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# THE PURIFICATION AND SOME PROPERTIES OF A β-LACTAMASE SENSITIVE TO INHIBITION BY *p*-CHLOROMERCURIBENZOATE

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SUMMARY

1 The β-lactamase from *Aerobacter cloacae* 53 has been purified and its activity measured against a range of substrates

2 The molecular weight of the enzyme is of the order of 24 000 and it has a net positive charge at pH 8.5

3 Amino acid analysis of the enzyme indicates the presence of two or three cysteine residues per molecule

4 Study of the SH groups suggest that both are deeply buried in the molecule

## INTRODUCTION

A wide range of types of β-lactamase is to be found among enteric bacteria. They range from enzymes that are predominantly penicillinases to those that primarily hydrolyse cephalosporins. These enzymes have been classified into a number of types[1,2] as follows:

Type 1. Enzymes active predominantly against cephalosporins. These enzymes are inhibited by cloxacillin but not by *p*-chloromercuribenzoate (PCMB).

Type 2. Enzymes active predominantly against penicillins. These enzymes are inhibited by cloxacillin but not by PCMB.

Type 3. Enzymes with a broad penicillinase/cephalosprinase profile which are inhibited by cloxacillin but not by PCMB.

Type 4. Enzymes with similar profiles to those of Type 3 but resistant to cloxacillin inhibition and sensitive to PCMB.

Examples of a number of these types of β-lactamase have been purified and their properties studied. These include the enzyme synthesised by strains of *Escherichia coli* carrying the R-factor $R_{TEM}$ (ref. 3), a Type 3 enzyme, the "cephalosporinase" synthesised by a strain of *Enterobacter cloacae*[4], a Type 1 enzyme, the "cephalosporinase" mediated by the ampicillin-resistance gene *amp*. A in strains of *E. coli*[5], a further Type 1 enzyme, and the enzyme from a strain of *E. coli* carrying the R-factor

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Abbreviations: PCMB, *p*-chloromercuribenzoate; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

$R_{GN238}$ (ref 6) The enzymes specified by the ampicillin-resistance genes on the R-factors R1 and $R_{GN14}$, which have been purified by LINDQUIST AND NORDSTROM[7] and YAMAGISHI *et al* [6], respectively, are probably identical with the enzyme specified by the R-factor $R_{TEM}$

None of the enzymes yet purified falls into the class sensitive to inhibition by PCMB and able to hydrolyse cloxacillin All data published so far on the amino acid composition of β-lactamases[8–10], whether from Gram-positive or Gram-negative bacteria, indicate that cysteine is absent from these enzymes This paper describes the purification of a β-lactamase sensitive to inhibition by PCMB, presumptive evidence for the presence of an SH group

## MATERIALS AND METHODS

### *Organism and medium*

Purification of the β-lactamase has been developed using *Aerobacter cloacae* strain 53 (ref 11) obtained from Dr J T Smith (Dept of Pharmaceutics, School of Pharmacy, London WC1) The organism was grown in 1% CY medium[12]

### *Column materials*

CM-cellulose powder (CM11) was obtained from Whatman Products, Reeve Angel, London Sephadex G-75 was obtained from Pharmacia, Uppsala, Sweden

### *Growth of organisms*

The organism was grown in 35-l batch culture from an inoculum of 3 l grown overnight at 37° with shaking in 1% CY medium The inoculum was added to 32 l fresh 1% CY medium containing 0 16% glucose, the glucose content being reduced to minimise mucus production by the organism The culture was grown for 5 h in a converted washing machine at 37°, foaming being controlled by the addition of 5 ml, M S Antifoam Emulsion F G (Midland Silicones, Barry, Glam ) The culture was harvested using a Sharples continuous flow centrifuge (Sharples Instruments, Camberley, Surrey) and the organisms stored at −20° until required

### *Enzyme assays*

β-Lactamase assays, substrate profile determinations and inhibition studies with PCMB, cloxacillin and carbenicillin were carried out as described previously[1]

### *Preparation of chromatography and Sephadex columns*

CM-cellulose was suspended in water and the fines removed It was then washed with 0 1 M $K_2HPO_4$–$KH_2PO_4$ buffer (pH 5 9), 400 ml per 50 g cellulose Excess phosphate was then washed out with water, till no phosphate could be detected in the washings

Sephadex was prepared as described in the manufacturer's booklet Columns were poured in water, then equilibrated against the appropriate buffer

### *Starch-gel electrophoresis*

The separation and detection of β-lactamases in starch-gels at pH 8 5 was performed as described previously[1]

*Amino acid analysis*

A 1-mg sample of purified β-lactamase was performic acid oxidised[13] prior to acid hydrolysis in 50% HCl at 105° for 24 h The HCl was removed in an evacuated desiccator at room temperature[14] Hydrolysed protein was analysed using a Technicon Autoanalyser[15]

## RESULTS

*Purification procedure*

An outline of the purification procedure and recovery after each step are shown in Table I Preliminary experiments showed that addition of 2-mercaptoethanol to

TABLE I

SUMMARY OF THE PURIFICATION OF THE β-LACTAMASE FROM *A cloacae* 53

For experimental detail see text The starting material was 35 l of culture containing about 140 g wet wt of organism, which yielded about 3 mg pure enzyme

| *Step* | *Procedure* | *Enzyme activity recovered (units)* | *Specific enzyme activity (units/μg protein)* | *Volume (ml)* | *Total protein* | *Recovery (%)* |
|---|---|---|---|---|---|---|
| 1 | Ultrasonic disruption | $2\ 3\ 10^6$ | 0 35 | 400 | 6 6 g | |
| 2 | Treatment with DNAase | $3\ 40\ 10^6$ | 0 31 | 575 | 11 g | 100 |
| 3 | Centrifugation and dialysis | $3\ 35\ 10^6$ | 1 3 | 600 | 2 6 g | 98 5 |
| 4 | Chromatography on CM-cellulose | $1\ 88\ 10^6$ | 10 1 | 204 | 186 mg | 55 |
| 5 | Sephadex G-75, 1st column | $1\ 26\ 10^6$ | 113 | 16 | 11 mg | 37 |
| | Sephadex G-75, 2nd column | $6\ 27\ 10^5$ | 240 | 6 | 2 6 mg | 18 5 |

buffer solution improved enzyme recovery in the course of purification and was therefore added, at 1 mM final concentration, to all buffer solutions unless stated otherwise All manipulations were carried out at 4°

*Step 1 Ultrasonic disruption*

The frozen organisms were thawed and resuspended to a density of about 100 mg/ml in 0 1 M phosphate buffer (pH 5 9) The organisms were disrupted with a 100 W Dawe Soniprobe (Dawe Instruments, London, Type 1130) at 20 kcycles/sec in a continuous flow cell The bacterial suspension was pumped through the cell at 6 ml/min and subjected to the maximum output of the disintegrator (6 A) The mean residence time for each bacterium in the flow cell was about 1 min After disruption the preparation was stored until required for Step 2

*Step 2 Treatment with deoxyribonuclease*

The disrupted cell suspension (400 ml) was treated with 100 μg deoxyribonuclease (Sigma Chemical Co , Deoxyribonuclease I, 1 × crystallised from beef pancreas) for 15 min to reduce viscosity then centrifuged at 15 000 × *g* for 20 min The supernatant was retained until required for Step 3 while the pellet was resuspended in half the original volume of 0 1 M phosphate buffer (pH 5 9) and disrupted once more, this time at a flow rate of 8 ml/min This preparation was centrifuged at 15 000 × *g* for 20 min and the supernatant combined with that obtained previously

*Step 3 High speed centrifugation and dialysis*

The pooled supernatants from Step 2 (575 ml) were centrifuged at 35 000 × *g* for 2 5 h and the pellet discarded The supernatant was dialysed overnight against 20 vol distilled water Mercaptoethanol was omitted from the dialysis system since it may interfere with the subsequent chromatography After dialysis, the non-diffusible material was centrifuged at 35 000 × *g* for 2 5 h to remove the small quantity of precipitate

*Step 4 Chromatography on CM-cellulose*

The material from Step 3 was absorbed with CM-cellulose, prepared as described in MATERIALS AND METHODS The cellulose was added stepwise till less than 10% of the initial β-lactamase activity remained unbound to the cellulose In a typical experiment 50 g of CM-cellulose were added to 450 ml of enzyme preparation in three steps over 2 h The cellulose, with absorbed enzyme, was poured as a column (a typical example was 2 9 cm × 40 cm) and washed with 1 l of distilled water Much material, but less than 10% of the enzyme activity was eluted from the column by this procedure The cellulose was then eluted with a gradient of phosphate buffer (pH 5 9) lacking mercaptoethanol When the column was 2 9 cm × 40 cm, the gradient was formed from 300 ml each of 10 mM and 0 5 M buffer Fractions (6 ml) were collected and assayed for enzyme activity and protein content as measured by absorption at 280 nm Fig 1 showed a typical elution profile About 80% of the enzyme activity recovered was found in 34 fractions (204 ml) while they contained only about 5% of the total protein added Fractions containing enzyme at a specific activity greater than 4 units/μg protein were pooled, dialysed overnight against distilled water and the non-diffusible material freeze-dried

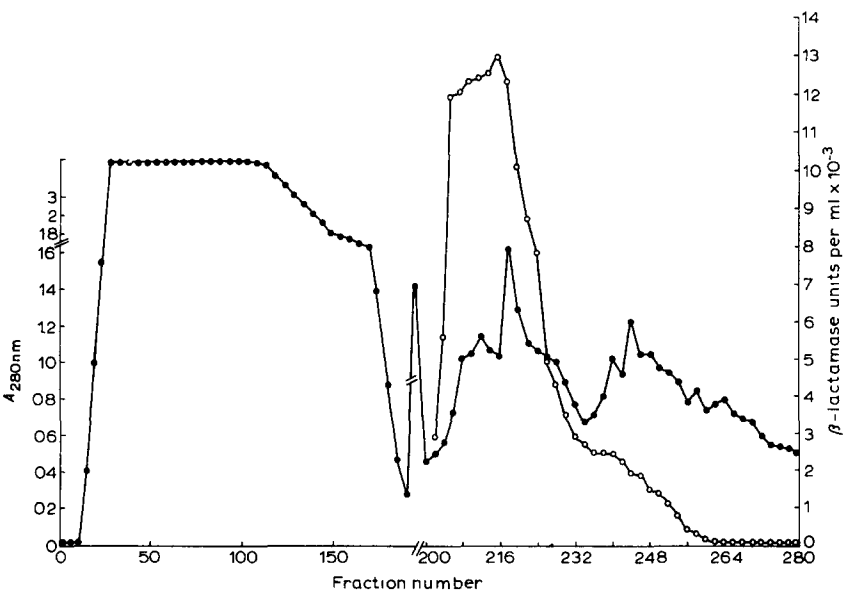


Fig 1 Chromatography of *A cloacae* 53 β-lactamase on CM-cellulose (2 5 cm × 40 cm) Fractions, 6 ml, were collected and assayed for enzyme activity (○) and protein content (●) as $A_{280\ nm}$ The gradient elution commenced at Fraction 180

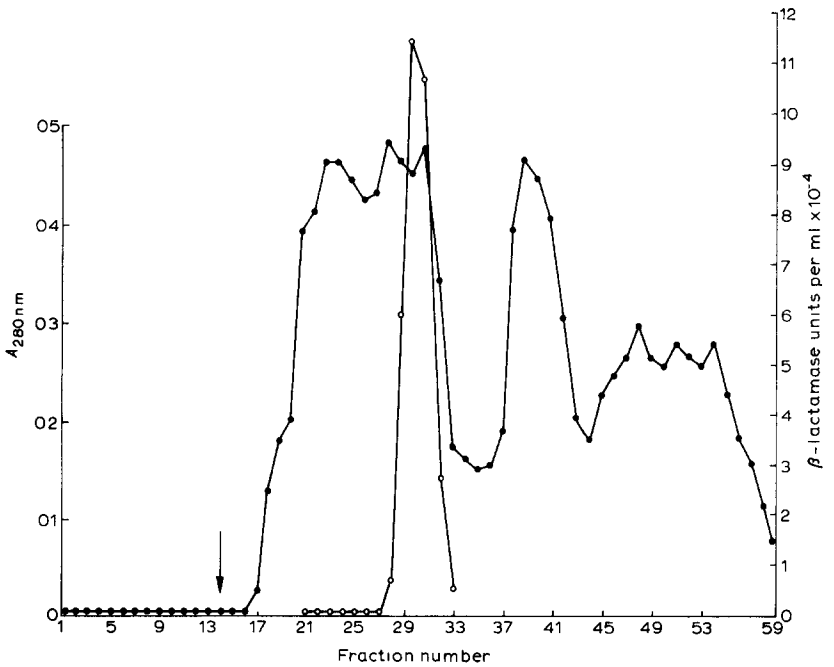


Fig 2 Separation of *A cloacae* 53β-lactamase through Sephadex G-75 (1 5 cm × 140 cm) equilibrated against 0 1 M phosphate buffer (pH 5 9) containing 1 mM 2-mercaptoethanol Fractions, 4 ml, were collected and assayed for enzyme activity (○) and protein content (●) as $A_{280\,nm}$ The arrow indicates the void volume of the column

*Step 5 Filtration through Sephadex G-75*

The freeze-dried material from Step 4 was dissolved in 4 ml 0 1 M phosphate buffer (pH 5 9), and loaded on a Sephadex G-75 column (1 5 cm × 140 cm) equilibrated against buffer of the same composition The column was eluted with similar buffer and fractions (4 ml) collected Fig 2 shows a typical elution pattern About 95% of the recovered activity was found in 4 fractions (16 ml total) and all had a specific activity of greater than 100 enzyme units/μg protein The pooled fractions were dialysed overnight against 40 vol distilled water and the non-diffusible material freeze-dried

The freeze-dried material was dissolved in 0 1 M phosphate buffer (pH 5 9) and re-run on the Sephadex G-75 column For this second run, the material was added in a total volume of 2 ml and fractions of 2 ml were collected Fig 3 shows the elution pattern obtained About 60% of the enzyme activity applied to the column was found in 3 fractions of specific activities 223, 262 and 234 enzyme units/μg protein, the constancy of these values suggesting that the enzyme eluted is of reasonable purity

The fractions were pooled and the purity of the product checked by starch-gel electrophoresis at pH 8 5 of 60 μg of material A single protein band was detected with a mobility identical to that observed for enzyme activity

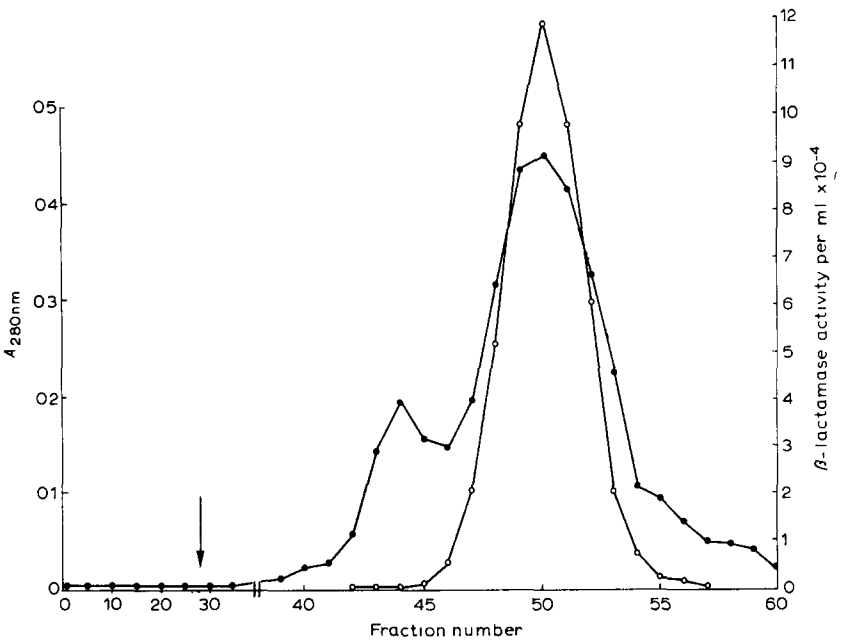


Fig 3 Re-run of *A cloacae* 53 β-lactamase through Sephadex G-75 (1 5 cm × 140 cm) equilibrated against o 1 M phosphate buffer (pH 5 9) containing 1 mM 2-mercaptoethanol Fractions, 2 ml, were assayed for enzyme activity (○) and protein content (●) as $A_{280\ nm}$ The arrow indicates the void volume of the column

*Properties of the enzyme*

*Substrate profile and susceptibility to inhibitors*

The substrate profile of the purified enzyme was determined against benzylpenicillin, ampicillin, cephaloridine, cephalexin, cloxacillin and carbenicillin as substrates (Table II) The enzyme had an approximately similar activity against benzylpenicillin, ampicillin and cephaloridine, was only about 10% as active against cloxacillin and carbenicillin and showed little detectable hydrolysis of cephalexin The enzyme is unusual among β-lactamases from Gram-negative bacteria in that it

TABLE II

THE SUBSTRATE PROFILE AND SUSCEPTIBILITY TO INHIBITORS OF *A cloacae* 53 β-LACTAMASE

All substrates were assayed at the same concentration, 6 mM, the rate of hydrolysis being expressed relative to an arbitrary set value of 100 for benzylpenicillin hydrolysis

| *Substrate* | *Relative rate of hydrolysis* |
|---|---|
| Benzylpenicillin | 100 |
| Ampicillin | 120 |
| Cephaloridine | 150 |
| Cloxacillin | 8 |
| Carbenicillin | 13 |
| Cephalexin | 0 |

can hydrolyse cloxacillin at a detectable rate[1] Normally such enzymes are inhibited by cloxacillin either competitively[16], or exceptionally, non-competitively[17]

Hydrolysis of cephaloridine (and probably also benzylpenicillin and ampicillin) by certain β-lactamases from Gram-negative bacteria is strongly inhibited competitively by cloxacillin[1] Hydrolysis of $10^{-4}$ M cephaloridine by the purified β-lactamases from *A cloacae* 53 was unaffected by the presence of either $10^{-4}$ M cloxacillin or $10^{-4}$ M carbenicillin Hydrolysis of cephaloridine in the presence of potential inhibitors was measured spectrophotometrically at 260 nm (ref 18)

*pH versus activity relationship*

The pH-activity curve of the enzyme was determined over a range of pH values

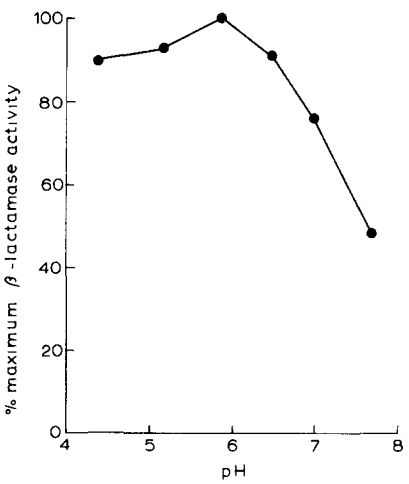


Fig 4 pH-activity curve of the purified β-lactamase from *A cloacae* 53 Sodium acetate buffers were used for the range pH 4 4–5 2 and $KH_2PO_4$–$K_2HPO_4$ buffers for the range pH 5 9–7 7 all buffers 0 1 M

from 4 4 to 7 7 using various buffer mixtures Fig 4 shows that the enzyme has a broad maximum activity over the pH range 5 2–6 5 with an optimum at pH 5 9 The enzyme retains over 80% of its activity at pH 4 4

*Molecular weight*

The molecular weight of the enzyme was determined by comparing its rate of filtration through Sephadex G-75 with the behaviour of proteins of known molecular weight on the same column The comparison was made against cytochrome *c* (mol wt 12 400), ribonuclease (13 700), myoglobin (17 800), chymotrypsinogen (25 000) and ovalbumin (45 000) This method gave a value of about 24 000 for the molecular weight of the β-lactamase

*Amino acid analysis*

Two samples, each 0 2 mg, of performic acid oxidised and hydrolysed β-lactamase were analysed for amino acid content The results were too variable to allow reliable conclusions to be drawn but confirmed the presence of 2 or 3 residues of cysteine per molecule This result supports the suggestion of SMITH[11] that the enzyme contains cysteine, deduced from inhibition studies with thiol reagents

*Reactions with chromophoric thiol reagents*

A freeze-dried sample of purified β-lactamase was dissolved in 2 ml 5 mM EDTA (pH 7 0) and treated overnight with 1 mM 2-mercaptoethanol to reduce any SH groups present capable of reduction under mild conditions The 2-mercaptoethanol was removed by gel-filtration on Sephadex G-25 to give an enzyme solution containing 0 1 mg/ml in 5 mM EDTA (pH 7 0)

Binding studies were carried out at 4° using the apparatus described by McMURRARY AND TRENTHAM[19] A sample of the β-lactamase, 0 05 mg, in saturated urea liberated 3 78 nmoles of 3-carboxy-4-nitrothiophenate after reaction with 5 μl 5 mM 5,5'-dithiobis-(2-nitrobenzoic acid), DTNB (ref 20) If the β-lactamase contains two thiols per monomer, this quantity of product corresponds to a molecular weight of 26 000

The native enzyme (0 03 mg/ml) showed no reactive groups on reaction for up to 50 sec with DTNB ($5\ 10^{-4}$ M) or 2,6-dichloromercuri-4-nitrophenol ($5\ 10^{-4}$ M) in 5 mM EDTA (pH 7) in the stop-flow apparatus described by McMURRARY AND TRENTHAM[19]

The time course of the reaction of the native enzyme, 0 01 mg/ml with 0 5 mM PCMB was followed by assaying residual enzyme activity iodometrically following incubation of the enzyme at 30° for up to 10 min Complete inhibition of the enzyme was only observed after 10 min pre-incubation, indicating a very slow rate of PCMB reaction, despite the high molar ratio of inhibitor to thiol groups in the enzyme

## DISCUSSION

The overall amino acid composition of the β-lactamase synthesised by *Aerobacter cloacae* strain 53 is very similar to that of other β-lactamases from enteric bacteria except that it appears to contain two cysteine residues/mole of enzyme whereas the other classes of enzyme lack this amino acid[10] These two cysteine residues presumably account for the relative sensitivity of this enzyme to sulphydryl reagents[1,11]

The fact that PCMB was capable of inactivating the enzyme suggested initially that one or both of the cysteine residues was part of the enzyme active centre, or at least close enough to impede the intercalation of enzyme and substrate The detailed kinetics reported here suggest, however, that the inactivation of the enzyme is due to denaturation, or at least to partial deformation, and that the –SH groups are only accessible after this has occurred Inactivation may follow the use of relatively high concentrations of PCMB ($5\ 10^{-4}$ M) for relatively long periods (10 min) at 30°, but pretreatment with 8 M urea is necessary for inhibition to occur with low concentrations of inhibitor for short periods, addition of substrate to the reaction provides no protection

The presence of cysteine residues in the penicillinase structure at a site away from the active centre, is easier to reconcile with current theories of penicillinase evolution than would have been the case if the residues were part of t substrate binding site The latter situation would have implied that a distinct type of β-lactam-hydrolysing site, using cysteine, had evolved among β-lactamases, a situation found in no other example from either Gram-positive or Gram-negative species The location of the cysteine residues away from the active centre may involve a less

fundamental difference Mutation in the $\beta$-lactamase structural gene may have led to the insertion of cysteine residues in place of other amino acids at two places deep in the enzyme structure, but these changes only produced minimal alterations in substrate profile (*cf* profiles of enzymes from Classes III and IV[2]) and the residues would be inaccessible to inhibitors High concentrations of sulphydryl reagents may disrupt the enzyme conformation sufficiently to allow alkylation of the cysteine sulphydryls to occur with consequent irreversible effects, both on enzyme conformation and activity

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