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Crystallization and preliminary X-ray diffraction of the surfactant protein *Lv*-ranaspumin from the frog *Leptodactylus vastus*

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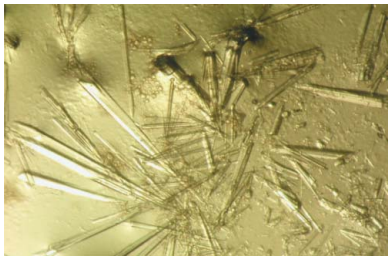

Lv-ranaspumin is a natural surfactant protein with a molecular mass of 23.5 kDa which was isolated from the foam nest of the frog *Leptodactylus vastus*. Only a partial amino-acid sequence is available for this protein and it shows it to be distinct from any protein sequence reported to date. The protein was purified from the natural source by ion-exchange and size-exclusion chromatography and was crystallized by sitting-drop vapour diffusion using the PEG/Ion screen at 293 K. A complete data set was collected to 3.5 Å resolution. The crystal belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 51.96$, $b = 89.99$, $c = 106.00$ Å. Assuming the presence of two molecules in the asymmetric unit, the solvent content was estimated to be 54%.

1. Introduction

Some amphibian species have developed a breeding strategy in which they deposit their eggs in environmentally stable foams in order to protect the eggs and larval development (Heyer, 1969). Recent studies have reported a rich diversity of proteins in amphibian foam nests (Fleming *et al.*, 2009; Hissa *et al.*, 2008; Cooper *et al.*, 2005) and a new class of proteins has emerged termed ranaspumins (proteins from frog foam nests). When the foam nests of the túngara frog (*Engystomops pustulosus*) were studied, it became clear that foam formation is only possible owing to the presence of surfactant proteins together with other functional proteins and carbohydrates (Fleming *et al.*, 2009; Cooper *et al.*, 2005).

These surfactant proteins have distinct hydrophobic and hydrophilic structural domains that adsorb naturally at the air–water interface, thus allowing foam formation. Proteins with natural foam and/or surfactant activities are rare in nature, as conventional surfactants may harm the cell membranes of biological tissues. In addition, foam formation requires a high energy input to overcome water surface tension (Cooper & Kennedy, 2010).

Currently, it is still not well known how the structures of surfactant proteins behave at interfacial or superficial surfaces, although some evidence for unfolding or conformational changes has been obtained when studying the surfactant proteins hydrophobins (de Vocht *et al.*, 2002) and ranaspumin-2 (Mackenzie *et al.*, 2009). In this study, we describe the crystallization of *Lv*-ranaspumin, a surfactant protein isolated from the foam nest of the tropical frog *Leptodactylus vastus*. It is a 23.5 kDa protein that shows a high and stable surfactant activity. The N-terminal amino-acid sequence is distinct from any other currently known protein, suggesting that *Lv*-ranaspumin may adopt a novel unique structure (Hissa *et al.*, 2008).

2. Materials and methods

2.1. Purification

Foam nests were collected and foam fluid was obtained according to a previously published procedure (Hissa *et al.*, 2008). *Lv*-ranaspumin is the main protein in the nest and it was purified by ion-exchange chromatography (Mono Q 4.6/100, GE Healthcare; eluted with a linear gradient of 20 m*M* Tris–HCl, 1 *M* NaCl) followed by

Table 1
Data-collection statistics.

Values in parentheses are for the outer resolution shell.

Beamline	BM14U, ESRF
Wavelength (Å)	0.9789
Unit-cell parameters (Å)	a = 51.96, b = 89.99, c = 106.00
Space group	$P2_12_12_1$
Resolution range (Å)	53.00–3.50 (3.69–3.50)
Completeness (%)	100.0 (100.0)
Multiplicity	6.8 (7.0)
$R_{\rm merge}$†	0.10 (0.52)
$\langle I/\sigma(I)\rangle$	9.6 (3.5)
Total reflections	45699 (6790)
Unique reflections	6710 (965)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

size-exclusion chromatography (HiLoad 26/60 Superdex 200, GE Healthcare; equilibrated with 50 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl). The size-exclusion chromatography column was calibrated with molecular-mass standards purchased from Bio-Rad. The single protein fraction of 23.5 kDa was concentrated to about 10 mg ml^{-1} (based on the absorbance at 280 nm) using Amicon centrifugal filter units (Millipore) with a 10 kDa cutoff membrane prior to crystallization trials. The homogeneity of the protein sample was determined by SDS–PAGE analysis (12% gel) under reducing conditions using molecular-mass markers purchased from Thermo Scientific.

2.2. Crystallization

A total of 384 initial crystallization conditions were tested utilizing various commercially available screens: Index (Hampton Research), Morpheus (Molecular Dimensions), JCSG+ Suite (Qiagen), PEG/Ion (Hampton Research) and PEG/Ion 2 (Hampton Research). The most promising crystal was grown at 293 K by the sitting-drop vapour-diffusion method in the PEG/Ion screen using an OryxNano robot (Douglas Instruments Ltd) to set up the drops. The drop consisted of 0.7 µl protein solution mixed with 0.7 µl reservoir solution and was allowed to equilibrate against 70 µl reservoir solution. The protein crystals grew within three weeks in condition No. 8 [0.2 *M* KCl, 20%(*w*/*v*) PEG 3350 pH 7.0]. These crystals were reproducible and the conditions are currently being optimized in order to obtain better diffracting crystals.

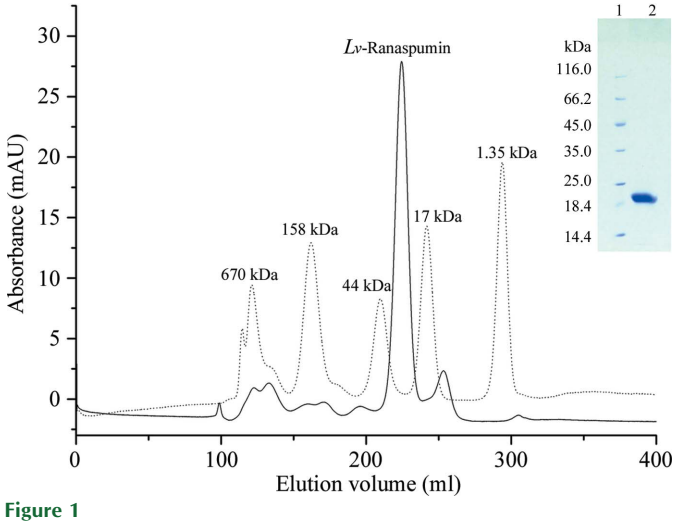

Figure 1
Size-exclusion chromatography and SDS–PAGE analysis of *Lv*-ranaspumin. A chromatogram from a Superdex 200 26/60 column of *Lv*-ranaspumin (solid line) and molecular-mass standards (dotted line) is shown. The inset shows an SDS–PAGE (run under reducing condition) of the major peak in the chromatogram.

2.3. Data collection and processing

Diffraction data were collected on beamline BM14U at the European Synchrotron Radiation Facility (ESRF), Grenoble equipped with a MAR 225 CCD detector. The data were processed using the programs *MOSFLM* (Leslie, 2006; Battye *et al.*, 2011) and *SCALA* (Evans, 2006). Details of data collection and processing are summarized in Table 1.

2.4. Mass-spectrometric analysis of the crystal

A crystal of *Lv*-ranaspumin was harvested and washed twice by transferring it into a fresh drop (1 µl) of reservoir solution. In the third drop the crystal was broken up and the solution was used for mass-spectrometric (MS) analysis using an UltrafleXtreme (Bruker Daltonics) matrix-assisted laser desorption–ionization time-of-flight (MALDI–TOF) mass spectrometer operating in positive linear mode acquiring spectra in the mass range m/z 6000–100 000. 1 µl sample was mixed with 1 µl 2% trifluoroacetic acid (TFA) and 1 µl matrix solution [15.2 mg ml^{-1} 2,5-dihydroxyacetophenone and 20 m*M* ammonium citrate dibasic in 75%(*v*/*v*) ethanol]. This mixture (1 µl) was spotted onto a MALDI target plate and left at room temperature (295 K) for drying. External mass calibration was performed using Protein Standard II (Bruker Daltonics).

3. Results and discussion

The protein *Lv*-ranaspumin was purified to apparent homogeneity by ion-exchange and size-exclusion chromatography as confirmed by SDS–PAGE (Fig. 1). The size-exclusion chromatogram indicated no tendency towards the formation of aggregates or oligomers. The elution volume of the most prominent peak is consistent with the protein being predominantly monomeric.

A large number of long needles appeared three weeks after crystallization setup (Fig. 2). In order to verify that the protein crystals consisted of *Lv*-ranaspumin, MS analyses of the obtained crystal were performed and showed the most prominent molecular mass signals to correspond to singly-, doubly- and triply-charged protein species. Therefore, we are confident that the crystals are indeed comprised of *Lv*-ranaspumin (Fig. 3). A closer inspection of the m/z range from 22 000 to 24 000 indicated the presence of six major protein species (inset in Fig. 3). The mass difference between the major proteins

Figure 2
Crystals of *Lv*-ranaspumin grown by the sitting-drop vapour-diffusion method at 293 K in 0.2 *M* KCl, 20%(*w*/*v*) PEG 3350 pH 7.0.

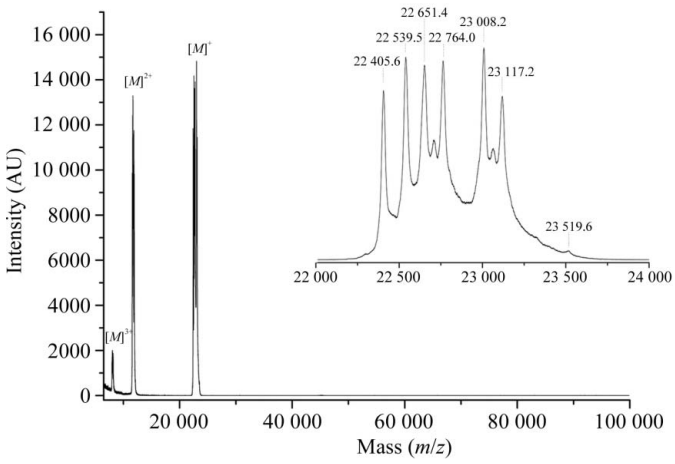

Figure 3
MALDI–TOF spectra (obtained in linear positive mode) of a dissolved *Lv*-ranaspumin crystal. Singly-, doubly- and triply-charged molecules are indicated. The inset shows an expanded view of the singly-charged peak.

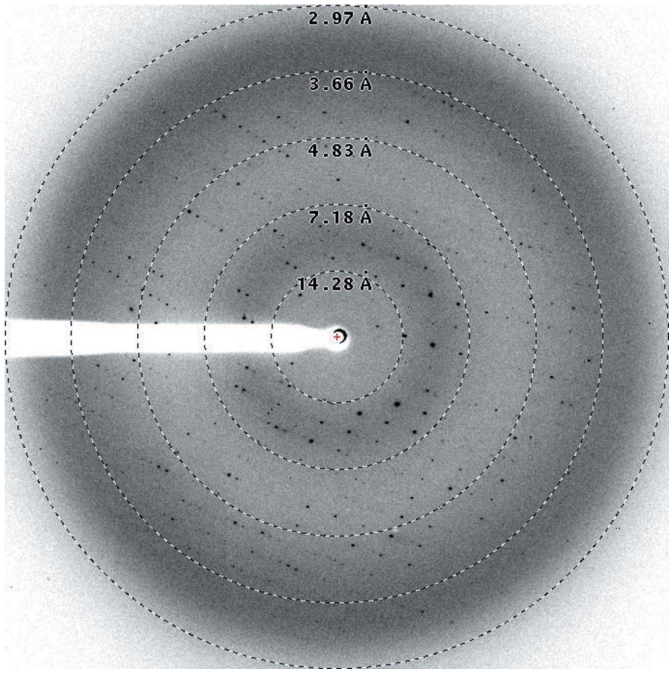

Figure 4
X-ray diffraction image of an *lv*-ranaspumin crystal.

species led to the hypothesis that the protein may have lost amino-acid residues during the crystallization process.

Crystals were harvested and flash-cooled in liquid nitrogen without any cryoprotectant. 180 diffraction images were collected at 100 K to a maximum resolution of 3.0 Å (Fig. 4), but were scaled to 3.5 Å based on $R_{\rm merge}$ and signal-to-noise ratios [$I/\sigma(I)$]. Diffraction images were integrated in the primitive orthorhombic space group $P2_12_12_1$ as confirmed by the program *POINTLESS* (Evans, 2006). Calculation of the Matthews coefficient (2.69 Å^3 Da^{-1}, corresponding to a solvent content of 54%) yielded two molecules in the asymmetric unit as the most probable arrangement [P(tot) = 0.92; Matthews, 1968; Kantardjieff & Rupp, 2003]. Analysis of the data set using the *phenix.xtriage* module of the *PHENIX* suite (Adams *et al.*, 2010) indicated the absence of twinning and the presence of translational pseudosymmetry. The latter may be produced by a noncrystallographic dyad more or less parallel to a crystallographic axis.

The lack of a complete amino-acid sequence hampered the search for a reliable molecular-replacement model and the phase problem still needs to be solved. Because *Lv*-ranaspumin must be purified from the natural source, selenomethionine incorporation is unattainable at present. Based on MS analysis of carboxymethylated protein samples it is anticipated that *Lv*-ranaspumin possesses eight cysteine residues (data not shown), which may just be enough to allow S-SAD phasing. The most promising approach, however, might still be MIR/MAD phasing by the incorporation of heavy atoms.

The behaviour of natural surfactant proteins is still not completely understood and structure elucidation of the protein *Lv*-ranaspumin will allow the correlation of its structure and its function, which could explain its long-term stability and its role in formation of the foam nest.

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