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Crystallization and preliminary X-ray crystallographic analysis of a non-specific lipid-transfer protein with antipathogenic activity from *Phaseolus mungo*

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A 9 kDa non-specific lipid-transfer protein (nsLTP) from mung bean (*Phaseolus mungo*) seeds, displaying antifungal activity, antibacterial activity and lipid-transfer activity, was crystallized at 297 K using ammonium sulfate as a precipitant by means of the hanging-drop vapour-diffusion method. Native X-ray diffraction data were collected to a resolution of 2.4 Å. The crystals are rhombohedral, belonging to space group $P2_12_12_1$, with unit-cell parameters $a = 38.671$, $b = 51.785$, $c = 55.925$ Å. Assuming the presence of one molecule in the crystallographic asymmetric unit results in a Matthews coefficient (V_M) of approximately 3.0 Å^3 Da^{-1}, corresponding to a solvent content of about 58%.

1. Introduction

Lipid-transfer proteins (LTPs) are ubiquitous polypeptides found in numerous living organisms, such as bacteria, yeast, plants and animals (Kader, 1996). Plant lipid-transfer proteins themselves form a multigenic family, which are well known for their ability to transfer *in vitro* various amphiphilic molecules, including phospholipids, glycolipids, fatty acids and steroids, between membranes (Garcia-Olmedo *et al.*, 1995). Because these proteins demonstrate a very broad specificity for various lipids, they are often prefixed non-specific (ns; Lerche & Poulsen, 1998). NsLTPs are subdivided into two subfamilies that differ in molecular weight: nsLTP1 (9–10 kDa) and nsLTP2 (7 kDa) (Douliez *et al.*, 2000). NsLTP1 is the best characterized group, with a basic pI and a conserved cysteine pattern, whose genes are spatially and temporally regulated (Kader, 1996). Plant nsLTPs are synthesized with an N-terminal signal peptide, which indicates that they follow the secretory pathway, in agreement with their location in extracellular layers, *i.e.* cell walls or cutin, and in vacuolar structures (Kader, 1996). Plant nsLTPs have been isolated from many plants, including wheat, rice, barley, maize, peach, apricot, radish, onion, cowpea, tobacco, spinach and mung bean (Douliez *et al.*, 2000; Kader, 1996, Cammue *et al.*, 1995; Terras *et al.*, 1992; Wang *et al.*, 2004).

In addition to having broad lipid-transfer activity, nsLTPs may also have additional biological functions. Meijer *et al.* (1993) suggested that nsLTPs are involved in the formation of cutin layers by transporting the hydrophobic cuticular components required. Surprisingly high stability towards denaturants, heat and proteases has been reported by Larsen & Winther (2001). Finally, the antimicrobial activities of nsLTP observed *in vitro* (Terras *et al.*, 1992; Garcia-Olmedo *et al.*, 1995; Cammue *et al.*, 1995; Wang *et al.*, 2004) suggest an important role for plant nsLTPs in the constitutive host defence mechanisms of plants against microbial pathogens. This may contribute to the development of biological control of fungal pathogens typical of the crop. Although these hypotheses are attractive and consistent with most of the biological data, there is no clear evidence for such roles *in vivo* (Kader, 1996).

The nsLTP family are often classified as hydrophobic proteins, because they possess eight highly conserved cysteine residues, which form four disulfide bonds (Douliez *et al.*, 2000). Three-dimensional structures of nsLTPs have been extensively studied and there are more than ten entries in the Protein Data Bank containing nsLTPs either in their unliganded states or in the form of complexes with ligands. The structures of unliganded nsLTPs were determined by X-ray and NMR spectroscopic techniques from four different plant sources, including maize (Shin *et al.*, 1995; Gomar *et al.*, 1996), wheat (Gincel *et al.*, 1994), barley (Heinemann *et al.*, 1996) and rice (Lee *et al.*, 1998; Poznanski *et al.*, 1999). Although the amino-acid sequence homology was not high, the structures were very similar. The common structural feature was a single compact domain with four α-helices and a long carboxyl terminal region, with the four disulfide bonds interconnecting the secondary-structure elements. There is a hydrophobic cavity surrounded by α-helices connected through disulfide bonds, while the hydrophobic cavity is the binding pocket for lipid or fatty-acid molecules (Han *et al.*, 2001). Close examination of the structure shows the existence of a

hydrophobic tunnel, which is involved in the prevention of microbial attack (Douliez *et al.*, 2000). In addition, three structures of nsLTPs complexes with ligands have been published (Lerche & Poulsen, 1998; Charvolin *et al.*, 1999; Han *et al.*, 2001). These studies indicated that not only do nsLTPs exert a wide range of properties on different lipids, but that the binding modes of these proteins also differ in spite of the fact that both the sequences and the three-dimensional structures are similar among nsLTPs.

The mung bean nsLTP showed strong antifungal activity toward *Fusarium solani*, *F. oxysporum*, *Pythium aphanidermatum* and *Sclerotium rolfsii* and exerted antibacterial activity toward the Gram-positive bacterium *Staphylococcus aureus* (Wang *et al.*, 2004). Examination of scanning electron micrographs revealed one possible antipathogenic mechanism, the transformation of the bacterial structure owing to destruction of the cell wall (Wang *et al.*, 2004). The full sequence of mung bean nsLTP was obtained by Lin *et al.* (2002). When it was compared with other nsLTPs from maize, rice, barley and wheat, whose structures are known, mung bean nsLTP showed a level of sequence identity of 52, 41, 38 and 39%, respectively, and also contained the pattern of eight cysteine molecules (Fig. 1). However, it remains unclear whether all nsLTPs, including those from the mung bean, possess a common array of disulfide bridges formed by the eight cysteines of the sequence and a similar hydrophobic cavity resulting from the disulfide bonds. It remains unclear whether this cavity is essential for nsLTP activity.

We report here the crystallization conditions and preliminary X-ray crystallographic data for the nsLTP from mung bean, in order to provide new insight into the biological function and significance of the nsLTP family and to allow a more detailed comparison with the known structures of nsLTPs.

2. Materials and methods

2.1. Protein preparation

The mung bean nsLTP was isolated from mung bean (*Phaseolus mungo*) seeds. The procedure entailed aqueous extraction, ammonium sulfate precipitation, ion-exchange chromatography on CM-Sephadex and high-performance liquid chromatography (HPLC) on POROS HS-20 (Wang *et al.*, 2004). The purified fraction was pooled and identified by SDS–PAGE (Laemmli, 1970) and by mass spectrometry. Crystallization screening was performed when lyophilized production had prepared more than 10 mg.

2.2. Crystallization of nsLTP and X-ray diffraction analysis

Crystallization was performed at 297 K by the hanging-drop vapour-diffusion method using 24-well tissue-culture plates. Initial crystallization conditions were established using screening kits from Hampton Research (Crystal Screens I, II and MembFac). Each hanging drop was prepared by mixing 3 µl protein solution (40 mg ml^{-1} protein in 10 m*M* Tris–HCl buffer pH 8.5) and 2 µl reservoir solution. Each hanging drop was placed over 500 µl reservoir solution. Small crystals were seen after about 7 d in crystallization drops using a reservoir solution containing 1.5 *M* ammonium sulfate as a precipitant, with 0.1 *M* Tris buffer pH 8.5 and 12%(*v*/*v*) glycerol. This condition was optimized by varying the glycerol concentration and by adding octyl β-D-glucopyranoside as a detergent. The crystals used in this study grew using a reservoir solution containing 1.5 *M* ammonium sulfate as a precipitant, 0.1 *M* Tris–HCl buffer pH 8.5, 8%(*v*/*v*) glycerol and 0.5%(*w*/*v*) octyl β-D-glucopyranoside, with drops consisting of 3 µl protein solution and 2 µl reservoir solution equilibrated against 500 µl reservoir solution at 297 K. Well ordered prisms grew over a period of 7 d (Fig. 2). A crystal was flash-cooled directly from the drop in a stream of nitrogen gas maintained at 100 K for data collection using a Bruker rotating-anode source (Cu $K\alpha$ = 1.5418 Å) with Montel mirror optics and a PROTEUM R CCD detector. Data were processed using *SAINT-Plus* and *PROSCALE* software (Bruker Co., Germany). Finally, a data set was collected to a resolution of 2.4 Å.

Table 1
Diffraction data statistics for the nsLTP crystal.

Values in parentheses refer to the highest resolution shell (2.51–2.40 Å).

X-ray wavelength (Å)	1.5418
Temperature (K)	100
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 38.671, b = 51.785, c = 55.925
Resolution range (Å)	52.0–2.4
Total/unique reflections	28538/4717
Data completeness (%)	99.3 (99.9)
R_{sym}	0.105 (0.172)
Average $I/\sigma(I)$	6.66 (4.02)

3. Results and conclusions

The mung bean nsLTP with both lipid-transfer activity and antimicrobial activity was prepared by a combination of extraction, ammonium sulfate precipitation and ion-exchange chromatography. The optimized crystals were obtained using a reservoir solution containing 1.5 *M* ammonium sulfate as a precipitant, 0.1 *M* Tris–HCl buffer pH 8.5, 8%(*v*/*v*) glycerol and 0.5%(*w*/*v*) octyl β-D-glucopyranoside as a detergent. Rhombohedral crystals grew to dimensions of 0.3 × 0.15 × 0.15 mm over a period of one week. The data statistics are shown in Table 1. The space group was

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Mung bean  1 MTCGQVQGNLAQCIGFLQKGGVVPPSCCTGVKNILNSSRTTADRRAVCSCLKAAAGAVRG  60
Maize      1 AISCGQVASAIAPCISYARGQGSGPSAGCCSGVRSLNNAARTTADRRAACNCLKNAAAGV  60
Rice       1 ITCGQVNSAVGPCLTYARGGAGPSAACCSGVRSLKAAASTTADRRTACNCLKNAARGIKG  60
Barley     1 LNCGQVDSKMKPCLTYVQGGPGPSGECCNGVRDLHNQAQSSGDRQTVCNCLKGIARGIHN  60
Wheat      1 IDCGHVDSLVRPCLSYVQGGPGPSGQCCDGVKNLHNQARSQSDRQSACNCLKGIARGIHN  60

Mung bean      61 INPNNAEALPGKCGVNIPYKISTSTNCNSIN 91      (100%)
Maize          61 SGLNAGNAASIPSKCGVSIPYTISTSTDCSRVN 93    (52%)
Rice           61 LNAGNAASIPSKCGVSVPYTISASIDCSRVS 91     (41%)
Barley         61 LNLNNAASIPSKCNVNVPYTISPDIDCSRIY 91     (38%)
Wheat          61 LNEDNARSIPPKCGVNLPYTISLNIDCSRV 90      (39%)
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Figure 1
Amino-acid sequence alignment of five plant nsLTPs, displayed using a BLAST search. Underlined characters show identical amino-acid residues.

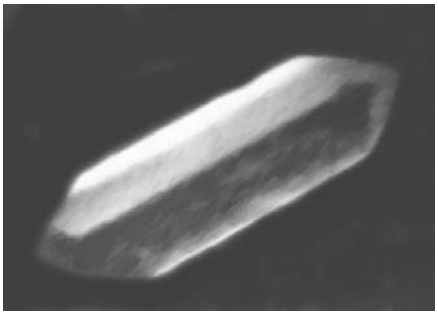

Figure 2
A crystal of nsLTP from *P. mungo* seeds. Its approximate dimensions are 0.3 × 0.15 × 0.15 mm.

identified as $P2_12_12_1$, with unit-cell parameters a = 38.671, b = 51.785, c = 55.925 Å. As the accurate molecular weight was determined to be 9292.97 Da by mass spectrography (Wang *et al.*, 2004), assuming the presence of one molecule in the asymmetric unit, $Z = 4$ gives a V_M value of approximately 3.0 Å^3 Da^{-1} and a solvent content of 58%. These values lie within the expected range for protein crystals (Matthews, 1968).

The overall completeness is adequate for solving the structure by molecular replacement. Currently, structure determination and model building are in progress. The structure, when completed, will explain the molecular basis of the antifungal activity, antibacterial activity and lipid-transfer activity of different products and provide new insight into the biological function and significance of nsLTPs.

In summary, crystals of mung bean nsLTP have been obtained. The crystals are ordered and diffract to 2.4 Å resolution using an in-house X-ray radiation source.

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