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Note

Use of chromatofocusing for separation of β-lactamases

II. β-Lactamase of a characterized *Pseudomonas aeruginosa* strain

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In the purification of β-lactamases from *Pseudomonas aeruginosa* strains, in general, complications begin immediately after successful cell disruption in that artefacts are produced, *e.g.*, by enzymatic formation of covalently modified species, proteolysis and deamination, which may be revealed only by isoelectric focusing (IEF).

For Class V d (ref. 1) β-lactamases of *P. aeruginosa* strains the isoelectric point, p*I*, ranges from 5.1 to 5.5 (ref. 2) and 5.9 (ref. 3), but multiple bonds are seen even within a single strain and without any differences in substrate profile. Class I d chromosomal enzymes of *P. aeruginosa* strains have p*I* values between 7.2 and 8.15 (refs. 4–12). In the plasmid carrier *P. aeruginosa* strains the Class III a TEM-1 type 1 and TEM-2 enzymes are similar to those of the enteric bacteria, having a p*I* range of 5.25–5.6 (refs. 3, 4, 10, 13 and 14).

The non-induced TEM-1 like β-lactamase of our *P. aeruginosa* MAR strain (p*I* 5.4) was released from the cells by a combination of freeze-thawing (twice) and sonication (described earlier[15]). We omitted induction of the enzyme, although it is inducible, because there was a change in the enzyme pattern[16] and a tendency to release into the medium. Some other characteristics of the enzyme which allowed us to classify it are assessed in the Experimental.

Class V e β-lactamase of *P. aeruginosa* ML 4295/Rms 149 has a neutral p*I* of 7.05 (ref. 17) and enzymes of some clinical *P. aeruginosa* isolates have a p*I* range of 7.0–7.1 (ref. 10) (Philippon type C oxacillinases).

By use of the chromatofocusing technique for the preparation of *P. aeruginosa* β-lactamase, we have been able to study its detectability and concentration by separation from proteins in a single step, exploiting the differences in p*I* values, as well as to reveal the effect of partial purification on the above features relative to fractionation of the crude enzyme extract.

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TABLE I

KINETIC CONSTANTS OF THE PARTIALLY PURIFIED β-LACTAMASES OF SUBGROUPS I–III

Carb = Carbenicillin; Oxa = oxacillin.

Subgroup	K_M* (μM)	V_{max}* (μmol min^{-1})	*Specific activity** (μmol min^{-1} mg^{-1})	*Nitrocefin end-point (mM)***
I	20	57	55.6	Carb <0.2–>0.04 Oxa <1.0–>0.2
II	22	55.5	98.5	Carb <0.2–>0.04 Oxa 1
III	18	56	13.8	Carb >0.04 Oxa <1.0–>0.2

* Measured with Nitrofecin as substrate.

** Inhibitory final concentration of the antibiotic in the standard checker-board test in which the yellow colour of the Nitrocefin substrate remains unchanged after 10 min[18,20,21].

EXPERIMENTAL

Bacterial strain

P. aeruginosa MAR, carrier of a TEM-1 like β-lactamase of p*I* 5.4, was kindly supplied by Dr. A. M. Philippon, Service de Biologie, C. H. U. Cohcin, 75014 Paris, and grown as originally described[7]. (Induction: 5 mg benzylpenicillin per ml caused an induction ratio of 30–40 with a lag of about 40 min.)

Partial purification and characterization of the β-lactamase

The enzyme was released from the harvested and washed cells by twice freeze-thawing the wet sediment, followed by sonication[15]. Partial purification of the crude enzyme solution was performed by CM-Sephadex and Sephadex G-100 chro-

TABLE II

KINETIC CONSTANTS OF THE PARTIALLY PURIFIED SUBGROUP II β-LACTAMASE

Activity measurements with various antibiotics as substrates were made according to the methods given in refs. 22–24. $\tau = K_M/V_{max}$, and corresponds to the half-life of the antibiotic.

Antibiotic	*Ref.*	K_M (μM)	*Rel.* V_{max}	$\tau_{rel.}$
Benzylpenicillin	22	20	100	100
Ampicillin	22	30	95	158
Carbenicillin	23	17.8	13.8	645
Methicillin	22	26.3	6	2191.5
Oxacillin	22	80	11.6	3450
Cloxacillin	22	200	5.9	$1.7 \cdot 10^4$
Cephaloridine	24	1000	125	4000

matography (stage 2) as described earlier[15]. Three subgroups of Sephadex G-100 enzyme fractions were pooled: prefractions (I) showing a positive Nitrocefin® droplet test[18] between 15 and 30 sec; main fractions (II) exhibiting a Nitrocefin colour change within 5 sec and postfractions (III) showing a colour change between 15 and 30 sec. The pooled Sephadex G-100 fractions I–III were lyophilized and stored at −30°C.

The crude enzyme preparation was precipitated from the dialysed sonicate at 100% $(NH_4)_2SO_4$ saturation and the precipitate was processed as described elsewhere[15].

Enzymatic constants of the partially purified preparations I–III are shown in Table I. The substrate profile of the partially purified preparation II is shown in Table II as an example. Those of subgroups I and III and the crude preparation were similar.

Chromatofocusing

Details of the chromatofocusing technique have already been published[15].

Protein estimation

The protein content of the solutions was estimated by measuring the absorbance at 280 nm following PB 74 Polybuffer exchange, and according to Lowry *et al.*[19].

Enzyme assay

β-Lactamase activity was assayed by measuring the absorbance of Nitrocefin in a cell of 1-cm pathlength at 486 nm as described earlier[15].

RESULTS AND DISCUSSION

Samples of partially purified extrachromosomally encoded β-lactamase of the *P. aeruginosa* MAR strain were applied to a C 10/20 column of PBE 94 and a pH gradient was developed with Polybuffer 74 in the tube, preincubated with 0.025 *M* imidazole–HCl, pH 7.4 and the proteins were successively eluted. The pH gradient showed the usual slightly biphasic linear character[15] in the range of pH 7–4.

The results of the first set of experiments, made with the stage 2 pooled prefractions, subgroup I, are shown in Fig. 1. The enzyme was eluted in one peak (fractions 13–15) which corresponded to pH 5.4. Of the total 28 units of enzyme applied to the column, 27 units (96.43%) were recovered in the three fractions. The amount of activity was distributed as follows: fraction 13, 6 units; 14, 20 units; 15, 1 unit.

The elution profile of the stage 2 pooled main fractions, subgroup II, is illustrated in Fig. 2. The β-lactamase of this preparation was eluted in a single peak (fractions 16–19) exhibiting a slight asymmetry and having a pI = 5.38 at fraction 17. Of the total 39 units of β-lactamase, 38 units (97.44%) were eluted in the following order: fraction 16, 3 units; 17, 18 units; 18, 14 units; 19, 3 units.

Fig. 3 shows the results of the experiment conducted with the stage 2 pooled enzyme postfractions, subgroup III. In fractions 14–16 the β-lactamase was concentrated and had a pI value of 5.37. Twenty-one units of enzyme were applied to the column and 20 units (95.24%) were recovered in the fractions: fraction 14, 6 units; 15, 12 units; 16, 2 units.

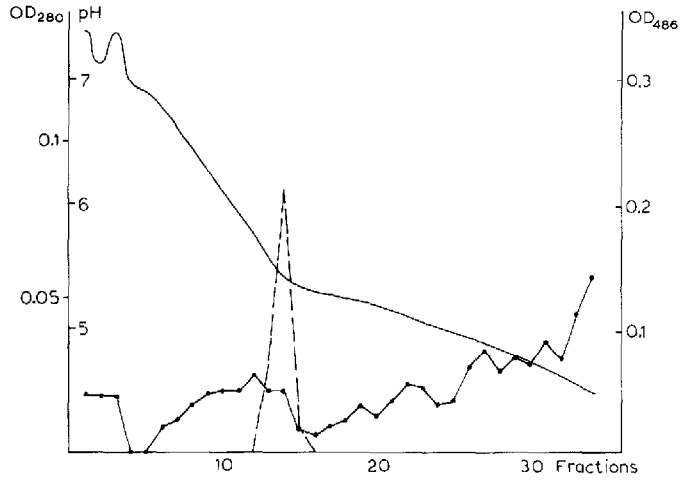

Fig. 1. Elution profile of partially purified β-lactamase of stage 2 pooled prefractions from PBE 94. A sample of 28 units of β-lactamase of stage 2 pooled prefractions from *P. aeruginosa* Marmot in 3 ml of 0.025 *M* imidazole–HCl start buffer, pH 7.4 was applied to a C 10/20 column of PBE 94. Elution conditions, pH (———), protein (●—●) and activity (— —) monitoring, were as described in the Experimental section. 2615 μg of total protein were applied to, and 1894 μg of protein eluted from, the column.

We supplemented the above experiments by using a crude enzyme preparation as a control (Fig. 4). The elution profile of the 100% $(NH_4)_2SO_4$ precipitate as a crude source of the β-lactamase also gave a single active peak in fractions 23–26 with a p*I* = 5.4 in fraction 25. Thirty-seven units of β-lactamase were put on the

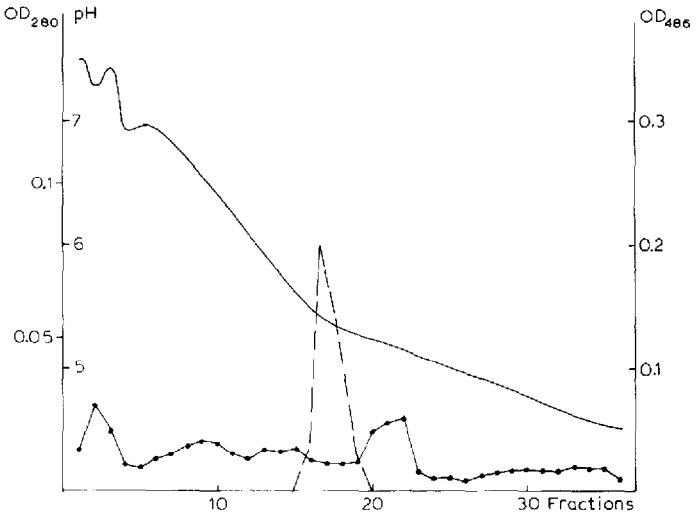

Fig. 2. Elution profile of partially purified β-lactamase of stage 2 pooled main fractions from PBE 94. A sample of 38 units of β-lactamase of stage 2 pooled main fractions from *P. aeruginosa* Marmot in 3 ml of 0.025 *M* imidazole–HCl start buffer, pH 7.4 was applied to a C 10/20 column of PBE 94. Elution conditions, pH (———), protein (●—●) and activity (— —) monitoring, were as described in the Experimental section. 2107 μg of total protein were applied to, and 1842 μg of protein eluted from, the column.

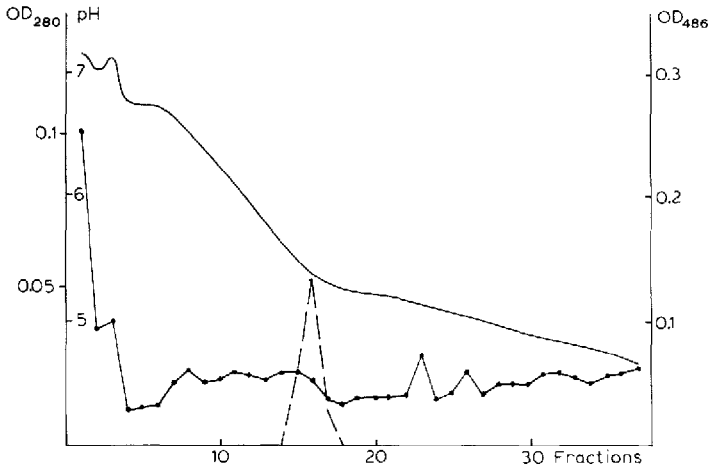

Fig. 3. Elution profile of partially purified β-lactamase of stage 2 pooled postfractions from PBE 94. A sample of 20 units of β-lactamase of stage 2 pooled postfractions from *P. aeruginosa* Marmot in 3 ml of 0.025 *M* imidazole–HCl start buffer, pH 7.4 was applied to a C 10/20 column of PBE 94. Elution conditions, pH (———), protein (●—●) and activity (— —) monitoring, were as described in the Experimental section. 2500 μg of total protein were applied to, and 1920 μg of protein eluted from, the column.

top of the column and 36 units (97.3%) were eluted in the following order: fraction 23, 3 units; 24, 14 units; 25, 15 units; 26, 4 units.

We think that our separation experiments demonstrate that the chromatofocusing technique is a useful tool to isolate the β-lactamase fraction of the *P. aeruginosa* MAR strain from most of the proteins simultaneously present, either in a partially purified or in a crude preparation. We suggest that the resolution power of

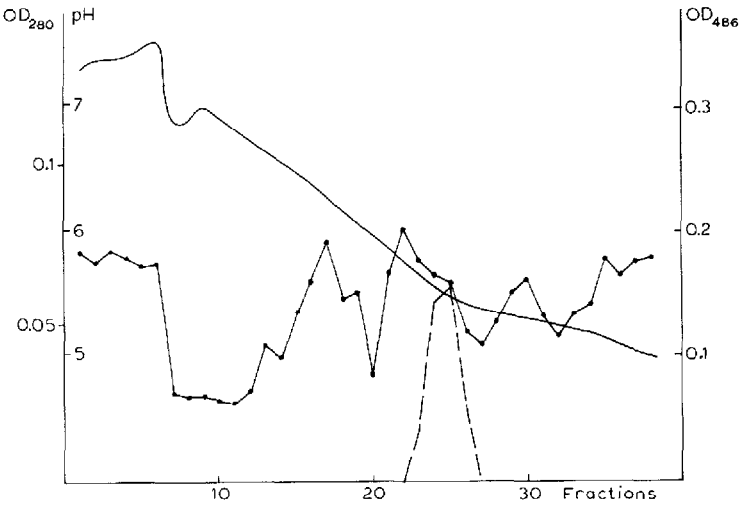

Fig. 4. Elution profile of crude β-lactamase from PBE 94. A sample of 37 units of β-lactamase of *P. aeruginosa* Marmot, in crude form, in 3 ml of 0.025 *M* imidazole–HCl start buffer, pH 7.4 was applied to a C 10/20 column of PBE 94. Elution conditions, pH (———), protein (●—●) and activity (— —) monitoring, were as described in the Experimental section. 4855 μg of protein were eluted from the total of 5250 μg protein applied to the column.

the PBE 94 column with Polybuffer 74 is so effective in concentrating the single β-lactamase of our strain that this separation system can be used directly, even for preparative purposes, without partial purification prior to chromatofocusing.

Accidental deviation of the furnished p*I* values towards lower pH, from the value of p*I* = 5.4, which was measured by IEF[14,16], is an inherent property of the chromatofocusing technique. The final value may even reach 0.1, depending on the rate of elution and the amount of the accompanying proteins with the same or nearly the same p*I* value. Nevertheless our p*I* results obtained with chromatofocusing are in good agreement with that assessed by IEF[14,16]. According to the data in Table II, the enzyme is a typical TEM-1 like β-lactamase[15].

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