and II

Homogeneity. Chromatography of samples of purified β-lactamase I and II separately in the Tris buffer on Sephadex G-75 (medium grade; bead form) gave in each case a single symmetrical peak, over which the change in extinction at 280nm paralleled exactly the change in β-lactamase activity. When subjected to electrophoresis (30V/cm) in polyacrylamide gel at pH7.0 for 3h both enzyme preparations migrated as single bands 1cm towards the cathode. In the sodium dodecyl sulphate buffer system of Weber & Osborn (1969) they both yielded single bands.

Isoelectric focusing in a wide-range pH gradient indicated that both β-lactamases I and II were isoelectric between pH8 and 10. In a narrow-range pH gradient β-lactamase I was resolved into three major components isoelectric at pH9.20, 9.47 and 9.68 respectively (Fig. 4). A shoulder on the peak shown by the component with pI9.20 corresponded to a protein

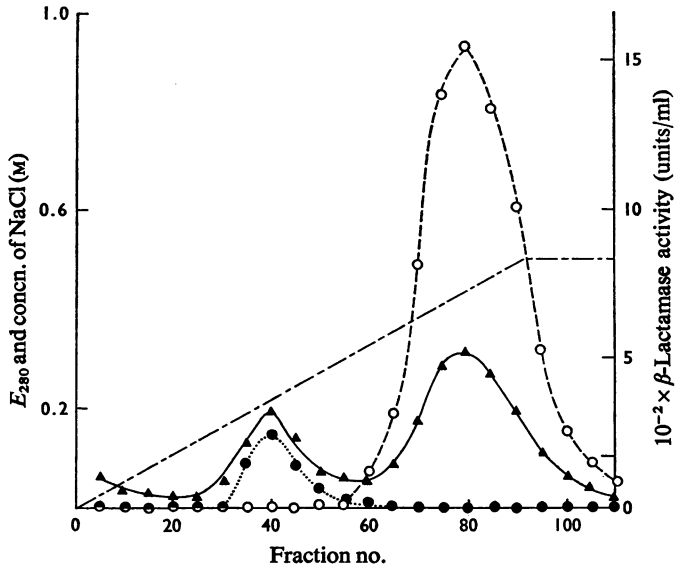

Fig. 3. *Elution of β-lactamase I and β-lactamase II from a column of Sephadex CM-50 in a NaCl gradient*

A linear NaCl gradient was formed by use of 500ml of Tris–Zn^{2+} buffer and 460ml of Tris–Zn^{2+}–salt buffer. Elution was completed with Tris–Zn^{2+}–salt buffer. The column was 10cm×6cm diam. The mixture of β-lactamases I and II applied was obtained from a Celite eluate. The flow rate was 175ml/h and fractions (10.6ml) were collected. ○, β-Lactamase I activity; ●, β-lactamase II activity; ▲, E_{280}; ———, concn. of NaCl (M).

with pI 9.10. The recovery of β-lactamase I was 88% and the three major peaks showed essentially the same specific activity. Under similar conditions β-lactamase II showed one main component, isoelectric at pH8.45, and a small amount of a second component, with a specific enzymic activity less than 10% of the first, which was isoelectric at pH10.11 (Fig. 5).

The properties described in the following sections were determined with purified β-lactamases which had not been subjected to isoelectric focusing.

Molecular weights. Dr. P. H. Lloyd of the Nuffield Department of Clinical Biochemistry, Oxford, kindly determined molecular weights by the sedimentation-equilibrium procedure of Lloyd & Peacocke (1970). Both enzyme preparations behaved as though they were homogeneous. The value obtained for β-lactamase I was 28000 and that for β-lactamase II was 22000. The values obtained for the molecular weights of β-lactamase I and β-lactamase II by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis were also 28000 and 22000 respectively. The two forms of β-lactamase I resolved by chromatography on Sephadex CM-50 were not distinguished by their mobilities in this system.

Amino acid composition. The results of amino acid analyses of the purified preparations of β-lactamases I and II (Table 1, stages 5A and 4B respectively) are given in Table 2, together with values reported by Kuwabara & Lloyd (1971) for a protein component of the β-lactamase II isolated as a protein–carbohydrate complex by Kuwabara (1970) and designated β-lactamase IIp by Dalgleish & Peacocke (1971). Table 3 gives the results of analyses of small samples (20–50μg) of the three components of β-lactamase I resolved by isoelectric focusing.

β-Lactamase I yielded no cysteic acid after oxidation and hydrolysis. However, a laboratory-scale and large-scale preparation of the enzyme gave 0.5mol/mol and 0.1mol/mol respectively of an amino acid which behaved like cysteic acid in the amino acid analyser, but was readily resolved from the latter by electrophoresis on paper at pH1.8. This substance was not resolved from homocysteic acid by electrophoresis at pH1.8, 4.5 or 8.9, or by chromatography in butan-1-ol–pyridine–water (1:1:1, by vol.). It was formed if oxidation either preceded or succeeded hydrolysis and was obtained in approximately equal amounts from the two components of β-lactamase I that were resolved during chromatography on Sephadex CM-50.

In its amino acid composition the β-lactamase II showed an overall similarity to earlier preparations (Kuwabara *et al.*, 1970; Kuwabara & Lloyd, 1971). Like the latter it contained relatively less alanine, isoleucine and arginine than β-lactamase I, relatively more valine and histidine, and yielded cysteic acid

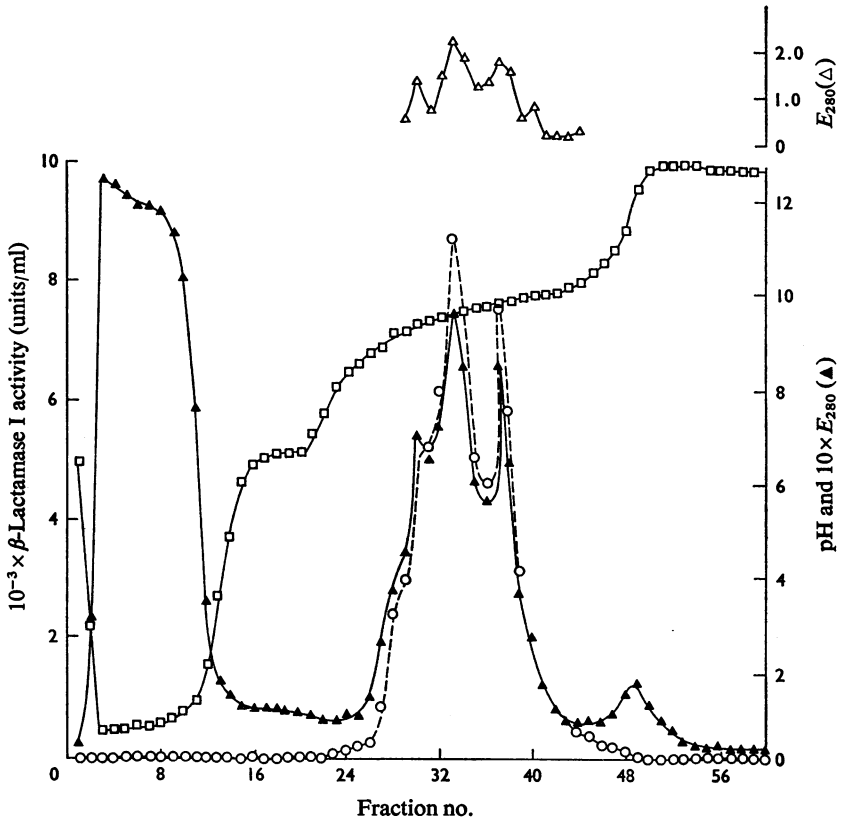

Fig. 4. *Isoelectric focusing of β-lactamase I*

○, β-Lactamase I activity; ▲, E_{280} of column eluate; △, E_{280} of eluate after dialysis; □, pH. For details see the text.

(1 mol/mol) after oxidation. Reaction of the denatured enzyme at pH 8.6 with iodo[2-^{14}C]acetate gave a protein with radioactivity corresponding to 0.72 mol of [^{14}C]carboxymethyl/mol. Acid hydrolysis gave a substance which behaved like [^{14}C]carboxymethylcysteine on electrophoresis at pH 1.8 and accounted for all the radioactivity of the protein.

Products of digestion with trypsin. Peptide 'maps' of the products obtained by digestion of oxidized β-lactamases I and II with trypsin under similar conditions showed the presence of 34 and 31 distinct peptides respectively. In addition there were several faint ninhydrin-positive spots corresponding to compounds with relatively high mobilities and R_F values, and a spot which appeared as a streak after chromatography and remained at the origin after electrophoresis. The two 'maps' showed little general similarity and a 'map' of a mixture of the two digests showed the presence of 47 peptides.

Enzymic activities. On the assumption that each enzyme contains a single catalytic site the catalytic-centre activity (mol of substrate hydrolysed/min per mol of enzyme) at 30°C and pH 7.0 of purified β-lactamase I with benzylpenicillin as substrate was 2.1×10^5. That of β-lactamase II was 0.80×10^5 with benzylpenicillin as substrate and 0.506×10^5 with cephalosporin C as substrate. The pH–activity curves for the two enzymes with benzylpenicillin as substrate were similar, showing a maximum in each case at pH 6 in routine assay solutions whose pH had been adjusted by addition of 12 M-HCl or 10 M-NaOH. The activities at pH 5.0, 6.0 and 9.0 were 80, 120 and 45% respectively of those at pH 7.0. A solution of β-lactamase I which had been kept at pH 4.0 at room temperature for 20 min showed about 90% of its original activity when assayed at pH 7.0. Under similar conditions β-lactamase II lost nearly 60% of its activity.

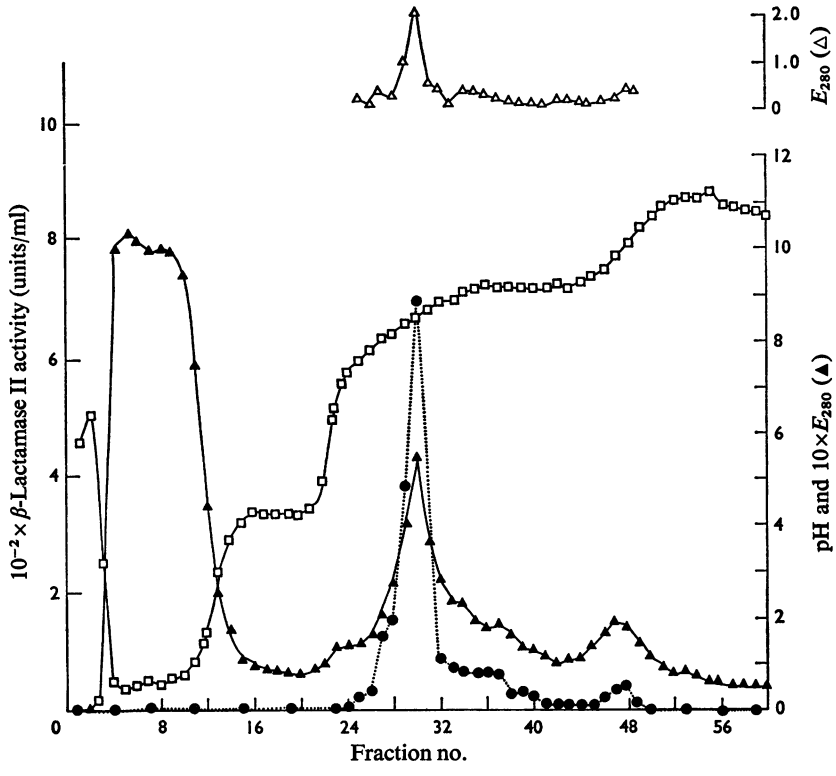

Fig. 5. *Isoelectric focusing of β-lactamase II*

●, β-Lactamase II activity; ▲, E_{280} of column eluate; △, E_{280} of eluate after dialysis; □, pH.

The substrate profile of β-lactamase I was similar to that reported previously (Kuwabara & Abraham, 1967) in that the rates of hydrolysis of the cephalosporins tested, with the exception of deacetylcephalosporin C lactone, were very much lower (<0.1%) than those of penicillins with similar *N*-acyl side chains. However, the rates for carbenicillin (D-α-carboxybenzylpenicillin) and methicillin (2,6-dimethoxyphenylpenicillin) were 22.4 and 6.0% respectively of that for benzylpenicillin when these substrates were tested in concentrations of 1.5mg/ml. Phenoxymethyl-6-α-methylpenicillin was hydrolysed in the concentration tested (1.5mg/ml) at less than 0.1% of the rate of phenoxymethylpenicillin itself, but phenoxymethylpenicillin sulphoxide was hydrolysed 1.4 times as fast as the latter. Desthiobenzylpenicillin appeared to be resistant to hydrolysis at the pH (9.0) at which measurements were made.

With β-lactamase II the values of $V_{max.}$ for a variety of penicillins and cephalosporins were of the same order as the value for benzylpenicillin. However, the rates of hydrolysis of 6-aminopenicillanic acid, Cephalexin (the 7-D-phenylglycyl derivative of deacetoxy-7-aminocephalosporanic acid) and Cefazolin {the 7-[1-(1H)-tetrazolylacetyl] derivative of an analogue of 7-aminocephalosporanic acid, in which the acetoxy group of the latter is replaced by 2-mercapto-5-methyl-1,3,4-thiadiazole} in concentrations of 1.5mg/ml were 1.5, 1.3 and 4.3% respectively of that of benzylpenicillin. The corresponding values for phenoxymethyl-6-α-methylpenicillin, 7-phenoxyacetamido-7-α-methyldeacetoxycephalosporanic acid and deacetylcephalosporin C lactone were less than 0.1% of that for benzylpenicillin. No hydrolysis of 7-methoxycephalothin [the 7-(2-thienylacetyl) derivative of 7-methoxy-7-aminocephalosporanic acid] or of the carbamate of 7-methoxydeacetylcephalosporin C was detected. Despite the relatively low rate of hydrolysis of Cephalexin, however, the activity of β-lactamase II in the mixture of the two enzymes eluted from Celite is sufficient for this mixture to be used in the assay of Cephalexin, as well as penicillins and other cephalosporins, by the method of Sabath *et al.* (1971), for the determination of these substances in blood in the presence of aminoglycoside antibiotics (L. D. Sabath, personal communication).

Table 2. *Amino acid composition of β-lactamases I and II*

The preparations of β-lactamase I and β-lactamase II were from stages 4B and 5A respectively (see Table 1). Values given for β-lactamase IIp are those of Kuwabara & Lloyd (1971) for the protein moiety obtained from a β-lactamase II–carbohydrate complex.

	Amino acid residues				
	In β-lactamase I		In β-lactamase II		In β-lactamase IIp
Amino acid	(per mol)	(%)	(per mol)	(%)	(per mol)
Asx	30	12.05	25	12.35	23
Thr	16	6.43	13	6.43	14
Ser	13	5.30	10	5.11	11
Glx	25	9.69	18	9.26	18
Pro	14	5.51	8	3.85	8
Gly	18	7.17	18	8.81	18
Ala	27	10.53	10	4.84	15
Val	16	6.34	22	11.27	15
Cys			1	0.53	1
Met	3	1.27	2	0.76	2
Ile	18	7.27	11	5.57	13
Leu	17	6.76	22	11.20	*
Tyr	8	3.10	4	2.15	5
Phe	6	2.21	3	1.65	4
His	3	1.08	5	2.40	4
Lys	23	9.04	19	9.41	16
Arg	12	4.59	4	2.19	4
Trp	4	1.66	5	2.32	2
Mol.wt.	28000		22000		22500

* No value reported.

Table 3. *Amino acid analysis of β-lactamase I and its component forms*

The first column gives values obtained for β-lactamase I at stage 4B of purification (Table 1). The other columns give the compositions of β-lactamase I components, separated by isoelectric focusing. Cysteine and tryptophan are not included.

	Amount of amino acid (residues/100 residues) in:			
		Components with pI:		
Amino acid	β-Lactamase I	9.20	9.47	9.68
Asx	12.06	12.52	12.10	11.79
Thr	6.44	6.77	6.22	6.09
Ser	5.31	4.26	3.75	3.63
Glx	9.70	8.66	9.47	9.39
Pro	5.52	6.28	6.31	5.63
Gly	7.18	7.57	7.28	6.76
Ala	10.54	10.25	10.11	10.21
Val	6.35	5.20	5.59	5.86
Met	1.27	1.22	1.43	1.74
Ile	7.28	8.45	8,23	8.75
Leu	6.77	6.49	6.63	7.02
Tyr	3.10	2.69	2.61	2.70
Phe	2.21	2.42	2.53	2.64
His	1.08	1.39	1.51	1.32
Lys	9.05	10.12	10.56	10.61
Arg	4.60	5.71	5.65	5.83

Determinations, from Lineweaver–Burk plots, of Michaelis constants for β-lactamase II with several cephalosporins as substrates gave the following values: cephalosporin C, 0.22mM; Cephalothin 0.11mM; Cephaloridine, 0.40mM; 7-aminocephalosporanic acid, 0.17mM. The values for cephalosporin C, Cephalothin and Cephaloridine are between five- and ten-fold lower than those reported previously for these compounds with the β-lactamase II–carbohydrate complex (Kuwabara & Abraham, 1967).

The rate of hydrolysis of benzylpenicillin (4mM) by β-lactamase I or II was not inhibited to the extent of 1% in the presence of approximately equimolar amounts of either desthiobenzylpenicillin, phenoxymethyl-6-α-methylpenicillin, or 7-phenoxyacetamido-7-α-methyldesacetoxycephalosporanic acid. Hence with benzylpenicillin as substrate and these β-lactamase-resistant compounds as potential inhibitors, $K_i/K_m > 95$.

Discussion

One or more of several factors may have been responsible for the earlier failures to recover β-lactamase II after its adsorption on to powdered glass or Celite, under conditions suitable for the purification

of β-lactamase I. The earlier procedures differed from that described here in that adsorption was carried out at pH4.5 (Miller *et al.*, 1965; Kuwabara, 1970), elution was attempted with buffers at pH8.0–8.5 (Kogut *et al.*, 1956; Miller *et al.*, 1965; Kuwabara, 1970; Sabath & Abraham, 1965) and/or the buffers used for elution contained phosphate (Kogut *et al.*, 1956; Miller *et al.*, 1965; Sabath & Abraham, 1965). β-Lactamase II is less stable than β-lactamase I at pH4.0. The insolubility of zinc phosphate may have led to a loss of the Zn^{2+} cofactor required by β-lactamase II and its subsequent irreversible inactivation. The use of the present method, in which both adsorption and elution were carried out at pH7.0 and the eluent contained only Tris–Zn^{2+}–citrate buffer and NaCl, resulted in the recovery of β-lactamase II, as well as β-lactamase I, in good yield under conditions suitable for large-scale preparation.

Kogut *et al.* (1956) reported that the isoelectric point of penicillinase (β-lactamase I) from *B. cereus* 569/H was slightly below pH5.5. The results of gel electrophoresis suggested that the isoelectric points of both β-lactamases I and II were, in fact, above pH7.0 and isoelectric focusing has shown that they lie between pH8 and pH10 in the solutions used. This is consistent with the finding that both enzymes are retarded, at pH7.0, on a column of carboxymethyl-Sephadex. On chromatography under these conditions most of the β-lactamase I emerged from the column uncontaminated by β-lactamase II, but the latter (with the lower isoelectric point) emerged first in a band containing a small proportion of β-lactamase I. However, since β-lactamase I could be selectively and quantitatively precipitated from solution by heat-treatment, a preparation of β-lactamase II free from β-lactamase I was readily obtained.

The amino acid composition of the penicillinase (β-lactamase I) from *B. cereus* has been determined previously by Jacob (reported by Citri & Pollock, 1966), Kuwabara (1970), Imsande *et al.* (1970) and Madaiah & Day (1971). The values given here (Table 2) for residues of amino acids/mol of protein lie, with minor exceptions, within the range of values reported earlier. However, with several amino acids, including serine, methionine, histidine, tyrosine and tryptophan, not all the determinations are in close agreement.

The separation of β-lactamase I into three main components by isoelectric focusing is consistent with the finding of Imsande *et al.* (1970) that a preparation of penicillinase from *B. cereus* 569 was resolved into three components by electrophoresis at pH9.2 in polyacrylamide gel. The failure to separate these components by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis and by gel filtration through Sephadex G-75 indicates that they have the same, or very similar, molecular weights. The similarities in their amino acid composition (Table 3) would be consistent with the hypothesis that the differences in their isoelectric points are associated with differences in their content of glutamine and/or asparagine residues.

The β-lactamase II isolated by the method described here differs from that prepared by Kuwabara (1970) in containing no carbohydrate moiety. The results of analyses of total hexose and of β-lactamase II activity at different stages of the purification process (Table 1) are consistent with the suggestion that a carbohydrate moiety was removed from the enzyme during adsorption on and/or elution from Celite.

In its Zn^{2+}-requirement, its substrate profile, its molecular weight and in much of its amino acid composition, the preparation of β-lactamase II obtained after elution from Celite resembled the protein moiety (IIp) obtained by Kuwabara & Lloyd (1971) from a purified protein–carbohydrate complex. However, the IIp protein of Kuwabara & Lloyd (1971) was reported to be thermolabile, like β-lactamase I, but unlike the protein–carbohydrate complex of Kuwabara (1970), or the β-lactamase II described here. The reason for this difference has not been established, but the present work has shown clearly that the relative stability of β-lactamase II to heat is not dependent on the presence of a carbohydrate moiety.

The presence of one cysteine residue in β-lactamase II, which seemed likely from the observations of Sabath & Finland (1968) and Kuwabara *et al.* (1970), has now been firmly established. It has also been confirmed that cysteine is absent from β-lactamase I, as it was reported to be by Imsande *et al.* (1970) and by Madaiah & Day (1971) from the penicillinase of *B. cereus* 569 and 569/H respectively. The finding of Kuwabara *et al.* (1970) that hydrolysis of oxidized β-lactamase I yielded less than 1 mol of a substance which behaved like cysteic acid/mol in the amino acid analyser can now be accounted for by the formation of small amounts of homocysteic acid under these conditions. This amino acid was obtained from each of the two components of β-lactamase I which had been separated by chromatography on Sephadex CM-50. Its origin is uncertain. Methionine yields homocystine on treatment with hot 9M-H_2SO_4 (Butz & du Vigneaud, 1932) and homocysteine thiolactone in hot conc. HI (Baernstein, 1934), whereas homocystine and homocysteic acid are found among the products of hydrolysis of methionine sulphoxide with hot 6M-HCl (Floyd *et al.*, 1963). However, no detectable amount of homocysteic acid was obtained from free methionine or from β-lactamase II under the conditions which led to its formation from β-lactamase I.

From the values obtained for the molecular weights and the lysine and arginine contents of β-lactamases I and II, the maximum numbers of peptides expected

to be formed from these enzymes on complete tryptic digestion are 36 and 24 respectively. The calculated maximum number for β-lactamase I is close to the number of peptides counted on tryptic-digest 'maps' (34) and to the numbers reported by Imsande *et al.* (1970) (35) and by Madaiah & Day (1971) (34). The calculated number for β-lactamase II is lower than the number found (31), possibly because hydrolysis was incomplete. If β-lactamase II were completely homologous with part of β-lactamase I, the calculated maximum number of peptides from a mixture of digests of the two enzymes would be 36, whereas, if there were no homology, the calculated number would be 60 and the number expected from those counted in the separate digests would be 65. The number of peptides counted in the mixture (47) appears to allow some degree of homology, but conclusions on the basis of the numbers of peptides detected in tryptic digests may be misleading (Harris & Hindley, 1965). In any event, the 'maps' do not suggest that the amino acid sequence of β-lactamase I can be represented by the sequence of β-lactamase II plus that of an additional peptide fragment. The latter possibility is also inconsistent with the relative amino acid compositions of the two enzymes. Thus β-lactamase II contains not only a cysteine residue which is absent from β-lactamase I, but also a larger number of residues of valine, leucine, histidine and tryptophan/mol. It appears that the synthesis of these two β-lactamases by the same organism is mediated by two different structural genes.

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