BIOCHEMICAL PROPERTIES OF \( \beta \)-LACTAMASE PRODUCED
BY *BACTEROIDES DISTASONIS*

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Three of 29 clinical isolates of *Bacteroides distasonis* strains, GAI2095, GAI2361 and GAI6270 were found to produce high levels of \( \beta \)-lactamase relative to the remaining 26 strains. The enzymes from these strains showed a broad substrate profile, hydrolyzing cephaloridine, cephalothin, cefazolin, cefuroxime, cefotaxime and cefmenoxime, and piperacillin at high rates. Examination of the substrate profiles indicated that the enzymes were mainly oxyimino-cephalosporinases. Inactivation of latamoxef over a long time by crude enzyme extracts of GAI2095 and GAI2361 was detected by a microbiological assay. The enzyme activities were inhibited by imipenem, cefoxitin, clavulanic acid and sulbactam but not by EDTA. The majority of \( \beta \)-lactamase activity was found in the cell extract prepared by ultrasonic treatment, especially in the precipitate by ultracentrifugation including membrane fraction. When the cells of *B. distasonis* were subjected to osmotic shock, negligible levels of \( \beta \)-lactamase activity were found in the supernatant fluid. The enzymes appeared to be tightly associated with the cell envelope since detergents were required to elute these activities.

Members of the *Bacteroides fragilis* group have been recognized as important opportunistic pathogens and are known to be moderately or highly resistant to \( \beta \)-lactam antibiotics.

\( \beta \)-Lactamases produced by Gram-positive and Gram-negative bacteria are considered to be the most important biochemical mechanism of resistance to \( \beta \)-lactam antibiotics. Several investigations have already described \( \beta \)-lactamases from *Bacteroides* species. However, only limited work has been done on the \( \beta \)-lactamase from *Bacteroides distasonis*.

In this paper, we deal with the biochemical properties of \( \beta \)-lactamase produced by *B. distasonis*, and also investigate the cellular location of the enzyme.

Materials and Methods

Bacterial Strains

Twenty-nine strains of *B. distasonis* isolated from clinical specimens were used. All of the strains were identified by the method of the Virginia Polytechnic Institute and were given our laboratory strain numbers. *B. fragilis* GAI558 (a clinical isolate) was chosen as representative control strain produced the typical oxyimino-cephalosporinase. These strains were stored in skim milk (10\%) at \(-70^\circ\)C.

Antibiotics

Benzylpenicillin, ampicillin, cloxacillin, piperacillin, sulbencillin, cephaloridine, cephalothin, cefazolin, cefuroxime, cefotaxime, cefmenoxime, cefoxitin and latamoxef were commercially available materials. The following compounds were gifts: Imipenem from Banyu Pharmaceutical Co., Ltd.; sulbactam from Pfizer Taito Co., Ltd.; and clavulanic acid from Beecham Research Laboratories.

Susceptibility Testing

Drug resistance was determined by an agar dilution method with GAM agar (Nissui Seiyaku
Co., Ltd., Tokyo). Of a diluted culture (ca. 10^6 cells per ml), one loopful (ca. 5 µl) was inoculated onto assay media containing serial 2-fold dilutions of drug. MICs of each drug were scored after incubation at 37°C for 24 hours in an anaerobic chamber.

**Culture Condition**
Cells were grown in GAM broth (Nissui Seiyaku Co., Ltd., Tokyo). Usually, a 10% inoculum from an overnight starter culture was added to bottles of broth and the cultures were grown statically at 37°C in an anaerobic chamber.

**Preparation of Crude β-Lactamase**
Cultures in the late exponential phase of growth were harvested by centrifugation at 5,000 × g for 15 minutes at 4°C and washed twice in 0.1 M phosphate buffer (pH 7.0). The cells were resuspended at about 0.05 of the volume of the original culture, in 0.1 M phosphate buffer (pH 7.0) and sonicated in an ultrasonic disruptor (TOMY SEIKO) for three periods of 1 minute each at 0°C. The supernatants after centrifugation (10,000 or 20,000 × g, 20 minutes at 4°C) constituted the crude enzyme.

**Enzyme Assay**
β-Lactamase activity was assayed by a modification of the micro-iodometric method of Novick with penicillins as substrates or by a direct spectrophotometric method of Waley with cephalosporins as substrates. One u of β-lactamase activity was expressed as 1 µmol of substrate hydrolyzed per minute at 30°C in 50 mM phosphate buffer. The Km and the maximum rate of hydrolysis (relative Vmax) values were determined from Lineweaver-Burk plots.

Alkaline phosphatase activity was measured by using p-nitrophenylphosphate as the substrate; µmol of end product (p-nitrophenol) were estimated.

**Antibiotic Assay**
Concentrations of cephapemycins and imipenem were measured by a microbiological assay with *Escherichia coli* NIHJ as the test strain.

**Inhibition Study**
The inhibition of the enzyme activity in the crude extract by various inhibitors was estimated by the micro-iodometric method using 200 µM piperacillin or cephaloridine. The inhibition activities of 0.1, 1.0 and 10.0 µM solutions of imipenem, cefoxitin, clavulanic acid and sulbactam, and 10 mM EDTA were determined after preincubation for 5 minutes at 30°C.

**Isoelectric Focusing**
Isoelectric focusing of crude sonicates was performed on a sucrose/ampholytes gradient (pH 3–10). The experiment was carried out at 4°C in the LKB8100 column over 24 hours at 900 V.

**Solubilization of β-Lactamase Activity**
To elute β-lactamase activity from the centrifugal precipitates, the precipitates were resuspended in each of the following: 1 M NaCl, 2% Triton X-100, 2% sodium deoxycholate, 2% sodium deoxycholate. After 20 minutes at room temperature, the mixture was centrifuged at 20,000 × g for 30 minutes at 4°C. The supernatant and resuspended pellet were assayed for β-lactamase activity.

**Purification of β-Lactamase**
Preliminary attempts to purify the crude β-lactamase preparations involved treatment of the enzyme with 2% sodium deoxycholate and elution through a Toyopearl HW-55F column (2.0 × 85 cm) which was equilibrated at 4°C in glycine-EDTA buffer (pH 9.0), containing 0.5% deoxycholate and eluted with the same buffer.

The β-lactamase was partially purified from the crude enzyme preparations described above by gel filtration on Toyopearl HW-65F and HW-55F columns. Toyopearl HW-65F was equilibrated at 4°C in 10 mM phosphate buffer (pH 7.0), containing 0.1 M KCl in a column (2.5 × 90 cm) and eluted with the same buffer. The concentrated elute from the Toyopearl HW-65F column was applied to the Toyopearl HW-55F column described above and eluted with the same buffer. Fractions showing β-lactamase activity were pooled and dialyzed against 10 mM phosphate buffer (pH 7.0). The
enzyme preparation was used for kinetics.

**Cellular Location of the Enzyme**

The cellular location of β-lactamase in *B. distasonis* was explored in two ways.

(i) Osmotic shock was performed by the method described by Neu and Chou (1970). The washed late exponential-phase cells were suspended in 20% sucrose - 30 mM Tris buffer (pH 7.3). EDTA was added to a concentration of 0.5 mM, and after 5 to 10 minutes of mixing, the cells were removed by centrifugation. The pellet of cells was resuspended in water at 0°C and mixed for 5 to 10 minutes. The cells were removed by centrifugation. To elute the enzyme from the centrifugal precipitate, the precipitate was resuspended in 2% Triton X-100.

Viability was determined on serial dilutions, made in GAM broth, which were plated on GAM agar.

(ii) The supernatants after centrifugation of the sonicated cell suspensions at 10,000 × g for 20 minutes at 4°C were ultracentrifuged at 140,000 × g for 2 hours at 4°C. To elute β-lactamase from ultracentrifugal precipitates, the precipitates were resuspended in 2% Triton X-100. After 30 minutes at room temperature, the mixture was ultracentrifuged at 140,000 × g for 2 hours at 4°C.

All supernatant fluids and cells were reserved for enzyme assay.

**Results**

**Susceptibility to β-Lactam Antibiotics**

Twenty-nine strains of *B. distasonis* were screened with a nitrocefin assay (1977) to determine their ability to produce β-lactamase. Strains GAI2095, GAI2361 and GAI6270 were the best producers of β-lactamase and were chosen for the biochemical studies. Resistance levels of these strains to β-lactam antibiotics are shown in Table 1. *B. distasonis* GAI2095, GAI2361 and GAI6270 were highly resistant to penicillins and cephalosporins except cefoxitin and imipenem. *B. fragilis* GAI558, which was chosen for comparison, was moderately resistant to penicillins and cephalosporins, except cefoxitin, latamoxef and imipenem.

**β-Lactamase Formation during the Growth**

The appearance of β-lactamase activity in the surrounding medium and crude extract prepara-

<table>
<thead>
<tr>
<th>Compound</th>
<th>GMI2095</th>
<th>GMI2361</th>
<th>GMI6270</th>
<th>GMI558</th>
</tr>
</thead>
<tbody>
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<td>Cephaloridine</td>
<td>1,600</td>
<td>1,600</td>
<td>800</td>
<td>400</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>1,600</td>
<td>1,600</td>
<td>800</td>
<td>400</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>1,600</td>
<td>1,600</td>
<td>400</td>
<td>400</td>
</tr>
<tr>
<td>Cefuroxime</td>
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<td>50</td>
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<td>12.5</td>
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<td>400</td>
<td>100</td>
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<td>100</td>
</tr>
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<td>3,200</td>
<td>1,600</td>
<td>400</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>3,200</td>
<td>3,200</td>
<td>3,200</td>
<td>200</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>&gt;3,200</td>
<td>&gt;3,200</td>
<td>3,200</td>
<td>400</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>&gt;3,200</td>
<td>&gt;3,200</td>
<td>1,600</td>
<td>400</td>
</tr>
<tr>
<td>Sulbenicillin</td>
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<td>3,200</td>
<td>1,600</td>
<td>200</td>
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<tr>
<td>Piperacillin</td>
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<td>200</td>
<td>400</td>
<td>50</td>
</tr>
<tr>
<td>Imipenem</td>
<td>1.56</td>
<td>1.56</td>
<td>1.56</td>
<td>0.39</td>
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</table>
tions during different phases of growth of *B. distasonis* GA12095 was studied. The maximal activity in the crude cell extracts was obtained in the late exponential phase after 7-hour incubation. Enzyme activity was not detected in the surrounding medium during cell growth.

## Solubilization of β-Lactamase Activity

The β-lactamase activities of *B. distasonis* GA12095, GA12361 and GA16270 could be extracted from the centrifugal precipitate at almost the same level with 2% Triton X-100 and 2% deoxycholate, but not with 1 M NaCl. The enzyme activities were lost when 2% sodium dodecyl sulfate was added to the pellet. Approximately 5% of β-lactamase activity still remained in the Triton X-100-treated precipitates. Triton X-100 and deoxycholate in these concentrations had no effect on the *B. distasonis* β-lactamase activity.

## Preliminary Purification of β-Lactamase

Fig. 1 (A) and (B) represent the UV absorption and β-lactamase activity profile during gel filtration of crude sonicates from *B. fragilis* GA1558 (A) and *B. distasonis* GA12095 (B) on Toyopearl HW-55F. Peak enzyme activity for the extract of strain GA1558 was eluted in fraction 71.

However, the enzyme activity from strain GA12095 was eluted in the void volume of the column. The range of the molecular weight calibration of Toyopearl HW-55F is $1 \times 10^3$ to $7 \times 10^5$. Therefore, the apparent molecular weight of the enzyme was $> 7 \times 10^5$.

Fig. 1 (C) shows the UV absorption and β-lactamase activity profile of the same crude sonicated extracts after treatment with deoxycholate as Fig. 1 (B). The β-lactamase activity peak shifted from the void volume of the column to fractions 58.

Calibration of the Toyopearl HW-55F column in the presence of deoxycholate with the protein standards indicated that the molecular weight of the deoxycholate-treated β-lactamase of *B. distasonis* GA12095 was approximately 43,000.

## Enzymatic Properties of the Enzyme

The kinetic parameters (*Km* and relative *Vmax*) of partially purified enzymes from *B. distasonis* and *B. fragilis* are shown in Table 2. The enzymes from *B. distasonis* GA12095 and GA12361 showed high activity against cephaloridine, cefazolin, cefuroxime, cefotaxime and piperacillin. Cephalothin, cefmenoxime and penicillins such as benzylpenicillin, ampicillin, carbenicillin, cloxacillin and sulbenicillin were also hydrolyzed, although the rates of hydrolysis were lower than that of cephaloridine. The enzymes from *B. distasonis* GA16270 and *B. fragilis* GA1558 showed high activity against cephalosporins, including oxyimino-cephalosporins. By the spectrophotometric method, detectable hydrolysis of cefoxitin, latamoxef and imipenem by the enzymes was not observed.

Hydrolysis of cefoxitin, latamoxef and imipenem was studied with crude enzyme extracts from *B. distasonis* strains. Each antibiotic (at a final concentration of 100 μM) was added to the crude enzyme extract. After 6 hours of incubation at 30°C, the residual amounts of the antibiotics were determined by a microbiological assay. Latamoxef was hydrolyzed by crude enzyme extracts from *B. distasonis* GA12095 and GA12361 (Table 3).

A number of enzyme inhibitors were tested for their inhibitory effect on the activity of the enzymes. The enzyme activities were almost completely inhibited by 0.1 μM clavulanic acid, sulbactam, cefoxitin and imipenem, but not by 10 mM EDTA.
Fig. 1. Toyopearl HW-55F column chromatography of crude sonicates from *Bacteroides fragilis* GAI558 (A) and *B. distasonis* GAI2095 (B), and the deoxycholate-treated enzyme of strain GAI2095 (C).

- UV absorbance (280 nm), ◦ β-lactamase activity.

(A)

(B)

(C)

Isoelectric Focusing

Crude sonicates of strains GAI2095 and GAI2361 showed two peaks of β-lactamase, with isoelectric points of 4.0 and 3.5, while strain GAI6270 displayed one peak at 3.7.
Table 2. Kinetic parameters of crude β-lactamaseα from Bacteroides distasonis strains.

<table>
<thead>
<tr>
<th>Compound</th>
<th>B. distasonis</th>
<th>B. fragilis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GAI2095</td>
<td>GAI2361</td>
</tr>
<tr>
<td>Vmaxb (μM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Km (μM)</td>
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<td></td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>34</td>
<td>40</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>270</td>
<td>225</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>201</td>
<td>252</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>104</td>
<td>93</td>
</tr>
<tr>
<td>Cefmenoxime</td>
<td>37</td>
<td>46</td>
</tr>
<tr>
<td>Benzylpenicillin</td>
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<td>21</td>
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<tr>
<td>Ampicillin</td>
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<td>38</td>
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<tr>
<td>Carbenicillin</td>
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<td>12</td>
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<tr>
<td>Cloxacillin</td>
<td>7.8</td>
<td>9.4</td>
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<tr>
<td>Sulbenicillin</td>
<td>5.6</td>
<td>5.4</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>238</td>
<td>220</td>
</tr>
</tbody>
</table>

a β-Lactamase activities for cephaloridine of B. distasonis GAI2095, GAI2361 and GA16270, and B. fragilis GAI558 were 0.36, 0.26, 0.78 and 1.71 μg/mg of protein, respectively.

b Hydrolysis is expressed as the relative rate of hydrolysis, taking the hydrolysis of cephaloridine as 100.

ND: Not detectable.

Table 3. Hydrolysis of antibiotics by crude enzymes from Bacteroides distasonis strains.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Latamoxef</th>
<th>Cefoxitin</th>
<th>Imipenem</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. distasonis GAI2095</td>
<td>4 (1,600)</td>
<td>78 (50)</td>
<td>75 (1.56)</td>
</tr>
<tr>
<td>B. distasonis GAI2361</td>
<td>6 (1,600)</td>
<td>82 (50)</td>
<td>72 (1.56)</td>
</tr>
<tr>
<td>B. distasonis GA16270</td>
<td>89 (100)</td>
<td>98 (50)</td>
<td>71 (1.65)</td>
</tr>
<tr>
<td>B. fragilis GAI558</td>
<td>95 (12.5)</td>
<td>95 (6.25)</td>
<td>77 (0.39)</td>
</tr>
<tr>
<td>Control‡</td>
<td>99</td>
<td>99</td>
<td>72</td>
</tr>
</tbody>
</table>

α Residual amounts of cephamycins and imipenem were determined by a microbiological assay.

β MIC (μg/ml).

Ge 50 mM phosphate buffer (pH 7).

Cellular Location of the Enzyme

β-Lactamases in aerobic Gram-negative rods are considered to be located in the periplasmic space, as well as other enzyme (alkaline phosphatase, 3'-nucleotidase and 5'-nucleotidase). Alkaline phosphatase was measured in parallel as a reference enzyme for periplasmically located enzymes.

Cells of B. distasonis GAI2095, GAI2361 and GA16270, and B. fragilis GAI558 were subjected to osmotic shock. Fig. 2 shows that a high level of alkaline phosphatase activity of strains GAI2095, GAI2361, GA16270 and GAI558 was found in the water shocking fluid (70~95% of the total activity), when the total enzyme was determined as a total of the activities in the water shocking fluid and Triton X-100-treated extracts from the sedimented materials. Similarly, strain GAI558 released a high level of β-lactamase in the water shocking fluid (60%), whereas strains GAI2095, GAI2361 and GA16270 released only 3~12% of total activity. Remaining cell-bound activity (88~97%) was found in the cell lysate. More than 99% of B. distasonis and B. fragilis cells were irreversibly damaged by the EDTA treatment and the cold shock.

When sonicated cell suspensions were centrifuged for 20 minutes at 10,000 x g and the supernatants were ultracentrifuged for 2 hours at 140,000 x g, 98% of β-lactamase activity of strain GAI558 ap-
peared in the supernatant, but in the case of strains GAI2095, GAI2361 and GAI6270, over 75% of the activity was found in the pellet (Fig. 3).

Discussion

Several authors have suggested that many strains of B. fragilis group produce β-lactamase that contributed to their resistance to β-lactam antibiotics, and there is a general correlation between the β-lactamase activity and the strain’s antibiotic MIC\(^3\text{--}^7\). Our results confirmed B. distasonis GAI2095, GAI2361 and GAI6270 were highly resistant to penicillins and cephalosporins, including oxyiminocephalosporins and latamoxef, and also β-lactamase high producer. The β-lactamases of GAI2095, GAI2361 and GAI6270 showed relatively high activity against cephalosporins, especially oxyiminocephalosporins. The substrate profiles and isoelectric points obtained for strains GAI2095 and GAI2361 seem almost identical, and are different from that of strain GAI6270. By the spectrophotometric method, detectable hydrolysis of latamoxef was not observed.

However, inactivation of latamoxef over a long time by crude enzyme extracts of GAI2095 and GAI2361 was detected by the microbiological assay. Mitsuhashi et al.\(^8\) suggested that inactivation due to enzymatic hydrolysis over a long time may play an important role in resistance to some cephamycins in strains of B. fragilis. We conclude that the enzymatic hydrolysis of latamoxef over a long time may contribute to the resistance of these strains.

The sensitivity to different β-lactamase inhibitors was similar to that of the enzymes from the other B. fragilis group. They were inhibited by cefoxitin, clavulanic acid and sulbactam at low concentrations but not by EDTA.

Mitsuhashi et al. reported that oxyimino-cephalosporins were hydrolyzed at high rates by β-lactamase from B. fragilis\(^9\), Proteus vulgaris\(^10\), Pseudomonas cepacia\(^11\), Pseudomonas maltophilia\(^12\), Flavobacterium odoratum\(^13\), Flavobacterium meningosepticum\(^14\) and Legionella gormanii\(^15\). These enzymes can be divided into two subgroups, type I (B. fragilis, P. vulgaris, P. cepacia, L-2 from P. maltophilia and F. meningosepticum) and type II (F. odoratum, L-1 from P. maltophilia and L. gormanii), by substrate and inhibitor profiles. According to this classification, the B. distasonis β-lactamase belongs to type I.

Results from the studies of cellular location of the enzyme suggest that the enzyme was tightly associated with the cell envelope, possibly membrane-bound. The enzyme was not released into the
surrounding medium during growth. Only levels of the enzyme were released by osmotic shock. The enzymatic activity remains associated with the sedimented material after ultracentrifugation and the enzyme could be solubilized with Triton X-100 from this precipitate. The cellular location of the *B. distasonis* enzyme seems to be distinct from that of the *B. fragilis* enzyme which was periplasmically located or in loose association with the cell envelope.

References