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Research Article

Purification and structural stability of the peach allergens Pru p 1 and Pru p 3

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Pru p 1 (a Bet v 1 homologue) and Pru p 3 (a nonspecific lipid transfer protein; nsLTP) are major allergenic proteins in peach fruit, but differ in their abundance and stability. Pru p 1 has low abundance and is highly labile and was purified after expression as a recombinant protein in *Escherichia coli*. Pru p 3 is highly abundant in peach peel and was purified by conventional methods. The identities of the proteins were confirmed by sequence analysis and their masses determined by MS analysis. The purified proteins reacted with antisera against related allergens from other species: Pru p 1 with antiserum to Bet v 1 and Pru p 3 with antiserum to Mal d 3 (from apple). The presence of secondary and tertiary structure was demonstrated by circular dichroism (CD) and high field NMR spectroscopy. CD spectroscopy also showed that the two proteins differed in their stability at pH 3 and in their ability to refold after heating to 95°C. Thus, Pru p 1 was unfolded at pH 3 even at 25°C but was able to refold after heating to 95°C at pH 7.5. In contrast, Pru p 3 was unable to refold after heating under neutral conditions but readily refolded after heating at pH 3.

Keywords: Bet v 1 homologue / Food allergy / Lipid transfer protein / Peach / Pru p 3

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1 Introduction

Peach (*Prunus persica*) is a native plant of China but is grown throughout the world, particularly in areas with a Mediterranean climate such as Southern Europe. Cultivated peaches are divided into two types, freestone and clingstone, depending on whether the flesh adheres to the stone, while nectarines are a group of cultivars in which the skin is smooth rather than downy. The genus *Prunus* comprises many species and interspecific hybridization is common. Related species, which may hybridize with peach, include plums and damsons (*P. domestica*, *P. insititia*, *P. cerasifera*, *P. salicina*, *P. americana*), apricot (*P. armeniaca*), almond (*P. dulcis*) and cherry (*P. avium*, *P. cerasus*) [1]. Peach is associated with two major allergy syndromes, in Central and Northern Europe and in Southern Europe, respectively.

In Central and Northern Europe peach allergy is strongly associated with birch pollinosis and crossreaction to peach fruit may occur only after primary sensitization *via* the inhalant route to the major birch pollen (BP) allergen Bet v 1, a 17 kDa protein [2, 3]. More than 50% of BP-allergic patients display allergic symptoms when eating fruits, vegetables and nuts [4, 5], with hazelnut, apple, walnut, cherry and

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Abbreviations: BP, birch pollen; **nsLTP,** nonspecific lipid transfer protein; **OAS,** oral allergy syndrome

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peach forming the 'top five' sources of BP-associated food allergens [6–8]. Elicited allergic symptoms are usually confined to the oral allergy syndrome (OAS), comprising local reactions of the mucosa of the upper aero-digestive tract with itching, inflammation and angioedema. The Bet v 1 homologue from peach is therefore termed Pru p 1. The biological role of Pru p 1 is not known but the fact that it and Bet v 1 belong to the PR-10 family of 'pathogenesis related proteins', which are synthesized in plants in response to damage or infection, indicates that they may be protective [9]. Other biological properties such as binding of plant steroids and lipids have also been reported [10, 11]. Pru p 1 is present in the fruit in low amounts and is highly labile to denaturation, a property shared with Mal d 1 of apple [12], Pru av 1 of sweet cherry [13] and other Bet v 1 homologues.

Peach is also the fresh fruit most frequently involved in allergic reactions in adult patients in the Mediterranean area, especially Spain [14] and Italy, the clinical presentation including both mild and severe symptoms. Local reactions such as OAS and contact urticaria are observed in more than 50% of patients and systemic reactions are frequently observed (18–44%), even in the absence of OAS [15, 16]. These symptoms, which may be severe and life-threatening, are elicited by Pru p 3, a member of the family of nonspecific lipid transfer proteins (nsLTPs). Pru p 3 was described as major peach allergen in 1999 [17] and is responsible for allergy to peach and other Rosaceae fruits without related pollinosis [15, 18]. Sensitization to Pru p 3 has been detected in more than 90% of patients allergic to peach in the Mediterranean area and probably occurs *via* the gastro-intestinal tract [19]. The prevalence of actual peach allergy established by double-blind, placebo-controlled food challenges (DBPCFCs) is 70–80% [16, 19].

The nsLTPs are related to other small sulphur-rich proteins such as the 2S albumins and cereal grain inhibitors of α-amylase [20, 21] and typically comprise about 90–95 amino acid residues. Different members vary in sequence identity from about 30–95% but all contain eight conserved cysteine residues which form four intrachain disulphide bonds [22, 23]. They are highly immunologically crossreactive, which has been demonstrated *in vivo* and *in vitro* [24, 25]. The nsLTPs have a highly conserved 3-D structure which is rich in α-helix and contains a hydrophobic cavity which can accommodate lipids [23]. They are also highly stable to denaturation and proteolysis, a characteristic shared with a number of related allergenic proteins including the 2S albumins and a-amylase inhibitors [21]. NsLTPs are major allergens in a number of plant foods, including other species of *Prunus* (Pru ar 3 in apricot, Pru av 3 in cherry, Pru d 3 in plum). They also have antifungal activity, belonging to the PR-14 protein family [26]. Unlike Pru p 1, Pru p 3 is abundant in the fruit, being present in approximately tenfold greater amounts in the peel than in the pulp [27, 28].

Pru p 1 and Pru p 3 together account for more than 95% of peach allergies in Europe. We therefore report their purification and characterization. In the case of Pru p 3 the abundance and stability allowed it to be purified as natural allergen using conventional biochemical approaches. In contrast, the low level and lability of Pru p 1 required a different approach to be adopted and the protein was expressed in *Escherichia coli* as a nonfusion recombinant protein.

2 Materials and methods

2.1 Generation of recombinant Pru p 1

2.1.1 Extraction and isolation of total RNA

Total RNA was extracted from 100 g fresh peach fruit (cultivar Red Haven) using the acidic guanidinium-Cl method including CsCl-purification [29]. Peel and pulp from peach were frozen in liquid nitrogen and ground to a fine powder in a grinding machine. Extraction buffer was added and the RNA was extracted as described [29].

2.1.2 cDNA synthesis, PCR and rapid amplification of 5′ and 3′ cDNA ends (RACE)

Total RNA (10 μg) was reverse transcribed using 40U Moloney murine leukemia virus reverse transcriptase (Fermentas, St. Leon-Roth, Germany). A synthetic oligonucleotide was used as a primer for first-strand cDNA synthesis (5′-GA GAA GGA $(T)_{25}$ AGC AGC T-3′) which was performed at 42°C for 60 min followed by heat denaturation at 95°C for 5 min. Based on the known cDNA sequences of the Bet v 1 homologues from cherry, apricot, apple and pear, a Mal d 1-specific primer pair (forward: 5′-ACA CCT CTG AGA TTC CAC CAC-3k; reverse: 5′-CAA CTT GGT YTC GTA AGA GAC-3′) was used for PCR, which was carried out in a final reaction volume of 40 μL containing 250 μM of dNTPs (Fermentas) and 12.5 μM of each primer. After a hot start, the PCR (35 cycles) was performed for 30 s 94°C, 45 s 59°C, 1 min 72°C. The RACE technology (GeneRacer Kit; Invitrogen, Paisley, UK) was used for amplification of the unknown 5′ and 3′ ends of the peach allergen according to the manufacturer's instructions. The 5′ cDNA end was amplified with a 5′ primer (5′-CGA CUG GAG CAC GAG GAC ACU GA-3′) purchased with the kit and a 3′ gene-specific primer (5′-GCT ACG TGA AGC ACA AGA TCG ACT C-3′) and the 3′cDNA end with a 5′gene-specific primer (5′-GCT ACG TGA AGC ACA AGA TCG ACT C-3′) and the 3′primer from the kit (5′-GCT GTC AAC GAT ACG CTA CGT AAC G-3′), respectively.

2.1.3 Cloning and sequencing of Pru p 1

PCR-fragments were purified from 1.2% w/v agarose gels using the QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany). DNA-fragments were ligated into pCR2.1-TOPO (TOPO TA Cloning Kit; Invitrogen) and the chemically competent *E. coli* strain XL1-Blue was transformed. Positive clones were selected by blue/white screening on plates containing X-gal and PCR screening with insert primer pairs.

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DNA sequence analysis of the subsequently isolated plasmids was performed with an automatic LI-COR fluorescent sequencer 4000 L (LI-COR, Lincoln, NE, USA).

2.1.4 Expression and purification of recombinant Pru p 1

To obtain full-length coding DNA, a *Nco*I restriction site was introduced at the 5′end and an *Eco*RI restriction site at the 3′end of the Pru p 1 encoding cDNA by PCR (forward Pru p 1: 5′-GGG *CCA TGG* GTG TCT TCA CAT ATG-3′; reverse Pru p 1: 5′-CCC *GAA TTC* TTA GTT GTA GGC ATC G-3′) and cloned into the expression plasmid pETBlue-2 (Novagen, Madison, USA). The initial cloning was done in the *E. coli* strain NovaBlue. For target gene expression, as a nonfusion protein, the *E. coli* strain Tuner (DE3)pILacI was transformed. Cells were grown in LB medium containing 1% w/v glucose, 100 µg/mL ampicillin and 20 µg/mL chloramphenicol to an OD of 0.6 measured at 600 nm. Expression of the recombinant protein was induced for 4 h at 30°C by inactivation of the *lac* repressor *via* addition of isopropyl-β-*d*-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Subsequently, cells were harvested by centrifugation ($3000 \times g$ for 20 min, 4°C) and disrupted using high pressure.

Biocryl precipitation (Tosohaas Bioseperation Specialists, USA) and ammonium sulphate precipitation (40% saturation) were carried out with the soluble cell fraction as prepurification steps. Recombinant Pru p 1 was enriched by anion exchange chromatography (10 mL Q Sepharose; GE Healthcare, Little Chalfont, UK) and eluted with a linear salt gradient (buffer A: 25 mM Tris-HCl, pH 8; buffer B: buffer A + 1 M NaCl; 0–25% B over 5 column volumes (CVs), flow rate 1 mL/min). Hydrophobic interaction chromatography (10 mL Phenyl Sepharose; GE Healthcare) was performed as a final purification step and rPru p 1 was eluted with a decreasing salt and an increasing pH gradient (buffer A: 20 mM sodium phosphate, 1 M ammonium sulphate, pH 6; buffer B: 80 mM Tris-HCl, 5% propan-2-ol, pH 9; 0–60% B over 5 CVs, 1 mL/min). To minimize the sample volume and to maximize the protein concentration, the anion exchange chromatography was repeated under the same conditions. Fractions containing rPru p 1 were pooled and dialysed against 10 mM sodium phosphate, pH 7.5, using a 6–8 kDa molecular weight cut off (MWCO) membrane (Spectrum Laboratories, Rancho Dominguez, USA). Purity was assessed by 15% SDS-PAGE under reducing conditions [30]. The concentration of the purified protein was determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, USA).

2.2 Purification of natural Pru p 3

Nonspecific lipid transfer protein (nsLTP) is abundant in the skin of peach fruit [31]. The peel from 5 kg of peaches (approximately 0.5 kg) was therefore flash frozen in liquid nitrogen and ground. The powder was then suspended in extraction buffer (46 mM sodium phosphate, 2 mM EDTA, 20 mM sodium diethyldithiocarbamate, 3 mM NaN_3, 3% w/v PVPP, pH 7.0) and stirred at 4°C for 60 min. The suspension was clarified by centrifugation ($10\,000 \times g$ for 10 min, 4°C) and the supernatant retained. The soluble protein was precipitated with ammonium sulphate to 95% w/v and the pellet resuspended in 300 mL of water. Salt was removed from the extract by dialysis, using 3.5 kDa MWCO dialysis tubing (Pierce), firstly against 10 L of water and then against 10 L of 20 mM 2-(*N*-morpholino) ethanesulphonic acid (MES buffer), pH 5.6. The dialysate was further clarified by centrifugation ($30\,000 \times g$ for 30 min, 4°C) and the supernatant was filtered to 0.45 µm.

The extract was loaded onto a 4.6 × 100 mm HS-Poros column (Perseptive Biosystems, Framingham, USA) prequilibrated with 20 mM MES buffer, pH 5.6, using a BioCad Sprint system (Perseptive Biosystems) and eluted with a linear salt gradient (buffer A: 20 mM MES, pH 5.6; buffer B: buffer A + 0.6 M NaCl; 0–100% B over 30 CVs, 5 mL/min). The absorbance was monitored at 220 and 280 nm and fractions analysed by SDS-PAGE. Fractions containing Pru p 3 (mass approximately 9 kDa) were concentrated by ultrafiltration with an MWCO of 5 kDa (Amicon).

The fractions from cationic exchange were loaded on to a Superdex™ 75 column (1.6 cm × 60 cm) equilibrated and eluted with 25 mM disodium hydrogen phosphate pH 7, 150 mM NaCl at a flow rate of 0.75 mL/min. The absorbance was monitored at 280 nm and the peak eluting at approximately 9 kDa was collected and concentrated by ultrafiltration, with an MWCO of 5 kDa (Amicon). This fraction was analysed by 15% SDS-PAGE under reducing conditions [30] and the protein concentration determined by a BCA assay (Pierce).

2.3 Immunoblotting

Western blotting of rPru p 1 was carried out with monoclonal and polyclonal anti-Bet v 1 antibodies using alkaline phosphatase(AP)-conjugated rabbit antimouse IgG (Jackson ImmunoResearch, West Grove, USA) and swine antirabbit IgG (Dako, Glostrup, Denmark), respectively, for detection.

Polyclonal antibodies against Mal d 3 and Cor a 8 were used for Western blotting of Pru p 3 using AP-conjugated swine anti-rabbit IgG for detection.

Detection of IgE binding proteins by immunoblotting was performed as described previously [32]. Briefly, the proteins were separated by 15% SDS-PAGE under reducing conditions and transferred to nitrocellulose (NC) membrane. After blocking, the membrane was incubated with 1:5 diluted sera from peach allergic patients. Bound IgE was detected using 1:40 diluted ^{125}I-labelled anti-human IgE (Demeditec Diagnostics, Germany). For control experiments, buffer and normal human serum (NHS) were tested, respectively.

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2.4 IgE ELISA

Microtiter plates (Nunc MaxiSorp, Roskilde, Denmark) were coated with 1 μg of proteins *per* well. After blocking with Tris-buffered saline (TBS) containing 0.5% v/v Tween-20 and 3% w/v nonfat dry milk, 1:5 diluted sera were applied onto the plates and incubated overnight at 4°C. After washing, incubation with a 1:1000 diluted AP-conjugated mouse anti-human IgE antibody (BD Pharmingen, San Diego, USA) was carried out for 2 h at room temperature in the dark. Color development was performed using *p*-nitrophenyl phosphate substrate tablets (Sigma–Aldrich, Steinheim, Germany) and the OD was measured at 405 nm. Buffer and sera of three nonallergic subjects were used as negative controls. OD values were counted positive if they exceeded the mean OD of the negative controls by more than three SDs.

2.5 *N*-terminal sequencing

N-terminal sequencing of purified proteins was performed with an Applied Biosystems Procise 491 sequencer (Applied Biosystems, Foster City, USA). rPru p 1 and nPru p 3 (50 pmol) were adsorbed on a Prosorb cartridge and subjected for sequence analysis, respectively. Sequence data were compared with protein databases using the BLAST program.

2.6 MS

For mass determination of intact rPru p 1, the protein was dissolved in 25% v/v ACN in 0.1% v/v aqueous formic at a concentration of 1 pmol/μL and directly infused into a Global Ultima Q-Tof instrument (Waters, Manchester, UK) with ESI. The infusion rate was 1 μL/min, using the Waters Nanoflow spray head with nitrogen as desolvation gas and a capillary voltage of 3.4 kV. The instrument was calibrated with horse heart myoglobin (Sigma, St. Louis, USA). Spectra of multiple charged ions were recorded for 3 min in a mass/charge range from 400 to 1900. Spectra were then combined and deconvoluted using the MaxEnt1 software supplied with the instrument. For sequence analysis, 5 μg of rPru p 1 were digested with the Proteoextract Trypsin Digestion Kit (Calbiochem, San Diego, USA). The resulting peptides were separated by capillary RP-HPLC (Waters, Milford, USA) directly coupled to the mass spectrometer (precolumn Waters Nanoease Symmetry300 trap column, separating column Waters Nanoease Atlantis dC18, connected *via* a 10 port stream select valve). The flow rate was adjusted to 300 nL/min by T-splitting. Peptides were eluted with an ACN gradient (solvent A: 0.1% v/v formic acid, 5% v/v ACN; solvent B: 0.1% v/v formic acid, 95% v/v ACN; 5–45% B in 90 min). For sequence analysis, the instrument was calibrated with the fragment ions of [Glu]-fibrinopeptide B (Sigma). Data were acquired in the Data Directed Analysis (DDA) mode. Survey and fragment spectra were analysed using the software PLGS version 2.2.5 (Waters) with automatic and manual data verification. For sequence identification a mini database comprising the trypsin and Pru p 1 sequences and a combined SwissProt/Trembl database were used.

Pru p 3 was subjected to RP-HPLC with on-line ESI-MS analysis using a modification of the method of Moreno *et al*. [33]. Pru p 3 was dissolved in water and applied to a Jupiter 5 μm, C4, 300 Å column (4.6 mm × 250 mm Phenomenex) equipped with a guard cartridge (3 mm × 4 mm, wide-pore C4, 'Security Guard', Phenomenex). Peptides were eluted with an ACN gradient (solvent A: 0.1% v/v TFA; solvent B: 0.085% v/v TFA 90% v/v ACN; 0.9–90% solvent B in 15 min). Native Pru p 3 was analysed by MALDI-TOF MS after in-gel trypsin digestion at the joint Institute of Food Research-John Innes Centre (IFR-JIC) proteomics facility as described by Moreno *et al*. [33].

2.7 NMR analysis

The allergens rPru p 1 and nPru p 3 were analysed by NMR, according to the following protocol. A 0.028 mM solution of rPru p 1 was prepared in 0.45 mL H_2O plus 0.05 mL of D_2O. The concentration of the purified nPru p 3 was increased by centrifugation coupled with a centrifugal filter unit (Centricon®) to a final concentration of 0.39 mM. The solutions were placed in high-quality NMR tubes with Argon as head-space gas. Two high resolution NMR experiments were carried out using a Bruker Avance 700 spectrometer operating at a proton resonance frequency of 700 MHz (11.7 Tesla) at 25°C. The two experiments differed in the method used to manage the water signal: the zgpr experiment minimizes it, while the zgesgp experiment suppresses the water peak. For each experiment 64 scans were programmed to analyse rPru p 1 and 256 for nPru p 3, respectively.

2.8 Circular dichroism (CD) spectroscopy

The secondary structures of the purified peach allergens were determined under varying conditions of pH and temperature using CD spectroscopy. For acidic conditions, the proteins were dialysed against 10 mM potassium phosphate, pH 3 and measured at concentrations of 0.23 mg/mL for rPru p 1 and 0.22 mg/mL for nPru p 3 in a Jasco J-810 spectropolarimeter (Jasco, Easten, USA) in a 0.1 cm quartz cuvette. Far UV spectra (250–190 nm) were recorded during heating (2°C/min) from 25 to 95°C at intervals of 10°C. The same procedure was performed for both allergens at pH 7.5 using a 10 mM sodium phosphate buffer (0.35 mg/mL rPru p 1 and 0.27 mg/mL nPru p 3). Data from three measurements were accumulated to calculate the mean spectra.

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2.9 Fourier transform-infrared (FT-IR) spectroscopy

Pru p 3 solution was prepared in distilled water and FT-IR spectra recorded according to Moreno *et al*. [33]. Briefly, FT-IR spectra were recorded using a Biorad FTS 175 C spectrometer equipped with a liquid N_2-cooled mercury cadmium telluride detector. Spectra were averages of 256 scans recorded at a resolution of 2 cm^{-1}. Self-deconvolution was carried out using spectrometer software (WINIR Pro).

2.10 Patients' sera and antibodies

Sera (15 for Pru p 1 and 5 for Pru p 3) were collected from patients with established food allergy to peach according to convincing case history (reported adverse reactions, *e.g.* OAS and in more severe cases even generalized symptoms such as urticaria and angioedema when eating fresh peach) and positive prick tests with fresh fruits. Murine mAb (BIP 1 [34]) raised against natural Bet v 1 and polyclonal rabbit antiserum raised against purified rBet v 1a [35] were used for rPru p 1 detection. Polyclonal rabbit antisera raised against nMal d 3 (apple LTP) and nCor a 8 (hazelnut LTP) were used for nPru p 3 detection (provided by J. Lidholm, Phadia AB, Uppsala, Sweden).

3 Results and discussion

3.1 Recombinant Pru p 1

3.1.1 Purification of rPru p 1

The Pru p 1 cDNA comprises 838 bp with an ORF of 483 nucleotides, coding for a protein of 159 amino acid residues (initiating methionine excluded) with a predicted molecular mass of 17516.6 and a calculated p*I* of 5.8. This sequence was entered into the EMBL database with the accession number AM 493970. The Pru p 1 coding sequence is identical to that reported by Lidholm *et al.* (EMBL accession no. DQ 251187), but minor differences are present in the 5′ and 3′ noncoding regions (C → T exchange at position 639 and 82 additional nucleotides at position 757–819 before the poly-A tail). One possible polyadenylation signal AATAAG is present, 115 nucleotides upstream of the poly-A tail. Pru p 1 shares 98% amino acid sequence identity with Pru av 1, the major allergen from sweet cherry, the two proteins differing only in three amino acid residues. Sequence identities with proteins from other members of the Rosaceae family are much lower: Mal d 1, Pyr c 1 and Pru ar 1 share 86, 83 and 76% sequence identity with Pru p 1, respectively (Table 1).

The Pru p 1 cDNA was cloned into the expression vector pETBlue-2 and expressed as a nonfusion recombinant protein in *E. coli* Tuner(DE3)pLacI. Induction with IPTG was carried out at 30°C (Fig. 1; lane 2) to reduce the formation of inclusion bodies, which formed at 37°C when higher cell densities and high protein expression levels were reached [36]. Recombinant Pru p 1 was isolated from the soluble fraction after disrupting the cells with a French press and purified to homogeneity by several precipitation steps, anion exchange chromatography and hydrophobic interaction chromatography. This purification protocol yielded 30 mg purified recombinant protein from 10 L of culture. SDS-PAGE (15%) under reducing conditions showed a single band of mass about 18 kDa (Fig. 1; lane 3).

Table 1. Amino acid sequence identity of Pru p 1 and related allergenic proteins

Allergen	Sequence identity %
Pru av 1	98
Pru ar 1	76
Mal d 1	86
Pyr c 1	83
Bet v 1	59

Pru av 1 (major cherry allergen; UniProt accession no. O24248), Pru ar 1 (from apricot; UniProt accession no. O50001), Mal d 1 (major apple allergen; UniProt accession no. Q9SYW3), Pyr c 1 (from pear; UniProt accession no. O65200) and Bet v 1 (major BP allergen; UniProt accession no. P15494).

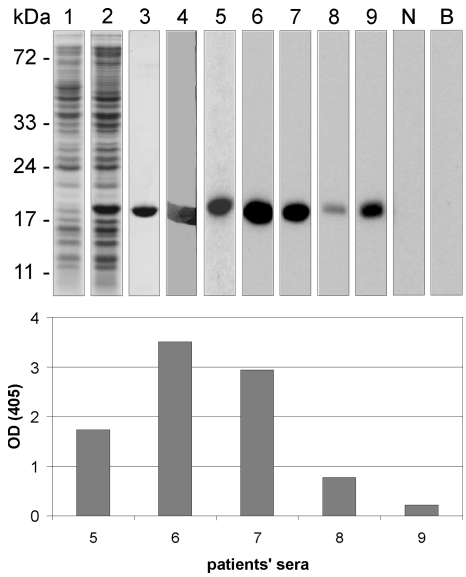

Figure 1. SDS-PAGE analysis using 15% acrylamide gels under reducing conditions and immunological characterization of rPru p 1. Top panel: lanes 1, 2 and 3 show CBB stained SDS-PAGE separations of crude lysates from control cells of *E. coli* (lane 1), cells expressing rPru p 1 (lane 2) and 1 μg purified rPru p 1 (lane 3). Lanes 4–9 show Western blots of 1 μg purified rPru p 1 reacted with polyclonal antiserum to Bet v 1 (lane 4) and sera of peach allergic patients (lanes 5–9). Lanes N and B show similar blots with control serum (N) and buffer (B). Bottom panel: results of IgE ELISA analysis with sera 5–9 used in the top panel.

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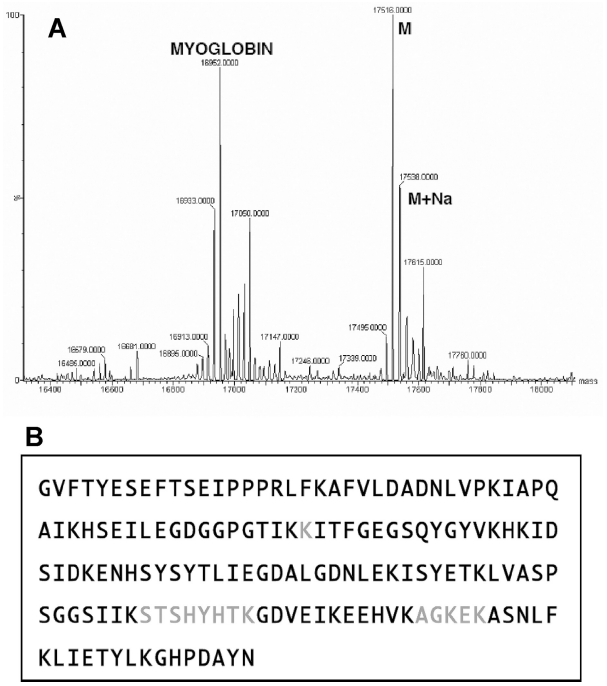

Figure 2. MS and peptide fingerprinting of rPru p 1. (A) Mass determination of intact rPru p 1 (1 pmol/μL) performed by ESI-TOF. Horse heart myoglobin (theoretical mass 16 952, accession number GI.2982074) was used as an internal standard. (B) 5 μg of rPru p 1 was digested with trypsin and the resulting peptides analysed and sequenced by LC-MS-MS (for details see Section 2). Amino acid residues verified by sequence analysis are shown in black.

3.1.2 Characterization of rPru p 1

The molecular mass of the purified intact rPru p 1 was determined by MS as 17 516, which was in excellent agreement with the theoretical mass of 17 516.6 Da (Fig. 2A). *N*-terminal sequencing of five amino acid residues (GVFTY) confirmed the identity of rPru p 1, showing that the initiating methionine was cleaved off according to the side chain length of the penultimate amino acid [37]. Further confirmation of the amino acid sequence was obtained after digestion with trypsin, the resulting peptides being analysed by LC-MS-MS. A total of 14 peptides with sequences matching that of Pru p 1 were identified, covering more than 90% of its sequence (Fig. 2B). Neither the mass measurements nor the peptide sequencing experiments showed evidence of contamination of the rPru p 1 preparation with other proteins. Similarly, no chemical modifications of amino acid residues that might have occurred during the expression and purification procedures were detected.

The NMR spectra of rPru p 1 have well-resolved peaks in the whole −0.5–10 ppm range. The spectrum derived from the zgesgp experiment (Fig. 3A) shows several peaks in the 5 ppm region, where they are obscured by the water peak in the spectrum without suppression of the water signal (zgpr

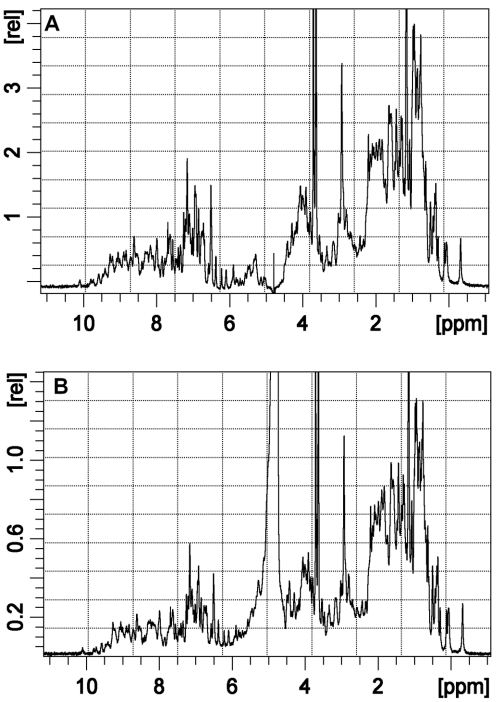

Figure 3. ^{1}H 700 MHz NMR spectra of rPru p 1. (A) Zgesgp experiment, (B) zgpr experiment.

experiment; Fig. 3B). These peaks come from the *alpha* protons, whose signals are dispersed above and below 4.4 ppm by the tertiary structure. Hence, it can be concluded that tertiary structure is present and the recombinant Pru p 1 is folded.

The purified rPru p 1 (1 μg *per* lane) reacted strongly with the polyclonal rabbit anti-Bet v 1 antibody (Fig. 1; lane 4) [35], but reacted only weakly with BIP 1, the monoclonal mouse anti-Bet v 1 antibody [34] (data not shown). It is possible that Pru p 1 either lacks some epitopes present on the Bet v 1 molecule, or that small differences were present between the structures of common epitopes on the two proteins, reducing crossreactivity. The respective buffer controls were negative. IgE immunoblot analyses were performed with sera from five peach-allergic patients. They all displayed IgE binding to rPru p 1 (Fig. 1; lanes 5–9) while no IgE reactivity was observed with NHS (Fig. 1; lane N) or with the buffer control (Fig. 1; lane B). The same sera and ten additional sera of peach allergic patients were all able to bind IgE to rPru p 1 in an ELISA experiment.

3.2 Natural Pru p 3

3.2.1 Purification of nPru p 3

Pru p 3 is highly enriched in the peel of the peach fruit so approximately 500 g peel was used for protein isolation. The extract was initially separated by cation exchange chromatography giving a fraction enriched in Pru p 3 but also containing proteins of higher molecular mass. The latter

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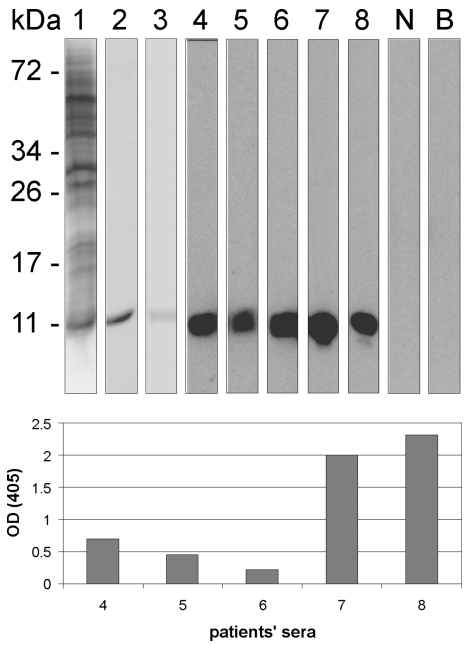

Figure 4. Purification and immunological characterization of nPru p 3. Top panel: lanes 1 and 2 show CBB stained SDS-PAGE separations using 15% acrylamide gels under reducing conditions of crude extract of peach peel (lane 1) and 1 μg purified nPru p 3 (lane 2). Lanes 3–8 show Western blots of 1 μg purified nPru p 3 reacted with polyclonal antiserum to Mal d 3 (lane 3) and sera of peach allergic patients (lanes 4–8). Lanes N and B show similar blots with control serum (N) and buffer (B). Bottom panel: results of IgE ELISA analysis with sera 4–8 used in the top panel.

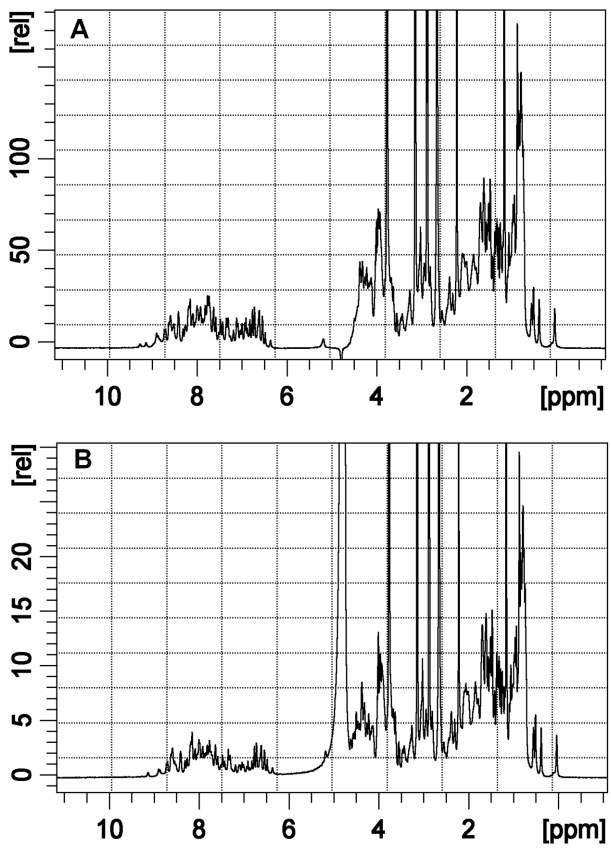

Figure 5. ^{1}H 700 MHz NMR spectra of nPru p 3. (A) zgesgp experiment, (B) zgpr experiment.

were removed by gel filtration chromatography, resulting in an essentially pure preparation (Fig. 4; lane 2). Pru p 3 migrates as an 11 kDa band on SDS-PAGE under reducing conditions. The yield from 500 g of peel was approximately 30 mg.

3.2.2 Characterization of nPru p 3

The molecular mass of the purified intact Pru p 3 was determined by LC-ES MS as 9132 Da, which was in agreement with the theoretical mass (9135 Da, UniProt accession no.: Q8H2B2). *N*-terminal sequencing of ten amino acid residues (ITCGQVSSSL) confirmed the identity as nPru p 3, showing 100% identity with the EMBL accession no. CAB96876 submitted by Diaz-Perales *et al*. Similarly, MALDI-TOF MS of tryptic peptides showed masses corresponding to those of Pru p 3, with a probability of identification of 100% based on the molecular weight search (MOWSE) score.

The NMR spectra of nPru p 3 are somewhat obscured by the buffer (see the truncated peaks in the 1–4 ppm region; Fig. 5) and could only be scanned when the protein concentration was increased from 0.013 to 0.392 mM. The spectra are characterized by many well-resolved peaks that are indicative of the presence of tertiary structure. On the other hand, only one small peak was observed between 4.5 and 6 ppm in the H-*alpha* region, and neither the amide/aromatic region (6.4–9.4 ppm) nor the aliphatic region (0–4.5 ppm) is very extended. This indicates that minor unstructured regions are also present.

The purified Pru p 3 reacted with the polyclonal rabbit anti-Mal d 3 antiserum (Fig. 4; lane 3), but not with the polyclonal rabbit anti-Cor a 8 (data not shown). IgE immunoblotting and IgE ELISA with a set of five sera from peach allergic patients showed specific IgE reactivity to nPru p 3 (Fig. 4; lanes 4–8).

3.3 Effects of pH and heating on the secondary structure

The CD spectrum of rPru p 1 measured at pH 7.5 showed a mixture of α- and β-secondary structures with a maximum at 194 nm and a minimum at 215 nm (Fig. 6A). On heating to 95°C, the spectrum changed to that of a typical random coil. This transition occurred above about 65°C and indicated that the protein was undergoing unfolding. Upon cooling to 25°C, rPru p 1 showed a CD spectrum similar in shape and peak heights to that recorded before heating, suggesting that rPru p 1 is able to correctly refold after thermal

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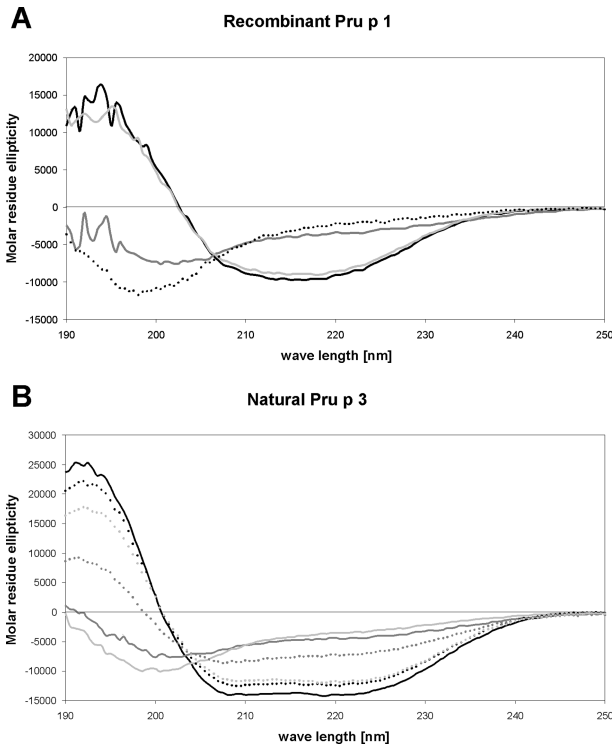

Figure 6. CD spectra showing the effects of pH and heating on the secondary structures of rPru p 1 (A) and nPru p 3 (B). Measurements were made at pH 7.5 (continuous lines —) and pH 3 (dotted lines · · ·) and at 25°C (black), on heating to 95°C (grey), and after heating and cooling to 25°C (light grey).

denaturation. This has also been observed for rPru av 1 and rMal d 1 [38, 39]. In contrast, the CD spectrum at pH 3 showed that the protein was denatured even at 25°C. This is consistent with the protein being located in the cytoplasm where the pH is close to neutral [40] and with previous observations that Bet v 1 homologues are more labile to denaturation at low pH than at neutral pH.

The CD spectrum of nPru p 3 at pH 7.5 was characteristic of an α-helical structure, with two negative minima at 208 and 219 nm and a maximum at 193 nm (Fig. 6B). On heating to 95°C, nPru p 3 underwent denaturation, starting at about 85°C. However, in contrast to Pru p 1, the denatured Pru p 3 protein was not able to refold when the temperature was returned to 25°C. Pru p 3 also differed from Pru p 1 in that the CD spectrum at pH 3 and 25°C was similar in shape and peak heights to that at pH 7.5, showing that it is stable under acidic conditions. When heated to 95°C at pH 3, Pru p 3 was not completely denatured and returned almost completely to its native state on cooling. This indicates that the disulphide structure of nPru p 3 is more stabile under acidic conditions than at near neutral ones. The cleavage of disulphide bonds at neutral pH and high temperature has previously been observed for thaumatin [41], a plant protein with eight conserved disulphide bridges, and in other proteins including hen egg lysozyme, insulin, ovalbumin, ribonuclease and transferrin. The most probable mechanism of this reaction is a β-elimination, where a base-catalysed subtraction of a β-proton from a cysteine results in cleavage of disulphide bonds. This allows the formation of new intermolecular disulphide linkages which stabilize aggregates. In contrast, thermal denaturation under acidic conditions leads to reversible unfolding of the protein.

The FT-IR spectra of nPru p 3 suggest that the protein contains α-helix (1655 cm-1), with two dominant amide bands appearing at around 1652 (amide I band) and 1541 cm-1 (amide II band). The secondary structure measured by FT-IR is therefore in agreement with the CD spectra (Fig. 6B).

4 Concluding remarks

Pru p 1 and Pru p 3 are responsible for over 95% of allergic reactions to peach fruit in Europe. We therefore report methods for the routine purification of these two proteins for use in allergy diagnosis and for studies of allergen structure and properties.

Pru p 1 was expressed and purified as recombinant non-fusion protein in *E. coli* because of its low abundance in peach fruits. Furthermore, Pru p 1 as well as other Bet v 1 homologues from plant-derived foods are rather labile and are readily degraded during purification from their natural source. This is well documented for a number of crude food extracts and commercial skin prick test solutions [12, 13]. Therefore, the expression of Pru p 1 as a recombinant protein in *E. coli* is the method of choice and results in a correctly folded protein as shown previously for other members of the Bet v 1 family. However, several considerations need to be addressed in order to achieve a product of high quality by expression in *E. coli*. In the case of Pru p 1, the formation of inclusion bodies was avoided by reducing the temperature during expression to 30°C. Thus, rPru p 1 could be purified from the soluble bacterial lysate under native conditions without refolding steps and avoiding the possibility of modifications during solubilization with urea.

In contrast, Pru p 3 is abundant in the fruit, being present in approximately tenfold greater amounts in the peel than in the pulp [27]. Because of its stability, it was possible to purify Pru p 3 as a native allergen using conventional biochemical approaches.

Heating at acidic pH was performed to simulate the conditions encountered by peach fruit products during thermal processing. The results of the CD spectroscopy show that Pru p 3 is able to refold under these conditions, indicating that Pru p 3 is correctly folded in processed peach products (*e.g.* juices, jams) and may be recognized by specific IgE antibodies from allergic patients [27]. This is consistent with the demonstration that the allergenic potential of

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peach LTP was not altered in heat-treated peach products [42]. On the other hand, the same authors reported that heating reduced IgE binding to all the other peach allergens, although the reactivity of IgE to Pru p 1 was only monitored in the BP-peach allergic patients' group.

Thus, Pru p 1 and Pru p 3 display different stabilities under varying conditions of pH and temperature. This means that the conditions used to process peach products may differentially affect the structures and hence allergenicities of the two proteins.

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