Notes

PURIFICATION AND PROPERTIES OF A CEPHALOSPORINASE FROM *ACINETOBACTER CALCOACETICUS*

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Cephalosporinase was purified about 150-fold from a strain *Acinetobacter calcoaceticus* ML4961. The isoelectric point was 9.9, and the molecular weight was about 38,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The optimal pH and optimal temperature were 8.5 and 45°C, respectively. Enzyme activity was inhibited by $HgCl_2$ and ferrous ion. The enzyme hydrolyzed cephaloridine, cefazolin, cephalothin, cephalexin, and cefoperazone at a high rate. Rabbit antiserum obtained against the purified enzyme showed no cross-reaction in neutralization test with cephalosporinases produced by strains of other bacterial species.

A. calcoaceticus, a member of a group of glucose-nonfermenting Gram-negative bacteria, has a wide distribution in nature and can be readily isolated from the environment such as soil and water[1]. *A. calcoaceticus* strains have recently isolated with increasing frequency from clinical specimens and shown to be resistant to many β-lactam antibiotics, including those that are newly developed[2~4]. It has long been recognized that *A. calcoaceticus* produces an inducible cephalosporinase, considered to play a significant role in resistance to β-lactam antibiotics[5].

However, no detailed report on a cephalosporinase from this species has been published. This paper deals with the enzymological, physicochemical, and immunological properties of purified enzyme from *A. calcoaceticus* ML4961. Benzylpenicillin, ampicillin, cephaloridine, cefazolin, cephalothin, cephalexin, cefuroxime, cefmetazole, cefoxitin, cefotetan, cefoperazone, latamoxef, ceftizoxime, and cefotaxime were commercial products. The following compounds were kindly provided by the respective manufactures: Cefmenoxime and carumonam from Takeda Chemical Industries, Ltd.; ceftazidime from Shin Nihon Jitsugyo Co., Ltd.; imipenem from Nippon Merck-Banyu Co., Ltd.; aztreonam from the Squibb Institute for Medical Research; clavulanic acid from Beecham Yakuhin K. K.; and sulbactam from Pfizer Taito Co., Ltd. The MICs of various β-lactam antibiotics were determined by using serial 2-fold dilutions in sensitivity disk agar-N (Nissui Seiyaku Co., Ltd.) with a final inoculum of about 5×10^4 cfu/plate. For the preparation of crude enzymes, we followed the method described previously[6~8].

Enzyme activity was determined by a direct spectrophotometric method as described previously[9,10] or by a modification of the NOVICK microiodometric method[11]. One unit of β-lactamase activity was defined as the amount of enzyme that hydrolyzed 1 μM of substrate per minute at 30°C in 50 mM phosphate buffer (pH 7.0). Determination of protein was carried out by the method of LOWRY *et al.*[12] with bovine serum albumin as the standard. The Michaelis constant (*Km*) and the maximum rate of hydrolysis (Vmax) were determined from Lineweaver-Burk plots. Dissociation constants of the enzyme-inhibitor complex (*Ki*) values for β-lactam antibiotics were determined from Dixon plots.

β-Lactamase inhibition was determined spectrophotometrically after 10 minutes of preincubation with an inhibitor at 30°C, using 100 μM cephaloridine as a substrate. The molecular weight was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, according to the method of LAEMMLI and FARVE[13]. The isoelectric point (pI) of the enzyme was determined in 0.5 mm thin-layer polyacrylamide gels (5% acrylamide) using broad-range ampholyte (pH 3.5 to 10.0; LKB-Produkter AB) and pI-marker (Oriental Yeast Co., Ltd.).

The preparation of rabbit antiserum and examination of immunological property were

Table 1. Kinetics of hydrolysis of various β-lactam antibiotics by cephalosporinase produced by *Acinetobacter calcoaceticus* ML4961 and resistance levels of *A. calcoaceticus* ML4961.

Substrate	*Km* (μM)	*Ki*[a] (μM)	Vmax[b]	Drug resistance[c] (μg/ml)
Cephaloridine	511		100	≧800
Cefazolin	766		345	≧800
Cephalothin	168		473	≧800
Cephalexin	400		202	≧800
Cefuroxime			0.6	400
Cefmetazole			0.6	200
Cefoxitin			0.4	400
Cefotetan			0.9	≧400
Cefoperazone	125		47	≧400
Latamoxef		0.9		50
Ceftizoxime	12		1.8	100
Cefotaxime		0.3	<0.1	50
Cefmenoxime		0.5	<0.1	50
Ceftazidime		116		12.5
Aztreonam		12		50
Carumonam		14		25
Imipenem		7		1.56
Clavulanic acid		>100		
Sulbactam		201		
Benzylpenicillin	4.8		0.46	≧800
Ampicillin	16		<0.1	≧800

[a] *Ki* values were determined with cephaloridine as a substrate by Dixon plots.
[b] Rates are given relative to an arbitrary value of 100 for hydrolysis of cephaloridine.
[c] Expressed as the MIC of each drug.

performed according to the procedures described previously[14,15]. The crude enzyme solution was purified by absorption and elution on a SP-Sephadex C-50 (Pharmacia Fine Chemicals) column, by gel filtration on a Toyopearl HW-50F (Tosoh Manufacturing Co., Ltd.) column. The active fractions were pooled and applied again to SP-Sephadex C-50 column.

The cephalosporinase from *A. calcoaceticus* ML4961 was purified about 150-fold by column chromatography. The molecular weight of the purified enzyme was estimated from electrophoretic mobility in sodium dodecyl sulfate-polyacrylamide gels to be about 38,000.

Analytical electrofocusing in thin-layer of polyacrylamide gels revealed the isoelectric point of the enzyme to be 9.9.

The optimum pH was approximately 8.5, and the enzyme showed the highest β-lactamase activity at 45°C.

The *Km*, *Ki*, and relative Vmax values are shown in Table 1. Relative Vmax values were obtained with cephaloridine, cefazolin, cephalothin, and cephalexin. As indicated by Vmax values, this enzyme easily hydrolyzes so-called 1st-generation cephalosporins. Cefoperazone was also hydrolyzed, although the rate of hydrolysis was lower than the above antibiotics.

On the other hand, cefotaxime, cefmenoxime, and ampicillin showed low Vmax values. Latamoxef, aztreonam, carumonam, imipenem, ceftazidime, clavulanic acid, and sulbactam also showed strong resistance to the hydrolysis by the enzyme.

However, the latter three compounds had no affinity to the enzyme. Thus the β-lactamase from *A. calcoaceticus* ML4961 presents a cephalosporinase profile as for typical cephalosporinase described by MITSUHASHI and INOUE[16].

The effects of inhibitors and ions on enzyme activity are shown in Table 2. Enzyme activity was inhibited by Hg^{2+} and Fe^{2+} ions but was not inhibited by other ions, iodine, *p*-chloromercuribenzoate, or EDTA. The neutralizing capacity of the antiserum against the purified enzyme was examined by using various cephalosporinases. As shown in Table 3, the neutralization tests of the antiserum against the cephalo-

Table 2. Effects of inhibitors and metal ions on the activity of cephalosporinase from *Acinetobacter calcoaceticus* ML4961[a].

Inhibitors and ions	Conc (mM)	Inhibition (%)
Iodine	0.1	0
p-CMB	0.1	0
Sodium thioglycollate	1.0	0
L-Cysteine	1.0	0
EDTA	1.0	0
$FeSO_4$	1.0	11.3
$CuSO_4$	1.0	0
$ZnCl_2$	1.0	0
$MnCl_2$	1.0	0
$CoSO_4$	1.0	0
$MgSO_4$	1.0	0
$CaCl_2$	1.0	0
$HgCl_2$	1.0	78.9

[a] The enzyme was preincubated in 50 mM tris-HCl buffer (pH 8.6) for 10 minutes at 30°C with each inhibitor and metal ion at the indicated concentration, and the remaining activity was assayed with cefoperazone (50 μM) as the substrate.

p-CMB: *p*-Chloromercuribenzoate.

Table 3. Inhibition of various cephalosporinase activities with antiserum of cephalosporinase from *Acinetobacter calcoaceticus* ML4961.

Enzyme source	Inhibition (%)
A. calcoaceticus ML4961	100
A. calcoaceticus IAM 12087	100
A. calcoaceticus NCTC 7844	78.3
A. calcoaceticus E2312	10.2
Escherichia coli GN5482	0
Citrobacter freundii GN7391	0
Enterobacter cloacae GN7471	0
Morganella morganii GN5407	0

sporinase from ML4961 showed that the enzyme was highly specific to *A. calcoaceticus* species, because anti-ML4961 serum could only cross-react with cephalosporinase from *A. calcoaceticus* ML4961, IAM 12087, and NCTC 7844, but did not with other cephalosporinases.

A. calcoaceticus ML4961 capable of producing cephalosporinase was resistant to various β-lactam antibiotics.

However, the resistance levels of this strain to different β-lactam antibiotics do not correlate with the hydrolysis rates for them. From this result, other factors, such as R-plasmid-mediated β-lactamase, binding affinity to penicillin-binding proteins, and permeability of drug seem to be involved in resistance mechanisms in addition to hydrolysis by cephalosporinase.

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