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A β-LACTAMASE OF *ESCHERICHIA COLI*

HIROSHI OGAWARA, KENJI MAEDA AND HAMAO UMEZAWA

Department of Antibiotics, National Institute of Health, Kamiosaki-2-chome, Shinagawa-ku, Tokyo 141 (Japan)

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SUMMARY

1. A β-lactamase of an *Escherichia coli* strain carrying an R factor derived from a *Proteus* strain, β-lactamase$_{75}$, was purified as a single peak on Sephadex and a single band on disc electrophoresis.

2. The molecular weight as determined by gel filtration and by disc electrophoresis was coincident and was 26 000.

3. The isoelectric point determined by the electrofocusing method was 5.26.

4. β-Lactamase$_{75}$ is quite sensitive to heat and is strongly inhibited by organic mercurials. In addition, the rate of hydrolysis of penicillin G and cephaloridine by this enzyme is greatly retarded by the presence of cloxacillin. From these results, it is concluded that β-lactamase$_{75}$ is a new type of β-lactamase.

5. Preliminary amino acid analysis showed the presence of cysteine. The significance of the cysteine residue is discussed in relation to the active site of this enzyme.

INTRODUCTION

Over the last few years there have been many reports describing the properties of β-lactamases such as penicillinase (penicillin amido-β-lactamhydrolase, EC 3.5.2.6) synthesized by a wide range of Gram-negative bacteria[1]. Jack and Richmond[2] examined β-lactamases from 46 strains of enteric bacteria, and Jenkins *et al.*[3] also studied those from 6 strains of *Shigella* and 4 strains of *Salmonella*. All the β-lactamases yet detected in Gram-negative bacteria can be classified into a number of types as follows[2–4]:

Type 1. Enzymes active mainly against cephalosporins or active mainly against penicillins. These enzymes are inhibited by cloxacillin but not by *p*-chloromercuribenzoate (PCMB).

Type 2. Enzymes with a broad substrate specificity which are inhibited by cloxacillin but not by PCMB.

Type 3. Enzymes with a similar broad substrate profile as those of Type 2 but

Abbreviation: PCMB, *p*-chloromercuribenzoate.

resistant to cloxacillin inhibition and sensitive to PCMB. These enzymes are sensitive to heat.

This report presents the purification and the properties including some chemical characteristics, of β-lactamase from an *Escherichia coli* strain carrying the R factor. This enzyme is sensitive not only to cloxacillin but also to organic mercurials and heat.

MATERIALS AND METHODS

Chemicals

Horse heart cytochrome *c*, sperm whale myoglobin, beef pancreatic chymotrypsinogen A and ovalbumin were purchased from Mann Research Laboratories. Trypsin (EC 3.4.4.4) was obtained from the Worthington Biochemical Co., pepsin (EC 3.4.4.1) from Sigma Co. and crystalline bovine serum albumin from Nutritional Biochemical Co. Penicillin G was a product of Takeda Pharmaceutical Co., ampicillin was obtained from Fujisawa Pharmaceutical Co., cephalothin from Eli Lilly Co. and cephaloridine from Glaxo Laboratories. DEAE-cellulose (0.96 mequiv/g) was obtained from Brown Co., and Sephadex G-75 (40–120 μm), Sephadex G-100 (40–120 μm) and Blue Dextran 2000 were obtained from Pharmacia Co. Acrylamide and *N,N'*-methylenebisacrylamide were purchased from Seikagaku Co. All inorganic salts and other organic reagents were of analytical reagent grade.

Growth of organisms

E. coli K12 W3630 carrying $R|_{75}^{+}|$, kindly supplied by Prof S. Mitsuhashi, Gunma University, was grown in 30 l of nutrient broth at 37 °C under aeration and stirring until the absorbance at 560 nm reached 0.7–1.0. Usually it took about 3 h. 4 l of an overnight culture were used as a seed culture. The culture was harvested at 0 °C using a Tomy Seiko continuous flow centrifuge at 21 500 × *g* (Tomy Seiko Co., Tokyo, Japan, Model RS-18P) and organisms stored at 5 °C until required.

Enzyme assay

β-Lactamase activity was determined iodometrically at pH 7.0 using a slight modification of the method of Perret[5], and the microiodometric assay of Novick[6] was used for the kinetic measurement of the enzyme reaction. Effect of cloxacillin on the rate of hydrolysis of cephaloridine was studied by the method of Jack and Richmond[2].

Molecular weight determination

Cytochrome *c* (mol. wt 11 700), myoglobin (17 200), trypsin (23 300), chymotrypsinogen A (25 700), pepsin (35 000) and ovalbumin (43 000) were used as molecular weight standards.

Gel filtration[7,8] was carried out at 3 °C using a 2 cm × 83 cm column of Sephadex G-100 equilibrated with 0.1 M sodium phosphate buffer, pH 7.0. The elution volume of β-lactamase$_{75}$ was determined by measuring the enzymic activity, while those of standard proteins were calculated from their protein concentrations. In each case the void volume was estimated using Blue Dextran 2000.

Disc electrophoresis was performed at room temperature by the method of Weber and Osborn[9].

Protein determination

Protein was determined using the method of Lowry *et al.*[10] with crystalline bovine serum albumin as a standard.

Amino acid analysis

Approximately 1 mg of the salt-free enzyme, which had been obtained by exhaustive dialysis and lyophilization, was dissolved in 1 ml of 6 M HCl and hydrolyzed at 110 ± 1 °C for 18 h in a sealed evacuated tube. The cysteine content was determined after performic acid oxidation and HCl hydrolysis[11]. The amino acid analysis was performed by the Hitachi KLA-3A analyzer. The tryptophan content was not determined.

RESULTS

Purification of the enzyme

All the procedures were carried out at 3 °C except where stated otherwise.

Step 1. Osmotic shock[12,13]. The cell pellet was subjected to osmotic shock by suspending the cells in 800 ml of 0.03 M Tris–HCl with 20% sucrose and 1 mM EDTA (pH 8.0), collecting the cells by centrifugation at 0 °C for 15 min at 13 000 × *g*, and then resuspending in 800 ml of ice–water. Cell debris was removed by centrifugation. This osmotic shock procedure was repeated two more times in the same volume and the supernatant was collected and lyophilized.

Step 2. Streptomycin sulfate treatment. The powder was dissolved in 500 ml of water and 5 g of streptomycin sulfate were added. After standing at 0 °C for 4 h, the precipitate was removed by centrifugation at 0 °C for 15 min at 13 000 × *g*. The supernatant was dialyzed against four changes of 5 l water and lyophilized.

Step 3. Sephadex G-75. The powder from Step 2 was dissolved in 50 ml of 0.001 M sodium phosphate buffer, pH 8.3, and passed through a 4 cm × 90 cm column of Sephadex G-75 equilibrated with the same buffer. The enzyme was eluted with the same buffer at a flow rate of about 50 ml/h. The active fractions (105 ml), detected by the spray method of Sykes and Richmond[14], were pooled and used directly for the next step.

Step 4. DEAE-cellulose. A 2.5 cm × 60 cm column of DEAE-cellulose was prepared by washing it with 2 M HCl and 2 M NaOH, and equilibrating with 0.01 M sodium phosphate buffer, pH 8.1. The enzyme solution from Step 3 was put on the column and washed with about 500 ml of the same buffer. A linear gradient (500 ml each) of 0–0.5 M NaCl in the same buffer was then applied at a flow rate of about 30 ml/h. The active fractions were pooled, dialyzed against three changes of 5 l of water and lyophilized.

Step 5. Sephadex G-100. The white powder from Step 4 was dissolved in 30 ml of water and placed on a 4 cm × 87 cm column of Sephadex G-100 which had been equilibrated with 0.01 M sodium phosphate buffer, pH 8.1. Elution was continued with the same buffer and fractions were collected in 15-g portions. The activity appeared from No. 35 to No. 43 with a maximum activity at No. 40.

Step 6. DEAE-cellulose. The enzyme solution from Step 5 was directly put on a 2.5 cm × 65 cm column of DEAE-cellulose equilibrated with 0.01 M sodium phosphate, pH 8.1. The column was washed with about 100 ml of the same buffer and eluted with a linear gradient (1 l each) of 0–0.5 M NaCl in the same buffer. The active fractions were dialyzed against three changes of 5 l of water and lyophilized.

Step 7. Sephadex G-100. The white powder from Step 6 was dissolved in 39 ml of water and passed through a 4 cm × 85 cm column of Sephadex G-100 equilibrated with 0.01 M sodium phosphate, pH 8.1. The same buffer was used as an elution fluid and 15-g portions of fractions were collected. The activity appeared from No. 33 to No. 41 with maximum at No. 36, which agreed well with the protein concentration.

The result of the purification was summarized in Table I.

TABLE I

PURIFICATION OF β-LACTAMASE$_{75}$

1 unit was defined as the amount of enzyme which hydrolyzed 1 μmole of penicillin G per h at 30 °C and pH 7.0.

Step	*Total protein (mg)*	*Total activity (units)*	*Specific activity (unit/μg)*	*Recovery (%)*
Extract	2170	$2.2 \cdot 10^6$	0.98	100
Sephadex G-75	315	$2.3 \cdot 10^6$	7.5	105
DEAE-cellulose	80.5	$1.1 \cdot 10^6$	14	50
Sephadex G-100	53.6	$4.8 \cdot 10^5$	9.0	22
DEAE-cellulose	14.3	$5.4 \cdot 10^5$	38	25
Sephadex G-100	12.3	$4.5 \cdot 10^5$	37	20

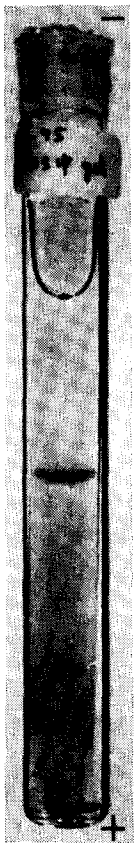
Physicochemical properties

The purity of the enzyme was checked by gel electrophoresis and gel filtration. In analytical polyacrylamide disc gel electrophoresis the enzyme migrated as a single band in a Tris–glycine buffer (pH 8.3) system. Disc gel electrophoresis in a sodium phosphate buffer (pH 7.0) containing sodium dodecyl sulfate system[9] also resulted in the appearance of a single band (Fig. 1).

Gel filtration with Sephadex G-100 (2 cm × 85 cm) in 0.01 M sodium phosphate buffer (pH 8.1) showed a single protein peak which coincided with the enzymic activity.

The molecular weight was calculated either from the mobilities in disc electrophoresis or from the elution volumes of a Sephadex G-100 column. The values obtained were 27 000 ± 1200 (S.E., 4 expts) and 25 500 ± 1400 (S.E., 2 expts), respectively. The former was calculated on the basis of protein migration, while the latter calculated on its enzymic activity. That these values were coincident within the error, again confirmed the purity of the enzyme.

The isoelectric point determined by the electrofocusing method (ampholine LKB 8131, pH 3–10) at 0 °C was 5.26. The pH–activity curve showed that the optimal pH was near 7 (Fig. 2). The activity decreased sharply in the acid region, slowly in the alkaline region.



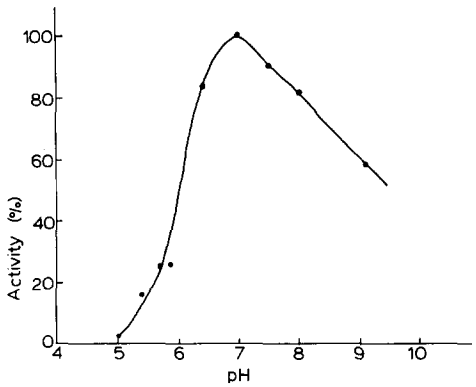


Fig. 1. Disc gel electrophoresis of β-lactamase$_{75}$. The disc gel electrophoresis shown in this figure was performed using the method of Weber and Osborn[9] for 5 h at room temperature.

Fig. 2. Effect of pH on the activity of β-lactamase$_{75}$. The enzyme was incubated at 37 °C for 30 min in a total volume of 1 ml with 6 μmoles of penicillin G and the reaction rate was measured iodometrically[5]. The buffers used were 0.1 M sodium acetate buffer for pH 5.0–5.7, and 0.1 M sodium phosphate buffer for pH 5.86–9.10.

Heat stability

The rate of heat inactivation of the enzyme was determined at 60 °C in 0.1 M sodium phosphate buffer, pH 7.0. The results showed that treatment for 1 min, 2 min and 5 min resulted in retention of only 10, 5 and 1% of the original activity, respectively. This fact indicates that the inactivation by heat is very rapid and irreversible for this enzyme.

Substrate specificity

The substrate specificity was determined against penicillin G, ampicillin, methicillin, oxacillin, cloxacillin, cephaloridine and cephalothin as substrates. The enzyme hydrolyzed penicillin G, ampicillin and cephaloridine fairly well, but methicillin, oxacillin, cloxacillin and cephalothin only slightly (Table II). Rate of hydrolysis of penicillin G was greatly retarded by the presence of cloxacillin, methicillin and oxacillin. The relative rate of hydrolysis of penicillin G (0.3125 mM) in 0.1 M sodium

TABLE II

RATE OF HYDROLYSIS OF PENICILLINS BY β-LACTAMASE$_{75}$

Rate of hydrolysis of penicillins was measured iodometrically[5] by incubating the mixture at pH 7.0 and 37 °C for 30 min. K_m and K_i values were determined at 25 °C using the Hitachi 124 recording spectrophotometer.

Penicillins	*Relative rate of hydrolysis*	*K_m or K_i (μM)*
Penicillin G	100	42.8
Ampicillin	123	
Methicillin	2.2	
Oxacillin	2.2	
Cloxacillin	0.2	43.7*
Cephaloridine	158	
Cephalothin	9.6	

* K_i for penicillin G.

phosphate buffer, pH 7.0, at 25 °C was found to be 1.00 (penicillin G only), 0.38 (*plus* cloxacillin, 0.3375 mM), 0.05 (*plus* methicillin, 0.3375 mM), and 0.12 (*plus* cloxacillin, 0.3375 mM). Hydrolysis of cephaloridine by β-lactamase$_{75}$ was also found to be inhibited by the presence of cloxacillin.

Effects of inhibitors

The effects of various organic and inorganic compounds on the activity of β-lactamase$_{75}$ were determined. From the results summarized in Table III, it is apparent

TABLE III

EFFECT OF VARIOUS SUBSTANCES ON THE ACTIVITY OF β-LACTAMASE$_{75}$

The enzyme was incubated in 0.1 M sodium phosphate buffer, pH 7.0, with various substances of the indicated concentrations for 10 min at 37 °C, except when *N*-ethylmaleimide and Ellman reagent (5,5'-dithiobis(2-nitrobenzoic acid)) were used. Incubations with these substances were carried out in 0.1 M sodium phosphate of pH 7.0 for *N*-ethylmaleimide or of pH 8.0 for Ellman reagent for 30 min at 37 °C. After the incubation, the remaining activities in aliquots were measured iodometrically. The following substances did not show the inhibition at the given concentration: $(NH_4)_2SO_4$, 1 mM; $ZnSO_4$, 1 mM; NaN_3, 5 mM; KCN, 1 mM; hydroxylamine, 1 mM; and 2,4-dinitrophenol, 1 mM.

Inhibitors or salts	*Concentration (mM)*	*Activity remaining (%)*
I_2	0.1	12
	0.02	—*
Urea	1.0	79
$CuSO_4$	0.5	41
$CuCl_2$	0.5	5
Copper (II) acetate	0.5	15
NaCl	100	—*
$NaNO_3$	100	—*
$CoSO_4$	1.0	70
Tetranitromethane	0.5	—*
N-Ethylmaleimide	1.0	—*
Ellman reagent	1.0	—*
PCMB	0.5	16
p-Chloromercuriphenylsulfonate	1.0	40

* No inhibition at the given concentration.

TABLE IV

EFFECT OF ORGANIC MERCURIALS ON THE ACTIVITY OF β-LACTAMASE$_{75}$

The enzyme was incubated in 0.1 M sodium phosphate buffer, pH 7.0, with organic mercurials, PCMB and *p*-chloromercuriphenylsulfonate, at the indicated concentrations for 10 min at 37 °C. The remaining enzymic activity was determined iodometrically with penicillin G as a substrate[5]. Figures in the table are expressed as the percentage of the remaining activity to the control.

Concentration (mM)	*Organic mercurials*	
	PCMB	*p-Chloromercuriphenylsulfonate*
1.0	6.3	38.5
0.75	—*	47.3
0.5	16.0	78.3
0.3	—*	73.9
0.25	29.4	—*
0.1	44.1	94.7

* Not determined.

that iodine and Cu^{2+} inhibit strongly the enzymic activity and Co^{2+} inhibits slightly. It should be pointed out here that PCMB also showed a strong inhibitory effect. This effect was observed even in treatment at 0 °C for 10 min with 0.832 mM PCMB in 0.1 M phosphate buffer, pH 7.0, which resulted in loss of 48% of the original activity. Another organic mercurial, *p*-chloromercuriphenylsulfonate, also produced inhibition of the β-lactamase activity (Table IV). In these cases, linear relationships were observed between the remaining enzymic activity and the logarithm of the concentrations. The inhibition by PCMB could be reversed by treatment with a 3 molar excess of glutathione, L-cysteine or dithiothreitol. Treatment with *N*-ethylmaleimide or with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman reagent) did not affect the enzymic activity (Table III).

Amino acid analysis

A sample of the enzyme was hydrolyzed in 6 M HCl for an amino acid analysis and the result is shown in Table V. Since the quantities of the purified protein available for analysis in this experiment were small, the amino acid composition shown in

TABLE V

AMINO ACID COMPOSITION OF β-LACTAMASE$_{75}$

The values given are the average of three determinations except for cysteine. This value is the average of two determinations.

Amino acids	*Relative molar ratio (leucine = 100)*	*Amino acids*	*Relative molar ratio (leucine = 100)*
Lysine	91.7	Alanine	131.0
Histidine	18.6	Cysteine	25.8
Arginine	48.2	Valine	74.7
Aspartic acid	146.9	Methionine	27.1
Threonine	67.3	Isoleucine	52.8
Serine	41.0	Leucine	100
Glutamic acid	125.2	Tyrosine	11.4
Proline	51.3	Phenylalanine	36.3
Glycine	79.5		

Table V is regarded as approximate. Preliminary experiments to measure the cysteic acid content[11] of the hydrolyzates of performic acid-oxidized samples of β-lactamase$_{75}$ gave values equivalent to about 3 moles of cysteine per mole of enzyme, assuming a molecular weight of 26 000. These results suggest that this enzyme has more than one residue of cysteine/mole.

DISCUSSION

A β-lactamase of an *E. coli* strain carrying the R factor, β-lactamase$_{75}$, was purified to the extent where it gave a single peak on Sephadex G-100 and a single band on disc electrophoresis. The agreement of the molecular weights, which were estimated either from the mobilities in disc electrophoresis or from the elution volumes from Sephadex G-100, confirmed the purity of the enzyme.

Among the β-lactamases reported, penicillinase$_{14}$ obtained from *E. coli*[15] is most similar to β-lactamase$_{75}$. Both enzymes have similar molecular weights and isoelectric points, and hydrolyze cloxacillin only slightly. However, β-lactamase$_{75}$ is quite sensitive to heat and is strongly inhibited by organic mercurials, PCMB and *p*-chloromercuriphenylsulfonate (Table IV). In addition, the rate of hydrolysis of penicillin G and cephaloridine by this enzyme is greatly retarded by the presence of cloxacillin. These facts, *i.e.* inhibition by organic mercurials and also by cloxacillin, strong lability in heat and little ability for hydrolyzing cloxacillin, are inconsistent with the suggestion[2] that β-lactamase enzymes sensitive to cloxacillin are resistant to inhibition by PCMB, and *vice versa*. They are also inconsistent with the finding of Jenkins and Drabble[3] that β-lactamases sensitive to heat hydrolyze cloxacillin rather rapidly. It is concluded, therefore, that β-lactamase$_{75}$ is a new type of β-lactamase. It is interesting in this respect that the synthesis of this enzyme is mediated by only one R factor (personal communication from Prof S. Mitsuhashi).

Two β-lactamases containing cysteine in their molecules have been isolated from *Aerobacter cloacae* 53[16] and from the Gram-positive bacterium, *Bacillus cereus* 569/H[17,18]. The presence of cysteine in β-lactamase$_{75}$ is shown by performic acid oxidation and HCl hydrolysis. This result, in addition to the inhibition by organic mercurials, suggests that the cysteine residue is concerned with the β-lactamase activity of this enzyme. The fact that PCMB inhibits the enzymic activity even at 0 °C supports this suggestion. However, *N*-ethylmaleimide and Ellman reagent do not show the inhibitory effect. This indicates that the cysteine residue might not be in direct connection with the active site of β-lactamase$_{75}$, as in the case of β-lactamase from *Aerobacter cloacae* 53[16]. Further studies are necessary for explaining this.

Csányi *et al.*[19] described that a tyrosine residue is concerned with the activity of a β-lactamase from *Bacillus cereus*, and Meadway[20], using an inhibition study of the enzyme from *Bacillus licheniformis* by tetranitromethane, also suggested that the tyrosine residue is associated with the enzymic activity. The data presented above (Table III) indicate that this is not the case in β-lactamase$_{75}$.

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