PROPERTIES OF A BROAD SPECTRUM β-LACTAMASE ISOLATED FROM *FLAVOBACTERIUM MENINGOSEPTICUM* GN14059

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A broad substrate-spectrum β-lactamase was purified from *Flavobacterium meningosepticum* GN14059. The purified enzyme gave a single protein band on SDS-polyacrylamide gel electrophoresis. The molecular weight was estimated to be about 30,000 and the isoelectric point was 5.1.

The enzyme hydrolyzed various β-lactam antibiotics including oxyiminocephalosporins and aztreonam. Relative rates, with cephaloridine as 100, were cephalothin 200, cefazolin 48, cefuroxime 153, cefotaxime 51, ceftazidime 20, ampicillin 26, carbenicillin 19, and aztreonam 20.

The enzyme activity was inhibited by clavulanic acid, sulbactam, imipenem and cephamycins.

Flavobacteria, glucose-nonfermenting, Gram-negative and yellow-pigmented rods, are widely distributed in natural ecosystems[1]. *Flavobacterium meningosepticum* is well known as a pathogen associated with neonatal meningitis, which has a poor prognosis[2,3]. Strains of *F. meningosepticum* isolated from clinical specimens have been shown to be resistant to many antibacterial agents including β-lactam antibiotics[3,4].

β-Lactamases produced by Gram-positive and Gram-negative bacteria are considered to be the most important biochemical mechanism of resistance to β-lactam antibiotics. The biochemical properties of a β-lactamase from *Flavobacterium odoratum* have been reported[5], however there have been limited analysis of β-lactamases from *F. meningosepticum*.

This paper deals with the study of some properties of a β-lactamase purified from a highly β-lactam-resistant *F. meningosepticum*.

Materials and Methods

Bacterial Strains

F. meningosepticum GN14059 was isolated from clinical specimens and was stored in glycerol at −70°C.

Media

Penassay broth (Bact antibiotic medium No. 3, Difco), sensitivity disk agar-N and sensitivity test broth (Nissui Pharm., Co., Ltd.) were used.

Antibiotics

Cephaloridine, cephalothin, cefazolin, cefoperazone, cefuroxime, ceftizoxime, cefoxitin, latamoxef and penicillins were commercially available materials. The following compounds were gifts: Cefmenoxime 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; sulbactam from Pfizer Taito Co., Ltd.; and clavulanic acid from Beecham Yakuhin K.K.

Susceptibility Testing

Drug resistance was determined by the agar dilution method with sensitivity disk agar-N. One loopful (*ca.* about 5 μl) of a diluted culture (*ca.* 10^6 cells per ml) was inoculated onto assay media containing serial 2-fold dilutions of a drug. MICs were scored after incubation at 30°C for 24 hours.

Enzyme Preparation

A culture in 6 liters of Penassay broth in the late logarithmic phase of bacterial growth was harvested by centrifugation at 5,500×*g* for 15 minutes at 4°C, and the bacteria were washed twice in 50 mM Na_2HPO_4 - KH_2PO_4 phosphate buffer (pH 7.0) by centrifugation. The cells were suspended in 25 mM of the same buffer and disrupted sonically in an ice-cold water bath. The supernatant, after centrifugation at 45,000×*g* for 30 minutes at 4°C, was used as the crude enzyme preparation. Streptomycin sulfate (Sigma) was added to the crude enzyme to a final concentration of 2% (w/v) to remove nucleic acids.

Purification of β-Lactamase

The streptomycin-treated enzyme solution was applied to a DEAE-Sepharose CL-6B (Pharmacia) column (2.5 by 40 cm) equilibrated with 25 mM phosphate buffer (pH 7.0). The enzyme was eluted at a flow rate of 40 ml/hour with a linear gradient of NaCl from 0 to 0.5 M in a total volume of 600 ml. The active fractions were pooled and concentrated by the addition of polyethylene glycol. The concentrated enzyme solution was loaded onto a Toyopearl HW-55F column (3.0 by 43 cm) and eluted at 20 ml/hour with 50 mM phosphate buffer (pH 7.0). The active fractions were dialyzed against distilled water, and purified by isoelectrofocusing.

Assay of β-Lactamase

Enzyme activity was assayed by a modification of the micro-iodometric method[6] or by a direct spectrophotometric method as described previously[7]. β-Lactamase activity (1 U) was expressed as 1 μM substrate hydrolyzed per minute at 30°C in 50 mM phosphate buffer. The *Km* and the maximum rate of hydrolysis (relative Vmax) were determined by Lineweaver-Burk plot. The dissociation constants of the enzyme-inhibitor complex (*Ki*) were determined by Dixon plot.

Determination of Protein

Protein determination was carried out with bovine serum albumin as standard[8].

Isoelectric Focusing

Determination of isoelectric point (pI) was carried out by using carrier Ampholine (pH 3.5~10) with a sucrose density gradient in 110 ml of column (LKB 8100). After focusing with a final potential of 300 V at 4°C for 3 days, fractions were collected and assayed for β-lactamase activity and pH.

Determination of MW

The MW was estimated by sodium dodecyl sulfate-polyacrylamide gel electro phoresis[9]. The purified enzyme and marker proteins were treated with 10% sodium dodecyl sulfate and 5% 2-mercaptoethanol at 100°C for 2 minutes, and then subjected to electrophoresis in a 12.5%-gel at 30 mA for 5 hours at room temp. Bovine serum albumin (67,000), ovalbumin (45,000), chymotrypsinogen A (25,000), and myoglobin (17,000) were used as marker proteins.

Inhibition Study

A solution of the enzyme was preincubated with an inhibitor in 50 mM phosphate buffer (pH 7.0) or distilled water at 30°C for 5 minutes, and the remaining enzyme activity was assayed spectrophotometrically with 0.1 mM cefuroxime as the substrate.

Results

Purification of β-Lactamase

The purification procedure for the enzyme from *F. meningosepticum* GN14059 is summarized in Table 1. The enzyme was purified over 110-fold from an extract of *F. meningosepticum* GN14059. The purified enzyme gave a single protein band on SDS-polyacrylamide gel electrophoresis and was about 5.1 mg. The MW of the enzyme was estimated to be *ca.* 30,000 by electrophoretic mobility on SDS-polyacrylamide gel compared to MW markers. The pI determined by electrofocusing was 5.1.

Enzymological Properties of the Enzyme

The kinetic parameters (*Km*, relative Vmax and *Ki*) of the enzyme and the levels of resistance of strain GN14059 against various β-lactam antibiotics are shown in Table 2. *F. meningosepticum* GN14059 was resistant to almost all β-lactam antibiotics tested. The MIC values of cefotaxime, cefoperazone, cephalothin, ampicillin and carbenicillin were 1,600 μg/ml or greater, respectively. Higher relative Vmax values were obtained with cephaloridine, cephalothin, cefazolin, cefuroxime, cefotaxime and cefmenoxime. Benzylpenicillin, ampicillin and carbenicillin were also hydrolyzed, al-

Table 1. Purification of β-lactamase from *Flavobacterium meningosepticum* GN14059.

Procedure	Total activity[a] (U)	Specific activity (U/mg of protein)	Purification (fold)	Recovery (%)
Ultrasonic disintegration	5,560	0.8	1.0	100
Streptomycin treatment	5,070	1.4	1.8	91
Chromatography on DEAE-Sepharose CL-6B	2,220	25	31	40
Gel filtration on Toyopearl HW-55F	1,450	44	55	26
Electrofocusing	450	88	110	8

[a] Activity was assayed spectrophotometrically with cefuroxime (100 μM) as substrate.

Table 2. Kinetic parameters of β-lactamase from *Flavobacterium meningosepticum* GN14059 and the resistance levels against various β-lactam antibiotics.

Substrates	Vmax[a]	*Km* (μM)	*Ki*[b] (μM)	MIC (μg/ml)
Cephaloridine	100	42		>1,600
Cephalothin	200	61		>1,600
Cefazolin	48	17		>1,600
Cefoperazone	12	15		>1,600
Cefuroxime	153	167		>1,600
Cefotaxime	51	71		>1,600
Ceftizoxime	26	28		800
Cefmenoxime	71	250		>1,600
Ceftazidime	20	95		400
Cefoxitin	<1		0.03	200
Latamoxef	<1		<0.01	200
Benzylpenicillin	51	42		>1,600
Ampicillin	26	30		>1,600
Carbenicillin	19	20		>1,600
Imipenem	<1		<0.01	25
Aztreonam	20	40		200
Clavulanic acid	<1		0.15	200

[a] Hydrolysis is expressed as the relative rate of hydrolysis, taking the hydrolysis of cephaloridine as 100.
[b] *Ki* values were determined with cefuroxime as substrate.

though the rates of hydrolysis were lower than those of the cephalosporins. Furthermore, the enzyme showed considerable activity against aztreonam. However, cefoxitin and latamoxef were resistant to hydrolysis by the enzyme. Against cefoxitin, latamoxef, imipenem and clavulanic acid, the GN14059 enzyme showed *Ki* values of 0.03, <0.01, <0.01 and 0.15 μM, respectively. These β-lactam antibiotics had high affinity for the enzyme as indicated by their low *Ki* values, resulting from competitive inhibition of the enzyme. The effects of some inhibitors and ions on enzyme activity were shown in Table 3. The enzyme activity was inhibited by clavulanic acid, sulbactam, cefoxitin, latamoxef, imipenem, Cu^{2+} and Hg^{2+}. However, the activity was not inhibited by EDTA.

Table 3. Effects of various inhibitors against β-lactamase activity of *Flavobacterium meningosepticum* GN14059.

Inhibitor and ion[a]	Concentration (μM)	Inhibiton (%)
Clavulanic acid	1	100
	10	100
Sulbactam	1	35
	10	85
Imipenem	1	100
	10	100
Cefoxitin	1	100
Latamoxef	1	100
EDTA	3,000	0
Iodine	100	100
Cu^{2+}	100	100
Hg^{2+}	100	100
Fe^{2+}	100	50
Zn^{2+}	100	22
Mg^{2+}	100	0

[a] The enzyme was preincubated in 50 mM phosphate buffer (pH 7.0) or distilled water for 5 minutes at 30°C with each inhibitor or ion at the indicated concentrations, and remaining activity was assayed with 100 μM cefuroxime as substrate.

Discussion

The purified β-lactamase of *F. meningosepticum* showed a broad substrate profile, including oxyiminocephalosporins, ampicillin and aztreonam, usually rather stable to β-lactamases. The activity of the GN14059 enzyme was inhibited by clavulanic acid, latamoxef and imipenem.

Our previous studies had shown that oxyiminocephalosporins were easily hydrolyzed at high rates by β-lactamases from *F. odoratum*[5], *Pseudomonas cepacia*[7], *Bacteroides fragilis*[10,11], *Proteus vulgaris*[12], *Pseudomonas aeruginosa*[13], *Legionella gormanii*[14]. In addition to penicillin β-lactamases and cephalosporin β-lactamases, therefore, these enzymes can be classified as oxyiminocephalosporin β-lactamases (CXases) in terms of substrate specificity[15]. CXases can be divided into two subgroups, *i.e.*, type I and type II, by substrate and inhibitor profiles[16]. Type I CXase rapidly hydrolyzed cephalosporins, oxyiminocephalosporins, ampicillin and aztreonam, and type II CXase hydrolyzed cephalosporins, oxyiminocephalosporins, ampicillin and imipenem. The activity of type I CXase was inhibited by clavulanic acid, latamoxef and imipenem, and type II was a metalloenzyme and was inhibited by EDTA but not by clavulanic acid.

Based on the substrate and inhibitor profiles, the GN14059 enzyme is classified into a type I of CXase. The β-lactamase produced by *F. odoratum*, the same genus *Flavobacterium* with *F. meningosepticum*, belongs to CXase type II, and its MW and pI were 26,000 and 6.9, respectively[5]. The GN14059 enzyme, however, was different from *F. odoratum* enzyme in MW, pI, substrate and inhibitor profiles. Furthermore, the enzyme from *F. meningosepticum* was different from type I CXases from *Pseudomonas maltophilia* (L-2 enzyme: MW 27,000, pI 8.4), *P. cepacia* (MW 24,000, pI 9.3) and *P. vulgaris* (MW 30,000, pI 8.8) in MW or pI.

Cefoxitin, latamoxef, imipenem and clavulanic acid, showed very low *Ki* values against GN14059 enzyme than *P. maltophilia* (*Ki* values; from 0.58 to 47.5 μM), *P. cepacia* (clavulanic acid: 1.72 μM) and *P. vulgaris* (cefoxitin and clavulanic acid: 17.8 and 1.07 μM) enzymes. The enzyme, similar to the penicillinase from *Alcaligenes denitrificans* subsp. *xylosoxydans*[17] and the cephalosporinase from *P. aeruginosa*[18], characteristically had an enzymic properties against these β-lactam antibiotics.

From these results, it was concluded that β-lactamase from *F. meningosepticum* GN14059 was a unique CXase type I.

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