Citrobacter diversus ULA-27 β -lactamases

Improved purification and general properties

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Two chromosome-encoded β -lactamases have been purified from *Citrobacter diversus* ULA-27. They exhibited slightly different isoelectric points (6.8 and 6.2) and very similar M_r values (≈ 29000). Their specificity spectrum was rather wide, since they hydrolysed some cephalosporins with k_{cat} values similar to those observed with the best penicillin substrates. Cloxacillin, methicillin and imipenem were hydrolysed very slowly. Hydrolysis of azthreonam could not be detected.

INTRODUCTION

Chromosomal β -lactamases are responsible for an increased resistance towards β -lactam antibiotics in many Gram-negative bacterial strains. In recent years the emergence of this phenomenon has become a relevant clinical problem. Resistance is also displayed against recent β -lactams (third-generation cephalosporins, for instance), because some of those enzymes can hydrolyse these compounds.

We have recently isolated and described a resistant strain of *Citrobacter diversus* which produces two chromosomal β -lactamases and exhibits a high level of resistance towards many penicillins and cephalosporins (Oliva *et al.*, 1987). Since little is known about the *Citrobacter diversus* β -lactamases, we have studied the enzyme produced by that bacterial strain to understand better their role in the resistance of this strain to antibiotics.

In the present work we have purified these two enzymes to homogeneity and characterized some of their molecular properties and substrate specificity.

MATERIALS AND METHODS

Materials

Benzylpenicillin was from Rhône-Poulenc (Paris, France), ampicillin, cloxacillin and oxacillin from Bristol Benelux (Brussels, Belgium), carbenicillin and methicillin from Beecham Research Laboratories (Brentford, Middx., U.K.), nitrocefin and cefuroxime from Glaxo Group Research (Greenford, Middx., U.K.), cephaloridine, cefazolin, cephalothin and cephalosporin C from Eli Lilly and Co. (Minneapolis, MN, U.S.A.), azthreonam from the Squibb Institute for Medical Research (Princeton, NJ, U.S.A.) and imipenem from Merck, Sharp & Dohme Research Laboratories (Rahway, NJ, U.S.A.). All these compounds were kindly given by the respective companies. The chromogenic cephalosporin PADAC was purchased from Hoechst (Scoppito, L'Aquila, Italy).

Aminophenylboronic acid, agarose and CH-Sepharose-4B were from Sigma; Sephadex G-75, Polybuffer exchanger 94 and Polybuffer 74 were from Pharmacia (Uppsala, Sweden).

Acrylamide, bisacrylamide and ammonium persulphate were from Bio-Rad Laboratories (Richmond, CA, U.S.A.), Ampholines (PAG-Plate 3.5–9.5) and M_r standards were from LKB (Bromma, Sweden).

Organism

Citrobacter diversus strain ULA-27, a clinical isolate which is highly resistant to cephalosporins and penicillins, was kindly given by Dr. Brunello Oliva, University of L'Aquila, L'Aquila, Italy. It was grown in a Minimal A Medium and harvested as previously described (Oliva *et al.*, 1987).

Protein and enzyme-activity determination

Protein concentration was determined as described by Bradford (1976), with bovine serum albumin as standard, or by measuring the A_{280} of the solution. The enzyme activity for form I (pI 6.8) was assayed in a 0.1 M-sodium phosphate buffer, pH 7.5, whereas form II (pI 6.2) was assayed in a 0.1 M-sodium acetate/acetic acid buffer, at pH 5.5, the pH at which the enzyme exhibited 'optimum stability'. The hydrolysis rates of both enzymes against PADAC or cephaloridine were recorded by using a Perkin-Elmer 550S spectrophotometer, at 25 °C and choosing the appropriate wavelengths for each substrate.

One unit of enzyme activity is the amount of enzyme which hydrolyses 1 μ mol of cephaloridine/min at maximal velocity and at pH 7.0 and 25 °C.

Purification of the enzymes

Bacteria were collected by centrifugation at 16000 *g* for 30 min at 4 °C, and the pellet was washed three times (with 20 mM-phosphate/0.15 M-NaCl, pH 7.2) and re-suspended in the same buffer. Bacteria were disrupted by four 2 min cycles of sonication (Ultratip Lab-Line System; power 75 W) in an ice bath. After centrifugation at 105000 *g* for 1 h, the clarified supernatant was

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extensively dialysed against 20 mM-acetate/acetic acid buffer, pH 5.7. This crude extract was concentrated and applied to a Sephadex G-75 (2.5 cm × 120 cm) column previously equilibrated with the buffer used for the dialysis. The active fractions were pooled and submitted to affinity chromatography. Phenylboronic-agarose with a hydrophobic spacer arm was used as described by Cartwright & Waley (1984) for the purification of similar β -lactamases. After washing the unbound inactive proteins, a single active peak was eluted with 0.5 M-borate buffer, pH 7.0. This enzymic fraction was further separated into two active peaks by chromatofocusing, using a pH gradient from 7 to 4 as previously described (Amicosante *et al.*, 1987). Each enzymic form was eluted at a pH corresponding to its isoelectric point (determined by isoelectric focusing).

Determination of M_r

The M_r of the enzymes was determined by SDS/polyacrylamide-gel electrophoresis as described by Laemmli (1970). The standard mixture contained the following pure proteins: ovotransferrin (M_r 78 000), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (30 000) and myoglobin (17 200).

Isoelectric focusing

This was performed using a Multiphor LKB apparatus on PAG-Plates pH 3.5–10 as described previously (Oliva *et al.*, 1987).

Amino acid analysis

Samples were dried *in vacuo* in 50 mm × 6 mm (internal diameter) Pyrex tubes and hydrolysed with 200 μ l of 5.7 M-HCl (Pierce, Rockford, IL, U.S.A.), containing 1% (w/v) phenol, in a Pico-Tag Work Station (Waters). After 24 h at 112 °C, they were dried *in vacuo*, and dissolved in 10 μ l of ethanol/water/triethylamine (2:2:1, by vol.; Pierce) and dried again. Samples were then derivatized to phenylthioisocyno-amino acids by using ethanol/water/triethylamine/phenyl isothiocyanate (7:1:1:1, by vol.; Pierce), as described by Bidlingmeyer *et al.* (1984), with minor modifications.

Temperature- and pH-stability of β -lactamases

Each enzyme was preincubated in the appropriate buffer for different periods of time at various temperatures; the enzymic activities were thereafter determined spectrophotometrically at 25 °C using 100 μ M-cefazolin as substrate. The enzyme activity was tested at different pH values at 25 °C, either directly or after a 10 min preincubation under the same conditions except for the absence of substrate. The residual activity was determined as described above. The following buffers were used: 0.1 M-sodium phosphate (pH 6.0–8.0), 0.1 M-Tris/HCl (pH 8.0–9.0); 3 M-KCl was added to all buffers, as described by Waley (1975), in order to minimize ionic-strength differences.

Kinetic parameters for penicillins and cephalosporins

The experiments were performed at 30 °C in 25 mM-sodium phosphate, pH 7.2, containing 0.2 M-NaCl and bovine serum albumin (0.05 mg/ml). The values of K_m and k_{cat} were derived from complete time courses obtained with a Beckman DU8 spectrophotometer interfaced to an Apple II microcomputer. The curves were analysed as described by De Meester *et al.* (1987). The conditions were chosen so that the reaction was virtually complete within 2 min, in order to avoid problems due to the progressive inactivation of the enzyme.

RESULTS

Purification, M_r values and pI values

Table 1 summarizes the results of a typical purification. The overall yield (form I + form II) was about 37%, form I representing about 78% of the total. Fig. 1 shows the elution pattern from the affinity-chromatography column and Fig. 2 the separation of the two forms by chromatofocusing. Fig. 3 shows an SDS/polyacrylamide gel of samples at various purification stages. From those data, M_r values of $28\,500 \pm 500$ and $29\,000 \pm 500$ were found for form I and II respectively. Isoelectric focusing (Fig. 4) indicated pI values of 6.8 ± 0.05 and 6.2 ± 0.05 for forms I and II respectively. A titration curve, obtained

Table 1. Purification of β -lactamases from *Citrobacter diversus* ULA-27 strain

Starting material was obtained from 45 litres of *Citrobacter diversus* ULA-27 culture.

Step	Total protein (mg)	Specific enzyme activity* (units/mg)	Total enzyme activity (units)	Purification	Yield (%)
Ultrasonic disruption	3381	1.9	6424	1	100
High-speed centrifugation	759	8	6072	4	95
Sephadex G-75 chromatography	157	24	3768	13	59
Phenylboronic acid-Sepharose 4B chromatography	14	234	3276	123	51
Chromatofocusing					
Form I, pI 6.8	3.5	532	1841	280	29
Form II, pI 6.2	1.1	457	521	240	8

* Cephaloridine was used as substrate.

Fig. 1. Affinity-chromatography step

About 120 ml of Sephadex G-75 eluate (1.4 mg of protein/ml) were loaded on to a column of phenylboronic acid-Sepharose-4B. The experimental conditions were similar to those reported by Cartwright & Waley (1984). The Figure shows A_{280} (—) (optical pathlength 2.5 mm) and β -lactamase activity (---), which was measured using cephaloridine as substrate.

Fig. 2. Chromatofocusing

A column (2.5 cm $\times$ 1.0 cm internal diameter) was filled with PBE94 and then equilibrated with 0.025 M-imidazole/HCl, pH 7.4. The pH gradient from 7 to 4 was developed by Polybuffer PB74 titrated with HCl at pH 4.0. For further details, see the Materials and methods section and Amicosante *et al.* (1987). After monitoring A_{280} with an optical pathlength of 2.5 mm (—), the pH of each fraction (---), and the activity of each fraction (·-·-) with cephaloridine as substrate, was determined.

on PAG-plates 3.5–10 indicated that form II behaved more acidically throughout the pH range 4–9.

Amino acid analysis

In agreement with its higher pI, form I contained four additional basic residues when compared with form II. It was thus possible to calculate that about 23 out of the 52 Asx+Glx residues of form I were Glu or Asp, whereas 20 or 21 of the 51 Asx+Glx of form II were Glu or Asp (Table 2).

Stability of the enzymes

Both proteins were stable during a 10 min incubation over the pH range 5–8 and lost 80% of activity after 10 min at pH 4.0 and 60% after 10 min at pH 9.0. Moreover, over the pH range 4–9, the stability profiles for both forms were practically superimposable. The influence of the temperature of incubation on the first-order rate constant for the decrease of activity at pH 5.7 is given in Table 3.

Kinetic parameters for various penicillins and cephalosporins

The experimental conditions and calculated values are given in Table 4. The same K_m and k_{cat} values were obtained when various initial concentrations of substrate and enzyme were utilized, which indicated that Henri-Michaelis kinetics applied and that no progressive enzyme inactivation occurred during the experiments (Joris *et al.*, 1986).

Both forms hydrolysed cloxacillin slowly and, at a substrate concentration of 100 μM , the accumulation of product remained linear throughout the observed time course. From those data, only the k_{cat} value was calculated. No hydrolysis of azthreonam was recorded

Fig. 3. SDS/polyacrylamide-gel-electrophoretic analysis after different purification steps

A 5–20%-(w/v)-polyacrylamide gradient was used. The proteins were stained with Coomassie Blue R-250. The following samples were examined: A, crude sonicated bacterial sample (120 μg); B, active peak from Sephadex G-75 chromatography (70 μg); C, phenylboronic acid-Sepharose-4B eluate (10 μg); D and E, forms at pI 6.8 (D, 8 μg) and pI 6.2 (E, 8 μg) from chromatofocusing. On the left a mixture of standard proteins was run as described in the Materials and methods section.

Fig. 4. Isoelectric-focusing analysis

The following samples were focused: A, eluate from phenylboronic acid-Sephadex-4B chromatography (14 μ g); B, form I after chromatofocusing (8 μ g); C, form II after chromatofocusing (8 μ g); the proteins were stained with Coomassie Blue R-250.

under our experimental conditions. The compound was used as inhibitor with cefazolin as substrate. By assuming competitive inhibition, a K_i value was computed from V_0/V_i -against-[I] plots, where V_0 and V_i were the steady-state rates of cefazolin hydrolysis in the absence or presence of the inhibitor (I) respectively. Imipenem was slowly hydrolysed. The K_m value was determined in a competition experiment, as described above, with azthreonam. The k_{cat} value was obtained by monitoring the linear decrease of A_{300} at a substrate concentration corresponding to 8-fold the K_m value. With the two chromogenic cephalosporins, nitrocefin and PADAC, as well as cefotaxime, biphasic phenomena were observed. Whether this pattern can be interpreted in terms of substrate-induced inactivation requires further investigation.

DISCUSSION

The β -lactamases of *Citrobacter diversus* are poorly investigated, but their contribution to the antibiotic-resistance phenomenon is an emerging clinical problem, probably on account of the presence of both chromosomal- and plasmid-mediated enzymes. The present paper focuses on the enzymes produced by a clinical isolate of *Citrobacter diversus* ULA-27 (Oliva *et al.*, 1987). This strain produces two forms of β -lactamase with pI values of 6.8 and 6.2 respectively and a very similar M_r value of about 29000. We previously reported that both enzymes were sensitive to *p*-chloromercuribenzoate and iodine and were strongly inhibited by clavulanic acid (Amicosante *et al.*, 1987). Both enzymes have a very similar amino acid composition and exhibit similar behaviours upon inactivation by extreme pH values and high temperature. Both β -lactamases show a broad substrate specificity and can inactivate very efficiently various penicillins and cephalosporins. Cloxacillin, azthreonam and imipenem were not hydrolysed, or hydrolysed very slowly, and further work is necessary in

Table 2. Amino acid composition of *Citrobacter diversus* ULA-27 β -lactamases

The composition of both enzymes in terms of residues/molecule is calculated on the basis of an M_r of 28500.

Amino acid	Amino acid composition			
	β -Lactamase pI 6.8		β -Lactamase pI 6.2	
	Experimentally obtained	Nearest integer	Experimentally obtained	Nearest integer
Asp + Asn	21.0	21	20.6	21
Glu + Gln	30.9	31	30.0	30
Ser	16.0	16	19.6	20
Gly	57.7	58	62.4	62
His	1.7	2	2.6	3
Thr	39.8	40	40.6	41
Pro	5.9	6	5.6	6
Ala	47.7	48	47.8	48
Arg	9.7	10	7.7	8
Val	12.6	13	12.6	13
Met	3.7	4	3.6	4
Ile	6.6	7	7.0	7
Leu	20.9	21	15.6	16
Trp*	2.8	3	2.9	3
Phe	2.3	2	3.7	4
Lys	7.8	8	6.4	6
Tyr	2.9	3	4.9	5
Cys†	4.9	5	5.2	5
Total	298 (M_r 28810)		302 (M_r 28973)	

* Determined on the basis of A_{280} .

† After oxidation with performic acid.

Table 3. Influence of temperature on the first-order rate constant for the decrease of activity (k_m) at pH 5.7

Temperature (°C)	Form ...	$10^3 \times k_m$ (min ⁻¹)	
		pI 6.8	pI 6.2
20		1.5 $\pm$ 0.4	8.7 $\pm$ 1
30		9 $\pm$ 1.4	21.5 $\pm$ 5
40		24 $\pm$ 2	31 $\pm$ 4
50		67 $\pm$ 11	82 $\pm$ 30
60		> 230	105 $\pm$ 13

order to clarify whether the most stable intermediate is the Henri-Michaelis complex (ES) or the acyl enzyme (ES*). Several cysteine residues were found in both proteins, confirming that the inhibitory effect of low *p*-chloromercuribenzoate concentrations on the enzymic activity could be due to the presence of a cysteine residue at or near the active site. For this purpose, a further study should be performed in the hope of isolating the active-site peptide using a specific molecular probe such as β -iodopenicillanic acid. (De Meester *et al.*, 1986). Those experiments should permit the identification of the various residues at or near the active site and the determination of the degree of similarity with β -lactamases of classes A and C. The production of several

Table 4. Substrate profile of *C. diversus* β -lactamases

Substrate	Wave-length (nm)	$\Delta\epsilon$ ($M^{-1} \cdot s^{-1}$)	[S] (mM)	Quantity of enzyme (μg)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/k_m ($M^{-1} \cdot s^{-1}$)
Form I							
Benzylpenicillin	230	800 ± 100	0.2–1	0.07–0.3	69 ± 10	570 ± 30	$(8.3 \pm 0.8) \times 10^6$
Ampicillin	240	600 ± 100	0.2–0.6	0.2	$22 \pm 4^*$	119 ± 4	$(5.4 \pm 0.8) \times 10^6$
Oxacillin	230	500 ± 100	1–2	0.7–2	230 ± 30	206 ± 20	$(0.9 \pm 0.2) \times 10^6$
Cloxacillin	230	500 ± 100	0.1–0.4	0.7–7	< 100	0.04 ± 0.004	ND†
Carbenicillin	230	750 ± 100	0.5–1.0	0.7–7	100 ± 10	58 ± 2	$(0.6 \pm 0.1) \times 10^6$
Methicillin	230	1000 ± 100	$(20-40) \times 10^{-3}$	0.14	5.5 ± 0.6	$< 10^{-3}$	< 200
Cephaloridine	260	10000 ± 500	1.0–2.0	0.2–0.4	740 ± 70	930 ± 100	$(1.25 \pm 0.25) \times 10^6$
Cephalothin	273	6300 ± 600	0.1–0.16	0.2–0.4	48 ± 3	63 ± 3	$(1.3 \pm 0.15) \times 10^6$
Cephalosporin C	260	4700 ± 100	0.1–0.3	0.9–1.8	126 ± 20	41 ± 5	$(0.3 \pm 0.06) \times 10^6$
Cefazolin	273	6600 ± 300	0.07–0.14	0.07–0.2	20 ± 2	166 ± 5	$(8.3 \pm 1.0) \times 10^6$
Cefuroxime	273	7500 ± 500	0.05–0.2	0.2–0.4	70 ± 7	54 ± 4	$(0.77 \pm 0.13) \times 10^6$
Azthreonam	—	—	—	0.04	$4.2 \pm 0.5^*$	ND	ND
Imipenem	300	6000 ± 300	0.200	0.04	24 ± 2	ND	ND
				7.5	ND	0.016 ± 0.003	660 ± 200
Form II							
Benzylpenicillin	230	800 ± 100	0.2–1	0.27–0.8	44 ± 3	132 ± 14	$(3 \pm 0.5) \times 10^6$
Ampicillin	240	600 ± 100	0.2–0.6	0.3	$24 \pm 6^*$	55 ± 2	$(2.3 \pm 0.4) \times 10^6$
Oxacillin	230	500 ± 100	1–2	1.5–4	300 ± 20	101 ± 5	$(0.33 \pm 0.04) \times 10^6$
Cloxacillin	230	500 ± 100	0.1–0.4	0.16	< 100	$(24 \pm 3) \times 10^{-3}$	ND
Carbenicillin	230	750 ± 100	0.5–1.0	1.6–16	115 ± 10	29 ± 2	$(0.25 \pm 0.05) \times 10^6$
Methicillin	230	1000 ± 100	$(20-40) \times 10^{-3}$	0.36	$4.2 \pm 0.4^*$	$< 10^{-3}$	< 250
Cephaloridine	260	10000 ± 500	1.0–2.0	0.2–0.4	740 ± 70	930 ± 100	$(1.25 \pm 0.25) \times 10^6$
Cephalothin	273	6300 ± 600	0.1–0.16	0.3–0.4	41 ± 2	55 ± 6	$(1.30 \pm 0.20) \times 10^6$
Cephalosporin C	260	4700 ± 100	0.1–0.3	2–4	123 ± 13	23 ± 9	$(0.19 \pm 0.09) \times 10^6$
Cefazolin	273	6600 ± 300	0.07–0.14	0.08–0.2	27 ± 5	57 ± 6	$(2.10 \pm 0.60) \times 10^6$
Cefuroxime	273	7500 ± 500	0.05–0.2	0.4–0.8	72 ± 5	30 ± 3	$(0.40 \pm 0.06) \times 10^6$
Azthreonam	—	—	—	0.08	$6.7 \pm 0.7^*$	ND	ND
Imipenem	300	6000 ± 300	0.200	0.16	$27 \pm 5^*$	ND	ND
				8.0	ND	0.071 ± 0.003	410 ± 150

* Determined as a K_i value using 100 μM -cefazolin as substrate.

† Abbreviation: ND, not determined.

different β -lactamases by the same strain is not exceptional. For example, *Serratia marcescens* GN7643 synthesizes a penicillinase (probably class A) and a class C cephalosporinase (Tajima *et al.*, 1981; Joris *et al.*, 1986). *Bacillus cereus* 569/H produces a soluble class B Zn^{2+} metallo-enzyme (Davies *et al.*, 1974) and soluble and membrane-bound forms of two distinct class A enzymes (Nielsen & Lampen, 1983; Hussain *et al.*, 1987). Strains of *Klebsiella pneumoniae* manufacture three different β -lactamases, TEM-1, TLE-2 and SHV-1, coded for by the same plasmid, pUK702 (Reid *et al.*, 1987). The exact relationships between the three enzymes have not been established, although some similarities appear between the TEM-1 (class A) and the TLE-2 enzymes. 'Ragged' N-termini, probably due to a poor specificity of the signal peptidase, are also observed with some class A β -lactamases (Ambler, 1970; Dehottay *et al.*, 1987).

The two β -lactamases are produced constitutively by *Citrobacter diversus*. In both cases, the genetic information is chromosome-encoded. The general properties of both enzymes, such as their pI values, M_r values and substrate profiles, appear to be those of class

A β -lactamases. However, the presently available data do not permit us to decide whether the two enzymes are the products of different genes or if they correspond to different processings of the same precursor.

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