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Natural and recombinant molecules of the cherry allergen Pru av 2 show diverse structural and B cell characteristics but similar T cell reactivity

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Clinical and Experimental Allergy

Summary

Background Cherry allergy is often reported in the context of allergy to other fruits of the Rosaceae family and pollinosis to trees because of cross-reactive allergens. Allergic reactions to cherry are reported by 19–29% of birch pollen-allergic patients. Pru av 2, identified as a thaumatin-like protein (TLP) from sweet cherry, was recognized by the majority of cherry-allergic patients in immunoblotting.

Objectives In order to investigate the structural characteristics and the immunoglobulin (Ig)E- and T cell reactivity of cherry-derived TLP, recombinant Pru av 2 was expressed in *Escherichia coli* and natural Pru av 2 was purified.

Methods Parallel-His and FLAG expression vectors were used for recombinant production of Pru av 2 in the cytoplasm and the periplasm of *E. coli*. Natural Pru av 2 was purified from fresh cherries and verified by N-terminal sequencing. Structural characterization was performed using circular dichroism (CD) measurements, and the biologic activity was measured in a glucanase assay. Using cherry-specific sera, the IgE-binding ability of recombinant and natural Pru av 2 was investigated in IgE-ELISA and the T cell reactivity was studied in proliferation assays.

Results Natural Pru av 2 revealed thaumatin-like structural features and bound IgE of 50% of cherry-allergic patients. It was demonstrated to be enzymatically active. Recombinant Pru av 2 expressed in the cytoplasm of *E. coli* exhibited a slightly different folding compared with the natural protein. It was not recognized by IgE from cherry-allergic subjects, but retained the ability to stimulate T lymphocytes. Periplasmic recombinant Pru av 2 was able to bind an anti-grape TLP antibody and cherry-specific IgE.

Conclusions We prepared two recombinant model TLPs from cherry, and compared their molecular characteristics as well as their IgE-binding activity and T cell interactions in relation to the natural counterpart. The cytoplasmic recombinant Pru av 2 can be used as a hypoallergenic variant in allergen-specific immunotherapy, whereas the periplasmic protein can be included in a component-resolved diagnosis.

Keywords cherry, circular dichroism, *E. coli* expression, food allergen, IgE binding, T cell reactivity, thaumatin-like protein

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Introduction

Pollinosis is one of the prevailing allergic diseases in adults throughout Europe. Allergy to pollen of trees of the order Fagales is often combined with food allergy to fruits of the Rosaceae family including cherries (*Prunus avium*). Based on a patient questionnaire, 29% of Swedish patients with

birch pollen allergy were defined as being allergic to cherries [1]. Even in Japan, it was reported that 61% of 54 birch-pollen-allergic patients were allergic to fruits and vegetables and 58% from the food-sensitive group stated adverse reactions after ingestion of cherries [2]. Oral allergy syndrome (OAS) was frequently reported as the most frequent clinical manifestation [2, 3]. Pollen-related cherry

allergy is caused by the presence of cross-reactive immunoglobulin (IgE) epitopes on homologous proteins [4].

Four allergens from sweet cherry have been identified so far. Pru av 1 and Pru av 4 are homologous to the birch-pollen allergens Bet v 1 and Bet v 2, respectively, and are in part responsible for the cross-reactivity between birch pollen and cherry [5, 6]. Pru av 3 is a non-specific lipid transfer protein (nsLTP) sharing high amino acid sequence identities with nsLTPs from other fruits of the Rosaceae family [6]. Pru av 2 was first discovered in 1996 as the most abundant soluble protein in ripe cherries accumulating during the ripening process [7]. It was purified and described as a non-sweet 29-kDa polypeptide. A high degree of sequence similarity to thaumatin, an intensely sweet-tasting protein of the fruits of a West African shrub, revealed its relation to thaumatin-like proteins (TLPs), family 5 of pathogenesis-related (PR) proteins [7]. These proteins are induced upon pathogen attack, infection, wounding or by chemicals as final products of the plant defence system [8].

Interestingly, many plant PR-proteins including TLPs were identified as allergens able to trigger allergic reactions in humans [9–11]. Mal d 2, a 31 kDa protein from apple (*Malus domestica*) sharing approximately 50% identity with PR-5 proteins, was the first TLP described as a food allergen [12]. Recombinant Mal d 2 was expressed in *Nicotiana benthamiana* [13]. Cap a 1 from bell pepper [14] and TLPs from kiwi [15], grape [16] and orange [17] were subsequently identified as food allergens. Inschlag et al. [18] described the TLP from cherry for the first time as a potential major allergen of 30-kDa and named it Pru a 2, later modified to Pru av 2. Expression of Pru av 2 in the cytoplasm of *Escherichia coli*, however, resulted in a protein with abolished IgE reactivity. Recombinant proteins with reduced IgE-binding capacity but with intact T cell reactivity have been proposed as candidates for therapeutic vaccines. Engineered hypoallergenic variants of the latex allergen Hev b 6.01 and of peptides of its hevein domain (Hev b 6.02) were shown to retain the ability to induce T cell proliferation without binding IgE or stimulating allergic effector cells [19]. Vaccination with recombinant (r)Bet v 1 fragments and rBet v 1 trimers had the potential to convert the pathological IgE response into a new, allergen-specific mixed T helper type (Th)2/Th1-like immune response [20, 21]. Site-directed mutagenesis of Pru av 1, the Bet v 1-homologue from cherry, was used to identify IgE epitopes and to reduce its IgE-binding capacity [22, 23]. In general, genetically engineered hypoallergenic proteins are required to display very low to no IgE binding but to retain T cell stimulatory capability.

In order to develop a future candidate vaccine against Rosaceae TLPs, Pru av 2 was produced by a cytoplasmic and periplasmic *E. coli* expression system. The aim of this study was to compare the structural characteristics, the IgE- and the T cell reactivity of cytoplasmic and periplasmic recom-

binant (r)Pru av 2 with natural (n)Pru av 2. The further intention of these IgE- and T cell studies was to create T cell reactive hypoallergenic derivatives for specific immunotherapy using simple bacterial expression techniques.

Materials and methods

Cloning and construction of the expression plasmids

Pru av 2 cDNA integrated into the pTAG vector [18] was used as a template for PCR amplification. PCR was performed using a sense 5'-primer matching the N-terminal sequence of mature Pru av 2 (5'-GAC GAC GCG ATG GCG ACA ATT TCT TTC AAA AAC-3', NcoI restriction site is underlined) and an antisense 3'-primer matching the C-terminal sequence (5'-GAC GAC CTC GAG TCA TGG GCA GAA AGT AAT GGC G-3', XhoI restriction site is underlined). Reaction mixtures were subjected to the following conditions: denaturation at 94 °C for 30 s, annealing at 62 °C for 45 s and extension at 72 °C for 1 min. After 30 cycles, a final elongation step of 7 min was added. PCR products were cloned into the pCR 2.1 TOPO-TA cloning[®] vector (Invitrogen Life Technologies, Groningen, Netherlands) and the plasmids propagated in *E. coli* XL-1 Blue cells. After digestion with NcoI and XhoI, the Pru av 2 coding sequence was ligated into either pHIS-Parallel2 [24] or pFLAG-Parallel2 [25] expression vectors (provided by F. Ferreira, Division of Allergy and Immunology, University of Salzburg, Salzburg, Austria) using the Fast-Link[™] DNA Ligation kit (Epicentre Technologies, Madison, WI, USA).

Cytoplasmic expression and purification of rPru av 2

For heterologous expression of recombinant cherry TLP in the cytoplasm, chemical competent *E. coli* BL21[DE3] cells were transformed with the expression vector pHIS-Parallel2 including the Pru av 2 sequence. Single positive colonies were grown in 20 mL Luria-Bertoni (LB) medium containing 100 mg/L ampicillin by continuously shaking at 37 °C overnight. After dilution (1 : 50) and incubation at 37 °C to an OD₆₀₀ of 0.9, expression was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The optimized incubation time for growing was 2.5 h. Cells were then harvested by centrifugation. The pellet was dissolved in 40 mL lysis buffer (100 mM Na-phosphate, 10 mM Tris, pH 8.0, 8 M urea) and lysed by stirring for 1 h at 4 °C. After centrifugation (4 °C, 40 000g, 30 min) and filtration, the lysate was shaken for 1.5 h at 4 °C with 5 mL of 50% w/v nickel-nitrilo-triacetic acid (Ni-NTA) agarose (Qiagen, Hilden, Germany) equilibrated with lysis buffer. The recombinant cytoplasmic cherry protein was purified by Ni-NTA affinity chromatography using buffers with decreasing pH from 8 to 4.5. Aliquots of protein fractions were analysed by 12% SDS-PAGE. The fractions containing purified but denatured

cherry TLP were pooled and refolded by several dialysis steps using a cut-off of 6–8 kDa at 4 °C with buffers (100 mM Na-phosphate, 10 mM Tris-HCl, 150 mM NaCl, 0.1 mM EDTA, pH 7.5) containing decreasing concentrations of urea (6–0 M) and reduced glutathione (5–0 mM).

Periplasmic expression and purification of rPru av 2

For periplasmic expression of rPru av 2 and secretion into the medium, chemically competent *E. coli* BL21(DE3) cells were transformed with the pFLAG-Parallel2 expression vector providing a signal peptide. 1.5 L of LB-medium containing ampicillin was inoculated with 30 mL of an overnight culture and grown with vigorous shaking at 20 °C to an OD₆₀₀ of 0.8. The cells were collected by centrifugation (4000 g, 10 min, 20 °C) and resuspended in LB-saccharose medium (1% peptone, 0.5% yeast extract, 1% NaCl, pH 7.4, 0.4 M saccharose). After induction by 1 mM IPTG and further incubation for 8 h at 20 °C, the cells were harvested by centrifugation (4000 g, 10 min, 4 °C). Soluble recombinant Pru av 2 was recovered from the medium by 80% ammonium sulphate precipitation. The ammonium sulphate pellet was dissolved in 50 mM Na-phosphate buffer (pH 7.2) and dialysed overnight. To recover the remaining non-secreted rPru av 2 from the periplasm, the cell pellet was placed on ice and carefully dissolved in resuspending buffer (50 mM Tris-HCl, pH 8.0, 20% saccharose, 1 mM EDTA). The suspension was kept on ice for 1 h and then centrifuged (40 000 g, 4 °C, 15 min). For purification of rPru av 2 from the periplasm, cell pellets were suspended in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM CaCl₂) and disrupted by pressure in a French Press at 10 000 psi. Insoluble material was removed by centrifugation (40 000 g, 20 min, 4 °C). The supernatant was loaded by gravitation up to five times onto a packed column of 1 mL anti-FLAG agarose (anti-FLAG[®] M1 agarose affinity gel, Sigma-Aldrich, St Louis, MO, USA). The recombinant periplasmic cherry protein was purified by immune-affinity chromatography under native conditions using 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM CaCl₂ as washing buffer. Elution of bound FLAG-tagged rPru av 2 proteins was performed with 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA after a 30 min incubation. All expression and purification steps were monitored by SDS-PAGE. The same purification procedures were performed with recombinant proteins from the saccharose-enriched medium after ammoniumsulphate precipitation.

Development of hen egg polyclonal antibodies to thaumatin

Purified natural thaumatin (provided by M. Witty, Cambridge University, Cambridge, UK) was purified from leaf

extracts of *Thaumatococcus daniellii* by conventional chromatographic methods. Two milligrams of the purified protein was used for the immunization of hens. After several rounds of immunizations, hen eggs were collected and protein was extracted. Hen egg antibodies were purified from the yolk prior to use, circumventing the blood collection procedure that is time consuming and stressful for the animal. The antibodies were evaluated for their specific binding to natural thaumatin.

Detection by thaumatin- and tag-specific antibodies

Proteins from crude *E. coli* lysates and purified recombinant Pru av 2 (1.5 µg/lane) were separated on a 12% SDS-PAGE gel under reducing conditions and subsequently transferred onto nitrocellulose membranes. The membranes were blocked in Tris-buffered saline+0.05% Tween-20 (TBST)+1% non-fat milk powder and incubated with diluted antibodies at room temperature. Total cell extracts were treated with the chicken anti-thaumatin antibody (1 : 1000) at 4 °C overnight and afterwards with alkaline phosphatase-conjugated affinity pure rabbit anti-chicken IgY (Merck, Darmstadt, Germany, 1 : 5000). After incubation for 2 h, the membranes were washed and developed for 1–10 min in the BCIP/NBT substrate solution.

For detection of His-tagged recombinant cytoplasmic (rc)Pru av 2, a penta-His-tag specific monoclonal antibody (Qiagen, 1 : 2000) was applied for 2.5 h. Mouse anti-FLAG[®] M2 monoclonal antibody (Sigma-Aldrich, 1 : 10 000) was used for detection of FLAG-tagged recombinant periplasmic (rp)Pru av 2 with an incubation time of 2 h. After washing the membranes three times with TBST, they were incubated in a 1 : 7500 dilution of alkaline phosphatase-conjugated AffiniPure rabbit anti-mouse IgG+IgM (Jackson ImmunoResearch, Cambridgeshire, UK) for 1 h. The blots were then washed again and stained with BCIP/NBT.

Purification and detection of nPru av 2

The TLP from sweet cherry was purified following, in principle, the methods described previously [26, 27]. Briefly, 1.5 kg ripe cherries (*Prunus avium*, cv. Germersdorfer) from Northern Austria were squeezed with 300 mL ice-cold water through Miracloth and diluted in sodium formate buffer with a final concentration of 20 mM. The cherry protein extract was adjusted to pH 3.8 with 100% formic acid and centrifuged. The supernatant was filtered and loaded onto a column of 25 mL SP-Sepharose Fast Flow (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with 20 mM sodium-formate buffer (pH 3.8). The column was washed with 20 mM sodium-formate buffer (pH 3.8). A 200 mL linear gradient of 0–200 mM NaCl in 50 mM Tris-HCl (pH 7.8) was used for elution. The Pru av 2 protein was detected by SDS-PAGE in the

flowthrough as well as in the washing step. This protein solution was precipitated by adding ammonium sulphate to a final concentration of 75%. After centrifugation, the precipitate was dissolved in 5 mL 20 mM sodium phosphate buffer (pH 7.2) and desalted by Sephadex G-25 prepacked PD-10 columns (Amersham Biosciences, Little Chalfont, UK). The protein concentration was determined by the BCA Protein Assay (PIERCE Biotechnology, Perbio Science, Erembodegem, Belgium) using bovine serum albumin as a standard. Proteins blotted onto nitrocellulose membranes were incubated with chicken anti-thaumatococcus antibody (1:1000) at 4 °C overnight, developed and stained as described above. For detection of possible glycosylation, a glycan-specific rabbit anti-horseradish peroxidase (HRP) antibody [28] (Sigma-Aldrich) was used in a 1:4000 dilution. Visualization was performed by alkaline phosphatase-conjugated swine anti-rabbit IgG (DAKO, Glostrup, Denmark) diluted 1:1000 and again BCIP/NBT solution.

N-terminal amino acid sequencing

Purified nPru av 2 was immobilized on a polyvinylidene difluoride membrane (ProSorb Sample Preparation Cartridge, Applied Biosystems, Foster City, CA, USA) and subjected to N-terminal sequencing on a Procise 491 sequencer (Applied Biosystems) using the Pulsed Liquid cleavage program and the manufacturer's chemistry version 1.1.1.

Circular dichroism measurements

The circular dichroism (CD) measurements were performed with nPru av 2 and rcPru av 2 in 10 mM Tris buffer (pH 7.5). The investigations were carried out on a Jasco J-715 spectropolarimeter using a 0.1 cm pathlength cell at room temperature. Spectra were recorded with 0.2 nm resolution at a scan speed of 50 nm/min and were the result of averaging three scans. CD spectra were smoothed with a 'running-average' algorithm and expressed as the mean residue ellipticity [Θ] at a given wavelength.

Enzyme-linked immunosorbent assay with anti-grape thaumatococcus-like protein antibody

Microtitre plates (Maxisorp, Nalge Nunc International, Denmark) were coated with 10 µg/mL periplasmic recombinant and natural Pru av 2 and incubated overnight at 4 °C. After blocking with TBST (Tris-buffered saline, pH 7.4%, 0.5% Tween 20) + 3% non-fat milk powder, rabbit antiserum raised against the TLP and allergen from grape berries [16] (anti-grape TLP antibody, provided by E. Waters, Australian Wine Research Institute, Glen Osmond, Australia) was diluted 1:1000 in TBST + 0.5% BSA and applied onto the plates overnight at 4 °C. After

washing three times with TBST, the plates were incubated with alkaline phosphatase-conjugated swine anti-rabbit immunoglobulins (DAKO) 1:1000 diluted in TBST + 0.5% BSA for 2 h at room temperature. Colour development was performed using *p*-nitrophenyl phosphate tablets (Sigma-Aldrich), and the optical density was measured at 405 nm (550 nm as reference wavelength) after 45 min using an ELISA reader (Dynatech MR 7000, Alexandria, VA, USA).

Endo-β1,3-glucanase activity tests

The endo-β1,3-glucanase activity of cherry TLP was assayed in two steps. Firstly, the enzymatic activity of natural Pru av 2 was evaluated. Hundred micrograms of nPru av 2 was incubated with substrate for 2 h. Subjected to the same conditions, 100 µg of Latex B extract from *Hevea brasiliensis* containing endo-β1,3-glucanases such as Hev b 2 was used in parallel for 2 h, as a positive control. In the second step, the biological activity of recombinant Pru av 2 expressed in the periplasm was investigated. Thereto, 18 µg of nPru av 2 and 18 µg of rpPru av 2 were incubated overnight. All proteins were diluted in 500 µL 50 mM sodium acetate, 100 mM NaCl, pH 5.5, mixed with 300 µL of azurine-crosslinked β-1,3 glucan (AZCL)-Pachyman substrate (Megazyme, Wicklow, Ireland) solved in the same buffer 0.5% (w/v) and incubated at 35 °C under continuous shaking. The reaction was stopped with 100% ethanol, and the enzymatic activity was measured at 590 nm in a spectrometer against substrate blanks (devoid of protein and treated similarly).

Patients and patient's sera

Sera from 20 adult patients recruited from Italy (Rome) with self-reported allergic reactions to cherry (*Prunus avium*), such as OAS or OAS-like itching, were selected for this study. The majority of patients reported additional symptoms to other fruits from the *Rosaceae* family and were sensitized to pollen or fungi. Cherry-specific serum IgE was measured with a Pharmacia UniCap device (Pharmacia, Uppsala, Sweden) and ranged from 0.0 to 27.6 kU/L (median: 13.8 kU/L). To define the limit value, sera from five non-allergic persons (control sera) were tested in parallel.

Immunoglobulin E-enzyme-linked immunosorbent assay experiments

Microtitre plates (Maxisorp, Nalge Nunc International) were coated with 1 µg of natural or recombinant Pru av 2 per well overnight at 4 °C. After blocking with TBS + 0.05% Tween-20 + 3% milk, diluted sera were applied onto the coated plates and incubated overnight at 4 °C. The wells were then washed with TBST and incubated with

alkaline phosphatase-conjugated mouse anti-human IgE antibodies (Pharmingen, San Diego, CA, USA) for 2 h at room temperature. Colour development was performed as described above and the optical density was measured after 30 min to 2 h. All samples were prepared in duplicate. For control experiments, normal human serum was used. The positive threshold for specific IgE binding was defined for each protein by adding 3 SD to the mean OD₄₀₅ value of the control sera.

Reduction and S-alkylation of nPru av 2

The eight disulphide bonds of purified nPru av 2 were completely reduced with dithiothreitol (DTT) and carbamidomethylated with iodoacetamide. In brief, 100 µg of the protein were dissolved in 50 µL of 0.3 M Tris/HCl, pH 8.0, containing 8 M guanidine HCl. After the addition of 5 µL of 0.5 M DTT, the sample was incubated at 50 °C for 4 h. The reduced cysteine residues were then S-alkylated by incubation with 12.5 µL of 0.4 M iodoacetamide for 1 h in the dark at 37 °C and the reaction was quenched by adding 5 µL of 0.5 M DTT. The modified proteins were dialysed against distilled water and subjected to IgE-ELISA, as described above.

T lymphocyte proliferation assays

Peripheral blood mononuclear cells (PBMC) were obtained from three birch-pollen-allergic individuals with OAS to cherry after testing for nPru av 2 in ELISA by Ficoll-Hypaque density gradient centrifugation (Pharmacia Diagnostics, Uppsala, Sweden). PBMC (2×10^5) were cultured in triplicate in 96-well plates (Nunc, Nunc) in 200 µL of serum-free Ultra Culture Medium (BioWhittaker, Walkersville, MD, USA) supplemented with 2 mM glutamine and 2×10^{-5} M 2-mercaptoethanol with and without allergen for 6 days. During the last 16 h of culture, 0.5 µCi [³H]-thymidine was added and the incorporated radioactivity was measured by scintillation counting.

Results

Purification of cytoplasmic and periplasmic rPru av 2

Cytoplasmic rPru av 2 was expressed in *E. coli* with a six-histidine tag for purification. Expression was monitored and confirmed by immunoblotting of total cell lysates using an anti-thaumatococcus antibody. The recombinant cherry TLP was found to be insoluble in inclusion bodies and could only be purified under denaturing conditions. Recombinant protein present in the elution steps was visualized by SDS-PAGE (Fig. 1, cytoplasmic, E1–E3) and the presence of rcPru av 2 was further detected by a penta-His-tag specific monoclonal antibody (Fig. 1, His). Under reducing conditions, the purified protein migrated as a

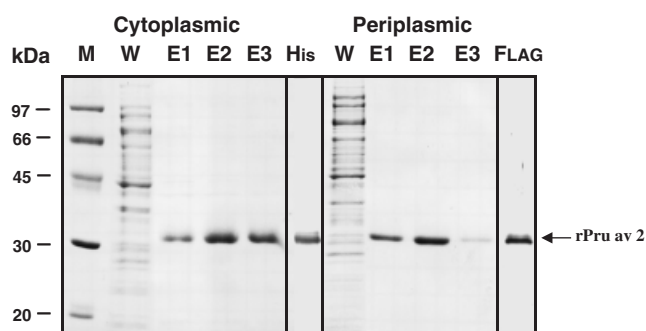


Fig. 1. Coomassie Brilliant Blue-stained SDS-PAGE analysis of purification steps of rPru av 2 expressed in the cytoplasm by means of pHIS-Parallel2 vector (Cytoplasmic, W, E1–E3) and in the periplasm by means of pFLAG-Parallel2 vector (Periplasmic, W, E1–E3). The purification of cytoplasmic rPru av 2 was achieved by Ni-NTA affinity chromatography, and refolding was carried out by dialysis. Purification of periplasmic rPru av 2 was performed by immune-affinity chromatography using anti-FLAG agarose. M, molecular weight marker; W, washing steps; E1–E3, elution steps of recombinant cytoplasmic and periplasmic rPru av 2. The presence of cytoplasmic rPru av 2 was verified by a penta-His-tag specific monoclonal antibody (His) and presence of periplasmic rPru av 2 by a mouse anti-FLAG[®] M2 monoclonal antibody (FLAG).

single band with a molecular weight 3–4 kDa higher than the predicted 30 kDa including the His-tag. The final concentration of rcPru av 2 was 0.7 mg/mL. Approximately 20 mg was obtained from 1.5 L culture.

For purification of periplasmic rPru av 2, a FLAG-tag was attached to the protein. Expression was followed by immunoblotting of total *E. coli* extracts using an anti-thaumatococcus antibody. *E. coli* cells were stimulated to secrete newly expressed protein from the periplasm into the medium by changing the osmotic conditions via an LB-saccharose medium. From the periplasm, rpPru av 2 was extracted without lysing the entire cells. Both the medium and the periplasmic fractions were analysed by SDS-PAGE and showed similar results. Periplasmic fractions are shown in Fig. 1 (periplasmic, E1–E3). The purified cherry protein was recognized by the mouse anti-FLAG[®] M2 monoclonal antibody (Fig. 1, FLAG). Purification under native conditions yielded about 150 µg from the medium and more or less the same quantity from the periplasmic extract on using 1.5 L culture. The concentration of both rpPru av 2 fractions was 0.03–0.05 mg/mL.

Purification and N-terminal sequence analysis of nPru av 2

Natural Pru av 2 was purified from ripe sweet cherries by cation exchange chromatography using increasing pH and salt concentrations. Coomassie Brilliant Blue (CBB)-stained SDS-PAGE analysis revealed a predominant band at about 30 kDa in the flowthrough and in the washing step with at least 98% purity (Fig. 2, lane: W_{CBB}). Using an anti-thaumatococcus antibody, the presence of nPru av 2 in the

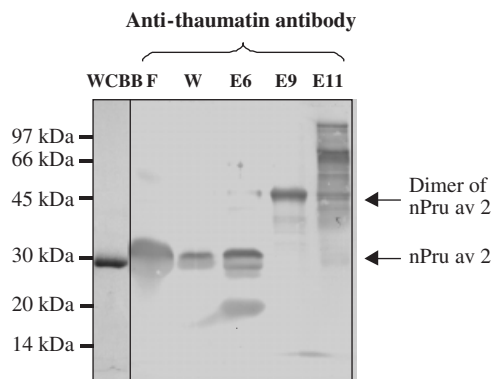


Fig. 2. Coomassie Brilliant Blue-stained SDS-PAGE analysis of purification steps of natural Pru av 2 from ripe cherries. Pure nPru av 2 was present in the washing step (lane: W_{CBB}). Thaumatin-like proteins were detected by Western blotting using the anti-thaumatin antibody. Lanes F, flowthrough; lane W, washing step; lanes E6, E9, and E11, elution steps.

flowthrough, the washing step and several fractions of elution was detected. The predominant band at 30 kDa was clearly recognized confirming the presence of TLPs from cherry (Fig. 2, lanes: F, W and E6). Fractions E9 and E11 most likely contained dimers of nPru av 2 (Fig. 2, lanes: E9 and E11). After desalting the enriched washing step and pooling the TLP-containing fractions, 17 mL solution of purified nPru av 2 was obtained. The protein concentration of the pooled fractions was 6.85 mg/mL. Altogether, approximately 120 mg of highly purified nPru av 2 were obtained from 1.5 kg ripe cherries. To verify the presence of the thaumatin-like cherry allergen, the N-terminal sequence of purified Pru av 2 was analysed. The resulting 5-amino acid N-terminus (ATISF) showed 100% identity to the deduced amino acid sequence of the N-terminus of the published mature TLP from cherry (SWISS-PROT: P50694) [7]. The presence of carbohydrate moieties on at least part of nPru av 2 was indicated by the binding of the glycan-specific rabbit anti-HRP antibody (data not shown).

Circular dichroism analysis of natural and cytoplasmic rPru av 2

Analysis of the far-UV CD spectrum of nPru av 2 showed a broad minimum at 210 nm and a maximum below 200 nm (Fig. 3, black curve). This is quite an unusual curve for a protein consisting mainly of a β -sheet and β -turn structure, but it appeared to be characteristic for the CD spectra of proteins from the TLP family (e.g. thaumatin [29, 30] and zeamatin [31]). The spectrum of rcPru av 2 was very similar in the 220–250 nm range, but showed a broadening of the peak and a shift of the minimum to 209 nm (Fig. 3, grey curve). These differences indicate a different fold of the recombinant protein as compared with the native one.

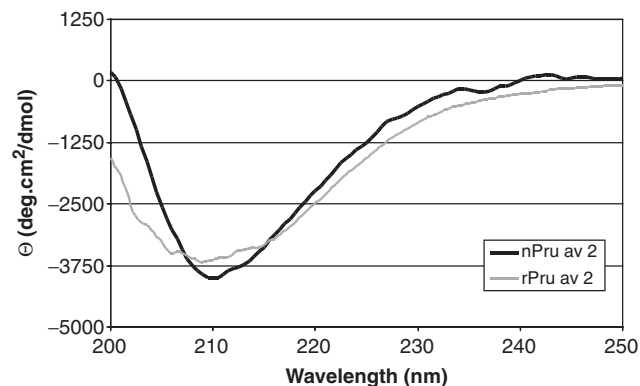


Fig. 3. Circular dichroism spectra of nPru av 2 (black curve) and cytoplasmic rPru av 2 (grey curve) between 200 and 250 nm at room temperature.

Reactivity of nPru av 2 and periplasmic rPru av 2 with anti-grape thaumatin-like protein antibody

Periplasmic recombinant Pru av 2 was tested in ELISA with anti-grape TLP antibody to evaluate the binding capacity of rpPru av 2 and to check the structural characteristics in comparison with nPru av 2. Both nPru av 2 (OD_{405} : 0.27 after 45 min) as well as the recombinant forms isolated from the periplasm (OD_{405} : 0.19) and from the culture medium of BL21 cells (OD_{405} : 0.26) showed similar binding capacities for the anti-grape TLP antibody.

Endo- β 1,3-glucanase activity tests

Using AZCL-Pachyman as a substrate, the endo- β 1,3-glucanase activity of Pru av 2 was determined in two assays. The first assay revealed the enzymatic activity of the natural cherry TLP. Natural Hev b 2 in latex B extract from *Hevea brasiliensis* served as a positive control and possessed high glucanase activity (Fig. 4, assay 1). In contrast, assay 2 demonstrated no enzymatic activity for periplasmic rPru av 2 performed under the same conditions as nPru av 2 (Fig. 4, assay 2). Cytoplasmic rPru av 2 was biologically inactive as well (data not shown).

Immunoglobulin E-enzyme-linked immunosorbent assay experiments

Purified nPru av 2 and rPru av 2 were tested for their reactivity with IgE antibodies in ELISA. IgE from the sera of 10 from 20 tested cherry-allergic patients recognized the natural cherry thaumatin-like allergen. Natural Pru av 2-positive sera were further assayed with the recombinant counterpart (Table 1). Periplasmic rPru av 2 was shown to be significantly IgE reactive in four patients (Fig. 5, nos. 1, 2, 6 and 8). Patients nos. 5, 6, 9 and 10 had high cherry-specific serum IgE levels and displayed strong IgE binding to nPru av 2, and patient no. 6 to rpPru av 2 as well. In contrast, specific IgEs to rcPru av 2 could not be detected

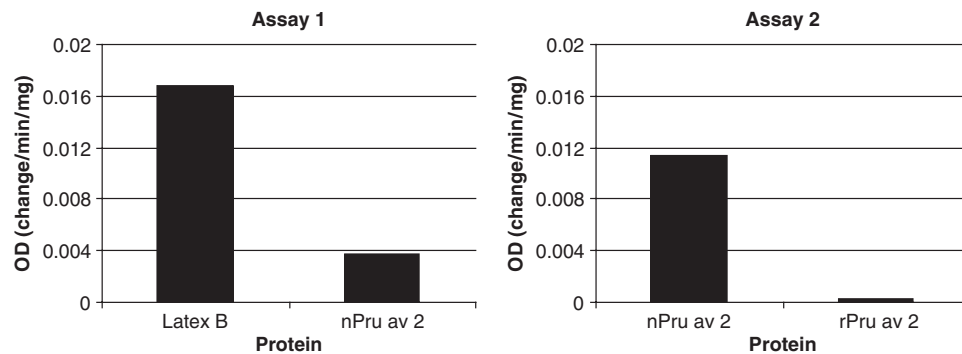


Fig. 4. The endo-β1,3-glucanase activity of Pru av 2 was tested in two assays. Assay 1: 100 µg of natural Pru av 2 and 100 µg of natural latex B extract as positive control were incubated for 2 h. Assay 2: 18 µg of nPru av 2 and 18 µg of periplasmic rPru av 2 were incubated overnight. The enzymatic activity is indicated as change of OD per minute incubation time per mg protein.

Table 1. Clinical characteristics and IgE-ELISA data of nPru av 2-positive patients with self-reported allergy to cherry

Patient number	Symptoms to cherry	Cherry CAP (kU/L)	OD ₄₀₅ for nPru av 2	OD ₄₀₅ for rPru av 2	OD ₄₀₅ for red. nPru av 2	Additional allergies or sensitizations
1	OAS	1.0	0.30	0.23	ND	SFS
2	OAS	0.9	0.51	0.46	0.06	OAS-OF, MPS
3	OAS	0.8	0.77	0.05	0.04	OAS-OF, MPFS
4	OAS	1.2	2.02	0.19	0.11	OAS-OF, MPS
5	OAS-I	27.6	2.80	0.11	ND	MPFS
6	OAS-I	8.6	3.10	1.65	0.36	MPFS
7	OAS-I	0.6	0.78	0.13	0.07	MPS
8	OAS	< 0.35	0.38	0.37	ND	MPS
9	OAS	4.3	2.07	0.11	0.08	OAS-OF, MPS
10	OAS	4.5	2.37	0.15	0.22	OAS-OF, MPS

ELISA values above the positive threshold are in bold.

red. nPru av 2, reduced and alkylated natural Pru av 2; OAS, oral allergy syndrome; OAS-I, OAS-like itching; ND, not determined; SFS, sensitization to Fagales pollens; MPS, multiple pollen sensitization; MPFS, multiple pollen and fungi sensitization; OAS-OF, OAS to other fruits.

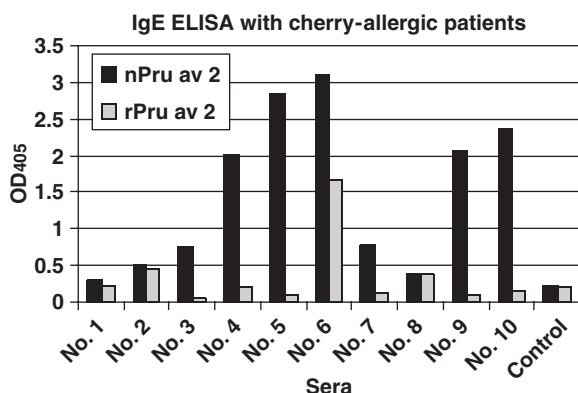


Fig. 5. The Immunoglobulin E-binding reactivity of sera from cherry allergic patients was tested in ELISA. Ten of 20 sera reacted with nPru av 2 (black bars) and four of the nPru av 2-positive sera (no. 1, 2, 6 and 8) reacted also with periplasmic rPru av 2 (grey bars). The threshold was defined as the OD₄₀₅ of 0.23 for nPru av 2 and of 0.21 for rPru av 2.

in any patient (data not shown). In order to evaluate the contribution of disulphide bonds to IgE-binding ability, reduced and alkylated nPru av 2 was tested with patients'

sera as well. IgE reactivity was detected in only one serum, which contained a very high amount of IgE directed to native nPru av 2 (patient 6, Table 1). Sera with OD values above the calculated threshold of 0.23 for nPru av 2, 0.21 for rPru av 2 and 0.22 for reduced and alkylated nPru av 2 were defined as positive.

Lymphoproliferative response

The T cell stimulatory activity was evaluated for the natural cherry TLP as well as for the recombinant counterpart isolated from the cytoplasm by inducing proliferation of PMBC from cherry-allergic nPru av 2-positive patients. PBMC were stimulated with titrated concentrations (25, 12.5, 6.25 and 3.13 µg/mL, respectively) of nPru av 2 as well as of rcPru av 2. The optimum concentration to induce proliferation was 12.5 µg/mL for nPru av 2 (mean cpm: 23 150) and 6.25 µg/mL for the recombinant allergen (mean cpm: 27 451). The mean value in cultures incubated with medium alone was 2529 cpm. These results showed that both proteins were able to induce

lymphoproliferation, and that the rcPru av 2 was not cytotoxic and contained the relevant T cell epitopes.

Discussion

Pru av 2 is a TLP from sweet cherry that belongs to family 5 of pathogenesis-related proteins [32]. IgE of the majority of cherry-allergic patients was shown to react strongly with this allergen among other cherry proteins in immunoblotting [18]. Cherry allergy is either caused by primary sensitization of homologous inhalant pollen allergens (Bet v 1 and Bet v 2) or directly by ingestion (Pru av 3). Pollen-related food allergens typically cause mild symptoms like OAS, whereas lipid transfer proteins (LTPs) frequently include systemic reactions [33]. The sensitization route of TLPs and the clinical relevance of this allergen family are still largely unknown.

In order to advance the investigations of molecular characteristics of cherry TLP and its activity in the human immune system, various recombinant allergens were generated. Preliminary expression trials of recombinant Pru av 2 in *E. coli* had not resulted in correct folding [18]. Conditions in the cytoplasm of *E. coli* do not favour the formation of the TLP's eight disulphide bridges. Therefore, expression in the periplasm was chosen to produce rPru av 2. Structural characteristics and IgE-binding abilities of periplasmically expressed rPru av 2 were compared with cytoplasmic rPru av 2 and with nPru av 2 purified from fresh cherries.

Periplasmic rPru av 2 possessed thaumatin-like secondary structures as deduced from its binding abilities to an anti-thaumatin antibody in immunoblot analysis and to an anti-grape TLP antibody in ELISA. These results indicate that rpPru av 2 retained thaumatin-like secondary structures and that cherry and grape TLP displayed cross-reactive epitopes. The anti-thaumatin antibody also bound cytoplasmic rPru av 2, indicating that certain conserved thaumatin-like features were present on this protein. CD spectra could only be analysed for rcPru av 2 isolated from the cytoplasm. The production of rpPru av 2 yielded only small quantities; thus, no evaluable CD measurements could be produced. CD analysis of rcPru av 2 resulted in a similar spectrum in the range of 220–250 nm as compared with nPru av 2. The expanded peak and the shift to the shorter wavelengths in the far UV region indicated an altered folding of the secondary structure probably because of incorrectly formed disulphide bridges in the cytoplasm of the expression host or upon *in vitro* refolding.

The biological activity was determined by an endo- β 1,3-glucanase assay. Natural Pru av 2 degraded the substrate efficiently, whereas periplasmic rPru av 2 exhibited no detectable enzymatic activity. Cytoplasmic rPru av 2 was also negative in the glucanase assay. Molecular modelling and docking studies showed that

the acidic cleft and a favourable positioning of the (1,3)- β -D-glucans towards the catalytically active acidic residues were required for the cleavage activity of fruit-specific TLPs [26]. The acidic cleft is highly electronegative and located between domains I and II. If the structural requirements of recombinant Pru av 2 for the cleft were missing because of improper folding, they consequently lacked glucanase activity. A structural relationship was found to exist between the glucanase and allergenic activity in fruit TLPs, as shown in the three-dimensional model built for the pollen allergen Jun a 3 from mountain cedar (*Juniperus ashei*) [34]. The main IgE-binding epitope coincided with the α -helix of the domain II being well conserved in PR-5 proteins [26].

The IgE binding from patients' sera to nPru av 2 was evaluated by ELISA experiments and only nPru av 2-positive sera were used to examine the IgE reactivity to the recombinant counterparts. IgE from 50% of the patients' sera (10/20) recognized nPru av 2 and 4 of 10 sera recognized rpPru av 2. This is strong evidence that the recombinant allergen expressed in the periplasm of *E. coli* possessed some B cell-epitopes. We speculate that rpPru av 2 is partly properly folded. The glycosylation present on at least a portion of nPru av 2 may contribute to the proper folding but was absent on the recombinant protein, further explaining the diminished IgE-binding ability. Cytoplasmic rPru av 2 did not bind IgE from any cherry-allergic patient, indicating that no B cell-epitopes were present. Incorrect formation of disulphide bonds during the refolding process and consequently the improper secondary structure compared with the natural TLP probably caused the missing IgE-epitopes.

The lack of serum IgE reactivity of cytoplasmic rPru av 2 offers an immense advantage for hypoallergenic immunotherapy. With the increasing number of allergens cloned and expressed as recombinant proteins, intensified interest has been generated in the development of hypoallergenic molecules for immunotherapy. A protein comprising no B cell epitopes that is able to retain T cell reactivity represents a potential candidate [20]. In this study, we report the hypoallergenic variant of rcPru av 2 expressed in the cytoplasm of *E. coli* with conserved T cell epitopes despite the loss of its IgE binding. Proliferative responses of rcPru av 2 assessed in PMBC of cherry-allergic subjects were determined. The ablation of B cell epitopes and the consequent abolishment of IgE response were probably because of the absence of multiple disulphide bonds. In contrast, periplasmic rPru av 2 contained at least some properly formed disulphide bridges, as it was able to bind IgE in a reduced way compared with the natural cherry TLP. Studies of other recombinant hypoallergens have demonstrated that mutation of multiple cysteine residues was required to inhibit IgE-reactivity completely [35, 36]. The decrease of the IgE-binding capacity of reduced and alkylated natural Pru av 2

observed in ELISA experiments confirmed that disulphide bridges contribute to immunological activity of the molecule.

In conclusion, this study presents two different kinds of recombinant Pru av 2 expressed in *E. coli*. Cytoplasmic rPru av 2 was not able to bind IgE, but its capacity of inducing T cell proliferation because of retained T cell epitopes was clearly demonstrated. This protein represents a well-defined model for studies of immune responses to TLPs in general and could be developed as a vaccine candidate for application in hypoallergenic immunotherapy in future. The other recombinant variant is the periplasmic rPru av 2 containing B cell epitopes and most likely correctly formed disulphide bonds. This thaumatin-like allergen displayed IgE-binding activity in the sera from cherry-allergic subjects and may be used for component-resolved *in vivo* diagnosis as standardized protein.

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References

- Eriksson NE, Formgren H, Svenonius E. Food hypersensitivity in patients with pollen allergy. *Allergy* 1982; **37**:437–43.
- Gotoda H, Maguchi S, Kawahara H, Terayama Y, Fukuda S. Springtime pollinosis and oral allergy syndrome in Sapporo. *Auris Nasus Larynx* 2001; **28** (Suppl.):S49–52.
- Ortolani C, Ispano M, Pastorello EA, Ansaloni R, Magri GC. Comparison of results of skin prick tests (with fresh foods and commercial food extracts) and RAST in 100 patients with oral allergy syndrome. *J Allergy Clin Immunol* 1989; **83**:683–90.
- Valenta R, Kraft D. Type 1 allergic reactions to plant-derived food: a consequence of primary sensitization to pollen allergens. *J Allergy Clin Immunol* 1996; **97**:893–5.
- Scheurer S, Metzner K, Haustein D, Vieths S. Molecular cloning, expression and characterization of Pru a 1, the major cherry allergen. *Mol Immunol* 1997; **34**:619–29.
- Scheurer S, Pastorello EA, Wangorsch A, Kastner M, Haustein D, Vieths S. Recombinant allergens Pru av 1 and Pru av 4 and a newly identified lipid transfer protein in the *in vitro* diagnosis of cherry allergy. *J Allergy Clin Immunol* 2001; **107**:724–31.
- Fils-Lycaon BR, Wiersma PA, Eastwell KC, Sautiere P. A cherry protein and its gene, abundantly expressed in ripening fruit, have been identified as thaumatin-like. *Plant Physiol* 1996; **111**:269–73.
- Stintzi A, Heitz T, Prasad V *et al.* Plant 'pathogenesis-related' proteins and their role in defense against pathogens. *Biochimie* 1993; **75**:687–706.
- Hoffmann-Sommergruber K. Pathogenesis-related (PR)-proteins identified as allergens. *Biochem Soc Trans* 2002; **30**:930–5.
- Hoffmann-Sommergruber K. Plant allergens and pathogenesis-related proteins. What do they have in common? *Int Arch Allergy Immunol* 2000; **122**:155–66.
- Ebner C, Hoffmann-Sommergruber K, Breiteneder H. Plant food allergens homologous to pathogenesis-related proteins. *Allergy* 2001; **56** (Suppl. 67):43–4.
- Hsieh LS, Moos M Jr, Lin Y. Characterization of apple 18 and 31 kd allergens by microsequencing and evaluation of their content during storage and ripening. *J Allergy Clin Immunol* 1995; **96**:960–70.
- Krebitz M, Wagner B, Ferreira F *et al.* Plant-based heterologous expression of Mal d 2, a thaumatin-like protein and allergen of apple (*Malus domestica*), and its characterization as an anti-fungal protein. *J Mol Biol* 2003; **329**:721–30.
- Fuchs HC, Hoffmann-Sommergruber K, Wagner B, Krebitz M, Scheiner O, Breiteneder H. Heterologous expression in *Nicotiana benthamiana* of Cap a 1, a Thaumatin-like protein and major allergen from bell pepper (*Capsicum Annuum*). *J Allergy Clin Immunol* 2002; **109**:S134.
- Gavrovic-Jankulovic M, Cirkovic T, Vuckovic O *et al.* Isolation and biochemical characterization of a thaumatin-like kiwi allergen. *J Allergy Clin Immunol* 2002; **110**:805–10.
- Pastorello EA, Farioli L, Pravettoni V *et al.* Identification of grape and wine allergens as an Endochitinase 4, a lipid-transfer protein, and a thaumatin. *J Allergy Clin Immunol* 2003; **111**:350–9.
- Ibanez MD, Sastre J, San Ireneo MM, Laso MT, Barber D, Lombardero M. Different patterns of allergen recognition in children allergic to orange. *J Allergy Clin Immunol* 2004; **113**:175–7.
- Inschlag C, Hoffmann-Sommergruber K, O'Riordain G *et al.* Biochemical characterization of Pru a 2, a 23-kD thaumatin-like protein representing a potential major allergen in cherry (*Prunus avium*). *Int Arch Allergy Immunol* 1998; **116**:22–8.
- Drew AC, Eusebius NP, Kenins L *et al.* Hypoallergenic variants of the major latex allergen Hev b 6.01 retaining human T lymphocyte reactivity. *J Immunol* 2004; **173**:5872–9.
- Ferreira F, Ebner C, Kramer B *et al.* Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. *Faseb J* 1998; **12**:231–42.
- Niederberger V, Horak F, Vrtala S *et al.* Vaccination with genetically engineered allergens prevents progression of allergic disease. *Proc Natl Acad Sci USA* 2004; **101** (Suppl. 2):14677–82.
- Neudecker P, Lehmann K, Nerkamp J *et al.* Mutational epitope analysis of Pru av 1 and Api g 1, the major allergens of cherry (*Prunus avium*) and celery (*Apium graveolens*): correlating IgE reactivity with three-dimensional structure. *Biochem J* 2003; **376**:97–107.
- Wiche R, Gubesch M, Konig H *et al.* Molecular basis of pollen-related food allergy: identification of a second cross-reactive IgE epitope on Pru av 1, the major cherry (*Prunus avium*) allergen. *Biochem J* 2005; **385**:319–27.
- Sheffield P, Garrard S, Derewenda Z. Overcoming expression and purification problems of RhoGDI using a family of "parallel" expression vectors. *Protein Expr Purif* 1999; **15**:34–9.

- 25 Wallner M, Gruber P, Radauer C *et al*. Lab scale and medium scale production of recombinant allergens in *Escherichia coli*. *Methods* 2004; **32**:219–26.
- 26 Menu-Bouaouiche L, Vriet C, Peumans WJ, Barre A, Van Damme EJ, Rouge P. A molecular basis for the endo-beta 1,3-glucanase activity of the thaumatin-like proteins from edible fruits. *Biochimie* 2003; **85**:123–31.
- 27 Dall'Antonia Y, Pavkov T, Fuchs H, Breiteneder H, Keller W. Crystallization and preliminary structure determination of the food allergen Pru av 2. *Acta Cryst* 2005; **F61**:186–8.
- 28 Wilson IB, Harthill JE, Mullin NP, Ashford DA, Altmann F. Core alpha1, 3-fucose is a key part of the epitope recognized by antibodies reacting against plant N-linked oligosaccharides and is present in a wide variety of plant extracts. *Glycobiology* 1998; **8**:651–61.
- 29 Korver O, van Gorkom M, van der Wel H. Spectrometric investigation of thaumatin I and II, two sweet-tasting proteins from *Thaumatococcus daniellii* Benth. *Eur J Biochem* 1973; **35**: 554–8.
- 30 Kaneko R, Kitabatake N. Structure–sweetness relationship in thaumatin: importance of lysine residues. *Chem Senses* 2001; **26**:167–77.
- 31 Batalia MA, Monzingo AF, Ernst S, Roberts W, Robertus JD. The crystal structure of the antifungal protein zeamatin, a member of the thaumatin-like, PR-5 protein family. *Nat Struct Biol* 1996; **3**:19–23.
- 32 van Loon LC, van Strien EA. The families of pathogenesis-related proteins, their activities and comparative analysis of PR-1 type proteins. *Phys Mol Plant Pathol* 1999; **55**:85–7.
- 33 Scheurer S, Lauer I, Foetisch K *et al*. Strong allergenicity of Pru av 3, the lipid transfer protein from cherry, is related to high stability against thermal processing and digestion. *J Allergy Clin Immunol* 2004; **114**:900–7.
- 34 Soman KV, Midoro-Horiuti T, Ferreón JC *et al*. Homology modeling and characterization of IgE binding epitopes of mountain cedar allergen Jun a 3. *Biophys J* 2000; **79**:1601–9.
- 35 Olsson S, van Hage-Hamsten M, Whitley P. Contribution of disulphide bonds to antigenicity of Lep d 2, the major allergen of the dust mite *Lepidoglyphus destructor*. *Mol Immunol* 1998; **35**:1017–23.
- 36 Bonura A, Amoroso S, Locorotondo G *et al*. Hypoallergenic variants of the *Parietaria judaica* major allergen Par j 1: a member of the non-specific lipid transfer protein plant family. *Int Arch Allergy Immunol* 2001; **126**:32–40.