Purification and Properties of β -Lactamases from *Serratia marcescens*

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Both a penicillinase and a cephalosporinase were purified from a strain of *Serratia marcescens* (GN7647) resistant to β -lactam antibiotics. The penicillinase was identical to the type I penicillinase, mediated by Rms212 and R-TEM. The purified cephalosporinase, a typical chromosomally mediated enterobacterial β -lactamase, gave a single protein band on polyacrylamide gel electrophoresis and immunoelectrophoresis; the pI was 9.56 and its molecular weight was approximately 37000. It contained tryptophan but not cysteine. The specific activity was 374 units (mg protein)⁻¹ for the hydrolysis of cephaloridine, and the optimal pH was 8.5. Rabbit antisera raised against the purified cephalosporinase showed no cross-reaction in a neutralization test with cephalosporinases produced by other species of *Enterobacteriaceae*.

INTRODUCTION

β -Lactam antibiotics showed relatively poor activity against *Serratia marcescens* (Verbist, 1976). Many strains produce an inducible cephalosporinase which is considered to be the 'species-specific' β -lactamase (Sawai *et al.*, 1968). Ito *et al.* (1980) reported that many strains of *S. marcescens* possess a penicillinase mediated by a plasmid. Some properties of the β -lactamases from *S. marcescens* have been investigated (Tsang *et al.*, 1975; Farrar & O'Dell, 1976) and compared with β -lactamases of other bacteria, using crude enzyme preparations (Sawai *et al.*, 1968).

In the present work, we have studied some of the physical, chemical and immunological properties of two β -lactamases produced by a strain of *S. marcescens*.

METHODS

Antibiotics. The β -lactam antibiotics used (see Results) were gifts from their respective manufacturers.

Bacterial strains and identification. All the strains of *Serratia marcescens* studied were isolated from clinical sources and were maintained in cooked meat medium (Eiken Chemical Co., Tokyo, Japan). Species were identified according to the scheme of Cowan & Steel (1974).

Antibiotic resistance. Minimum inhibitory concentrations of various β -lactam antibiotics were determined by using twofold dilutions in heart infusion agar (Eiken Chemical Co.) with an inoculum of 10⁴ organisms.

Culture and harvesting of organisms. Trypto-soy broth (2 l; Eiken Chemical Co.) in a 5 l flask was inoculated with 100 ml of an overnight culture at 37 °C and incubated on a rotary shaker at 37 °C. After 4 h, the bacteria were harvested by centrifuging and washed once with 0.15 M-NaH₂PO₄/Na₂HPO₄ buffer, pH 7.5. The packed

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cells were resuspended in 200 ml 1 mM-phosphate buffer, pH 7.5, and the suspensions were treated for 4 min in an ultrasonic disintegrator (80 W, 25 kHz). The disrupted suspensions were centrifuged at 10000 *g* for 20 min at 5 °C to remove unbroken cells and cell debris; the cell-free extract was retained as the crude enzyme preparation. Protein was determined by the Lowry method.

Purification of β -lactamases. (All buffer solutions were $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ unless otherwise stated.) To the cell-free extract, streptomycin sulphate was added to give 1.5% (w/v). After removing the precipitate by centrifuging (10000 *g*, 20 min), the supernatant was dialysed overnight against distilled water and then against 2 l 5 mM buffer, pH 7.2. The precipitate formed during dialysis was removed by centrifuging and the supernatant was passed through a CM-Sephadex C-50 column (2.3×30 cm) equilibrated with 5 mM buffer, pH 7.2. The eluate was monitored by recording the absorbance at 280 nm. The first peak contained a large amount of inactive protein and a plasmid-mediated penicillinase. The chromosomal cephalosporinase appeared in the second peak after elution with 0.1 M buffer, pH 7.2; the active fraction was retained as the 'partially purified' enzyme preparation.

The first peak was applied to a DEAE-Sephadex A-50 column (2.3×20 cm) and after washing with 200 ml 0.01 M buffer, pH 7.2, was eluted with a 400 ml linear gradient of 0.01 to 0.1 M-phosphate buffer, pH 7.2. The second peak was applied to another column of CM-Sephadex C-50 (2.3×20 cm) and after washing with 200 ml 0.01 M buffer, pH 7.2, was eluted with a 400 ml linear gradient of 0.01 to 0.1 M-phosphate buffer, pH 7.2. The active fractions (for penicillinase, those eluted with 0.048 to 0.055 M buffer; for cephalosporinase, those eluted with 0.060 to 0.068 M buffer) were pooled and then dialysed against distilled water. The dialysed solutions were used for isoelectric focusing (see below). In each case, fractions around the peak of enzyme activity were pooled and applied to a Sephadex G-200 column (2.3×86 cm) equilibrated with 0.05 M-Tris/HCl/0.1 M-NaCl buffer, pH 8.0, and eluted with the same buffer. Active fractions were dialysed against distilled water and then lyophilized.

Isoelectric focusing. Isoelectric focusing was performed on a sucrose/ampholytes gradient (pH 3.5 to 10). The experiments were carried out at 5 °C in the LKB 8100 column as previously reported (Tajima *et al.*, 1980).

Polyacrylamide gel electrophoresis. The purity of the enzyme preparations was checked by discontinuous gel electrophoresis as described by Davis (1964), using 7.5% (w/v) polyacrylamide gel, pH 4.3, as the separation gel. Electrophoresis was carried out in duplicate gels: one was stained with Coomassie Brilliant Blue R250 and the other was cut into slices 4 mm thick. The slices were disrupted in 1 ml 0.01 M buffer, pH 7.5, and the β -lactamase activity was measured.

Determination of molecular weight. The molecular weight of the enzymes was determined by gel filtration through Sephadex G-200 with 0.05 M-Tris/HCl/0.1 M-NaCl buffer, pH 8.0, as described by Andrews (1964), and by sodium dodecyl sulphate (SDS)–polyacrylamide slab gel electrophoresis according to Weber & Osborn (1969), using bovine serum albumin (mol. wt 67000), ovalbumin (45000), chymotrypsinogen (25700) and lysozyme (14400) as molecular weight standards.

Assay of β -lactamase. Hydrolysis of β -lactam antibiotics was assayed spectrophotometrically (O'Callaghan *et al.*, 1969; Waley, 1974; Tajima *et al.*, 1980) by measuring the decrease in absorbance at the substrate specific wavelength at 30 °C in 3 ml 0.01 M- $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 7.5. One unit of β -lactamase activity is defined as the amount catalysing the hydrolysis of 1 μmol substrate min^{-1} , and activities are expressed as units (mg enzyme protein) $^{-1}$.

Substrate profile. The substrate profile of 'partially purified' enzyme preparations was determined with cephaloridine, cefazolin, cephalothin, cephalixin, cefotiam, cefmetazole and penicillin G. The rate of hydrolysis of each antibiotic was calculated relative to the rate of hydrolysis of cephaloridine which was given an arbitrary value of 100.

Estimation of kinetic parameters. Using the purified enzyme preparation, the K_m and $V_{\max}$ values for several substrates were estimated from Lineweaver–Burk plots. The K_m values are expressed as μM and the $V_{\max}$ values as μmol substrate hydrolysed min^{-1} (mg protein) $^{-1}$. The K_i values were determined from Lineweaver–Burk plots at various concentrations of inhibitors, using cephaloridine as the substrate.

Immunological methods. Rabbit antisera were raised against the purified cephalosporinase as previously reported (Tajima *et al.*, 1980). The effect of the antibodies on β -lactamase was determined by a neutralization test of the hydrolysing activity of the enzyme (Jack & Richmond, 1970; Tajima *et al.*, 1980). Immunoelectrophoresis was done in 1.2% (w/v) agar containing veronal buffer, pH 8.6.

Amino acid analysis. The amino acid composition of the enzymes was determined as described by Spackman *et al.* (1958) and as previously reported (Fujii-Kuriyama *et al.*, 1977), with an amino acid analyser (Hitachi model KLA-2).

RESULTS

Resistance levels and β -lactamase activities

All of 21 strains of *Serratia marcescens* examined had a high degree of resistance to cefazolin and ampicillin (minimum inhibitory concentrations $>400 \mu\text{g ml}^{-1}$). Their

Table 1. *Summary of the purification of cephalosporinase from S. marcescens strain GN7647*

These results were obtained for purification from a 16 l culture (100 μ g cefmetazole ml⁻¹ added as an inducer) of strain GN7647. Activity was determined with 100 μ M-cephaloridine as substrate.

Procedure	Total protein (mg)	Specific activity (units mg ⁻¹)	Recovery (%)
1. Ultrasonic disintegration	7700	6.0	100
2. Streptomycin treatment	3455	10	74.8
3. Chromatography on CM-Sephadex	162.2	178	62.5
4. Re-chromatography on CM-Sephadex	79.5	251	43.2
5. Isoelectric focusing	50.8	332	36.5
6. Gel filtration on Sephadex G-200 and lyophilized	37.5	374	30.4

β -lactamase activities ranged from <0.1 to 1.72 units (mg protein)⁻¹: 10 of the strains had activities of more than 1 unit (mg protein)⁻¹.

The substrate profiles of the 'partially purified' enzyme preparations (see Methods) from these strains were all similar. The β -lactamases hydrolysed cephaloridine (rate of hydrolysis = 100), cefazolin (289 to 330), cephalothin (334 to 413), cephalixin (20 to 30) and cefotiam (52 to 77). Cefmetazole and penicillin G were not hydrolysed.

Serratia marcescens GN7647 had the highest enzyme activity [1.72 units (mg protein)⁻¹], and therefore this strain was used as a source for purification of β -lactamase.

Separation and purification of two β -lactamases

The cephalosporinase and the penicillinase were separated by chromatography on a column of CM-Sephadex (see Methods). The cephalosporinase was further purified as indicated in Table 1 and as previously reported (Tajima *et al.*, 1980). The purity of the lyophilized preparation was checked by polyacrylamide gel electrophoresis, SDS-polyacrylamide slab gel electrophoresis and immunoelectrophoresis. In polyacrylamide gel electrophoresis the enzyme activity and the stained band coincided, and SDS-polyacrylamide slab gel electrophoresis gave a single protein band. Agar gel immunoelectrophoresis using the anti-GN7647-cephalosporinase sera and the crude enzyme preparation of *S. marcescens* GN7647 gave a single precipitin arc, showing that the antisera contained a single component reacting with crude enzyme preparation; moreover, the precipitin arc for the antisera and the purified enzyme preparation was symmetrical with that of the crude enzyme preparation. These results indicated that the purified enzyme preparation was homogeneous.

Likewise, for the purified penicillinase [specific activity 1506 units (mg protein)⁻¹], the enzyme activity and the stained band coincided after polyacrylamide gel electrophoresis and a single protein band was detected after SDS-polyacrylamide slab gel electrophoresis.

Physicochemical properties of the purified enzymes

The molecular weight of the purified cephalosporinase determined by gel filtration through Sephadex G-200 was 37000, and from its electrophoretic mobility in the SDS-polyacrylamide slab gel the subunit molecular weight of the enzyme was also estimated to be 37000. Neutral sugar was not detectable in the purified enzyme by the phenol/sulphuric acid method (Dubois *et al.*, 1956). These results show that the purified enzyme is composed of a single polypeptide of molecular weight 37000. The amino acid composition of the purified enzyme is given in Table 2: the enzyme contained tryptophan but not cysteine. Its pI was 9.56.

The penicillinase was found to be a single polypeptide with a molecular weight of about 26000. It contained cysteine but not tryptophan, and its pI was 5.10.

Table 2. *Amino acid composition of cephalosporinase from S. marcescens strain GN7647*

Amino acid	Content ($\mu\text{mol mg}^{-1}$)	Residues*
Aspartic acid	0.827	35
Threonine	0.436	18
Serine	0.396	17
Glutamic acid	0.817	34
Proline	0.601	25
Glycine	0.677	28
Alanine	0.921	39
Cysteine	0	0
Valine	0.535	19
Methionine	0.296	12
Isoleucine	0.365	15
Leucine	0.659	28
Tyrosine	0.479	20
Phenylalanine	0.226	10
Tryptophan	0.102	4
Lysine	0.437	18
Histidine	0.096	4
Arginine	0.288	12
Total	8.160	338

* The calculated numbers of residues are based on assumed molecular weight of 37000.

Table 3. *Kinetics of hydrolysis of β -lactam antibiotics by β -lactamases from S. marcescens strain GN7647*

Antibiotic	Cephalosporinase			Penicillinase		
	$V_{\max}^*$	K_m (μM)	$K_i^\dagger$ (μM)	$V_{\max}^*$	K_m (μM)	$K_i^\dagger$ (μM)
Cephaloridine	100	267	—	46	286	—
Cefazolin	645	571	—	8.4	182	—
Cephalothin	87	56	—	4.0	—	—
Cephalexin	4.3	—	—	0.1	—	—
Cephadrine	4.0	—	—	0.1	—	—
Cefamandole	30	174	—	12	46	—
Cefoperazone	14	8	—	17	167	—
Cefotiam	56	17	—	1.6	—	—
Cefuroxime	0.5	—	0.22	0	—	>100
Cefotaxime	0.6	—	1.1	0	—	—
Ceftizoxime	2.2	—	7.7	0	—	—
Cefmenoxime	0.2	—	0.45	0.1	—	—
Cefoxitin	0	—	0.45	0	—	>100
Cefmetazole	0	—	0.41	0	—	—
Moxalactam	0	—	7.8	0	—	>100
Penicillin G	2.5	—	—	100	63	—
Ampicillin	0	—	0.005	99	49	—
Carbenicillin	0	—	0.07	9.0	—	—
Cloxacillin	0	—	0.001	0.1	—	25
Methicillin	0	—	0.34	0	—	0.29

* $V_{\max}$ values are given relative to an arbitrary value of 100 for hydrolysis of cephaloridine or penicillin G, as appropriate.

† K_i values were determined from Lineweaver–Burk plots at various concentrations of inhibitors, using cephaloridine as the substrate.

Enzymic properties of the purified enzymes

The pH–activity curve for the cephalosporinase was relatively sharp and had a maximum near pH 8.5. The curve for the penicillinase was rather broad and had a maximum at about pH 6.5.

The kinetic parameters of the enzyme activities towards cephalosporins, cephamycins and penicillins are given in Table 3. For the cephalosporinase, the relative $V_{\max}$ with cefazolin, cephaloridine, cephalothin, cefotiam, cefamandole and cefoperazone was higher than with penicillins. Among the cephalosporin derivatives tested, cefuroxime, cefotaxime and cefmenoxime were resistant to hydrolysis by the cephalosporinase, and cephamycins and semi-synthetic penicillins were not hydrolysed at all. Moreover, cephamycins inhibited the enzyme activity of the cephalosporinase competitively. It is interesting to note that cloxacillin completely inhibited the activity of the cephalosporinase but only partially inhibited the activity of the penicillinase. The penicillinase hydrolysed ampicillin and carbenicillin, and also cephalosporins such as cephaloridine, cefoperazone, cefamandole, cefazolin and cephalothin.

Immunological properties of the cephalosporinase

The neutralizing capacity of the antisera was checked with chromosomally mediated enterobacterial β -lactamases obtained from various clinical isolates (five *Serratia marcescens*, two *Pseudomonas aeruginosa*, two *Enterobacter cloacae*, one *Proteus morganii*, one *Proteus vulgaris*, one *Proteus rettgeri*, one *Bacteroides fragilis*) and with four types of R-plasmid-mediated penicillinase from R-plasmid-carrying *Escherichia coli*. The antisera neutralized completely (98 to 100%) cephalosporinase from *S. marcescens* strains; none of the other β -lactamases were affected at all.

DISCUSSION

Farrar & O'Dell (1976) reported that strains of *S. marcescens* fall into two groups with respect to their resistance to β -lactam antibiotics. Strains in one group produce small amounts of an inducible cephalosporinase, which appears to be chromosomally mediated. Strains in the other group produce large amounts of a non-inducible penicillinase-cephalosporinase, which is plasmid-mediated.

In the present study, both a penicillinase and a cephalosporinase were purified from a strain of *S. marcescens* GN7647. By combining several methods, including isoelectric focusing and neutralization with antisera, we have characterized differences in isoelectric point, molecular weight and immunological properties of β -lactamases in *S. marcescens*.

The purified cephalosporinase from *S. marcescens* GN7647 showed the same general properties as for typical cephalosporinases described by Sawai *et al.* (1968) and for class I β -lactamases described by Richmond & Sykes (1973). As indicated by $V_{\max}$ values, this enzyme has a high activity on cephalosporin derivatives and a low activity for semi-synthetic penicillins, some novel cephalosporins and cephamycins. It has been reported by numerous investigators (O'Callaghan & Muggleton, 1967; Jack & Richmond, 1970; Yaginuma *et al.*, 1973; Richmond & Sykes, 1973; Fujii-Kuriyama *et al.*, 1977; Tajima *et al.*, 1980; Minami *et al.*, 1980) that semi-synthetic penicillins, particularly cloxacillin, methicillin and carbenicillin, are not only resistant to hydrolysis by cephalosporinase but are strong inhibitors of the enzyme activity. These semi-synthetic penicillins inhibited the purified enzyme of *S. marcescens* strain GN7647 in a competitive manner, as did cefuroxime, cefmenoxime and cephamycins such as cefoxitin and cefmetazole. The K_i values of these β -lactamase-stable cephalosporins and cephamycins were comparable with that of methicillin.

The purified cephalosporinase had a molecular weight of 37 000 and its isoelectric point was 9.56. The chromosomally mediated cephalosporinases purified from *Pseudomonas aeruginosa* GN918 (Yaginuma *et al.*, 1973), *Proteus morganii* 1510 (Fujii-Kuriyama *et al.*, 1977), *Citrobacter freundii* GN7391 (Tajima *et al.*, 1980) and *Escherichia coli* GN5482 (Minami *et al.*, 1980) have molecular weights of 34 000, 38 000, 38 000 and 39 000, respectively. The molecular weight of the purified enzyme of *S. marcescens* GN7647 is thus close to those of other cephalosporinases. However, the isoelectric point for the purified

enzyme was more basic than those of other cephalosporinases (pI 7.2 to 8.7). Matthew & Harris (1976) reported that β -lactamases from *S. marcescens* strains 1324E and 1331E had pI values of 8.2 and 8.5, respectively. Sykes & Matthew (1976) reported that the β -lactamase from strain 1331E hydrolysed cloxacillin, but our purified enzyme did not hydrolyse it significantly.

The cephalosporinase protein of strain GN7647 was detected among other strains of *S. marcescens*, such as 1823, 1831, 1840, 1845 and GN10857, isolated from clinical material, by the immunoelectrophoretic analysis and neutralization test.

The penicillinase from *S. marcescens* strain GN7647 was identical to type I penicillinases described by Sawai *et al.* (1970) and class III β -lactamases described by Richmond & Sykes (1973).

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