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Ragged *N*-termini and other variants of class A β-lactamases analysed by chromatofocusing

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Four β-lactamases excreted by Gram-positive bacteria exhibited microheterogeneity when analysed by chromatofocusing or ion-exchange chromatography. Ragged *N*-termini were in part responsible for the charge variants, but deamidation of an asparagine residue was also involved, at least for the *Bacillus licheniformis* enzyme. The activity of a contaminating proteinase could also be demonstrated in the case of the *Actinomadura* R39 β-lactamase. With that enzyme, proteolysis resulted in partial inactivation, but the inactivated fragments were easily separated from the active forms. With these, as with the other enzymes, the kinetic parameters of the major variants were identical with those of the mixture within the limits of experimental error, so that the catalytic properties of these enzymes can be determined with the 'heterogeneous' preparations.

INTRODUCTION

Excretion of extracellular β-lactamases exhibiting various extents of microheterogeneity by Gram-positive bacteria seems to be a rather common phenomenon (Ambler & Meadway, 1969; Thatcher, 1975*a*; Dehottay *et al.*, 1987; Lenzini *et al.*, 1988). The most widely studied case is that of the enzyme produced by *Bacillus licheniformis*, where a 'ragged' *N*-terminus was first demonstrated (Ambler & Meadway, 1969; Thatcher, 1975*b*; Simons *et al.*, 1978; Izui *et al.*, 1980). The same phenomenon appeared to be at least partially responsible for the microheterogeneity of the β-lactamases of *Streptomyces albus* and *S. cacaoi* cloned and produced in *S. lividans* (Dehottay *et al.*, 1987; Lenzini *et al.*, 1988). The present study was undertaken after discovering that distinct active fractions of various Gram-positive β-lactamases could be separated by chromatofocusing on a MonoP column (Dehottay *et al.*, 1987; Matagne *et al.*, 1990). Although we were interested in elucidating the origin of the variations at the molecular level, we thought that it was even more important to compare the kinetic parameters of the individual variants with those of the mixtures to determine if meaningful results could be obtained without resolving pure preparations into individual components.

To allow easy reference to the sequences of other class A β-lactamases, we use the ABL numbering scheme. In that system, a residue can be referred to by two numbers: one is its position in the natural protein or precursor sequence and the second (called ABL) is based on Ambler's (1979) alignment of the first four enzymes whose sequence was determined. For instance, the active serine residue in the *Actinomadura* R39 β-lactamase is no. 86 in that enzyme and ABL 70.

MATERIALS AND METHODS

β-Lactam compounds

Benzylpenicillin was from Rhône–Poulenc (Paris, France), ampicillin from Bristol Benelux (Brussels, Belgium), β-iodopenicillanic acid from Pfizer Central Research (Sandwich, Kent, U.K.), and cephaloridine and cephalothin were from Eli Lilly and Co. (Indianapolis, IN, U.S.A.). These antibiotics were kindly given by the respective companies. Penicillin V was a gift from Professor H. Vanderhaeghe and Professor P. Claes (Katholieke Universiteit Leuven, Leuven, Belgium). Nitrocefin was purchased from Oxoid (Basingstoke, Hants., U.K.).

Enzymes

***Actinomadura* R39 β-lactamase.** The enzyme was produced by *Streptomyces lividans* TK24 harbouring plasmid pIJ424 containing the gene coding for the *Actinomadura* R39 β-lactamase (Piron-Fraipont *et al.*, 1989) and was purified as described by Piron-Fraipont *et al.* (1989).

***Bacillus licheniformis* 749/C β-lactamase.** The enzyme was produced by the original strain and purified as described by Matagne *et al.* (1990).

***S. albus* G β-lactamase.** The enzyme was produced by *S. albus* G strain R2 harbouring plasmid pDML6 containing the gene coding for the *S. albus* G β-lactamase (Dehottay *et al.*, 1987) and was purified as described by Matagne *et al.* (1990).

***S. cacaoi* β-lactamase.** The enzyme was produced by *S. albus* G strain R2 harbouring plasmid pDML51 containing the gene coding for the *S. cacaoi* β-lactamase (Lenzini *et al.*, 1987) and purified as described by Matagne *et al.* (1990).

The four β-lactamase preparations were the same as those used for the study of the catalytic properties of these enzymes (Matagne *et al.*, 1990).

Isoelectric focusing and pI calculations

Isoelectric focusing was performed in a Bio-Rad model-111 Mini IEF cell with LKB ampholytes in the pH ranges 3–10 and 3.5–5 or in a Pharmacia Fast System apparatus using the pH range 4–6.5.

The pI calculations were done with the help of the 'isoelectric' algorithm from the GCG package version 6 (Devereux *et al.*, 1984).

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Chromatographic techniques

Chromatofocusing. Chromatofocusing experiments were performed on a MonoP HR5/20 column connected to a Pharmacia f.p.l.c. system. The pH gradient went from 5.7 to 4, except with the *B. licheniformis* β-lactamase, for which it started at pH 6.1. Buffer A was 25 mM-*N*-methylpiperazine/HCl (Janssen), pH 5.7 or 6.1, and buffer B, was a 10-fold dilution of Polybuffer 74 (Pharmacia) adjusted to pH 4 with HCl. Buffer B used in the experiments with the *S. albus* G and *S. cacaoi* β-lactamases also contained 5% (v/v) glycerol and 5% (v/v) ethylene glycol. Elution was performed with 34 ml of buffer B at a flow rate of 0.7 ml/min. The amount of enzyme used in each experiment was of the order of 1 mg.

Ion exchange. A MonoQ HR5/5 and a MonoS HR5/5 columns connected to a Pharmacia f.p.l.c. system were used for ion-exchange experiments. Elution of the *Actinomadura* R39 enzyme from the MonoQ column was performed with a linear NaCl gradient (0.4–0.6 M) over 36 ml in 20 mM-Tris/HCl buffer, pH 7.2. The experiment was performed at a flow rate of 1 ml/min with about 0.5 mg of enzyme.

Amino acid sequencing

Amino acid sequencing was done with a 470-A Applied Biosystems gas-phase sequenator as described previously (Joris *et al.*, 1985).

β-Lactamase activity

Routinely, β-lactamase activities were measured using nitrocefin as substrate in 50 mM-sodium phosphate, pH 7, at 30 °C. When necessary, enzyme samples were diluted in the same buffer containing 0.1 mg of BSA/ml. One unit represents the amount of enzyme hydrolysing 1 μmol of substrate/min at maximal velocity.

Kinetic parameters and thermal stability of β-lactamases

The determination of the kinetic parameters and the analysis of the thermal stability of the enzymes were performed as described by Matagne *et al.* (1990). The thermal inactivation of the *B. licheniformis* β-lactamase was monitored by continuously measuring the hydrolysis of a reporter substrate (nitrocefin) as described by De Meester *et al.* (1987).

RESULTS

pI values and chromatofocusing

PAGE, when performed under non-denaturing conditions, indicated a charge heterogeneity for the four β-lactamases. Five bands were also observed upon gel isoelectric focusing of the β-lactamases of *S. albus* G and *S. cacaoi*. In both cases, the pI of the main band was chosen as representative of that of the protein (Table 1).

The pI of the *Actinomadura* R39 β-lactamase was so low that it could not be determined; over the pH range 3.5–5.0 all the protein material concentrated near the anode. This was in good agreement with the strong interaction observed between that enzyme and anion-exchangers [see Matagne *et al.* (1990) and below].

The pI of the *B. licheniformis* enzyme was not redetermined, and the value shown in Table 1 is from Meadway (1969). By using the amino acid compositions deduced from the complete amino acid sequences of the four β-lactamases (Ambler & Meadway, 1969; Dehottay *et al.*, 1987; Lenzini *et al.*, 1988; Houba *et al.*, 1989), the pI values were calculated and found to agree well with the experimental ones (Table 1).

Three of the enzyme preparations were resolved into several catalytically active and distinct fractions by chromatofocusing on MonoP. As discussed below, with the *Actinomadura* R39 enzyme, another method had to be used, owing to its particularly low pI. With the *B. licheniformis* β-lactamase preparation, four active fractions were obtained, which were eluted respectively at pH values of 5.12, 5.0, 4.95 and 4.8 (Fig. 1*a*). Alternatively, distinct active fractions were also obtained with the same enzyme by chromatography on MonoS at pH 3.0 (results not shown), confirming the charge heterogeneity.

Table 1. pI values of the four β-lactamases studied

The measured pI values are those of the major component seen on the gel for each enzyme. The other pI values were calculated using the amino-acid-sequence data from Dehottay *et al.* (1987) (*S. albus* G), Lenzini *et al.* (1988) (*S. cacaoi*), Houba *et al.* (1989) (*Actinomadura* R39) and Ambler & Meadway (1969) (*B. licheniformis*). In each case, the *N*-terminal residue is indicated (using the one-letter notation).

β-Lactamase	pI value		First residue
	Measured	Calculated	
S. albus G	5±0.1	4.89	G-42 (ABL 22)
S. cacaoi	4.5±0.1	5.08	H-25 (ABL 23)
Actinomadura R39	< 4	3.8	A-32 (ABL 15)
B. licheniformis	5*	5.09	K-1 (ABL 26)

* From Meadway (1969).

The pure preparation of the *S. albus* G β-lactamase was resolved into three major peaks, which were eluted respectively at pH values of 4.64, 4.52 and 4.49 (Fig. 1*b*). Similar chromatofocusing experiments were performed with a crude preparation of the same enzyme. After reaching the maximum of enzyme production, a sample of culture medium, freed from the bacterial mycelium by centrifugation, was directly applied to the column. The measurement of enzymic activity in the collected fractions indicated the presence of several peaks, demonstrating that charge heterogeneity of the enzyme was already present in the culture medium and was thus not due to modifications occurring during the purification or upon long-term storage of the purified enzyme. The major peaks were present in both the crude and pure preparations, but the relative quantities were somewhat modified, probably reflecting the fact that fractions with lower specific activities were sometimes discarded during the purification. Variations in the culture conditions also altered the chromatographic patterns of the crude supernatant. This was probably due to modifications in processing of the exoenzyme, a phenomenon which is known to be very sensitive to the exact composition and pH of the growth medium (Mézes *et al.*, 1985).

With the *S. cacaoi* enzyme, chromatofocusing resolved the pure preparation into at least three active fractions, eluted at pH values of 4.35, 4.2 and 4.15 (Fig. 1*c*). With both *Streptomyces* enzymes, several active peaks were also separated by applying an NaCl gradient to the MonoQ column.

Chromatofocusing failed to separate distinct fractions of the *Actinomadura* R39 β-lactamase. Under standard conditions (pH gradient from 5.7 to 4), the enzyme was not eluted. Adjustment of the Polybuffer 74 to pH 3 yielded a regular pH gradient down to that pH, but failed to elute the enzyme. The ionic strength of the eluent was then increased in an attempt to decrease the electrostatic interactions between the enzyme and the ion-exchanger. At a constant pH of 4.5, the enzyme could be eluted from the MonoP column by adding 650 mM-NaCl to the Polybuffer. However, an NaCl concentration as low as 100 mM

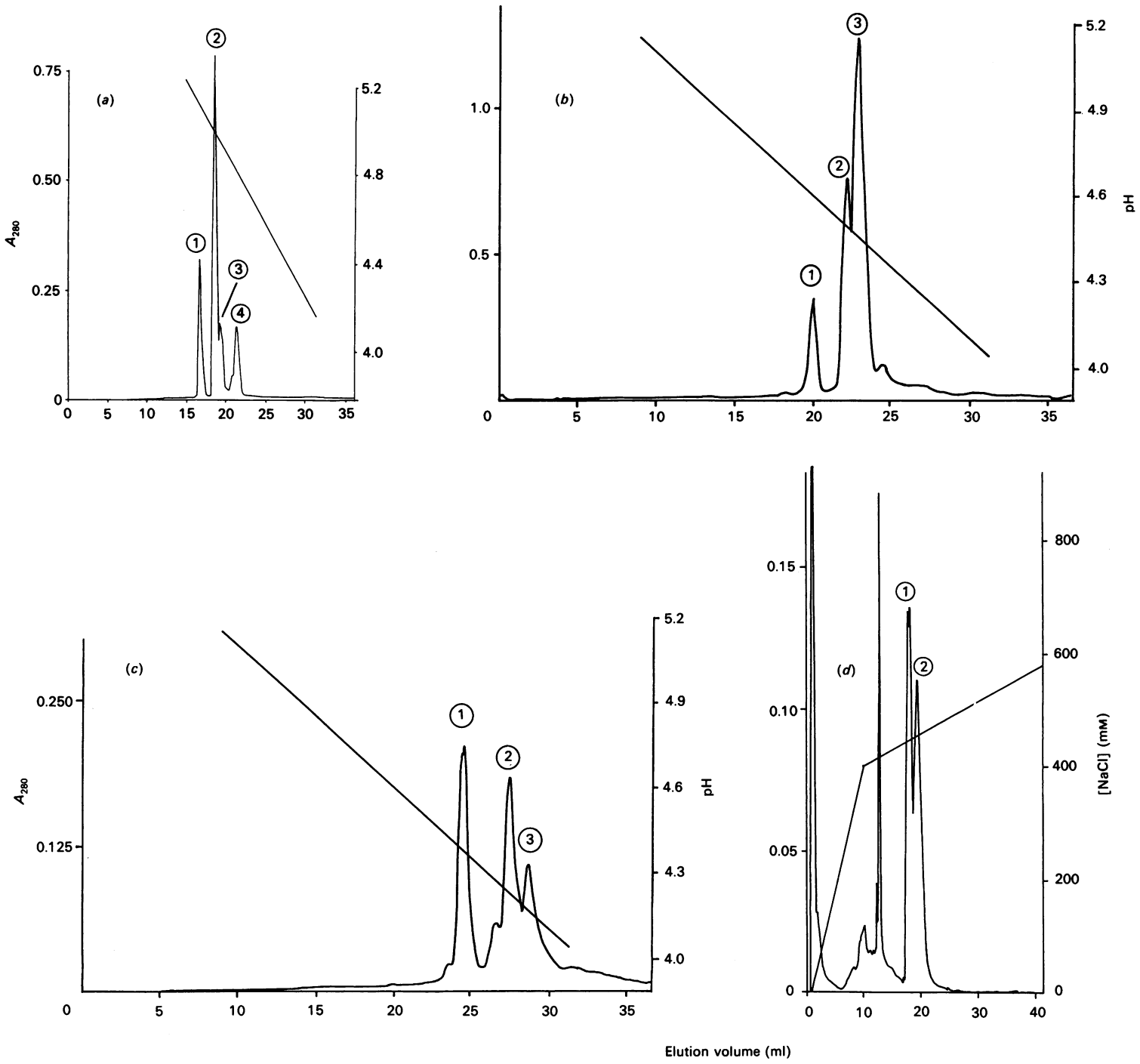

Fig. 1. Resolution of the various purified enzymes into distinct catalytically active fractions

(*a*), (*b*) and (*c*) Chromatofocusing on MonoP of the enzymes from *B. licheniformis* (0.7 mg, *a*); *S. albus* G (2 mg, *b*) and *S. cacaoi* (1 mg, *c*). The pH gradient is represented by the diagonal line. (*d*) Elution of the *Actinomadura* R39 enzyme (0.5 mg) from the MonoQ column by an NaCl gradient (the two diagonal lines). Ringed numbers refer to fractions described in the Tables.

grossly perturbed the formation of the pH gradient, making it impossible to utilize a combination of pH gradient and high salt concentration to elute the enzyme. Finally, an anion-exchanger (MonoQ HR5/5) was used at pH 7.2, and elution was performed with a shallow salt gradient. The preparation thus yielded two active fractions (Fig. 1*d*, peaks 1 and 2). The u.v.-absorbing material eluted before these two peaks consisted of inactive protein; in fact they were degradation products of the enzyme (see below). The high salt concentration needed to elute the enzyme from the MonoQ (500 mM-NaCl as against only 200 mM and 150 mM respectively for the *S. cacaoi* and the *S. albus* G β-lactamases) and its retention on MonoP at very low pH (pH 3) all agreed with the very low value of the pI of the *Actinomadura* R39 β-lactamase.

Structural analysis of the variants

The heterogeneity of the pure *S. albus* G β-lactamase had already been shown to be due to a ragged *N*-terminus (Dehottay

Table 2. *N*-terminal amino acid sequences of the various *B. licheniformis* 749/C β-lactamase species

Fraction numbering refers to Fig. 1(*a*). The vertical arrow at the bottom indicates the beginning of the α_1-helix (Moews *et al.*, 1990).

Fraction	Sequence	Calculated pI		Observed elution pH
1	+ 10 - 15 K T E M K D D F A K	5.09		5.12
	20 M K D D F A K L E E	5.08		
2	- + 10 - 15 E K T E M K D D F A	5.00	Asn-273	5.0
	- 20 Ē M K D D F A K L E	4.98		
	1 5 + - + S Q P A E K N E K T	5.01		
3	+ 10 - 15 K T E M K D D F A K L	4.99		4.95
	20 M K D D F A K L E E	4.98		
4	- + 10 - 15 E K T E M K D D F A	4.91	Asp-273	4.8
	- 20 Ē M K D D F A K L E	4.90		
	1 5 + - + S Q P A E K N E K T	4.93		
ABL numbering...	20 25 30 35			

Table 3. *N*-Terminal amino acid sequences of the various *Actinomadura* R39 β-lactamase species

Fraction numbering refers to Fig. 1(*d*). The arrows indicate the positions where the α_1-helix begins in the enzymes of *S. albus* G (A; Dideberg *et al.*, 1987) and *B. licheniformis* (B; Moews *et al.*, 1990).

Fraction	Sequence	Calculated pI
Shortest and longest forms observed in the initial preparation	35 A V D	3.84
	1 - - 5 - 10 - 15 20 25 30 A E A E P A S A E V (T A E D L S G E F E R L E S E F D A R L G V Y)	3.80
1	- - - Ē P A S A Ē V T A Ē D L S	3.81
	- - S A Ē V T A Ē D L S G E F	3.82
	- V T A Ē D L S G E F E R L	3.83
	- T A Ē D L S G E F E R L E	3.83
	10 15 20	
2	- V T A Ē D L S G E F E R L	3.83
	- T A Ē D L S G E F E R L E	3.83
	- A Ē D L S G E F E R L E	3.83
	D L S G E F E R L E	3.84
ABL numbering...	15 20 25 ↑ 30 ↑ 35 40 45 50 A B	

et al., 1987). In that case the enzyme had been produced by *Streptomyces lividans* PD6. Upon production by *S. albus* G, as in the present study, a similar pattern, with similar pI values, was observed. It was thus quite likely that the new preparation contained a family of variants similar to that observed before. Indeed, the loss of histidine-41 (ABL 21), observed by Dehottay *et al.* (1987) nicely explained the ΔpH (0.12) observed by us between peaks 1 and 2 (Fig. 1*b*). In consequence, the sequences of the *S. albus* G variants were not further analysed. Similarly, Lenzini *et al.* (1988) had already obtained various active species

of the *S. cacaoi* β-lactamase when produced by *S. lividans* ML1 and shown that the loss of histidine-25 (ABL 23) was one of the contributing factors. In the case of both *Streptomyces* enzymes, it thus appeared that ragged *N*-termini were obtained whether the host producing cells were *S. lividans* or *S. albus* G.

The determination of *N*-terminal sequences of the four active fractions obtained by chromatofocusing of the *B. licheniformis* enzyme (Fig. 1*a*) indicated that each fraction was still heterogeneous and that the mature β-lactamase consisted of at least five different molecular species (Table 2). This *N*-terminal heterogeneity could not completely account for the charge heterogeneity of the *B. licheniformis* β-lactamase. Indeed, fractions 1 and 3, which were distant by more than 0.1 pH unit, contained variants exhibiting the same *N*-terminal sequences (Table 2). The same observation was made for fractions 2 and 4. Moreover, the distance (in retention volume) between fractions 1 and 3 was the same as that between fractions 2 and 4. It was thus likely that the same factor was responsible for the separation of molecular species with the same *N*-termini into two distinct groups. The more acidic properties of the enzymes in fractions 3 and 4 could be explained by the presence of one additional negative charge, which might originate from the spontaneous deamidation of the antepenultimate asparagine residue, a position that was shown by Ambler (1979) to be particularly sensitive to that type of modification.

The effects of the asparagine → aspartic acid modification on the pI values of the various forms were computed (Table 2) and found to explain nicely the elution pattern from the MonoP column. Moreover, the pI values calculated for the ten molecular variants were in good agreement with their distribution into the different fractions separated by chromatofocusing.

With the *Actinomadura* R39 enzyme, the situation was somewhat different. Upon completion of the purification, two major *N*-terminal residues were found, alanine-32 (ABL 15) and alanine-65 (ABL 48), accompanied by three minor forms: valine-41, phenylalanine-57 and aspartic acid-58. At that stage, chromatography of the preparation on the MonoQ column yielded no indication of the presence of inactive protein peaks. The peak of active enzyme was not symmetrical, which suggested a possible heterogeneity. That all the molecular species present in the preparation represented active enzyme was, however, demonstrated by the homogeneous behaviour on the phenylboronate–agarose affinity column (Piron-Fraipont *et al.*, 1989).

The enzyme was stored at 4 °C in 50 mM-sodium phosphate, pH 7.0, or 100 mM-Tris/HCl, pH 7.2, and a slow decrease of activity was observed. After 4 months only 53% of the original activity was retained. When the partially inactivated enzyme was analysed on MonoQ (Fig. 1*d*), several u.v.-absorbing peaks appeared which were not present when the same experiment had been performed just after completion of the purification. The specific activity of these new peaks was less than 0.5% of that of the newly purified enzyme, whereas the two peaks numbered 1 and 2 on Fig. 1(*d*) also exhibited the same maximum specific activity. The protein content of the inactive fractions, determined on the basis of the A_{280} value, corresponded to 46% of the total, a value agreeing well with the proportion of activity lost upon storage.

Sequencing of the active proteins corresponding to peaks 1 and 2 (Table 3) yielded at least six different *N*-terminal residues: glutamic acid-36 (ABL 19), serine-38, valine-41, threonine-42, alanine-43 and aspartic acid-45. As shown by the calculated values in Table 3, the loss of one negative charge had a very limited effect on the pI value, probably due to the very acidic properties of the protein.

As Table 3 shows, the pattern of *N*-terminal residues had changed during the 4 months after the purification. In the case of the *Actinomadura* R39 β-lactamase, the heterogeneity of the preparation was, at least partially, due to the presence of a proteinase which modified the enzyme upon storage. One of the remarkable features was the disappearance, after 4 months, of

Table 4. *B. licheniformis* β-lactamase: kinetic parameters of the starting mixture and of the various fractions

	Fraction 1				Fraction 2				Fraction 4				Mixture		
Substrate	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	Relative $k_{cat.}/K_m$ (%)*	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	Relative $k_{cat.}/K_m$ (%)*	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	Relative $k_{cat.}/K_m$ (%)*	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)
Benzylpenicillin	72	1510	21100	57	74	2470	32900	88	75	2890	37200	100	71	2710	38200
Ampicillin	93	1130	12200	56	91	1850	20300	93	94	2060	21800	100	86	1810	21300
Nitrocefin	50	690	13600	76	49	880	17800	100	48	760	15900	89	42	930	22200
Cephaloridine	89	510	5700	79	92	590	6400	89	89	640	7200	100	89	680	7600

* For each substrate, the maximum value of $k_{cat.}/K_m$ is arbitrarily set as 100%; the values were determined with an S.D. of ±10%.

Table 5. *S. albus* β-lactamase: kinetic parameters of the starting mixture and of the various fractions

	Fraction 1				Fraction 2				Fraction 3				Mixture		
Substrate	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	Relative $k_{cat.}/K_m$ (%)*	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	Relative $k_{cat.}/K_m$ (%)*	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	Relative $k_{cat.}/K_m$ (%)*	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)
Benzylpenicillin	1100	860	760	33	1300	3000	2320	100	1300	2800	2150	93	1000	2800	2800
Ampicillin	510	980	2020	36	610	3100	5130	91	560	3100	5610	100	650	3900	6100
Penicillin V	890	880	1030	36	1100	3100	2890	100	1100	3100	2840	98	1000	2800	2900
Cephaloridine	400	50	121	26	320	150	464	100	330	150	458	99	320	200	620
Cephalothin	830	70	80	25	800	250	310	97	820	260	320	100	720	260	370

* For each substrate, the maximum value of $k_{cat.}/K_m$ is arbitrarily set as 100%; the values were determined with an S.D. of ±10%.

Table 6. *S. cacaoi* β-lactamase: kinetic parameters of the starting mixture and of the various fractions

Substrate	Fraction 1 K_m (μM)	Fraction 1 $k_{cat.}$ (s^{-1})	Fraction 1 $10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	Fraction 1 Relative $k_{cat.}/K_m$ (%)*	Fraction 2 K_m (μM)	Fraction 2 $k_{cat.}$ (s^{-1})	Fraction 2 $10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	Fraction 2 Relative $k_{cat.}/K_m$ (%)*	Fraction 3 K_m (μM)	Fraction 3 $k_{cat.}$ (s^{-1})	Fraction 3 $10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	Fraction 3 Relative $k_{cat.}/K_m$ (%)*	Mixture K_m (μM)	Mixture $k_{cat.}$ (s^{-1})	Mixture $10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)
Benzylpenicillin	132	1150	8400	78	141	1530	10800	100	137	1270	9300	86	96	1050	11000
Ampicillin	56	310	5500	79	53	380	7000	100	52	280	5400	77	52	310	5700
Penicillin V	79	840	10800	78	85	1160	13900	100	79	760	9600	69	50	770	15000
Nitrocefin	–	–	775	74	–	–	1040	100	–	–	755	72	1300	1050	800
Cephaloridine	1080	220	205	69	1110	330	295	100	1160	230	195	66	1050	260	250

* For each substrate, the maximum value of $k_{cat.}/K_m$ is arbitrarily set as 100%; the values were determined with an S.D. of ±10%.

Table 7. *Actinomadura* R39 β-lactamase: kinetic parameters of the starting mixture and of the various fractions

Substrate	Fraction 1 K_m (μM)	Fraction 1 $k_{cat.}$ (s^{-1})	Fraction 1 $10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	Fraction 1 Relative $k_{cat.}/K_m$ (%)*	Fraction 2 K_m (μM)	Fraction 2 $k_{cat.}$ (s^{-1})	Fraction 2 $10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	Fraction 2 Relative $k_{cat.}/K_m$ (%)*	Mixture K_m (μM)	Mixture $k_{cat.}$ (s^{-1})	Mixture $10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)
Ampicillin	111	1660	15000	96	91	1420	15700	100	120	1900	16000
Nitrocefin	100	650	6500	100	112	690	6300	97	70	600	8500
Cephaloridine	34	430	12500	97	32	420	12900	100	38	440	11600

* For each substrate, the maximum value of $k_{cat.}/K_m$ is arbitrarily set as 100%; the values were determined with an S.D. of ±10%.

Table 8. Thermal inactivation of the β-lactamases from *B. licheniformis* (*a*) and *S. albus* G (*b*): comparison of the fractions

See Figs. 1(*a*) and 1(*b*). k_a^{66} and k_a^{45} are the first-order inactivation rate constants at 66 °C and 45 °C respectively.

(*a*) *B. licheniformis*

Parameter	Heterogeneous preparation	Fraction 1	Fraction 2	Fraction 4
$10^3 \times k_a^{66}$ (s^{-1})	17.3	17.8	19.4	18.2
$t_{\frac{1}{2}}$ (s)	40	39	36	38

(*b*) *S. albus* G

Parameter	Heterogeneous preparation	Fraction 1	Fraction 2	Fraction 3
$10^3 \times k_a^{45}$ (s^{-1})	0.21	0.195	0.225	0.215
$t_{\frac{1}{2}}$ (min)	55	59	51	54

the shorter forms of the enzyme (phenylalanine-57, aspartic acid-58 and alanine-65), suggesting that those forms were probably more susceptible to digestion by the endogenous proteinase.

Kinetic properties and thermal stability

The kinetic properties and thermal stability of the individual fractions were compared with those of the original mixtures. The results are summarized in Tables 4–8.

With the *B. licheniformis* enzyme, the K_m values were very similar for each fraction. Catalytic-centre activities were identical for peaks 2 and 4, but were significantly lower for peak 1. Table 8 also shows that the thermostabilities of the various fractions did not differ from that of the mixture. It was thus difficult to decide whether the lower specific activity of peak 1 was an intrinsic characteristic of that molecular variant or if it could be due to a partial inactivation during the chromatofocusing experiment. Nevertheless, Table 4 also shows that the smaller activity of peak 1 did not significantly influence the $k_{cat.}$ of the mixture.

A similar situation prevailed for the *S. albus* G enzymes. The minor fraction 1 exhibited decreased $k_{cat.}$ values, but a similar thermostability. Again, the properties of that minor fraction did not appear to have a major impact on those of the mixture (Tables 5 and 8).

With the *S. cacaoi* and *Actinomadura* R39 enzymes, the properties of the different fractions were not significantly different from those of the mixture (Tables 6 and 7).

Interaction with β-iodopenicillanate

6-β-Iodopenicillanate is a mechanism-based inactivator of class A, class C and class D β-lactamases; it inactivates stoichiometrically the β-lactamase of *B. licheniformis* (De Meester *et al.*, 1986). The inactivation is characterized by the rearrangement of the penicilloyl moiety of the acyl-enzyme into a dihydrothiazine chromophore.

After partial (about 50%) inactivation of the *B. licheniformis* β-lactamase by 6-β-iodopenicillanate, the enzyme was submitted to chromatofocusing (Fig. 2). The attachment of the inactivator to the active site of the enzyme added one negative charge to the protein. This new negative charge displaced the whole chromatographic profile towards the lower pH values. Fig. 2 shows that each peak was split into an active fraction (high pH) and an inactive fraction (low pH). This experiment confirms the form-

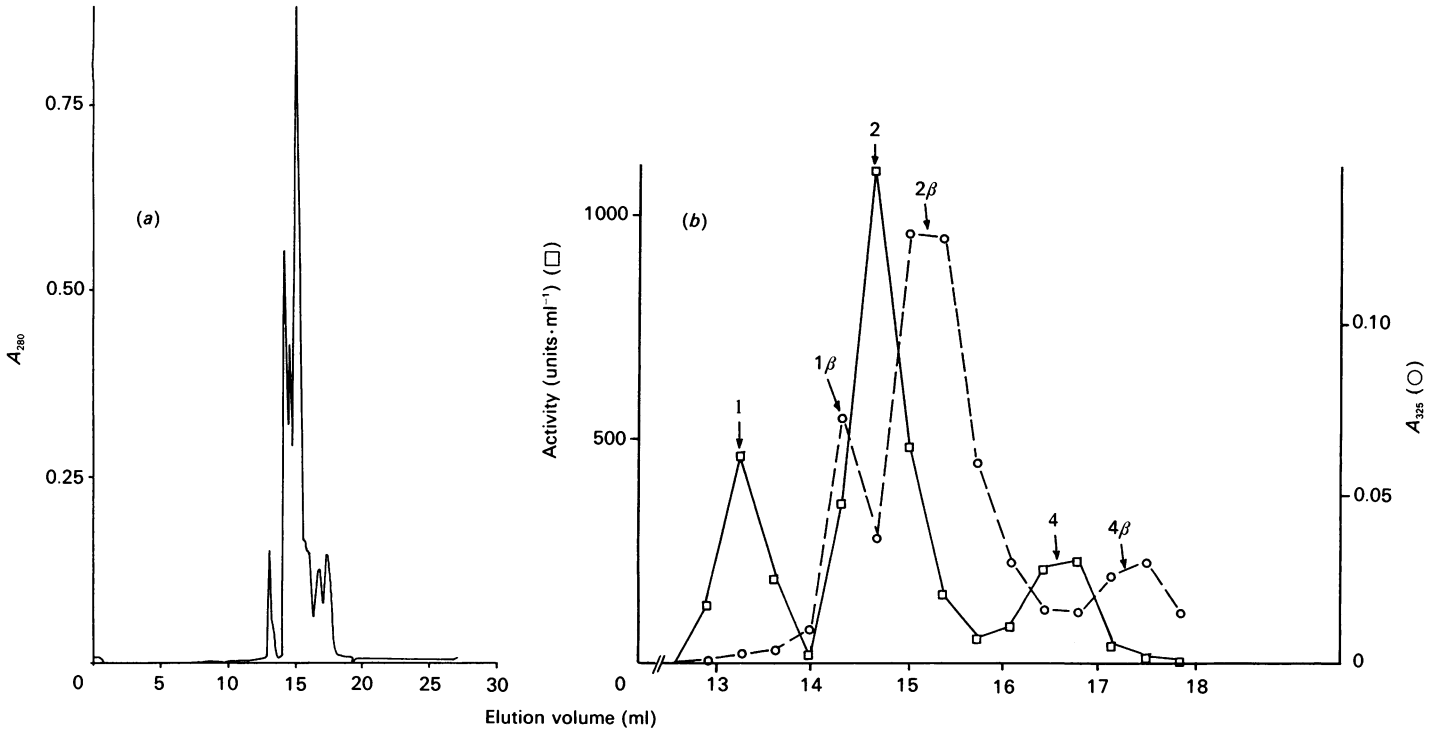

Fig. 2. Chromatofocusing on MonoP of the *B. licheniformis* β-lactamase after partial inactivation by β-iodopenicillanate

Pure enzyme (0.7 mg, 23 nmol) was allowed to react with 10 nmol of β-iodopenicillanate in a total volume of 700 μl of buffer A for 10 min at 30 °C. The sample was then injected into the column. (*a*) Protein profile. (*b*) Activity and labelling profiles; 350 μl fractions were collected and analysed as follows. A_{325} allows one to detect the inactivated enzyme, whereas catalytic activity, measured with a 'good' substrate (nitrocefin), reveals the intact enzyme (active enzyme). The arrows show the relationship between the intact and inactivated forms of variants.

ation of the irreversibly inactivated dihydrothiazine-containing rearrangement product. It especially underlines the sensitivity of the method used, as it clearly appears that the appearance of one single new charge, even in the active site of the protein, could be easily detected by chromatofocusing.

DISCUSSION

Chromatofocusing was found to be a method of choice for the separation of protein variants originating from post-transcriptional modifications. The technique only failed in the case of the *Actinomadura* R39 β-lactamase, which exhibits an exceptionally low pI. Separation of the molecular variants of that enzyme was only possible by simple ion-exchange chromatography with the help of a very shallow gradient. Even under those conditions, the resolution remained poor. For the proteins with pI values of about 5, the addition of one negatively charged or the removal of one positively charged group decreased the pI by at least 0.1 unit, allowing a rather easy separation. By contrast, with the R39 enzyme (calculated pI 3.8), the loss of three glutamic acid residues only modified the calculated pI value (3.8) by 0.03 unit. That value could not be experimentally confirmed. In other studies (A. Matagne, unpublished work), the technique was also found very useful to purify small quantities of modified enzymes obtained by site-directed mutagenesis. Indeed, recording similar specific activities in different active peaks supplied good indication of purity.

The kinetic parameters of the variants were generally not significantly different from each other. The only exception was fraction 1 of the *S. albus* G enzyme, which exhibited only 30% of the activity of the other fractions. However, since the K_m values were similar for all variants, it was quite possible that the lower $k_{cat.}$ value was due to partial inactivation of that variant during the separation process. Another observation in favour of that hypothesis was that, whenever a lower activity was measured, it was similarly decreased for all tested substrates. The most important observation was that the kinetic parameters determined on the mixture were not significantly different from those of the major individual species, indicating that the conformation of all the molecules was probably the same. This was also confirmed by the similar thermostabilities. For the *B. licheniformis* and *S. albus* G enzymes, it is also important to stress the fact that good crystals, allowing the collection of high-resolution data, have been obtained from the mixtures (Dideberg *et al.*, 1987; Moews *et al.*, 1990), clearly indicating that all the variants presented identical conformations. Interestingly, however, the crystallographic studies have shown that the first *N*-terminal residues of those proteins have no rigid structure but seem to be freely floating in the solvent. The first well-defined residues are aspartic acid-31 (ABL) in the *B. licheniformis* and serine-27 (ABL) in the *S. albus* G enzymes. In both cases, those residues are at the beginning of the α_1-helix, and the shortest forms which have been found start at methionine-29 for the former and serine-27 for the latter. It thus seems that the residues preceding the *N*-terminus of the α_1-helix are expendable. In agreement with those results, the major form of the *S. cacaoi* enzyme starts at histidine-23 (ABL) and the shortest form found after storing the *Actinomadura* R39 β-lactamase for 4 months starts at aspartic acid-28 (ABL). In both cases, the α_1-helix is certainly not drastically shortened. However, sequencing of the fresh *Actinomadura* R39 β-lactamase preparation indicated an important proportion of more markedly shortened forms, starting mainly at alanine-48 (ABL) and, in much smaller proportions, phenylalanine-40 and aspartic acid-41. In all these variants, the

α_1-helix would be absent and, in the first one, a portion of the β-1 strand would also be deleted. This could result in a much decreased stability of these proteins, making them more susceptible to the proteinase(s) seemingly present in the *Actinomadura* R39 β-lactamase preparation. It should be remembered that the initial preparation lost about 45% activity after 4 months of storage, but that the rate of activity decrease became much lower thereafter.

A few indications have been obtained on the phenomena which are responsible for the appearance of those variants. As stated above for the *Actinomadura* R39 β-lactamase, the presence of a proteinase in the final preparation is extremely likely, since different *N*-terminal residues are found before and after long-term storage of the solutions (those solutions cannot be frozen without important losses of enzyme activity). The activity of proteinases can also probably explain the results of Brive *et al.* (1977), who observed the appearance of new satellite bands correlated to a significant loss of enzyme activity during conservation of Gram-negative cells at 37 °C. With the *B. licheniformis* enzyme, deamidation of the antepenultimate asparagine residue, which had been observed previously (Ambler, 1979), nicely explains the separation of molecules exhibiting the same *N*-termini into fractions differing by more than 0.1 pH unit after chromatofocusing. But the numerous variations observed in the *N*-terminal part of the enzymes must be explained by a lack of specificity of the signal peptidase(s) or by the presence of one or more proteinases in the culture supernatant. Erpicum *et al.* (1990) have reported low, but significant, proteinase activities in the culture supernatants of *Streptomyces lividans* TK24 and *S. albus* G. On the other hand, many of the hydrolysis sites do not appear to reflect the usual 'consensus' sequences characteristic of signal peptidases. We believe that one of the important conclusions of the present work is that the kinetic properties of the mixtures are not significantly different from those of the isolated forms. With the R39 enzyme, where more extensive degradation occurred, the enzymic activity seemed to be either totally conserved or completely lost.

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REFERENCES

Ambler, R. P. (1979) in β-Lactamases (Hamilton-Miller, J. M. T. & Smith, J. T., eds.), pp. 99–125, Academic Press, New York and London
Ambler, R. P. & Meadway, R. J. (1969) Nature (London) **222**, 24–26
Brive, C., Barthelemy, M., Bouanchaud, D. H. & Labia, R. (1977) Ann. Microbiol. (Paris) **128B**, 309–317
Dehottay, P., Dusart, J., De Meester, F., Joris, B., Van Beeumen, J., Erpicum, T., Frère, J. M. & Ghuysen, J. M. (1987) Eur. J. Biochem. **166**, 345–350
De Meester, F., Joris, B., Reckinger, G., Bellefroid-Bourguignon, C., Frère, J. M. & Waley, S. G. (1987) Biochem. Pharmacol. **36**, 2393–2403
De Meester, F., Frère, J. M., Waley, S. G., Cartwright, S. J., Virden, R. & Lindberg, F. (1986) Biochem. J. **239**, 575–580
Devereux, J., Haeberli, P. & Smithies, O. (1984) Nucleic Acids Res. **12**, 387–395
Dideberg, O., Charlier, P., Wery, J. P., Dehottay, P., Dusart, J., Erpicum, T., Frère, J. M. & Ghuysen, J. M. (1987) Biochem. J. **245**, 911–913
Erpicum, T., Granier, B., Delcour, M., Lenzini, M. V., Ngugen-Disteche, M., Dusart, J. & Frère, J. M. (1990) Biotechnol. Bioeng. **35**, 719–726
Houba, S., Willem, S., Duez, C., Molitor, C., Dusart, J., Frère, J. M. & Ghuysen, J. M. (1989) FEMS. Microbiol. Lett. **65**, 241–246
Izui, K., Nielsen, J. B. K., Caulfield, M. P. & Lampen, J. O. (1980) Biochemistry **19**, 1882–1886
Joris, B., De Meester, F., Galleni, M., Reckinger, G., Coyette, J., Frère, J. M. & Van Beeumen, J. (1985) Biochem. J. **228**, 241–248
Lenzini, M. V., Nojima, S., Dusart, J., Ogawara, H., Dehottay, P., Frère, J. M. & Ghuysen, J. M. (1987) J. Gen. Microbiol. **133**, 2915–2920
Lenzini, M. V., Ishihara, H., Dusart, J., Ogawara, H., Joris, B., Van Beeumen, J., Frère, J. M. & Ghuysen, J. M. (1988) FEMS Microbiol. Lett. **49**, 371–376
Matagne, A., Misselyn-Bauduin, A. M., Joris, B., Erpicum, T., Granier, B. & Frère, J. M. (1990) Biochem. J. **265**, 131–146
Meadway, R. J. (1969) Ph.D. Thesis, University of Edinburgh
Mèzes, P. S. F., Blacher, R. W. & Lampen, J. O. (1985) J. Biol. Chem. **260**, 1218–1223
Moews, P. C., Knox, J. R., Dideberg, O., Charlier, P. & Frère, J. M. (1990) Protein Struct. Funct. Genet. **7**, 156–171
Piron-Fraipont, C., Duez, C., Matagne, A., Molitor, C., Dusart, J., Frère, J. M. & Ghuysen, J. M. (1989) Biochem. J. **262**, 849–854
Simons, K., Sarvas, M., Garoff, H. & Helenius, A. (1978) J. Mol. Biol. **126**, 673–690
Thatcher, D. R. (1975*a*) Biochem. J. **147**, 313–326
Thatcher, D. R. (1975*b*) Methods Enzymol. **43**, 653–664

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