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Crystallization and preliminary structure determination of the plant food allergen Pru av 2

Yuliya Dall'Antonia,[a] Tea Pavkov,[a] Heidemarie Fuchs,[b] Heimo Breiteneder[b] and Walter Keller[a]*

[a]Institute of Chemistry, Structural Biology, Karl-Franzens-University Graz, Heinrichstrasse 28, 8010 Graz, Austria, and [b]Medical University of Vienna, Department of Pathophysiology, AKH-EBO-3Q, Waehringer Guertel 18-20, Vienna 1090, Austria

Correspondence e-mail: walter.keller@uni-graz.at

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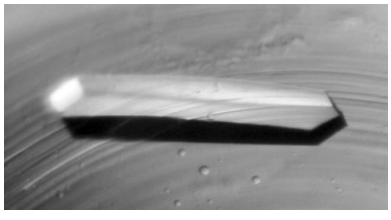


Thaumatin-like proteins (TLPs) have mostly been investigated in the context of their function as pathogenesis-related proteins and only in recent years have some of them been classified as allergens. Here, the purification and crystallization of the first allergenic TLP, Pru av 2, a 23.3 kDa protein isolated from ripe cherries, is reported. The crystals diffracted to 2.1 Å resolution at a rotating-anode generator and were found to belong to space group $P2_1$, with unit-cell parameters a = 44.48, b = 41.04, c = 59.16 Å, β = 106.61° and one molecule per asymmetric unit. In order to obtain high-resolution data, an annealing protocol was applied that improved the resolution limit from 1.6 to 1.3 Å at a synchrotron.

1. Introduction

Almost 4% of the human population suffer from food allergy. Interestingly, most plant food allergens belong to very few protein families and superfamilies (Breiteneder & Radauer, 2004). Of those, members of the pathogenesis-related proteins (PRs) have frequently been identified as allergens (Ebner *et al.*, 2001; Hoffmann-Sommergruber, 2000). PRs are defined as proteins that are encoded by the plant genome and induced specifically in response to infections by fungi, bacteria or viruses or by adverse environmental conditions. PRs have been classified into 14 families by van Loon & van Strien (1999). The family 5 PRs share significant amino-acid sequence similarity with thaumatin, an intensely sweet-tasting protein from the fruit of the West African ketemfe plant (*Thaumatococcus daniellii*; Van der Wel & Loeve, 1972). Therefore, these proteins are called thaumatin-like proteins (TLPs). They have been isolated from a wide variety of plant species (Selitrennikoff, 2001). A number of allergenic TLPs from fruit and pollen have already been described (Breiteneder & Radauer, 2004). TLPs for which specific IgE reactivity has been described include apple Mal d 2 (Hsieh *et al.*, 1995; Krebitz *et al.*, 2003), cherry Pru av 2 (Inschlag *et al.*, 1998), bell pepper Cap a 1 (Fuchs *et al.*, 2002), the kiwi TLP Act c 2 (Gavrović-Jankulović *et al.*, 2002) and the pollen TLPs Jun a 3 (Midoro-Horiuti *et al.*, 2000) and Cup a 3 (Cortegano *et al.*, 2004).

To date, the structures of four plant TLPs have been solved (Batalia *et al.*, 1996; Koiwa *et al.*, 1999; Min *et al.*, 2004; Ogata *et al.*, 1992), but nothing is known about their allergenic potential and IgE cross-reactivity within the TLP family in general. In order to close this gap in our knowledge, we have initiated structural and immunological investigations of TLP proteins. Here, we present the purification and crystallization of Pru av 2, a 23.3 kDa allergen (222 amino acids) which was isolated from sweet cherry. Pru av 2 is the first allergenic fruit TLP to be crystallized.

2. Protein purification

The TLP from sweet cherry was purified according to a previously described protocol (Menu-Bouaouiche *et al.*, 2003), with some modifications. In brief, 1.5 kg ripe cherries (*Prunus avium* cv. Germersdorfer) grown in Northern Austria were squeezed with 300 ml ice-cold water through Miracloth and diluted in sodium formate buffer to a final concentration of 20 m*M*. The extract was

adjusted to pH 3.8 with 100% formic acid and then centrifuged at 9000*g* for 30 min at 277 K. After filtration of the supernatant, the cherry protein extract was further diluted with distilled water to 1200 ml and loaded onto a column of 25 ml SP-Sepharose Fast Flow (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with 20 m*M* sodium formate buffer pH 3.8 and 1 *M* NaCl. All chromatographic steps on the ÄKTA-Prime System (Amersham) were performed at 277 K. After loading the sample, the column was washed with 20 m*M* sodium formate buffer pH 3.8. Proteins were subsequently eluted using a 200 ml linear gradient of 0–200 m*M* NaCl in 50 m*M* Tris–HCl pH 7.8. 10 ml fractions were collected and analyzed by SDS–PAGE.

The Pru av 2 protein was detected in the flowthrough and was highly purified after the washing step. All other proteins were retained on the column. The Pru av 2 present in 600 ml of the washing step was precipitated by adding ammonium sulfate to a final concentration of 75%. After centrifugation, the precipitate was dissolved in 5 ml 20 m*M* sodium phosphate buffer pH 7.2 and desalted using Sephadex G-25 prepacked PD-10 columns (Amersham Biosciences, Uppsala, Sweden), resulting in a diluted volume of 17 ml. The fractions containing the purified Pru av 2 protein were pooled. Prior to crystallization experiments, the buffer was replaced by water and the protein was concentrated to 9 mg ml^{-1} using Ultrafree-MC filter units (Millipore). The protein concentration was determined by the BCA Protein Assay (Pierce Biotechnology, Perbio Science, Erembodegem, Belgium) using bovine serum albumin as standard.

3. Crystallization

Initial crystallization conditions for Pru av 2 were screened at 293 K using the Index Screen (Hampton Research Inc.) and the microbatch method with the ORYX crystallization robot (Douglas Instruments). Small crystals were obtained from a condition consisting of 25%(*w*/*v*) PEG 3350 and 0.1 *M* citric acid pH 3.5. The drops contained 0.5 µl protein and 0.5 µl precipitant solution. The precipitant concentration and the pH of the citric acid were optimized and the best crystals were obtained using solutions consisting of 26–30%(*w*/*v*) PEG 3350 and 0.1 *M* citric acid pH 3.35–3.5. Plate-like crystals formed within a week and grew to maximum dimensions of 0.55 × 0.15 × 0.1 mm (Fig. 1).

4. Data collection and processing

The first complete data set was collected using a system with a MAR 345 image-plate detector and a rotating-anode generator operating at 40 kV and 80 mA. Crystals were cooled using flash-cooling in liquid nitrogen without cryoprotectant. Data were collected at 100 K by the standard oscillation method using a crystal-to-detector distance of 80 mm, an oscillation range of 1.0° and an exposure time of 20 min per image. The crystal diffracted to 2.1 Å and no radiation damage was detected during data collection. The crystal belonged to space group $P2_1$, with unit-cell parameters a = 44.48, b = 41.04, c = 59.16 Å, β = 106.61°, and had a mosaicity of 0.75°. Assuming the presence of one molecule of Pru av 2 in the asymmetric unit, the Matthews coefficient (Matthews, 1968) is 2.1 Å^3 Da^{-1} and the solvent content is 44.1%. Data processing and reduction were performed with *DENZO*

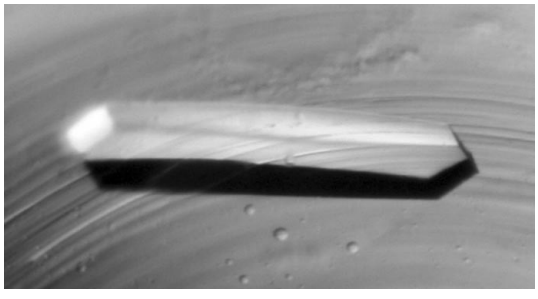
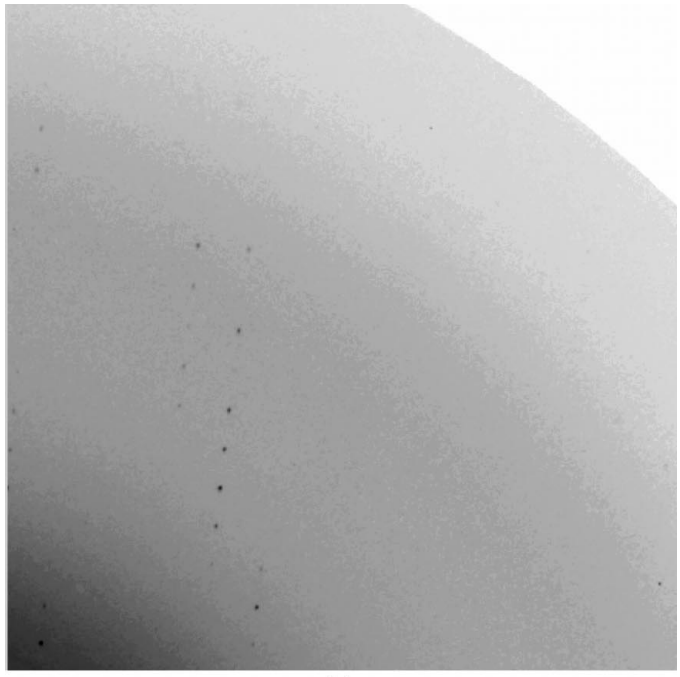
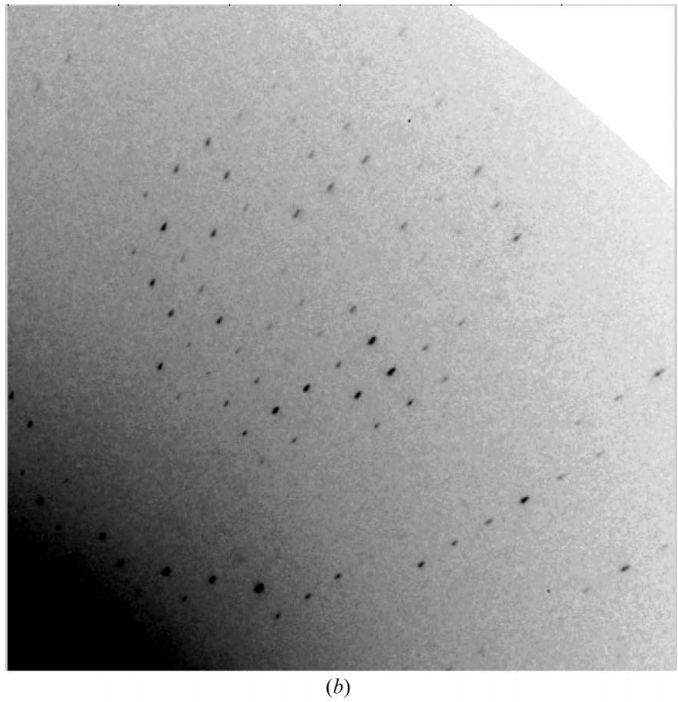


Figure 1
A representative crystal of Pru av 2 grown using the microbatch method at 293 K in 26%(*w*/*v*) PEG 3350, 0.1 *M* citric acid pH 3.35.



(*a*)



(*b*)

Figure 2
The diffraction pattern of a Pru av 2 crystal using synchrotron radiation at beamline X11, DESY, Hamburg (*a*) before annealing (the outer edge of the plate is at 1.6 Å), (*b*) after annealing (the outer edge of the plate is at 1.15 Å).

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

	In-house data	Data obtained at beamline X11
Detector	MAR 345 IP	MAR CCD
Wavelength (Å)	1.5418	0.8126
Resolution range (Å)	26.0–2.1 (2.14–2.10)	20–1.3 (1.32–1.30)
Space group	$P2_1$	$P2_1$
Unit-cell parameters (Å, °)	a = 44.48, b = 41.04, c = 59.16, β = 106.61	a = 44.29, b = 41.15, c = 58.94, β = 107.08
No. of observations	35715 (843)	187498 (9143)
No. of unique reflections	12211 (413)	49833 (2457)
Completeness (%)	96.2 (66.6)	100 (100)
Average $I/\sigma(I)$	15.80 (4.28)	26.0 (6.95)
$R_{\rm sym}(I)$† (%)	6 (20.8)	5.1 (16.6)

† $R_{\rm sym} = \sum_{hkl}\sum_i |I_i - I_{\rm avg}|/\sum_{hkl}\sum_i |I_i|$.

and *SCALEPACK* (Otwinowski & Minor, 1997). Crystal parameters and diffraction data statistics are summarized in Table 1.

In order to obtain a high-resolution structure, data collection was carried out at beamline X11, EMBL/DESY Hamburg, Germany equipped with a MAR Research CCD detector. For data collection, the crystals were flash-cooled in liquid nitrogen after soaking in cryoprotectant solution containing 20%(*w*/*v*) glycerol. Under these conditions, the crystals diffracted to 1.6 Å. Repeated cycles of annealing (warming up the crystal by interrupting the cold stream, followed by recooling to 100 K in the gaseous nitrogen stream) resulted in the extension of the resolution limit to 1.3 Å, with a simultaneous increase of the mosaicity to 0.4°. Fig. 2 shows a comparison of the diffraction pattern before and after the annealing procedure.

Database searches with the program *GenTHREADER* (Jones, 1999) revealed that thaumatin is the structure closest related to Pru av 2, exhibiting 41.5% sequence identity. The whole thaumatin structure (PDB code 1thv) was used as a model for molecular replacement. A clear solution was obtained with *MOLREP* (Collaborative Computational Project, Number 4, 1994) using data from 18 to 3 Å, where the first peak in the translation function showed a relative height (TF/σ) of 11.6, compared with 5.3 for the second peak.

Model building and refinement is in progress and upon completion the Pru av 2 structure will form the basis for an analysis of allergenicity and cross-reactivity within the TLP family of allergens.

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