Proteolytic interconversion and *N*-terminal sequences of the *Citrobacter diversus* major β -lactamases

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The *N*-terminal sequences of the two major β -lactamases produced by *Citrobacter diversus* differed only by the absence of the first residue in form II and the loss of five amino acid residues at the *C*-terminal end. Limited proteolysis of the homogeneous form I protein yielded a variety of enzymatically active products. In the major product obtained after the action of papain, the first three *N*-terminal residues of form I had been cleaved, whereas at the *C*-terminal end the treated enzyme lacked five residues. However, this cannot explain the different behaviours of form I, form II and papain digestion product upon chromatofocusing. Form I, which was sequenced up to position 56, exhibited a very high degree of similarity with a *Klebsiella oxytoca* β -lactamase. The determined sequence, which contained the active serine residue, demonstrated that the chromosome-encoded β -lactamase of *Citrobacter diversus* belong to class A.

INTRODUCTION

β -Lactamases are widely distributed bacterial enzymes that hydrolyse β -lactam antibiotics and represent one of the most important factors of microbial resistance to those therapeutic agents. We have investigated the β -lactamases produced by a clinical isolate of *Citrobacter diversus* ULA 27 strain. This strain showed a high resistance towards both penicillins and cephalosporins, owing to the synthesis of large amounts of class A chromosome-encoded β -lactamases which, upon isoelectric focusing, behaved as a family of enzymes with pI values ranging from 5.7 to 6.8. The two main bands exhibited pI values of 6.8 (form I) and 6.2 (form II) respectively and represented about 92% of the total quantity of β -lactamase. Those two major components were isolated (Amicosante *et al.*, 1988a) and showed similar physico-chemical and substrate-specificity features. On the basis of their pI values, M_r values, substrate specificity, active-site sequence and interaction with β -iodopenicillanic acid, both forms were proposed to be class A β -lactamases (Amicosante *et al.*, 1988b).

Several phenomena, such as chemical instability during enzyme storage or self-association, are reported by some authors to be responsible for the generation of satellite bands (Simpson & Pledst, 1983). These bands could also be due to the removal or modification of specific amino acids by proteinases of low specificity (Ambler & Meadway, 1969; Brive *et al.*, 1977). In the present study we report the effects of limited proteolysis on the main β -lactamase of *C. diversus* ULA-27 (form I).

Proteolysis was performed either with papain or with crude membrane preparations of the *C. diversus* ULA-27 strain itself and generated patterns of satellite bands similar to those observed *in vivo*. We also obtained extended *N*-terminal sequences of both form I and form II β -lactamases.

MATERIALS AND METHODS

Antibiotics and chemicals

Benzylpenicillin, papain, phenylmethanesulphonyl fluoride (PMSF), carbonic anhydrase and carboxypeptidase Y were from

Sigma Chemical Co. (St. Louis, MO, U.S.A.); cephalothin was from Eli Lilly and Co. (Indianapolis, IN, U.S.A.); carbenicillin was from Beecham Research Laboratories (Brentford, Middx., U.K.); cefazolin was from commercial sources, and PADAC {7-(thienyl-2-acetamido)-3-[2-(4-*NN*-dimethylaminophenylazo)-pyridinium methyl]-3-cephem-4-carboxylic acid} was purchased from Hoechst (Scoppito, L'Aquila, Italy). All other chemicals were of the purest available analytical grade.

Organism

Citrobacter diversus ULA 27, was a clinical isolate. The organism was grown and the β -lactamases purified as previously described (Oliva *et al.*, 1987; Amicosante *et al.*, 1988a).

Membrane preparation

C. diversus ULA 27 cells were sonicated and centrifuged for 60 min at 105 000 *g*, in a Beckman L8-70 Ultra-centrifuge. Pellets were re-suspended in 0.1 M-sodium phosphate buffer, pH 7.0, and washed three times in the same buffer. Aliquots of the final suspension were used for proteolysis experiments, after addition of 2 mM-MgCl₂.

Electrophoretic analysis

SDS/PAGE was performed in a discontinuous system, as described by Laemmli (1970), with a fixed 15%-(w/v)-acrylamide concentration.

M_r values for native and treated enzymes were determined in this system, using carbonic anhydrase (M_r 29 000) and native form I as reference standard. The proteins were stained with AgNO₃ as described by Heukeshoven & Dernick (1985).

Analytical isoelectric-focusing assays were performed as previously reported (Oliva *et al.*, 1987) in a Multiphor II apparatus, cooled at 10 °C.

A Whatman 2MM paper, dipped in 500 μ M-PADAC and layered on the gels, was used to reveal β -lactamase activity. The proteins were stained with Coomassie Blue R-250.

Fast chromatofocusing

This was performed with the help of a Perkin–Elmer Series 10

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; PADAC, 7-(thienyl-2-acetamido)-3-[2-(4-*NN*-dimethylaminophenylazo)pyridinium methyl]-3-cephem-4-carboxylic acid.

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chromatography apparatus equipped with a Mono P HR 5/20 column (Pharmacia, Uppsala, Sweden). The column was equilibrated with 0.025-M-2,2-nitrotriethanol/Bistris buffer, pH 7.0. Proteins were eluted with 25 ml of a 10-fold-diluted Polybuffer 74, adjusted to pH 5.0, at a flow rate of 1 ml/min.

β -Lactamase assay

β -Lactamase activity was monitored using 100 μ M-cefazolin as substrate, monitoring the initial rates of substrate disappearance at 273 nm with the help of a Perkin-Elmer 550 S spectrophotometer. One unit of β -lactamase activity was the amount of enzyme with hydrolysed 1 μ mol of cephaloridine/min at 30 °C and pH 7.0.

k_{cat} and K_m determinations

Kinetic parameters were determined following the previously described procedure (Amicosante *et al.*, 1988a), using an ULTROSPEC II spectrophotometer connected to an Apple II microcomputer via a RS232 interface (De Meester *et al.*, 1987).

For the determination of k_{cat} values, the enzyme concentration in the various samples was estimated by titration with β -iodopenicillanic acid.

Proteolysis

Limited proteolysis of β -lactamase form I was performed at 37 °C by incubating the enzyme with papain (enzyme/papain ratio = 40:1, w/w) in 0.1 M-sodium phosphate buffer, pH 7.0, containing 1 mM-cysteine.

Samples were withdrawn at different times and stored at -70 °C until use. In control experiments, form I was also incubated under the same conditions but in the absence of papain.

In parallel experiments form I was incubated for 15 h with crude membrane preparations under the same experimental conditions with a β -lactamase form I: membrane protein ratio of 1:40 (w/w). After incubation, the mixture was centrifuged for 30 min at 105 000 *g* and the supernatant was dialysed against 20 mM-triethanolamine, pH 7.0, and loaded on a phenylboronic acid-Sepharose column. After elution, the different forms were separated by fast chromatofocusing as described above. The same sample were also analysed by isoelectric focusing.

N-Terminal sequencing

Protein sequence analyses were carried out on a 477A pulsed-liquid sequencer (Applied Biosystems, Foster City, CA, U.S.A.).

The native β -lactamase form I was sequenced twice, once for 19 cycles on 150 pmol of protein still containing ampholytes, and a second time for 60 cycles on 3 nmol of protein freed from ampholytes by ultrafiltration through a Centricon 10 micro-concentrator (Amicon, Danvers, MA, U.S.A.).

The protein fraction of β -lactamase form I obtained after proteolysis (two samples of 200 and 225 pmol) were subjected to sequence analysis without removing the ampholytes. β -Lactamase form II was sequenced starting with 1.5 nmol of protein.

C-Terminal sequencing

Pure enzymes (4 nmol) were incubated at 25 °C in 0.1 M-phosphate buffer (pH 7.4) containing 0.1 % SDS (final concn.) and carboxypeptidase Y at a β -lactamase:carboxypeptidase ratio of 80:1 (w/w).

Samples were withdrawn after 0, 15, 30, 60 and 90 min. Digestion was stopped by adding 0.1 M-HCl and acetone. After centrifugation the supernatants were dried in a Pico-Tag workstation and the samples derivatized with phenyl

isothiocyanate. The resulting phenyl isothiocyanate derivatives were analysed by reverse-phase h.p.l.c. as previously reported (Amicosante *et al.*, 1988a).

RESULTS

Using the fast-chromatofocusing technique we could separate two satellite bands (Fig. 1a, peaks b and d) with good resolution. The recovery of the form eluted at pH 6.3 was sufficient for a detailed study.

Papain treatment

Incubation of a homogeneous form I sample with papain generated a heterogeneous pattern on isoelectric focusing (not shown). The zymogram bands revealed by PADAC focused at pI values of 6.8, 6.3, 6.2 and 5.7.

The same phenomenon did not occur when form II was subjected to the same treatment. The disappearance of form I and concomitant generation of new bands was a time-dependent phenomenon. Figs. 1(b) and 1(c) show that form I was progressively transformed into a new form with a pI of 6.3. A longer incubation induced a progressive disappearance of the enzymic activity

Possible spontaneous degradation of the enzyme was excluded by parallel experiments performed under the same conditions, but in the absence of papain.

Proteolytic effect of crude membrane preparations

After complete removal of β -lactamase activity, membrane preparations of *C. diversus* ULA 27 were incubated with form I β -lactamase. After increasing periods, aliquots of the mixture

Fig. 1. Fast-chromatofocusing analyses

(a) β -Lactamases naturally occurring in *C. diversus* ULA 27. Protein eluate (1 mg) from phenylboronic acid-Sepharose chromatography was analysed. (b) Form I (400 μ g) after 2 h of digestion with papain. x = papain. (c) Form I (400 μ g) after 6 h of digestion with papain. x = papain. (d) Form I (1.0 mg) after treatment with crude membrane. The elution pH values are: (a) 6.8; (b) 6.3; (c) 6.8; (d) 5.7. For experimental details, see the Materials and methods section.

Table 1. Kinetic parameters of *C. diversus* ULA 27 β -lactamases

Substrate	Naturally occurring						Product of digestion			
	Form I (pI 6.8)		Form II (pI 6.2)		Form (pI 6.3)		Membrane (pI 6.3)		Papain (pI 6.3)	
	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})
Benzylpenicillin	64 $\pm$ 5	510 $\pm$ 20	51 $\pm$ 3	415 $\pm$ 30	67 $\pm$ 7	525 $\pm$ 40	81 $\pm$ 9	571 $\pm$ 60	72 $\pm$ 10	361 $\pm$ 30
Carbenicillin	94 $\pm$ 8	96 $\pm$ 8	106 $\pm$ 10	47 $\pm$ 10	63 $\pm$ 5	49 $\pm$ 8	54 $\pm$ 7	43 $\pm$ 7	Biphasic	80 $\pm$ 12
Cephalothin	23 $\pm$ 3	183 $\pm$ 16	28 $\pm$ 2	90 $\pm$ 9	29 $\pm$ 4	144 $\pm$ 20	23 $\pm$ 3	173 $\pm$ 15	26 $\pm$ 4	375 $\pm$ 25
Cefazolin	44 $\pm$ 4	144 $\pm$ 7	44 $\pm$ 4	124 $\pm$ 14	16 $\pm$ 6	178 $\pm$ 8	49 $\pm$ 5	145 $\pm$ 15	Biphasic	45 $\pm$ 10

Fig. 2. SDS/PAGE analysis of purified isoforms

A fixed 15% acrylamide concentration was used. The following samples were examined: A, carbonic anhydrase (M_r 29000); B, form I (pI = 6.8); C, form II (pI = 6.2); D, papain-treated form I (pI = 6.3); E, naturally occurring minor form (pI = 6.3).

were centrifuged and the supernatants were tested by analytical isoelectric focusing. This analysis showed that all the bands with β -lactamase activity were superimposable on those obtained after papain treatment. In the presence of 1 mM-PMSF or 2 mM-EDTA, no changes in the number of active bands were observed (results not shown). Experiments performed following the procedure reported in the Materials and methods section allowed the recovery of the main product formed after digestion by membrane preparations as shown in Fig. 1(d).

Kinetic results

The two forms with a pI of 6.3 obtained by the different proteolytic procedures were analysed for their kinetic properties and compared with the naturally occurring forms with pI values of 6.8, 6.3 and 6.2 (Table 1). The k_{cat} and K_m values calculated for two different penicillins and two cephalosporins showed no significant differences in the overall picture, if one excepts the product of papain digestion, which exhibited biphasic kinetics with carbenicillin and cefazolin.

M_r determination

SDS/PAGE analysis showed no significant differences between the M_r values of the naturally occurring forms (pI 6.8, 6.3 and 6.2) and the papain-treated form (Fig. 2).

	1	5	10	15	20
TEM-2	L	P	V	F	A
<i>K. pneumoniae</i>	L	V	V	Y	A
<i>K. oxytoca</i>	S	L	W	A	S
Form I (6.8)	G/T	T	Q	G	E
Form II (6.2)		T	Q	G	E
p-Form I (6.3)				G	E
Class A numbering	21	25	30	35	40
TEM-2	G	A	R	V	G
<i>K. pneumoniae</i>	S	G	R	V	G
<i>K. oxytoca</i>	G	G	R	L	G
Form I (6.8)	G	G	R	L	G
Form II (6.2)	G	G	R	L	G
Class A numbering	41	45	50	55	60
TEM-2	P	E	E	R	F
<i>K. pneumoniae</i>	A	D	E	R	F
<i>K. oxytoca</i>	G	D	E	R	F
Form I (6.8)	G	D	E	R	F
Active-site peptide					
Class A numbering	66	70	73		

Fig. 3. N-Terminal sequences of forms aligned I and II and of the major papain digestion product aligned with those of the *K. oxytoca*, *K. pneumoniae* and TEM-2 enzymes

Residues which are conserved in all known class A β -lactamases are boxed with a solid line. Those which are conserved in five other β -lactamases or more are boxed with a discontinuous line. The numbers in the bottom line give Ambler's consensus numbering of class A β -lactamases. Abbreviation: p-Form I, papain-treated form I.

N-Terminal sequence

The *N*-terminal amino acid sequences of forms I and II and of the major papain digestion product (pI 6.3) of form I are reported in Fig. 3. Starting with residue F⁴⁵, the sequence overlapped with that of the peptide containing the active serine residue, which had been isolated previously (Amicosante *et al.*, 1988b).

C-terminal sequences

Analyses performed on the various enzymes, gave the following C-terminal amino acid sequences:

Native form I Leu-Met-Pro-Asp/Asn-Arg-Gly-Tyr-
Asp/Asn-OH

Native form II Leu-Met-(Gly)-OH

Papain-treated Leu-Met-Pro-OH

The results first indicated a loss of five C-terminal residues in the papain-treated form I and in the naturally occurring form II. Some technical problems arose during the sequence determination of form II owing to the presence of glycine as contaminant, although this did not invalidate the meaning of the experimental data.

DISCUSSION

Microheterogeneity of apparently homogeneous β -lactamase preparations appears to be a common phenomenon (Brive *et al.*, 1977; Dehottay *et al.*, 1987; Matagne *et al.*, 1991). This observation has been explained by self-association of the proteins and, in the absence of a signal peptidase, deamidation of particularly unstable asparagine residue or enzymic proteolysis. The two latter mechanisms might already occur in the periplasm of Gram-negative bacteria or in the culture supernatant in Gram-positive bacteria, but also during the purification steps and upon storage. Both proteolysis and non-specific signal peptidases result in the production of proteins with ragged *N*-termini (Matagne *et al.*, 1991).

Our results indicate, that, in the case of the *C. diversus* β -lactamase, post-synthesis proteolytic degradation and/or a limited specificity of the signal peptidase are responsible for the observed pattern of active forms. Indeed, treatment of the major form with papain or *C. diversus* membranes also resulted in the generation of enzymically active β -lactamases, exhibiting a variety of pI values, the patterns being reminiscent of those observed with the naturally occurring mixture. In the papain and membrane treatments, the main degradation product exhibited a pI of 6.3.

Table 2. Comparison matrix between the *C. diversus*, *K. oxytoca*, *K. pneumoniae* and TEM-2 β -lactamases

The comparison was made on the basis of the 56 *N*-terminal residues of the *C. diversus* enzyme, but the last two β -lactamases have an additional residue between positional 37 and 38 (residues 58 in Ambler's numbering).

Organism	Identities					
	<i>K. oxytoca</i>		<i>K. pneumoniae</i>		TEM-2	
	No.	%	No.	%	No.	%
<i>C. diversus</i>	39/56	70	15/57	26	14/57	25
<i>K. pneumoniae</i>	15/57	26	—	—	31/57	54
<i>K. oxytoca</i>	—	—	15/57	26	14/57	25

Determination of the *N*-terminal sequences indicated that form II was only one residue shorter than form I and that papain treatment removed the first three *N*-terminal residues of form I (none of residues as charged).

After the papain treatment of form I, the C-terminal sequence indicated the loss of two aspartic acid/asparagine and one arginine residue among the amino acids removed. This result can only explain the difference between the pI values of the form II, form I and the digested form I if we assume that aspartic acid residues were asparagine; in that case, the total charge of the treated enzyme becomes more negative, shifting the pI towards a more acidic value. Moreover, M_r values estimated by SDS/PAGE failed to reveal larger modifications such as internal nicking of the protein after proteolytic treatment. It was noteworthy that the chromatofocusing pattern observed after treatment of form I with membrane preparations was very similar to that obtained with the naturally occurring mixture of β -lactamase.

The modifications did not result in important alterations of the catalytic properties (Table 1), the only surprising feature being the biphasic time course observed with the papain digestion product. If one excepts the first residue, which was absent in form I, the *N*-terminal sequences of the naturally occurring forms I and II were identical, indicating that both proteins were probably encoded by the same gene, and that form II was derived of form I by proteolytic action and/or modification as discussed above. The sequence could be determined up to position 56, covering, at positions 45–52, the active-site-serine-containing peptide which had been isolated and sequenced before (Amicosante *et al.*, 1988b). Since that peptide had been obtained by digestion with trypsin, it was quite likely that the unidentified residue in position 44 was arginine.

Fig. 3 shows that the *N*-terminal sequence confirms that the *C. diversus* β -lactamases belong to class A, as was suggested by the rate of inactivation by β -iodopenicillanate and the sequence of the active-site peptide (Amicosante *et al.*, 1988b). Besides the FXXXSXXX sequence around the active serine residue, residues in positions 17 and 25 were respectively glutamic acid and glycine as in all class A enzymes (numbers 37 and 45 in Ambler's consensus numbering). Comparisons with various class A sequences yielded an unexpected result: a very high sequence identity with that recently published for a β -lactamase of *Klebsiella oxytoca* (Arakawa *et al.*, 1989). Indeed, after the first nine residues, the sequences are nearly identical, exhibiting, between positions 10 and 56, 39 strict identities (83%). Surprisingly, the *K. oxytoca* enzyme exhibits a much higher similarity to the *C. diversus* enzyme than to the *K. pneumoniae* β -lactamase (Arakawa *et al.*, 1986) which, in turn, is very similar to the TEM-2 β -lactamases. Table 2 shows that the four enzymes form two distinct groups which have only about 25% of common residues and the sequence portion of the *C. diversus* enzyme. Our results thus confirm the high degree of heterogeneity in class A sequences and the fact that enzymes produced by taxonomically distant species might exhibit larger proportions of conserved residues than enzymes produced by bacteria of the same genus.

Further work is needed to clone the β -lactamase gene of *C. diversus*. It will be interesting to see if the high degree of similarity with the *K. oxytoca* enzyme extends further to the C-terminal part of the proteins.

The work in L'Aquila was supported by the C.N.R. (grant no. 88.00492.04; a bilateral project with J.M.F.) and the Funds of the Italian Ministry of Education. The work in Liège was supported in parts by the Funds de la Recherche Scientifique Médicale (Contract no. 3.4507.83), an Action Concertée with the Belgian Government (Convention 86/91-90), a convention with the Region Wallonne (C2/c16/Conv. 246/20428), the Funds de Recherche de la Faculté de Médecine ULg and a contract

with the E.E.C. (B.A.P.-0197-B). The work in Gent was supported by the Belgian National Incentive Program on Fundamental Research in Life Sciences, initiated by the Belgian State Prime Minister's Office, Science Policy Programming Department.

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Received 14 September 1990/1 November 1990; accepted 7 November 1990