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Purification and Characterization of a New β-Lactamase from *Clostridium butyricum*

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A highly penicillin-resistant β-lactamase-producing strain of *Clostridium butyricum*, strain NBL 3, was isolated. The specific activity of the unpurified β-lactamase was 0.29 U/mg of protein. The enzyme was purified 1,130-fold by QAE Zetaprep, Sephacryl S-300, and Mono Q column passages. The purified enzyme was judged homogeneous by sodium dodecyl sulfate gradient gel electrophoresis and fast protein liquid chromatography. The enzyme hydrolyzed phenoxymethylpenicillin, benzylpenicillin, ampicillin, and carbenicillin more rapidly than piperacillin and cephaloridine. Cephalothin, cephalexin, cefoxitin, moxalactam, cefotaxime, and imipenem were only slightly hydrolyzed, at less than 1% the rate for phenoxymethylpenicillin. The enzyme was inhibited by clavulanic acid, sulbactam, tazobactam, and *p*-chloromercuribenzoate. The molecular weight was determined by gel filtration and sodium dodecyl sulfate gradient gel electrophoresis to be 32,000. The isoelectric point was 4.4. Aspartic acid-asparagine, glutamic acid-glutamine, leucine, lysine, alanine, and serine dominated the amino acid composition.

Several investigators have reported that *Bacteroides fragilis* and closely related species produce β-lactamases with penicillinase and/or cephalosporinase activities and that the enzymes play a significant role in the resistance of these organisms to various beta-lactam antibiotics (17, 20, 21, 23). In contrast to the *B. fragilis* group of organisms, the clostridia are in general susceptible to beta-lactam antibiotics (7, 15, 25). A few reports have indicated that *Clostridium ramosum*, *Clostridium clostridiiforme*, and *Clostridium butyricum* produce β-lactamases (4, 13, 29) and that relatively high concentrations of penicillin are required to inhibit some strains of *Clostridium perfringens* (14).

Recently, we found that three *C. butyricum* strains isolated from patients with intra-abdominal infections were highly resistant to penicillins. The resistance was caused by β-lactamase production. The aim of the present investigation was to purify and characterize the β-lactamase from one of these *C. butyricum* strains (NBL 3).

MATERIALS AND METHODS

Bacterial strain. *C. butyricum* NBL 3, a clinical strain isolated at The National Bacteriological Laboratory, Stockholm, Sweden, was used. The strain was identified by Gram staining, biochemical tests, and gas-liquid chromatographic analysis according to the criteria of the *Anaerobe Laboratory Manual* (9). β-Lactamase activity was detected with nitrocefin by the method of Kammer et al. (11).

β-Lactamase production. β-Lactamase was produced in fermentors with a 3-liter working volume (FL 103; BioTec, Stockholm, Sweden). Temperature was controlled with an accuracy of ±0.01°C, and pH was controlled with an accuracy of ±0.05 pH unit. The impeller speed was 50 rpm (BioTec LP-300). The medium used was a prereduced medium containing the following per liter: 5 g of yeast extract, 3 g of meat extract powder, 10 g of starch, 15.6 mmol of $Na_2HPO_4 \cdot 12H_2O$, 1.4 mmol of $NaH_2PO_4 \cdot H_2O$, and 5 g of L-cysteine. Cultivation was performed at 37°C and pH 7.0 under an N_2 atmosphere. The culture was harvested in the late log phase (18 h) by centrifugation at 10,000 × *g* for 30 min at 4°C.

Determination of β-lactamase activity. β-Lactamase activity was assayed spectrophotometrically with nitrocefin (0.10 mM) in 50 mM sodium phosphate buffer (pH 7.0, 37°C) as described previously (18). One unit of β-lactamase was defined as the amount which formed 1.0 μmol of product per min under these conditions. All spectrophotometric measurements were made with a model 24 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.).

Determination of protein. Protein was assayed by the method of Lowry et al. (12) with bovine serum albumin as the standard. The protein concentrations in samples from the last purification step were calculated from the amino acid analysis.

Purification of β-lactamase. Purification steps 1, 4, and 5 were carried out at 20°C, while steps 2 and 3 were carried out at 4°C.

(i) Step 1: mass anion exchange. The supernatant of the culture (2,900 ml) was passed through a QAE Zetaprep 250 column (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) equilibrated with 15 mM sodium phosphate buffer (pH 7.0). The column was washed with 350 ml of sodium phosphate buffer (15 mM, pH 7.0) containing 0.1 M NaCl. The β-lactamase was eluted with the same buffer containing 0.6 M NaCl. The flow rate was 25 ml/min. Three fractions (50 ml each) containing β-lactamase activity were collected and concentrated to 50 ml by ultrafiltration (Pellicon membrane disc; nominal molecular weight exclusion limit, 10,000; Millipore Corp., Bedford, Mass.).

(ii) Step 2: gel filtration. The concentrated β-lactamase sample from step 1 was passed through a Sephacryl S-300 column (5.0 by 71 cm; Pharmacia) with 20 mM Tris hydrochloride buffer (pH 8.0) containing 0.1 M NaCl as the eluent. The fractions containing β-lactamase activity were pooled.

(iii) Step 3: mass anion exchange. The β-lactamase-containing sample (110 ml) from step 2 was passed through a QAE Zetaprep 15 column (Pharmacia) equilibrated with 20 mM Tris hydrochloride buffer (pH 8.0) containing 0.1 M NaCl. After sample passage, the column was washed with

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the same buffer (100 ml) as before and the β-lactamase was eluted with 20 mM Tris hydrochloride buffer (pH 8.0) containing 0.3 M NaCl. The fractions containing β-lactamase activity were dialyzed against 20 mM Tris hydrochloride buffer (pH 8.0) containing 80 mM NaCl.

(iv) Step 4: anion-exchange chromatography. Chromatography was carried out on a Mono Q column (HR 5/5; fast protein liquid chromatography [FPLC] system; Pharmacia) equilibrated with 20 mM Tris hydrochloride buffer (pH 8.0) containing 80 mM NaCl. The β-lactamase sample from step 3 was processed in five consecutive runs. Separation was achieved by elution with increasing concentrations of NaCl and two solutions: 20 mM Tris hydrochloride (pH 8.0) (solution A) and 1.0 M NaCl in 20 mM Tris hydrochloride (pH 8.0) (solution B). One milliliter of 8% solution B was passed through the column after loading. This was followed by 2 ml of a linearly increasing concentration of solution B up to 18% and then 5 ml at 18%. The concentration of solution B was then linearly increased to 30% in the next 6 ml, followed by 1 ml of 100% solution B. Active fractions from each run were collected and diluted with 2 volumes of solution A, which lowered the NaCl concentration to that used initially in step 5.

(v) Step 5: anion-exchange chromatography. Diluted samples from each run of step 4 were chromatographed once on a Mono Q column under the same conditions, except for the NaCl gradient (Fig. 1). The most active fractions were collected, either lyophilized after desalting against milli-Q water (Millipore) by gel filtration on Sephadex G-25 columns (PD-10; Pharmacia) or diluted with 9 volumes of sodium phosphate buffer (50 mM, pH 7.0) containing 1% (wt/vol) gelatin, and stored at −70°C for later use.

Determination of molecular weight and tests for enzyme purity. (i) Gel filtration. The purified β-lactamase was chromatographed on Superose 12 (HR 10/30; Pharmacia) with the FPLC system. The eluent was 20 mM Tris hydrochloride buffer (pH 8.0) containing 0.1 M NaCl, and the flow rate was 0.5 ml/min. The column was calibrated with molecular weight markers chromatographed under the same conditions.

(ii) SDS gradient gel electrophoresis. Lyophilized samples and molecular weight markers were dissolved in 10 mM Tris hydrochloride (pH 8.0) containing 1 mM EDTA, 5% β-mercaptoethanol, and 1% sodium dodecyl sulfate (SDS) and then heated for 6 min at 100°C. Electrophoresis was carried out in 0.04 M TRIS–0.02 sodium acetate–2 mM EDTA–0.2% SDS (pH 7.4) with commercially obtained gradient gels (PAA 4/30; Pharmacia). Running conditions were 150 V for 2 h at 15°C.

Analytical isoelectric focusing. Isoelectric focusing was carried out with commercially available ampholine gels providing a pH gradient of 4.0 to 6.5 (Pharmacia). The samples were applied to the ampholine gels and subjected to a constant power of 50 W (initially 2,000 V and 25 mA) for 2.5 h at 7°C. The pH gradient of the focused plate was measured at 0.5-cm intervals with a surface electrode. Demonstration of β-lactamase activity in the gels was achieved with nitrocefin as a substrate.

Determination of amino acid composition. The amino acid composition of the purified enzyme was determined in a Stein-Moore amino acid analyzer at the Central Amino Acid Analysis Laboratory, University of Uppsala, Uppsala, Sweden.

Substrate profile. Purified enzyme diluted in 50 mM sodium phosphate buffer (pH 7.0) containing 1% gelatin was used for kinetic studies. K_m values were determined indirectly by competitive interaction between the antibiotic and nitrocefin (1, 27). Briefly, the initial rate of hydrolysis of nitrocefin at six different concentrations (40 to 300 μM) was determined in the presence of 1.2 nM β-lactamase. Each nitrocefin concentration was combined with five antibiotic concentrations in ratios from 1 to 5 (1 = a K_m of 0.5 to 1 μM). The apparent K_m values for nitrocefin were plotted against the antibiotic concentrations to obtain the K_m for the antibiotic graphically.

V_{max} values for the different antibiotics were calculated from the Henri-Michaelis-Menten equation by using the K_m values obtained by the indirect determination and the initial rates obtained from the hydrolysis of benzylpenicillin, phenoxymethylpenicillin, and ampicillin at final concentrations of 0.50 mM, carbenicillin at 0.40 mM, and piperacillin, cephaloridine, cephalothin, cephalexin, cefoxitin, moxalactam, cefotaxime, and imipenem at 0.10 mM. The initial rates were obtained by direct spectrophotometric assays based on the difference in light absorption between substrate and product at a specified wavelength (5, 16, 22, 28). The relative maximal hydrolysis rate was obtained by setting V_{max} for benzylpenicillin at 100. Turnover (k_3) was calculated from V_{max} by assuming a molecular weight of 32,000.

Inhibition studies. The degree of inactivation of β-lactamase activity by various inhibitors was determined with nitrocefin as a substrate in the spectrophotometric assay. Clavulanic acid, sulbactam, tazobactam, and *p*-chloromercuribenzoate (pCMB) were preincubated together with various concentrations of enzyme for different times (2 to 80 min) at 37°C before determination of the remaining β-lactamase activity. Percent inhibition was calculated as $100 \times [(c - r)/c]$, where c is the activity in control samples incubated without inhibitor and r is the remaining activity in samples incubated with inhibitor.

Chemicals. Molecular weight markers were from Sigma Chemical Co., St. Louis, Mo., and from Pharmacia. Benzylpenicillin, phenoxymethylpenicillin, ampicillin, carbenicillin, cloxacillin, and clavulanic acid were provided by Astra, Södertälje, Sweden. Piperacillin and tazobactam were from Lederle Laboratories, Wayne, N.J. Cefoxitin and imipenem were from Merck Sharp & Dohme, Rahway, N.J. Cephalothin, cephalexin, and moxalactam were from Lilly, Fegersheim, France. Cefotaxime was from Hoechst, Frankfurt, Federal Republic of Germany. Cephaloridine and nitrocefin were from Glaxo Pharmaceuticals, Ltd., Greenford, United Kingdom. Sulbactam was from Pfizer Inc., Groton, Conn. pCMB was from Sigma.

RESULTS

β-Lactamase formation. The β-lactamase was produced during the growth period up to 18 h. Approximately 90% of the enzyme was extracellular, and the remaining part was intracellular. The specific activity in the supernatant was 0.29 U/mg. The formation of β-lactamase did not change significantly when benzylpenicillin (64 μg/ml) or cefoxitin (0.5 μg/ml) was added to the medium.

MICs. The strain was highly resistant to benzylpenicillin (MIC, 2,048 μg/ml), phenoxymethylpenicillin (MIC, 2,048 μg/ml), and ampicillin (MIC 1,024 μg/ml); moderately resistant to cloxacillin (MIC, 64 μg/ml), cephaloridine (MIC, 64 μg/ml), cephalothin (MIC, 16 μg/ml), cephalexin (MIC, 16 μg/ml), cefotaxime (MIC, 32 μg/ml), and moxalactam (MIC, 16 μg/ml); and susceptible to cefoxitin (MIC, 4 μg/ml) and imipenem (MIC, 0.5 μg/ml).

Purification. The results of each purification step are

TABLE 1. Purification of extracellular β-lactamase from *C. butyricum* NBL 3

Step	Total activity (U)	Sp act (U/mg of protein)	Purification (fold)	Recovery (%)
Culture medium	2,190	0.285		
1 Mass anion exchange on QAE Zetaprep 250	1,940	5.52	19.4	88.6
2 Gel filtration on Sephacryl S-300	1,500	34.6	121	68.5
3 Mass anion exchange on QAE Zetaprep 15	1,490	48.0	168	68.0
4 Anion exchange on first Mono Q column	644	235	825	29.4
5 Anion exchange on second Mono Q column	569	412 (916)[a]	1,130 (3,200)[a]	26.0

[a] Values in parentheses were based on the protein concentration calculated from the amino acid analysis.

presented in Table 1. QAE Zetaprep 250 was used for the isolation of the enzyme from large volumes of culture medium. In addition, large amounts of contaminating proteins were removed from the enzyme. In step 2, Sephacryl S-300 chromatography was used to separate the enzyme from proteins of other molecular sizes and to change the solvent to a more useful one for the next step. Passage through QAE Zetaprep 15 eliminated further contaminants from fractions containing β-lactamase. From these three steps, the enzyme was purified about 170-fold, with a yield of 68%. Two cycles of anion-exchange chromatography on Mono Q columns resulted in a single peak of β-lactamase with a high specific enzyme activity (Fig. 1). The material recovered after the last step was a homogeneous enzyme, as judged by SDS gradient gel electrophoresis (Fig. 2) and gel filtration (Fig. 3). The protein yield was 1.4 mg when determined by the Lowry method with bovine serum albumin as a standard but 0.62 mg when calculated from the amino acid analysis.

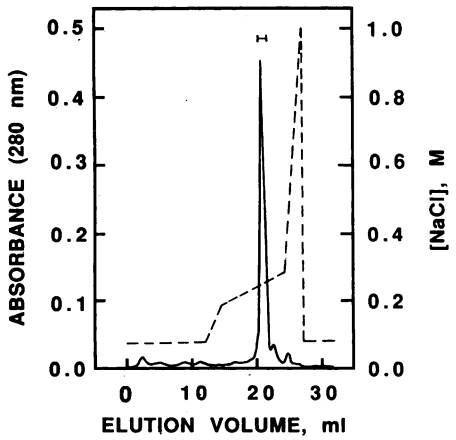

FIG. 1. Last step in the purification of β-lactamase by anion exchange on a Mono Q column. The effluent was collected in 0.3-ml fractions, and the flow rate was 1.0 ml/min. β-Lactamase activity was found in the main protein peak. Fractions with the same ratio of activity to A_{280} were collected. ——, A_{280}; – – –, NaCl concentration.

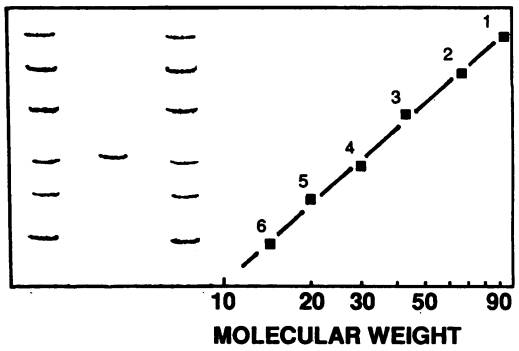

FIG. 2. SDS-polyacrylamide gradient gel electrophoresis of the purified β-lactamase (2.5 μg) from *C. butyricum* (middle lane) and low-molecular-weight calibration kit proteins: 1, phosphorylase *b*; 2, bovine serum albumin; 3, ovalbumin; 4, carbonic anhydrase; 5, trypsin inhibitor; and 6, α-lactalbumin (3 μg each). Electrophoresis was done at 150 V for 2 h at 15°C. Staining was done with 0.02% Coomassie brilliant blue and 7% acetic acid for 18 h, and destaining was done with 30% ethanol and 10% acetic acid for 24 h. Molecular weights are shown in thousands.

Molecular weight and isoelectric point. SDS gradient gel electrophoresis of SDS-treated and SDS-reduced enzyme revealed a single band which corresponded to a molecular weight of approximately 32,000 (Fig. 2). Gel filtration on Superose 12 also revealed a molecular weight of 32,000 (Fig. 3). The isoelectric point of the enzyme was estimated to be 4.4.

Amino acid composition. The amino acid composition of the purified enzyme is shown in Table 2. The amino acids aspartic acid-asparagine, glutamic acid-glutamine, leucine, lysine, alanine, and serine dominated the composition (60 mol%). The enzyme contained 0.84 mol% half-cystine, the lowest-quantity amino acid present. No histidine was present. Assuming a molecular weight of 32,000, the enzyme consisted of 287 amino acid residues.

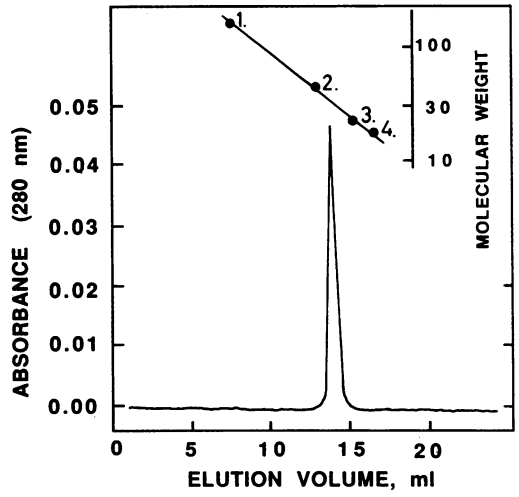

FIG. 3. Gel filtration on a Superose 12 column (1 by 30 cm) of 0.1 ml of the pooled β-lactamase from step 5 (marked in Fig. 1). The insert is the calibration curve established by a plot of the elution volume against molecular weight (log scale) for the calibration marker proteins; 1, bovine gamma globulin; 2, ovalbumin; 3, trypsin inhibitor; and 4, myoglobin. Molecular weights are shown in thousands.

TABLE 2. Amino acid composition of the purified β-lactamase from *C. butyricum* NBL 3

Amino acid	Mol%	No. of residues[a]
Asx	15.66	45
Thr	5.15	15
Ser	8.00	23
Glx	11.03	32
Pro	4.12	12
Gly	6.94	20
Ala	8.21	24
Cys[b]	0.84	2
Val	3.95	11
Met	1.07	3
Ile	6.60	19
Leu	8.83	25
Tyr	4.47	13
Phe	2.83	8
Lys	8.73	25
Arg	2.23	6
Trp	1.35	4
NH_3	22.3	64

[a] The number of amino acid residues was calculated by assuming a molecular weight of 32,000. The total number of residues is 287.
[b] Determined after performic acid oxidation.

Substrate profile. The kinetic parameters for various beta-lactam antibiotics hydrolyzed by the purified β-lactamase are shown in Table 3. The enzyme hydrolyzed benzylpenicillin, phenoxymethylpenicillin, ampicillin, and carbenicillin more effectively than the other beta-lactam antibiotics tested. The K_m value for ampicillin was in the concentration range possible to determine by direct measurement. There was no difference in the K_m values obtained by the two methods. K_m (micromoles per liter), V_{max} (micromoles per minute per milligram), and turnover (k_3, per second) as well as the ratio k_3/K_m are also shown in Table 3.

TABLE 3. Kinetic parameters of the purified β-lactamase from *C. butyricum* NBL 3 with different beta-lactam agents and the chromogenic substrate nitrocefin[a]

Beta-lactam agent (substrate)	K_m (μM)	V_{max}		k_3 (s^{-1})	k_3/K_m
		μmol/min per mg	Relative		
Benzylpenicillin	54	7,600	100	4,050	75
Ampicillin	150	12,100	159	6,430	43
Phenoxymethylpenicillin	65	4,800	63.9	2,570	40
Piperacillin	11	590	7.8	315	29
Carbenicillin	900	13,200	175	7,060	7.8
Cloxacillin	4,200	ND[b]			
Cephaloridine	690	1,400	17.6	714	1.0
Cephalothin	1,400	26	0.3	13.9	0.01
Cephalexin	1,700	0.9	0.01	0.5	<0.001
Cefoxitin	21,000	ND			
Moxalactam	9,900	4.9	0.07	2.6	<0.001
Cefotaxime	9,300	1.9	0.03	1.0	<0.001
Imipenem	180	0.08	0.001	0.04	<0.001
Nitrocefin	240	3,100	41.0	1,660	6.9

[a] The activity with each substrate was determined in 50 mM sodium phosphate buffer (pH 7.0) at 37°C. The final concentrations of β-lactamase used for the determination of V_{max} were 1.2 nM (benzylpenicillin, phenoxymethylpenicillin, ampicillin, piperacillin, nitrocefin, and carbenicillin), 3.5 nM (cephaloridine), and 70 nM (cephalothin, cephalexin, cefoxitin, moxalactam, cefotaxime, and imipenem).
[b] ND, Not determined.

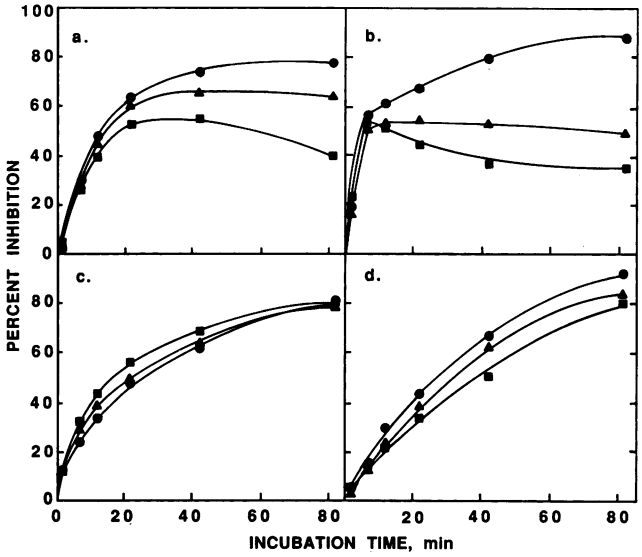

FIG. 4. Effect of time on the inhibition of β-lactamase activity by clavulanic acid (40 nM) (a), tazobactam (400 nM) (b), sulbactam (20 μM) (c), and pCMB (20 μM) (d). The β-lactamase (9 nM, ●; 27 nM, ▲; and 45 nM, ■) was incubated with the inhibitors for different times at pH 7.0 and 37°C before determination of the remaining activity.

Inhibition studies. Various concentrations of the enzyme (9, 27, and 45 nM) were preincubated for up to 80 min with 40 nM clavulanic acid, 400 nM tazobactam, 20 μM sulbactam, or 20 μM pCMB. With clavulanic acid the degree of inactivation initially proceeded as a mole-to-mole binding but, depending on the enzyme concentration, the inactivation was reversed with time (Fig. 4). With tazobactam there was a fast inactivation period, followed by a slow period of inactivation or reactivation, depending on the enzyme concentration (Fig. 4). In the presence of sulbactam or pCMB, the degree of inactivation increased during the whole incubation period (Fig. 4).

DISCUSSION

The known mechanisms of beta-lactam resistance in anaerobic bacteria involve the production of β-lactamases, the alteration of penicillin-binding proteins, and the blocking of the penetration of beta-lactams through the outer membranes. The most important factor in beta-lactam resistance is the production of β-lactamases. β-Lactamases of anaerobic bacteria, especially *B. fragilis*, have been extensively investigated during recent years (3, 19, 23, 24).

In contrast to *B. fragilis*, however, reports on resistance in gram-positive anaerobic bacteria are limited. Weinrich and Del Bene reported β-lactamase activity in *C. ramosum* and *C. clostridiiformis* (29). Carlson et al. demonstrated high resistance to penicillins in three strains of penicillinase-producing *C. butyricum* (4).

The properties of β-lactamases from different bacteria vary to a great extent, making investigations of highly purified enzymes justified. The present communication reports on the purification and properties of a new β-lactamase from *C. butyricum*. With the progress of analytical techniques for proteins, purification methods for β-lactamases have been substantially improved during recent years (6, 27). Two new techniques, mass anion exchange on QAE

Zetaprep and anion exchange on a strong anionic exchanger (Mono Q) with the FPLC system, were used for the purification of the enzyme. By mass anion exchange, the β-lactamase was initially isolated and highly purified (20-fold) within a few hours from 3 liters of untreated culture supernatant. The Mono Q column, which has a very good resolution but a limited load, was used in the last purification steps. In a previous investigation dealing with the purification of a β-lactamase from *Fusobacterium nucleatum* (27), chromatofocusing was shown to be useful. However, with chromatofocusing the proteins are eluted at a pH around the isoelectric point. To avoid such a low pH (4.4), we chose anion exchange on Mono Q for the purification of the *C. butyricum* β-lactamase. FPLC-gel filtration (Superose 12) made it possible to determine the molecular weight rapidly and was simultaneously a criterion for purity.

Kinetic parameters for different beta-lactam antibiotics revealed that the purified β-lactamase from *C. butyricum* is a typical penicillinase. Weinrich and Del Bene (29) classified the enzyme from *C. ramosum* as class IV β-lactamase according to Richmond and Sykes (21). Sykes and Smith classified the enzyme from *C. clostridiiformis* as class II β-lactamase (26). It has been reported that the substrate profile of the enzyme from *C. butyricum* resembles that of the enzyme from *C. clostridiiformis* (2, 8). Weinrich and Del Bene, however, stated that the enzyme from *C. clostridiiformis* was unusual since it was not affected by any of the inhibitors cloxacillin, cefoxitin, and pCMB (29). In our inhibition studies, the enzyme from *C. butyricum* was susceptible to inactivation by clavulanic acid, sulbactam, tazobactam, and pCMB. The low molar ratio of clavulanic acid to enzyme for inactivation indicates that hydrolysis or dissociation of clavulanic acid occurs at a very low rate. Since pCMB is a reagent that mainly reacts with cysteine, the inactivation of *C. butyricum* β-lactamase activity by this inhibitor is probably due to the presence of the two cysteines in the enzyme structure.

The molecular weight of the purified enzyme measured by both gel filtration and SDS gradient gel electrophoresis was 32,000. Hart et al. (8) reported a molecular weight of 80,000, which may represent an aggregated form of the enzyme. The isoelectric point of the purified enzyme was 4.4, and similar values have been reported by other investigators (2, 8).

On the basis of the results, it can be concluded that the β-lactamase from *C. butyricum* does not resemble any other previously described β-lactamases from anaerobic bacteria.

C. butyricum is not a common isolate like *B. fragilis* in anaerobic infections. However, this microorganism has been reported to be associated with bacteremia, traumatic wound infections, and neonatal necrotizing colitis (4, 7, 10). The novel β-lactamase from *C. butyricum* may have important clinical implications.

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