The amino acid sequence of the zinc-requiring β-lactamase II from the bacterium *Bacillus cereus* 569

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The amino acid sequence of the zinc-requiring β-lactamase II from *Bacillus cereus* strain 569 has been determined. It consists of a single polypeptide chain of 227 residues. It is the only example so far fully characterized of a class B β-lactamase, and is structurally and mechanistically distinct from both the widely distributed class A β-lactamases (such as the *Escherichia coli* RTEM enzyme) and from the chromosomally encoded class C enzymes from Gram-negative bacteria.

Class B β-lactamase *Zinc enzyme* *Primary structure* Bacillus cereus

1. INTRODUCTION

β-Lactamases are extracellular or periplasmic enzymes produced by bacteria which confer resistance to natural penicillin and cephalosporin antibiotics. β-Lactamases can be divided into classes on the basis of their primary structure [1]. The enzymes of class A (M_r ~30000) and class C (M_r ~39000 [2]) have a serine residue at the active site, but otherwise are not similar in sequence to each other. The class B enzymes (M_r ~25000) require zinc for activity. Their only well-characterized occurrence is in *Bacillus cereus* [3], which also produces 2 class A enzymes [4–8]. The other characterized zinc-dependent β-lactamase is from *Pseudomonas maltophilia* [9] and differs markedly from the *B. cereus* enzyme [10].

In this paper we report evidence for the amino acid sequence of a class B enzyme, the β-lactamase II from *B. cereus* strain 569. As described below, difficulties were encountered in the sequence determination because a stable aminopeptidase copurified with the β-lactamase. To avoid this problem, our main effort has now been changed to the study of the β-lactamase II from another strain of *B. cereus*, 5/B/6 [11]. However, as other laboratories are using the 569 enzyme for studies of gene sequence [12] and mechanism of action [13], we are now reporting our amino acid sequence evidence.

2. EXPERIMENTAL

β-Lactamase II was isolated from the culture supernatant of *B. cereus* 569/H/9 (NCIB 12054), a magnoconstitutive derivative of strain 569, as described by Davies et al. [5]. The amino acid sequence was investigated by the methods we have used for the study of bacterial cytochromes *c* [14,15] and other β-lactamases [16]. The protein

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Dedicated to E.P. Abraham and M.R. Pollock, with apologies for how long they have had to wait for these results

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was denatured, by either performic acid oxidation or aminoethylation under reducing conditions, and digested with a proteinase. The resulting peptides were separated by gel filtration followed by paper electrophoresis and chromatography, and analysed quantitatively for amino acid composition and purity. Peptide sequences were investigated by the dansyl-phenylisothiocyanate method. Amide groups were mostly assigned from the electrophoretic mobilities of small peptides. The products of CNBr cleavage of the native protein have also been studied, and the N-terminal sequence of the whole protein determined in a spinning-cup automatic sequencer.

3. RESULTS

The evidence for the amino acid sequence proposed is summarized in fig.1. The peptides shown were derived from 7 independent digests. These

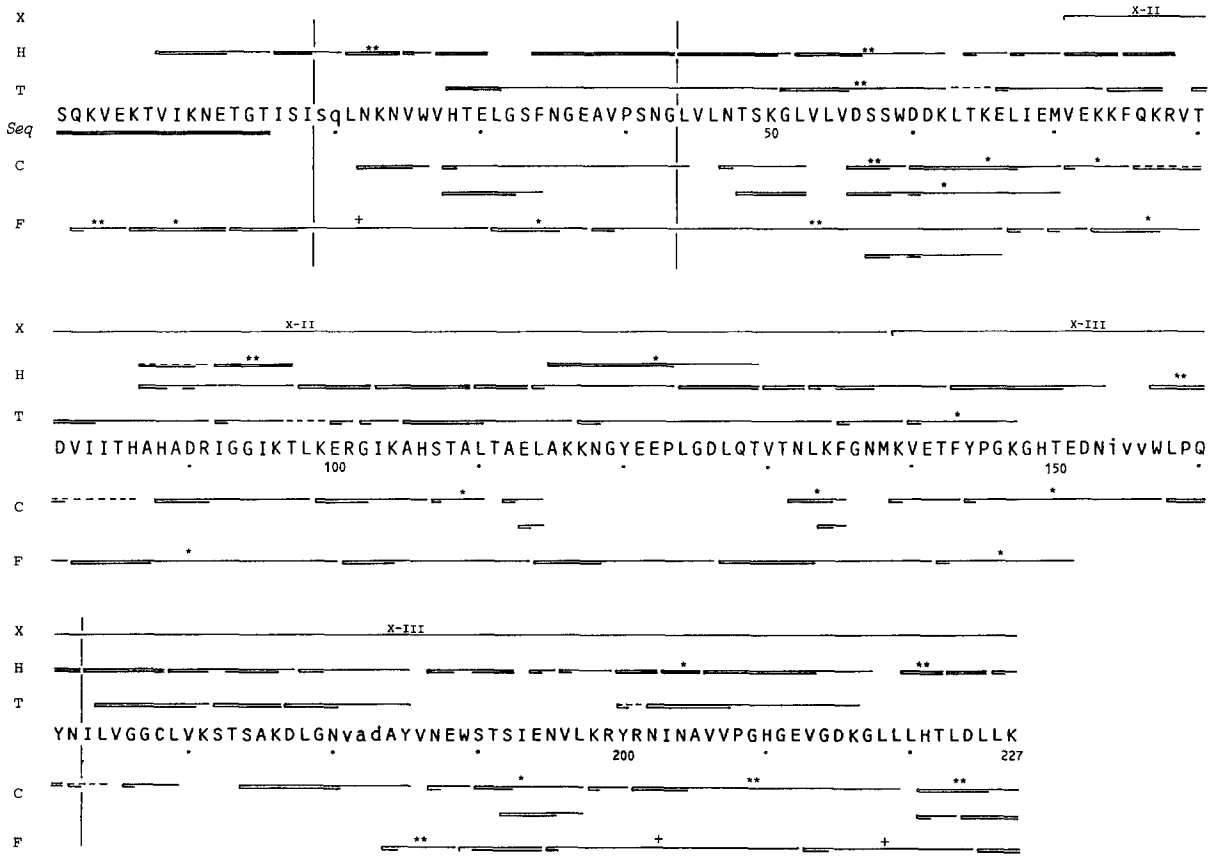

Fig.1. Proposed amino acid sequence for *Bacillus cereus* 569 β-lactamase II. Peptides derived from CNBr cleavage (X), thermolysin (H) or trypsin (T) digestion are shown above the sequence, and by automatic degradation of the whole protein (*Seq*), chymotrypsin (C) or staphylococcal proteinase (F) digestion below the sequence. Solid lines indicate peptides that have been purified and analysed quantitatively. The analyses for the CNBr peptides X-II and X-III are given in table 1. Substandard analyses [14] are marked *, and particularly bad ones ** (0.3–0.45 mol/mol of an unexpected amino acid, usually glycine and serine). Peptides that were found to be present but not isolated in a pure form are shown with dashed lines. The lines are doubled where the sequence has been determined by phenyl isothiocyanate degradation. Lower case letters indicate residues that had not been put into sequence at the time the DNA sequence determination [12] was started, and vertical lines overlaps that were then regarded as dubious. Homologous peptides from a staphylococcal proteinase digest of *Bacillus cereus* 5/B/6 β-lactamase II that have been characterized, and which strengthen the evidence for these doubtful regions are marked + (J. Fleming, M. Daniel and R.P. Ambler, unpublished work).

were: (a) 2.0 μmol oxidized protein digested with trypsin, (b) 1.8 μmol oxidized protein digested with thermolysin, (c) 1.6 μmol aminoethylated protein digested with chymotrypsin, (d) 1.2 μmol aminoethylated protein digested with staphylococcal proteinase, (e) 1.7 μmol native protein treated with 90 mg CNBr in 4 ml of 50% (v/v) HCOOH for 24 h at 28°C, (f) 1.8 μmol aminoethylated protein digested with trypsin, (g) 1.8 μmol aminoethylated protein digested with thermolysin. The denatured protein was difficult to dissolve at pH 8.5, and in digest (f) insoluble peptides appeared by the end of the incubation period. Aminoethylation was the method chosen for denaturation in most of the digests, so as to make characterization of the tryptophan-containing peptides easier. However, considerable difficulty was found in reconciling the peptides characterized from different digests with the specificities expected for endopeptidases, and it was eventually realized that the β-lactamase II preparation was contaminated with an aminopeptidase that remained active despite reduction with dithiothreitol in the presence of EDTA and 8 M urea, treatment with ethylene imine, and gel filtration into 5% (v/v) HCOOH. The effect of this contamination was the removal of one or more amino acids from the N-terminus of many peptides by the time that they were isolated. The effect was most marked for peptides with hydrophobic N-terminal sequences. In the thermolysin digest (g) the peptide N-termini found were so uncharacteristic and the amounts of free hydrophobic amino acids so large that the explanation of the phenomena was realized. Assays

Table 1

Amino acid composition of *Bacillus cereus* 569 β-lactamase II and of CNBr peptides derived from it

	Whole protein			CNBr fragments			
	Sequence	Analysis		X-II		X-III	
		[5]	[6]	Sequence	Analysis	Sequence	Analysis
Glycine	20	20.4	21.6	6	6.2	9	9.6
Alanine	11	11.4	12.5	6	5.9	4	4.2
Valine	23	25.0	22.7	4	3.9	11	10.0
Leucine	25	25.0	26.1	6	6.2	11	10.7
Isoleucine	13	12.5	12.5	5	4.5	4	3.7
Serine	13	11.4	11.4	1	1.2	4	4.1
Threonine	18	14.8	17.0	7	6.4	5	4.7
Aspartic acid	11	28.4	27.2	3	6.2	5	11.5
Asparagine	16			3		7	
Glutamic acid	16	20.4	20.4	5	6.0	5	6.0
Glutamine	5			2		1	
Phenylalanine	4	3.4	4.5	2	2.0	1	1.1
Tyrosine	5	4.5	5.7	1	1.1	4	3.6
Tryptophan	4	5.7	2.3	0	n.d.	2	n.d.
Cysteine	1	1.1	1.1	0	n.d.	1	0.4
Methionine	2	2.3	2.3	1	0.8	0	(<0.15)
Proline	5	9.1	5.7	1	0.9	3	2.5
Lysine	23	21.6	21.6	9	9.3	7	7.3
Histidine	7	5.7	6.8	3	2.8	3	2.8
Arginine	5	4.5	5.7	3	3.2	2	2.2
Total	227			68		89	

The analyses from [5] and [6] have been recalculated to fit a 227-residue protein. n.d., not determined

of the preparation using an L-leucine-*p*-nitroanilide spectrophotometric assay [17] showed detectable amounts of aminopeptidase both in the β-lactamase II preparations (1 mU/mg) and in the aminoethylated material (0.08 mU/mg). It is noteworthy that zinc-requiring extracellular aminopeptidases are known in bacteria [17].

Despite these difficulties, it was eventually possible to arrange the peptides isolated from the *B. cereus* 569 enzyme into a sequence (fig.1) which was consistent with the amino acid compositions of the whole protein and of the two out of a theoretical three CNBr fragments which had been purified (table 1). There were a small number of places where the overlap evidence was weak, or where the order of 2 or 3 amino acids had not been determined, and the sequence at the extreme N-terminus was not completely known. This sequence hypothesis was then communicated to the laboratory that had just started to clone and sequence the gene coding for the enzyme [12]. Subsequent experiments have mainly been concerned with the β-lactamase II from another strain of *B. cereus*, for which a mutant has been isolated which produces no β-lactamase I (strain 5/B/6 [11]), from which the β-lactamase II can readily be purified in large amounts, and free from aminopeptidase contamination. Homologous peptides from the 5/B/6 enzyme have been characterized that establish the weak overlaps in the 569 enzyme (fig.1), and suggest the likely sequence of the unordered amino acids. An automatic sequencer degradation has also established the N-terminal sequence of the 569 enzyme.

4. CONCLUSIONS

The results of the amino acid sequence investigation are in complete agreement with those from DNA sequencing [12]. The amino acid sequence contains several regions that were difficult to study by the methods chosen, as the peptides necessary to establish some overlaps could not be readily isolated. The evidence for the sequence was not considered strong enough to justify publication until supported by more results from the 5/B/6 enzyme. However, the completely independent DNA sequence analysis [12] has again demonstrated the general reliability of our amino acid sequencing approach, and in particular the accuracy of the amide group assignments and the absence of errors through missed or imagined small pieces of sequence.

The amino acid sequence of the β-lactamase II consists of 227 residues giving, without zinc, an M_r of 24946. This compares with previous estimates of 22000 from ultracentrifugation and SDS-PAGE [5]. The only cysteine residue is located at position 168, while the histidine residues that have been recognized as being zinc ligands [18] are found at positions 86 and/or 88 and 210. Evidence has recently been obtained that the carboxyl group of Glu-37 is catalytically essential [13].

The protein contains a pentapeptide sequence that also occurs in the class A β-lactamase of *Staphylococcus aureus* (Ile-Gly-Gly-Ile-Lys [16]), but no other sequence similarity has been detected. No similarity is apparent to the zinc-containing D-alanyl-D-alanine carboxypeptidase of *Streptomyces albus* [19] or to the N-terminal sequence of the zinc-dependent β-lactamase from *Pseudomonas maltophilia* [10].

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