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Expression, purification, crystallization and preliminary X-ray analysis of the cathelicidin motif of the protegrin-3 precursor

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Numerous precursors of antibacterial peptides with unrelated sequences share a similar prosequence which belongs to the cathelicidin family of proteins. The three-dimensional structure of this cathelicidin motif, which contains two disulfide bonds, has not yet been reported. The cathelicidin motif (ProS) of the protegrin-3 precursor was overexpressed in *Escherichia coli* as a His-tagged protein. The His₆ tag was removed by thrombin cleavage. ProS was purified to homogeneity and single crystals were obtained by the hanging-drop vapour-diffusion method at pH 3–4. Preliminary X-ray diffraction analysis indicated that these crystals belong to the hexagonal space group *P*6₂22 or *P*6₅22, with unit-cell parameters $a = b = 51.42$, $c = 134.25$ Å. These crystals diffracted beyond 2.75 Å (1.9 Å at ESRF) and contain one molecule per asymmetric unit.

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1. Introduction

Numerous precursors of antibacterial peptides with unrelated sequences, including the protegrin precursors, share a similar prosequence of 96–101 residues and belong to cathelicidin family (Gennaro *et al.*, 1998; Gennaro & Zanetti, 2000; Ritonja *et al.*, 1989; Scocchi *et al.*, 1997; Storici *et al.*, 1996; Wang *et al.*, 1998; Zanetti *et al.*, 1995, 2000; Zhao *et al.*, 1994). Protegrins (PG-1 to PG-5, 16–18 residues) are a family of five antibacterial peptides isolated from porcine leucocytes (Kokryakov *et al.*, 1993). They are initially synthesized as a 149-residue precursor (147 residues for PG-2) devoid of antibacterial activity, in which three regions have been identified: the signal peptide (sequence 1–29), the prosequence (sequence 30–130) and the protegrin sequence (sequence 131–148 for PG-1 and PG-3). The Gly149 is removed in a well known amidation step (Merkler, 1994; Suzuki *et al.*, 1990; Zhao *et al.*, 1997). The protegrin prosequence, referred to as ProS, contains four cysteines engaged in two disulfide bonds. Their 1–2, 3–4 arrangement was clearly established for probactenecin-7, a bovine cathelicidin (Storici *et al.*, 1996). Taking into account the high degree of sequence identity between the probactenecin and ProS sequences (74–78%) and the alignment of the four cysteines, the disulfide-bond arrangement of ProS was assumed to be identical to that of probactenecin. Therefore, the two disulfide bonds of ProS are Cys85–Cys96 and Cys107–Cys124. The detailed three-dimensional structure of this widespread cathelicidin motif is not yet known. To determine the structure of the cathelicidin motif of the protegrin-3 precursor,

ProS was overexpressed in *E. coli*. Here, we report the overexpression, purification, crystallization and preliminary X-ray diffraction analysis of the recombinant ProS.

2. Materials and methods

2.1. Overexpression and purification of the His-tagged ProS

A PCR product containing the coding sequence of ProS was generated from the plasmid pBluescript including the pro-protegrin-3 cDNA (Panyutich *et al.*, 1997). The cDNA encoding for the ProS sequence (residues 30–130) was subcloned into the *Nde*I/*Bam*HI sites of the pET-15b plasmid vector (Novagen) leading to the N-terminus in frame fusion with the His tag. *E. coli* strain BL21(DE3), transformed with the recombinant plasmid, was grown in 400 ml of medium (20 g l⁻¹ tryptone, 10 g l⁻¹ yeast extract, 5 g l⁻¹ sodium citrate, 5 g l⁻¹ KH₂PO₄ adjusted to pH 7.0) containing 100 mg l⁻¹ ampicillin. After overnight growth at 310 K, the culture was used to inoculate 4 l of the same medium supplemented with ampicillin (100 mg l⁻¹), MgSO₄ (10 mM), glucose (5 g l⁻¹), biotin (1 mg l⁻¹), thiamine (10 mg l⁻¹) and nicotinamide (10 mg l⁻¹) and grown at 310 K for 3 h. Expression was induced for 4 h by addition of 1 mM IPTG. The cells were harvested by centrifugation for 15 min at 8000 rev min⁻¹. Cells, 20 g wet weight, were resuspended in 50 ml of ice-cold buffer A (100 mM Tris–HCl pH 7.5, 150 mM NaCl) containing 5 mM benzimidazole. The mixture was homogenized and frozen/thawed three times. The lysate was

Table 1
Data-collection statistics.

Values in parentheses refer to data in the last resolution shell (2.80–2.75 Å).

Space group	<i>P</i> ₆ ,22 or <i>P</i> ₆ ,22
Unit-cell parameters (Å)	
<i>a</i> = <i>b</i>	51.42
<i>c</i>	134.25
Resolution range (Å)	23–2.75
Asymmetric unit content	Monomer
Total No. of reflections	21019
No. of unique reflections	3110
Redundancy	6.6
Average <i>I</i> /σ(<i>I</i>)	16.1 (3.6)
Completeness (%)	91.3 (58)
<i>R</i> _{merge} † (%)	6.2 (28.6)

$$\dagger R_{\text{merge}} = \sum |I_h - \langle I_h \rangle| / \sum I_h.$$

kept on ice and probe-sonicated for 1 min with 0.1 s bursts at 340 W. Particulate material was then removed by centrifugation at 20 000*g* for 30 min at 277 K. The supernatant was further clarified by centrifugation at 90 000*g* for 30 min and applied to a Q-Sepharose (Amersham Pharmacia Biotech) column (20 × 2.6 cm) equilibrated with buffer A.

The flowthrough containing the protein was applied to an Ni-NTA Superflow (Qiagen) column (2 × 2.5 cm) equilibrated with buffer A. The column was washed with 30 ml of buffer A, 50 ml of buffer A containing 1 M NaCl and then re-equilibrated with buffer A. The protein was eluted with 0.5 M imidazole in the above buffer and 10 ml fractions of eluent were analyzed by Coomassie-staining SDS-PAGE gels. The protein-containing fractions 1–4 were pooled and concentrated 20 times using an Ultrafree (Millipore) filter with a MW cutoff of 5000 Da. Imidazole was removed by

several concentration/dilution cycles with buffer A. The concentration of the sample was estimated by UV–visible spectroscopy using a calculated extinction coefficient of 2800 mol^{−1} cm^{−1} at 280 nm.

2.2. His-tag cleavage and purification of ProS

The protein was digested at 298 K in the presence of 2 mM CaCl₂ with 10 units of thrombin per milligram of protein. The cleavage was complete after 1 h. The protein was purified by gel filtration on a Sephadex HR-100 (Pharmacia Biotech) column (100 × 1.6 cm) equilibrated with buffer B (50 mM sodium phosphate, 50 mM NaCl pH 7.0). 1 ml fractions were collected at a flow rate of 0.6 ml min^{−1}. The protein was eluted as a single peak and the corresponding fractions pooled and concentrated. About 10–12 mg of purified ProS were obtained from 20 g wet weight of cells. Purity was controlled by Coomassie-staining SDS-PAGE gels, ¹H NMR and mass spectrometry (Fig. 1).

2.3. Crystallization

The hanging-drop vapour-diffusion method was used to screen and to optimize the crystallization conditions. The drops contained equal volumes (1 µl) of ProS protein solution (35 mg ml^{−1} in 10 mM Tris–HCl buffer pH 7.0) and reservoir solution. The optimized reservoir solution consisted of 1.2–1.4 M ammonium sulfate and 0.1 M sodium citrate buffer at pH 3.8. Two types of hexagonal diffraction-quality crystals were reproducibly obtained within a week at 291 K. From drops containing 1.2 M ammonium sulfate reservoir solution, crystals reached dimensions of about 0.2 × 0.1 × 0.05 mm, whereas larger crystals were obtained from drops containing 1.4 M ammonium sulfate reservoir solution (Fig. 2). Prior to data collection, crystals were transferred to cryoprotectant mother liquor supplemented with 30% glycerol and flash-frozen.

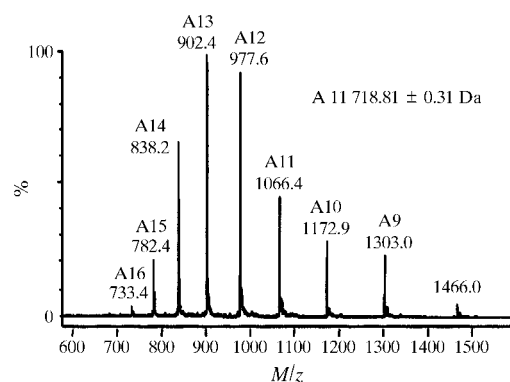
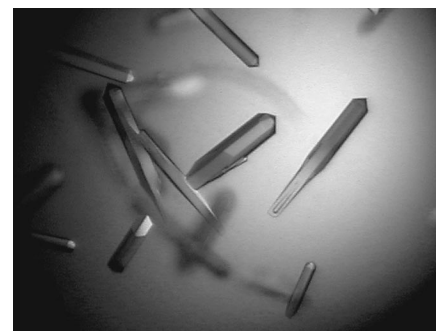


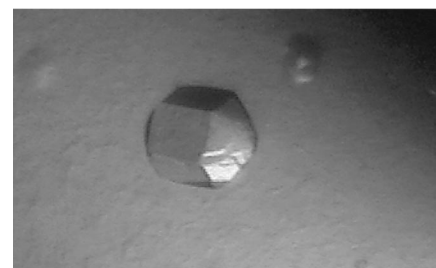
Figure 1
Molecular mass spectrum of the recombinant ProS. Mass analysis of the recombinant ProS was performed using the electrospray mass spectrometry technique (ES-MS) on a VG Bio-Q quadrupole with a mass range of 4000 Da (Bio-Tech, Manchester, England) in the positive mode. The protein was desalted on Zip-Tip (Millipore) and 10 pmol were used for mass analysis. The calibration was performed using the multiply charged ions produced by a separate introduction of horse heart myoglobin (16 951.4 Da).

90 mA. The crystal-to-detector distance was 140 mm and the oscillation range was 0.5° per film (25 min exposure time). The images were processed and scaled with the *HKL* suite of programs (Otwinowski & Minor, 1997). The autoindexing procedure indicated that the two types of crystals belong to a primitive hexagonal space group, with unit-cell parameters *a* = *b* = 51.42, *c* = 134.25 Å. The lattice was assigned to one of the enantiomorphic space groups *P*₆,22 or *P*₆,22. Considering a molecular weight of 11 718 Da for the protein, the Matthews coefficient was determined to be 2.19 Å³ Da^{−1}, assuming one molecule per asymmetric unit, with a corresponding solvent content of 44%. Table 1 summarizes the data-collection statistics. While good-quality data could be collected to 2.75 Å resolution on a conventional X-ray source, the same crystal diffracted beyond 1.9 Å resolution using synchrotron radiation (ESRF, Grenoble, FIP beamline).

Initial attempts to solve the structure by molecular replacement using various structures supposed to share a similar global fold (PDB entries 1cew, 1stf, 1dvd, 1mnl, 1eqk, 1g96) as search models have so far proven unsuccessful. Screening for heavy-atom derivatives is now under way.



(a)



(b)

Figure 2
Photomicrographs of the two types of crystals obtained from drops containing (a) 1.2 M and (b) 1.4 M ammonium sulfate of the recombinant ProS overexpressed in *E. coli*. Typical dimensions are about 0.1 × 0.1 × 0.08 mm and 0.2 × 0.05 × 0.05 mm for A and B, respectively.

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