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Note

Chromatofocusing for separation of β-lactamases

I. Microscale separation of RTEM- and chromosomally mediated β-lactamases of *Escherichia coli* J6-2

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Penicillin resistance in association with β-lactamase production in Gram-negative bacteria was recognized by Anderson and Datta[1]. The RTEM* plasmid-carrying bacteria seem to be very common in a wide range of compatibility groups from a broad taxonomic range[2]. Matthew and Hedges[3] classified TEM**-type β-lactamases into two subclasses TEM-1 and TEM-2 according to their isoelectric point of p*I* 5.6. The subclass TEM-1 was characterized further by immunology and isoelectric focusing (IEF)[3–5]: TEM-1 type 1, p*I* = 5.4; TEM-1 type 2, p*I* 7.7. New details of the molecular pharmacology of the transposable elements (Tn 3 type), responsible for the mediation of RTEM-1 β-lactamases of strains other than *Escherichia coli*, have recently been published[6]. In *E. coli* strains a common, low activity, chromosomally mediated β-lactamase can also be detected with a p*I* > 8 in the alkaline range[7].

RTEM type β-lactamases have been isolated from different microorganisms by using miscellaneous combinations of separation techniques[8–12]. Recently, screening (identification and classification) of these enzymes based on their substrate profiles and p*I* values has become general practice[13–16].

We have extended the arsenal of separation and isolation methods for β-lactamases simultaneously present in bacteria by introducing the technique of chromatofocusing (Pharmacia, Uppsala, Sweden)[18]. Preparation of the β-lactamases from the crude extract on the basis of their p*I* values enables their detection and separation in a single step. In the present study chromatofocusing was used for analytical/microscale purification of β-lactamases of an RTEM$^+$ *E. coli* J6-2 strain and of its RTEM$^-$ variant for comparison.

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* RTEM: drug resistance plasmid (R), carrying a TEM[19,20].
** TEM: transposable element mediator = transposon.

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EXPERIMENTAL

Bacterial strains

E. coli J6-2 $Pro^- His^- Trp^- Lac^-$ RTEM-1 type 1^+ and its $RTEM^-$ variant was obtained from Dr. J. T. Smith, The Microbiology Section, The School of Pharmacy, University of London, London, Great Britain. The organisms were grown in normal bouillon which has the following composition: beef extract (Difco), 0.3%; bacto pepton (Difco), 0.5%; NaCl, 0.5%; glucose, 0.1%. The glucose was autoclaved separately and added immediately before use. For routine growth a colony was transferred from an agar plate to 100 ml of medium on a shaker at 38°C and left overnight. In the morning, 150-ml portions of the medium in 500-ml conical flasks were inoculated with 5 ml of the culture and shaken at 38°C for 4–5 h.

Partial purification of β-lactamases

The cells were harvested by low-speed centrifugation and washed twice with 0.05 *M* sodium phosphate, pH 7.0 buffer. The sediment was resuspended in the same buffer to about 4–5 times of its wet weight. The cells were broken in the macro flow cell of a Braunson Sonifier Model B-15P in ten 1-min runs, with 1-min cooling intervals, at 0°C and maximum power. The supernatant from the low-speed centrifugation was centrifuged again at 80,000 *g* for 60 min at 4°C. The supernatant was then dialysed overnight against 0.05 *M* sodium phosphate, pH 7.0 buffer. The crude enzyme solution was loaded on to a DEAE-cellulose column (18 × 3 cm) and eluted with the same buffer at 5 ml per 10 min, at 4°C (stage 1). Fractions showing the highest β-lactamase activity with Nitrocefin®, kindly supplied by Dr. R. T. Hotston, Glaxo Group Research, Greenford, Great Britain, were combined and loaded on to a Sephadex G-50 superfine column (18 × 3 cm) (stage 2), eluted and tested in the same way as for the β-lactamase fractions showing the highest activity. The most active fractions were then pooled and dialysed extensively to reduce the sodium phosphate molarity to 10^{-3} *M*, and finally were freeze dried in small portions.

Crude, dialysed, sonicated samples of both the $RTEM^+$ and $RTEM^-$ strains were saturated with $(NH_4)_2SO_4$ and the precipitate redissolved in a small volume of 0.05 *M* sodium phosphate, pH 7.0, followed by low-speed centrifugation and extensive dialysis against the same buffer. Besides these, stage 2 samples of the $RTEM^+$ β-lactamase preparation were also used for chromatofocusing studies.

Chromatofocusing

Before application of the samples to the PBE 94 column[18], buffer exchange was carried out on a Bio-Gel P-2 column (7 × 1 cm) using 0.025 *M* imidazole–HCl buffer[18], pH 7.4. The PBE 94 column (20 × 1 cm) was equilibrated before use with 0.025 *M* imidazole–HCl buffer, pH = 7.4. The enzyme was eluted with 150 ml Polybuffer 74[18]. The original Polybuffer 74 was diluted eight-fold by volume, adjusted to pH = 4.0 with HCl, degassed and 5 ml were applied to the column[18]. The protein (about 500–5000 μg per 3 ml) was applied to the top of the column, followed by the Polybuffer 74, pH 4.0, 3-ml fractions were collected, and the pH and β-lactamase activity of each fraction were estimated. The pH was measured with a PHM 62 Standard pH Meter (Radiometer, Copenhagen, Denmark). Chromatofocusing reagents were separated from the isolated enzyme by filtration on Bio-Gel P-2 in 0.01 *M* sodium phosphate buffer, pH 7.0.

Protein estimation

After buffer exchange, the protein content of the solutions was estimated by measuring the absorbance at 280 nm. In all other cases the method of Lowry *et al.*[17] was employed.

Enzyme assay

β-Lactamase activity was assayed in 0.01 *M* sodium phosphate, pH 7.0 with Nitrocefin, at 30°C. One unit is that amount of enzyme which is able to hydrolyze 1 μmol of Nitrocefin in 1 min at 30°C.

RESULTS AND DISCUSSION

Partially purified β-lactamase preparation from *E. coli* RTEM-1 type 1^+ was obtained as described in the Experimental section. The results of the experiment with the stage 2, partially purified material are shown in Fig. 1. The pH gradient is fairly linear in the range pH 7–4, showing a slightly biphasic character. The enzyme was eluted as one peak (fractions 16–19), at a pH of 5.42. Of the total 117 units of enzyme applied to the PBE 94 column, 112 units (97 %) were recovered in the four fractions. The activities in the fractions were as follows: fraction 16, 26 units; 17, 27 units; 18, 30 units; 19, 29 units. The chromosomally mediated common β-lactamase was not

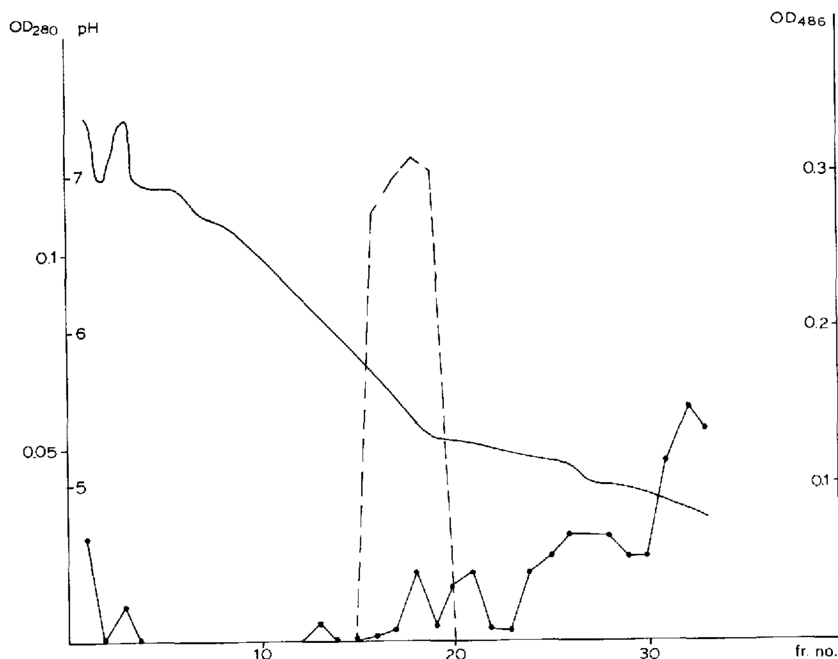

Fig. 1. Elution profile on PBE 94 of partially purified β-lactamase (stage 2). About 113 units of β-lactamase from the stage 2 sample of *E. coli* J6-2 $RTEM^+$ in 3 ml of 0.025 *M* imidazole–HCl buffer, pH 7.4 were applied to a C 10/20 column[18], 20 cm × 10 mm, of PBE 94. Elution conditions, pH (——), protein (●—●) and activity (---) monitoring were as described in the Experimental section. 490 μg of total protein were applied to and 408 μg of protein were eluted from the column.

found. This enzyme would have been eluted in the initial fraction because its p*I* value is greater than 8[7], and it is not bound to the column. The reason for the absence of this enzyme might be that it was eliminated by DEAE-cellulose and Sephadex G-50 chromatography.

The elution profile of the crude β-lactamase preparation of *E. coli* RTEM-1 type 1$^+$, obtained after 100% saturation with $(NH_4)_2SO_4$, is illustrated in Fig. 2. The pH gradient obtained is similar to that of Fig. 1. As expected, two enzyme peaks were eluted. The enzyme in the first peak (fractions 1–3) has a p*I* > 7.4, since it is not bound to the column. It is therefore probably identical with the chromosomally mediated β-lactamase[7]. The second peak (fractions 13–15) has p*I* 5.42. Of the total of 60 units of enzyme applied to the column, 5.6 units were eluted in the first peak (fraction 1, 2.2 units; 2, 2.4 units; 3, 1 unit) and 52.6 units were eluted in the second peak (fraction 13, 15.6 units; 14, 19 units; 15, 18 units), *i.e.*, 58.2 units (97%) in all. The first peak corresponds to the chromosomal enzyme and the second to the TEM-1 type 1 β-lactamase.

Similarly to isoelectric focusing chromatofocusing is a good method for separation of both the chromosomally and the transposon (Tn 3 like) coded β-lactamases.

Fig. 3 shows the elution profile of the crude enzyme preparation of *E. coli* RTEM-1 type 1$^-$ obtained after 100% saturation with $(NH_4)_2SO_4$. The pH gradient is similar to those of Figs. 1 and 2. Because of the lack of RTEM-1 type 1, only the chromosomally coded enzyme was eluted in a single peak (fractions 1 and 2). The

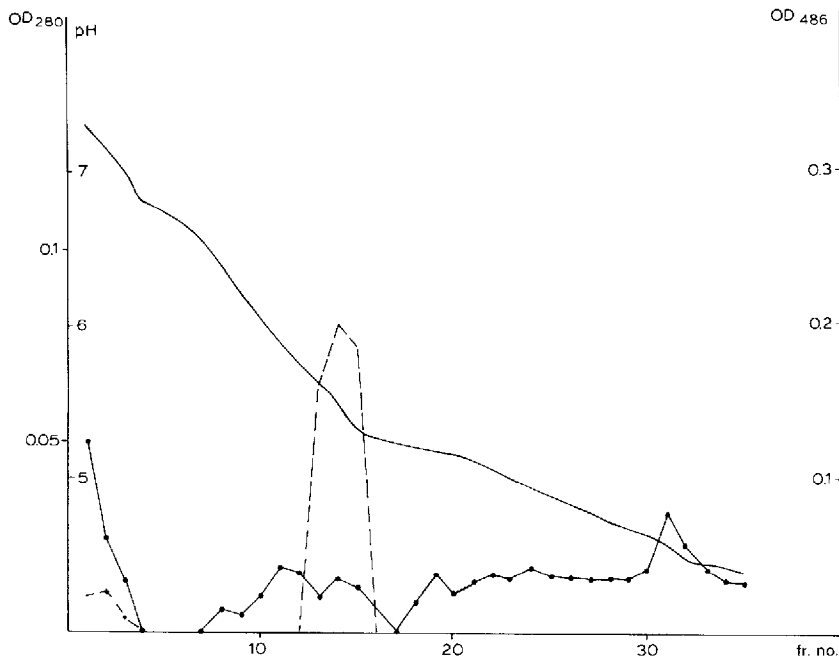

Fig. 2. Elution profile from PBE 94 of crude β-lactamase. About 58 units of crude β-lactamase from *E. coli* J6-2 RTEM$^+$ in 3 ml of 0.025 *M* imidazole–HCl buffer, pH 7.4 were applied to a C 10/20 column of PBE 94. Elution conditions, pH (——), protein (●–●) and activity (– – –) monitoring were as described in the Experimental section. 1800 μg of total protein were applied to and 1380 μg of protein were eluted from the column.

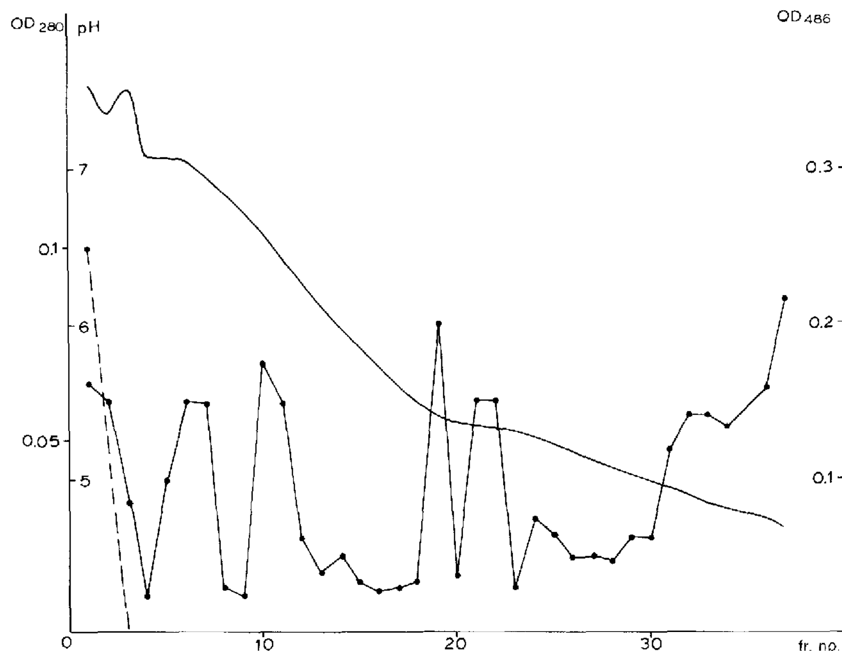

Fig. 3. Elution profile from PBE 94 of crude β-lactamase. About fourteen units of crude β-lactamase from *E. coli* J6-2 RTEM$^-$ in 3 ml of 0.025 *M* imidazole–HCl buffer, pH 7.4 were applied to a C 10/20 column of PBE 94. Elution conditions, pH (——), protein (●—●) and activity (– – –) monitoring were as described in the Experimental section. 4890 μg of total protein were applied to and 4215 μg of protein were eluted from the column.

position of the peak corresponds to that in Fig. 2, thus the p*I* of the enzyme is above pH 7.4 (ref. 7). Of the total of 15 units of enzyme applied to the column, 13.5 units (90%) were eluted (fraction 1, 9.5 units; 2, 4 units).

It can be concluded from our experiments that chromatofocusing is a useful and rapid method for the separation and identification on the analytical/micropreparative scale of β-lactamases of different origins.

Good separation of the plasmid mediated and the chromosomal β-lactamases from both crude and partially purified enzyme preparations of *E. coli* RTEM-1 type 1^+ and RTEM-1 type 1^- was achieved.

We suggest that the method will be applicable also to large scale preparations. However, because of the presence of other bacterial proteins having identical or closely similar p*I* values, it does not give completely pure enzyme preparations; nevertheless, a substantial degree of purification could be achieved in a single step.

Good evidence of the effective separation is that the p*I* values (5.42) obtained by chromatofocusing are in very good agreement with that published for the RTEM-1 type 1 plasmid mediated β-lactamase[3–7].

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