Purification and characterization of an extracellular β -lactamase produced by *Acinetobacter calcoaceticus*

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(Received 16 January 1992; accepted 11 February 1992)

A β -lactamase was purified 430-fold from the culture supernatant of *Acinetobacter calcoaceticus* by ion exchange chromatography on CM-Sephadex and affinity chromatography on phenylboronic-acid-agarose. The purified enzyme was homogeneous as judged by SDS-PAGE, and was characterized with respect to molecular mass (38 and 41 kDa by gel filtration on Sephadex G-75 and SDS-PAGE, respectively), pH optimum (pH 7.0), temperature optimum (45 °C) and isoelectric point (9.3). The β -lactamase showed mainly cephalosporinase activity. It was inhibited by cloxacillin, carbenicillin, penicillanic acid sulphone (sulbactam) and aztreonam. It was not inhibited by clavulanic acid up to a concentration of 0.25 mM. Neither EDTA nor *p*-chloromercuribenzoate, up to concentrations of 1 or 100 mM, respectively, affected activity. According to these characteristics, it is a typical CEP-N cephalosporinase.

Introduction

Acinetobacter calcoaceticus is an increasingly important cause of nosocomial infection (Bergogne-Bérézin *et al.*, 1987; Dijkshoorn *et al.*, 1987*a, b*, 1989), and one of the particular attributes of isolated strains is resistance to many antimicrobial agents, including β -lactam antibiotics. The resistance of these strains against β -lactams probably results from the production of β -lactamases (Joly-Guillou *et al.*, 1987, 1988). Joly-Guillou *et al.* (1988) screened one hundred clinical isolates of *A. calcoaceticus* for enzymic resistance to β -lactam antibiotics, and showed that 81% of the strains produced a β -lactamase. They observed penicillinases of the TEM and CARB type and also cephalosporinase activity.

TEM and CARB enzymes are plasmid-encoded and well-characterized (Medeiros, 1984). The exact nature and character of the presumed chromosomal cephalosporinases of *Acinetobacter* spp. is not fully clarified (Joly-Guillou *et al.*, 1987, 1988; Joly-Guillou & Bergogne-Bérézin, 1986; Hood & Amyes, 1989, 1991). There is general agreement that they belong to the Group 1 enzymes (CEP-N) of the classification of Bush (1989*a*) i.e., they are cephalosporinases not inhibited by clavulanic acid.

However, upon closer examination, Hood & Amyes

(1991) found considerable heterogeneity among the *Acinetobacter* cephalosporinases. On the basis of isoelectric focusing, molecular mass and biochemical properties, they described four different *Acinetobacter* Chromosomal Enzymes (ACEs) with molecular masses of up to 1000 kDa, and they envisaged an even more complex situation when all genospecies of *Acinetobacter* were investigated.

Furthermore, conflicting views exist with respect to the inducibility of these enzymes. Morohoshi & Saito (1977) and Medeiros (1984) found the chromosomal β -lactamases of *A. anitratum* to be inducible enzymes, whereas Bauernfeind (1986) described them as either inducible or constitutive. For all eight strains investigated by Hood & Amyes (1991) no induction was observed with cefoxitin as the inducer.

A wild-type *A. calcoaceticus* strain CCM 5593 was previously reported to be resistant to a great variety of penicillins and cephalosporins and to produce a cephalosporinase (Blechschiidt *et al.* 1989). The enzyme could be induced by various β -lactams, including ampicillin and imipenem. Best results with respect to enzyme yield were obtained with the inducer cefotaxime and long (24 h) cultivation times (Blechschiidt *et al.*, 1991). Under conditions of maximum enzyme production, more than 90% of the enzyme was found in the culture medium. This extracellular location is not due to cell lysis or periplasmic leakage, but could be attributed to a semi-

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selective export process across the outer cell membrane (Borneleit *et al.*, 1991). However, there might be another type of β -lactamase which is not inducible and which is retained within the cells.

In view of the multiple β -lactamases observed in *Acinetobacter* this study was performed to characterize and classify the β -lactamase exported into the extracellular medium by *A. calcoaceticus* CCM 5593.

Methods

Micro-organism. *Acinetobacter calcoaceticus* CCM 5593 was obtained from the Czechoslovak Collection of Micro-organisms, Brno, Czechoslovakia and used throughout this study.

Antibiotics. Antibiotics were obtained from commercial suppliers as follows: cephalosporin C, cephaloridine, carbenicillin, nafcillin, oxacillin, cloxacillin and cefuroxime from Sigma; penicillin G and cefalexin from Jenapharm; cefotiam (Halospor) and cefsulodine (Monaspor) from Ciba-Geigy; cephalothin from Eli Lilly; amoxycillin (Amoxypen) from Grünenthal; ampicillin from Serva; azlocillin (Securopen) from Bayer; cefotaxime (Claforan) from Hoechst; aztreonam from ICN. Clavulanic acid was used as a component of Augmentan (clavulanic acid/amoxycillin in a molar ratio of 1:2) (Smith Kline Beecham).

Culture conditions. *A. calcoaceticus* CCM 5593 was stored on nutrient agar I (Immunpräparate) at 4 °C. For β -lactamase production, a minimal salts medium (Kleber *et al.*, 1973) with 1% (w/v) sodium acetate, supplemented with 0.5% yeast extract was used. Cultivation was carried out in 500 ml Erlenmeyer flasks containing 100 ml medium. An induction culture was prepared by making a 1:25 dilution of an overnight culture in fresh medium and incubating it at 30 °C with shaking. Cefotaxime (50 μ g ml⁻¹) was added as inducer immediately after inoculation of the culture. After 24 h cultivation, the cells were harvested by centrifugation at 5000 g for 15 min at 4 °C.

Determination of β -lactamase activity. β -Lactamase activity was determined by a spectrophotometric method (Samuni, 1975; Waley, 1974) measuring the decrease in absorbance at an appropriate wavelength, and with a substrate concentration of 480 μ M at 20 °C (Specord M42; Carl Zeiss). The wavelength used was that which gave a maximum in the difference spectrum when a non-hydrolysed substrate was scanned against a hydrolysed one. The millimolar absorbancy difference was used to calculate the rate of hydrolysis. One unit of enzyme activity (U) is defined as the amount of enzyme that hydrolysed 1 μ mol substrate in 1 min at 20 °C in 100 mM-potassium phosphate buffer, pH 7.0.

Effects of temperature and pH on β -lactamase activity. Thermal stability and temperature optimum were determined in 100 mM-potassium phosphate buffer, pH 7.0. To investigate pH dependence, a 50 mM-glycylglycine/piperazine buffer (pH 4–10) was used. Activity was determined by the macro-iodometric assay of Perret (1954) with cefalexin (480 μ M) as substrate.

Purification of β -lactamase. Cell-free culture medium was dialysed against 20 mM-glycylglycine/piperazine buffer (pH 8.0) or 16 h at 4 °C. Then CM-Sephadex C-50 gel [10 g (1 medium)⁻¹] was added and the mixture was stirred carefully. After 90 min, the gel was removed by filtration, washed with buffer and packed into a column (20 $\times$ 2.5 cm). The column was eluted with a 0 to 1 M linear gradient of NaCl in

20 mM-glycylglycine/piperazine buffer, pH 8.0. Fractions (4 ml) were collected and assayed for β -lactamase activity. β -Lactamase positive fractions were pooled, concentrated to 10 ml by pressure filtration (Amicon PM 10 filter) and dialysed against 20 mM-triethanolamine hydrochloride (pH 7.0) containing 1 M-NaCl for 16 h at 4 °C.

For affinity chromatography, a boronic acid column with a hydrophilic spacer arm was prepared according to Cartwright & Waley (1984). The gel was washed with 200 ml NaCl/sorbitol (1 M/0.5 M; pH 7.0), 200 ml 0.5 M-sodium borate (pH 7.0) and finally 20 mM-triethanolamine hydrochloride (pH 7.0) containing 1 M-NaCl (loading buffer). Enzyme preparation was applied to the column and run at a flow rate of 30 ml h⁻¹. The column was washed with loading buffer until the A_{280} of washings was zero. The β -lactamase was eluted with 0.5 M-sodium borate (pH 7.0) containing 1 M-NaCl. For further use, β -lactamase was lyophilized and stored at -15 °C.

Determination of K_m and V_{max} . In order to calculate K_m and V_{max} , enzyme activities were measured in duplicate over a range of different substrate concentrations at different wavelengths: cephalothin (from 8–480 μ M; λ = 263.3 nm), cephalosporin C (from 8 to 480 μ M; λ = 263 nm), cefalexin (from 8 to 480 μ M; λ = 262.5 nm), cephaloridine (from 8 to 440 μ M; λ = 259.8 nm), cefotiam (from 8 to 480 μ M; λ = 276 nm), penicillin G (from 1 to 240 μ M; λ = 233 nm), ampicillin (from 1 to 240 μ M; λ = 235 nm), amoxycillin (from 1 to 240 μ M; λ = 243.2 nm) and azlocillin (from 1 to 240 μ M; λ = 231.5 nm). The reaction was started by adding enzyme to the substrate, which had been preincubated in 100 mM-potassium phosphate buffer, pH 7.0. Change of absorbance was recorded for 2 min to calculate the initial rate of hydrolysis at 20 °C.

Protein determination. Protein content was determined by the Lowry method with bovine serum albumin as the standard.

Results

Purification of β -lactamase

The β -lactamase liberated into the culture medium by *Acinetobacter calcoaceticus* CCM 5593 could be purified by a two-step procedure (Table 1). After dialysis of the cell-free culture supernatant against 0.02 M-glycylglycine/piperazine buffer (pH 8.5), within 90 min 80–90% of the enzyme bound to CM Sephadex C-50. After elution by a linear salt gradient the enzyme was purified 78-fold. A final purification of about five-fold was achieved by an affinity chromatography step using *m*-aminophenylboronic-acid-hemisulphate-modified Affi-Gel 10. The purified enzyme had a specific activity of 104.9 U (mg protein)⁻¹ and was homogeneous according to SDS-PAGE (Fig. 1).

Molecular mass and isoelectric point

The molecular mass of the purified β -lactamase was estimated to be 38 kDa by Sephadex G-75 gel filtration, and 41 kDa by SDS-PAGE (Fig. 1). The isoelectric point was 9.3 (Fig. 2).

Fig. 1. SDS-PAGE and molecular mass determination for purified β -lactamase. 25 μ g protein was dissolved in SDS (2%, w/v; 100 $^{\circ}$ C, 10 min). Electrophoresis was done using the Laemmli (1970) buffer system in 12.5% (w/v) polyacrylamide slab gels. Protein was stained with Coomassie Blue G-250. Molecular mass markers were phosphorylase B (92.5 kDa), bovine serum albumin (67 kDa), hen egg ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (soya bean) (21 kDa) and cytochrome *c* (13 kDa).

Effect of temperature and pH on the activity of the enzyme

Activity and stability at pH 7.0 were measured at different temperatures between 20 and 60 $^{\circ}$ C. The apparent temperature optimum was 45 $^{\circ}$ C. When the enzyme was incubated for 30 min at 55 $^{\circ}$ C, 70% of the activity determined after incubation at 45 $^{\circ}$ C was recovered.

Fig. 2. Isoelectric focusing of β -lactamase. (a) β -lactamase (1 and 0.25 μ g) and (b) standard marker proteins (5 μ g). Isoelectric focusing was performed in Phastgel (IEF 3-9) using the Phastsystem (Pharmacia). For separation and silver-staining, computer programs (Dombach, 1989) were used. Marker proteins (pI 3-10; Pharmacia): amyloglucosidase (3.5), trypsin inhibitor (soya bean) (4.55), β -lactoglobulin A (5.2), carbonic anhydrase B (bovine) (5.85), carbonic anhydrase B (human) (6.55), myoglobin, acid band (horse) (6.85), myoglobin, basic band (horse) (7.35), lectin, acid band (*Lens culinaris*) (8.15), lectin, medium band (*L. culinaris*) (8.45), lectin, basic band (*L. culinaris*) (8.65), trypsinogen (9.3).

The pH optimum was 7.0. The β -lactamase was inactive with cefalexin as the substrate at pH values below 4.3.

Table 1. Purification of the extracellular β -lactamase from *A. calcoaceticus* CCM 5593

Values are related to a volume of 2.5 l culture supernatant. β -Lactamase activity was determined spectrophotometrically with cefalexin (480 μ M) as substrate.

Step	Protein (mg)	Total activity (U)	Specific activity (U mg ⁻¹)	Yield (%)	Purification (-fold)*
1. Cell-free culture medium	8653	1610	0.186	100	—
2. Dialysis	6357	1550	0.244	96	1
3. CM-Sephadex C-50-Batch (pH 8.5)	60.2	1143	19.0	71	78
4. <i>m</i> -Aminophenylboronic-acid-hemisulphate-modified Affi-Gel 10	9.4	987	104.9	61	430

* Purification factors were related to dialysed culture supernatant because the yeast extract contained in the medium caused artificially high values in protein determination.

Table 2. Kinetic parameters of the β -lactamase from *A. calcoaceticus* CCM 5593 with various β -lactam substrates

β -Lactamase activity was determined spectrophotometrically. Michaelis constants (K_m) and V_{max} values were determined by Eadie-Hofstee plots.

Substrate	V_{max} (U mg protein ⁻¹)	K_m (μ M)	V_{max}/K_m
Cephalothin	655.0	267.1	2.45
Cephalosporin C	341.2	288.4	1.18
Cefalexin	191.5	399.7	0.48
Cephaloridine	79.3	204.6	0.39
Cefotiam	23.5	94.6	0.25
Penicillin G	18.7	3.1	6.04
Ampicillin	4.0	8.7	0.46
Amoxycillin	5.7	14.4	0.39
Azlocillin	2.7	14.6	0.14

Substrate specificity and kinetic parameters

The substrate profile of the purified enzyme is represented by the V_{max} and K_m values for various cephalosporins and penicillins listed in Table 2. The V_{max} values indicate that cephalosporins are generally hydrolysed more quickly than penicillins. For the newer cephalosporins cefsulodine and cefuroxime, no activity was determined. The same was found for the penicillins nafcillin, carbenicillin, oxacillin and cloxacillin. Cephalothin was hydrolysed best of the cephalosporins, and penicillin G, best of the penicillins. For the cephalosporins tested up to 550 μ M, no substrate inhibition was observed.

The K_m values reveal a higher affinity of penicillins for the enzyme than that of the cephalosporins. Furthermore, the ratio V_{max}/K_m indicates that under physiological conditions (no substrate saturation of the enzyme) penicillins are hydrolysed comparably to cephalosporins. The best substrate under these conditions should be penicillin G.

β -Lactamase inhibition

Both cloxacillin and carbenicillin were effective in inhibiting the hydrolysis of the most susceptible substrate cephalothin. The inhibitor constants estimated by Dixon plots were $K_i=0.074 \mu$ M for cloxacillin and $K_i=0.135 \mu$ M for carbenicillin (Fig. 3). In the corresponding Lineweaver-Burk plots (not shown) an intercept of the regression lines at the ordinate indicated competitive inhibition.

Sulbactam exerts its inhibitory effect in a progressive manner. A preincubation time of the enzyme with the inhibitor of about 180 min is needed to reach the full extent of inhibition (Fig. 4). From the initial rates obtained after this period an I_{50} value of 116 nM was determined.

Fig. 3. Inhibition of cephalothin hydrolysis by cloxacillin (a) and carbenicillin (b) (Dixon plots). Substrate concentrations: $\circ$, 15 μ M; $\bullet$, 30 μ M; $\square$, 60 μ M; $\blacksquare$, 120 μ M; $\triangle$, 240 μ M; and $\blacktriangle$, 480 μ M. β -Lactamase was assayed spectrophotometrically. Initial rates plotted were derived after a 1 min preincubation of the enzyme with substrate and inhibitor. A linear reaction course was obtained for a further 5 min recording period.

Fig. 4. Inhibition of cephalothin hydrolysis by sulbactam. β -Lactamase (0.01 mg ml⁻¹) was incubated with sulbactam at different concentrations: $\circ$, 0 nM; $\bullet$, 10 nM; $\square$, 50 nM; $\blacksquare$, 100 nM; $\triangle$, 200 nM; $\blacktriangle$, 400 nM; and ∇ , 800 nM. At times indicated, samples were withdrawn and β -lactamase activity was assayed spectrophotometrically (substrate: cephalothin, 480 μ M).

The inhibitory effect of aztreonam on cephalothin hydrolysis was fully established only after a preincubation period of 10 min. Under these conditions, I_{50} was 2.0 μ M.

Neither *p*-chloromercuribenzoate (pCMB) (0.1–100 mM) nor EDTA (0.05–1 mM) had a significant inhibitory effect on the purified β -lactamase.

Clavulanic acid at concentrations between 5 and 250 μ M did not inhibit amoxycillin hydrolysis.

Discussion

We purified and characterized a cephalosporinase produced as an extracellular enzyme by *A. calcoaceticus* CCM 5593. For the purification procedure, the key step was affinity chromatography on *m*-amino-phenyl-boronic-acid-hemisulphate-modified Affi-Gel 10. Cartwright & Waley (1984) found a perfect correlation between a β -lactamase being inhibited by boronic acids and its retention on boronic acid columns, supplementing the correlation between a β -lactamase being a serine enzyme and its inhibition by boronic acids. This suggests that the β -lactamase of *A. calcoaceticus* CCM 5593 may be regarded as a serine enzyme, and this characterization is supported by the fact that it is not inhibited by EDTA, which excludes the possibility of a metalloenzyme.

The β -lactamase from *A. calcoaceticus* CCM 5593 hydrolyses cephalosporins better than penicillins and therefore is to be designated a cephalosporinase. β -Lactamases of class I according to the Richmond & Sykes (1973) classification (R&S I β -lactamases) are also commonly referred to as cephalosporinases, since hydrolysis rates ($V_{\max}$) are often much larger for cephalosporins than for penicillins. However, this is rather a misnomer, since these enzymes, like the enzyme of *A. calcoaceticus* CCM 5593, are capable of hydrolysing certain penicillins with high efficiency ($V_{\max}/K_m$) (Richmond & Sykes, 1973). Even the more detailed examination of the substrate specificity of the enzyme purified from *A. calcoaceticus* CCM 5593 is in accordance with several reports for other R&S I enzymes, e.g. from *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Citrobacter freundii*, *Morganella morganii* (Sanders & Sanders, 1986), *Escherichia coli* (Sawai *et al.*, 1982), *Proteus rettgeri* (Ohya *et al.*, 1980) and *Serratia marcescens* (Yang *et al.*, 1990).

For the *Acinetobacter* cephalosporinase, the kinetics of hydrolysis for various β -lactam antibiotics have been described by Morohoshi & Saito (1977) and, subsequently, for some newer agents by Hikida *et al.* (1989). On the basis of these data, together with the inhibitor studies performed by these authors it was classified by Bush (1989 *b*) in Group 1, subtitled CEP-N. Group 1 cephalosporinases correlate primarily with the Sykes & Richmond classes Ia, Ib, and Id. They are almost exclusively chromosomally encoded and are often inducible. They are potently inhibited by cloxacillin and

aztreonam but poorly inhibited by clavulanic acid or sulbactam.

All these characteristics apply for the *A. calcoaceticus* CCM 5593 cephalosporinase with the exception of sulbactam inhibition. Similarly, Joly-Guillou *et al.* (1988) found inhibition by cloxacillin and sulbactam and resistance to clavulanic acid for various *Acinetobacter* strains which exhibited cephalosporinase activity only. However, while sulbactam sensitivity suggests a group 2c enzyme according to Bush (1989 *c*), these enzymes are usually inhibited by pCMB. Furthermore, sulbactam inhibition (K_i 3.8 μ M) was also reported for the Group 1 enzyme from *Citrobacter freundii* GN 7391 (Bush 1989 *c*).

The Group 1 enzymes usually have basic isoelectric points and molecular masses greater than 30 kDa. Joly-Guillou *et al.* (1987) described 30 strains of *Acinetobacter* with a chromosomal cephalosporinase of $pI > 8$. For 14 strains investigated by Medeiros *et al.* (1985), the pI ranged from 8.8 to > 10 . Among the 8 strains investigated by Hood & Amyes (1991), seven partially purified cephalosporinases did not focus on conventional isoelectric focusing systems, which was attributed to extremely basic isoelectric points together with exceptionally high molecular masses, which were found to be > 1000 kDa. Our data are more similar to the parameters reported by Morohoshi & Saito (1977) who determined a molar mass of 30 kDa, an optimal temperature of 40 °C and an optimal pH 7.25–7.50.

On the basis of their characterization of the cephalosporinases from 8 strains belonging to 3 different genospecies, Hood & Amyes (1991) postulated 4 different *Acinetobacter* Chromosomal Enzyme (ACE) subgroups, all of them belonging to the general group 1 β -lactamases. Among them, the subgroup 3 enzyme from *A. lwoffii* H 126 closely resembled the enzyme investigated in this study. A molecular mass of 32 500 Da, a pI of 9.1, and a K_m for cephaloridine of 380 μ M agree fairly well with our results. The I_{50} values for cloxacillin and aztreonam (0.003 and 0.08 μ M, respectively) are lower than those found for the *A. calcoaceticus* CCM 5593 enzyme. However, Hood & Amyes (1991) have already pointed out that the degree of heterogeneity among the *Acinetobacter* cephalosporinases might turn out to be even higher when the full spectrum of the 17 genospecies was investigated.

The authors acknowledge the financial support of the BMFT (project number 0319886A6) and would like to thank Janett Leischnig for expert technical assistance.

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