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Note

Use of chromatofocusing for separation of β-lactamases

IV*. β-Lactamases of the *E. coli* K12 strain carrying RP4 plasmid

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Hedges *et al.*[1] named "TEM-like" enzymes as the most common group of R-factor mediated β-lactamases that belong to Class IIIa in the Richmond–Sykes classification system[2]. This group of enzymes has also been extensively studied by Labia *et al.*[3], who pointed out that the plasmid RP4 produces TEM-2 β-lactamase, expressing it constitutively. Previously Matthew *et al.*[4] had shown that this enzyme was less frequently found than the TEM-1 type 1 β-lactamse, which is specified by R-factors of nearly all compatibility groups. The TEM-2 β-lactamase is determined only by a minority of R-plasmids within the groups of compatibility of FI, C and P. It has been shown that β-lactamases produced by RP1, R46-1, JR66-1, R667 and some other plasmids were identical with the TEM-2 enzyme[1,4–6].

Analytical isoelectric focusing of the RP4 mediated TEM-2 β-lactamase on thin-layer polyacrylamide gel (PA-IEF) furnished an isoelectric pH (*i.e.*, p*I*) of 5.6[3]. PA-IEF also revealed the presence of the chromosomally mediated β-lactamase (p*I* > 8.0) common to the *E. coli* strains[3,7]. A reappraisal of the p*I* determinations of Matthew and Hedges[6] by comparing the data with the sucrose density gradient IEF (SD-IEF) and PA-IEF results of Barthélémy *et al.*[8] showed that the p*I* value of 5.6 for any TEM-2 β-lactamase producing plasmid harboured in any bacterium species was convincingly reproducible and they did not show alterations in their substrate profiles[2,9,10]. Starch gel electrophoresis in 0.03 *M* borate buffer (pH 8.5) also resolved TEM-1 type 1 (p*I* = 5.4[3,4]) and TEM-2 enzymes, exhibiting mobilities of −17 and −14 mm, respectively[9].

In this article we report on our experience gained with the micro-scale separation of TEM-2 enzyme from the chromosomally coded β-lactamse of an RP4 carrying *E. coli* K12 strain and on the remarkable enrichment and separation of the enzymes from accompanying proteins using a chromatofocusing procedure that exploits differences in their p*I* values.

* For Part III, see S. Gál, B. L. Toth-Martinez and L. Kiss, *J. Chromatogr.*, 264 (1983) 170.

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EXPERIMENTAL

Bacterial strain

E. coli K12 J5-3 RP4$^+$ was kindly provided by Dr. J. T. Smith, Microbiology Section, School of Pharmacy, University of London, London, U.K. Details of culturing have been described elsewhere[11].

Partial purification of β-lactamases

The three steps in the partial purification of the β-lactamases were DEAE-cellulose column chromatography (stage 1[11]), molecular sieving on a Sephadex G-50 superfine column (stage 2[11]) and DEAE-Sephadex A50 column chromatography (18 × 3 cm column) (stage 3). The sub-groups of the DEAE-Sephadex A50 β-lactamase fractions were pooled into prefractions (I) showing a positive PADAC strip/droplet test[12] between 15 and 30 sec, main fractions (II) showing a colour change of the chromogenic β-lactamase substrate within 5 sec and postfractions (III) exhibiting a colour change between 15 and 30 sec. The pooled DEAE-Sephadex A50 fractions I–III were freeze-dried and stored at −30°C.

A crude enzyme preparation was precipitated from the dialysed sonicate at 100% ammonium sulphate saturation and the precipitate was processed as described earlier[11].

PADAC (Lot no. 110235, λ_{max} 572 nm, ε_{max} 58,000 l mol^{-1} cm^{-1}), a chromogenic indicator substrate of β-lactamases, was kindly supplied by Calbiochem-Behring (Lucerne, Switzerland).

Chromatofocusing, enzyme assay and protein determination

Details of the chromatofocusing technique have been published elsewhere[11]. β-Lactamase activity was assayed in 0.01 *M* sodium phosphate (pH 7.0) with PADAC at 37°C[13–15]. One unit is that amount of enzyme that is able to hydrolyse 1 μmol of PADAC in 1 min at 37°C. A high accuracy in determining the enzyme activity was possible because the ε_{max} of PADAC is about three times higher than that of Nitrocefin. Protein was determined either by measuring the absorbance at 280 nm or according to Lowry *et al.*[16].

RESULTS AND DISCUSSION

Partially purified pre-, main- and postfractions of RP4 coded TEM-2 β-lactamase were compared with a crude preparation of *E. coli* K12 J5-3 host organism to assess the capability of the PBE 94 (in 0.025 *M* imidazole–HCl cycle, pH 7.4)–Polybuffer 74 chromatofocusing system to separate the plasmid-mediated enzyme from the chromosomal β-lactamase.

The results of the experiment with the stage 2 partially purified prefraction (I) are shown in Fig. 1. The two enzymes separated well. The enzyme of the first peak (fractions 1–3) had a pI > 7.4 as it was not bound to the column. It is therefore probably identical with the chromosomally mediated β-lactamase[3]. The second peak (fractions 17–21) had pI = 5.63 in fraction 20, which is in good agreement with the value published for the RTEM-2 enzyme[2,3,9,10]. Fraction 19 has pH = 5.75, which is high, but we have already pointed out that the distribution of the activity in asym-

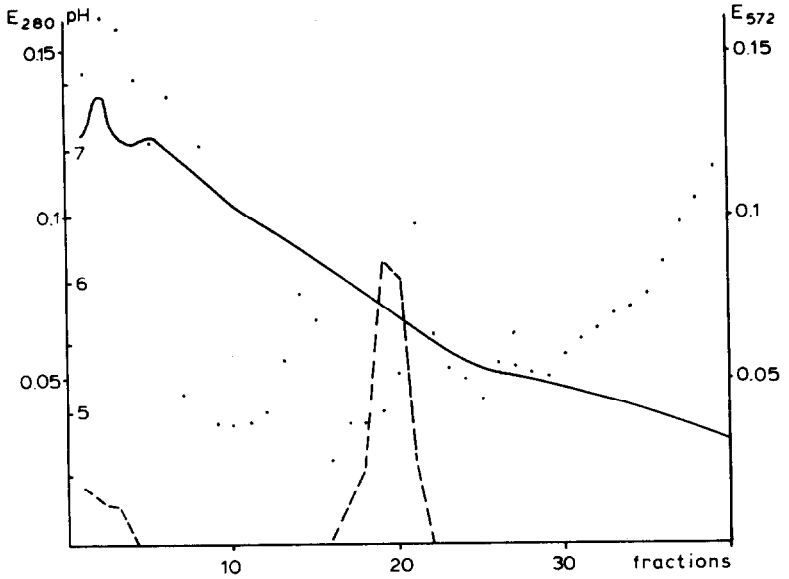

Fig. 1. Elution profile of stage 2 partially purified prefraction (I) on PBE 94. A sample of 0.603 unit of β-lactamase mixture in 2.5 ml of 0.025 *M* imidazole–HCl start buffer (pH 7.4) was applied to a C 10/20 (*i.e.* 20 cm × 10 mm I.D.) column of PBE 94[11]. Elution conditions, pH (——), protein (· · ·) and activity monitoring (– – –) as described under Experimental. Activity was measured in 200-μl aliquots of each fraction. 3955 μg of total protein were applied to and 3041 μg eluted from the column.

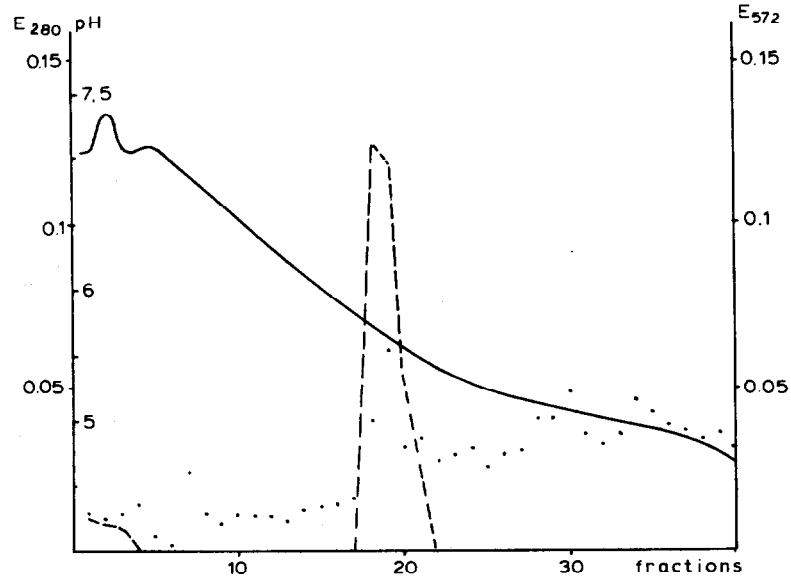

Fig. 2. Elution profile of stage 3 partially purified main fraction (II) on PBE 94. A sample of 87.24 units of β-lactamase mixture in 3.5 ml of 0.025 *M* imidazole–HCl start buffer (pH 7.4) was applied to a C 10/20 column of PBE 94[11]. Elution conditions, pH (——), protein (· · ·) and activity (– – –) monitoring as described under Experimental. Activity of the chromosomal enzyme was measured in 200-μl aliquots per fraction and that of the TEM-2 enzyme fractions was assessed in 2-μl aliquots. 1242 μg of total protein were applied to and 1049 μg eluted from the column.

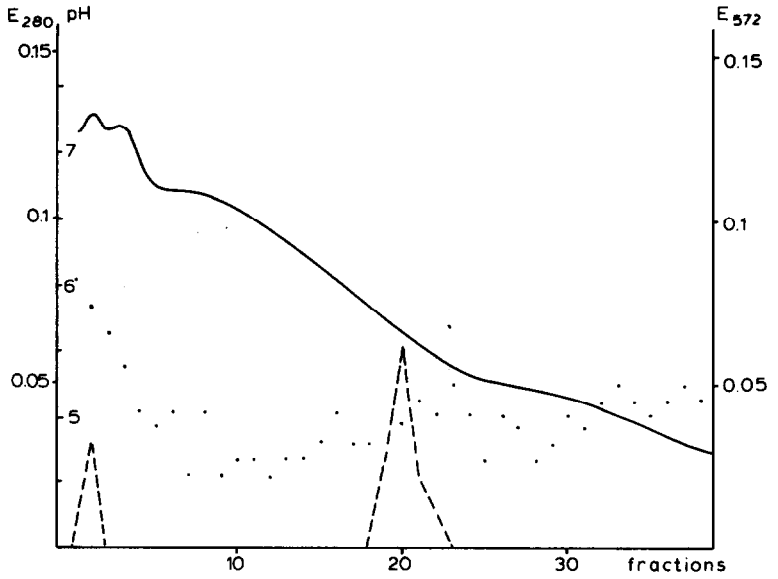

Fig. 3. Elution profile of stage 3 partially purified postfraction (III) on PBE 94. A sample of 0.902 unit of β-lactamase mixture in 2 ml of 0.025 *M* imidazole–HCl start buffer (pH 7.4) was applied to a C 10/20 column of PBE 94[11]. Elution conditions, pH (——), protein (· · ·) and activity (– – –) monitoring as described under Experimental. Activities of both the chromosomal enzyme and the TEM-2 β-lactamase elution fractions were assayed in 200-μl aliquots. 2140 μg of total protein were applied to and 1565 μg eluted from the column.

metric peaks depends on the amount of enzyme present[11]. Re-runs of an analytical amount from any fraction belonging to the same peak always resulted in a sharp peak of p*I* = 5.6 ± 0.02. Of the total of 0.603 unit of enzyme applied to the column 0.089 unit were eluted in the first peak (fraction 1, 0.039 unit; 2, 0.026 unit; 3, 0.03 unit) and 0.487 unit were eluted in the second peak (fraction 17, 0.026 unit; 18, 0.05 unit; 19, 0.185 unit; 20, 0.172 unit; 21, 0.054 unit), *i.e.*, 0.576 unit (95.5%) in all.

Fig. 2 shows the elution profile of the stage 3 partially purified main fraction (II). The chromosomal enzyme appeared in fractions 1–3. The position of the peak corresponded to that in Fig. 1, hence the p*I* of the enzyme was above pH 7.4[3]. The TEM-2 enzyme, however, was eluted in fractions 18–21. The second peak had p*I* = 5.64 in fraction 19 and p*I* = 5.75 in fraction 18. Of the total of 87.240 units of enzyme applied to the column, 0.065 unit was found in the first peak (fraction 1, 0.026 unit; 2, 0.021 unit; 3, 0.018 unit) and 82.824 units were eluted in the second peak (fraction 18, 32.328 units; 19, 30.517 units; 20, 13.966 units; 21, 5.948 units), *i.e.*, 82.889 units (95%) in all.

The elution profile of the stage 3 partially purified postfraction (III) is illustrated in Fig. 3. The chromosomal β-lactamase[3] of fraction 2 (0.010 unit) and the TEM-2 enzyme peak of fractions 19–22 (fraction 19, 0.145 unit; 20, 0.183 unit; 21, 0.390 unit; 22, 0.126 unit) represent 0.844 unit in all. Of the total of 0.902 unit of enzyme applied to the column, 0.854 unit (94.7%) were regained. The p*I* value of the plasmid mediated enzyme was found to be 5.66 in fraction 21.

For comparison we supplemented the experiments by using a crude enzyme

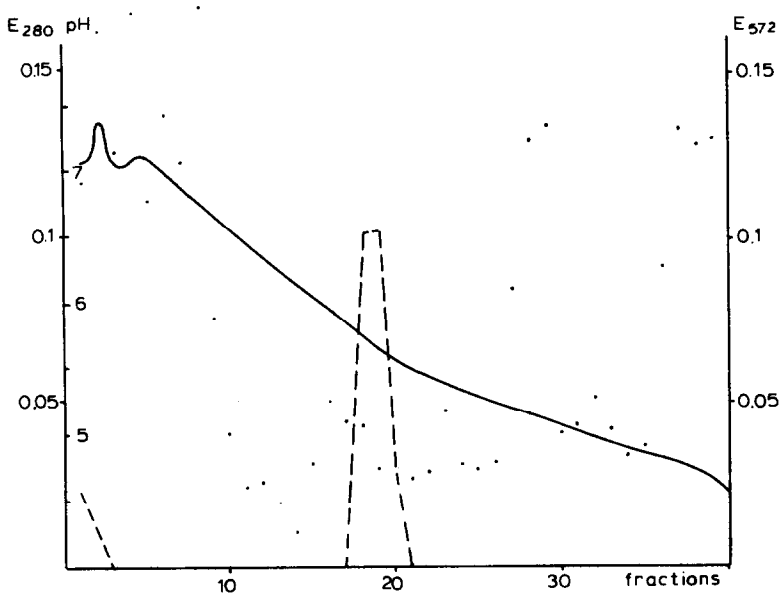

Fig. 4. Elution profile of crude β-lactamase mixture from PBE 94. A sample of 9.414 units of β-lactamase mixture in 3 ml of 0.025 *M* imidazole–HCl start buffer (pH 7.4) was applied to a C 10/20 column of PBE 94[11]. Elution conditions, pH (——), protein (· · ·) and activity (– – –) monitoring as described under Experimental. Activities of the elution fractions for both the chromosomal and the TEM-2 enzyme were assessed in 15-μl aliquots. 3276 μg of total protein were applied to and 3045 μg eluted from the column.

preparation (Fig. 4). As the elution profile shows in fraction 2–3 we found the chromosomal enzyme[3]. The TEM-2 enzyme, however, was found in fractions 18–20. Of the total of 9.414 units of β-lactamase mixture applied to the column, 0.793 unit were found in fraction 1 and 0.379 unit in fraction 2, *i.e.*, 1.172 units in all as chromosomal enzyme of p*I* > 7.4. The second peak showed p*I* = 5.63 in fraction 19 and p*I* = 5.7 in fraction 18. The distribution of TEM-2 β-lactamase activity of the second peak was as follows: fraction 18, 3.483 units; 19, 3.517 units; 20, 0.966 unit, *i.e.*, 7.966 units in all; 9.138 units of enzymes (97%) were eluted from the column in two peaks.

It can be concluded from the results that chromatofocusing is an excellent and rapid method for the separation and identification of β-lactamases simultaneously present in bacterial cell extracts. Good separation of the RP4 plasmid mediated TEM-2 enzyme from the chromosomally coded β-lactamase of *E. coli* K12 J5-3 was achieved because of the large differences in p*I* values. At the same time, most of the accompanying proteins were also removed from the enzyme fractions.

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