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Note

Use of chromatofocusing for separation of β-lactamases

V*. Inducible chromosomally mediated β-lactamase of the *Enterobacter cloacae* 53 strain

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In the Enterobacter species the β-lactamases are apparently closely related chromosomally mediated cephalosporinases. Their production is inducible in many strains[1–8]. Plasmid-mediated cephalosporinases have not been found frequently, but TEM-1[1,9,10], SHV-1 + PSE-2, PSE-2 alone[11] and CARB-1[4] plasmid-mediated β-lactamases have already been reported in Enterobacteriaceae.

The first Enterobacter cephalosporinase was isolated from *E. cloacae* P99[12] and studied by Hennessey[3] among other Enterobacter strains. It proved to be inducible [3]. Inhibition[13,14] and immunological studies[15] of the P99 enzyme have also been published. Along with the *E. cloacae* P99 β-lactamase[16], another Enterobacter cephalosporinase was purified to homogeneity from the *E. cloacae* 214 strain and studied in some detail[4]. These two enzymes had similar characteristics but were different regarding both their isoelectric points, p*I* (*E. Cloacae* P99: p*I* 7.8[13] or <8.0[9], 8.25[17], 8.5[12]. *E. cloacae* 214: p*I* 7.5[18] or 7.6[3,4,8]), and isoelectric focusing patterns[6,19].

The chromosomally mediated inducible and non-inducible cephalosporinases of the Enterobacter species have p*I* values between 7.5 and 8.9. Table I shows some characteristic results for clinical isolates obtained by the analytical isoelectric focusing (IEF) screening technique. In this article we report the micro-scale separation of the *E. cloacae* 53 chromosomally mediated inducible cephalosporinase from accompanying proteins using a chromatofocusing procedure that exploits differences in the p*I* values of protein fractons in preparations of different purities.

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* For Part IV, see B. L. Toth-Martinez, S. Gál. F. J. Hernádi, L. Kiss and P. Nánási, *J. Chromatogr.*, 287 (1984) 413.

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TABLE I

REPRESENTATIVE p*I* VALUES OF CEPHALOSPORINASES OF SOME *E. CLOACAE* STRAINS

Strain			*pI*	*Ref.*
	53		7.5	2
	214		7.6	3,4,8
	P99		7.8	13
			8.0	9
			8.25	17
			8.5	12
	5		7.8	20
	150 M		7.8	20
	352 M		7.8	20
	2091		7.8	20
	2	Wild type, non-induced	8.0	21
	2	Wild type, induced	8.0	21
	2	Cefamandole-resistant mutant	8.0	21
	1685 E		8.2	20
GN	7471		8.4	20
R	140		8.7	11
R	156		8.7	11
R	178		8.7	11
R	248		8.7	11
	149 M		8.8	20
	314 M		8.8	20
	988 M		8.8	20
	159		8.8	20
	163		8.8	20
	208		8.8	20
	966/81		8.8–8.9	22
	363		8.9	23

EXPERIMENTAL

Bacterial strain

E. cloacae 53 was kindly supplied by Dr. J. T. Smith, Microbiology Section, School of Pharmacy, University of London. Details of the culturing procedure have been described elsewhere[24].

Partial purification of β-lactamase

The enzyme was released from the harvested and washed cells by sonication[24]. Partial purification of the crude enzyme solution was performed by CM-Sephadex C-50, Sephadex G-50 and QAE-Sephadex 50 chromatography as described earlier (stages 1, 2 and 3, respectively). Three subgroups of enzyme fractions from QAE-Sephadex 50 were pooled: the prefraction (I), main fraction (II) and postfraction (III), showing positive Nitrocefin droplet tests between 30 and 50 sec, within 5 sec and between 30 and 50 sec, respectively. In these experiments only fraction II was analysed.

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 estimation

Details of the chromatofocusing technique have already been described[24]. β-Lactamase activity was assayed by measuring the absorbance of Nitrocefin in a cell of 1-cm pathlength at 486 nm according to the procedure of O'Callaghan *et al.*[25]. One unit is that amount of β-lactamase 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.*[26].

RESULTS AND DISCUSSION

The partially purified main subfraction (II) from the third stage of the enzyme preparation on QAE-Sephadex 50 was compared with the crude β-lactamase fraction of *E. cloacae* 53 to assess the ability of the PBE 94 (equilibrated with buffer 0.025 *M* ethylamine–acetic acid buffer, pH 9.4)–Polybuffer 96 (diluted ten-times and adjusted to pH 6.0 with acetic acid) chromatofocusing system to separate the chromosomal enzyme from other protein fractions. The results of the experiments are shown in Fig. 1. The β-lactamase was well separated from most of the accompanying proteins except from those exhibiting about the same p*I* values. The peak fraction had a p*I* value of 7.54. Of the total of 81 units of enzyme applied to the column, 77.3 units (95.4%) were eluted in a single peak (fraction 12, 5.65 units; 13, 11.31 units; 14, 30.17 units; 15, 23.89 units; 16, 6.28 units).

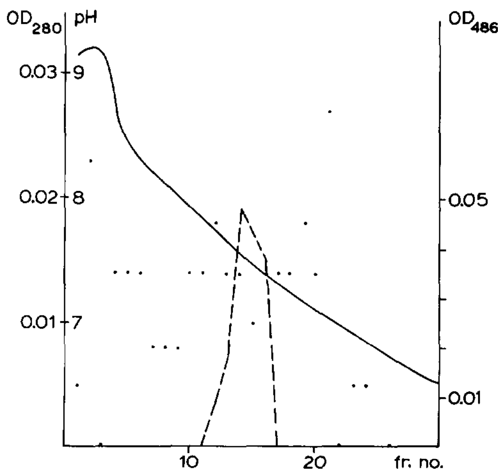

Fig. 1. Elution profile of stage 3 partially purified main fraction (II) on PBE 94. A sample of 81 units of β-lactamase in 1.5 ml of 0.025 *M* ethylamine–acetic acid starting buffer (pH 9.4) was applied to a C 10/20 column of PBE 94[24]. Elution conditions, pH (———), protein (···) and activity (– – –) monitoring as described in Experimental. The activity was measured in 100-μl aliquots of each fraction. A 292-μg amount of total protein was applied to and 244 μg were eluted from the column. fr. no. = Fraction number.

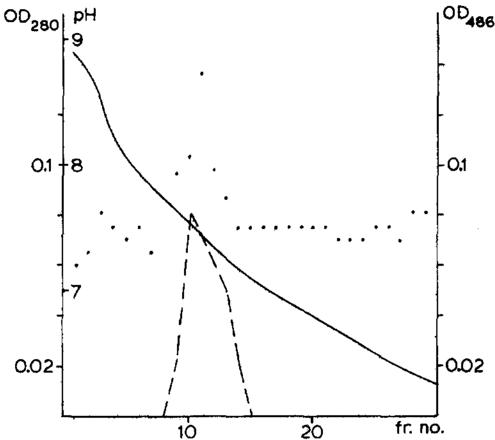

Fig. 2. Elution profile of crude β-lactamase from PBE 94. A sample of 200 units of β-lactamase in 1 ml of 0.025 *M* ethanolamine–acetic acid starting buffer (pH 9.4) was applied to a C 10/20 column of PBE 94[24]. Elution conditions, pH (———), protein (···) and activity (– – –) monitoring as described in Experimental. The activities of the eluted fractions were assessed with 100-μl aliquots. A 2500-μg amount of total protein was applied to and 2204 μg were eluted from the column.

The crude enzyme preparation served as a control (Fig. 2) and also gave a single active peak in fractions 9–14 with p*I* 7.52 in fraction 10. Two hundred units of β-lactamase were placed on top of the column and 187 units (93.5%) were eluted in the following order: fraction 9, 13.5 units; 10, 50.3 units; 11, 40.8 units; 12, 37.7 units; 13, 31.4 units; 14, 13.8 units.

We conclude that chromatofocusing is a good and rapid method for concentration of β-lactamase and its separation from most of the accompanying proteins of *E. cloacae* 53. The distribution of β-lactamase activity in more than one fraction can be related either to the fact that the amount of β-lactamase is more than enough to saturate a narrow section of the column, or to the multiplicity and partial separation of the active enzyme species as is frequently observed in analytical AIEF[19]. The p*I* values of the peak fractions are in good agreement with those found by analytical AIEF[2].

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