Biochemical Properties of a Penicillin Beta-Lactamase Mediated by R Factor from *Bordetella bronchiseptica*

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The β -lactamase specified by an R_{te16} plasmid in *Bordetella bronchiseptica* was purified 200-fold by carboxymethyl-Sephadex column chromatography and electrofocusing. The enzyme has a molecular weight of $46,000 \pm 3,000$ and an isoelectric point of 8.3 and was highly active against phenethicillin, oxacillin, and propicillin. The enzyme activity was inhibited by sodium chloride but not by ferrous ion. The maximal enzyme activity to benzylpenicillin was observed at pH 7.0 to 7.5 and at 40 C. It is concluded that this enzyme is different from the R-mediated β -lactamases, i.e., the type I and type II β -lactamases which have been classified in previous papers.

It was reported that ampicillin resistance in many enteric bacteria is due to the presence of R factors (2) which mediate the formation of a penicillin β -lactamase (7, 9). Many papers have been presented concerning the purification of penicillinases (PCases) from *Escherichia coli* carrying R_{TEM} , R_1 , R_{GN14} (renamed R_{ms212}), R_{GN238} (renamed R_{ms213}), or R_{1818} (4, 5, 8, 10, 16, 18). It was reported from this laboratory that the R factor-mediated PCases were classified into two groups, i.e., type I (R_{ms212}) and type II (R_{ms213}), according to their substrate specificity and other enzymological properties. The R_{TEM} - and R_1 -mediated PCases could be grouped into the type I PCase (16).

From the nasal cavities of young pigs suffering from infectious atrophic rhinitis, Terakado et al. (17) isolated *Bordetella bronchiseptica* strains resistant to sulfonamide, streptomycin, and ampicillin. The resistances were mediated by R factors.

We have selected randomly one of the R plasmids (R_{te16}) demonstrated in *B. bronchiseptica* and describe in this paper the enzymological properties of R_{te16} -mediated PCase to compare them with those of other R plasmid-mediated PCases.

MATERIALS AND METHODS

Bacterial strain. An R_{te16} factor was demonstrated from *B. bronchiseptica* of swine origin and transferred by conjugation to *E. coli* ML1410. The R_{te16} factor can confer resistances to ampicillin, streptomycin, and sulfonamide to its host strain (17).

Culture. *E. coli* ML1410 R_{te16}^+ was grown overnight in 400 ml of nutrient broth at 37 C. The culture was diluted with 4 liters of fresh broth and then grown

at the same temperature under aeration until the optical density at 620 nm (Hitachi Perkin-Elmer spectrophotometer) reached from 0.7 to 0.9. The cells were then harvested by centrifugation and washed once with 0.1 M sodium phosphate buffer (pH 7.5). The yield of *E. coli* ML1410 R_{te16}^+ was 18 g (wet weight).

Antibiotics. Benzylpenicillin, ampicillin, oxacillin, cloxacillin, dicloxacillin, and cephalixin were supplied by the Toyo Jozo Co., Ltd., Tokyo. Carbenicillin, phenethicillin, methicillin, and 6-aminopenicillanic acid were kindly provided by the Meiji Seika Co., Tokyo. Cephaloridine, cephalothin, and cephaloglycin were contributed by Shionogi & Co., Osaka. Cefazolin was obtained from the Fujisawa Pharmaceutical Co., Ltd., Osaka. Cephapirin was a gift from Bristol Laboratories, Inc., Syracuse, N.Y.

Preparation of column. Carboxymethyl-Sephadex c-50 (Pharmacia, Uppsala, Sweden) was equilibrated with 0.05 M sodium phosphate buffer (pH 6.0) and then packed in a column. This column (2.0 by 45 cm) was used at 5 C.

Enzyme assay. PCase activity was determined iodometrically at 30 C according to the method of Perret (13), except that 0.1 M phosphate buffer (pH 7.5) was used instead of 0.2 M phosphate buffer (pH 6.5). Cephalosporinase activity was measured similarly except that the amount of hydrolyzed cephaloridine was calculated on the basis of 1 mol being equivalent to four equivalents of iodine (1). The microiodometric assay method devised by Novick (12) was employed for kinetic measurement of the enzyme reaction. One unit of PCase and cephalosporinase activities is defined as the amount of enzyme which hydrolyzes 1 μ mol of benzylpenicillin or cephaloridine, respectively, in 1 h at 30 C in 0.1 M phosphate buffer (pH 7.0) with 8 mM substrate.

Protein determination. Protein determination was routinely carried out by method of Lowry et al. (11), with bovine serum albumin as the standard.

Determination of molecular weight. The molecu-

lar weight of the enzyme was estimated by thin-layer gel filtration on Sephadex G-100 (superfine), equilibrated with 0.05 M sodium phosphate buffer (pH 7.0) containing 0.1 M ammonium sulfate, according to the method of Andrews (3). The plate used was 20 by 20 cm. Bovine serum albumin (molecular weight, 67,000), ovalbumin (molecular weight, 45,000), pepsin (molecular weight, 35,500), chymotrypsin (molecular weight, 25,000), trypsin (molecular weight, 20,000), and cytochrome c (molecular weight, 12,000) were used as the markers. The thin-layer gel was run for 8 h at 5 C, and the locations of the markers were followed by staining the protein with 1% amido black. The location of the enzyme was detected by spraying the gels with a 0.1 M sodium phosphate buffer (pH 7.0) solution containing 5 mg of benzylpenicillin per ml, followed by spraying with a 0.1 M sodium acetate buffer (pH 4.0) containing 0.016 M I₂ and 0.12 M KI.

Agar-gel electrophoresis. The purified enzyme was submitted to agar-gel electrophoresis. Agar-gel (0.8% agarose) was prepared (16) and run with the reservoir adjusted to pH 7.0 phosphate buffer (ionic strength, 0.04). The gel with 150 μ g of purified enzyme was run for 3 h in a potential gradient of 20 V/cm; after the run was completed, the agar-gel was longitudinally divided into two portions. A part of the smaller portion was stained for protein with 1% amido black in 7% (vol/vol) acetic acid. Another part of the agar-gel was sliced transversely, 2 mm in thickness, on slide glass for the assay of PCase activity. Each slice was suspended in 1 ml of 0.1 M phosphate buffer (pH 7.5) and incubated overnight at 4 C. The supernatant thus obtained was used for the assay of PCase activity.

RESULTS

Purification of the enzyme. The culture of the organism was harvested by centrifugation at the mid-exponential phase of growth and washed with 0.1 M phosphate buffer at pH 7.5. Washed cells (18 g, wet weight) were suspended in 65 ml of 0.1 M phosphate buffer (pH 7.5) and disintegrated by ultrasonication (20 kilocycles/s) for 4 min in an ice-water bath. The broken cells were then centrifuged at $10,000 \times g$ for 30 min below 5 C, and the resulting supernatant fraction (stage 1) was further centrifuged at $105,000 \times g$ for 2 h at 4 C. The supernatant fraction was dialyzed for 18 h at 5 C against distilled water (stage 2). The resulting enzyme solution was applied to a carboxymethyl-Sephadex c-50 column equilibrated with 0.05 M sodium phosphate buffer (pH 6.0), and the column was washed with 70 ml of the same buffer. A linear gradient (1,200 ml) of 0.1 to 0.5 M sodium phosphate buffer (pH 6.0) was then applied. The enzyme was eluted at a phosphate buffer concentration of about 0.3 M, with 5-ml fractions being collected (stage 3). The active fractions from 115 to 133 were pooled (95 ml in total), dialyzed three times for 8-h periods at

5 C against distilled water, and concentrated to 5 ml by addition of 10 g of Sephadex G-100 powder. The concentrated enzyme was used for electrofocusing in an Ampholine electrofocusing apparatus, model LKB 8100 (LKB-Produkter AB, Stockholm-Bromma, Sweden). A carrier ampholyte was used which gave a pH gradient between pH 7 and 9, and electrofocusing was carried out below 4 C in a column of 110-ml capacity. The total amount of the carrier ampholytes (40%) was 3 ml. After focusing for 96 h, the contents of the column were distributed into 2-ml fractions, and each fraction was assayed for its protein concentration, PCase activity, and pH (stage 4). Peaks of the enzyme activity completely agreed with those of protein in stage 4 (Fig. 1). Results of the purification procedure with R_{te16} PCase are shown in Table 1. The specific enzyme activity at the final stage showed an increase of 217 times that of stage 1.

Isoelectric point. The isoelectric point of the enzyme was determined from the electrofocusing (Fig. 1), indicating that R_{te16} PCase was a basic protein whose isoelectric point was 8.3.

The purified enzyme showed a single band on agar-gel electrophoresis and moved towards the cathode with a mobility of 12 mm/3 h at pH 7.0, and a single protein band corresponding to PCase activity was demonstrated (Fig. 2).

FIG. 1. Electrofocusing of R_{te16} PCase (stage 4). Broken line indicates pH. Each fraction (2 ml) was assayed for protein concentration (●) and activity (○). Benzylpenicillin was used as a substrate.

TABLE 1. Summary of the purification of PCase R_{te16} from *B. bronchiseptica*

Stage no.	Procedure	Activity recovered (U)	Sp act (U/mg of protein)	Purification factor	Recovery (%)	
					Per stage	Overall
1	Ultrasonic disintegration	5,170	4.3	1.0	100.0	100.0
2	Centrifugation at $105,000 \times g$	4,810	6.9	1.7	93.0	93.0
3	Chromatography on CM-Sephadex ^a	2,310	720.0	167.0	48.0	44.7
4	Electrofocusing	1,680	935.0	217.0	72.7	32.5

^a CM, Carboxymethyl. FIG. 2. Agar-gel electrophoresis pattern of purified PCase R_{te16} . The agar-gel was longitudinally divided into two portions. A part of the small portion was stained for protein. Another part was used for the assay of PCase activity.

Molecular weight. The approximate molecular weight of the R_{te16} PCase was estimated to be $46,000 \pm 3,000$, the molecular weight of R_{te16} PCase being considerably larger than those of PCases mediated by other R plasmids, except R_{1816} (5, 8, 10, 16, 18).

Effects of pH and temperature. The pH activity curve of the enzyme is shown in Fig. 3. R_{te16} PCase shows a sharp pH activity curve with a maximum within the range of pH 7.0 to 7.5. This enzyme retained 70% of its maximal activity within the range of pH 6.6 to 8.2. The activity versus temperature profile of the enzyme is shown in Fig. 4. A temperature of 40 C was optimal for the enzyme, with benzylpenicillin as the substrate.

Inhibition. A number of inhibitors and ions

FIG. 3. Effect of pH on the activity of R_{te16} PCase. Sodium phosphate buffer (0.1 M) was used for the pH range from 5.5 to 8.0, and 0.1 M borate buffer was used for the pH range from 8.0 to 9.0.

were tested for their effects on enzyme activity. The enzyme preparation was preincubated in 0.1 M phosphate buffer (pH 7.5) in the presence or absence of inhibitor (or ion) for 5 min at 30 C, and then enzyme activity was assayed using benzylpenicillin as a substrate. Iodine (0.5 mM) inhibited the activity by more than 90%. Ferrous, nitrate, and chloride ions also markedly inhibited the R_{te16} PCase activity, whereas *p*-chloromercuribenzoate, ethylenediaminetetraacetate, or cupric ion showed no effect (Table 2). The relationship of enzyme activity to chloride ion concentration is shown in Fig. 5.

Substrate specificity and K_m . The relative activities and K_m values of the enzyme for a

FIG. 4. Effect of temperature on the activities of R_{te16} PCase. The enzyme solution in 0.1 M phosphate buffer (pH 7.5) containing 8 mM benzylpenicillin as substrate was incubated for 15 min at the indicated temperatures.

TABLE 2. Effects of inhibitors and ions on the activities of PCase R_{te16}

Inhibitor and ion ^a	Concn (mM)	Inhibition (%)
Iodine	0.5	92
<i>p</i> -CMB	1.0	— ^b
EDTA	1.0	—
CuSO ₄	1.0	—
FeSO ₄	1.0	99
KNO ₃	10.0	36
NaCl	100.0	67

^a *p*-CMB, *p*-Chloromercuribenzoate; EDTA, ethylenediaminetetraacetate.

^b —, Effects of less than $\pm 5\%$ on enzyme activity were defined as no effect.

number of substrates were determined and are shown in Table 3. It is significant that R_{te16} PCase showed much higher enzyme activity toward phenethicillin, oxacillin, cloxacillin, and propicillin, but had rather lower activity against carbenicillin and methicillin. However, the enzyme has a lower affinity for oxacillin and cloxacillin. It also has lower activity toward cephalosporins than toward the penicillins. Of the substrates tested, oxacillin was most rapidly hydrolyzed by R_{te16} PCase, whereas R_{ms212} PCase was most active against methicillin and ampicillin (18) under these conditions.

DISCUSSION

It can be concluded from these results that the R_{te16} PCase is active against the semisynthetic penicillins, with the exception of methicillin. This enzyme has the following properties: a molecular weight of about 46,000, an isoelectric point of 8.3, and sensitivity to ferrous and chloride ions.

The type I PCase (R_{ms212}) has a low activity against semisynthetic penicillins (except ampicillins) and a molecular weight of about 20,600 (16), and the type II PCase (R_{ms213}) has a high activity against methicillin and a molecu-

FIG. 5. Effect of chloride concentration on the activity of R_{te16} PCase. The purified PCase preparation in phosphate buffer was used.

TABLE 3. Kinetics of the hydrolysis of various penicillins and cephalosporins by PCase R_{te16}

Substrate	K_m (mM)	Relative rate of hydrolysis (%) ^a
Benzylpenicillin	4	100
Ampicillin	9	131
Carbenicillin	11	54
Phenethicillin	18	413
Oxacillin	81	682
Cloxacillin	192	310
Dicloxacillin		106
Methicillin		38
Propicillin		582
6-Aminopenicillanic acid	73	100
Cephaloridine	61	23
Cephalotin		10
Cephalexin		3
Cefazolin		23
Cephaloglycin		12
Cephapirin		45

^a Rates of hydrolysis of the 16 substrates are expressed in percentage of hydrolysis of benzylpenicillin.

lar weight of about 25,400 (18). The R_{ms213} PCase was much more temperature sensitive (optimal temperature, 30°C) than was the enzyme mediated by R_{te16} . Thus, the R_{te16} PCase appears to be distinct from the other two and is classified as type III PCase.

Dale and co-workers (4-6) described the properties of the R_{1818} PCase, and the R_{1818} PCase was found to belong to a class Vb β -lactamase classified by Richmond and Sykes (14). The molecular weight, isoelectric point, and optimal pH of R_{1818} PCase are 44,600, 8.3, and 7.2 to 7.4, respectively (4, 5). The R_{1818} PCase is therefore very similar to the type III PCase reported here, and it is also similar on the basis of its unique substrate specificity, i.e., high activity with cloxacillin, dicloxacillin, oxacillin, propicillin, and phenethicillin, which are known to be very stable to many other PCases from gram-negative bacteria (5, 6). It should also be noted that the R_{te16} and R_{1818} PCases are quite sensitive to chloride ion. The R_{1818} was demonstrated in *Salmonella typhimurium*, whereas the R_{te16} was isolated from *B. bronchiseptica* (17). PCases mediated by these two R factors are quite similar in substrate profile, K_m values, molecular weight, optimal pH, isoelectric point, and sensitivity to chloride ion, although their host origins are different. It might be speculated from these facts that the origin of the PCase gene on the R_{te16} factor may be the same as that of the R_{1818} PCase gene.

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