Amino-terminal heterogeneity of *E. coli* TEM-β-lactamase secreted from *Bacillus subtilis*

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E.coli TEM-β-lactamase, secreted from *Bacillus subtilis* after transformation with three different hybrid plasmids, was purified and subjected to direct amino-terminal sequence analysis. The results show that the signal sequence cleavage site varies depending on the hybrid plasmid construction and cannot be exactly predicted from the DNA sequences. The results are of general interest if recombinant DNA technology is used to synthesize, e.g. pharmaceutical products where the preservation of the authentic amino-terminal structure is highly desirable.

Secretion vector *Signalase cleavage* *Hybrid protein* (Bacillus subtilis)

1. INTRODUCTION

We have previously shown that *Bacillus subtilis* is able to secrete enzymatically active *E. coli* TEM-β-lactamase (penicillin amido-β-lactamhydrolase, EC 3.5.2.6) after transformation with 3 different hybrid plasmids [1]. The plasmid pKTH78 (−1 construction) contains the complete region coding for the *B. amyloliquefaciens* α-amylase promoter and signal sequence joined to the modified structural gene of TEM-β-lactamase lacking its own signal sequence. Plasmids pKTH83 (+4 construction) and pKTH84 (+14 construction) are similar but contain in addition the parts coding for 4 and 14 amino acids from the amino terminus of mature α-amylase (see fig.4). All these constructions are able to secrete over 90% of the synthesized β-lactamase to the culture medium. The yield of β-lactamase from the +14 construction is, however, only about 20% of that produced by the −1 and +4 constructions [1]. We have previously shown that correct cleavage takes place (31 amino acids from the initiation methionine) when *B. amyloliquefaciens* α-amylase is cloned in the plasmid pUB110 and secreted from *B. subtilis* [2,3]. The size estimations of β-lactamase secreted from *B. subtilis* by the −1 and +4 vector constructions suggest signal sequence cleavage at or within a few amino acids from the original site [1]. Secreted β-lactamase from the +14 construction, however, migrates slightly faster than would be expected if the signal sequence were cleaved at the original distance from the initiation methionine.

Here, we have purified β-lactamase synthesized from the −1, +4 and +14 constructions and subjected the purified proteins to direct amino-terminal sequence analysis. The information obtained shows that the specificity of signal sequence cleavage varies depending on the construction used and cannot be exactly predicted from the deduced amino acid sequence.

2. MATERIALS AND METHODS

2.1. *Bacterial strains and culture conditions*

B. subtilis strains IH6211 (pKTH78), IH6221 (pKTH83) and IH6222 (pKTH84) were grown in SMS medium containing 1% soluble starch (Difco)

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with vigorous aeration at 37°C to early stationary growth phase. Phenylmethylsulfonyl fluoride (PMSF) and EDTA were added to a final concentration of 1 and 10 mM, respectively, and the cell suspension was cooled on crushed ice. The bacterial cells were removed by centrifugation at 4°C and the supernatant kept frozen until used for isolation of β-lactamase. For radiolabelling, a 1.0 ml aliquot was taken at early stationary growth phase and transferred to a large prewarmed test tube to which 150 μCi carrier-free [^{35}S]Cys (NEN, 600 Ci/mmol) and 50 μg L-methionine were added. After shaking for 30 min at 37°C the suspensions were treated as above and the supernatants frozen until use.

2.2. *Purification of unlabelled β-lactamase*

Unlabelled β-lactamase was purified from the culture medium of *B. subtilis* strains, harbouring the plasmids with −1, +4 and +14 constructions, by ammonium sulfate precipitation, gel filtration, high-performance anion-exchange chromatography and reverse-phase high-performance liquid chromatography (HPLC). The ammonium sulfate precipitation was performed from 50 ml culture medium, using 52% saturation at 0°C for 1 h, and followed by centrifugation at 10000 × *g* for 30 min at 4°C. The precipitate was dissolved in water and the proteins subjected to gel filtration (Bio-Gel P100, 20 mM triethanolamine acetate, pH 7.7). β-Lactamase active fractions were detected by the method of Callaghan et al. [4]. The active fractions from the gel filtration were pooled and subjected to high-performance anion-exchange chromatography (Mono Q, 0.5 × 5 cm, Pharmacia). The column was equilibrated with 20 mM triethanolamine acetate, pH 7.7, and chromatography was performed with a 15 min linear gradient of 0–0.5 M sodium acetate in equilibration buffer. The proteins were detected at 280 nm and the β-lactamase active fractions detected as above [4]. Reverse-phase HPLC was on Vydak 218TP wide pore C_{18} column (0.46 × 25 cm), using a 30 min linear gradient (0–70%) of acetonitrile in 0.1% trifluoroacetic acid. The detection was performed at 218 nm. Analytical SDS-polyacrylamide gel (12.5%) electrophoresis was carried out in the presence of 2-mercaptoethanol [5] and the gel stained with Coomassie brilliant blue.

2.3. *Purification of radiolabelled β-lactamase*

Radiolabelled β-lactamase was precipitated from the culture medium (1 ml) by trichloroacetic acid (15%, 0°C, 30 min). The precipitate was centrifuged, washed with acetone at −20°C and subjected to preparative SDS-polyacrylamide gel (12.5%) electrophoresis (SDS-PAGE) in the presence of 2-mercaptoethanol, as above. The β-lactamase was localized by autoradiography of the wet gel overnight, and electrophoretically eluted from the gel slice by using an ISCO model 1750 eluator [6]. The eluted β-lactamase was precipitated with trichloroacetic acid (15%, 0°C, 30 min).

2.4. *Amino-terminal sequence analysis*

For amino-terminal sequence analysis, purified unlabelled and [^{35}S]Cys-labelled proteins were degraded manually [7]. Released amino acid derivatives were converted to the corresponding phenylthiohydantoin derivatives (90 μl of 25% trifluoroacetic acid containing 0.01% dithiothreitol, 25 min at 55°C, drying with nitrogen) and dissolved in 50 μl methanol. For unlabelled proteins 20 μl of the sample from each cycle was analyzed on a Varian 5020 high-performance liquid chromatograph (flow 1.0 ml/min, 41°C, detection at 269 nm), using a Spherisorb S5 ODS2 column (0.46 × 25 cm) and a gradient of acetonitrile (24–44% in 6 min, then isocratic) in 40 mM sodium acetate, pH 4.9 [8]. In radio-sequence analysis 40 μl of the sample from each cycle was measured for radioactivity after addition of 4 ml Insta-Gel (Packard) and the remaining 10 μl analyzed by HPLC to check the performance of the degradation cycles.

3. RESULTS AND DISCUSSION

3.1. *Purification and amino-terminal sequence analysis of unlabelled β-lactamase*

Unlabelled β-lactamase from all 3 constructions (−1, +4 and +14) was purified essentially by the same procedure, based on ammonium sulfate precipitation, gel filtration, high-performance anion-exchange chromatography and reverse-phase HPLC. About 99% of the enzyme activity was recovered in the ammonium sulfate precipitation step, which also resulted in some purification of β-lactamase, as shown in fig.1A and B. The

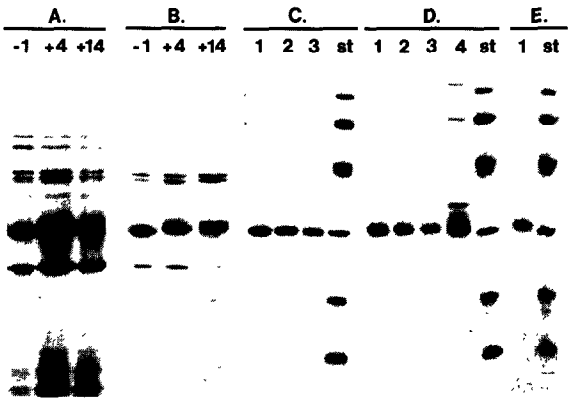

Fig.1. Analytical SDS-polyacrylamide gel (12.5%) electrophoresis in the presence of 2-mercaptoethanol. (A) Proteins precipitated with trichloroacetic acid from 500 μl culture medium of *B. subtilis*, harbouring the secretion vector constructions −1, +4 and +14; (B) proteins precipitated with ammonium sulfate from 250 μl culture medium as previously; (C,D,E) proteins obtained from β-lactamase active peaks (numbers refer to the corresponding peaks in fig.2), when samples from constructions −1, +4 and +14, respectivesly, were subjected to high-performance anion-exchange chromatography. A standard protein mixture (st) containing phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and α-lactoglobulin was run in lanes as indicated.

following gel filtration step on Bio-Gel P100 was mainly to remove residual ammonium sulfate from the dissolved precipitate, and to change the buffer to the equilibration buffer used in the following purification step. High-performance anion-exchange chromatography of samples from constructions −1 and +4 gave several β-lactamase active peaks, whereas a single active peak was obtained from the +14 construction (fig.2). Analytical SDS-PAGE of the multiple active peaks from the −1 and +4 constructions, however, gave protein bands with essentially the same mobility for each construction (fig.1C,D), excluding the possibility that the multiple peaks represent enzymatically active β-lactamase fragments of different sizes. The result also shows that β-lactamase from the +4 construction migrates slightly more slowly than that from the −1 construction. All the multiple active peaks were also essentially pure β-lactamase, as judged from the SDS-polyacrylamide gel. The sample from the +14 construction gave only one β-lactamase active peak in anion-exchange chromatography (fig.2). On SDS-PAGE this peak gave a single protein band (fig.1E) migrating slightly more slowly than β-lactamase from the +4 construction. At this stage we cannot explain the reason for the occurrence of the multiple peaks. β-Lactamase from the construction

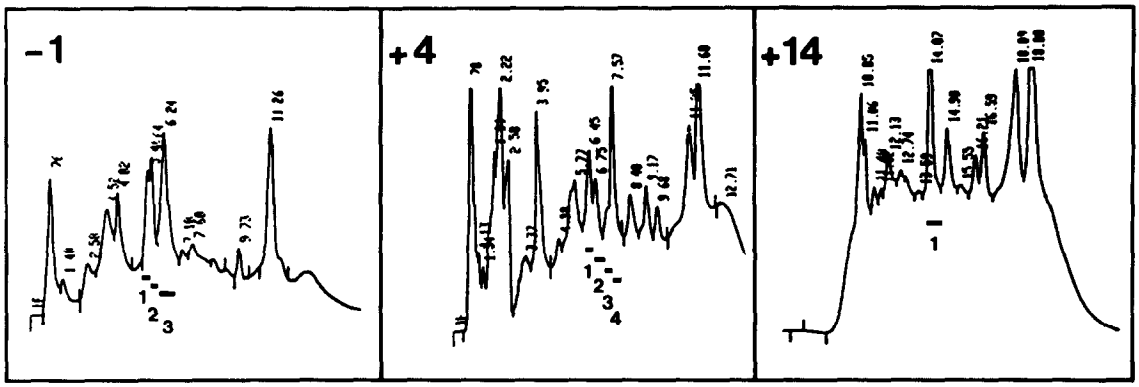

Fig.2. High-performance anion-exchange chromatography of pooled β-lactamase active fractions from Bio-Gel P100. Proteins were separated on a Mono Q column using a 15 min linear gradient of 0–0.5 M sodium acetate in 20 mM triethanolamine acetate, pH 7.7. The gradient was started at the time of injection (−1 and +4 constructions) or 5 min after injection (+14 construction). The flow rate was 1.0 ml/min, and the detection at 280 nm. The panels show chromatograms of samples originating from the constructions −1, +4 and +14. β-Lactamase active fractions, as detected by the nitrocefin reaction, are indicated by numbered bars. Numbers refer to lanes in fig.1C–E.

−1 behaves as if it had 3 different net charges, and that from construction +4 as if it had 4. This charge difference is due neither to heterogeneity in their amino termini (see later) nor to the size (fig.1C,D). It is possible that these molecules differ in their glutamic acid/glutamine and aspartic acid/asparagine content, or that the protein exists in several conformations, resulting in different net charges at the protein surface.

All β-lactamase active peaks from anion-exchange chromatography (fig.2, peaks 1–3 from construction −1, peaks 1–4 from construction +4 and peak 1 from construction +14) were further analyzed and purified by reverse-phase HPLC. Peaks with identical elution positions (24.5 min) were obtained from all samples, as shown for the analyzed anion-exchange peak 1 from construction +4 as an example (fig.3). Thus, reverse-phase HPLC does not show any difference in the hydrophobicity of the purified β-lactamases.

The β-lactamase peak from each reverse-phase separation was collected and subjected to amino-terminal sequence analysis, the results being shown in fig.4. The samples originating from the −1 construction all gave the same result (fig.4A). Two amino acid derivatives were released in each degradation cycle, indicating that the samples contained a mixture of two cleavage products of the same precursor, differing from each other by two amino-terminal amino acids. In the −1 construction the original α-amylase signal sequence

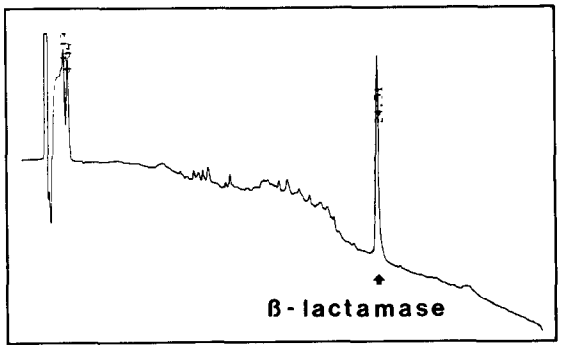

Fig.3. Reverse-phase HPLC of one β-lactamase active peak from anion-exchange chromatography. An aliquot of peak 1 (+4 construction, fig.2) was injected. Separation was on a Vydak 218 TP column (0.46 × 25 cm), using a 30 min linear gradient of acetonitrile (0–70%) in 0.1% trifluoroacetic acid. The flow rate was 1.0 ml/min and the detection at 218 nm.

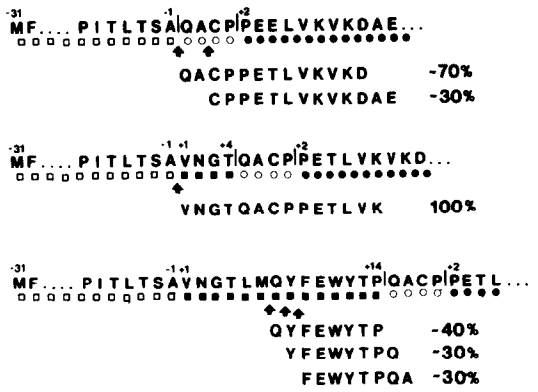

Fig.4. Results of the amino-terminal sequence analysis of β-lactamase from secretion vector constructions −1, +4 and +14. All enzymatically active fractions from anion-exchange chromatography were further purified by reverse-phase HPLC and individually subjected to Edman degradation. Arrows show processing sites obtained in the amino acid sequences deduced from the vector constructions. Amino acids derived from (□) α-amylase signal sequence, (■) α-amylase structural gene, (○) linker and (●) β-lactamase structural gene regions in the constructions are shown. (A) −1 construction: the heterogeneous structure shown, consisting of two sequences in indicated proportions, was obtained for all 3 analyzed preparations. (B) +4 construction: the single amino acid sequence shown was obtained for all 4 analyzed preparations. (C) +14 construction: the heterogeneous structure consisting of 3 sequences was obtained for the reverse-phase purified preparation.

cleavage site Ala-Val is changed to Ala-Gln. It seems that the cleaving enzyme(s) predominantly recognizes and hydrolyzes this site, but also the Ala-Cys bond located two amino acid residues later in the sequence.

All 4 samples originating from the +4 construction gave identical results. A single pure sequence (fig.4B) was obtained, indicating that the cleaving enzyme recognizes and hydrolyzes the original α-amylase signal sequence cleavage site also present in this construction.

β-Lactamase from the +14 construction gave a heterogeneous amino-terminal structure (fig.4C), corresponding to cleavage products of the same molecule but differing from each other by one and two amino acids, respectively. The different molecules were present in the mixture in approximately equimolar amounts. The sequences obtained could be localized on the deduced sequence, indicating cleavage sites as shown in fig.4C. In-

terestingly, the +14 construction codes for a protein where the original α-amylase signal sequence cleavage site Ala-Val, as well as the site Ala-Cys cleaved in the −1 construction, are present. However, no active β-lactamase indicating cleavage at these sites could be found from the +14 construction.

3.2. *Radiosequence analysis*

The results with the −1 and +4 constructions were also confirmed by radio-sequence analysis. β-Lactamase from all 3 constructions was labelled with [^{35}S]Cys, an amino acid available in high specific activity and coded by the linker nucleotide sequence used to join the α-amylase and β-lactamase genes in the constructions. β-Lactamase from the +14 construction was poorly labelled, and was thus excluded from the radio-sequence analysis. However, on analytical SDS-PAGE after autoradiography, it gave a band with the same relative mobility to the other β-lactamases (not shown) as that which was obtained in the analysis of the unlabelled ones. Radiolabelled β-lactamase from constructions −1 and +4 was purified from 1 ml culture medium by preparative SDS-PAGE.

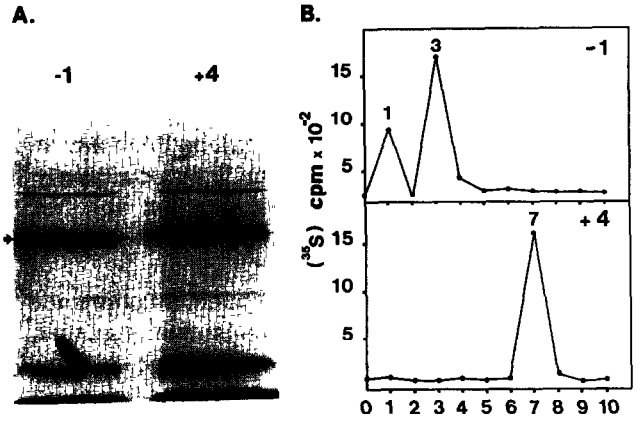

Fig.5. (A) Preparative SDS-polyacrylamide gel (12.5%) electrophoresis of [^{35}S]Cys-labelled proteins from the growth medium of *B. subtilis* harbouring the plasmids with secretion vector constructions −1 and +4. β-Lactamase was detected by autoradiography at positions shown by an arrow and eluted from the gel. (B) Radiosequence analysis of purified [^{35}S]Cys labelled β-lactamase. Results show recovery of ^{35}S radioactivity in Edman degradation cycles of β-lactamase from constructions −1 and +4. 0-cycle was identical to the others but without addition of phenylisothiocyanate.

The protein was detected from the gel by autoradiography, as shown in fig.5A, eluted from the gel, and precipitated together with 10 nmol carrier apomyoglobin. The radiochemically pure protein was then subjected to 10 cycles of Edman degradation. 80% of the sample from each cycle was counted for radioactivity with results as shown in fig.5B. The remaining 20% of the sample was analyzed on HPLC to control the degradation cycles. The radio-sequence results obtained fully correlate with the results obtained for purified unlabelled proteins.

Preservation of the authentic N-terminus is highly desirable when, for instance, pharmaceutical products are synthesized. As shown here it is not possible to predict signal peptidase cleavage site from the DNA sequences, a fact that places even more emphasis on the N-terminal amino acid sequencing. Since *Bacillus* is known to have more than one type of processing, care should be taken in interpreting the results. It cannot be ruled out that, in addition to the primary signal peptidase cleavage of the hybrid protein, secondary processing may take place during secretion or in the culture medium.

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