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Purification, crystallization and initial crystallographic characterization of brazil-nut allergen Ber e 2

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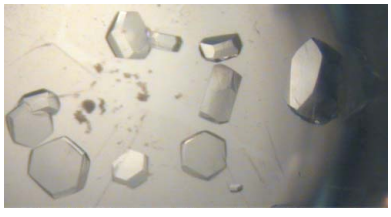
Peanut and tree-nut allergies have attracted considerable attention because of their frequency and their lifelong persistence. Brazil-nut (*Bertholletia excelsa*) allergies have been well documented and the 11S legumin-like seed storage protein Ber e 2 (excelsin) is one of the two known brazil-nut allergens. In this study, Ber e 2 was extracted from brazil-nut kernels and purified to high purity by crystalline precipitation and gel-filtration chromatography. Well diffracting single crystals were obtained using the hanging-drop vapour-diffusion method. A molecular-replacement structural solution has been obtained. Refinement of the structure is currently under way.

1. Introduction

Allergies are hypersensitive reactions in allergic patients, most of which are initiated by interactions between antigens and IgE molecules (Johansson *et al.*, 2001). Food allergies are among the most common causes of hypersensitivity. They affect as many as 2–8% of infants and young children and more than 90% of all food allergies are caused by eight food sources: peanuts, soybeans, milk, eggs, fish, crustacean shellfish, wheat and tree nuts (US Food and Drug Administration, 2005). To date, the structural basis underlying the allergenicity of food allergens has been relatively under-explored. Structural information on food allergens may help to establish a structure–function relationship, which in turn may help us to understand the physicochemical basis of the allergenicity of food allergens. Unlike allergies to other food allergens, such as eggs and milk, which mostly occur during childhood and tend to disappear in adults, nut-induced allergies are often permanent (Hourihane, 1998; Sampson, 1999; Schreiber & Walker, 1989; Sicherer, 2002).

Brazil nuts are a valuable food source with a huge market in Europe and North America, with annual sales of over 33 million US dollars (Peres *et al.*, 2003), but they are also a source of allergens (Gillespie *et al.*, 1976; Teuber *et al.*, 2003). Based on data from consecutive patients seen by a doctor over a year in an allergy clinic in England, the brazil nut was shown to be the second commonest cause of nut allergies (Ewan, 1996) after peanut. Recently, the first case of a sexually triggered allergic reaction was reported in a female subject arising from the consumption of brazil nuts by her male partner (Bansal *et al.*, 2007). Known allergens from brazil nuts include a 2S seed storage protein Ber e 1 (Pastorello *et al.*, 1998) and the 11S seed storage protein Ber e 2 (Bartolome *et al.*, 1997). The 11S seed storage proteins have also been identified as a food allergens in a number of other edible nuts and crops; examples include Ara h 3 in peanuts (Rabjohn *et al.*, 1999), Cor a 9 in hazelnuts (Beyer *et al.*, 2002), Jug r 4 in walnuts (Wallowitz *et al.*, 2006), Ana o 2 in cashew nuts (Wang *et al.*, 2003), Sin a 2 in mustard seeds (Palomares *et al.*, 2005), the globulin in sesame seeds (Hsiao *et al.*, 2006) and glycinin in soybeans (Helm *et al.*, 2000).

The Ber e 2 brazil-nut allergen is also known as excelsin and was the second protein to be crystallized in history, more than one and a half centuries ago (Hartig, 1855). The unit-cell parameters of Ber e 2 crystals obtained by dialysis were determined nearly a quarter of a century ago (Kamiya *et al.*, 1983), but its structure has not been



reported, presumably owing to poor crystal quality. Currently, limited information is available on the 11S allergens in general. Only the soybean (*Glycin max*) glycinin (Adachi *et al.*, 2003) and proglycinin (Adachi *et al.*, 2001) structures have been reported; the PDB coordinates (2evx) of the 11S protein of winter squash (*Cucurbita maxima*) have also been released. We are interested in understanding the structural basis of the allergenicity and the allergenic cross-reactivity of nut allergens. Here, we report the crystallization and X-ray data collection of Ber e 2 and the initial solution of its structure.

2. Meterials and methods

2.1. Protein purification

The procedures used for protein extraction from brazil nuts and purification of Ber e 2 by recrystallization were based on methods reported in the literature (Hartig, 1855; Kamiya *et al.*, 1983). Briefly, to extract and purify Ber e 2, brazil nuts were purchased from a local store. 30 g of raw shelled nuts were ground using a KitchenAid blender in 25 ml 1 *M* NaCl solution and incubated at 333 K for 20 min with stirring at 2 min intervals. The sample was then centrifuged at 2000*g* for 15 min at 298 K. The supernatant was collected as crude protein extract. To purify Ber e 2, 10 ml crude protein extract was mixed with an equal volume of hexane and incubated for 2 h at room temperature with stirring. The sample was then centrifuged at 2000*g* for 15 min at 298 K and the lower aqueous solution was collected and extracted with hexane four more times. The defatted protein extract was then dialyzed against 1000 ml 100 m*M* NaCl solution at 293 K. Ber e 2 was 'desalted out' as microcrystals in the dialysis tubing after 12 h and the crystalline precipitate was collected by centrifugation at 300*g* for 5 min at 293 K. The protein was redissolved in 1 *M* NaCl solution to a final volume of 5 ml and 5 ml hexane was added to further defat the protein. The defatted sample was collected by centrifugation at 2000*g* as described above. This crystalline precipitation and defatting procedure was repeated three more times. All the above procedures were carried out with protease inhibitors (100 n*M* aprotinin, 50 µ*M* antipain, 50 µ*M* leupeptin and 0.5 µg ml^{-1} pepstatin) and antibiotics (50 µg ml^{-1} ampicillin and 50 µg ml^{-1} kanamycin) present in the sample. To further purify the four-times crystallized Ber e 2, a 300 ml Superose 6 column (XK 26/70, GE Healthcare, Piscataway, NJ, USA) was used. The Ber e 2 protein was dissolved in 10 m*M* Tris buffer (10 m*M* Tris–HCl pH 7.5) containing 1 *M* NaCl to a final concentration of 10 mg ml^{-1}. 20 ml of the sample was loaded onto the column, which was pre-equilibrated and eluted with the same buffer that was used to dissolve the protein. Ber e 2 eluted as a single peak with a small shoulder in the leading edge. The shoulder was spared when the (8 ml) fractions of the main peak were pooled. The Ber e 2 in the pooled sample was recrystallized again by dialysis and pelleted by centrifugation. The pellet was collected and stored at 253 K or used directly for purity analysis and for crystallization setups.

2.2. SDS–PAGE analysis

SDS–PAGE was run with 4–20% polyacrylamide gels in Tris–HEPES–SDS running buffer (100 m*M* Tris, 100 m*M* HEPES, 3 m*M* SDS pH 8.0). Pre-stained protein molecular-weight markers (Precision Plus All Blue, Bio-Rad) containing protein standards of 10, 15, 20, 25, 37, 50, 75, 150 and 250 kDa were used as references. For reducing SDS–PAGE, 288 m*M* 2-mercaptoethanol was added to 2× nonreducing sample buffer [20% glycerol, 150 m*M* Tris–HCl pH 6.8, 4 m*M* EDTA, 4% SDS, 0.4%(*w*/*v*) bromophenol blue dye]. Ber e 2 samples were first dissolved in 8 *M* urea and then mixed with the reducing/nonreducing sample buffer at a ratio of 1:1 and boiled for 5 min. The boiled samples were cooled on ice before loading into the gel wells. After electrophoresis, the gel was stained in 0.1% Coomassie Brilliant Blue R-250, 40% ethanol, 10% acetic acid for 2 h and then destained with 40% ethanol, 10% acetic acid. Images of the gel were taken using a Nikon (Coolpix-995) digital camera and analyzed with the *ImageJ* software (Abramoff *et al.*, 2004).

2.3. Determination of Ber e 2 solubility

The solubility of Ber e 2 as a function of NaCl concentration was determined by dissolving the protein to saturation in 10 m*M* Tris buffer containing 1 *M* NaCl. The sample was then subjected to centrifugation to pellet the undissolved protein. An aliquot of the supernatant was then diluted with Tris buffer containing 1 *M* NaCl to bring the final absorbance at 280 nm to less than 1.0 as determined using a Cary 300 UV–visible spectrophotometer. Additional aliquots of the supernatant were diluted with Milli-Q water to the desired final NaCl concentrations and all samples were then subjected to centrifugation to pellet the 'desalted out' Ber e 2 microcrystals. The absorbance at 280 nm of the supernatants was determined as described. Because the starting microcrystals were not dry, the NaCl concentration of the first sample was determined by diluting an aliquot of it ten times with de-ionized water and measuring its refractive index at 589 nm at 293 K after the 'desalted out' Ber e 2 had been pelleted. The NaCl concentrations of the other samples were calculated based on that of the first sample and the dilution factors of the samples.

2.4. Crystallization of Ber e 2

A 0.55 *M* NaCl solution containing 280 mg ml^{-1} Ber e 2 was used as a starting solution for crystallization trials. 4 µl aliquots of protein sample were spotted onto EasyXtal CrystalSupports (Qiagen, Valencia, CA, USA) and screw-sealed over reservoir solutions of 0–0.5 *M* NaCl at 0.05 *M* intervals. Ber e 2 crystals were readily

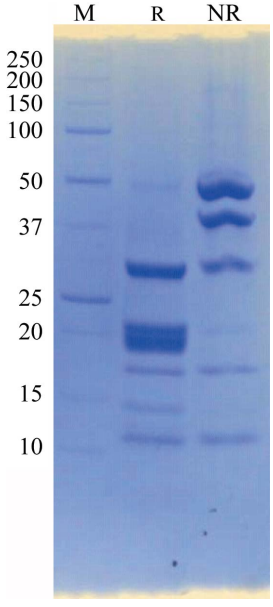


Figure 1
Purification of Ber e 2. Purified Ber e 2 was analyzed by SDS–PAGE under both reducing (lane R) and non-reducing (lane NR) conditions. The molecular weights (in kDa) of the protein standards contained in the marker (lane M) are shown on the left-hand side of the gel image.

obtained in all the drops. However, after several rounds of optimization, the best crystals were obtained in drops starting from 0.45 *M* NaCl solution containing ∼100 mg ml^{-1} Ber e 2 over pure water.

2.5. X-ray diffraction experiments and crystal characterization

In order to collect X-ray diffraction data at low temperature, sucrose, glycerol, PEG 400 and ethylene glycol at 30%(*v*/*v*) in water were tested for their ability to protect Ber e 2 crystals. Single crystals were picked up with Mounted CryoLoops (Hampton Research) that were attached to a CrystalCap Copper Magnetic (Hampton Research), briefly plunged into one of the cryoprotectant solutions, flash-frozen in liquid nitrogen and checked for diffraction using the SER-CAT 22BM beamline at the Advanced Photon Source (APS), Argonne National Laboratory. The glycerol solution was used as a cryoprotectant for the final data collection. X-ray data collection was performed using a MAR225 CCD detector. The diffraction data were processed using the *HKL*-2000 suite of programs (Otwinowski & Minor, 1997) and *XGEN* (Howard, 2000). A structural model was derived by molecular-replacement calculations using the program *Phaser* (McCoy *et al.*, 2005; Storoni *et al.*, 2004), starting with a Ber e 2 homology model constructed using the program *SCWRL* (Canutescu *et al.*, 2003) from chain *A* of the structure of glycinin from a mutant soybean cultivar composed of only the A3b4 subunit (Adachi *et al.*, 2003; PDB code 1od5) as a template.

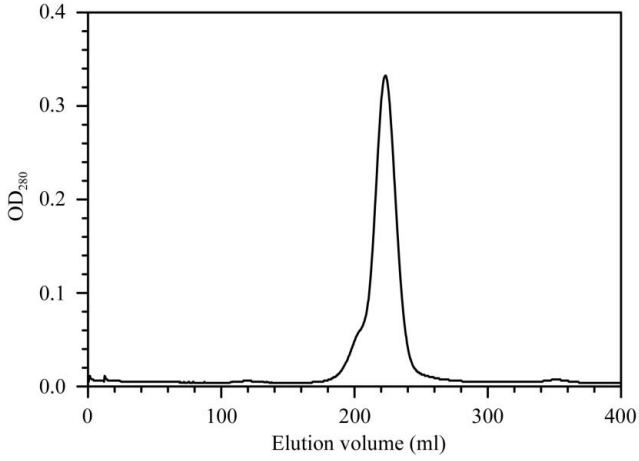


Figure 2
Elution profile of Superose 6 gel-filtration chromatography of fourfold-crystallized Ber e 2. 8 ml fractions of the major peak (excluding the front shoulder) were collected for crystallization trials.

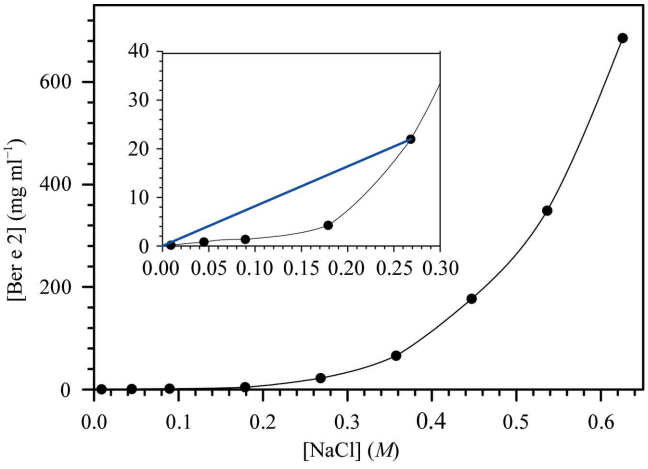


Figure 3
Solubility of Ber e 2 as a function of NaCl concentration. The absorbance values at 280 nm of the supernatants of saturated Ber e 2 solutions after centrifugation were measured. The concentration of Ber e 2 was then determined using a theoretical extinction coefficient of ε_{280} = 42 570 cm^{-1} mol^{-1} calculated according to the Ber e 2 sequence (Entrez protein sequence AAO38859).

3. Results and discussion

Ber e 2 was crystallized more than a century and a half ago as a means of purifying the protein (Hartig, 1855; McPherson, 1999). In order to ensure the purity of the protein, the protein was crystallized four times and defatted in this study. As shown in Fig. 1, purified Ber e 2 contained multiple bands on SDS–PAGE. This is consistent with the reported SDS–PAGE patterns for Ber e 2 and for 11S proteins from other species (Dickinson *et al.*, 1989), and indicated that Ber e 2 was post-translationally cleaved, as pro-Ber e 2 consists of 465 amino acids with a theoretical molecular weight of ∼52 kDa and other 11S proteins are also known to be cleaved, yielding an N-terminal acidic subunit and a C-terminal basic subunit (Kitamura *et al.*, 1976). However, whether all the bands separated from the purified protein result from post-translational cleavage of Ber e 2 awaits the identification of the peptide by N-terminal sequencing. Fig. 1 also shows that there is/are interchain disulfide-bond link(s) between the cleaved peptides of Ber e 2. This is also consistent with observations on 11S proteins from other species (Kitamura *et al.*, 1976). The front shoulder of the gel-filtration peak (Fig. 2) apparently contained the same peptide components as the main peak as judged by SDS–PAGE analysis (data not shown). However, for the purpose of obtaining diffraction-quality crystals, the fractions containing the shoulder were not used.

To obtain information on the behavior of Ber e 2 in solution, its solubility as a function of salt concentration was determined. As shown in Fig. 3, the solubility increases rapidly with the concentration of NaCl. Above the saturation line, Ber e 2 will be supersaturated and below the line it will be below saturation. If a sample is allowed to absorb water, both the salt concentration and the protein concentration will decrease, but as long as the curvature of the saturation line is positive ($d^2y/dx^2 > 0$), the absorption of water by a sample on the saturation line will result in supersaturation of the protein. This is also represented by a straight line connecting a point on the saturation line and the origin of the plot going through the supersaturation region (insert in Fig. 3). This indicates that a Ber e 2 sample close to saturation at an NaCl concentration of >0.20 *M* can be used for a crystallization trial in a vapor-diffusion setup using a 'desalting-out' mode, where water molecules will 'diffuse' from the reservoir to the sample. Thus, crystallization trials were set up using reservoir solu-

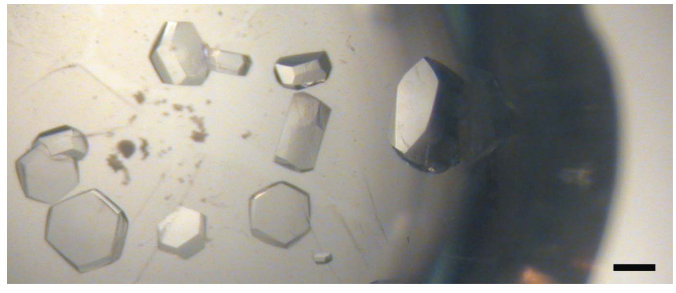


Figure 4
Single crystals of Ber e 2 obtained by vapor diffusion using the hanging-drop method. Bar, 200 µm.

Table 1
X-ray data-collection statistics.

Values in parentheses are for the outer shell.

Wavelength (Å)	1.0
Data-collection temperature (K)	110
Resolution (Å)	46.2–2.04 (2.10–2.04)
Unit-cell parameters (Å)	$a = b = 92.44$, $c = 213.22$
No. of observed reflections	177787
No. of unique reflections	42858
Completeness (%)	98.4 (93.4)
Mean $I/\sigma(I)$	7.5 (2.0)
$R_{\rm sym}$† (%)	15.3 (56.1)

† $R_{\rm sym} = \sum_{hkl}\sum_i |I_{hkl,i} - \langle I_{hkl}\rangle| / \sum_{hkl}\sum_i I_{hkl,i}$.

tions with lower salt concentrations than that in the starting crystallization drops. As shown in Fig. 4, single crystals as large as ∼0.4 × 0.4 × 0.2 mm were obtained using the hanging-drop vapor-diffusion method.

Diffraction data were collected using the SER-CAT 22BM beamline at APS. The Ber e 2 crystals diffracted to 2.04 Å and a complete data set (150 1° frames with 5 s exposure) was collected (Table 1). Data processing with *HKL*-2000 (Otwinowski & Minor, 1997) and *XGEN* (Howard, 2000) revealed the hexagonal space group *H*3, with unit-cell parameters $a = b = 92.44$, $c = 213.22$ Å. Assuming the presence of two Ber e 2 monomers in an asymmetric unit, the Matthews coefficient was 1.68 Å^3 Da^{-1}, corresponding to protein crystals containing 27% solvent, given an average partial specific volume of 0.74 cm^3 g^{-1} for proteins; assuming the presence of one Ber e 2 monomer per asymmetric unit, the Matthews coefficient is 3.35 Å^3 Da^{-1}, with a solvent content of 63%.

A BLAST search showed 38% sequence identity between Ber e 2 and the soybean glycinin A3b4 subunit. A homology model of Ber e 2 was constructed using the program *SCWRL* (Canutescu *et al.*, 2003) and the monomeric Ber e 2 model was used in a molecular-replacement calculation using *Phaser* (McCoy *et al.*, 2005; Storoni *et al.*, 2004). This generated a molecular-replacement solution with two Ber e 2 molecules in the asymmetric unit and a log-likelihood gain of 2510. The *R* factor of the solution after one round of rigid-body and restrained refinement with the program *REFMAC* (Murshudov *et al.*, 1997) was 28.2% to 2.04 Å resolution, with an $R_{\rm free}$ of 35.7% using 5% test refractions selected from thin shells.

A preliminary inspection of the map calculation and refinement led to an experimental electron-density map at 2.04 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. Preliminary inspection of the model suggested that there are two disulfide bonds in Ber e 2, between Cys31 and Cys64 and between Cys107 and Cys286, and an expected hexamer of Ber e 2 can be generated by crystallographic symmetry operations. Currently, model building and refinement of the structure is under way. Structure determination of Ber e 2 will provide information necessary for determining the similarities and differences between 11S protein allergens from different food sources in order to infer the structural basis of their allergenicity and allergic cross-reactivity.

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