Biochem. J. (1988) 251, 73–79 (Printed in Great Britain)

Complete amino acid sequence of p453-plasmid-mediated PIT-2 β-lactamase (SHV-1)

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The complete amino acid sequence of the p453-plasmid-mediated PIT-2 β-lactamase (SHV-1) was determined. The protein contains 265 residues. Peptides resulting from digestions with trypsin, *Staphylococcus aureus* V8 proteinase, chymotrypsin and Lys-C proteinase and cleavage with CNBr were separated and purified by using reverse-phase h.p.l.c. The amino acid sequence of each peptide was manually determined with the dimethylaminoazobenzene isothiocyanate/phenyl isothiocyanate double-coupling method. The primary structure of PIT-2 β-lactamase was compared with those of two closely related enzymes, namely TEM-1 β-lactamase and the β-lactamase of *Klebsiella pneumoniae* strain LEN-1. The PIT-2 β-lactamase amino acid sequence was strongly retained, with respectively 68% and 88% homology. Thus PIT-2 enzyme could represent an evolutionary step between a chromosomally encoded β-lactamase and the plasmid-mediated TEM β-lactamases.

INTRODUCTION

The β-lactamases, which include a large number of chromosomally and plasmid-mediated enzymes, play a major role in bacterial resistance to penicillins and cephalosporins. On the basis of their biochemical properties and primary structures, β-lactamases have been divided into three evolutionary distinct classes (Ambler, 1980; Jaurin & Grundström, 1981; Bergström *et al.*, 1982). TEM-type enzymes, the most widespread β-lactamases (Matthew, 1979), belong to class A and are plasmid-encoded. Plasmid-determined β-lactamases hold an important place in bacterial resistance since they are widely distributed throughout the world in bacterial species (Matthew, 1979; Medeiros, 1984). More than 20 such well-characterized enzymes have been reported (Medeiros *et al.*, 1985). Among them, a penicillinase encoded by a transposon (Nugent & Hedges, 1979), frequently encountered in *Klebsiella pneumoniae* where it was first detected by Pitton (1972), was named PIT-2 by O'Callaghan *et al.* (1978) and further designated SHV-1 by Matthew *et al.* (1979). On the basis of determination of kinetic constants and amino acid composition analysis, PIT-2 enzyme appeared to be closely related to TEM-type penicillinases (Barthélémy *et al.*, 1986), although no evolutionary relationship was found between them by DNA hybridization analysis (Levesque *et al.*, 1987). An important feature of PIT-2 β-lactamase was described by Kliebe *et al.* (1985), who isolated laboratory mutants that showed high broad-spectrum hydrolytic activity towards cephalosporins. Moreover, a clinical isolate of cefotaxime-resistant *Klebsiella ozaenae* was also found to produce a transferable β-lactamase (called SHV-2) that could be a natural mutant of PIT-2.

This set of properties of PIT-2 β-lactamase made it an attractive candidate for structural analysis. We have previously published the determination of the first 107 residues of the *N*-terminal sequence (Barthélémy *et al.*, 1987). In the present paper the complete amino acid sequence of PIT-2 β-lactamase is described and compared with those of other related enzymes.

EXPERIMENTAL

Purification of the PIT-2 β-lactamase

The PIT-2 β-lactamase was purified from a hyperproducing variant of an *Escherichia coli* K12 strain carrying plasmid p453, as previously described (Barthélémy *et al.*, 1986). The protein was found to be homogeneous by SDS/polyacrylamide-gel electrophoresis and analytical isoelectric focusing.

Carboxymethylation

The β-lactamase, dissolved in 6 M-guanidinium chloride/2 mM-EDTA/0.1 M-Tris/HCl buffer, pH 8.3, was reduced with a 3-fold molar excess of dithiothreitol for 1 h at room temperature under N_2. A 6-fold molar excess of iodoacetic acid over dithiothreitol was added and the solution was left under N_2 for 1 h in the dark at room temperature. After the addition of 1% (v/v) of 2-mercaptoethanol the *S*-carboxymethylated β-lactamase was extensively dialysed against 50 mM-NH_4HCO_3/0.01% (v/v) thiodiglycol and then freeze-dried.

H.p.l.c. purification of proteinase-digest peptides and CNBr-cleavage fragments

All the peptides were purified by h.p.l.c. with an LKB 2152 controller, two LKB 2150 pumps, an LKB 2238 detector and a Rheodyne injector. Peptides separated on a Waters C_{18} μBondapak column (10 μm particle size, 4 mm × 300 mm) were detected at 226 nm and collected manually. The flow rate was 1 ml/min at room temperature. Peptide elutions were performed with 0.1% (v/v) trifluoroacetic acid in water (solvent A) and acetonitrile (solvent B). A three-step linear gradient from solvent A to solvent B was used. In some instances peptides were further chromatographed with a linear gradient from 0.25 mM-ammonium acetate buffer, pH 6.0, to 0.5 mM-ammonium acetate buffer (pH 6.0)/acetonitrile (2:3, v/v). Finally all peptides were rechromatographed with isocratic elution with 0.1% (v/v) trifluoroacetic acid in acetonitrile.

Tryptic and chymotryptic cleavages

The digestion of the *S*-carboxymethylated protein (90 nmol) by trypsin (Sigma Chemical Co.) and of the long staphylococcal-proteinase-digest peptide V13 (10 nmol) by chymotrypsin (Sigma Chemical Co.) were performed for 4 h and 2 h respectively at 37 °C in 0.25 M-NH_4HCO_3 at an enzyme/substrate ratio of 1:50 (w/w).

Staphylococcal-proteinase digestions

The digestion of the *S*-carboxymethylated β-lactamase (90 nmol) and tryptic peptide T11 (25 nmol) by *Staphylococcus aureus* V8 proteinase (Sigma Chemical Co.) were performed for 8 h at 37 °C in 0.25 M-NH_4HCO_3 at an enzyme/substrate ratio of 1:25 (w/w). A second equivalent portion of proteinase was added to the peptide T11 solution, and the reactions were allowed to proceed for an additional 12 h.

Lysine-specific proteolytic cleavage

The long staphylococcal-proteinase-digest peptide V15 (20 nmol) was dissolved in 8 M-urea and then digested in 4 M-urea/0.25 M-ammonium bicarbonate buffer, pH 9, by endoproteinase Lys-C from *Lysobacter enzymogenes* (Boehringer Mannheim) at 37 °C for 18 h at an enzyme/substrate ratio of 1:100 (w/w).

CNBr cleavage

S-Carboxymethylated protein (75 nmol) dissolved in 500 μl of 70% (v/v) formic acid was digested with CNBr (7.5 mg) for 20 h at 37 °C. In order to convert *C*-terminal residues into a single form (homoserine lactone) the peptide mixture was treated before h.p.l.c. analysis with anhydrous trifluoroacetic acid for 1 h at 20 °C (Ambler & Brown, 1967).

Carboxypeptidase digestions of β-lactamase and tryptic peptide T26

S-Carboxymethylated β-lactamase (10 nmol) or tryptic peptide T26 (15 nmol) in 1 ml of 0.2 M-*N*-ethylmorpholine/acetate buffer, pH 8.5, containing 20 nmol of norleucine as an internal standard, was digested at 25 °C with a mixture of carboxypeptidases A and B (Boehringer Mannheim). At specified times (30 min to 4 h) samples (200 μl) were withdrawn and subjected to amino acid analysis.

Amino acid analysis

Peptides (1–2 nmol) were hydrolysed at 110 °C under vacuum in the presence of 200 μl of 6 M-HCl for 23 h. For large peptides amino acid analysis was performed for 48 h and 72 h. Hydrolysates of peptides from CNBr cleavage were treated with aq. 10% (v/v) pyridine/acetic acid buffer, pH 6.5, at 105 °C for 1 h, to allow conversion of homoserine lactone into homoserine (Ambler, 1965). Amino acids were separated by using a Kontron Liquimat 2 amino acid analyser incorporating a Waters 420 fluorimeter and detected after the formation of derivatives with *o*-phthalaldehyde.

Amino acid sequence determinations

Sequential degradation of 2–10 nmol of peptides was performed according to Chang's manual diaminoazobenzene isothiocyanate/phenyl isothiocyanate double-coupling method, essentially as described by Allen (1981), with slight modifications. After the coupling step the excess reagents were removed by three succesive extractions with 600 μl of heptane/ethyl acetate (3:1, v/v). The cleavage step was performed with anhydrous trifluoroacetic acid at 58 °C under N_2 for 5 min and for a further 5 min if proline was the expected *N*-terminal residue.

Peptide nomenclature

Peptides were numbered in the order in which they occur in the sequence starting from the *N*-terminus. Tryptic peptides are designated by a T, *S. aureus* V8 proteinase peptides with a V, Lys-C proteinase peptides with a K, chymotryptic peptides with a C and CNBr-cleavage peptides with CN. Peptides obtained from subdigestions are designed by indicating first the parent peptide followed by a second letter indicating the cleavage method and numbered according to their position in the sequence of the parent peptide.

RESULTS

The *N*-terminal analysis of the purified protein revealed only one amino acid derivative at each position among the first six residues. Following analyses were perturbed by non-specific cleavages of peptide bonds, which occurred during the trifluoroacetic acid treatment. A limitation of the reaction time for the cleavage step allowed identification of the first 15 *N*-terminal amino acid residues (Barthélémy *et al.*, 1987). The whole sequence of PIT-2 β-lactamase was established by

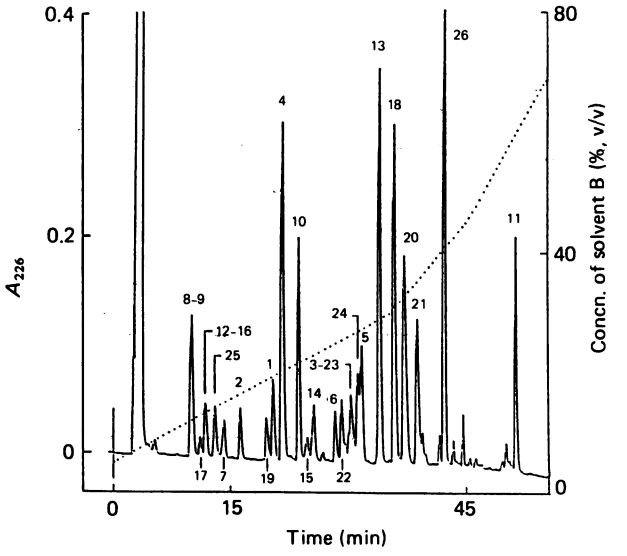


Fig. 1. Separation of tryptic-digest peptides from PIT-2 β-lactamase by h.p.l.c.

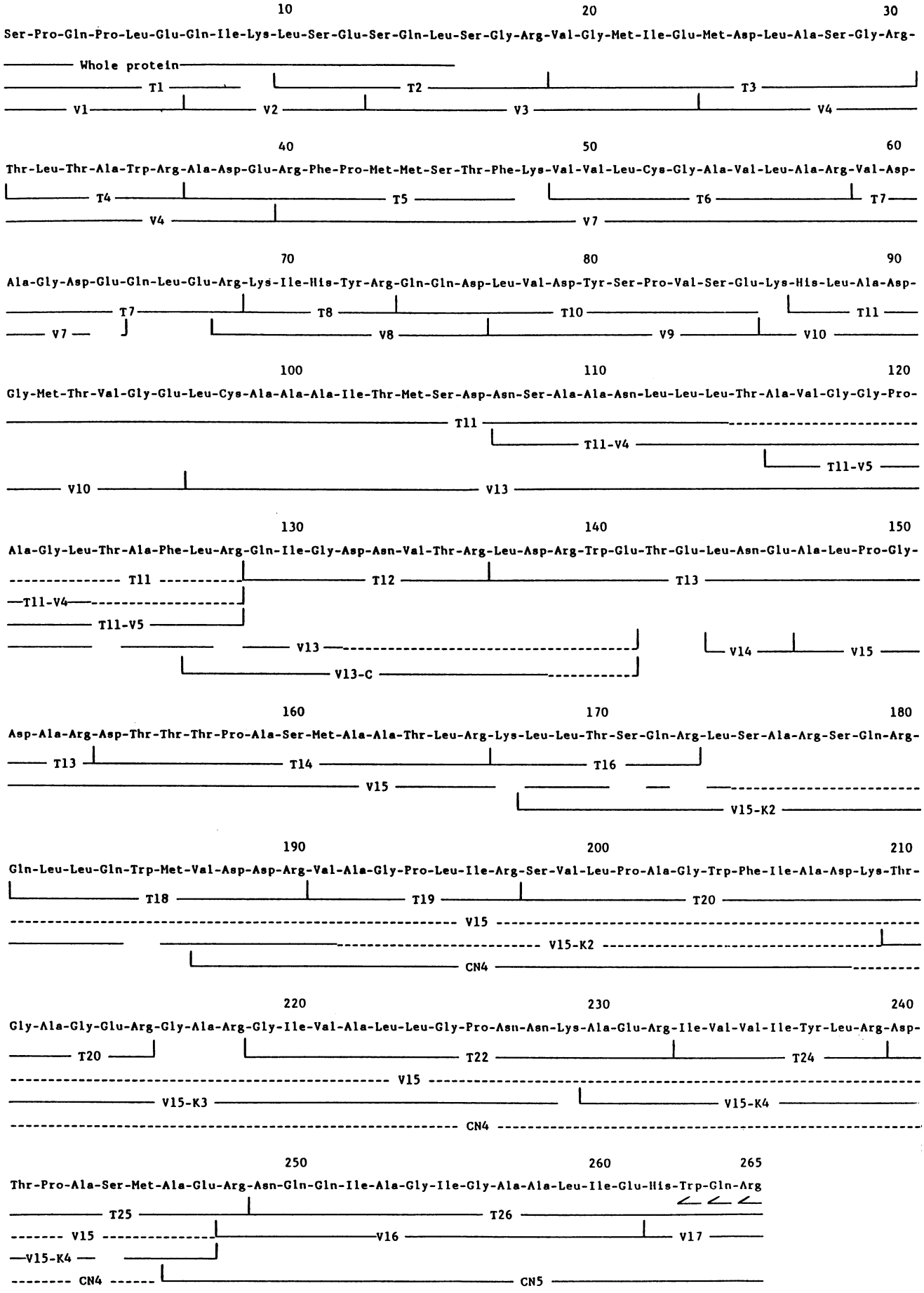
The tryptic digest of PIT-2 enzyme (90 nmol) adjusted to pH 2 with trifluoroacetic acid was filtered through a 0.45 μm-pore-size filter, then concentrated to 400 μl. Portions (100 μl) were applied to a C_{18} μBondapak column and eluted as described in the text. ——, A_{226}; · · · ·, concn. of solvent B. The numbering of the peaks refers to the numbering of the corresponding peptides described under 'Peptide nomenclature' in the Experimental section. Those peaks that are indicated by two numbers were rechromatographed at pH 6.

Table 1. Amino acid compositions of PIT-2 β-lactamase and of peptides from its tryptic digest

The results are expressed as the number of residues per molecule of peptide, without correction for destruction or incomplete hydrolysis. Values less than 0.2 are omitted. Cysteine was determined as the *S*-carboxymethyl derivative, and proline was not detected. Values in parentheses are obtained from the sequence. Amino acid composition data of purified PIT-2 β-lactamase are taken from Barthélémy *et al.* (1986). Abbreviations: Cm, carboxymethyl; N.D., not determined.

		Amino acid composition (residues/molecule)												
Amino acid	Peptide ... Residues ...	T1 1–9	T2 10–18	T3 19–30	T4 31–36	T5 37–48	T6 49–58	T7 59–68	T8 69–73	T9 70–73	T10 74–86	T11 87–128	T12 129–136	T13 137–153
Asx				1.0 (1)		1.0 (1)		2.0 (2)			2.0 (2)	4.0 (4)	2.1 (2)	2.9 (3)
Thr					1.9 (2)	1.0 (1)						3.6 (4)	1.0 (1)	1.0 (1)
Ser		0.9 (1)	2.7 (3)	0.9 (1)		0.9 (1)					1.8 (2)	2.1 (2)		
Glx		3.1 (3)	2.0 (2)	1.0 (1)		1.0 (1)		3.0 (3)			3.0 (3)	1.1 (1)	1.1 (1)	3.0 (3)
Pro		N.D. (2)	N.D.	N.D.	N.D.	N.D. (1)	N.D.	N.D.	N.D.	N.D.	N.D. (1)	N.D. (1)	N.D.	N.D. (1)
Gly			1.0 (1)	1.9 (2)			1.1 (1)	1.0 (1)				4.8 (5)	1.0 (1)	1.1 (1)
Ala				1.0 (1)	1.0 (1)	1.0 (1)	2.0 (2)	1.0 (1)				8.5 (9)		1.9 (2)
Cm-Cys							0.7 (1)					0.8 (1)		
Val				1.0 (1)			2.7 (3)	1.0 (1)			1.9 (2)	2.0 (2)	1.0 (1)	
Met				1.5 (2)		1.4 (2)						1.6 (2)		
Ile		0.9 (1)		0.9 (1)					1.0 (1)	1.0 (1)		1.0 (1)	1.0 (1)	
Leu		1.0 (1)	1.9 (2)	1.0 (1)	1.0 (1)		2.0 (2)	0.9 (1)			0.9 (1)	6.7 (7)		2.9 (3)
Tyr									0.9 (1)	1.0 (1)	0.9 (1)			
Phe						1.9 (2)						1.1 (1)		
His									1.0 (1)	1.0 (1)		1.0 (1)		
Lys		1.0 (1)				1.0 (1)			1.0 (1)		1.0 (1)			
Arg			1.0 (1)	1.0 (1)	1.0 (1)	0.9 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.1 (1)		1.1 (1)	0.9 (1)	1.9 (2)
Trp		N.D.	N.D.	N.D.	N.D. (1)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D. (1)
Yield (%)		38	46	43	34	41	32	48	30	20	54	55	29	35

		Amino acid composition (residues/molecule)													
Amino acid	Peptide ... Residues ...	T14 154–166	T15 154–167	T16 167–173	T17 168–173	T18 181–190	T19 191–197	T20 198–215	T21 198–209	T22 219–232	T23 219–229	T24 233–239	T25 240–248	T26 249–265	PIT-2 1–265
Asx		1.1 (1)	1.1 (1)			2.0 (2)		1.1 (1)	1.0 (1)	2.0 (2)	1.9 (2)		1.0 (1)	1.1 (1)	24.0 (23)
Thr		3.6 (4)	3.9 (4)	0.9 (1)	0.9 (1)			0.9 (1)					0.9 (1)		17.7 (16)
Ser		1.0 (1)	1.1 (1)	0.9 (1)	1.0 (1)			1.0 (1)	0.9 (1)				0.8 (1)		16.6 (16)
Glx				1.0 (1)	1.0 (1)	2.1 (2)		1.1 (1)		1.1 (1)			1.0 (1)	3.9 (4)	30.3 (30)
Pro		N.D. (1)	N.D. (1)	N.D.	N.D.	N.D.	N.D. (1)	N.D. (1)	N.D. (1)	N.D. (1)	N.D. (1)	N.D.	N.D. (1)	N.D.	12.1 (11)
Gly		0.2 (0)					1.0 (1)	3.0 (3)	1.1 (1)	1.9 (2)	2.0 (2)			2.0 (2)	21.2 (21)
Ala		2.8 (3)	2.8 (3)				1.0 (1)	3.1 (3)	1.9 (2)	2.0 (2)	1.0 (1)		1.9 (2)	3.0 (3)	34.0 (33)
Cm-Cys															1.9 (2)
Val						0.9 (1)	0.9 (1)	0.9 (1)	1.0 (1)	0.9 (1)	1.1 (1)	1.7 (2)			15.9 (16)
Met		0.7 (1)	0.7 (1)			0.9 (1)							0.8 (1)		8.8 (9)
Ile							1.0 (1)	0.9 (1)	0.9 (1)	0.8 (1)	0.8 (1)	1.6 (2)		2.7 (3)	12.7 (13)
Leu		1.0 (1)	1.0 (1)	1.8 (2)	1.9 (2)	2.0 (2)	1.0 (1)	0.9 (1)	0.9 (1)	2.0 (2)	2.1 (2)	1.1 (1)		1.1 (1)	31.8 (31)
Tyr												0.8 (1)			3.0 (3)
Phe								0.8 (1)	0.9 (1)						4.0 (4)
His														1.0 (1)	3.0 (3)
Lys			1.0 (1)	1.0 (1)				1.0 (1)	1.0 (1)	1.1 (1)	1.0 (1)				7.0 (7)
Arg		1.0 (1)	1.1 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.1 (1)		1.0 (1)		1.1 (1)	0.9 (1)	1.0 (1)	23.3 (22)
Trp		N.D.	N.D.	N.D.	N.D.	N.D. (1)	N.D.	N.D. (1)	N.D. (1)	N.D.	N.D.	N.D.	N.D.	N.D. (1)	3.7 (5)
Yield (%)		30	10	21	12	40	35	30	15	22	14	13	50	35	


10
20
30
Ser-Pro-Gln-Pro-Leu-Glu-Gln-Ile-Lys-Leu-Ser-Glu-Ser-Gln-Leu-Ser-Gly-Arg-Val-Gly-Met-Ile-Glu-Met-Asp-Leu-Ala-Ser-Gly-Arg-
Whole protein
T1
T2
T3
V1
V2
V3
V4
40
50
60
Thr-Leu-Thr-Ala-Trp-Arg-Ala-Asp-Glu-Arg-Phe-Pro-Met-Met-Ser-Thr-Phe-Lys-Val-Val-Leu-Cys-Gly-Ala-Val-Leu-Ala-Arg-Val-Asp-
T4
T5
T6
T7
V4
V7
70
80
90
Ala-Gly-Asp-Glu-Gln-Leu-Glu-Arg-Lys-Ile-His-Tyr-Arg-Gln-Gln-Asp-Leu-Val-Asp-Tyr-Ser-Pro-Val-Ser-Glu-Lys-His-Leu-Ala-Asp-
T7
T8
T10
T11
V7
V8
V9
V10
100
110
120
Gly-Met-Thr-Val-Gly-Glu-Leu-Cys-Ala-Ala-Ala-Ile-Thr-Met-Ser-Asp-Asn-Ser-Ala-Ala-Asn-Leu-Leu-Leu-Thr-Ala-Val-Gly-Gly-Pro-
T11
T11-V4
T11-V5
V10
V13
130
140
150
Ala-Gly-Leu-Thr-Ala-Phe-Leu-Arg-Gln-Ile-Gly-Asp-Asn-Val-Thr-Arg-Leu-Asp-Arg-Trp-Glu-Thr-Glu-Leu-Asn-Glu-Ala-Leu-Pro-Gly-
T11
T12
T13
T11-V4
T11-V5
V13
V14
V15
V13-C
160
170
180
Asp-Ala-Arg-Asp-Thr-Thr-Thr-Pro-Ala-Ser-Met-Ala-Ala-Thr-Leu-Arg-Lys-Leu-Leu-Thr-Ser-Gln-Arg-Leu-Ser-Ala-Arg-Ser-Gln-Arg-
T13
T14
T16
V15
V15-K2
190
200
210
Gln-Leu-Leu-Gln-Trp-Met-Val-Asp-Asp-Arg-Val-Ala-Gly-Pro-Leu-Ile-Arg-Ser-Val-Leu-Pro-Ala-Gly-Trp-Phe-Ile-Ala-Asp-Lys-Thr-
T18
T19
T20
V15
V15-K2
CN4
220
230
240
Gly-Ala-Gly-Glu-Arg-Gly-Ala-Arg-Gly-Ile-Val-Ala-Leu-Leu-Gly-Pro-Asn-Asn-Lys-Ala-Glu-Arg-Ile-Val-Val-Ile-Tyr-Leu-Arg-Asp-
T20
T22
T24
V15
V15-K3
V15-K4
CN4
250
260
265
Thr-Pro-Ala-Ser-Met-Ala-Glu-Arg-Asn-Gln-Gln-Ile-Ala-Gly-Ile-Gly-Ala-Ala-Leu-Ile-Glu-His-Trp-Gln-Arg
T25
T26
V15
V16
V17
V15-K4
CN4
CN5

analysis of peptides generated by a tryptic digestion. The alignment of tryptic peptides was found by using overlapping fragments isolated from staphylococcal-proteinase and CNBr cleavages or from subfractionation of large peptides by chymotrypsin, Lys-C proteinase and staphylococcal proteinase. All peptide mixtures were submitted to reverse-phase h.p.l.c. A typical elution pattern, corresponding to the tryptic digest, is indicated in Fig. 1. The amino acid compositions of the 26 tryptic peptides are listed in Table 1. Except for peptide T11, all the peptides were completely sequenced by Edman degradation. However, the *C*-terminal lysine residues of peptides T1, T5 and T10 were not identified. The unsequenced part of peptide T11, of which only the first 28 residues were determined, was elucidated by analysis of subpeptides T11–V1 to T11–V5 obtained from fractionation of peptide T11 with an excess of *S. aureus* proteinase. The results of sequences analysis are given in Fig. 2. By comparison with the *N*-terminal sequence of the intact *S*-carboxymethylated protein, we positioned peptide T1 at the *N*-terminus. As all peptides ended in an arginine or lysine residue, because of the tryptic cleavage, no particular one could be suspected to be located at the *C*-terminus. Peptides T9, T15, T17, T21 and T23, which result from partial cleavages at lysine residues, are not shown, but their positions in the sequence are indicated in Table 1. Three small peptides (residues 174–177, 178–180 and 216–218), which most probably were eluted with the buffer peak, were not recovered by h.p.l.c. The Arg–Phe bond at position 40–41 and the Arg–Trp bond at position 139–140 were apparently resistant to tryptic cleavage.

Staphylococcal V8 proteinase cleavages mainly occurred at peptide glutamyl bonds, although one aspartyl bond (position 76–77) was fully hydrolysed (Fig. 2). Peptide V6 resulted from an unusual cleavage of a Thr–Ala bond in position 33–34. Another Thr–Ala bond cleavage (position 115–116) was also observed during subfractionation of tryptic peptide T11 by staphylococcal proteinase. H.p.l.c. analysis of a reaction mixture sample removed after 1 h digestion revealed preferential peptide glutamyl bond cleavages at residues 143, 146, 247 and 261. That, mainly, yielded the fragment V15, which formed an insoluble precipitate, even in the presence of 4 M-urea. So the aggregation of peptide V15 did not allow the Glu–Arg bond proteolysis at positions 214–215 and 231–232. Partial amino acid sequence of peptides V13 and V15 (35 and 28 residues respectively) were performed, whereas all the other peptides were fully sequenced. Peptide V17 was suspected to be the *C*-terminal fragment of the β-lactamase as it ends in a non-specific arginine residue. Structural analysis of peptides isolated from staphylococcal proteinase digestions provided evidence for the alignment of tryptic peptides spanning residues 1–131.

In order to elucidate the unsequenced part of peptide V13, the latter was subdigested by chymotrypsin, the reaction being stopped before complete digestion. The peptide V13–C was purified from the chymotryptic digest by C_{18} μBondapak h.p.l.c. Its partial amino acid sequence (Fig. 2) overlaps tryptic peptides T12 and T13. Moreover the sequenced part of the *N*-terminal sequence of peptide V15 spans peptides T13, T14, T15 and T16. Therefore the sequence of the first 174 *N*-terminal residues of the protein was determined.

From sequence analysis of tryptic peptides we knew that the 101-residue peptide V15 contained three lysine residues residing in strategic positions. In order to obtain overlapping sequence at positions 170–248, the fragment V15 was subjected to Lys-C endoproteinase digestion. Pure peptides V15–K1 to V15–K4 were removed from a single reverse-phase h.p.l.c. Amino acid sequence analysis allowed identification of the first 24 residues of peptide V15–K2 (Fig. 2). The other three peptides were fully sequenced, although *C*-terminal lysine derivatives were not identified. Sequencing of peptide V15–K2 established the sequence at positions 174–180 and confirmed the structure of fragments 168–173 and 181–191. Lastly, the positions 216–218 were deduced from the structure of peptide V15–K3.

Furthermore fragments overlapping tryptic peptides T18, T19 and T20 were obtained from CNBr cleavage of the PIT-2 β-lactamase. Five fragments at positions 1–21, 25–43, 162–186, 187–245 and 246–265, isolated by reverse-phase h.p.l.c., spanned the first 43 *N*-terminal residues and the last 105 *C*-terminal residues. No additional fragment from the protein core was recovered pure in useful amount. The *N*-terminal sequences of peptides CN4 and CN5, which provide new data for structure elucidation, are shown in Fig. 2.

Sequence determinations of subpeptides from Lys-C proteinase digestion and of peptides from CNBr cleavage provided overlaps for tryptic peptides T18 to T26. Thus the PIT-2 β-lactamase sequence was extended from position 174 to residue 265.

The *C*-terminal sequence of PIT-2 β-lactamase was investigated by the use of carboxypeptidases A and B. After incubation with carboxypeptidase B, only arginine (0.8 mol/mol of protein) was found in the digest. With a mixture of the carboxypeptidases A and B, the amino acids released were arginine, glutamine and tryptophan. It is noteworthy that no trace of histidine was detected. A time-course study of amino acid release yielded the *C*-terminal sequence -Trp-Gln-Arg, which likewise was deduced from digestion of the tryptic peptide T26 with carboxypeptidases A and B. The results confirmed the findings from Edman degradation of the proteolytic peptides.

Fig. 2. Amino acid sequence of PIT-2 β-lactamase

Residues identified by manual sequence determinations are underlined by continuous lines. Blanks denote residues not identified. Broken lines indicate peptides only checked by amino acid composition analysis. *C*-Terminal residues determined after carboxypeptidases digestion are underlined by ∠.

DISCUSSION

The complete 265-residue amino acid sequence of PIT-2 β-lactamase is summarized in Fig. 2. The M_r value calculated from the sequence is 28835. This is close to the value of 27500 that was obtained by SDS/polyacrylamide-gel electrophoresis (Barthélémy *et al.*, 1986). The amino acid composition determined from the purified protein is in agreement with the sequence (Table 1).

In Fig. 3 the amino acid sequence of PIT-2 β-lactamase is compared with that of TEM-1 β-lactamase deduced from the nucleotide sequence of the ampicillin-resistance gene carried by plasmid pBR 322 (Sutcliffe, 1978). TEM-1 and TEM-2 β-lactamases differ only by a

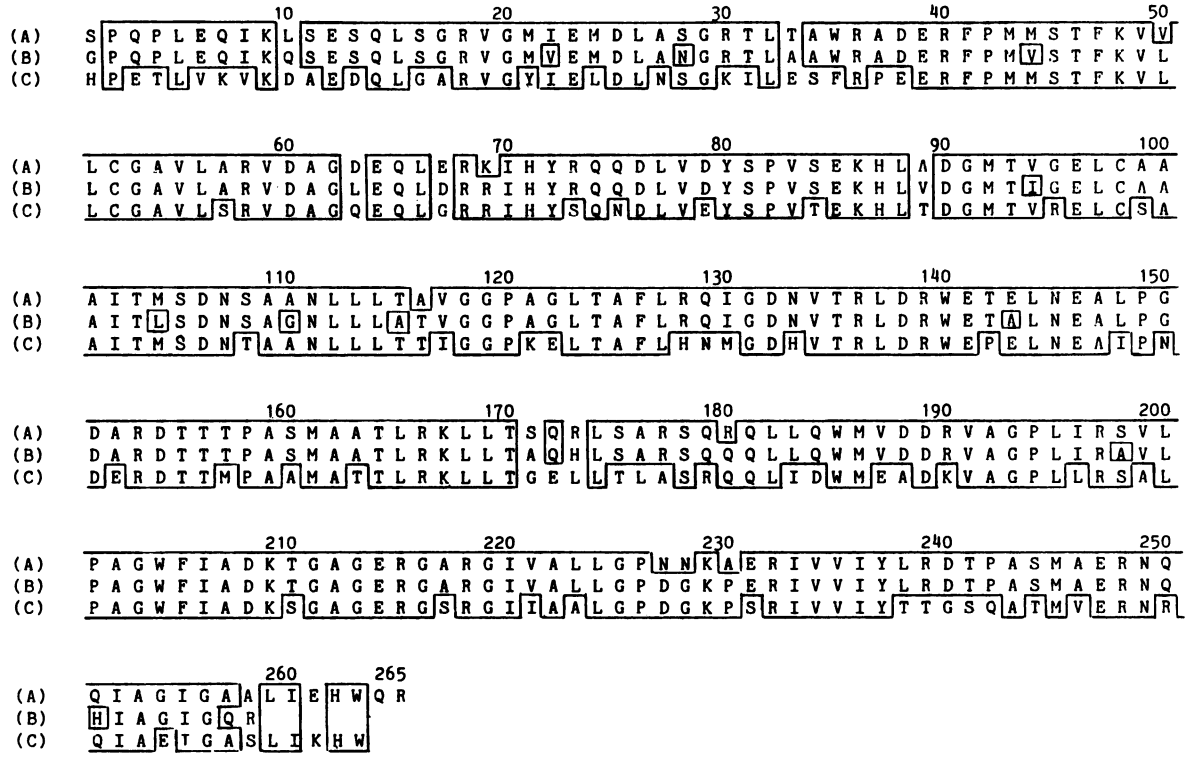


Fig. 3. Amino acid sequence comparison of the three evolutionarily related β-lactamases

(A), PIT-2 β-lactamase (present work); (B), β-lactamase of *K. pneumoniae* strain LEN-1 (Arakawa *et al.*, 1986); (C), TEM-1 β-lactamase (Sutcliffe, 1978). Arakawa *et al.* (1986) proposed a phenylalanine residue at position 64 of the expected mature *Klebsiella* protein (position 85 of the pre-penicillinase), but the corresponding codon of the nucleotide sequence indicated a glutamic acid residue. Boxed residues hold identical amino acid residues.

single amino acid substitution at position 14 (Ambler & Scott, 1978; Barthélémy *et al.*, 1985). The PIT-2 enzyme is two residues longer at the *C*-terminus than is the TEM-1 protein. A 68% amino acid sequence homology was observed between the two β-lactamases. Residues 1–40, 171–190 and 240–265 are poorly retained. In contrast, the sequences spanning residues 39–56, and hence surrounding the active-site serine residue at position 45 of the TEM β-lactamases (Fisher *et al.*, 1980), are strongly conserved, 17 out of 18 residues being the same. We therefore conclude that plasmid-mediated PIT-2 β-lactamase and TEM-1 β-lactamase are closely evolutionarily related and, consequently, that the PIT-2 enzyme belongs to the class A β-lactamases. Levesque *et al.* (1987) have developed a probe to the TEM-1 β-lactamase consisting of 424 bp from a pBR 322 DNA fragment. This probe encodes the amino acid sequence covering residues 60–199 of the β-lactamase. Unexpectedly, no hybridization occurred between this probe and the DNA of the PIT-2-β-lactamase-encoding plasmid R974, even under low-stringency conditions. This could be indication of a greater degeneracy at the DNA level. In addition, it should be noted that Levesque *et al.* (1987) used plasmid R974 as source of PIT-2-β-lactamase-encoding gene whereas we sequenced the p453-encoded β-lactamase.

Sawai *et al.* (1973) found some immunological homology between chromosomally mediated β-lactamase of *Klebsiella* and the TEM-1 enzyme. They proposed that the genetic determinant of the β-lactamase was made up from a DNA fragment of the *Klebsiella* group. This suggestion was supported by Arakawa *et al.* (1986), who prepared a 500 bp probe from the chromosomal DNA gene encoding the β-lactamase of *K. pneumoniae* strain LEN-1. The amino acid sequence predicted from the nucleotide sequence of this gene is shown in Fig. 3. The probe, which coded for the amino acid sequence covering residues 14–184, hybridized with the TEM-1 β-lactamase-encoding plasmid pBR 322. When we compared in Fig. 3 the amino acid sequence of the *K. pneumoniae* β-lactamase with that of PIT-2 β-lactamase, we observed 88% homology. No region of poorly retained sequence appeared except at the *C*-terminus, the *Klebsiella* enzyme being seven residues shorter than the PIT-2 protein. This difference can be explained by a single-base deletion occurring in the nucleotide sequence of the *K. pneumoniae* chromosome fragment prepared by Arakawa *et al.* (1986). Indeed, one deoxyguanine insertion at nucleotide sequence position 1117 (i.e. between the triplets encoding Gly-256 and Gln-257 of the mature protein) would yield the following amino acid sequence: A(257) A L I E H W Q R, which fits perfectly with the *C*-terminus sequence of PIT-2 β-lactamase.

Our findings strongly support the hypothesis of Nugent & Hedges (1979), who assumed that the PIT-2-β-lactamase-encoding gene, originally carried by the chromosome in *Klebsiella* group, could have been later incorporated into a plasmid. Thus the rather *K.*

pneumoniae-confined PIT-2 (SHV-1) β-lactamase could represent an evolutionary step between a chromosomally encoded β-lactamase of *K. pneumoniae* and the widespread plasmid-mediated TEM β-lactamases.

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Received 22 April 1987/30 September 1987; accepted 23 November 1987