Purification and Properties of Inducible Penicillin β-Lactamase Isolated from Alcaligenes faecalis

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An inducible penicillin β-lactamase was purified from a strain of Alcaligenes faecalis resistant to β-lactam antibiotics. The purified enzyme preparation gave a single protein band on polyacrylamide gel electrophoresis, and its molecular weight was 29,000 based on sodium dodecyl sulfate-acrylamide gel electrophoresis. Its isoelectric point was 5.9. The enzyme more rapidly hydrolyzed penicillins, such as penicillin G, ampicillin, carbenicillin, piperacillin, and cloxacillin, than it hydrolyzed cephalosporins. For the hydrolysis of penicillin G, the optimal pH was 5.5, and the optimal temperature was 35°C. The enzyme activity was inhibited by iodine, Cu²⁺, Hg²⁺, and EDTA but was not inhibited by clavulanic acid and sublactam.

Alcaligenes faecalis, a glucose-nonfermenting, gram-negative rod, has recently been isolated from clinical specimens (13).

We collected 39 strains of A. faecalis from urinary tract infections and found that most of these strains were resistant to β-lactam antibiotics. β-Lactamase has been considered to be one of the important biochemical mechanisms of resistance to β-lactam antibiotics in bacteria. Many investigations have already described β-lactamase from glucose-nonfermenting, gram-negative rods (2, 11). However, there have been no reports on the β-lactamase of A. faecalis.

We report here the purification and biochemical properties of penicillinase (PCase) produced by A. faecalis GN14061.

MATERIALS AND METHODS

Bacterial strains. From patients with urinary tract infection, 39 strains of A. faecalis were isolated. All strains were maintained until use at −80°C in skim milk containing 5% glucose (pH 7.4).

Media. Brain heart infusion broth (Difco Laboratories) and sensitivity disk agar N and sensitivity test broth (Nissui Pharmaceutical Co., Ltd.) were used. Medium B (5) was used for large-scale culture.

Drugs. Penicillin G (PCG), ampicillin, carbenicillin, cloxacillin, cephaloridine, cephalothin, and cefazolin were commercially available materials. We received the following compounds as gifts from manufacturers: piperacillin, cefturoxime, cefotaxime, cefizoxime, cefoperazone, cefpiramide, aztreonam, clavulanic acid, imipenem, sulbactam, and cefotixin.

Determination of antibacterial activity. MICs were determined by a standard agar dilution method with sensitivity disk agar N. Of diluted culture (ca. 10⁶ CFU/ml), one loopful (ca. 5 μl) in sensitivity test broth was inoculated onto assay media containing serial twofold dilutions of drug. MICs were determined after incubation at 37°C for 18 h. Inducibility of β-lactamase production. An overnight culture grown in brain heart infusion broth was diluted 20-fold with medium B and incubated with shaking at 37°C until the optical density of the culture at 570 nm reached ca. 0.4 (mid-log phase). PCG was added to a final concentration of 25 μg/ml as an inducer for β-lactamase production, and incubation was continued. Portions were harvested by centrifugation at 1, 2, 3, 4, and 5 h after the addition of inducer, and β-lactamase activity in sonically disrupted cells was assayed spectrophotometrically with 200 μM PCG as a substrate.

Preparation of crude enzyme. An overnight culture in 500 ml of brain heart infusion broth was diluted 20-fold with medium B and cultured with shaking at 37°C for 2 h. PCG was added as an inducer for β-lactamase production at a final concentration of 400 μg/ml, and growth with shaking was continued. After 3 h of incubation, the bacterial cells were harvested by centrifugation at 4°C, washed twice with 50 mM phosphate buffer (pH 7.0), and suspended in the same buffer at 5% of the original volume. The cell suspension was sonicated in an ultrasonic disrupter (UR-150; Tomy Seiko Co., Ltd.) at 75 W for 3 min in an ice bath. The disrupted cell suspension was centrifuged at 30,000 × g for 40 min at 4°C. Streptomycin sulfate was added to the resulting supernatant fluid in a final concentration of 2% (wt/vol) for the removal of nucleic acid. After standing for 10 h, the precipitate was removed by centrifugation, and the supernatant was dialyzed against 10 mM Tris-hydrochloride buffer (pH 8.8). The precipitate formed during dialysis was removed by centrifugation, and the resulting supernatant was used as the crude enzyme preparation.

Purification of β-lactamase. The crude enzyme solution was applied to a DEAE-Toyopearl 650 M column (3.0 by 35 cm) equilibrated with 10 mM Tris-hydrochloride buffer (pH 8.8). The enzyme was eluted at a flow rate of 80 ml/h with a linear gradient of NaCl from 0 to 0.5 M in a total volume of 400 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 50 ml/h with 50 mM phosphate buffer (pH 7.0) containing 0.1 M KCl. The active fractions were dialyzed against distilled water and purified with an ampholine electrofocusing apparatus (model 8100; LKB Instruments, Inc.). Electrophoresing was carried out by
using carrier ampholine (pH 3.5 to 10) with a sucrose density gradient at 4°C for 72 h.

**Assay for β-lactamase.** β-Lactamase activity was determined by a direct spectrophotometric method described previously (2, 6), a modification of the Novik microiodometric method (8). The \( K_m \) and the maximum rate of hydrolysis (relative \( V_{max} \)) were determined by the Lineweaver-Burk plot. The dissociation constants \( (K_i) \) of the enzyme-inhibitor complex were determined by the Dixon plot.

**Determination of protein.** Protein determination was carried out by the method of Lowry et al. (4) with bovine serum albumin as the standard.

**Determination of the pl.** For determination of the pl, gel electrophoresis was carried out with a gel of 6% polyacrylamide (in 2% ampholine [pH 3.5 to 10]). Portions of acetylated cytochrome \( c \) from horse heart tissue (pl 4.1, 4.9, 6.4, 8.3, 9.7, and 10.6) were used as pl markers. Gel electrophoresis with 20 μl of the purified enzyme was performed at 8 mA per plate for 6 h.

**Determination of M.W.** The molecular weight (M.W.) was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Laemmli and Farve (3). The purified enzyme and marker proteins were treated with 10% sodium dodecyl sulfate and 5% 2-mercaptoethanol at 100°C for 2 min and then subjected to the electrophoresis in a 10% gel with a current of 30 mA at room temperature. Marker proteins used (and their M.W.s) were bovine serum albumin (67,000), ovalbumin (45,000), chymotripsinogen A (25,000), and myoglobin (17,000).

**Inhibition study.** A solution of enzyme was preincubated with a test compound in 50 mM phosphate buffer (pH 7.0), and the remaining enzyme activity was assayed spectrophotometrically with 200 μM PCG as the substrate.

## RESULTS

**Inducibility of β-lactamase production.** The kinetics of β-lactamase induction in *A. faecalis* GN14061 are shown in Fig. 1. Without the addition of PCG, β-lactamase activity was not detected during any phase of growth. The maximum specific activity (micromoles of PCG hydrolyzed per minute per milligram of protein) was obtained at 1 h after the addition of PCG.

**Purification of β-lactamase.** The purification procedure for the enzyme from *A. faecalis* GN14061 is summarized in Table 1. The β-lactamase was purified from the crude enzyme solution (100 ml) obtained from 22.5 g (wet weight) of washed cells. The enzyme was purified ca. 128-fold and gave a single protein band on polyacrylamide gel electrophoresis. The purified enzyme was 1.2 mg of protein.

**Physicochemical properties of the purified enzyme.** The approximate M.W. of the purified enzyme was 29,000, as estimated by gel electrophoresis. The pl was 5.9.

**Enzymological properties of the purified enzyme.** The kinetic parameters \((K_m, \text{ relative } V_{max}, K_i)\) of the enzyme and the levels of resistance of strain GN14061 to various β-lactam antibiotics were determined. *A. faecalis* GN14061 was resistant to PCG, ampicillin, cloxacillin, and carbenicillin (Table 2). The enzyme showed much higher hydrolyzing activity against penicillins such as PCG, ampicillin, piperacillin, cloxacillin, and carbenicillin than against any of the cephalosporins tested. The optimal pH for activity was around 5.5. A temperature of 35°C was optimal for this enzyme. The effects of some inhibitors and ions on enzyme activity are shown in Table 3. The enzyme activity was almost completely inhibited by iodine, Cu\(^{2+}\), or Hg\(^{2+}\), but was not inhibited by clavulanic acid or sulbactam. Enzyme

### Table 1. Summary of purification of β-lactamase from *A. faecalis* GN14061

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total activity* (U)</th>
<th>Sp act (U/mg of protein)</th>
<th>Purification fold</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultrasonic disintegration</td>
<td>9,870</td>
<td>3.8</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Streptomycin treatment</td>
<td>9,297</td>
<td>3.8</td>
<td>1.0</td>
<td>93</td>
</tr>
<tr>
<td>Chromatography on DEAE-Toyopearl</td>
<td>6,110</td>
<td>232</td>
<td>61</td>
<td>62</td>
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<tr>
<td>650 M column</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Gel filtration on Toyopearl HW-55F</td>
<td>1,311</td>
<td>471</td>
<td>124</td>
<td>13</td>
</tr>
<tr>
<td>Electrofocusing</td>
<td>581</td>
<td>486</td>
<td>128</td>
<td>6</td>
</tr>
</tbody>
</table>

* Activity was assayed spectrophotometrically with PCG (200 μM) as the substrate. Activity (1 U) is expressed as 1 μM of PCG hydrolyzed per min at 30°C in 50 mM phosphate buffer (pH 7.0).
activity was not 100% inhibited by 3 mM EDTA treatment. After inhibition by EDTA, 100% of the enzyme activity was regained after dialysis against distilled water.

**DISCUSSION**

β-Lactamase from *A. faecalis* GN14061 was an inducible enzyme and seemed to be a penicillin β-lactamase, based on its substrate specificity. There have been few reports of inducible PCase in gram-negative bacteria (12, 14).

The purified β-lactamase showed a unique substrate profile and high activity against penicillin G, ampicillin, carbenicillin, piperacillin, and cloxacillin. The substrate profile of enzyme from *A. faecalis* GN14061 showed it to be typical of the PCase type; that is, this enzyme did not hydrolyze cephalosporins including cephaloridine, cephap tololin, and cefazolin. The substrate profile of the GN14061 enzyme is different from any other PCase types, such as I, II, III, IV, and V (7), TEM types (14), or PCase type II (9), in cloxacillin and cephaloridine hydrolysis. Although a PCase, its activity was not inhibited by clavulanic acid or sublactam, which are known to inhibit the activity of PCase (7). The *K* values of clavulanic acid against PCase types I, II, III, and IV were 0.47, 18.3, 21.7, and 2.50 μM, respectively, and those of sulbactam ranged from 0.47 to 3.57 μM. However, clavulanic acid and sulbactam had high *K* values of 100 μM or greater against the GN14061 enzyme, indicating that clavulanic acid could not inhibit the enzyme.

Enzyme activity was inhibited by inhibitors such as EDTA, iodine, Cu²⁺, and Hg²⁺. Although activity was inhibited by EDTA, which is known to be a chelating agent, activity was not 100% inhibited by treatment with 3 mM EDTA. After inhibition by EDTA, enzyme activity was regained 100% by dialysis against distilled water. It is suggested that the GN14061 enzyme is not a metalloenzyme such as β-lactamase from *Pseudomonas maltophilia* (11) or *Bacillus cereus* 569 (10).

The M.W. of *A. faecalis* GN14061 enzyme was estimated to be 29,000, and the pI was 5.9. The M.W.s and pIs of other PCase types (7, 9, 14) are as follows: type I (M.W. 20,600, pI 5.4); type II (M.W. 23,000, pI 8.3); type III (M.W. 46,000, pI 8.3); type IV (M.W. 21,000, pI 5.7); type V (M.W. 29,600, pI 8.7); TEM-1 (M.W. 22,000, pI 5.4), and TEM-2 (M.W. 23,500, pI 5.4); these were different from the M.W. and pI of the GN14061 enzyme, except for those of PSE-1 (M.W. 28,500, pI 5.7 [14]). However, the enzyme from *A. faecalis* GN14061 is different from PSE-1 in cloxacillin and cephaloridine hydrolysis.

These properties of the *A. faecalis* GN14061 enzyme are different from other β-lactamases previously classified by Richmond and Sykes (9), Sykes and Matthew (14), and Mitsushashi and Inoue (7). Therefore, we consider the enzyme from *A. faecalis* GN14061 to be a new type of PCase.

**LITERATURE CITED**


