CHARACTERIZATION OF CEPHALOSPORINASES FROM *BACTEROIDES FRAGILIS*, *BACTEROIDES THETAIOTAOMICRON* AND *BACTEROIDES VULGATUS*

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The susceptibility of 80 *Bacteroides fragilis* group strains isolated from clinical specimens to β-lactam antibiotics was investigated by agar dilution method. Twenty strains showed high resistance to the antibiotics. The resistance level of the isolates to cephaloridine was related to the amount of β-lactamase activity (cephalosporinase; CSase) produced. *B. fragilis* GN-11477, *B. thetaiotaomicron* GN11478 and *B. vulgatus* GN11479 were selected from among the CSase producing strains, and the enzymes were purified about 300-fold by affinity chromatography employed ampicillin as ligand bound to activated CH Sepharose 4B. The enzyme preparations gave a single protein band on polyacrylamide gel electrophoresis. The molecular weights of the three enzymes were estimated to be approximately 32,000 and their isoelectric points were 5.2, 4.9 and 4.5, respectively. The optimal pH and the optimal temperature of the enzymes were 7.2 and 37°C, respectively. The enzyme activities were inhibited by iodine, some divalent ions, *p*-chloromercuribenzoate, clavulanic acid, cephamycin derivatives and cloxacillin. The enzymes showed hydrolytic activity against cephaloridine, cephalothin, cefazolin, cefuroxime and also newly introduced cephalosporins such as cefotaxime, cefoperazone and cefmenoxime. Each mouse antisera obtained against the purified enzymes showed cross-reactions with its each enzyme and others in neutralization test.

Organisms of the *Bacteroides fragilis* group such as *B. fragilis*, *B. thetaiotaomicron* and *B. vulgatus*, which were formerly classified as subspecies of *B. fragilis*[1], have been isolated from clinical specimens with increasing frequency[2~5], and are the single most common pathogens[6,7]. These isolates almost all exhibited moderate or high resistance to β-lactam antibiotics[8,9] as well as tetracycline[10] and aminoglycosides[11].

Their resistance against β-lactam antibiotics were ascribed by several investigators to the production of β-lactamase[12~17]. These enzymes were shown to be membrane associated, constitutively produced, and present in all strains which were resistant to β-lactam antibiotics[16,17]. The enzymes hydrolyzed cephalosporins more readily than penicillins and were sensitive to inhibitors such as cloxacillin and *p*-chloromercuribenzoate[14~17]. DARLAND and BIRNBAUM[12] found that β-lactamases from *B. fragilis* hydrolyzed cefuroxime and cefamandole, and the enzyme activities were inhibited by cefoxitin. Our previous paper[13] also showed that the purified enzyme from *B. fragilis* was able to hydrolyze cefoperazone and cefotaxime, which are known to be resistant to hydrolysis by many β-lactamases. However, the β-lactamase activities of the enzymes from other *B. fragilis* group such as *B. thetaiotaomicron* and *B. vulgatus* against the novel cephalosporins have not been studied extensively. Furthermore, there is disagreement about whether *B. thetaiotaomicron* and *B. vulgatus* produce different β-lactamases[14,15].

This paper deals with the comparison of the enzymological and physicochemical properties of the CSases from *B. fragilis*, *B. thetaiotaomicron* and *B. vulgatus*. The susceptibility of the clinical isolates

against newly introduced β-lactam antibiotics, and the relationship between the resistance levels and the amount of β-lactamase produced in the organisms are also investigated.

Materials and Methods

Bacterial Strains

Eighty strains of the *B. fragilis* group, including *B. fragilis, B. thetaiotaomicron* and *B. vulgatus*, were isolated from clinical specimens at the following three microbiological laboratories in Japan: Juntendo Hospital in Tokyo; Tokyo Metropolitan Geriatric Hospital; and Institute of Physical and Chemical Research in Wako, Saitama. All the strains were identified by the method of Virginia Polytechnic Institute[18], and were given strain numbers by our laboratory. These strains were stored in skim milk (10%) at −70°C.

Drugs

Cephaloridine, cephalothin, cefazolin, cephalexin, benzylpenicillin, ampicillin, carbenicillin and cloxacillin were commercially available materials. Other compounds were kindly provided by the following manufacturers: cefsulodin[19], cefotiam[20], cefmenoxime[21] (Takeda Chemical Industries, Ltd.), cefamandole (Eli Lilly & Co.), cefuroxime (Glaxo Group Research Ltd.), cefotaxime (Hoechst Japan)[22], cefoperazone (Toyama Chemical Co., Ltd.)[23], ceftizoxime (Fujisawa Pharm. Co., Ltd.)[24], cefpiramide (SM-1652) (Sumitomo Chemical Co., Ltd.)[25], cefoxitin (Daiichi Seiyaku Co., Ltd.), cefmetazole (Sankyo Co., Ltd.), cefotetan (YM-09330) (Yamanouchi Pharm. Co., Ltd.)[26], latamoxef (Shionogi & Co., Ltd.)[27], clavulanic acid (Beecham Yakuhin Co., Ltd.)[28], sulbactam (CP-45,899) (Pfizer Inc.)[29], and *N*-formimidoylthienamycin (Merck Banyu Co., Ltd.)[30].

Antibiotic Susceptibility Testing

Bacterial susceptibility to antibiotics was determined by GAM agar (Nissui Pharm., Co., Ltd.)-dilution method, and expressed as minimal inhibitory concentration (MIC; μg/ml). The plates containing serial two-fold dilutions of antibiotic were inoculated with one loopful (about 5 μl) of 10^6 colony forming units (cfu/ml) of overnight cultures in GAM broth at 37°C. The MIC values were scored after 18 hours of incubation at 37°C in GasPac jars.

Culture Conditions

The growth medium was Brain heart infusion broth (Difco) supplemented with 0.5% yeast extract, 0.05% cystein-HCl and 0.001% hemin (BHI-CH medium). The medium was prepared anaerobically by a modification of the method of NORD *et al.*[31] Bacteria were grown overnight at 37°C in BHI-CH medium. The cultures were diluted 50-fold with the same fresh medium and sealed with a rubber stopper under the anaerobic conditions, and then grown at the same temperature by the shaking method.

Preparation and Purification of β-Lactamase

The enzyme was extracted from 2 liters cultured cells of the each strains. Cells at middle logarithmic growth phase were harvested by centrifugation, suspended in 0.05 M phosphate buffer (pH 7.0), and disruptured by ultrasonic treatment. The supernatants after ultracentrifugation (45,000 rpm for 30 minutes at 4°C) constituted the crude enzyme preparations. The crude preparations were purified by a modification of the method of ERIQUEZ *et al.*[32] Activated CH-Sepharose 4B (Pharmacia Fine Chemical, Uppsala) (5 g) was suspended in 100 ml of 1 mM HCl and washed with 1 liter of 1 mM HCl. Ampicillin (200 mg) was dissolved in 50 ml of 0.1 M $NaHCO_3$ (pH 8.0) and mixed with the gel suspension by shaking at a low rate of speed for 6 hours at 4°C. The excess ligand was washed away with 0.1 M $NaHCO_3$ (pH 8.0), and the remaining reactive spacers were blocked with 1 M ethanolamine. The ampicillin-Sepharose was washed with three cycles of buffer, first at high pH (0.05 M tris-HCl, pH 8.0, 0.5 M NaCl) and then at low pH (0.05 M acetate, pH 4.0, 0.5 M NaCl), and finally equilibrated with 0.05 M phosphate buffer (pH 7.0). The primary elution of the crude enzyme was performed with the 0.05 M phosphate buffer (pH 7.0), and to this buffer was added 0.5 M NaCl for the secondary elution.

Enzyme Assay

β-Lactamase activity was determined by the spectrophotometric method of WALEY *et al.*[33], or by a

modified microiodometric method[34] using penicillins as substrates. One unit of the enzyme activity was defined as that which hydrolyzed 1 μmole substrate per minute at 30°C in 0.05 M phosphate buffer (pH 7.0).

Determination of Kinetic Parameters

The MICHAELIS constant (*Km*) and the maximum rate of hydrolysis (relative *Vmax*) values for several substrates were estimated from LINEWEAVER-BURK plots. The *Ki* values for several compounds were also determined from LINEWEAVER-BURK and DIXON plots with cephaloridine as substrate.

Inhibitory Study

The inhibition of enzyme activity by various compounds was assayed by the spectrophotometric assay method, using 0.1 mM of cephaloridine as substrate. The enzyme dialyzed against running water was preincubated in distilled water with each of the compounds at indicated concentrations for 10 minutes at 30°C, and then the remaining enzyme activity was assayed.

Protein Determination

The concentration of protein in the column elutes was estimated by measuring the absorbance at 280 nm. A more accurate estimation was carried out by LOWRY's method[35] with bovine serum albumin as the standard.

Polyacrylamide Gel Electrophoresis

To determine the approximate molecular weight of the enzyme, sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out according to the method of WEBER and OSBORN[36], using bovine serum albumin (molecular weight, 68,000), ovalbumin (45,000), chymotrypsinogen (25,000), and cytochrome C (12,500) as molecular weight standards. The acrylamide gel concentration was 10%. The purity of the enzyme preparation was also checked by discontinuous gel electrophoresis as described by MAURER[37], using 7.5% acrylamide gel, pH 4.0, as the separation gel.

Isoelectric Focusing

Isoelectric focusing was performed on a sucrose/ampholytes gradient (pH 3.5~10). The experiment was carried out at 4°C in the LKB 8100 column over 48 hours at 300 V. The column contents were then fractionated into 3 ml per tube.

Production of Antiserum and Immunological Study

Mouse antisera were produced against each of the purified enzymes from GN11477, GN11478 and GN11479 by the method of MURATA *et al.*[38]. The enzyme preparations (100 μg of protein) were dissolved in 0.1 ml of saline and emalsified with the same volume of complete FREUND's adjuvant. The enzyme solutions (each of 0.2 ml) were injected intraperitoneally into 5 male mice (ICR, weighing 20~25 g). After 4 weeks following the first stimulation, booster injections containing 100 μg of protein were administrered intraperitoneally. Antisera were collected 2 weeks after the last injection. The effects of antisera on enzyme activity were examined by testing the neutralization of the hydrolyzing activities of the enzymes. The enzyme solution (0.1 ml, 1.0 unit/ml) was inoculated with various amounts of antisera in 0.1 ml of 0.05 M phosphate buffer (pH 7.0) at 37°C for 1 hour and then left at 4°C for 18 hours. The mixture solution was centrifuged at 3,000 rpm for 10 minutes and the residual enzyme activity of the supernatant was assayed spectrophotometrically, using cephaloridine (0.1 mM) as substrate.

Results

Susceptibility to β-Lactam Antibiotics

The susceptibility of 60 clinical isolates of *B. fragilis* to cefazolin and ampicillin is shown in Table 1. It was found that the MICs against 38 isolates were 50 μg/ml or less, but 22 isolates were resistant to 100 μg/ml of cefazolin and ampicillin. Five strains of *B. thetaiotaomicron* and one of *B. vulgatus* also exhibited high resistance to both the antibiotics (Table 1). These resistant strains also showed high resistance to novel β-lactam antibiotics such as cefotaxime, cefoperazone, cefmenoxime and cefpiramide

Table 1. Susceptibility of *B. fragilis* group against cefazolin and ampicillin.

Drugs	Organisms	No. of isolates	MICs (μg/ml)									
			≦0.20	0.39	0.78	1.56	3.13	6.25	12.5	25	50	100≦
Cefazolin	*B. fragilis*	60				2[a]	2	9	25			22
	B. thetaiotaomicron	12					1				6	5
	B. vulgatus	8				1			2	3	1	1
Ampicillin	*B. fragilis*	60			1	3	2	2	30			22
	B. thetaiotaomicron	12						1	2	4		5
	B. vulgatus	8		1					2	4		1

a) Number of isolates inhibited.

Table 2. Susceptibility of the resistant strains[a] of the *B. fragilis* group to novel cephalosporins.

Drugs	MICs (μg/ml)						
	≦25	50	100	200	400	800	1600≦
Cefotiam			5[b]	2	21		
Cefmenoxime		1	6	3	18		
Cefuroxime	1	5	5	16	1		
Cefotaxime	6	5	11	3	3		
Ceftizoxime	6	16	3	3			
Cefoperazone			1	5	3	16	3
Cefpiramide			4	1	9	11	3
Cefoxitin	26	1	1				
Cefmetazole	26	1	1				
Cefotetan	24	1	3				
Latamoxef	25	2	1				
N-Formimidoyl-thienamycin	28						

a Twenty-eight strains were resistant to cefazolin and ampicillin.
b Number of isolates inhibited.

(Table 2). In contrast, cefoxitin and other cephamycin derivatives were relatively active against the resistant strains.

β-Lactamase Activity

On the basis of the enzyme activities and MICs for the antibiotics, the strains used fell into four groups. Table 3 showed the activities of the crude enzyme preparations from the strains which were selected randamly from the each group, and their resistance levels. The first group (Group I) comprised the cephalosporin-resistant strains whose enzymes hydrolyzed cephaloridine at high rates but not ampicillin (CSase type). The enzyme activity of the Group I strains was directly proportional to the MICs for cephaloridine (Fig. 1). The second group was represented by only one strain which was highly resistant to ampicillin. The β-lactamase produced by the strain hydrolyzed ampicillin more readily than cephaloridine. The third group consisted of two strains, whose enzymes hydrolyzed both ampicillin and cephaloridine. The members of the fourth group were strains sensitive to the antibiotics; their enzyme activities were not detectable.

β-Lactamase Formation during the Growth

To determine the kinetics of β-lactamase formation, the relationship between bacterial growth and the enzyme activity were investigated. The appearance of the CSase activity in the surrounding medium, intact cells and crude extract preparations during different phase of growth of *B. fragilis* GN11477 was studied (Fig. 2). The maximal specific activity in both the intact cells and crude extracts was obtained after 4-hour incubation of the cells (middle log phase cells). Accordingly, the activity in the crude extract was more than twice that in the intact cells. Thereafter, the enzyme activity decreased rapidly with further incubation. After 24-hour incubation, the activity had fallen to about 10% of the maximal value. The enzyme was released from bacterial cells into surrounding medium and was maintained in the medium during the cell growth.

Table 3. Susceptibility of the *B. fragilis* group against β-lactam antibiotics and their β-lactamase activities.

Group	Organisms		MICs (μg/ml)		β-Lactamase activities[a]	
			CER[b]	APC	CER	APC
I	*B. fragilis*	GN11477	400	400	0.50	<0.01
		GN11480	400	200	0.51	<0.01
		GN11482	400	200	0.48	<0.01
		GN11483	400	200	0.51	<0.01
		GN11486	400	200	0.53	<0.01
	B. thetaiotaomicron	GN11478	800	400	1.15	<0.01
	B. vulgatus	GN11479	200	100	0.20	<0.01
II	*B. fragilis*	GN11499	100	≧1600	0.06	0.25
III	*B. fragilis*	GN11487	400	≧1600	0.58	0.11
	B. thetaiotaomicron	GN11487	800	≧1600	1.24	0.19
IV	*B. fragilis*	GN11495	6.25	3.13	<0.01	<0.01
	B. vulgatus	GN11496	3.13	1.56	<0.01	<0.01

[a] β-Lactamase activity expressed as units/mg of protein.
[b] Abbreviations: CER, cephaloridine; and APC, ampicillin.

Fig. 1. Correlation between the MIC and the cephalosporinase activity in the strains used.

MICs (μg/ml) and enzyme activities for *B. fragilis* (●, and GN11477, ○), *B. thetaiotaomicron* (■, and GN11478, □) and *B. vulgatus* GN11479 (△).

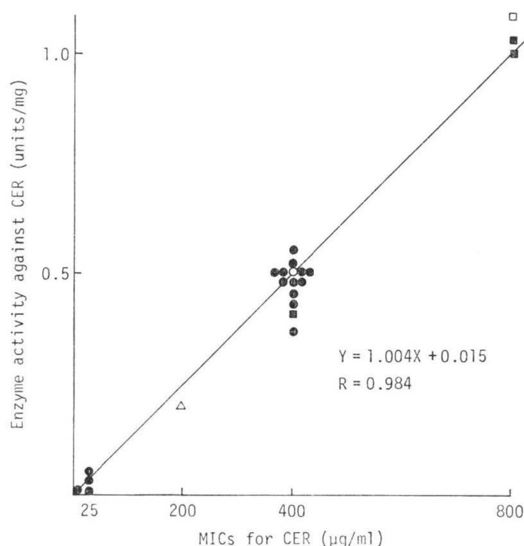
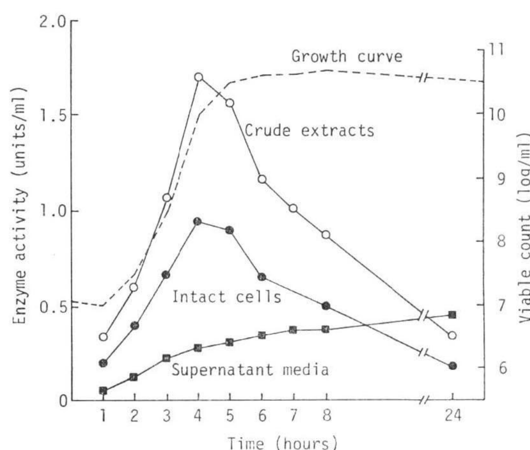


Fig. 2. Relationship between the bacterial growth and the formation of cephalosporinase in *B. fragilis* GN11477.

To determine the effect of the growth phase on the enzyme activity, cells growing in 1-liter BHI-CH medium (– – –) were harvested at 1 hour interval after inoculation and centrifuged (4,000 rpm for 15 minutes at 4°C).

The supernatant medium (■), intact cells (●) and crude extract (○) were assayed for enzyme activity using 0.1 mM of cephaloridine as substrate.



The maximal activities in the crude enzymes and intact cells of *B. thetaiotaomicron* GN11478 and *B. vulgatus* GN11479 were also examined, and found to appear 3 hours and 4 hours after incubation of growth, respectively. Both strains released the enzymes into the media at the same rates of GN11477.

Table 4. Purification of cephalosporinases by affinity chromatography from *B. fragilis* GN11477, *B. thetaiotaomicron* GN11478 and *B. vulgatus* GN11479.

Procedure	GN11477		GN11478		GN11479	
	Recovery[a]	Sp[b]	Recovery	Sp	Recovery	Sp
Ultrasonic disintegration	1,822	0.51 (1.0)[c]	4,550	1.15 (1.0)	905	0.20 (1.0)
Ultracentrifugation (45,000 rpm)	1,700	2.20 (4.3)	4,115	2.95 (2.6)	800	1.33 (6.65)
Affinity chromatography on activated Sepharose 4B (Ligand, APC, 0.5 M NaCl)	873	155.5 (311)	2,320	406.9 (330)	362	47.3 (270)

[a] Recovery of total activity (units).
[b] Specific activity (units/mg of protein).
[c] Purification factor.

Table 5. Kinetics of hydrolysis of β-lactam antibiotics by purified cephalosporinases from *B. fragilis* GN11477, *B. thetaiotaomicron* GN11478 and *B. vulgatus* GN11479.

Substrates	Cephalosporinase from								
	GN11477			GN11478			GN11479		
	Km[a]	*Ki*[b]	*Vmax*[c]	*Km*	*Ki*	*Vmax*	*Km*	*Ki*	*Vmax*
Cephaloridine	166		100	83		100	200		100
Cephalothin	100		50	91		81	111		79
Cefazolin	125		29	125		60	455		118
Cephalexin	ND[d]		<1	ND		<1	ND		<1
Cefuroxime	59		39	143		50	100		48
Cefamandole	48		20	10		6	19		12
Cefotiam	42		16	28		7	48		23
Cefsulodine	166		7	148		3	128		14
Cefmenoxime	22		8	69		6	29		9
Cefotaxime	77		21	63		7	135		22
Ceftizoxime	63		10	87		5	43		20
Cefoperazone	101		23	56		7	105		26
Cefpiramide	225		56	22		26	177		49
Cefoxitin		0.5			0.8			0.6	
Cefmetazole		0.2			0.2			0.2	
Cefotetan		0.2			0.3			0.2	
Latamoxef		0.1			0.1			0.2	
Benzylpenicillin	59		3	7		1	ND		<1
Ampicillin		0.6			0.6			2.1	
Carbenicillin		0.6			1.7			1.3	
Cloxacillin		0.4			0.4			0.6	
Clavulanic acid		0.2			0.1			0.2	
Sulbactam		0.3			0.2			0.5	
N-Formimidoyl-thienamycin		<0.1			<0.1			<0.1	

[a] *Km* values (μM).
[b] Rate of hydrolysis of substrate expressed as percentage of rate of cephaloridine as 100.
[c] *Ki* values (μM) were determined using cephaloridine as substrate.
[d] Not detectable.

Purification of Cephalosporinase by Affinity Chromatography

The enzyme from GN11477 was purified about 300-fold with a recovery rate of 48% (Table 4). The enzymes from GN11478 and GN11479 were also purified about 300-fold in the same way. Their recovery rates were about 50%. Each of the enzyme preparations gave a single protein band on sodium dodecyl sulfate-acrylamide gel electrophoresis.

Enzymatic Properties of the Purified Enzyme

The MICHAELIS constant (*Km*), the maximum rate of hydrolysis (relative *Vmax*) and the dissociation constant of enzyme-inhibitor complex (*Ki*) are shown in Table 5.

The enzymes of GN11477, GN11478 and GN11479 showed relatively high activity against cephaloridine, cephalothin, cefazolin, cefuroxime and cefpiramide. Cefotaxime, cefamandole, cefoperazone, ceftizoxime, cefotiam and cefmenoxime were also hydrolyzed by the enzymes, though the rates were lower than those of the antibiotics mentioned above. However, the kinetic parameters of the three enzymes to some substrates, such as cefazolin, cefuroxime, cefamandole, cefotiam, cefmenoxime and cefoperazone, were different from one another (Table 5). Cefoxitin, cefmetazole, cefotetan and latamoxef (cephamycin derivatives) showed resistance to hydrolysis by the three enzymes. Ampicillin, carbenicillin and cloxacillin also showed such resistance. Furthermore, clavulanic acid, sulbactam and *N*-formimidoylthienamycin exhibited high affinity and resistance to the enzymes (low *Ki* values).

Inhibitory Action of Inhibitors

A number of enzyme inhibitors, divalent ions and several β-lactams which are resistance to the hydrolysis by the enzymes, were tested for their inhibitory effects on the activities of the enzymes. Iodine, *p*-chloromercuribenzoate (PCMB) and Hg^{++} ion completely inhibited the enzyme activities, but Co^{++}, Zn^{++} and Mg^{++} ions failed to inhibited the activities at a concentration of 0.5 mM.

Physicochemical Properties

Molecular weights of the enzymes from GN11477, GN11478 and GN11479 were estimated to be 32,000, 29,500 and 32,200, respectively, on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The isoelectric points of the enzymes of GN11477, GN11478 and GN11479 were 5.2, 4.9 and 4.5, respectively. The temperature of 37°C was optimal for the activities of all three enzymes. The optimal pH was about 7.2, and the enzymes showed less than 50% of their maximal activities at pH 5.5 and 8.5 when cephaloridine was used as the substrate.

Immunological Properties

The neutralizing capacity of the antisera was checked with the three enzymes obtained from *Bacteroides* strains. Each of the antisera to GN11477, GN11478 and GN11479 completely neutralized not only the activities of their own enzymes, but also those of the other enzymes from the *B. fragilis* group. However, none of the other types of cephalosporinases from *Escherichia coli* and *Enterobacter cloacae*, and the plasmid-mediated penicillinases (type 1~4)[39] were affected by the antisera.

Discussion

Several investigators have reported that β-lactamases from Gram-positive and Gram-negative bacteria including *B. fragilis* have a significant role in the resistance of the organisms against β-lactam antibiotics[13,39,40]. The enzymes produced by *B. fragilis* mainly hydrolyzed cephalosporins more readily than penicillins[12~17]. The work presented in this paper confirmed the relationship between MICs for

cephaloridine and the amount of CSase in the *B. fragilis* group, which includes *B. thetaiotaomicron* and *B. vulgatus* (Group I). The enzyme-producing strains were highly resistant to novel cephalosporins such as cefotaxime, cefmenoxime, cefoperazone and cefpiramide which possess greater potency against Gram-negative bacteria[21~23,25]. These findings will complicate the treatment of *B. fragilis* infections which are refractory to chemotherapy by many commonly employed antibiotics.

The CSases of *B. thetaiotaomicron* GN11478, *B. vulgatus* GN11479 and *B. fragilis* GN11477 were released into the surrounding media during the growth. This result was similar to that reported by OLSSON *et al.*[16]. They found that in the stirred stationary-phase culture of the strains of *B. fragilis*, CSase was released into the extracellular fluid at a rate of 50%. The maximal activities of the enzyme in the intact cells and the crude extracts were observed 3 or 4 hours after initiation of the growth, and thereafter the enzyme activities decreased rapidly. This fact might be due to proteolytic enzymes produced by the organisms or to a spontaneous bacteriolytic effect during the growth.

Physicochemical properties of the three enzymes such as molecular weights and isoelectric points were found to be similar to one another. The enzymes hydrolyzed not only cephaloridine, cephalothin and cefazolin but also members of cefuroxime group such as cefuroxime, cefotaxime and ceftizoxime, cefoperazone, cefmenoxime and cefpiramide. However, the kinetic parameters of the enzymes against some substrates were different. The enzymes hardly hydrolyzed cephalexin, cefoxitin, cefmetazole, cefotetan, latamoxef and penicillins. Each mouse antiserum against the purified enzymes showed cross-reactions with its enzyme and the others in the neutralization test. From these enzymological and immunological properties, it is assumed that there is a close mutual resemblance among the CSases from *B. fragilis*, *B. thetaiotaomicron* and *B. vulgatus*.

MITSUHASHI and INOUE[39] surveyed a wide range of β-lactamases from Gram-negative and Gram-positive bacteria to compare the enzymatic, physicochemical and immunological properties of the enzymes, and classified them into three groups: 1) species-specific cephalosporinase, 2) cefuroximase, and 3) penicillinase. Properties of the enzymes produced by *B. thetaiotaomicron* and *B. vulgatus* as well as *B. fragilis* were found to be similar to that of cefuroximase. RICHMOND and SYKES[40] also classified β-lactamases from Gram-negative bacteria into 15 types on the basis of the substrate and sensitivity to inhibitors (PCMB or cloxacillin). The enzymes from the strains used did not fall into any of the 15 types.

The species-specific cephalosporinase (RICHMOND type I) was inhibited by cefoxitin and other cephamycins, but clavulanic acid and sulbactam did not inhibit it completely[39,40]. On the other hand, the activities of the penicillinases (RICHMOND type II, III, IV and V) were strongly inhibited by clavulanic acid, but not by cefoxitin[39,40]. However, the activities of purified GN11477, GN11478 and GN11479 enzymes were inhibited by cefoxitin and clavulanic acid. β-Lactamases of the cefuroximase type from *Proteus vulgaris* and *Pseudomonas cepacia* hydrolyzed cefuroxime-group cephalosporins at high rates[41,42]. From this point of view, the CSases from *B. fragilis*, *B. thetaiotaomicron* and *B. vulgatus* are classified as being of the cefuroximase type. However, the both β-lactamases from *P. vulgaris* and *P. cepacia* hydrolyzed benzylpenicillin and ampicillin at relatively high rates[41,42], though the CSases from the *B. fragilis* group strains did not hydrolyze penicillins. In addition, the isoelectric points of the enzymes from the *B. fragilis* group ranged from 4.5 to 5.2, and lower than those of *P. vulgaris*, *P. cepacia* and other CSases (7.8 ~ 8.5)[39].

On the basis of the above evidence, we conclude that the CSases produced by *B. thetaiotaomicron* and *B. vulgatus* as well as *B. fragilis* are a unique type of β-lactamase.

Furthermore, we found penicillinase-producing strains (Group II and III) which showed a high resistance to ampicillin not previously reported. It is planned to study the properties of the penicillinase and the transferability of the genetic determination for the enzyme.

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