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Biochemical Properties and Purification of Metallo-β-Lactamase from *Bacteroides fragilis*

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The β-lactamase from *Bacteroides fragilis* GAI-30144 hydrolyzed imipenem, oxyiminocephalosporins, cephamycins, and penicillins. Enzyme activity was inhibited by EDTA. Zinc completely reversed inactivation of the enzyme by EDTA. The molecular mass of purified enzyme was estimated to be 33,000 daltons.

Carbapenems such as imipenem were initially believed to be stable to the hydrolytic action of β-lactamases. However, some bacterial strains have been reported to be resistant to imipenem by producing β-lactamase (2, 7, 8, 10).

Bacteroides fragilis is frequently isolated from clinical specimens. While the clinical isolates generally contain β-lactamase (3), resistance to imipenem is rare in this organism. Recently, we discovered that two *B. fragilis* strains isolated from clinical specimens (GAI-30079, GAI-30144) were highly resistant to imipenem and investigated the biochemical properties of the β-lactamases extracted from these organisms.

The MICs were determined by an agar dilution method by using modified GAM (5) agar (Nissui Seiyaku Co. Ltd., Tokyo, Japan). Crude extracts prepared from approximately 10 g (wet weight) of *B. fragilis* GAI-30144 cells were loaded onto a DEAE-cellulose (DE 52: Whatman Ltd., Kent, England). The combined active fractions from the DE 52 column were concentrated and chromatographed on a Sephacryl S-200 column (Pharmacia AB, Uppsala, Sweden). Active fractions were pooled and subjected to a Mono Q column (Pharmacia AB, Uppsala, Sweden). The fractions from the Mono Q column containing the highest specific activity were used as the final enzyme preparations. The enzyme activity was assayed by a spectrophotometric technique (6). The kinetic parameter, Michaelis constants (K_m), and maximum velocities of hydrolysis (V_{max}) were calculated with the Lineweaver-Burk plot by the method of least-squares analysis. For partially purified enzyme solution, isoelectric focusing was performed. The presence of β-lactamase was detected by soaking the gel in nitrocefin solution. Molecular weight was estimated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Protein was measured by the method of Bradford (1), with gamma globulin used as a standard.

A total of 145 strains of *B. fragilis* were isolated from clinical specimens over a period from December 1986 to December 1987. *B. fragilis* GAI-30079 and GAI-30144 were found to be highly resistant to imipenem. MICs of imipenem against GAI-30079 and GAI-30144 were 50 and 100 μg/ml, respectively. GAI-30079 and GAI-30144 were also highly resistant to cephamycins, ceftizoxime, and moxalactam.

Some enzymological properties were studied by using the crude enzyme solution from GAI-30144. The kinetic parameters for cephaloridine, ceftizoxime, penicillin G, moxalactam, and imipenem are shown in Table 1. The enzyme showed a high level of activity to both cephalosporins and penicillins. Furthermore, the substrates usually considered to be stable to *B. fragilis* β-lactamases, i.e., ceftizoxime, moxalactam, and imipenem, were significantly hydrolyzed by this enzyme. The ratios of relative V_{max} to K_m suggested that cephaloridine is the best substrate, followed by penicillin G, ceftizoxime, imipenem, and moxalactam, in that order. In addition, the enzyme hydrolyzed cephamycins, while aztreonam was quite stable (data not shown). These observations suggested that characteristic resistance to ceftizoxime, moxalactam, cephamycins, and imipenem is involved in β-lactamase activity. The substrate profile of the GAI-30079 enzyme was quite similar to that of the GAI-30144 enzyme. The imipenem-hydrolyzing β-lactamase from GAI-30144 was subjected to further investigation.

The β-lactamase from GAI-30144 was not inhibited by 1 mM clavulanic acid or sulbactam. It was completely inhibited by 1 mM *p*-chloromercuribenzoic acid and EDTA. When the enzyme was completely inactivated by EDTA, 0.5 mM $ZnCl_2$ was added. The inhibitory effects of EDTA were completely reversed by the addition of Zn^{2+} (Fig. 1). A similar study was then conducted with several divalent cations (Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Ba^{2+}). Co^{2+} and Mn^{2+} partially restored enzyme activity, but Mg^{2+}, Ca^{2+}, and Ba^{2+} did not (Table 2). The effect of zinc on enzyme stability was also studied. Enzyme solutions containing 110 μg of crude enzyme were incubated with or without 1 mM $ZnCl_2$ in 100 μl of 200 mM morpholinepropanesulfonic acid (MOPS)–KOH (pH 7.2) at room temperature. After 28 h, 100% of the initial activity (0.07 μmol/min/mg of protein) still continued in the sample containing zinc, while only approximately 12% continued in a control group. It was suggested that zinc stabilized β-lactamase activity, although protease action on enzyme activity remained a possible factor. Note that zinc was added during enzyme purification.

TABLE 1. Kinetic parameters of the β-lactamase from *B. fragilis* GAI-30144

Substrate	K_m (μM)	V_{max} (μmol/min/mg of protein)	Relative V_{max} (%)[a]	Relative V_{max}/K_m
Cephaloridine	15	0.08	100	6.8
Ceftizoxime	154	0.23	293	1.9
Penicillin G	359	0.60	766	2.1
Moxalactam	58	0.05	59	1.0
Imipenem	108	0.12	150	1.4

[a] Relative V_{max} values are expressed relative to the V_{max} for cephaloridine, which was considered to be 100%.

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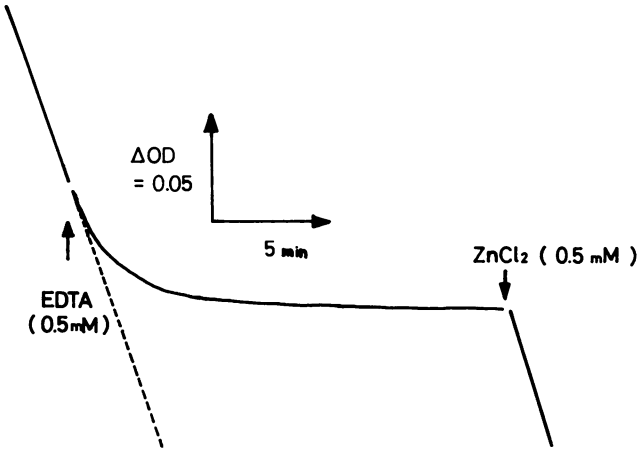

FIG. 1. Effect of EDTA and zinc ion on the β-lactamase activity of *B. fragilis* GAI-30144. The decrease in the optical density (OD) of imipenem by β-lactamase action was monitored continuously at 299 nm.

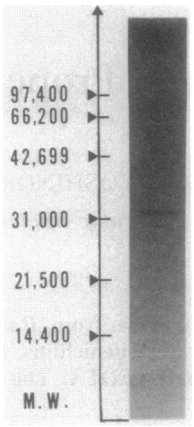

FIG. 2. SDS-polyacrylamide gel electrophoresis of purified β-lactamase from *B. fragilis* GAI-30144. Molecular weights (MW) are indicated on the left.

The β-lactamase was purified approximately 800-fold from crude extract and appeared to be homogeneous on the basis of SDS-polyacrylamide gel electrophoresis (Fig. 2). The specific activity, which was determined with imipenem as a substrate, was 15.4 μmol/min/mg of protein, and the yield was 3.6% of the initial activity in the crude enzyme preparation. The molecular mass of purified β-lactamase from GAI-30144 was estimated to be 33,000 daltons on the basis of SDS-polyacrylamide gel electrophoresis (Fig. 2). The isoelectric point, as estimated by electrofocusing, was about 4.7.

Yotsuji et al. (10) reported an imipenem-hydrolyzing β-lactamase from *B. fragilis* G-237. This enzyme had a substrate and inhibitor profile similar to that of the GAI-30144 enzyme. However, the effect of EDTA was not explained in their report. By contrast, β-lactamase from *B. fragilis* TAL-2480 was reported by Cuchural et al. (2) to be completely inhibited by EDTA. This enzyme also hydrolyzes carbapenems, cephamycins, cephalosporins, and penicillins and is not susceptible to either clavulanic acid or sulbactam. Its properties are similar to those of the GAI-30144 enzyme. However, its molecular mass, as estimated by genetic study, was 25,249 daltons (9), which is different from that of the GAI-30144 enzyme. It remains of great interest to define the relationships among TAL-2480, G-237, and GAI-30144 enzymes.

Our results suggest that the GAI-30144 enzyme is a metallo-β-lactamase which requires Zn^{2+} as a physiological cofactor. This type of β-lactamase has been reported to be produced by *Xanthomonas maltophilia*, *Flavobacterium odoratum*, and *Legionella gormanii* (4, 7, 8). These β-lactamases have various distinct characteristic properties, but the following three features are common to them: (i) they are not inhibited by typical β-lactamase inhibitors; (ii) the metal ion is essential to enzyme activity; and (iii) they can hydrolyze imipenem (2, 4, 7, 8). It is possible that the metallo-β-lactamase has a catalytic mechanism different from those of other widespread non-metallo-β-lactamases and that this is why metallo-β-lactamase can hydrolyze a β-lactamase-stable compound like imipenem and yet is not inhibited by typical β-lactamase inhibitors.

TABLE 2. Effects of metal ions on recovery of GAI-30144 β-lactamase activity inhibited by EDTA

Metal	% Recovery[a]
Zn^{2+}	112.2
Co^{2+}	42.3
Mn^{2+}	23.3
Mg^{2+}	0
Ca^{2+}	0
Ba^{2+}	0

[a] Enzyme activity was assayed with imipenem (100 μM) as the substrate and is expressed as the percentage of hydrolysis relative to that of the control sample. The control sample was preincubated in the same manner as the test samples (see text), except for the absence of EDTA and metal ions.

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