Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Crystallization and initial crystallographic characterization of a vicilin-type seed storage protein from *Pinus koraiensis*

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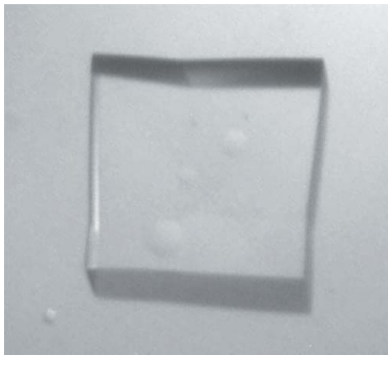
Received 14 August 2007
Accepted 30 October 2007



The cupin superfamily of proteins includes the 7S and 11S seed storage proteins. Many members of this family of proteins are known allergens. In this study, the Korean pine (*Pinus koraiensis*) vicilin-type 7S seed storage protein was isolated from defatted pine-nut extract and purified by sequential gel-filtration and anion-exchange chromatography. Well diffracting single crystals were obtained by the vapor-diffusion method in hanging drops. The crystals belong to the primitive cubic space group $P2_13$, with unit-cell parameters $a = b = c = 148.174$ Å. Two vicilin molecules were present in the asymmetric unit and the Matthews coefficient was determined to be 2.90 Å^3 Da^{-1}, with a corresponding solvent content of ∼58%. A molecular-replacement structural solution has been obtained using the program *Phaser*. Refinement of the structure is currently under way.

1. Introduction

Pine nuts are the edible seeds of pine trees (family Pinaceae, genus *Pinus*) and are among the ingredients of many traditional vegetable dishes and other foods (Nergiz & Dönmez, 2004; Özgüven & Vursavuş, 2005). It is believed that in some regions, people could have survived winter by consuming only pine nuts (Cordenunsi *et al.*, 2004). Pine nuts and other tree nuts are highly nutritious and their consumption has important health benefits associated with reduction of the risk of coronary heart disease and nonfatal myocardial infarction (Feldman, 2002; Ryan *et al.*, 2006). However, similar to other tree nuts and peanuts, pine nuts are also known to be a source of food allergens (Garcia-Menaya *et al.*, 2000; Ma *et al.*, 2002). The 7S and 11S seed storage proteins in a number of tree nuts and peanuts are known to be major allergens (Roux *et al.*, 2003). Whether the 7S protein in pine nut is also an allergen remains to be determined. Structural characterization of allergens plays an important role in the understanding of the molecular basis of protein allergenicity. The structures of the 7S seed storage proteins from soybean (*Glycine max*; Maruyama *et al.*, 2001, 2004), jack bean (*Canavalia pubescens*; Ko *et al.*, 1993, 2000), mung bean [*Vigna radiata* (L.) Wilczek] and French bean (*Phaseolus vulgaris*; Lawrence *et al.*, 1990, 1994) have been determined. Additional three-dimensional structures of 7S seed storage proteins, especially those from tree nuts, are needed for characterization of the stability and allergenicity of these groups of proteins. Here, we report the crystallization, X-ray data collection and initial phase determination of a 7S vicilin-type protein from pine nut.

2. Methods

2.1. Protein purification

The detailed purification procedures along with the biochemical and biophysical characterization of Korean pine-nut vicilin by circular dichroism, differential scanning calorimetry, thermo-induced and urea-induced unfolding and fluorescence spectroscopy will be reported elsewhere (Jin *et al.*, in preparation). Briefly, raw whole Korean pine-nut kernels were purchased from a local grocery store and the kernels were homogenized in ten volumes of phosphate-buffered saline. Fat was removed from the crude preparation by

Table 1
X-ray data-collection statistics.

Values in parentheses are for the outer shell.

Resolution (Å)	50–2.70 (2.80–2.70)
Wavelength (Å)	1.0
Data-collection temperature (K)	110
No. of observed reflections	158155
No. of unique reflections	29133
Completeness (%)	97 (96.3)
Redundancy (%)	5.4 (4.4)
Mean $I/\sigma(I)$	16.9 (4.04)
$R_{\rm merge}$† (%)	6.5 (39.3)
Reflections rejected (%)	0.06

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

extracting three times with a 1:1(*v*:*v*) water–cyclohexane mixture. Protease inhibitors (100 n*M* aprotinin, 50 m*M* antipain, 50 m*M* leupeptin and 0.5 mg ml^{-1} pepstatin) and antibiotics (50 mg ml^{-1} ampicillin and 50 mg ml^{-1} kanamycin) were added to the defatted sample, which was then dialyzed against 10 m*M* Tris–HCl buffer pH 7.9 before chromatographic purification. Vicilin was purified by sequential gel-filtration chromatography using an XK 26/70 column packed with Superdex 200 (GE Healthcare, Piscataway, USA) and anion-exchange chromatography using an HR 10/10 column packed with 8 ml Source Q15 medium (GE Healthcare). The gel-filtration column was equilibrated and eluted with the Tris–HCl buffer plus 100 m*M* NaCl and vicilin fractions were loaded onto the Source Q15 column after a buffer change back to the Tris–HCl buffer. The protein bound to the anion-exchange column was eluted using the Tris–HCl buffer plus a 0–0.6 *M* NaCl gradient.

2.2. Crystallization

Purified pine-nut vicilin was concentrated to 60 mg ml^{-1} and the buffer was replaced with distilled water by repeated dilution/concentration with Ultracel-5k filter devices (Millipore, Bedford, USA). A crystallization screen was performed at room temperature with the Crystal Screen kit from Hampton Research (Aliso Viejo, USA). Using the hanging-drop vapor-diffusion method, 1 µl protein solution was mixed with 1 µl crystallization reservoir solution and sealed against 1 ml reservoir solution in 24-well Linbro plates. Numerous crystals appeared in the drop hanging over solution No. 34 (2 *M* sodium formate, 0.1 *M* sodium acetate trihydrate pH 4.6) of the Crystal Screen kit within a week. After optimization of the precipitant concentration, pH and protein concentration, a starting protein concentration of 30 mg ml^{-1} was used for the final crystallization set up with the original solution No. 34 of the Crystal Screen kit as the reservoir solution. The hanging-drop vapor-diffusion method was used, but the drops were made by mixing 3 µl protein solution and 3 µl reservoir solution.

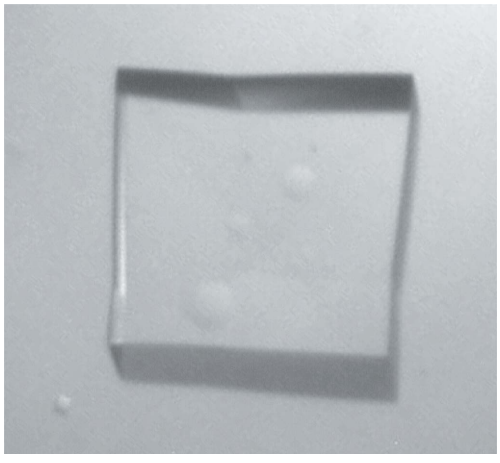


Figure 1
Single crystal of pine-nut vicilin obtained by vapor diffusion using the hanging-drop method. The dimensions of the crystals are ~50 × 250 × 250 µm.

2.3. X-ray data collection and analysis

To screen for a cryoprotectant, single crystals were immersed in different cryoprotectant solutions containing varying concentrations of sucrose, glycerol or PEG 400 for 5 min and the crystals were examined under a stereomicroscope for signs of cracking and deformation. The final cryoprotectant used was 30%(*w*/*v*) glycerol in the crystallization reservoir solution. Single crystals were immersed briefly in the cryoprotectant solution, flash-cooled and stored in liquid nitrogen. X-ray data collection was performed using a MAR225 CCD detector on the SER-CAT 22BM beamline at the Advanced Photon Source (APS), Argonne National Laboratory. The diffraction data were processed using the *HKL*-2000 suite of programs (Otwinowski & Minor, 1997) and a structural model was derived by molecular-replacement calculations using the program *Phaser* (McCoy *et al.*, 2005; Storoni *et al.*, 2004).

3. Results and discussion

Under the optimized conditions, single crystals of dimensions $\simeq 50 \times 250 \times 250$ µm were obtained (Fig. 1). SDS–PAGE analysis of crystals redissolved in water showed the same bands as the purified vicilin sample (data not shown). Noticeable radiation damage to the vicilin crystals was observed during data collection, but we were able to collect a complete 2.70 Å data set (Fig. 2). Processing of the diffraction data revealed a primitive cubic crystal system with unit-

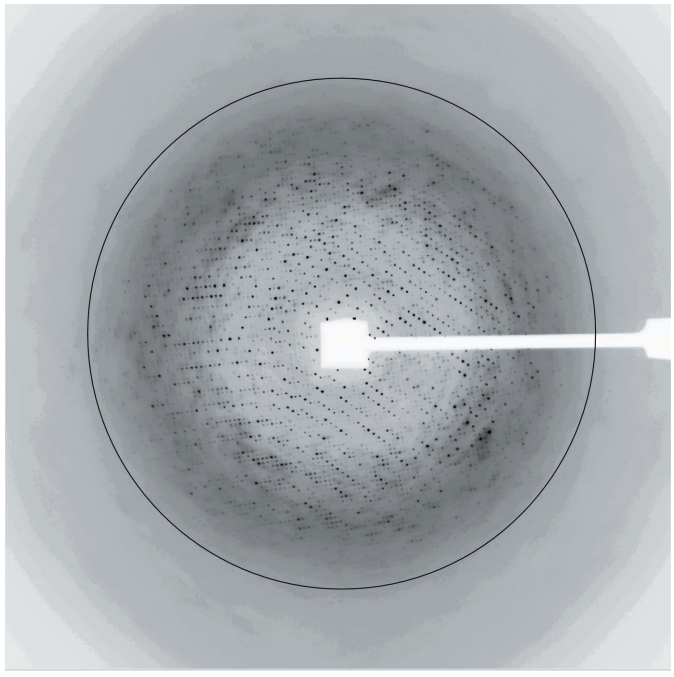


Figure 2
A typical frame of the pine-nut vicilin X-ray diffraction data. 60 frames with 12 s exposures were collected with a crystal-to-detector distance of 220 mm. The 2.70 Å resolution circle is shown.

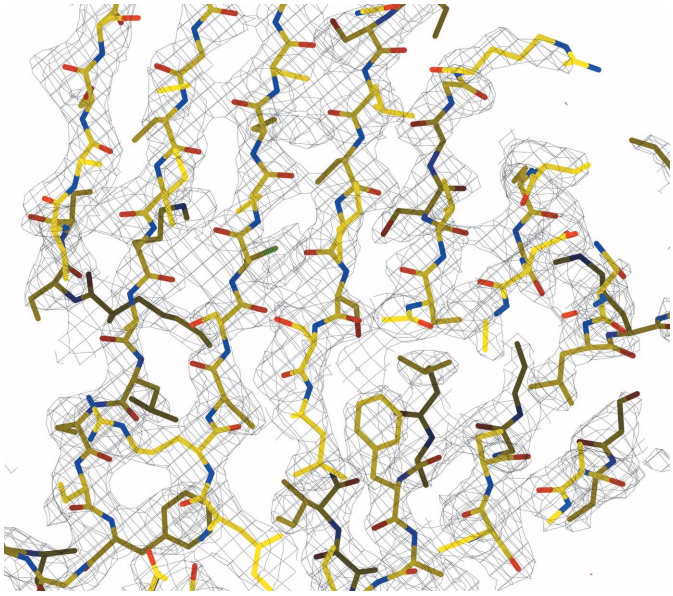


Figure 3
A portion of a $2F_o - F_c$ electron-density map. The map was calculated after a rigid-body and restrained refinement cycle starting from a *Phaser* molecular-replacement solution. The map was contoured at 1.5σ and the structure model is shown in stick representation using the CPK coloring scheme.

cell parameters $a = b = c = 148.174$ Å. Based on systematic absences in specific reflections in the diffraction, the space group was determined to be $P2_13$ (Table 1). Assuming the presence of two monomers of vicilin in the asymmetric unit and an average partial specific volume of 0.74 $cm^3\ g^{-1}$ for proteins, the Matthews coefficient was determined to be 2.90 $Å^3\ Da^{-1}$, with a corresponding solvent content of the crystal of ∼58%.

A database search with *BLAST* showed 30% sequence identity between Loblolly pine (*P. taeda*) vicilin and soybean β-conglycinin. Thus, we constructed a vicilin structure model using chain *A* of the structure of the recombinant conglycinin structure (PDB code 1ipk; Maruyama *et al.*, 2001) as a template with the program *SCWRL* (Canutescu *et al.*, 2003; data not shown). Molecular-replacement calculations starting with the monomeric vicilin model resulted in a structural solution with two vicilin molecules in the asymmetric unit and a log-likelihood gain of 2431. The *R* factor of the solution after one round of rigid-body and restrained refinement using the program *REFMAC* (Murshudov *et al.*, 1997) was 39.5%, with an R_{free} of 47.1% using 5% randomly chosen test reflections.

A preliminary inspection of the map calculation and refinement led to an experimental electron-density map at 2.70 Å resolution with a clear protein–solvent boundary. The electron-density map allowed modeling of most of the main-chain atoms for both of the monomers (Fig. 3). The two protomers in the asymmetric unit are perpendicular to each other. Currently, model building and refinement of the structure are under way. Structural determination of the vicilin-type seed protein from pine nuts will provide information required for comparison of the similarities and differences among the 7S proteins from various food sources in order to understand seed protein allergenicity.

This work was supported by a grant from Illinois Institute of Technology. X-ray diffraction data were collected at Southeast Regional Collaborative Access Team (SER-CAT) 22-BM beamline at the APS, Argonne National Laboratory. Use of the APS was supported by the US Department of Energy, Office of Science, Office of Basic Energy Sciences under contract W-31-109-Eng-38.

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