

Unexpected influence of a C-terminal-fused His-tag on the processing of an enzyme and on the kinetic and folding parameters

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Abstract The addition of a poly-His C-terminal extension, designed to facilitate the purification of the protein, to the β -lactamase of a thermophilic *Bacillus licheniformis* strain modified the site of action of the signal peptidase. This resulted in the secretion of a protein with a different N-terminus, showing that this type of protein engineering might not always be as 'neutral' as generally assumed.

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Key words: His-tag; β -Lactamase; *Bacillus*; Thermophile

1. Introduction

The addition of poly-His terminal extensions, designed to allow the easy purification of the target protein on a metal-chelating gel, has become a standard practice in molecular biology. In this study, we produced and purified a β -lactamase from a thermophilic *Bacillus licheniformis* strain [1] with and without a poly-His C-terminal extension and observed an unexpected modification of the cleavage site of the signal peptide in the latter.

2. Materials and methods

2.1. Recombinant DNA techniques, bacterial strains, plasmids and growth conditions

The procedures used were essentially those described by Sambrook et al. [2]. The BS3 strain, isolated from a traditional hot bath in the Meknes area (Morocco) was grown in liquid M4 medium at 50°C [1]. The genomic DNA was extracted as described in [3]. The cloning experiments were performed in *E. coli* RRI or Top 10 F' as hosts [4,5] using the pUC_{BM}20 vector [6,7] from Boehringer (Mannheim, F.R.G.). The *E. coli* overexpression strains BL21DE3 or HMS174DE3 [8] were from Novagen, Oxon, UK. The ampicillin resistance gene of the pET22b plasmid (Novagen) was inactivated by insertion of a Kanamycin resistance cartridge (Pharmacia, Roosendaal, The Netherlands) at the *Pst*I site. The resulting vector (pET22bK^r) was used to overproduce the BS3 β -lactamase.

Kanamycin, chloramphenicol or ampicillin were added to growth media at 50 μ g ml⁻¹, 30 μ g ml⁻¹ or 50 μ g ml⁻¹, respectively.

2.2. Identification of the strain

Identification of the *Bacillus* BS3 strain was performed at the BCM/MLG Culture Collection, Laboratorium voor Microbiologie, Universiteit Gent, Belgium, on the basis of an ARDRA test [9], a fatty acid analysis and an API test. All the criteria identified the

isolate as a *Bacillus licheniformis* strain with excellent identification scores.

2.3. Oligonucleotides and PCR experiments

The oligonucleotides were purchased from Eurogentec (Liège, Belgium). Their sequences were deduced from that of the *B. licheniformis* 749/Cbl gene obtained by Neugebauer et al. [10]. Further details on the molecular biology and sequencing techniques are available from the authors.

2.4. Protein purification and enzyme chemistry

The Q-Sepharose and Mono-S columns were from Pharmacia (Uppsala, Sweden) and the Ni²⁺-nitrilotriacetate-agarose column from Affiland (Ans, Belgium). The kinetic parameter were determined as described previously [1,11]. All experiments were performed at 30°C in 50 mM sodium phosphate, pH 7. Thermal unfolding curves were obtained as previously described [12] in 50 mM sodium acetate (pH 5), 50 mM sodium phosphate (pH 7) and 50 mM glycine/NaOH (pH 9) buffers.

2.5. Internal peptide isolation

After gel electrophoresis of a 30 pmol sample, the enzyme was transferred to a nitrocellulose membrane with the help of a Bio-Rad Semi-dry DB Trans-Blot SD and hydrolysed by the Lys-C endoprotease (10% w/w) during 24 h at 37°C. The peptides were purified by HPLC on a Reverse Phase column and the sequence determined with 10 pmoles of each peptide.

2.6. Sequence analysis

N-Terminal sequence analysis was carried out on a pulsed-liquid sequencer with on-line analysis of the PTH-amino acids (Perkin Elmer, Applied Biosystems Division, Foster City, CA). C-Terminal sequencing was performed on a Procise 494C sequencer (Perkin Elmer) using a slight modification of the protocol described by Boyd et al. [13]. The alkylated thiohydantoin were identified by reversed phase HPLC [13]. Prior to sequence analysis, the lysine side chains were modified with phenylisocyanate under basic conditions [14].

2.7. Mass spectrometry

Electrospray ionisation mass spectrometry was carried out on a Bio-Q quadrupole mass spectrometer (Micromass, Altrincham, UK). Ten microliters of a sample solution in 50% acetonitrile/1% formic acid were injected manually in the 20 μ l loop of the Rheodyne injector and pumped with a Harvard syringe pump (South Natick, MA) at a flow rate of 5 μ l/min. Scans of 9 s over the mass range of 600–1500 a.m.u. were collected over 2 min. Mass calibration was done with horse heart cytochrome c.

2.8. Crystallographic techniques

The initial crystallization conditions for the rBS3 β -lactamase were determined using the Gridscreen, Crystal Screen and Crystalscreen II sparse matrix crystallization screening kits (Hampton Research).

The best crystals were obtained using the hanging drop vapour diffusion method from drops containing 5 μ l of protein solution (10 mg/ml) and 5 μ l of 25% PEG 6000 in 100 mM sodium acetate buffer pH 5.0, equilibrated against 1 ml of the latter solution at 20°C.

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Diffraction intensities were collected using a Siemens X1000 area detector system using FRAMBO/ASTRO collection software. The X-ray source was a graphite-monochromated CuK α radiation produced by a Rigaku RU-200 rotating-anode generator operating at 40 kV and 80 mA. The detector was positioned at 130 mm from the crystal. Intensities from 500 frames were recorded. In a single frame, reflections resulting from a 0.3° oscillation of the crystal were recorded in 90 s. A single crystal yielded a complete dataset with a total of 40 503 observations to 2.4 Å. Data evaluation was done with SAINT [15].

3. Results

3.1. Cloning and sequencing

Since the N-terminus of the enzyme purified from the culture supernatant of *Bacillus* BS3 appeared to be blocked, the protein was digested with the Lys-C endopeptidase. Four internal peptides were sequenced and found to correspond to analogous peptides in the *B. licheniformis* 749/C β -lactamase with only two mutations.

In consequence, a PCR experiment was performed with 1.5 μ g of genomic BS3 DNA and primers corresponding to nt 392–410 and 1167–1186 in the sequence of the *B. licheniformis* 749/C gene, yielding the expected 820-bp product. Several templates were completely sequenced on both strands. The sequence, available under the Y10006 accession number in the EMBL bank revealed 36 mutations when compared to that of the 749/C gene, generating the 7 following modifications in the primary structure of the protein: A59T, A133T, V187A, R191Q, D227E, A238G and M287V (ABL numbering, [16]). Two of these (A133T and A238G) are located in the vicinity of the active site (Fig. 1).

3.2. Overproduction

For overproduction and purification of the protein, two strategies were utilized. The protein was produced both with and without a C-terminal (His)₆-tag. PCR reactions yielded the genes encoding respectively the protein with the wild-type C-terminus or with an eleven residue A₃LEH₆ C-terminal extension, as a result of cloning between the *Mse*I and *Not*I restriction sites in pET22bK⁺. In both cases, the signal peptide was that of pullulanase (*pelB*) present in the expression plasmid and the first residue of the periplasmic, mature protein was expected to be Lys²⁶. Good production was obtained with BL21DE3 cells grown at 37°C, induced at an A₆₀₀ value of 1.0 with 1 mM IPTG during 3 h. About 10 mg of enzyme exhibiting the wild-type C-terminus were purified to homogeneity by Q-Sepharose chromatography at pH 7.2 from 1 liter of culture. The rBS3 protein exhibited the expected N-terminal sequence (KTEMKDDEAK) and the *M_r* value measured by ESMS was 29 476.8 $\pm$ 2 in excellent agreement with that of the 265 residue polypeptide deduced from the gene sequence (29 475). The periplasmic fraction containing the His-tagged protein was purified on Ni²⁺-NTA agarose but only 50% of the enzymatic activity was retained by the column. A new periplasmic extract was purified by Q-Sepharose chromatography, yielding upon SDS-PAGE, a doublet of proteins with very similar apparent *M_r* values, which could be separated as two fractions of identical specific activities by FPLC on a Mono S column at pH 4.5. The two proteins exhibited identical N-terminal sequences (AAQPAMAKTEM) where the first seven residues corresponded to the seven last residues of the *pelB* signal peptide. This indicated an incorrect N-ter-

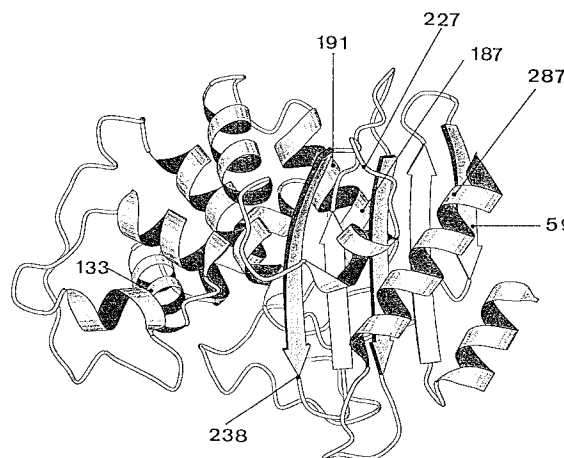


Fig. 1. Alpha-carbon trace (Molscript, [19]) of the *B. licheniformis* 749/C β -lactamase (PDB entry code 4BLM). The positions of the residues which are different in the BS3 are labelled.

минаl processing of the proteins and suggested a heterogeneous C-terminus.

C-Terminal sequencing of the first fraction (A) revealed the sequence –MNGKAAAL with some contamination (about 20%) by the sequence –MNGKA. C-Terminal sequence analysis of the second fraction (B) also yielded the above major sequence, but with a much lower initial yield than for fraction A (13 vs 143 pmoles). However, a Glu residue was also detected at degradation step 7. Since the chemistry of the C-terminal sequence analysis so far does not allow to reproducibly identify the His residue, it was concluded that the Glu could only be the residue preceding the 6 histidines of the His-tag sequence and that this was the major polypeptide in fraction B. This conclusion is corroborated by the fact that the major peaks in the electrospray spectra of fractions A and B differed by 952.0 Da which is nearly identical to the theoretical mass of the sequence EH₆ (951.0 Da).

3.3. Properties of the recombinant enzymes

Within the limits of experimental errors, the *K_m* values for a set of 2 penicillin and 5 cephalosporin substrates were not significantly different from those of the original enzyme. The *k_{cat}* values were 2–3-fold higher with the recombinant proteins, which could be explained by a loss of activity of the WT enzyme upon storage of rather dilute solutions, a situation which often results in progressive denaturation. The kinetic properties of the rBS3 enzyme were also very similar to those of the 749/C β -lactamase, the only significant differences being observed with cefazolin (6-fold decrease of *k_{cat}/K_m*), 7-amino cephalosporanic acid and cefotaxime (6- and 10-fold increase, respectively).

Thermal unfolding, monitored at pH 5, 7 and 9 was fully reversible in all cases. The rBS3 protein exhibited a *T_m* value of 64.7°C, very close to that (64.4°C) observed with the WT enzyme. The two other proteins with the N- and C-terminal extensions were somewhat less stable, exhibiting *T_m* values lowered by 1.4 to 3.0°C depending upon the conditions.

3.4. Preliminary crystallographic data

Crystals of the rBS3 protein grew as thin and twinned plates or as hollow elongated needles. Larger prisms (0.6 $\times$ 0.15 $\times$ 0.15 mm) diffracting to 2.4 Å were obtained by

adding 1% of *n*-octyl- β -D-glucoside. X-ray data collected with the area detector and processed with the SAINT software indicated that the crystals belong to space group $P2_12_12_1$ with unit-cell dimensions $a=54.97$ Å, $b=87.03$ Å and $c=110.76$ Å. The *R* factor for merging data was 11.9%. The structure of the BS3 β -lactamase will be solved by molecular replacement, using the refined structure of the *B. licheniformis* 749/C β -lactamase (PDB entry code 4BLM) as starting model.

4. Discussion

The properties of the rBS3 enzyme were very similar to those of the *B. licheniformis* 749/C β -lactamase [11]. The rBS3 protein was somewhat more heat-stable than its 749/C counterpart ($\Delta T_m = 1.5^\circ\text{C}$), which makes it the most heat-stable serine β -lactamase isolated so far. Solving the 3D structure of the rBS3 enzyme might supply clues to the understanding of these small differences.

The addition of a C-terminal poly-His tag, designed to facilitate the purification, resulted in a modified processing of the N-terminus of the protein. As shown by Fig. 1, the N- and C-termini of the folded protein are very close to each other and the C-terminal extension clearly affects the site at which the signal peptidase exerts its activity. This also demonstrates that the latter enzyme liberates the soluble extracellular protein after it has acquired a native or near native structure. The observed heterogeneity of the C-terminal extension could be due to a periplasmic protease, specific for non-polar C-termini and designated Tsp [17] or Prc [18]. The addition of a poly-His tag has become a rather common practice in molecular biology, most often when the target protein is produced in small quantities and difficult to purify. Our results should serve as a warning that this addition might not always be as 'neutral' as usually assumed. In the present case, the modified processing did not seriously influence the functional properties of the protein, but this should not be taken as granted. In consequence, a careful comparison of the wild-type and 'His-tagged' proteins remains necessary to avoid the dangers of blindly assuming that the latter always behave exactly as the former.

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