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Hydrophobic character of surface regions and total hydrophobicity of four variants of chromosomal class C β-lactamase from *Pseudomonas aeruginosa* are identical. Chromatographic comparison of the hydrophobic character of the variants and the effect of focusing buffer composition on the separation of the variants by chromatofocusing with internal and external pH gradients

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Abstract

The hydrophobic character of class C β-lactamase molecular variants from *Pseudomonas aeruginosa* was compared by hydrophobic interaction chromatography and reversed-phase liquid chromatography, respectively. Separation of the variants by hydrophobic interaction chromatography was not achieved by modifying salt and pH of mobile phases. Reversed-phase liquid chromatography of the variants resulted in almost identical retention times. The results showed that the hydrophobic character of surface regions as well as total hydrophobicity of the variants are identical. The resolving power of external, internal and gradient chromatofocusing of the variants on strong and weak anion exchangers using low-molecular-mass buffers was compared to that of commercial ampholytes and showed no difference in separation pattern of the variants. Comparisons of variant isoelectric point (p*I*) values determined by chromatofocusing and isoelectric focusing showed that p*I* values determined by gradient chromatofocusing were most similar to the p*I* values determined by isolectric focusing.

Keywords: pH gradient; Chromatofocusing; β-Lactamase; Enzymes

1. Introduction

Separation of proteins is based on differences in their physical and chemical properties, and these diversities include size, charge, hydrophobicity and specific affinities. Two chromatographic techniques, hydrophobic interaction chromatography (HIC) and reversed-phase liquid chromatography (RPLC), are methods both based on interactions between immobilized hydrophobic ligands and intrinsic hydrophobic moieties of the proteins. Chromatofocusing is

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a separation technique which discriminates between heterogeneities in accessible surface charges of proteins.

In HIC high concentrations of salt are utilized to facilitate interactions between immobilized hydrocarbonaceous ligands and exposed hydrophobic amino acid residues at the surface of the proteins. Separation of the adsorbed native proteins are based on the hydrophobicity of the surface contact areas of the proteins.

In RPLC the binding of proteins to a hydrophobic stationary phase is usually very strong because of the high ligand substitutions in RPLC matrices, and this requires the use of non-polar, organic solvents for desorption of the proteins. In 1978 Sluytermann and co-workers [1,2] introduced chromatofocusing, and like ion-exchange chromatography, it is based on protein surface charges. During the elution pH of the mobile phase changes progressively, and the adsorbed proteins are eluted in the order of their isoelectric point (p*I*) values [1]. The pH gradient may be established either internally or externally. The external pH gradient is produced in the mixing chamber at the inlet of the column in contrast to the internal pH gradient which is generated inside the column by the mutual buffering action of the stationary and the mobile phase. Recently, gradient chromatofocusing has been developed where an externally formed pH gradient is introduced into a weak ion-exchange column and thus superimposed on an internally generated gradient within the column [3,4].

β-Lactamases (E.C. 3.5.2.6) are enzymes that provide bacteria with resistance against β-lactam antibiotics, i.e., penicillins, cephalosporins, and related compounds [5]. Based on their primary structure β-lactamases are divided into four molecular groups, A, B, C and D [6–8], and the predominant molecular class encountered in the Gram-negative bacterium, *Pseudomonas aeruginosa*, is a class C β-lactamase.

Previously, β-lactamases from other bacteria have been separated by chromatofocusing [9–13]. Recently, the different isoforms of the class C β-lactamase from the *P. aeruginosa* 258a strain have been purified by dye-affinity chromatography, size-exclusion chromatography (SEC), HIC, and finally resolved by conventional chromatofocusing [14]. In analytical IEF the p*I* values of the variants were determined as 8.7, 8.3, 8.2 and 7.6, respectively.

Chromatofocusing ensuing SEC of *P. aeruginosa* β-lactamase resulted in a single peak comprising all molecular variants (unpublished observation). However, a successful separation of the isoforms by chromatofocusing required a preceding step of HIC. The charge heterogeneities of the *P. aeruginosa* β-lactamase were found to be due to terminal truncations of a single gene product [14]. The variant with p*I* 8.7 represents the primary gene product, in this paper denoted variant 1, and the remaining isoforms are designated variants 2, 3, and 4 in the order of descending p*I*.

In the present study we have employed different sodium salts and pH values of the mobile phase to reveal putative differences in hydrophobic character of the β-lactamase variants by the use of Phenyl Superose as the hydrophobic resin. Due to the different p*I* values of the molecular variants the purpose was to observe if separation of the variants occurred with different salts and pH values of the mobile phases. In addition, differences in general hydrophobicity of the variants were analyzed by RPLC.

Usually, pH gradients in chromatofocusing are generated by commercially available mixtures of synthetic ampholytes. However, elution by buffers of low-molecular-mass constituents has been used [15–18], and since most proteins have p*I* values in the range 5–7, focus has been on the development of buffer compositions covering this pH range. As the majority of identified class C β-lactamases have p*I* values in the range 7–9 [19] this prompted us to investigate the effect of focusing buffers consisting of common buffers on the separation of the β-lactamase variants by chromatofocusing. For elution, focusing buffers composed of the non-polymeric buffers, Bis-Tris propane and Tris, were used and compared to the commercially available Polybuffer 96. Fractionations were performed by internal, external and gradient chromatofocusing.

2. Experimental

2.1. Materials

Bis-Tris propane {1,3-bis[tris(hydroxymethyl)-methylamino]propane} is a cationic, low-molecular-mass buffer with two pK_a values of 9.0 and 6.8,

respectively. Tris is also a cationic buffer with a pK_a of 8.1. All chemicals except when mentioned otherwise were purchased from Sigma (St. Louis, MO, USA).

Except for low-pressure dye-affinity chromatography, all other chromatographic techniques were performed on a fast protein liquid chromatography (FPLC) system (Amersham Pharmacia Biotech, Uppsala, Sweden). The following columns, HiLoad 16/60 Superdex 75 prep grade, Phenyl Superose HR 5/5, Fast Desalting Column HR 10/10, MonoP HR 5/20, MonoQ HR 5/5, and ProRPC HR 5/10 were all purchased from Amersham Pharmacia Biotech. The ProRPC column is substituted with C_1 and C_8 alkyl ligands. MonoQ is a strong and MonoP a weak anion exchanger with quarternary ammonium groups and mixed quarternary and tertiary amines, respectively, as ligands. The ionic capacities of the MonoQ and MonoP columns are 0.27–0.37 and 0.15–0.21 mmol/ml gel, respectively (product information from Amersham Pharmacia Biotech).

All eluates were continuously monitored for UV absorbance at 280 nm with a 2510 Uvicord SD recorder (Amersham Pharmacia Biotech).

2.2. Purification of the β-lactamase

The *P. aeruginosa* strain 258a was isolated from a chronically lung infected patient suffering from cystic fibrosis. The bacteria were grown, harvested, and subsequently treated according to Giwercman et al. [20].

Initially, the β-lactamase was purified as described [14] by dye-affinity chromatography on a Cibacron Blue 3GA-Agarose 3000-CL column and bound proteins were eluted with 25 m*M* Tris, 1 *M* NaCl, 5 m*M* EDTA, pH 8.0. The eluate was concentrated by ultrafiltration in a stirred cell (Amicon, Danvers, MA, USA) and further purified by SEC in a HiLoad 16/60 Superdex 75 prep grade column preequilibrated with 25 m*M* Tris, 150 m*M* NaCl, 5 m*M* EDTA, pH 8.0. Qualitative tests of β-lactamase activity of chromatographic fractions were performed with nitrocefin (Becton Dickinson, Cockeysville, MD, USA). Fractions exhibiting β-lactamase activity were analyzed by the standard discontinuous sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [21], and pure fractions were pooled and used for further analyses.

Protein concentrations were determined by use of the micro method of the Bio-Rad protein assay (Bio-Rad, Richmond, CA, USA) with bovine serum albumin (BSA) as a reference.

2.3. Hydrophobic interaction chromatography of the β-lactamase

Pooled fractions from SEC were divided into equal aliquots and analyzed by HIC in a Phenyl Superose HR 5/5 column equilibrated in 50 m*M* buffer+1.5 *M* sodium sulfate, 50 m*M* buffer+3 *M* sodium acetate, or 50 m*M* buffer+4 *M* sodium chloride. The used buffer components were Bis-Tris propane at pH 7 and 9 and Tris at pH 8, and depending on the anion constituent of the salt pH was adjusted with 2 *M* of either sulfuric acid, acetic acid or hydrochloric acid. The pH was adjusted to the desired value and subsequently the modifying salt was added. Just prior to use the mobile phase was filtered through a 0.20-μm filter. Immediately prior to application a solution of the water-structuring salt was added to the sample at a final concentration equal to the equilibrating mobile phase. The injected sample amount per run was 0.38 mg in 1.2 ml. At a flow-rate of 0.5 ml/min adsorbed β-lactamase was eluted by a descending linear salt gradient from 100% to 0% in 40 min. All runs were performed at ambient temperature.

2.4. Chromatofocusing of the β-lactamase

All chromatofocusing buffer solutions were degassed prior to pH-adjustment and before use filtered through 0.20-μm filters and once more degassed. All buffers, except Polybuffer 96 and 75 m*M* Tris, were pH-adjusted to pH 9.00 and 6.80, respectively, with 2 *M* hydrochloric acid. As recommended by the manufacturer, 75 m*M* Tris and Polybuffer 96 (Amersham Pharmacia Biotech) were adjusted to pH 9.30 and 6.00, respectively, with 2 *M* acetic acid.

The low-molecular-mass buffer components were Bis-Tris propane and Tris, and they were used in concentrations of 5, 10 and 25 m*M*, respectively. Solutions of Bis-Tris propane have a minimum buffer capacity of around pH 8, and since buffer capacities are additive [22], Tris was included to ensure a more even buffer capacity of the mobile phase.

Immediately after elution the pH of collected fractions (1 ml) was measured by a pHC 2701 pH electrode on a PHM 80 pH meter (Radiometer Danmark, Rødovre, Denmark). The determined pH profiles were averages ($n=3$) of blank pH gradients.

The β-lactamase containing peak from HIC with ammonium sulfate as the modifying salt [14] was divided into equal aliquots. Prior to chromatofocusing the aliquot of 0.31 mg β-lactamase in 1 ml was buffer-changed to the high pH equilibrating buffer in a Fast Desalting Column and applied directly to the pre-saturated ion-exchange column.

Initially, the β-lactamase molecular isoforms were fractionated by chromatofocusing on the MonoQ column and eluted by an external gradient formed in the mixing chamber of the FPLC apparatus. At a flow-rate of 0.5 ml/min the pH gradients were generated from pH 9.00 to 6.80 in 50 min succeeded by 100% of the focusing buffer for 8 min. Alternatively, the β-lactamase isoforms were separated on the MonoP column by gradient chromatofocusing according to Liu and Anderson [3,4]. A solution of 25 m*M* Bis-Tris propane+25 m*M* Tris, pH 9.00 was used as the equilibrating buffer and the same buffer composition at pH 6.80 as the focusing buffer.

The externally generated gradient with a increase of 2.5% per min of the focusing buffer was introduced into the MonoP column at a flow-rate of 0.5 ml/min.

Finally, conventional chromatofocusing on the MonoP column was performed as recommended by the manufacturer with 75 m*M* Tris–acetate, pH 9.30 as the equilibrating buffer. The variants were resolved by a descending pH gradient with a 10-fold dilution of Polybuffer 96 adjusted to pH 6.0 as the focusing buffer. Elution was done with 34 ml of the focusing buffer at a flow-rate of 0.5 ml/min.

The sample used in conventional chromatofocusing was from another harvest of *P. aeruginosa* 258a.

2.5. Reversed-phase liquid chromatography of the β-lactamase variants

The separated β-lactamase variants from chromatofocusing were each subjected to RPLC on a ProRPC column. The variants were eluted by a linear 30 min gradient from 0 to 70% (v/v) acetonitrile (Merck, Darmstadt, Germany) containing 0.1% (v/v) trifluoroacetic acid (TFA) (Merck) at a flow-rate of 0.5 ml/ml. Acetonitrile and TFA were of HPLC grade.

2.6. Analytical isoelectric focusing

The β-lactamase variants were electrofocused in agarose IEF gels, the focused proteins were passively transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA) and then immunodetected by the use of polyclonal rabbit anti-β-lactamase antibodies as described [14]. Samples were supplemented with 0.1% BioLyte 7/9 (Bio-Rad) and 15 ng of β-lactamase was applied per lane. Focused and blotted samples and IEF standards (myosin and Lentil lectin) were also colloidal gold stained with AuroDye according to Ref. [23].

2.7. Measurement of β-lactamase activity

The biological activities of purified variants were quantified by a direct spectrophotometric method [24]. In a total sample volume of 1 ml 0.1 m*M* nitrocefin dissolved in 100 m*M* phosphate, pH 6.9 at 37°C was used as substrate. The absorbance was monitored at 482 nm with a Shimadzu UV-160A spectrophotometer (Shimadzu, Kyoto, Japan). One unit is defined as 1 μmol nitrocefin hydrolyzed per min per mg β-lactamase. Measurement of activity was performed six times.

3. Results and discussion

3.1. Hydrophobic interaction and reversed-phase liquid chromatography

The hydrophobic behavior of the β-lactamase was analyzed by HIC and RPLC, respectively. The HIC chromatograms of the *P. aeruginosa* β-lactamase molecular variants are shown in Fig. 1, and separation of the variants has not occurred. HIC of the β-lactamase revealed only minor differences in elution profiles. An additional minor peak eluting later than the main peak was observed consistently only with sodium sulfate. Analytical IEF of aliquots from the minor peaks seen with sodium sulfate and

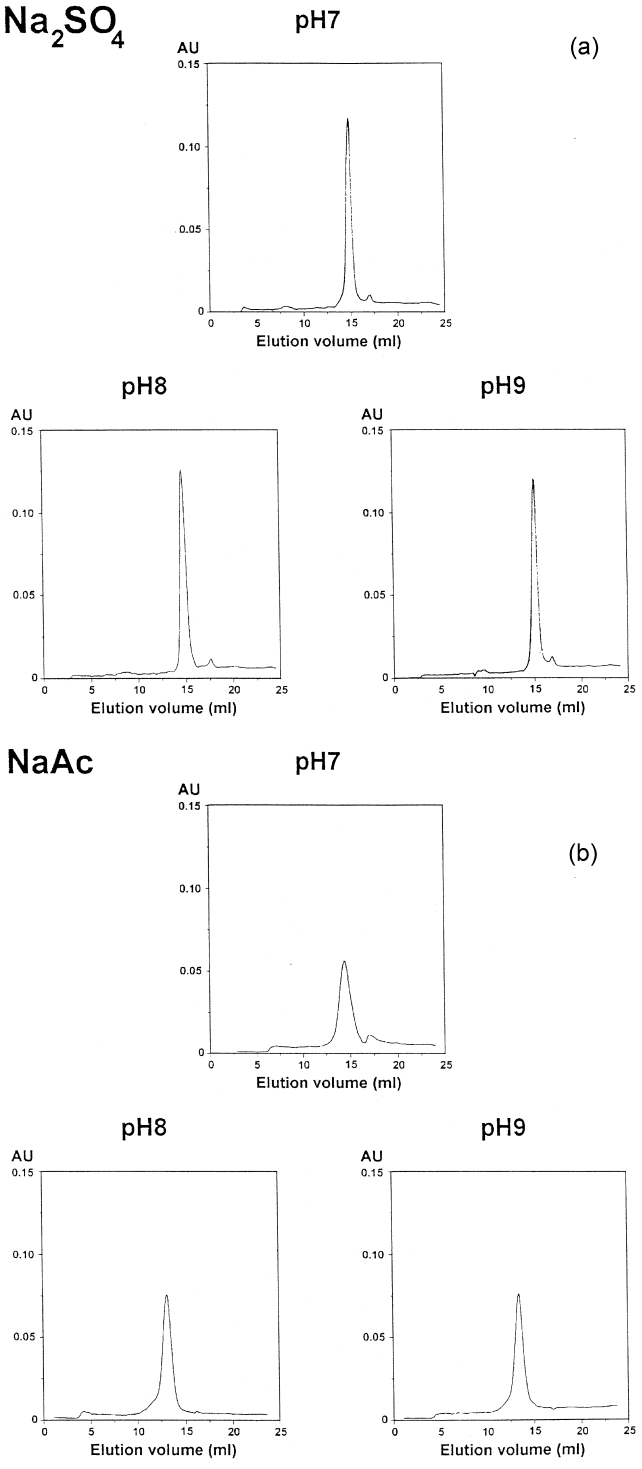


Fig. 1. HIC chromatograms on Phenyl Superose of β-lactamase from *P. aeruginosa*. (a) 1.5 *M* sodium sulfate; (b) 3 *M* sodium acetate; (c) 4 *M* sodium chloride. The buffer component in mobile phases of pH 7 and 9 was 50 m*M* Bis-Tris propane, while 50 m*M* Tris was the buffer constituent in mobile phases of pH 8. The flow-rate was 0.5 ml/min. The gradient was developed in 40 min.

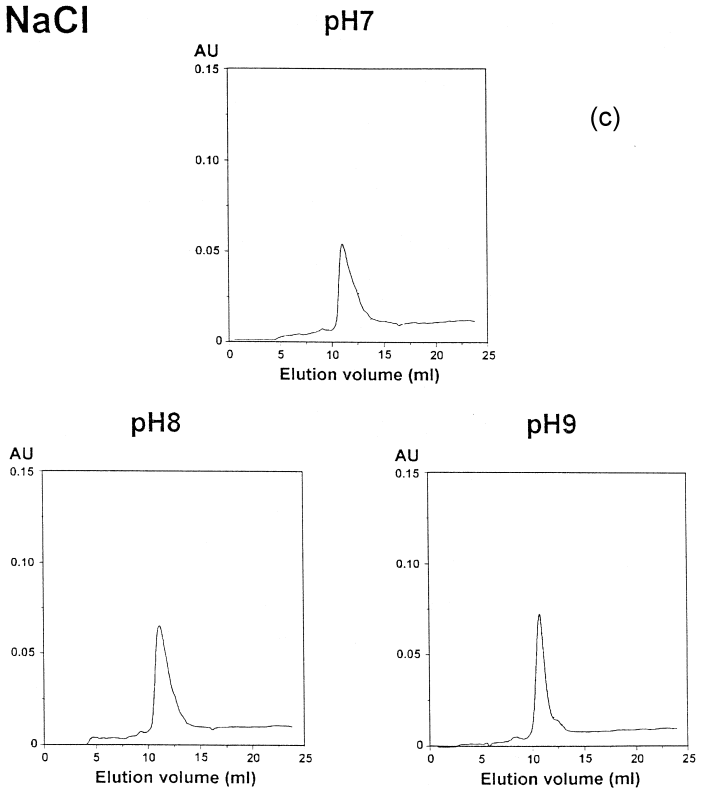


Fig. 1. (*continued*)

all main peaks showed that all variants were present in each sample (data not shown).

Retention times for the used sodium salts correlate with the lyotropic properties of the water-structuring salts (sodium sulfate>sodium acetate>sodium chloride).

The influence of mobile phase pH on protein retention in HIC is not well defined. However, we were not able to demonstrate any significant influence of pH on the retention of the variants. Thus, the results from HIC indicate that the exposed surface hydrophobicities of the β-lactamase molecular variants are similar.

Fig. 2 illustrates the RPLC elution profiles of the variants. RPLC of a pool which contained all variants resulted in a single peak (data not shown). RPLC of the separated molecular variants gave peaks with scarcely indistinguisable retention times which is compatible with the conclusions from HIC. The combined results from HIC and RPLC therefore suggest, that all variants share exterior and interior regions of equal hydrophobicity.

3.2. Chromatofocusing

In the present study and following HIC with a descending gradient of ammonium sulfate the molecular variants were separated by external, internal and gradient chromatofocusing, respectively, and compared to the elution profile from conventional chromatofocusing.

Fig. 3 shows the comparison of pH gradients established by the different focusing buffers. None of the gradients are strictly linear in the entire pH range, but the pH gradients generated by 5 m*M* Bis-Tris propane+5 m*M* Tris in the MonoQ column and by 25 m*M* Bis-Tris propane+25 m*M* Tris in gradient chromatofocusing both approach linearity. The other blank pH gradients are slightly sigmoid, convex or concave. None of gradients display any

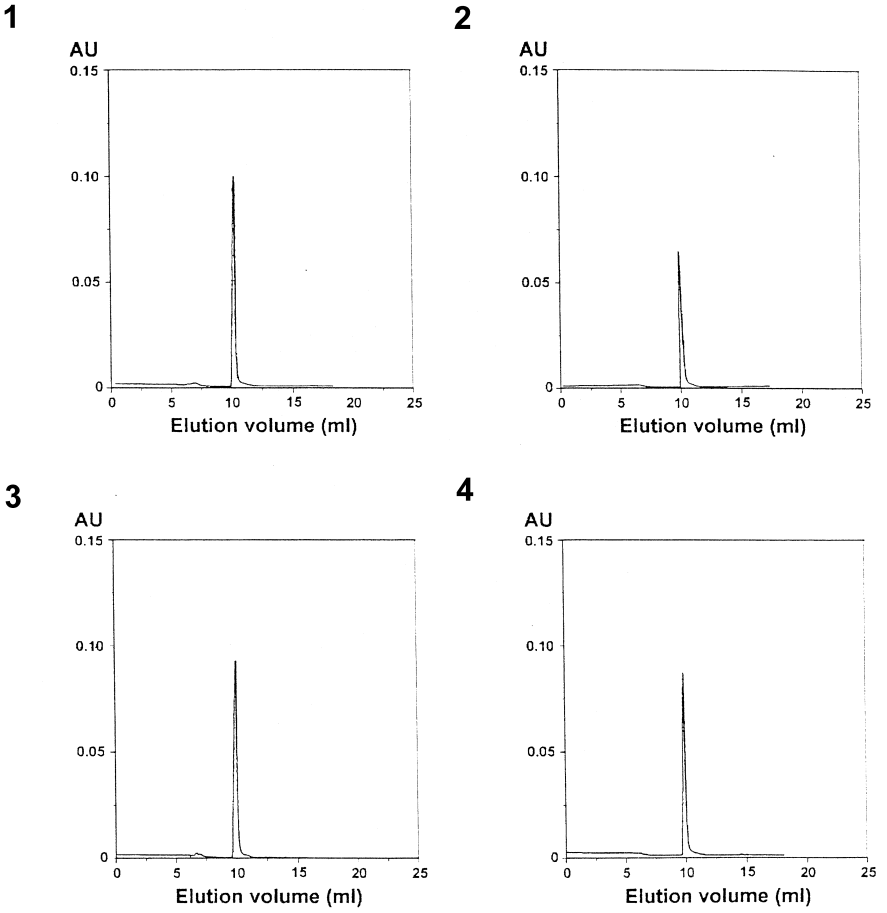


Fig. 2. RPLC on a ProRPC column of the separated molecular variants of *P. aeruginosa* β-lactamase. The A-solvent was 0.1% (v/v) TFA and the B-solvent was 0.1% (v/v) TFA in 70% (v/v) acetonitrile. The gradient was developed from 0 to 100% B-solvent in 30 min at a flow-rate of 0.5 ml/min. Figs. 2.1–2.4 represent the variant Nos. 1 to 4.

distinct spikes or plateaus, but the most irregular gradient is actually produced by Polybuffer 96. A gradient from pH 9 to 6.8 generated by Polybuffer was even more irregular (not shown).

Theoretically, lower ionic strength of the focusing buffer should enhance chromatofocusing resolution. However, peak resolutions are identical irrespective of the used ion exchanger and focusing buffer which is demonstrated in Fig. 4. Displacements of the elution profiles relative to each other have occurred. In the MonoQ column the retention of the enzyme species decreased constantly in correlation with an increasing ionic strength of the mobile phase. As the resolution of the variants is identical despite the used buffer, the buffer capacity does not seem to influence the separation, neither does the ionic strength. Compared to conventional chromatofocusing, buffers with higher ionic strength may be used in gradient chromatofocusing without compromising the resolution. This is advantageous, since *P. aeruginosa* β-lactamase is extensively retained by some stationary phases equilibrated in low ionic strength buffers (unpublished observation). This adherence to surfaces in low ionic strength buffers may be reflected in the enlarged retention times (lower apparent p*I*) on the MonoQ column.

Compared to the other chromatographic peaks, the peak obtained by the use of Polybuffer 96 (Fig. 4g) exhibited the smallest peak width. This may be due to the steeper pH gradient generated by Polybuffer

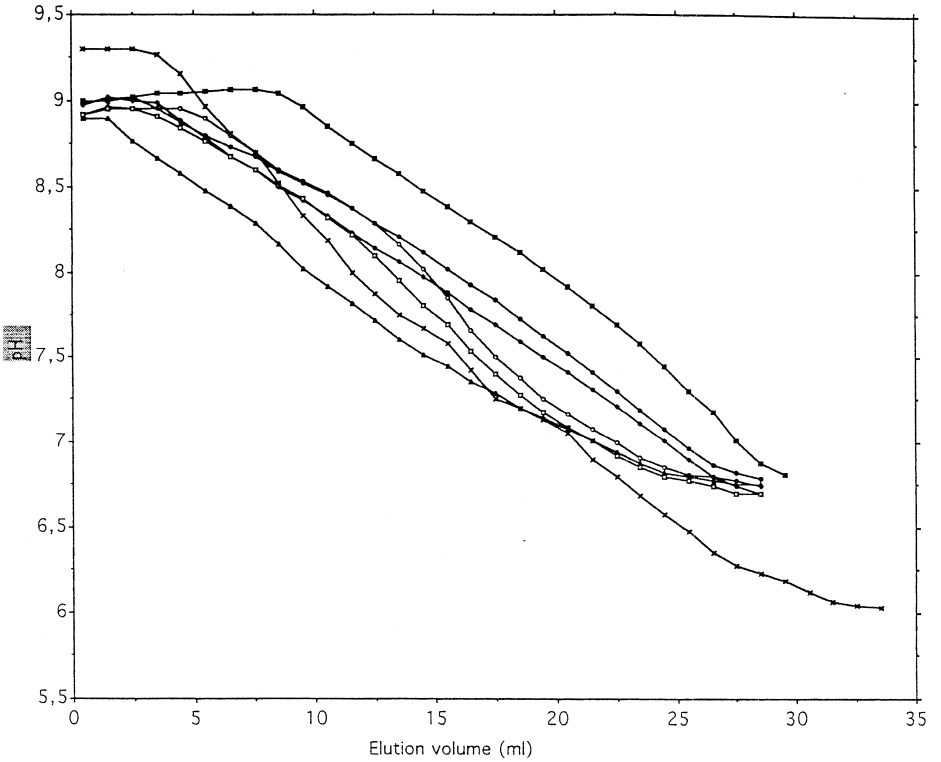


Fig. 3. Comparison of blank pH gradients on the strong anion exchanger, MonoQ, and the weak anion exchanger, MonoP. The used focusing buffers are ○ 5 m*M* Bis-Tris propane; ◇ 5 m*M* Bis-Tris propane+5 m*M* Tris; □ 10 m*M* Bis-Tris propane; ▲ 25 m*M* Bis-Tris propane; ● 25 m*M* Bis-Tris propane+25 m*M* Tris (external pH gradient); ■ 25 m*M* Bis-Tris propane+25 m*M* Tris (gradient chromatofocusing); × 1:10 dilution of Polybuffer 96. The pH profiles are averages ($n=3$) of pH gradients generated by external chromatofocusing on the MonoQ column (○–●), gradient chromatofocusing on the MonoP column (■), and conventional chromatofocusing on the MonoP column (×). The flow-rate was 0.5 ml/min, and the gradients (○–●) were developed by external mixing of equilibrating buffer, pH 9 with focusing buffer, pH 6.8 in 25 min.

96 (Fig. 3) as more shallow pH gradients will lead to larger dilution factors.

Based on the blank pH gradients the p*I* values of the separated molecular variants (pI_{chrom}) were measured and the values are given in Table 1 in comparison with the p*I* values (pI_{IEF}) determined by analytical agarose IEF [14]. The Table also includes the specific activities of the molecular variants. Table 2 shows the differences between the p*I* values determined by IEF and chromatofocusing using the different focusing buffers.

Sluyterman and Elgersma [1] have derived an equation in which the deviation of the observed p*I* (in chromatofocusing) from the real p*I* of a protein is partly dependent on the Donnan potential of the ion exchanger and partly on the ratio of buffer capacity of the stationary phase and the mobile phase. Since MonoQ is a strong anion exchanger it means that the exchanger is positively charged in the entire pH range, 2–12, and thus exerts no buffering action. When the buffer capacity of the exchanger is zero, then according to the equation [1], the $pI_{IEF}-pI_{chrom}$ difference becomes directly proportional to the Donnan potential of the ion exchanger. A mobile phase with a low ionic strength results in a high Donnan potential and consequently a larger $pI_{IEF}-pI_{chrom}$ difference. During a run, the pH in the column alters progressively, and the decrease in pH is simultaneously accompanied by an increase of the ionic strength of the mobile phase. Concurrently with the gradual elevation of the ionic strength of the mobile phase, the Donnan potential of the ion exchanger

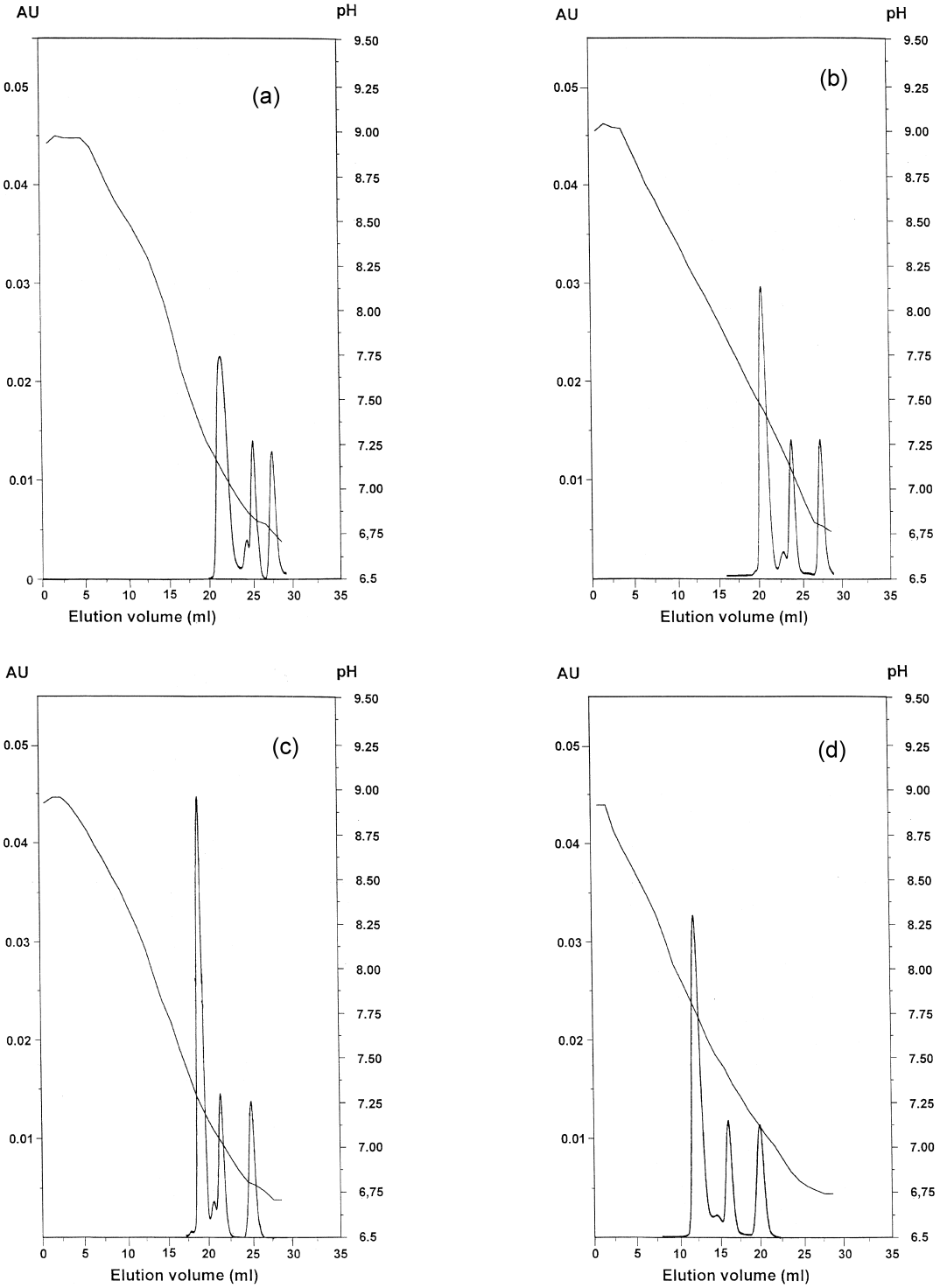
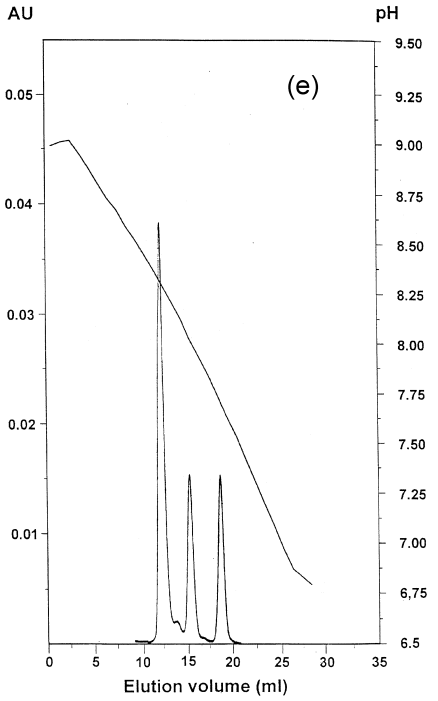
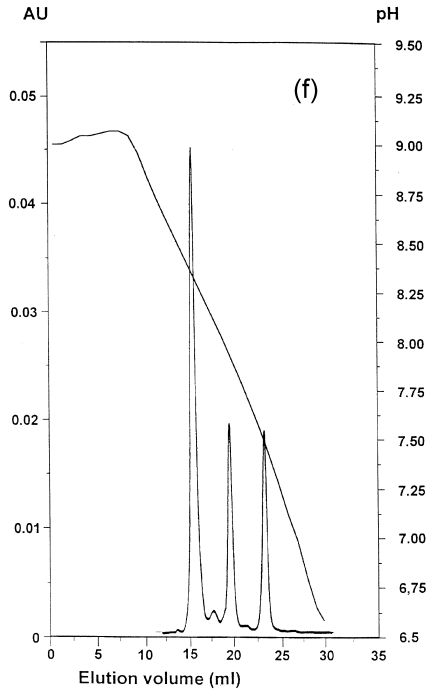


Fig. 4. Chromatofocusing elution profile comparisons of β-lactamase molecular variants separated on the MonoQ column by external pH gradients (a–e), gradient (f) and conventional (g) chromatofocusing on the MonoP column. The focusing buffers (a–g), gradients, and flow-rate as in Fig. 3.





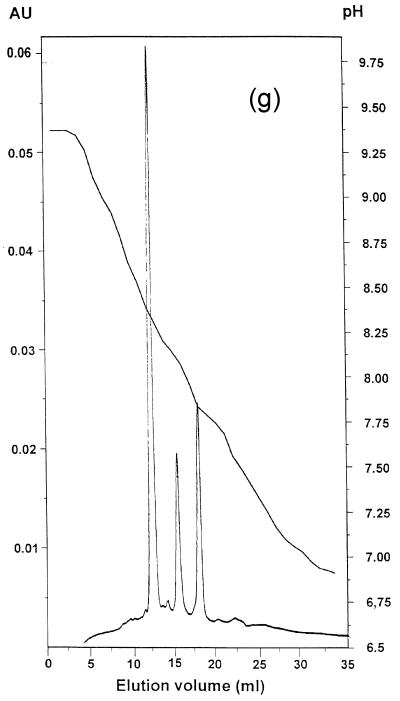


Fig. 4. (*continued*)

Table 1
Apparent p*I* values of the β-lactamase molecular variants determined by external chromatofocusing on a MonoQ column, gradient and internal chromatofocusing, respectively, on a MonoP column in relation to the p*I* values measured by analytical IEF

Peak No.	IEF p*I*	Specific activity[a] (units $\cdot 10^{-3}$) ± SD	Apparent p*I* measured						
			on MonoQ					on MonoP	
			Buffer 1[b]	Buffer 2[b]	Buffer 3[b]	Buffer 4[b]	Buffer 5[b]	Buffer 6[b]	Buffer 7[b]
1	8.7	2.77±0.09	7.1	7.5	7.4	7.8	8.3	8.4	8.0
2	8.3	1.81±0.04	6.9	7.2	7.2	7.6	8.1	8.2	7.7
3	8.2	1.92±0.06	6.8	7.1	7.1	7.5	8.0	8.1	7.6
4	7.6	3.30±0.06	6.7	6.8	6.8	7.2	7.7	7.7	7.3

[a] Values from Ref. [14].
[b] Buffer 1: 5 m*M* Bis-Tris propane on MonoQ. Buffer 2: 5 m*M* Bis-Tris propane+5 m*M* Tris on MonoQ. Buffer 3: 10 m*M* Bis-Tris propane on MonoQ. Buffer 4: 25 m*M* Bis-Tris propane on MonoQ. Buffer 5: 25 m*M* Bis-Tris propane+25 m*M* Tris on MonoQ. Buffer 6: 25 m*M* Bis-Tris propane+25 m*M* Tris on MonoP by gradient chromatofocusing. Buffer 7: 1:10 dilution of Polybuffer 96 on MonoP.

declines, and according to the equation [1] this results in reduced values of $pI_{IEF} - pI_{chrom}$.

For every buffer composition in Table 2 it is evident that the difference between pI_{IEF} and pI_{chrom} is diminished in relation to the decreasing pH of the mobile phase; the lower pH, the smaller the difference is. From Table 2 it also appears that irrespective of MonoQ or MonoP is used the buffer composed of 25 m*M* Bis-Tris propane+25 m*M* Tris produces the best correspondence to p*I* values determined by IEF and is in this respect superior to Polybuffer 96.

In a previous study [14] mass spectrometry (MS) of variant 2 (p*I* 8.3) showed that the variant actually contained two subforms with observed molecular masses of 40 047 and 40 752, respectively. None of the buffer compositions employed for chromatofocusing in this study nor a less steep pH gradient with Polybuffer 96 (data not shown) are able to resolve the subforms. As the two subforms also comigrated in IEF [14] this connotes identical or almost identical surface charges of the subforms.

Table 2
Differences ($\Delta pI = pI_{IEF} - pI_{chrom}$) between p*I* values determined by analytical IEF and by chromatofocusing[a]

Peak No.	MonoQ					MonoP	
	ΔpI_1	ΔpI_2	ΔpI_3	ΔpI_4	ΔpI_5	ΔpI_6	ΔpI_7
1	1.6	1.2	1.3	0.9	0.4	0.3	0.7
2	1.4	1.1	1.1	0.7	0.2	0.1	0.6
3	1.4	1.1	1.1	0.7	0.2	0.1	0.6
4	0.9	0.8	0.8	0.4	−0.1	−0.1	0.3

[a] The subscripts indicate the buffer No. used for chromatofocusing as described in Table 1. The buffer Nos. 1 to 5 and Nos. 6 and 7 are used in the MonoQ and MonoP columns, respectively.

Analytical IEF and immunodetection of the eluted molecular variants from chromatofocusing (data not shown) demonstrated that all peaks were pure and uncontaminated which indicated that the molecular variants were separated irrespective of the used buffer and anion exchanger.

As the majority of class C β-lactamases have p*I* values in the range 7 to 9 this implies that pH gradient elution in strong anion exchangers by Bis-Tris propane+Tris buffers may be an easy and less costly alternative for separation of molecular variants of this molecular class of β-lactamases.

4. Conclusions

Separation of the molecular variants of β-lactamase from *P. aeruginosa* by HIC and RPLC has not occurred which implies that the hydrophobic character of surface regions as well as total hydrophobicity of the variants are identical.

Separation of the molecular variants by external, internal and gradient chromatofocusing on strong and weak anion exchangers showed no qualitative differences. The resolution power of the low-molecular-mass buffers compared to Polybuffer 96 is equal, but the p*I* values measured in gradient chromatofocusing using a mixture of 25 m*M* Bis-Tris propane and 25 m*M* Tris as buffer deviated less from the p*I* values determined by IEF.

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