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Note

Use of chromatofocusing for separation of β-lactamases

III. β-Lactamases of the *Bacillus cereus* 569/H strain

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β-Lactamase I and II of *Bacillus cereus* 569/H, a spontaneous mutant of strain 569, have been found to be constitutive, inducible exoenzymes. β-Lactamase I (E.C. 3.5.2.6) is a heat-labile typical penicillinase and β-lactamase II (E.C. 3.5.2.8) is a Zn^{2+}-requiring heat-stable cephalosporinase[1].

The isolation and characterization of these enzymes were accomplished some time ago[2–12], including the amino acid sequence of β-lactamase I[13]. Sequence studies on β-lactamase II are in progress[14]. There is, however, still a controversy regarding the net charge of these molecules and the corresponding isoelectric pH values, p*I*[1]. For β-lactamase I, p*I* values of < 5.5[15], > 5.0[2], about 7.0[1], 9.2, 9.47 and 9.68[9] (multiplicity of active fractions) and > 9.2[8,12] have been reported but these were measured under very different experimental conditions. For β-lactamase II, p*I* values of 8.45 and 10.11[9] (the latter for a minor fraction) were reported. In general, the authors agree that this enzyme is a basic protein[1,4].

The great differences in p*I* values and the multiplicity of both enzymes might be ascribed to the presence of different molecular species during their maturation, although there is no evidence available for this hypothesis[1,4,8]. On the other hand, we suggest that strong binding of carbohydrate moieties to the enzyme species might lead to minimum differences in p*I* values by masking charge(s) without influencing the amino acid composition (in cases of multiplicity).

The aim of our study was the micro scale separation of *B. cereus* 569/H β-lactamases I and II from accompanying proteins by chromatofocusing and by exploiting differences in the p*I* values.

EXPERIMENTAL

Bacterial strain and purification of the β-lactamases

B. cereus 569/H (NCTC 9945) was grown to produce β-lactamase I by omitting

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$ZnSO_4$ in the medium or in the presence of a final concentration of 1 m*M* $ZnSO_4$ to produce mainly β-lactamase II, otherwise as described by Kuwabara[5]. The first $(NH_4)_2SO_4$ precipitates were processed twice on Celite 535 columns[9]. Crude enzyme was prepared by precipitation in cold acetone as described by Pollock *et al.*[2].

Chromatofocusing, enzyme assay and protein estimation

Details of the chromatofocusing technique have been published[16]. β-Lactamase activity measurements for both enzymes were made by the Nitrocefin® method[16]; however, Polybuffer 74, imidazole–HCl and phosphate on a Bio-Gel P-2 column (7 × 1 cm) were replaced by 0.05 *M* Tris–HCl, pH = 7.0 prior to adding 1 m*M* $ZnSO_4$ when assessing β-lactamase II activity. When both enzymes were present in the same solution, β-lactamase activity was first estimated without 1 m*M* $ZnSO_4$ (activity of β-lactamase I) and then β-lactamase I + β-lactamase II activity was assessed in the presence of 1 m*M* $ZnSO_4$. Controls were set up either by treating the enzyme solution at 65°C for 60 min (heat deactivation of β-lactamase I) or by adding EDTA to a final concentration of 10 m*M* (chelating Zn^{2+}). One unit is that amount of enzyme which is able to hydrolyze 1 μmol of Nitrocefin in 1 min, at 30°C. β-Lactamase II hydrolyzes twice as much (±5%) Nitrocefin as does β-lactamase I under the test conditions in the presence of Zn^{2+}. Protein was estimated according to Lowry *et al.*[17].

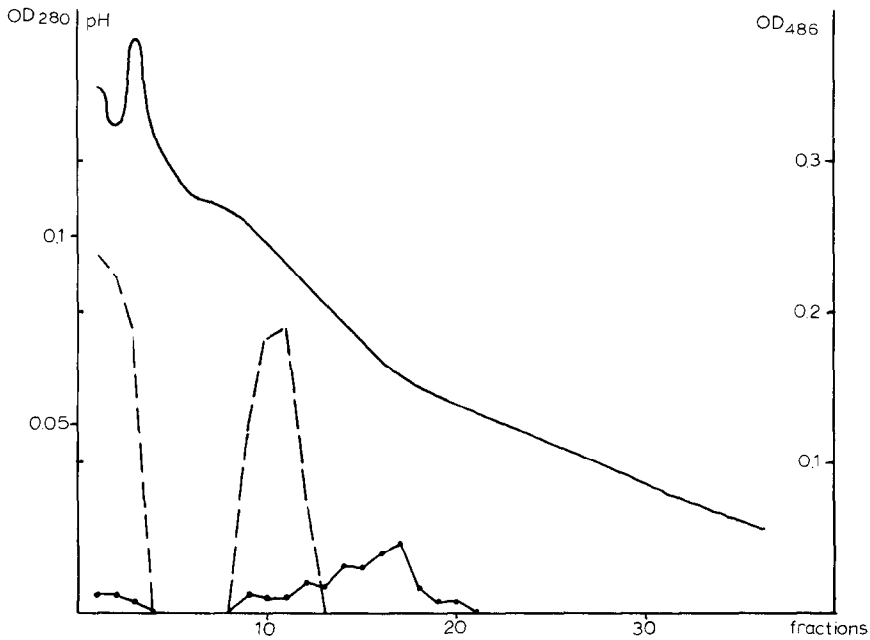

Fig. 1. Elution profile of purified β-lactamase I + II from PBE 94. A mixture of 53.5 units of β-lactamase I + 74 units of β-lactamase II from *B. cereus* 569/H in 3 ml of 0.025 *M* imidazole–HCl start buffer, pH 7.4 was applied to a C 10/20 column of PBE 94. Elution conditions, pH (———), protein (●—●) and activity (– – –) monitoring, were as described in the Experimental section. 304 μg of total protein were applied to, and 220 μg eluted from, the column.

RESULTS AND DISCUSSION

Purified β-lactamase I and II were simultaneously applied to a C 10/20 column of PBE 94, preincubated with 0.025 *M* imidazole–HCl, pH 7.4, and a pH gradient was developed with Polybuffer 74[16].

The elution profile is shown in Fig. 1. Both β-lactamases were eluted as single peaks. β-lactamase II exhibited p*I* > 7.4 and β-lactamase I had a p*I* of 6.37. Of the total 75 units of β-lactamase II added to the mixture of enzymes, 72.5 units (97.97%) were eluted. Corresponding data for the β-lactamase I preparation were: total 53.5 units added and 52 units (97.2%) eluted. The fractions containing β-lactamase II were: fraction 1, 36 units; 2, 23.5 units; 3, 13 units. Those showing β-lactamase I activity were: fraction 9, 11.5 units; 10, 17 units; 11, 16.5 units; 12, 7 units.

Fig. 2 demonstrates the separation of β-lactamase activities, namely 54 units of β-lactamase I + unknown β-lactamase species and 22 units of β-lactamase II. Seventy-six units of activity in the form of a crude enzyme preparation were applied to the column, of which 21.5 units (97.73%) of β-lactamase II were found in the active fractions 1–3 and 52.3 units (96.85%) of β-lactamase I + unknown β-lactamase species in fractions 11–18. The distributions of the respective enzyme activities were as follows: β-lactamase II, fraction 1, 10.5 units; 2, 6 units; 3, 5 units; β-lactamase I + unknown β-lactamase species, fraction 11, 1.5 units; 12, 7.8 units; 13, 10 units; 14, 6 units; 15, 8 units; 16, 10 units; 17, 6 units; 18, 3 units.

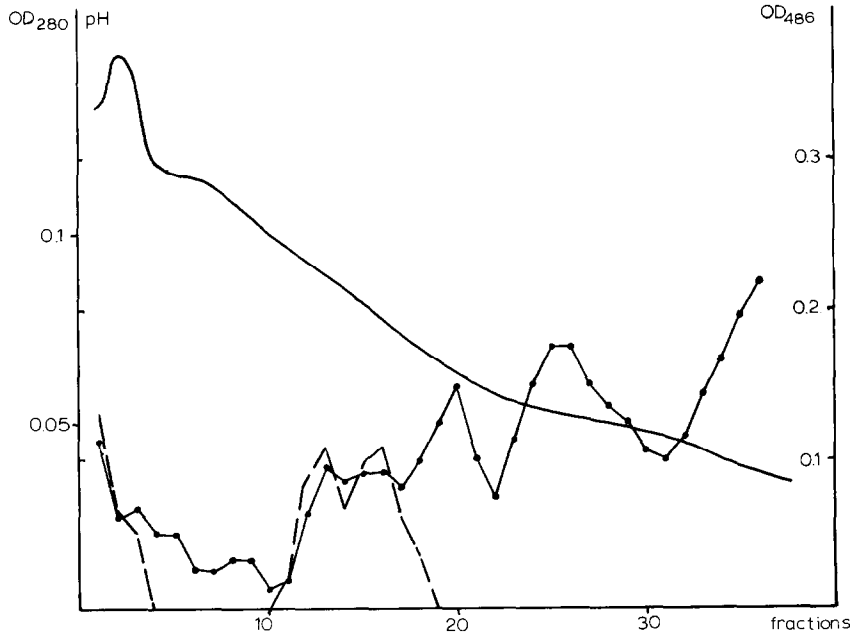

Fig. 2. Elution profile of crude β-lactamase mixture from PBE 94. A sample of 76 units of a β-lactamase mixture of *B. cereus* 569/H crude preparation in 3 ml of 0.025 *M* imidazole–HCl start buffer, pH 7.4 was applied to a C 10/20 column of PBE 94. Elution conditions, pH (———), protein (●—●) and activity (– – –) monitoring were as described in the Experimental section. 4750 μg of total protein were applied to, and 4323 μg eluted from, the column.

The β-lactamase II activity had pI > 7.4, but in the mild acidic range we observed two peaks which did not separate well from each other. The peak having a pI value of 6.38 clearly corresponded to that of the β-lactamase I fraction. The second relative activity maximum had a pI of 5.96. The appearance of this double peak requires further investigation to identify these activities. It may be possible to achieve a better separation of the species by producing a smoother pH gradient in the column. In order to provide sufficient purity for specific studies of the subfractions isoelectric focusing (IEF) probes are being introduced.

Taking into consideration the inherent nature of chromatofocusing, *i.e.*, the lower apparent pI values obtained, we may safely conclude that the difference from the actual values may be 0.1 units to higher pI values, but would never be significant with respect to our β-lactamase I, having a pI of not greater than 6.1. This is in qualitative agreement with Pollock *et al.*[2]. Our pI > 7.4 for β-lactamase II also confirms in a qualitative sense that the enzyme is an alkaline protein[1,4,9].

From the point of view of our separation, the most important facet of the above results is that exo β-lactamase I and II fractions of our strain can be resolved and there is an indication for the presence of a (probably covalently) modified enzyme species. In addition, the separation of the β-lactamase activities from most of the contaminating proteins can be accomplished in a single step.

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