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Single amino acid substitution between SHV-1 β-lactamase and cefotaxime-hydrolyzing SHV-2 enzyme

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SHV-2 β-lactamase was purified from an overproducing variant of a clinical isolate of *Escherichia coli* resistant to cefotaxime. Pure protein was digested by trypsin and Lys-C endoproteinase. Proteolytic peptides, isolated by reverse-phase HPLC, were submitted to manual Edman degradation and aligned by homology with the sequence of SHV-1 β-lactamase. A putative amino acid sequence was deduced. Structural comparison revealed that SHV-2 differed from SHV-1 by only one amino acid, Gly → Ser, at position 213 of the mature protein.

β-Lactamase; Cefotaxime resistance; Amino acid sequence

1. INTRODUCTION

β-Lactamases are bacterial enzymes that confer resistance to β-lactam antibiotics. Plasmid and chromosomally-encoded β-lactamases are numerous [1,2] and most of penicillins and cephalosporins are affected by their catalytic activity. So, the new methoxy-imino cephalosporins, such as cefotaxime, believed to be 'β-lactamase stable' antibiotics, were extensively used in therapy of clinical infectious diseases. But, rapid emergence of resistance to these broad-spectrum cephalosporins was observed in several bacterial strains overproducing a chromosomally-mediated β-lactamase [3–5]. The first report of transferrable resistance to cefotaxime in a few bacterial strains was published in 1983 [6]. Two years later, in one of these strains, the involvement of a plasmid-mediated β-lactamase capable of hydrolyzing cefotaxime and other third-generation cephalosporins was demonstrated [7]. This β-lactamase was named SHV-2, as it was closely related to a well-known SHV-1 enzyme, on the basis of biochemical studies and DNA hybridization. The authors claimed that 'only a few mutational steps can convert an SHV-1 producing strain into a strain producing a cefotaxime-hydrolyzing enzyme'. Recently we described the complete amino acid sequence of SHV-1 β-lactamase [8]. Here we report structural features of SHV-2 β-lactamase that buttress the above assumption.

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2. MATERIALS AND METHODS

2.1. *β-Lactamase purification and assay*

Escherichia coli A2302 was a clinical isolate from Tunis [9] which produced an SHV-2 β-lactamase whose genetic determinant was carried by a conjugative plasmid (Philippon, personal communication). Protein was purified, from a mutant strain overproducing the SHV-2 enzyme (Ben Yaghlane, unpublished), according to the procedure previously described for SHV-1 purification [10]. Enzymatic activity was measured by using a microacidimetric method [11]. One unit of β-lactamase activity was defined as the amount of enzyme which hydrolyzed 1 μmol of benzyl penicillin at 37°C and pH 7.0.

2.2. *Proteolytic cleavages and peptide isolation*

60 nmol of carboxymethylated protein (2 mg/ml, 0.25 M NH_4HCO_3) were digested at an enzyme/substrate ratio of 1:50 (w/w) at 37°C for 2 h at pH 8.0 with trypsin (Sigma) or for 20 h at pH 9.0 with endoproteinase Lys-C from *Lysobacter enzymogenes* (Boehringer, Mannheim). Subfractionation of tryp-

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tic peptide T10 by *Staphylococcus aureus* V8 protease (Sigma) was performed in the same buffer at pH 8.0 at an enzyme/substrate of 1:50 (w/w) for 18 h at 37°C. Peptides resulting from cleavages were purified on a 5 μm Nucleosil C18 column (4 × 250 mm), by using an LKB HPLC system, at room temperature at a flow rate of 1 ml/min, with 0.1% trifluoroacetic acid in water as solvent A and acetonitrile as solvent B. Tryptic and *S. aureus* V8 protease digests were separated by using a three-step linear gradient of solvent B (0 min, 5%; 35 min, 30%; 45 min, 45%; 60 min, 80%). With Lys-C endoproteinase digest a linear gradient of solvent B was increased from 17% to 55% within 50 min. In some instances peptides were further chromatographed at pH 6.0 on the same column, with a linear gradient from 0.25 mM ammonium acetate buffer to 0.5 mM ammonium acetate buffer/acetonitrile (2:3, v/v), within 65 min. Peptides were detected at 226 nm.

2.3. *Amino acid and sequence analyses*

Carboxymethylated protein or peptides (1–2 nmol) were hydrolyzed at 110°C under vacuum with 200 μl of 6 M HCl for 23 h. For the larger peptide T10 and protein additional analyses were performed for 48 h and 72 h. Amino acids were separated as described [8]. Manual degradation of peptides (2–10 nmol) was performed according to the Chang's double coupling method [12] as described by Allen [13] with slight modifications [8].

3. RESULTS AND DISCUSSION

On SDS-gel electrophoresis purified protein gave a single band whose mobility corresponded to an M_r value of 28000 ± 500. The first N-terminal amino acid degradations each yielded one residue with the sequence Ser-Pro-Gln-Pro-Leu-Glu-. Pure enzyme showed a specific activity of 600 U/mg and behaved as SHV-2 β-lactamase [7] in kinetic constants and analytical isoelectric focusing. The amino acid composition did not differ markedly from that of the SHV-1 protein [10] and fitted well with that deduced from the amino acid sequence analysis.

From tryptic digestion 24 peptides were recovered by reverse-phase HPLC at pH 2.0 (fig.1). Peaks 1, 3, 8, 11, 14, 17 and 21 were further rechromatographed at pH 6.0. Peptide 19 which eluted with the buffer peak was separated by additional isocratic chromatography at pH 2.0 with solvent A. After amino acid composition determinations all the peptides except T10 were fully sequenced by manual Edman degradation (fig.2). Primary structure of the 42-residue long peptide T10 was elucidated by analysis of subpeptides T10-V1, T10-V3 and T10-V5 resulting from fractionation of the parent peptide by *S. aureus* V8

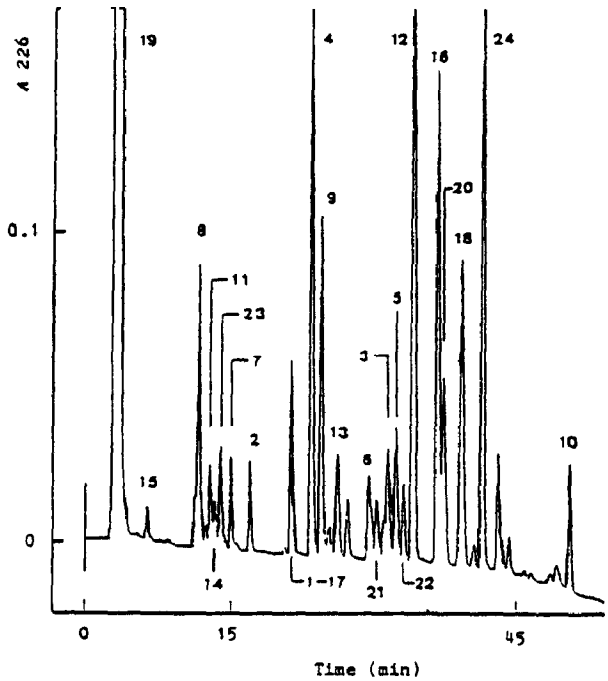

Fig.1. Separation of peptides (18 nmol) after digestion of SHV-2 β-lactamase with trypsin. Peptide mixture, adjusted to pH 2.0 with trifluoroacetic acid, was applied to a Nucleosil C18 column and eluted as described in section 2. Peptides are numbered in the order in which they occur in the sequence, starting from the N-terminus. Peptide 20 is the parent fragment of peptides 18 and 19.

protease. T10-V5 resulted from an unexpected cleavage at a threonine residue. From sequence analysis of tryptic peptides, SHV-2 and SHV-1 β-lactamases appeared quite related. So, the 24 peptides yielded by trypsin digestion of the SHV-2 protein were aligned in fig.2 according to the sequence of the SHV-1 enzyme [8]. No attempt to find spanning fragments from another proteolytic cleavage was made. However, as tryptic peptides corresponding to positions 178–180 and 216–218 were not recovered, the SHV-2 protein was further cleaved by Lys-C endoproteinase and the HPLC isolated peptides K5 and K6 were subjected to Edman degradation.

From those results we deduced the putative amino acid sequence shown in fig.2 and demonstrated that SHV-2 β-lactamase differed from the SHV-1 enzyme by one amino acid substitution, Gly ⟶ Ser at position 213. This mutation, which may result from a single nucleotide base change in DNA structure, allows

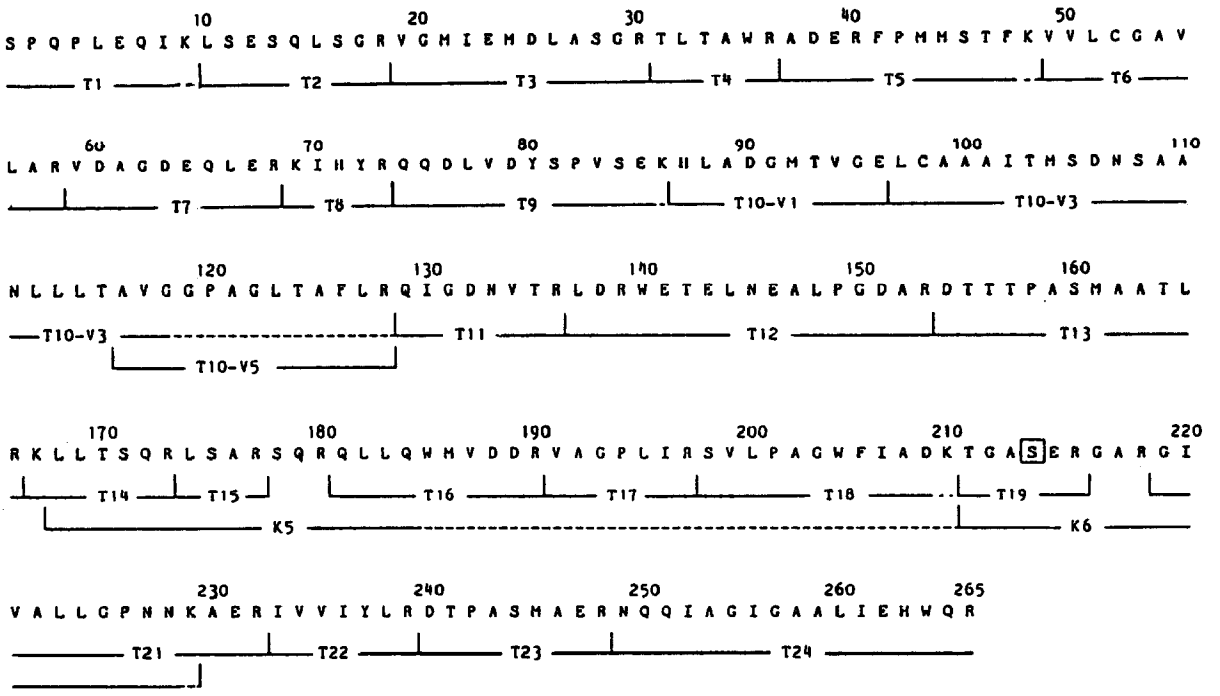

Fig.2. Putative amino acid sequence of SHV-2 β-lactamase. T and K indicate peptides derived from digestion with trypsin and Lys-C endoproteinase, respectively. T10-V designate peptides obtained from subfractionation of T10 with *S. aureus* V8 protease. Peptide alignment was supported by strong homology with the sequence of SHV-1 enzyme [8]. Residues identified by manual Edman degradation are underlined by continuous lines. Dotted lines indicate residues only checked by amino acid analysis. Boxing position 213 holds a glycine residue in SHV-1 amino acid sequence.

the new SHV-2 β-lactamase to efficiently hydrolyze cefotaxime and related compounds. Recently [14], it was demonstrated that the CTX-1 enzyme, another plasmid-mediated β-lactamase with cefotaxime activity [15], differed from the TEM-2 enzyme by only two amino acids. The substitutions occurred at positions 102 (Glu ⟶ Lys) and 236 (Gly ⟶ Ser) of the unprocessed TEM-2 protein, i.e. at positions 79 and 213 of the mature enzyme. So, it is likely that residue 213 must interfere with the active site of these β-lactamases. SHV-1 and TEM enzymes, found to be closely related to a chromosomally-mediated β-lactamase of *Klebsiella pneumoniae* [8,16], both belong to class A as defined in [17]. Recently, the crystal structure of β-lactamase from *S. aureus* PC1 strain, another class A β-lactamase, has been reported [18]. The proposed three-dimensional structure of the substrate-enzyme complex allowed a direct interaction between an alanine at position 238 and the side chain of the β-lactam antibiotic. Position 238 of the *S. aureus* enzyme corresponds to position 213 of SHV and TEM β-lactamases. Then, in the same way, residue 213 could interplay with the oxime group of cefotaxime bound to the active site. In the case of CTX-1 and SHV-2 β-lactamases, whose position 213 holds a serine residue, a new hydrogen bond may occur in the enzyme-substrate complex so that the catalytic activity towards cefotaxime antibiotic may be markedly increased.

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