Journal of Chromatography, 545 (1991) 189–195
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 066

Short Communication

Use of chromatofocusing for separation of β-lactamases

IX. Analytical chromatofocusing for the separation of a chromosomal cephalosporinase from *Proteus vulgaris* 1028

SUSANNE GÁL, ANDREA TAR, BELA L. TOTH-MARTINEZ* and FERENC J. HERNADI
Chemotherapy Section, Department of Pharmacology, University Medical School of Debrecen, Debrecen H-4012 (Hungary)
(First received January 29th, 1990; revised manuscript received October 30th, 1990)

ABSTRACT

Simultaneous purification and isoelectric point (p*I*) determination was carried out at analytical scale of the chromosomal cephalosporinase from the *Proteus vulgaris* 1028 strain. Comparison of the enzyme to the purification results with *m*-aminophenylboronic acid–agarose affinity chromatography with sodium dodecyl sulphate–polyacrylamide gel electrophoresis revealed that minute amounts of accompanying proteins having identical p*I* values but different molecular masses were found in the chromatofocused preparation. The molecular mass of the enzyme was 24 000 dalton. The p*I* was found to be 8.3.

INTRODUCTION

The frequent β-lactam resistance of *Proteus vulgaris* strains is due to their intense β-lactamase production. *P. vulgaris* is usually resistant to ampicillin, first-generation cephalosporins and cefuroxime, and there are some isolates that exhibit resistance against ureidopenicillins and third-generation cephalosporins [1]. The β-lactamase enzymes produced by *P. vulgaris* spp. were classified by Richmond and Sykes [2] as class I enzymes, but owing to their physico-chemical properties and their substrate profile they should be reclassified as class III enzymes.

The *P. vulgaris* β-lactamases have a wide range of isoelectric points (pI) but their substrate specificities are very similar [3]. Matsubara *et al.* [4] characterized a special *P. vulgaris* enzyme that had a molecular mass of 29 000 dalton and alkaline p*I* of 8.8. As the enzyme was able to hydrolyse cefuroxime very rapidly, Matsubara *et al.* classified it

0021-9673/91/$03.50

separately under the name cefuroximase. The V_{max} value for penicillins is lower, but penicillins exhibit a higher affinity to the *P. vulgaris* enzyme. Thus resistance can be explained by hydrolysis. Tajima *et al.* [5] described another specific enzyme with a molecular mass of 28 000 dalton and p*I* = 7.8, which did not hydrolyse the 7-α-methoxy-substituted congener cephalosporins. Okonogi *et al.* [3] investigated *P. vulgaris* clinical isolates and found the p*I* values of these β-lactamases to be within the range 6.9–9.0. According to Aspiotis *et al.* [1], the *P. vulgaris* specific β-lactamases can be grouped into three classes according to three p*I* values: 7.4, 8.8 and 9.5. Yang and Livermore [6] found five clinical isolates with inducible β-lactamases; their production could be stably derepressed in the basal mutants and the enzymes had pI values of 7.8, 8.0, 8.2, 8.6 and 8.9. Pagani *et al.* [7] investigated *P. vulgaris* 85, which proved to be strongly resistant against the new generation cephalosporins and its β-lactamase was acidic with p*I* = 5.6.

According to Matthew and co-workers [8,9], analytical isoelectric focusing is a suitable method for the identification of β-lactamases and for bacterium taxonomic purposes. As minute changes in the amino acid composition in the *Proteus* spp. enzymes by mutation can result in significant changes in the p*I* values without essential changes in the substrate profile, the above observation does not seem to apply to that species [3].

We have been able to use the chromatofocusing technique successfully in a comparative methodological project for the assessment of p*I* values of β-lactamases, and therefore we carried out the present experiments with the same aim of establising at the same time whether this technique is suitable for the isolation and purification of the cephalosporinase from crude extracts of *P. vulgaris* 1028.

EXPERIMENTAL

Bacterial strain

Constitutive cephalosporinase-producing *P. vulgaris* 1028 strain was kindly provided by Dr. R. Then (Hoffmann-La Roche, Basle, Switzerland).

Crude enzyme preparation, purification of the β-lactamase, chromatofocusing, enzyme assay and protein determination

In order to obtain a crude enzyme preparation, the strain was cultured in nutrient broth at 37°C with low-speed shaking (80 rpm). Portions of 150 ml of medium were inoculated in 500-ml conical flasks and cultured overnight. The cells were spun down and treated by freezing–thawing three times. Complete disruption of the cells was achieved in a Braun Labsonic 2000 sonifier by 30-s sonications at 0°C three times with 1-min cooling intervals. Cells debris was ultracentrifuged at 105 000 *g* for 60 min in a Janetzki VAC-601 ultracentrifuge. The supernatant was regarded as crude enzyme.

In order to prepare pure enzyme, *m*-aminophenylboronic acid–agarose affinity chromatography was used (type B column) according to Cartwright and Waley [10]. A 3 × 0.9 cm I.D. column was used. The enzyme was applied to the column in 20 m*M* triethylamine hydrochloride–0.5 *M* sodium chloride buffer (pH 7.00). The affinity material was pre-equilibrated with the same buffer. The column was then washed with the above buffer until accompanying protein-free eluates were collected, however,

elution of the enzyme was carried out with 0.5 *M* borate–0.5 *M* sodium chloride elution buffer (pH 7.00). Enzyme fractions of 3 ml were collected and the high specific activity fractions were pooled for chromatofocusing.

Chromatofocusing was carried out in a Pharmacia C10/20 column with PBE 94 in the pH range 9.00–6.00 [11]. Fractions of 4 ml were collected. The starting buffer was 0.025 *M* ethanolamine–acetic acid (pH 9.6) and the elution buffer was Polybuffer 96 in tenfold dilution (pH 6). Enzyme activity, pH and protein content were monitored.

β-Lactamase activity was determined with nitrocefin (NC) as substrate at 486 nm according to O'Callaghan *et al.* [12]. One unit was that amount of enzyme which was capable of hydrolysing 1 μmol of NC per minute at 37°C.

Protein was measured according to Lowry *et al.* [13].

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Weber and Osborn [14] and used for molecular mass determination. A molecular mass standard mixture (SDS-7) was purchased from Sigma (St. Louis, MO, U.S.A.).

The stability of the β-lactams was measured according to Ross and O'Callaghan [15].

RESULTS AND DISCUSSION

The chromosomal β-lactamase enzyme produced by our *P. vulgaris* 1028 strain belongs to class III of the Richmond–Sykes classification system. The substrate profile data, shown in Table I, support this conclusion.

For the purification of the crude enzyme, *m*-aminophenylboronic acid–agarose affinity chromatography was used. According to our experience, N-acetyl-D-penicillamine–Sepharose 4B gel was capable of binding only penicillinase character β-lactamases [16].

Fig. 1 shows the results of the purification of the enzyme on a type B *m*-aminophenylboronic acid–agarose affinity column. In 2 ml, 3.3 U of crude enzyme were administered to the column. Unbound enzyme was not detected in the wash. About 96% of the activity was eluted from the column with the elution buffer. The affinity material is able to purify the enzyme in a single step by eliminating the accompanying proteins, as opposed the multi-step classial methods.

TABLE I

STABILITY OF THE β-LACTAM ANTIBIOTICS AGAINST THE ISOLATED AND PURIFIED *PROTEUS VULGARIS* 1028 β-LACTAMASE

β-Lactam[a]	Rel. v_{max}[b]	β-Lactam[a]	Rel. v_{max}[b]
AMP	60	CTA	25
CPH	120	CPER	30
CXI	< 1	CTRI	75
CMA	150	CTAZ	< 1
CFUR	150		

[a] AMP = ampicillin; CPH = cephalotin; CXI = cefoxitin; CMA = cefamandole; CFUR = cefuroxime; CTA = cefotaxime; CPER = cefoperazone; CTRI = ceftriaxone; CTAZ = ceftazidime.

[b] Rel v_{max} = relative to cephaloridine = 100.

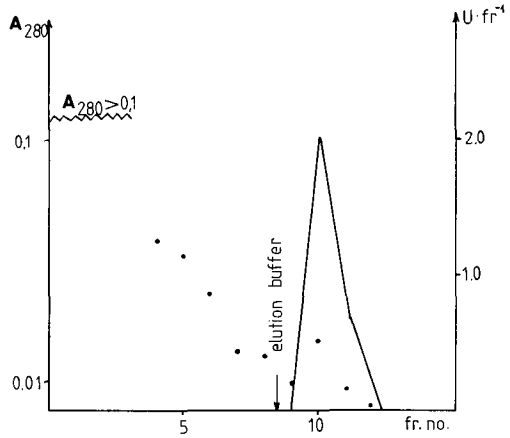

Fig. 1. Elution profile of the rude extract of β-lactamase from *P. vulgaris* 1028 strain on a type B phenylboronic acid–agarose affinity column. Protein (dots) and activity (solid line) monitoring were as described under Experimental; fr.no. = fraction number; U fr^{-1} = units per fraction.

In order to establish whether chromatofocusing was suitable not only for p*I* determination but also for the purification of this chromosomally coded cephalosporinase, the crude enzyme sample was chromatofocused. Fig. 2 depicts the chromatogram of 2.83 U of crude enzyme preparation in a 2-ml volume. The activity was eluted in four fractions: No. 8, 0.29 U; No. 9, 1.55 U; No. 10, 0.70 U; and No. 11, 0.18 U. These represent 96% of the total activity applied to the column. Fig. 2 demonstrates that the majority of the accompanying proteins were separated from

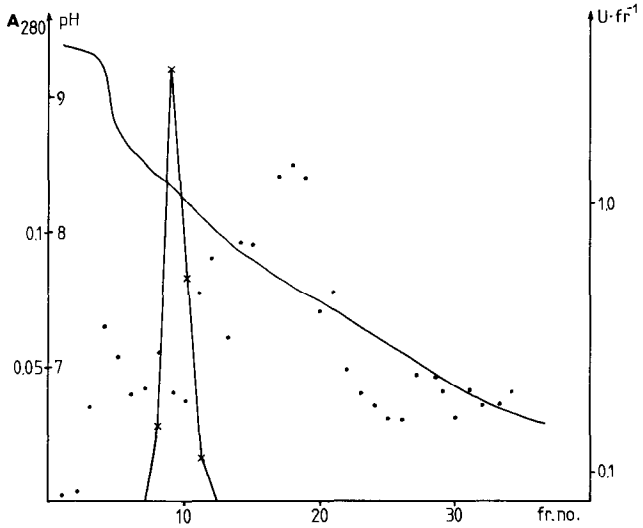

Fig. 2. Elution profile of the crude *P. vulgaris* 1028 enzyme after analytical chromatofocusing. Elution conditions: solid line, pH; ×, activity; and dots, protein monitoring as described under Experimental; fr. no. = fraction number; U fr^{-1} = units per fraction.

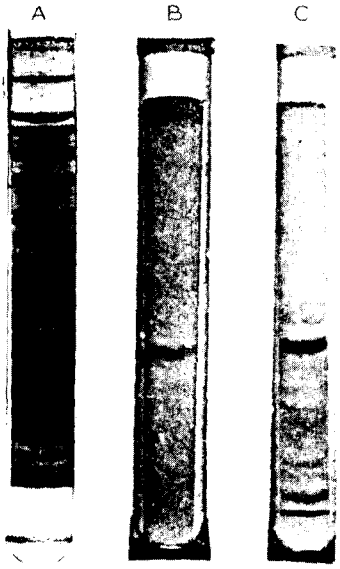

Fig. 3. SDS-PAGE of *Proteus vulgaris* β-lactamase. (A) Crude extract (200 μg of protein); (B) sample of the enzyme purified by affinity chromatography (30 μg of protein); (C) sample of the chromatofocused β-lactamase (50 μg of protein). The gels were subjected to 110 V, 70 mA for 4 h. Protein was detected with Coomassie Brilliant Blue R-250. The anode was at the bottom of the gels.

the activity according to their different p*I* values. The main fraction emerged from the column at pH 8.32.

The purity of the enzyme preparations was determined by SDS-PAGE (Fig. 3). Fig. 3 reveals that both (Fig. 3B and C, respectively) methods resulted in the production of pure enzymes. Two faint bands of the chromatofocused sample indicate the presence of minute amounts of accompanying proteins having identical p*I* values but different masses of the enzyme. The molecular mass of the enzyme was 24000.

Table II shows specific activity data (U mg^{-1}) expressing the extent of purification related to the crude enzyme.

In order to check the p*I* value obtained with the crude enzyme, chromatofocusing of the purified β-lactamase from the affinity gel was also performed. Fig.

TABLE II

COMPARISON OF PURITY PARAMETERS OBTAINED BY AFFINITY CHROMATOGRAPHY AND CHROMATOFOCUSING OF THE *PROTEUS VULGARIS* 1028 ENZYME

Parameter	Crude extract	Affinity chromatography	Chromatofocusing
Specific activity (U mg^{-1})[a]	0.47	28	22
Purification (fold)	1	60	47

[a] Specific activity was calculated as the number of units per mg of total protein.

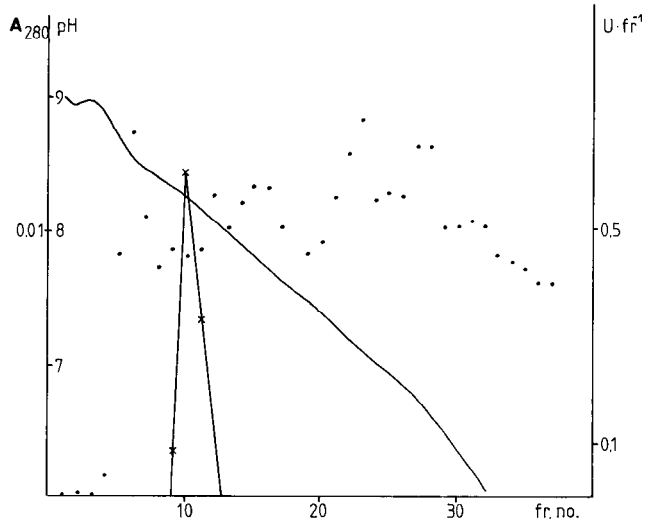

Fig. 4. Elution profile of the pure *P. vulgaris* 1028 enzyme after analytical chromatofocusing. Elution conditions: solid line, pH; ×, activity; and dots, protein monitoring as described under Experimental; fr. no. = fraction number; U fr^{-1} = units per fraction.

4 shows the chromatogram of the purified enzyme. The very low absorbances at 280 nm are due to the Polybuffer ampholytes as described in ref. 11. These values overall are negligible on comparing them with the absorbance values at 280 nm in Fig. 2, which represent Polybuffer ampholyte values together with those of the protein fractions. A 1.03 U amount of enzyme was applied to the PBE 94 column in 2 ml, and it emerged in three fractions: No. 9, 0.07 U; No. 10, 0.6 U; No. 11, 0.34 U; alltogether 98% of the total activity was regained. The peak enzyme fraction had p*I* = 8.26.

The p*I* values for the purified and the crude enzyme preparations agree well, and therefore we may conclude that the cephalosporinase of the *P. vulgaris* 1028 strain has its p*I* value at 8.3.

Finally we concluded that both affinity chromatography and chromatofocusing are suitable methods for the purification of this enzyme, but chromatofocusing also provides the p*I* value.

Our previous experiments with standard β-lactamases [17–20] demonstrated that p*I* values determined using other methods provided data identical with those obtained with chromatofocusing. Therefore, we may conclude that the p*I* value of *P. vulgaris* 1028 β-lactamase is also independent of other factors, *i.e.*, only its p*I* is decisive during chromatofocusing.

REFERENCES

1 A. Aspiotis, W. Cullmann, W. Dick and M. Stieglitz, *Chemotherapy*, 32 (1986) 236.
2 M. H. Richmond and R. B. Sykes, *Adv. Microb. Physiol.*, 9 (1973) 31.
3 K. Okonogi, M. Kuno and L. Higashide, *J. Gen. Microbiol.*, 132 (1986) 143.
4 N. Matsubara, A. Yotsuji, K. Kumano and M. Inoue, *Antimicrob. Agents Chemother.*, 19 (1981) 185.
5 M. Tajima, Y. Takenouchi, S. Ohya and S. Sugawara, *Microbiol. Immunol.*, 26 (1982) 531.
6 Y. Yang and D. M. Livermore, *Antimicrob. Agents Chemother.*, 32 (1988) 1385.

7 L. Pagani, M. Perduca and E. Romero, *Microbiologica*, 6 (1983) 163.
8 M. Matthew, A. M. Harris, M. J. Marshall and G. W. Ross, *J. Gen. Microbiol.*, 88 (1975) 169.
9 M. Matthew and A. M. Harris, *J. Gen. Microbiol.*, 94 (1976) 55.
10 S. J. Cartwright and S. G. Waley, *Biochem. J.*, 221 (1984) 505.
11 *Chromatofocusing with Polybuffer™ and PBE™ (Technical Handbook)*. Pharmacia, Uppsala, 1980.
12 C. H. O'Callaghan, A. Morris, M. Kirby and A. H. Shingler, *Antimicrob. Agents Chemother.*, 1 (1972) 283.
13 O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
14 K. Weber and M. Osborn, *J. Biol. Chem.*, 244 (1969) 4406.
15 G. W. Ross and C. H. O'Callaghan, *Methods Enzymol.*, 33 (1975) 69.
16 L. Kiss, A. Tar, S. Gál, B. L. Tóth-Martinez and F. J. Hernádi, *J. Chromatogr.*, 448 (1988) 109.
17 B. L. Toth-Martinez, S. Gál and L. Kiss, *J. Chromatogr.*, 262 (1983) 373.
18 B. L. Toth-Martinez, S. Gál, F. Hernádi, L. Kiss and P. Nánási, *J. Chromatogr.*, 287 (1984) 413.
19 L. Kiss, B. L. Toth-Martinez, S. Gál and F. Hernádi, *J. Chromatogr.*, 333 (1985) 244.
20 A. Tar, S. Gál, B. L. Toth-Martinez, F. Hernádi and L. Kiss, *J. Chromatogr.*, 368 (1986) 427.