Biochem. J. (1968) **109**, 469
Printed in Great Britain

The Purification and Some Properties of a β-Lactamase (Cephalosporinase) Synthesized by *Enterobacter cloacae*

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(*Received* 26 *April* 1968)

1. A β-lactamase has been purified from a strain of *Enterobacter cloacae*. 2. This enzyme is about eighty times as active against cephaloridine as against benzylpenicillin or ampicillin. 3. The enzyme has a net positive charge at pH 8·0 and a molecular weight of about 14000. 4. An approximate amino acid composition of the enzyme is reported.

So far only one β-lactamase (EC 3.5.2.6) has been purified from a Gram-negative species. This was the enzyme synthesized by *Escherichia coli* strain TEM, in which the genes specifying enzyme synthesis and structure were found to be carried on a resistance factor (R-factor) (Datta & Kontomichalou, 1965; Datta & Richmond, 1966). This enzyme was about half as active when the cephalosporin derivative cephaloridine was used as substrate as it was with benzylpenicillin or ampicillin.

This paper reports the isolation of a β-lactamase from a second Gram-negative species, *Enterobacter cloacae*. The enzyme produced differs from the one purified from *Escherichia coli* strain TEM in a number of respects. Although it has an approximately similar molecular weight (about 14000), its physical properties are distinctly different, as it carries a net basic charge at pH 8·0. Further, the enzyme is primarily a cephalosporinase, being about eighty times more active against cephaloridine and cephalosporin C than against benzylpenicillin.

The genes involved in β-lactamase synthesis in *Enterobacter cloacae* strain 214 appear to be chromosomal; at least there is no evidence that they are lost from the cells at high frequency or are transferred from cell to cell by conjugation, both of which are characteristics of genes carried on R-factors (Datta, 1965).

METHODS AND MATERIALS

Organism and medium. Enterobacter cloacae strain 214 was chosen for this work as it produced relatively large amounts of enzyme compared with other strains of this species (Hennessey, 1967). It is a distinct strain from that studied by Smith & Hamilton-Miller (1963) and by Hamilton-Miller, Smith & Knox (1965) and described as *Escherichia coli* strain 214.

The organism was grown in 1% CY medium of the following composition: acid-hydrolysed casein (Difco), 1·0%; yeast extract (Difco), 1·0%; sodium β-glycerophosphate, 0·12M; $MgSO_4,7H_2O$, 1·0mM; trace-metal solution, 0·02ml./l. The trace-metal solution contained: $CuSO_4,5H_2O$, 0·5%; $ZnSO_4,7H_2O$, 0·5%; $FeSO_4,7H_2O$, 0·5%; $MnCl_2,4H_2O$, 0·2%; conc. HCl, 1% (final concn.). No glucose was added to this medium, since its presence caused *Enterobacter cloacae* strain 214 to produce a slime that hindered successful harvesting.

Culture and harvesting of organisms. Cultures (12l.) were grown in a fermenter (Microferm; New Brunswick Scientific Co. Inc., New Brunswick, N.J., U.S.A.) in 1% CY medium. The medium was inoculated, to give an initial culture density of about 0·05mg. dry wt. of bacteria/ml., from a stationary-phase culture that had been grown overnight in 1% CY medium. The culture was aerated with 2–6l. of air/min. and stirred at 200rev./min. Antifoam (tripentyl citrate) was added, as necessary, to check foaming. After about 1½hr. growth, benzylpenicillin (0·5g./l.) was added to the culture, as this induced the culture to some extent and therefore increased the final yield of enzyme (Hennessey, 1967). The culture was harvested by pumping it through a continuous-flow centrifuge after about 5hr. growth. The organisms collected in this way were resuspended in about 200ml. of 0·1M-KH_2PO_4–Na_2HPO_4 buffer, pH 7·0, centrifuged down again, and resuspended in 100ml. of similar buffer.

Enzyme assay. Enzyme activities are expressed in units similar to those originally defined by Pollock & Torriani (1953) for penicillinase. With the enzyme studied here, the unit is expressed as μmoles of cephaloridine hydrolysed/hr. at 30° and pH 5·9. Hydrolysis of the cephaloridine was followed iodometrically by the method used by Perret (1954) for penicillin hydrolysis. The calculation of enzyme activity, however, took account of the fact that after

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hydrolysis 1 mole of a cephalosporin derivative is equivalent to about 4g.equivalents of iodine, rather than to 8g.equivalents of iodine, which are found on hydrolysis of a penicillin (Alicino, 1961). The cephaloridine solution used in the assay was 7mM in 0·2M-KH_2PO_4–Na_2HPO_4 buffer, pH 5·9.

Protein determinations. The approximate concentration of proteins in column eluates was followed spectrophotometrically. A protein solution of 1·0mg./ml. was assumed to have an extinction of 1·0 at 280mμ. For more accurate protein estimations the method of Lowry, Rosebrough, Farr & Randall (1951) was used.

Preparation of columns. (*a*) Sephadex C-50. This material was equilibrated against 10mM-phosphate buffer, pH 6·2, in a conical flask, degassed and poured as a column (2·5cm. × 30cm.).

(*b*) Sephadex G-75. This material was equilibrated against 33mM-phosphate buffer, pH 7·0, containing 1·0mM-sodium azide as a preservative, degassed and poured as a column (2·0cm. × 115cm.).

Buffer solutions. All phosphate buffer solutions were KH_2PO_4–Na_2HPO_4 unless otherwise stated.

Materials. Benzylpenicillin and cephaloridine, which is 7-[(2-thienyl)acetamido]-3-(1-pyridylmethyl)cephalosporanic acid, were obtained commercially from Glaxo Laboratories Ltd. (Greenford, Middlesex).

CM-Sephadex C-50 and Sephadex G-75 were obtained from Pharmacia (Uppsala, Sweden).

Ultrasonic disintegrator. The organisms were disrupted in a 100w ultrasonic disintegrator (Measuring and Scientific Equipment Ltd., London, S.W. 1). In certain experiments the cells were disrupted in a continuous-flow cell provided by the manufacturers.

Amino acid analysis. The method of Spackman, Stein & Moore (1958) was used with an amino acid analyser (Evans Electroselenium Ltd., Halstead, Essex) fitted with the accelerated modification described by Spackman (1963). Sample loadings were about 0·5mg. of protein hydrolysate/column.

Samples for analysis were evaporated to dryness in 16mm. × 125mm. Pyrex test tubes and then dissolved in 0·5ml. of freshly diluted 6N-HCl. The contents of the tube were frozen in acetone–solid CO_2, and the tubes were evacuated to a pressure of 0·1–0·05mm. Hg while the acid thawed. After 5min. evacuation, the tubes were sealed and heated at 105±0·5° for 24hr. The acid was then removed in a desiccator at room temperature.

Starch-gel electrophoresis. This was done as described by Smithies (1955), with a bridge solution of 0·33M-sodium borate buffer, pH 8·45, and run for 110min. The gel itself was prepared in 50mM-glycine–NaOH buffer, pH 8·9. The presence of protein bands in the gel was detected by staining with Amido Black B solution, as described by Grassman & Hanning (1950). The Amido Black solution was made up by dissolving 13g. of Amido Black B/l. of solution containing methanol (450ml.), acetic acid (100ml.) and water (450ml.). The location of cephalosporinase activity was detected by spraying the gel with a solution containing 1·0g. of cephaloridine dissolved in 10ml. of 10mM-I_2–30mM-KI soln. The presence of enzyme activity was shown by the presence of a white band against the dark-blue background caused by the interaction of the iodine solution with the starch of the gel.

RESULTS

Purification procedure

The quantity of enzyme activity remaining in the preparation after each stage of the purification procedure, together with the percentage recovery of enzyme activity at each stage, are shown in Table 1.

Stage 1*: Ultrasonic disintegration.* The suspension of organisms in 0·1M-phosphate buffer, pH 7·0, which was obtained when the culture from the fermenter was centrifuged and washed (see the Methods and Materials section) was cooled to 4° and disrupted for 1min. in the ultrasonic disintegrator; 5ml. of the suspension was treated at a time and the broken cells were stored at 4° until the entire cell suspension had been processed. This usually took about 1hr. Rise in temperature during disruption was minimized by immersing the tube in which the suspension was treated in a bath of chipped solid carbon dioxide. Subsequently it was found possible to break the bacteria in a continuous-flow cell coupled to the disintegrator, and then the bacteria were disrupted so that the mean residence time of bacteria in the flow cell was about 1min.

The suspension of broken cells was centrifuged at 5000*g* at 4° for 20min. to remove unbroken cells

Table 1. *Summary of purification of β-lactamase from* Enterobacter cloacae

Experimental details are given in the text. The starting material was 12l. of culture containing 27·8g. dry wt. of bacteria.

Stage no.	Procedure	Activity recovered (units)	Recovery (%) Per stage	Recovery (%) Overall
1	Disintegration	3585400	100	100
2	Centrifugation	2587200	72·1	72·1
3	Dialysis	2482000	95·9	69·2
4	Chromatography on CM-Sephadex C-50	1476096	59·1	41·1
5	Dialysis and gel filtration on Sephadex G-75	1330560	90·1	37·1

and cell debris, and the supernatant fluid was either stored at 4° or used immediately for the next stage of the purification.

Stage 2: Centrifugation. The enzyme preparation from stage 1 was centrifuged at 105000*g* at 2° for 2hr. and the sediment discarded.

Stage 3: Dialysis. The enzyme solution from stage 2 (about 250ml.) was dialysed twice against 1mM-phosphate buffer, pH 7·0, at 4° for 16hr. each. The contents of the dialysis bags were then pooled, freeze-dried and reconstituted in 30ml. of 50mM-phosphate buffer, pH 6·0. During this stage a precipitate formed; this was removed by centrifugation at 105000*g* at 2° for 2hr.

Stage 4: Chromatography on CM-Sephadex C-50. To prepare the enzyme solution obtained from stage 3 for chromatography, the material was dialysed for 16hr. at 4° against 1mM-phosphate buffer, pH 6·0. The dialysate was then loaded on to a Sephadex C-50 column (see the Methods and Materials section), and the column was washed with about 150ml. of 10mM-phosphate buffer, pH 6·0. Much protein, nucleic acid and pigmented material but less than 1% of the enzyme activity applied to the column passed straight through. This material was discarded. The enzyme was eluted with a concentration gradient of phosphate buffer, pH 6·2. The gradient was constructed from 180ml. each of 0·5M and 10mM buffer. About 90% of the total enzyme activity recovered was found in five fractions (30ml. total), which were then pooled.

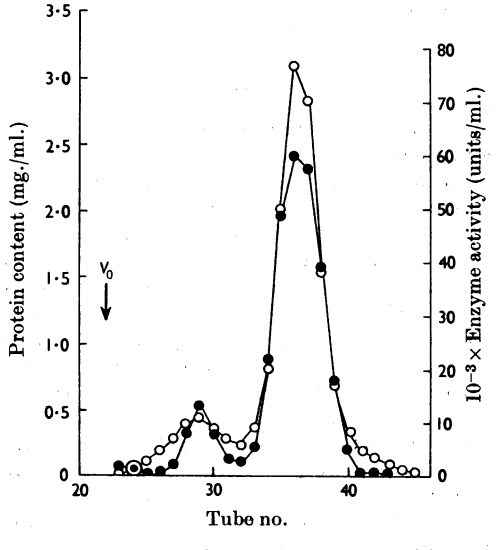

Fig. 1. Gel filtration of *Enterobacter cloacae* β-lactamase through Sephadex G-75. ○, Protein content; ●, enzyme content; V_0, exclusion volume of the column.

Stage 5: Dialysis and gel filtration. The pooled fractions from stage 4 were dialysed at 4° for 16hr. against water and freeze-dried. The enzyme was reconstituted in about 2ml. of water and applied to a Sephadex G-75 column which had previously been equilibrated against 33mM-phosphate buffer, pH 7·0. Fig. 1 shows the elution pattern obtained when the enzyme was eluted by the same buffer. Although two peaks of very similar specific enzyme activity were found, fractions from the main peak only were pooled (eight fractions, 27·2ml. total). These combined fractions were dialysed against water and freeze-dried.

To check the purity of this preparation, a small sample of the enzyme was submitted to starch-gel electrophoresis at pH 8·0, and a single protein band corresponding to the cephalosporinase activity was found. The enzyme migrated towards the anode at this pH value. The purity of the material obtained by stage 5 was further confirmed by rechromatography on CM-cellulose. About 65% of the total enzyme activity was recovered in seven 3·0ml. fractions with a specific enzyme activity against cephaloridine of 27·2, 26·8, 25·3, 25·7, 26·6, 27·1 and 27·1 units/μg. of protein respectively.

Molecular weight

The approximate molecular weight of the protein was determined by passing it down a Sephadex G-50 column in the presence of ox heart cytochrome *c* (mol.wt. 14000) and myoglobin (mol.wt. 17000) as markers. The cephalosporinase was eluted in the same position as the cytochrome *c*; this suggests that the enzyme has a molecular weight of about 14000.

Amino acid composition

A sample (approx. 1mg.) of the enzyme was hydrolysed as described in the Methods and Materials section, and the amino acid content of the hydrolysate was determined with an amino acid analyser. The quantity (μmoles) of each amino acid recovered is shown in Table 2. These results are also expressed as residues/mol. on the basis of a molecular weight of 14000. This value was chosen primarily because of the results reported above, but also because it agreed well with the presence of two histidine residues/mol. of enzyme. The two other values for the molecular weight that are feasible on the basis of the amino acid analysis were 7000 (one histidine residue/mol.) and 21000 (three histidine residues/mol.). Neither of these two values were considered likely because of the behaviour of the enzyme on the Sephadex G-50 column.

Since the quantities of purified protein available for analysis in this experiment were small, the amino acid composition shown in Table 2 is regarded

Table 2. *Amino acid composition of* Enterobacter cloacae *β-lactamase*

The quantity of enzyme hydrolysed was approx. 1 mg. For experimental details see the text. The number of residues/mol. was calculated on the basis of a molecular weight of 14000. The quoted value for methionine is the sum of the quantity of methionine and that of methionine sulphoxide in the analysis. The quoted values for serine and threonine are respectively 3% and 6% higher than the measured values to allow for the partial destruction known to occur with these amino acids under the hydrolysis conditions used (Ambler & Brown, 1967).

	Amino acid composition	
	(μmole)	(residues/mol.)
Lysine	0·39	9
Histidine	0·09	2
Arginine	0·20	5
Aspartic acid	0·50	10
Threonine	0·37	8
Serine	0·35	7
Glutamic acid	0·56	12
Proline	0·44	9
Glycine	0·47	10
Alanine	0·72	15
Valine	0·46	10
Methionine	0·23	5
Isoleucine	0·26	6
Leucine	0·48	10
Tyrosine	0·24	5
Phenylalanine	0·15	3
Cysteine	?	?
Tryptophan	?	?

as approximate. Further, the analysis contains no value for cysteine nor for tryptophan since both these amino acids are likely to have been totally destroyed by the hydrolysis conditions. Preliminary experiments to measure the cysteic acid content of hydrolysates of performic acid-oxidized samples of *Enterobacter cloacae* β-lactamase (Hirs, 1956) gave values equivalent to about 0·4 mole of cysteic acid/mole of enzyme, assuming a molecular weight of 14000. These results suggest that this *Enterobacter cloacae* β-lactamase has at the most one residue of cysteine/mole, but that in all probability, as in the β-lactamases from Gram-positive species, cysteine is lacking.

Substrate profile

The purified enzyme preparation was tested for its activity against various substrates, and values for the turnover number of the enzyme against these substrates are shown in Table 3. The relative rates of hydrolysis found are very similar to those reported by Hennessey (1967). The Michaelis constant of the enzyme was measured with benzylpenicillin only and was found to be 53 μM. Determination of Michaelis constants with cephalosporin derivatives as substrates proved difficult as commercial preparations of these compounds always contain some contaminating iodine-reacting material, and the presence of this material means that the micro assay for penicillinase (Novick, 1962) cannot be used. Even recrystallization of the substrate leaves enough contamination to make the method unreliable.

Table 3. *Rate of hydrolysis of various substrates by* Enterobacter cloacae *β-lactamase*

The rate of hydrolysis of these substrates was determined iodometrically by the method of Perret (1954) as modified by Novick (1962). Initially the results were calculated in terms of μmoles of substrate destroyed/hr./μg. of enzyme protein; they were then recalculated as turnover numbers on the basis of a molecular weight for the enzyme of 14000. For the purposes of this calculation, 1 mole of a penicillin derivative was taken to be equivalent to 8 g.equivalents of iodine (Perret, 1954) and 1 mole of a cephalosporin derivative to be equivalent to 4 g.equivalents of iodine (Alicino, 1961).

Substrate	$V_{max.}$ (turnover number)
Benzylpenicillin	71
Ampicillin	8
Phenoxymethylpenicillin	61
Cephaloridine	$5{\cdot}8 \times 10^3$
Cephalothin	$8{\cdot}2 \times 10^2$
Cephalosporin C	$5{\cdot}1 \times 10^3$
7-(Phenylacetamido)cephalosporanic acid	$1{\cdot}3 \times 10^3$

DISCUSSION

Apart from its approximately similar molecular weight, the enzyme studied here is clearly different from the only other β-lactamase yet purified from a Gram-negative species (Datta & Richmond, 1966). Whereas the *Escherichia coli* enzyme was about twice as active against benzylpenicillin (turnover number $2{\cdot}0 \times 10^4$) as against cephaloridine (turnover number $1{\cdot}4 \times 10^4$), the enzyme from *Enterobacter cloacae* was about eighty times as active against cephaloridine (turnover number $5{\cdot}8 \times 10^3$) as against benzylpenicillin. Further, the two enzymes migrated in opposite directions on electrophoresis at pH 8·0 in starch gel. Despite these differences, however, it should be stressed that it does not inevitably follow that the amino acid sequence of the two proteins will be found to be unrelated.

Hamilton-Miller *et al.* (1965) found that two strains of *Aerobacter* (*Enterobacter*) *cloacae* hydro-

lysed cephaloridine at about 60% of the rate of hydrolysis of benzylpenicillin, compared with about 8000% found for the strain studied here. The identity of these strains is in doubt (J. M. T. Hamilton-Miller, personal communication). Extracts from these two strains attacked ampicillin as readily as benzylpenicillin, whereas *Enterobacter cloacae* strain 214 hydrolysed ampicillin at less than 10% of the rate of hydrolysis of benzylpenicillin.

Whether these two sets of results indicate that more than one type of β-lactamase can be found among strains of *Enterobacter cloacae*, or whether they just reflect the difficulty of distinguishing the various species of Enterobacteriaceae by their cultural characteristics (cf. Cowan & Steel, 1965), is not clear. There is no doubt, however, that the strain of *Enterobacter cloacae* studied in detail by Fleming, Goldner & Glass (1963) and studied further by Hennessey (1967), makes a β-lactamase very similar to, if not identical with, that examined in the present paper.

Although the method used in this work to determine the molecular weight of the enzyme only gives an approximate value, it seems probable that the *Enterobacter cloacae* β-lactamase has a molecular weight similar to that of the penicillinase purified from *Escherichia coli* strain TEM, the only other Gram-negative species to be examined in detail (Datta & Richmond, 1966). Both these lactamases from Gram-negative species have molecular weights of about 15000, and they are therefore probably quite distinct molecules from the lactamases that have been purified from Gram-positive species, where the molecular weights appear to lie in the range 28000–35000 (Citri & Pollock, 1966).

We thank Dr R. P. Ambler for carrying out the amino acid analyses and for his advice over many other points that arose in the course of the work. M.H.R. is an honorary member of the Medical Research Council Group on Bacterial Enzyme Variation.

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