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Note

Use of chromatofocusing for separation of β-lactamases

VI*. Comparative studies on two chromatofocusing polybuffer exchanger matrices with the R46 plasmid coded β-lactamase carried by the *Escherichia coli* K12 J5-3 strain

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The oxacillin-hydrolysing R46 (R1818) factor-mediated constitutive β-lactamase was recognized by Datta and Kontomichalou[1]. It is able to hydrolyse isoxazolyl penicillins and methicillin like other oxacillin-hydrolysing enzymes (of the OXA type, one of which, R46, is an OXA-2 β-lactamase[2,3]), but is resistant to inhibition by *p*-chloromercuribenzoate (pCMB) and cloxacillin and is inhibited by chloride ions[2,4,5]. It has a high molecular weight, 44 600 daltons (Sephadex gel filtration)[3] and comprises a dimer of two subunits of molecular weight 28 400 daltons (polyacrylamide gel + 1.0% sodium dodecyl sulphate + 1.0% 2-mercaptoethanol in 0.01 *M* phosphate buffer, pH 7.0)[6].

The enzyme proved to be a basic protein in its dimeric form with a mobility of 0.7 cm/h towards the cathode[6] in starch gel electrophoresis. Analytical isoelectric focusing (IEF) revealed a doublet of bands accompanied by satellites between p*I* values of 7.0 and 7.6[7]. In a comparative analytical IEF study, Vecoli *et al.*[8] found that the p*I* values of the two main fractions of the R46 enzyme were 7.6 and 7.8 in agarose gel or 7.5 and 7.7 in polyacrylamide gel, respectively.

Labia and Barthélémy[9] revealed that analytical IEF in polyacrylamide gels and preparative electrofocusing in a density gradient may give very different p*I* values for a given β-lactamase, particularly if it is a strongly basic protein as is the case with the R46 enzyme. This is due to a retardation of the enzyme migration at the cathodic end-field of the gel caused by gathering of the protein molecules, where the linearity of the pH gradient also suffers from convection streaming. Therefore, proteins having absolute p*I* values higher than 8.0 may show values lower by as much as one pH unit when measured by electrofocusing methods.

When Dale and Smith[10] used a modified reservoir buffer at pH 8.6 to induce

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the R46 β-lactamase to enter the gel it proved to be unsatisfactory since the p*I* of the enzyme was very near to that of the pH; therefore the band become diffuse because of inefficient stacking.

The aim of this study was a comparison of two chromatofocusing polybuffer exchangers from the point of view of their abilities to separate and enrich the R46 β-lactamase from accompanying proteins and the reproducibility of the p*I* values.

EXPERIMENTAL

Bacterial strain

Escherichia coli K12 J5-3 strain carrying the R46 plasmid, capable of producing constitutive OXA-2 β-lactamase, was kindly provided by Dr. J. T. Smith, Microbiology Section, School of Pharmacy, University of London, London, U.K. Details of the culturing procedure have been published elsewhere[11].

Partial purification of β-lactamase

The three steps in the partial purification of the β-lactamase were DEAE-cellulose chromatography (stage 1), molecular sieving on a Sephadex G-100 column (stage 2) and DEAE-Sephadex A-50 column chromatography (stage 3) as described earlier[11]. The subgroups of the fractions from DEAE-Sephadex A-50 were pooled in pre- (I), main (II) and postfractions (III) according to their enzyme activities assessed with Nitrocefin[11].

The crude enzyme fraction was made by sonication of the resuspended washed cells in 0.05 *M* phosphate buffer, pH 7.0. Following treatment of the sonicate with RNase and DNase ultracentrifugation was performed for 1 h at 105000 *g* and the supernatant was extensively dialysed against the above buffer.

Chromatofocusing, enzyme assay and protein determination

Two different types of chromatogrocussing polybuffer exchanger matrices were used, although the same Polybuffer 96 (diluted ten times in distilled water and adjusted to pH 6.0 with acetic acid; Pharmacia, Uppsala, Sweden) was applied to both of them. One of the columns was made up of the original PBE 94 (equilibrated with 0.025 *M* ethylamine–acetic acid buffer, pH 9.4; Pharmacia), but the other matrix was prepared in Dr. Kiss' laboratory by linking polyamines to Reactigel (6X, Pierce Eurochemie, Rotterdam, The Netherlands) and brought into the ethanolamine–acetic acid buffer, pH = 9.4 as was the former. Preparative studies of the latter chromatofocusing matrix are in progress. Otherwise, the pretreatment of β-lactamase samples and chromatofocusing was conducted as described earlier[11].

β-Lactamase activity was assayed by measuring the absorbance of Nitrocefin in a cell of 1-cm pathlength at 486 nm as described by O'Callaghan *et al.*[12]. One unit is that amount of enzyme which is able to hydrolyse 1 nmol of Nitrocefin in 1 min at 30°C.

The protein content of the solutions was estimated by measuring the absorbance at 280 nm or as described by Lowry *et al.*[13].

RESULTS AND DISCUSSION

The crude fraction of R46-coded OXA-2 β-lactamase was subjected to chromatofocusing in the PBE 94–Polybuffer 96 system and the results compared to those obtained by chromatofocusing the same crude enzyme preparation in a recently available Reactigel (6X)-based polybuffer exchange–Polybuffer 96 system. Comparison was also made with the results obtained with partially purified stage 3 main fraction (II) in the Reactigel (6X)-based polybuffer exchanger–Polybuffer 96 system.

The results of the experiment with the crude enzyme in the original Pharmacia system are shown in Fig. 1. Only traces of the chromosomally coded strongly basic *E. coli* β-lactamase were detected in fraction 1. The OXA-2 enzyme was found in fractions 4–8. Fraction 5 had p*I* 8.45. Of the total of 45.5 units of enzyme applied to the column, 41.85 units (92%) were eluted (fraction 4, 3.95 units; 5, 13.83 units; 6, 10.06 units; 7, 9.16 units; 8, 4.85 units).

The elution profile of the crude enzyme in the new Reactigel (6X)-based polybuffer exchanger–Polybuffer 96 system is illustrated in Fig. 2. The common *E. coli* chromosomal β-lactamase was detected in the first fraction eluted and the OXA-2 enzyme peaks of fractions 3–7 (fraction 3, 7.0 units; 4, 17.24 units; 5, 32.33 units; 6, 9.7 units) represent 66.27 units in all. Of the total of 68.46 units of β-lactamase applied to the column, 97% were recovered. The p*I* value of the plasmid-mediated enzyme was found to be 8.48.

For comparison, partially purified stage 3 main fraction (II) of OXA-2 β-lactamase was examined in the new Reactigel (6X)-based polybuffer exchanger–Polybuffer 96 system. The results are shown in Fig. 3. The minimum amount of chromosomal enzyme was eliminated during purification[14]. The OXA-2 enzyme was eluted in one peak (fractions 3–6) at p*I* 8.49. Of the total of 68.6 units of enzyme applied to the column, 66.27 units (96.6%) were recovered. The distribution of the activity

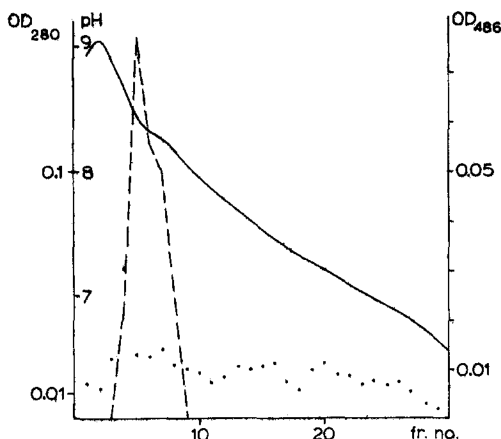

Fig. 1. Elution profile of crude β-lactamase from PBE 94, A sample of 45.5 units of β-lactamase in 0.5 ml of 0.025 *M* ethanolamine–acetic acid starting buffer (pH 9.4) was applied to a C 10/20 column of PBE 94[11]. Elution conditions, pH (———), protein (···) and activity (– – –) monitoring as described in Experimental. The activities of the eluted fractions were assessed with 300-μl aliquots. A 562-μg amount of total protein was applied to and 525 μg were eluted from the column. fr. no. = Fraction number.

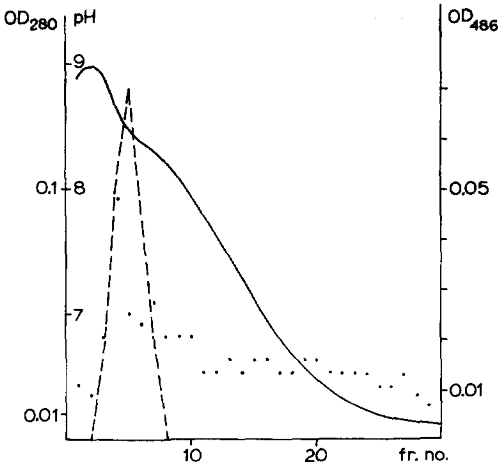

Fig. 2. Elution profile of crude β-lactamase from the Reactigel (6X)-based chromatofocusing matrix. A sample of 68.46 units of diluted β-lactamase in 1 ml of 0.025 *M* ethylamine–acetic acid starting buffer (pH 9.4) was applied to a C 10/20 column of Reactigel (6X)-based matrix. Elution conditions, pH (———), protein (···) and activity (— — —) monitoring as described in Experimental. The activities of the eluted fractions were measured with 50-μl aliquots. A 845-μg amount of total protein was applied to and 790 μg were eluted from the column.

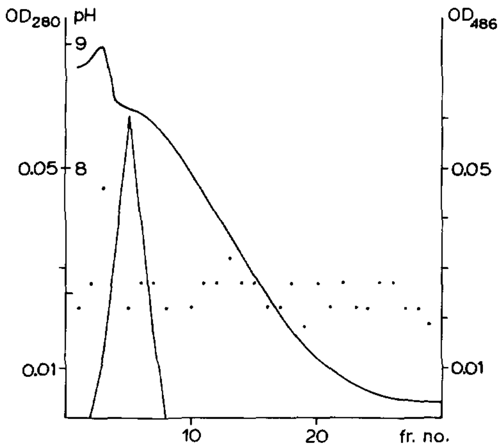

Fig. 3. Elution profile of stage 3 partially purified main fraction (II) from the Reactigel (6X)-based chromatofocusing matrix. A sample of 68.6 units of β-lactamase in 2 ml of 0.025 *M* ethylamine–acetic acid starting buffer (pH 9.4) was applied to a C 10/20 column of Reactigel (6X)-based chromatofocusing matrix. Elution conditions, pH (———), protein (···) and activity (— — —) monitoring as described in Experimental. The activities of the eluted fractions were measured with 100-μl aliquots. A 600-μg amount of total protein was applied to and 578 μg were eluted from the column.

in the fractions was as follows: fraction 3, 7.0 units; 4, 17.24 units; 5, 32.33 units; 6, 9.7 units.

Good concentration of the R46 plasmid-mediated enzyme from both crude and partially purified preparations was achieved by both polybuffer exchangers. The pH profile of the Reactigel (6X)-based matrix was as good as that of the Pharmacia PBE 94 matrix when the commercial Pharmacia Polybuffer 96 was used. The p*I* values obtained by chromatofocusing are higher than those measured by isoelectrofocusing techniques. The distribution of the R46 plasmid-coded OXA-2 β-lactamase activity in more than one fraction may reflect the doublet nature of the two enzyme subunits frequently observed by analytical IEF.

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