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Note

Use of chromatofocusing for separation of β-lactamases

VII. Analytical and medium scale preparative chromatofocusing of the constitutive chromosomal cephalosporinase P99 from *Enterobacter cloacae*

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An Enterobacter-specific constitutive chromosomal cephalosporinase, isolated from an *E. cloacae* strain called P99, was first reported by Fleming *et al.*[1]. The enzyme is produced abundantly without induction, and has therefore attracted the attention of biochemists, and recently of clinicists, because of an increase in infections due to various *E. cloacae* strains[2].

The enzyme has been characterized in inhibition studies[3,4], with regard to its substrate profile[1,5–7] and its M_r was found to be 39 000[6,8].

It was shown to be a weak basic protein following its preparation[6,7,9,10] and chemical characterization[8,10]. Although its isoelectric focusing (IEF) pattern exhibited only one major band with an isoelectric point, p*I*, of 7.9, several minor bands, all of which showed β-lactamase activity, could be detected in its proximity at both higher and lower pH values[6,8]. Seeberg *et al.*[11] determined a value p*I* = 7.8 by analytical IEF (AIEF). The value p*I* = 8.25 was also reported for β-lactamase P99[10].

In view of the practical importance of the characterization and classification of β-lactamases by their p*I* values during routine laboratory screening of β-lactam resistance in clinical isolates[12,13], we have introduced analytical chromatofocusing[14–19]. This method enables a one-step micro scale separation of β-lactamases simultaneously present in the crude extract of the microorganism, *e.g.*, the chromosomally mediated common β-lactamase of the plasmid carrier *Escherichia coli* strains and a transposonal enzyme[14,15,17] or the constitutive inducible exoenzymes of *Bacillus cereus* 569/H: β-lactamase I (E.C. 3.5.2.6), II (E.C. 3.5.2.8) and III (if solubilized)[16]. We have also investigated the effects of different polybuffer exchangers on the p*I* value[19]. Besides the separation of the enzymes from each other, their separability from accompanying proteins is another important aspect of the use of this technique, mainly in the case of single β-lactamase-producing bacteria[18].

The aim of the present study was to evaluate the p*I* value by analytical chro-

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matofocusing, to determine the ability of this method to isolate and purify the enzyme from proteins, and to scale-up the procedure.

EXPERIMENTAL

Bacterial strain

Samples of *E. cloacae* P99 strain were kindly provided by various laboratories: Drs. G. W. Ross and I. N. Simpson, Glaxo Group Research, Greenford, Middlesex, U.K.; Dr. J.-M. Frère, Université de Liège, Faculté de Médecine, Service de Microbiologie, Institute de Chimie, Liège, Belgium; Dr. K. Bush, Squibb Institute for Medical Research, Princeton, NJ, U.S.A.; Dr. C. A. Cooper, Beecham Pharmaceuticals, Research Division, Brockham Park, Betchworth, Surrey, U.K.; Dr. G. Waley, Sir William Dunn School of Pathology, University of Oxford, Oxford, U.K. Details of the culturing have been published elsewhere[6,10].

Purification of β-lactamase

A sample of a crude extract from *E. cloacae* P99 cephalosporinase was purified for analytical purposes according to the method of Ross[6]. The rest of the extract was used either for comparative analytical or medium scale preparative purposes.

Chromatofocusing, enzyme assay, protein determination and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

A Pharmacia C 10/20 (20 cm × 1 cm) column packed with PBE 94 chromatofocusing material and equilibrated with 0.025 *M* ethanolamine–acetic acid buffer, pH 9.4, was used for analytical purposes. The preparative column (23 cm × 2.5 cm) was also packed with PBE 94 and equilibrated as above. In order to elute the enzyme, the same Polybuffer 96, diluted ten times in distilled water and adjusted to pH 6.0 with acetic acid, was applied to the columns. Fractions of 4 and 3 ml were collected from the analytical columns, of 6 ml from the preparative column. The flow-rate was the same for all columns, 1 ml/min.

β-Lactamase activity was assayed by measuring the absorbance of Nitrocefin at 486 nm in a cell of pathlength 1 cm as described by O'Callaghan *et al.*[20]. One unit is that amount of enzyme which is able to hydrolyse 1 μmol of Nitrocefin in 1 min at 37°C.

The protein content of the fractions was estimated by measuring their absorbance at 280 nm or as described by Lowry *et al.*[21].

SDS-PAGE was performed according to the method of Weber and Osborn[22].

RESULTS AND DISCUSSION

The results of the analytical experiment with the largely prepurified enzyme are shown in Fig. 1. β-Lactamase P99 was found in fractions 8–12. Fraction 10 had p*I* = 7.88. Of the total of 13.0 units of enzyme applied to the column, 12.6 units (97%) were eluted: fraction 8, 0.61 units; 9, 4.31 units; 10, 7.18 units; 11, 0.36 units; 12, 0.14 units.

The analytical elution profile of the crude enzyme is illustrated in Fig. 2. The enzyme peak of fractions 9–13 (fraction 9, 4.85 units; 10, 4.31 units; 11, 24.25 units;

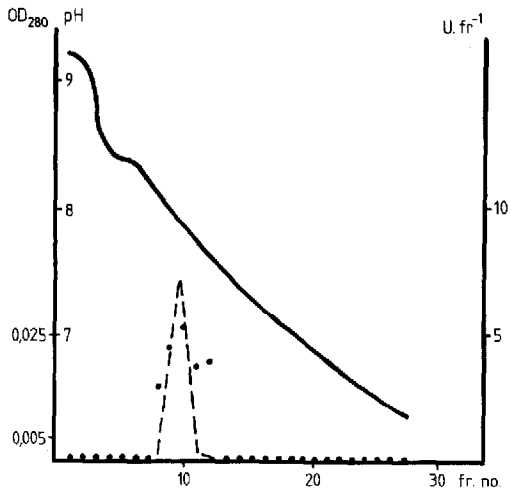

Fig. 1. Elution profile of the prepurified β-lactamase P99 from PBE 94. A sample of 13.0 units of β-lactamase in 2 ml of 0.025 *M* ethanolamine–acetic acid starting buffer pH 9.4 was applied to a C 10/20 column of PBE 94. Elution conditions: pH (———), protein (.....) and activity (--------) monitoring as described in the Experimental. A 450-μg amount of total protein was applied to and 438 μg were eluted from the column. fr.no. = Fraction number; U.fr^{-1} = units per fraction.

12, 5.0 units; 13, 0.7 units) represents 39.11 units in all. Of the total of 40.3 units of β-lactamase P99 applied to the column, 97% were recovered. The p*I* value of the chromosomally mediated constitutive enzyme was found to be 7.9.

For preparative purposes, the crude enzyme fraction was used (Fig. 3). The PBE 94 matrix was 80–90% saturated with the enzyme according to the results of a

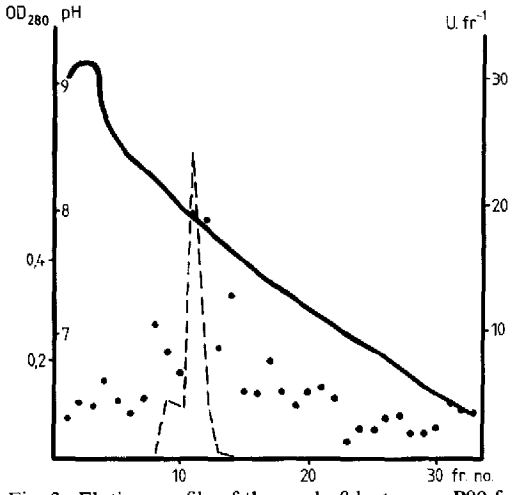

Fig. 2. Elution profile of the crude β-lactamase P99 from PBE 94. A sample of 40.3 units of β-lactamase in 2 ml of 0.025 *M* ethanolamine–acetic acid starting buffer pH 9.4 was applied to a C 10/20 column of PBE 94. Elution conditions as in Fig. 1. A 16.0-mg amount of total protein was applied to and 13.1 mg were eluted from the column.

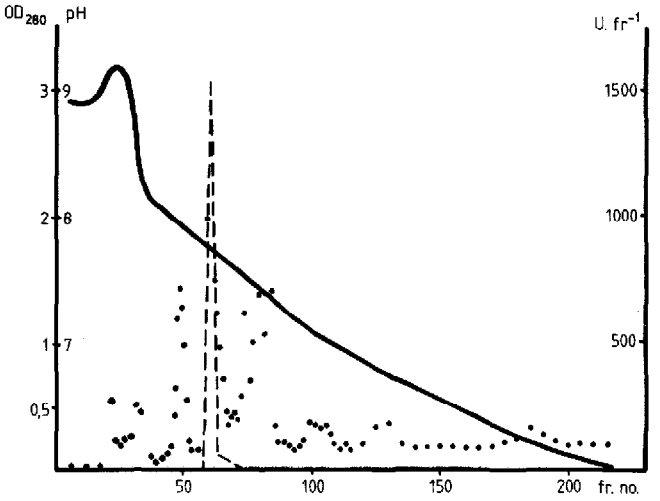

Fig. 3. Elution profile of the crude β-lactamase P99 from the preparative PBE 94 column. A sample of 4715 units of β-lactamase in 25.0 ml of 0.025 *M* ethanolamine–acetic acid starting buffer pH 9.4 was applied to a 23 cm × 2.5 cm column of PBE 94. Elution conditions as in Fig. 1. A 500-mg amount of total protein was applied to and 450 mg were eluted from the column.

TABLE I

COMPARISON OF PREPARATIVE CHROMATOFOCUSING OF THE CHROMOSOMALLY CODED CONSTITUTIVE CEPHALOSPORINASE OF *E. CLOACAE* P99 WITH THE CLASSICAL PURIFICATION PROCEDURE[6]

Parameter	*Crude extract*	*Main elution peak*	*Classical purification*
Total units	4715	3896	646 (from initial 1390 units)
Specific activity (units per mg protein)			
Nitrocefin as substrate	4.7	136.3*	
Cephaloridine as substrate			134.3
Protein (mg/ml)	20.0	0.96	0.07
Volume (ml)	25.0	96.0	71.0
Purification to specific activity	1	29	40.7 (from initial activity of 3.3)
Overall yield from total units (%)	*100*	*82.6*	*47.4*

* A three-fold increase in the specific activity for Nitrocefin occurred when 0.05 *M* phosphate buffer, pH 7.0, was replaced by 0.025 *M* ethanolamine–acetic acid starting buffer, pH 9.4, on a Sephadex *G-25 column (21 cm × 3.5 cm) before chromatofocusing* was started.

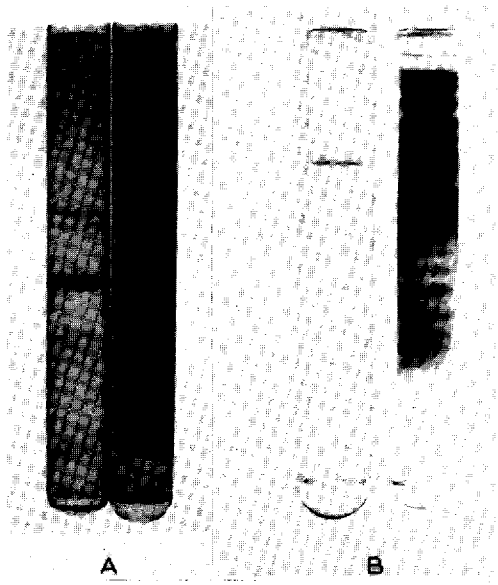

Fig. 4. (A) SDS-PAGE of pre-purified β-lactamase P99 and the crude extract. A 50-μl portion of enzyme at a concentration of 0.3 and 2.0 mg/ml, respectively, was applied to each track and the gels were subjected to 90 V, 70 mA, for 4 h. Protein was detected with Coomassie Brilliant Blue R250. The anode is at the bottom of the gels. (B) SDS-PAGE of chromatofocused β-lactamase P99 (fraction 10 from the analytical column or fraction 60 from the preparative column) and crude extract (as above). A 50-μl portion of enzyme at a concentration of 0.45 and 2 mg/ml, respectively, was applied to each track and the gels were subjected to 110 V, 40 mA, for 3 h. All other conditions as in (A).

preliminary experiment. Elution was effected directly with Polybuffer 96. Of the total of 4715 units of enzyme applied to the column, 3896 units (82.6%) were recovered in the main peak and 370 units (7.98%) were distributed between all the other 200 fractions before and after this peak; therefore, this portion of the β-lactamase was considered lost from a preparative point of view. The distribution of the activity in the fractions of the main peak was a follows: fraction 57, 52.9 units; 58, 175.6 units; 59, 1215 units; 60, 1552.6 units; 61, 429.3 units; 62, 145.8 units, 63, 61.6 units; 64, 35.1 units; 65, 34.0 units; 66, 45.4 units; 67; 34.6 units; 68, 26.0 units; 69, 26.0 units; 70, 24.3 units; 71, 23.3 units; 72, 14.5 units. The p*I* value for fraction 60 was 7.85. Table I gives the purification parameters, and Fig. 4 shows the results of SDS-PAGE of the prepurified, the chromatofocused and the crude enzyme, respectively.

Excellent concentration of the chromosomally mediated constitutive β-lactamase P99 from prepurified and crude preparations can be achieved. The minimum amount of accompanying rapidly- and slowly migrating proteins which still remained together with the prepurified enzyme (Fig. 4A) is eliminated in the single-step chromatofocusing technique (Fig. 4B). The p*I* values of the peak fractions are in good agreement with those assessed by AIEF.

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