

Characterization of Wild-Type Recombinant Bet v 1a as a Candidate Vaccine against Birch Pollen Allergy

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Key Words

Bet v 1 · Birch allergy · Specific immunotherapy · Vaccine

Abstract

Background: We describe the production in *Escherichia coli* as a recombinant protein of clinical grade wild-type Bet v 1a (rBet v 1a), to be used as a candidate vaccine against birch pollen allergy. **Methods:** This recombinant protein was purified by hydrophobic interaction and ion exchange chromatography and characterized by SDS-PAGE, immunoprint and circular dichroism in parallel with natural Bet v 1 (nBet v 1) purified from a birch pollen extract. We also compared rBet v 1 and nBet v 1 for their capacity to induce histamine release from basophils and to stimulate T lymphocyte proliferation. **Results:** rBet v 1a appears in SDS-PAGE as an 18-kDa monomeric protein, whereas purified nBet v 1 comprises a mixture of isoforms (resolving as three distinct bands and six spots after 1-dimensional and 2-dimensional electrophoresis, respectively). Both recombinant and natural purified Bet v 1 molecules are recognized by IgE from birch

pollen-allergic patients as well as anti-Bet v 1 murine monoclonal antibodies, suggesting that the recombinant protein is correctly folded in a native configuration. Circular dichroism analysis confirmed that the two Bet v 1 molecules exhibit similar 3-dimensional structures, even if rBet v 1a appears more compact and stable in thermodenaturation/renaturation experiments. Both rBet v 1 and nBet v 1 induce the degranulation of sensitized basophils and proliferation of Bet v 1-specific T lymphocytes in a similar manner. **Conclusions:** On the basis of these structural and biological properties, rBet v 1a is a valid candidate vaccine against birch pollen allergy, currently evaluated in humans.

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Introduction

Allergen-specific immunotherapy is broadly recognized as the only etiologic treatment for type I IgE-mediated allergies [1–4]. This approach has been used for almost a century [2, 3], and its efficacy has been established by multiple double-blind placebo-controlled stud-

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ies [4, 5]. As of today, immunotherapeutic approaches are based on the administration through the subcutaneous or sublingual routes of aqueous extracts prepared from raw allergenic materials (e.g. house dust mite cultures, pollens, animal hair). These extracts represent complex mixtures whose composition is hardly known at the molecular level and which are difficult to standardize.

More than 400 allergens have been characterized thus far, providing the opportunity to rely on the power of genetic engineering to replace biological extracts with recombinant allergen proteins, both for diagnostic and therapeutic purposes [1, 6, 7]. Recombinant allergens should be preferred to corresponding purified natural molecules, in order to cope with reproducibility, traceability and yield issues. Vaccines based on recombinant allergens are particularly suitable when the number of major allergens incriminated is limited. This, for example, is the case for birch pollen allergies, for which more than 95% of patients display IgE reactivity against Bet v 1, and up to 60% of such patients react solely to Bet v 1 [8–14]. This allergen is well-characterized in molecular terms [13–15]. It belongs to the ubiquitous family of pathogenesis-related plant proteins, possibly acting as a plant steroid carrier [14, 16]. Both crystallographic and NMR studies revealed a 3-dimensional structure for Bet v 1 associating a seven-stranded β sheet with three α helices, creating a y-shaped hydrophobic cavity [13–15].

One open question regarding the design of molecular allergens for specific immunotherapy treatment is whether they should bear IgE binding epitopes [6, 7]. Hypoallergenic forms of recombinant allergens or peptide fragments have been developed which do not induce degranulation of IgE+ mast cells or basophils, while maintaining a capacity to elicit IgGs and stimulate T lymphocytes [17–20]. An alternative is to use allergens in their wild-type conformation for desensitization purposes since, as of today, efficacy has been mostly demonstrated with extracts containing native allergens [21].

Herein, we describe the production and characterization of a vaccine candidate against birch pollen allergy, based on a wild-type (i.e. unmodified) Bet v 1 molecule expressed as a recombinant protein (rBet v 1). The Bet v 1a isoform has been selected since it is quantitatively the most represented isoform (i.e. accounting for more than 50% of the total Bet v 1 mass [22]) in the natural extract. We demonstrate that rBet v 1a shares similar structural and biological properties with the natural Bet v 1 (nBet v 1) molecule purified from birch pollen extract.

Materials and Methods

Production and Purification of GMP Grade Wild-Type rBet v 1a

The *Escherichia coli* strain BL21(DE3) (Stratagene) was used as a bacterial expression system. The Bet v 1a gene was obtained as an RT-PCR fragment in which the coding sequence is bracketed between an *NdeI* site at the start and an *EcoRI* site immediately after the stop codon [23, 24]. The DNA sequence encoding for rBet v 1a was cloned in the expression vector pMW172 [25], a derivative of pRK172 [26]. The Bet v 1a coding sequence (corresponding to amino acids 1–483) was inserted in pMW172 between *NdeI* and *EcoRI* restriction sites, under the control of the bacteriophage promoter T7. In this expression system, the T7 RNA polymerase was provided by the prophage λ (DE3) under the conditional *lacUV5* promoter control. This construction expresses Bet v 1a after induction with IPTG. In addition to the Bet v 1a gene, this plasmid vector also contains the ampicillin resistance gene (Amp). *E. coli* BL21 (DE3) cells transformed with the pMW172-Betv1a plasmid were grown in a 5-liter stirred bioreactor (Prelude, Biolafite). A 200-ml preculture was inoculated from a thawed working cell bank vial and grown overnight at 37 °C in YPN-selective medium (yeast extract 1%, vegetable peptone 2%, NaCl 2%) in the presence of ampicillin at 100 μ g/ml, up to an absorbance of ~ 5 . The 5-liter bioreactor was inoculated with 200 ml of preculture at a starting absorbance of around 0.2 and incubated during 4 h in order to reach the log growth phase with an absorbance of 4.0 ± 0.5 . The culture was performed without antibiotic in YPNKG medium (yeast extract 1.5%, vegetable peptone 0.66%, NaCl 1.5%, KH_2PO_4 0.3% supplemented with 2% glucose). Fermentation settings were as follows: 37 °C, pH 7.0 regulated by the addition of NaOH 5 M, constant stirring at 300–1,000 rpm, 20% O_2 , aeration at 750 ml/min with pure O_2 .

When the culture reached the log phase, the expression of Bet v 1a was induced by the injection of 50 ml of 0.1 M IPTG with a sterile syringe through the septum of the reactor and the culture was continued for 3 h. Three hours after IPTG induction, a complement of 100 ml of a 50% glucose solution was injected through the septum. At 4 h postinduction, the culture was harvested and centrifuged at 3,000 rpm for 30 min. Bacteria pellets were then stored at -20°C . After thawing at $+4^\circ\text{C}$, these pellets were pooled and resuspended in a final volume of 1 liter of lysis buffer (Tris 50 mM, NaCl 50 mM, EDTA 1 mM, pH 8.0). The pool of bacteria was vigorously homogenized using an Ultra-Turrax apparatus during 15 min at 10,000 rpm. Bacterial cells were then destroyed with a cell disrupter at 1,200 bar (Panda 2 K). The viscosity of the lysate was reduced by nuclease treatment (using 10 U/ml Benzonase during 1 h at room temperature). The lysate was centrifuged at 8,000 rpm for 60 min at $+4^\circ\text{C}$. Salts (0.76 M NaCl and 0.65 M $\text{NaH}_2\text{PO}_4 \cdot 2 \text{H}_2\text{O}$) were added to the supernatant for an overnight precipitation at $+4^\circ\text{C}$. After centrifugation (20 min at 18,000 rpm) and filtration onto 0.22 μm , the supernatant was loaded on a phenylsepharose FF (Pharmacia, high capacity) XK26/40 column. Chromatography was performed at room temperature on a QuantaSep 1000 LXR Process System (Sepragen) with the following buffers: equilibration buffer (0.2 M $\text{NaH}_2\text{PO}_4 \cdot 2 \text{H}_2\text{O}$, 1 M NaCl, pH 4.5), buffer A (0.8 M NaCl, 10 mM acetic acid, 15 mM $\text{NaH}_2\text{PO}_4 \cdot 2 \text{H}_2\text{O}$, pH 4.5), buffer B (25 mM Tris-HCl pH 9.3, 8% isopropanol), and elution buffer (70% buffer A; 30% buffer B). After immobilization and washing with buffer A, the recombinant protein was eluted with the elution buffer. The protein eluate was diafiltrated by tangential ultrafiltration at $+4^\circ\text{C}$ against 25 mM Tris-HCl, pH 8.0 buffer with the ProFlux M12 system (Millipore), using a Pelli-

con2 regenerated cellulose UFT cartridge with a cutoff of 5 kDa and a filtration area of 0.1 m² (Millipore). The precipitate produced during the diafiltration was eliminated by filtration onto 0.22-μm filters. The filtrate was then loaded at room temperature on a DEAE-Sepharose FF (Pharmacia, high capacity) XK26/40 column. The buffers used were loading buffer (25 mM Tris-HCl, pH 8.0), washing buffer (25 mM Tris-HCl, pH 7.4), and elution buffer (25 mM Tris-HCl, 12.5 mM NaCl, pH 7.4). The rBet v 1a protein was then concentrated/diafiltered by tangential ultrafiltration against NaCl 155 mM (Proflux M12/Pellicon 2, Millipore) and diluted to obtain a final concentration of 1 mg/ml. Finally, the purified rBet v 1a protein was sterilized by filtration onto a 0.22-μm sterilizing MiniKleenpak DFL filter (PALL), and stored at -80 °C.

Purification of nBet v 1 from Birch Pollen Extracts

Briefly, defatted birch pollen (Pharmallerga, Czech Republic) was extracted at a 5% (m/v) ratio in 50 mM ammonium bicarbonate, pH 8.0, under gentle stirring for 16 h at +4 °C. After centrifugation at 6,000 g for 80 min, the supernatant was filtered onto a 0.22-μm Polyssep cartridge (Millipore, Bedford, Mass., USA). The extract obtained was diluted twice in 0.2 M sodium phosphate, 1.8 M ammonium sulfate buffer, pH 6.0, before loading onto a 500-ml Butyl Sepharose 4 Fast Flow gel (Amersham Biosciences) equilibrated with 0.1 M sodium phosphate, 0.9 M ammonium sulfate, pH 6.0 (buffer C). After washing the gel with buffer C, preelution was performed using the following gradient, applied on 6 column volumes: 0–22% of buffer D (0.02 M imidazole, 8% isopropanol, pH 7.4) diluted in buffer C. nBet v 1 was eluted by maintaining the 78% buffer C–22% buffer D combination. It was then concentrated onto a 3-kDa cassette (Omega, Pall Corporation, New York, N.Y., USA) up to 1 mg/ml based on high-performance liquid chromatography (HPLC) quantification. In order to eliminate unwanted chemicals, nBet v 1 was loaded onto a 350-ml G25 Sephadex column (Amersham Biosciences) for desalting against 155 mM NaCl. Purified nBet v 1 was then sterilized by filtration over a 0.22-μm Millipack filter (Millipore) and stored at -80 °C.

Quality Control of rBet v 1 and nBet v 1 Proteins

Standard quality controls performed in order to qualify rBet v 1 and nBet v 1 for in vivo administration to humans included sterility, identity (by immunoreactivity with specific antibodies on immunoblots from 1- and 2-dimensional gels, and NH₂-terminal sequencing), purity [following Coomassie staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis] and potency (measuring IgE binding by RAST inhibition). Levels of endotoxin were determined using the Limulus amebocyte lysate test with the Endochrome kit (Charles River Laboratories, L'Arbresle, France). For protein determination on the basis of ultraviolet (UV) absorbance, extinction coefficients of purified rBet v 1 and nBet v 1 were obtained using the ProtParam tool of ExPASy, after entering the corresponding sequences (accession No. P15494 and CAB02156, respectively). Protein concentrations of r- and nBet v 1 were calculated from UV absorbance values obtained at 280 nm, under denaturing conditions using the deduced extinction coefficient. Protein determination by micro-BCA assay was performed using the micro-BCA kit (Pierce, Rockford, Ill., USA). Bet v 1 quantification by reversed-phase HPLC method was performed on a Symmetry 300, C4 column (4.6 × 150 mm; Waters, Milford, Mass., USA). UV absorbance was monitored at 214 nm.

Peptide Mass Fingerprinting

Purified Bet v 1 was submitted to electrophoresis on a 10–15% PHAST System gradient gel (Amersham Biosciences) which was Coomassie-stained. The in-gel digestion protocol of the EMBL Bio-analytical Research Group was followed (<http://www.mann.embl-heidelberg.de/GroupPages/PageLink/activities/protocols/ingeldigest.html>). Protein bands were digested with 5 μl of 40 ng/μl porcine trypsin (Promega) for 30 min at 58 °C as previously described [27]. One microliter of the digestion solution was spotted on a stainless steel MALDI plate and mixed with 1 μl of a saturated solution of α-cyano-4-hydroxy-cinnamic acid (Sigma) recrystallized in 50% acetonitrile/0.3% TFA in water. After complete drying, spots were washed 2 times with 10 μl of water/0.1% TFA, and analyzed on an Applied Biosystem Voyager DE-RP instrument. Databases were queried at 300 ppm with the following programs on the web: Protein Prospector (<http://prospector.ucsf.edu/>) and Profound (<http://prowl.rockefeller.edu/>).

Circular Dichroism Spectroscopy

Circular dichroism (CD) experiments were carried out as described previously [28]. The measurements were done at 22 °C with constant N₂ flushing. Far-UV and near-UV CD spectra were measured in 1- and 10-mm path length quartz cells, respectively. Protein concentrations were 35 μM for near-UV CD and 6 μM for far-UV acquisitions, based on UV determination. The scans were recorded using a bandwidth of 2 nm and an integration time of 1 s at a scan rate of 0.5 nm·s⁻¹. Each spectrum represents the average of 10 and 20 scans for far- and near-UV regions, respectively. Whatever UV region was investigated, the blank was determined using sodium phosphate 5 mM, pH 7.5. Spectra were corrected for blank and a smoothing algorithm was applied with the minimum filter in the CD6 software (CDMax, filter 5). The mean residue ellipticity in far-UV [θ]_F and the molar ellipticity in near-UV [θ]_N were calculated, respectively, from the relations [θ]_F = (100 × θ_m)/(C × l × N) and [θ]_N = (100 × θ_m)/(C × l), where θ_m is the measured ellipticity in degrees, C is the concentration in moles per liter, l is the path length of the cell in centimeters and N is the number of peptide links. The units of the mean residue ellipticity [θ]_F and that of the molar ellipticity [θ]_N are expressed in degrees square centimeter per decimole residue (deg·cm²·dmol⁻¹·res⁻¹) and in degrees square centimeter per decimole (deg·cm²·dmol⁻¹), respectively.

Thermodenaturation/Renaturation Studies

CD spectra of rBet v 1 and nBet v 1 were recorded on a JASCO J-810 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) fitted with a Peltier type temperature control system (model PTC-423S/L). Far-UV CD spectra were recorded in a 0.1-cm quartz cuvette at a protein concentration of 15 μM, based on UV and micro-BCA determination. Spectra were recorded from 25 to 95 °C every 5 degrees with a band width of 1 nm, a response time of 1 s, with results obtained from an average of 3 scans. The reversibility of the unfolding process was determined by monitoring the spectra after cooling to the starting temperature. Final spectra were baseline-corrected by subtracting the corresponding spectra obtained with the buffer alone under identical conditions. All measurements were performed in 20 mM KCl, 20 mM NaCl, pH 7.0. The results were expressed as the mean residue ellipticity at a given wavelength.

Immunogenicity Studies

Reactivity with Murine Monoclonal Antibodies

Briefly, to generate monoclonal antibodies (mAbs) 5B4 and 6H4 to Bet v 1, BALB/c mice were injected intraperitoneally with 100 µl of complete Freund's adjuvant and 100 µl of a birch pollen extract containing 1 mg/ml of proteins. Mice were injected twice at J19 and J41 with the same amount of birch pollen extract and 100 µl of incomplete Freund's adjuvant. Sera from immunized animals were analyzed at J34 by direct ELISA for reactivity with birch pollen extract. Splenocytes from the mouse displaying the highest antibody titer were used for lymphocyte hybridization [29]. Hybridomas were screened by ELISA for reactivity with a birch pollen extract, and subsequently by immunoprint after SDS-PAGE or isoelectric focusing (IEF) to confirm their reactivity with Bet v 1. Five hybridomas were selected, all producing IgG1, and cloned. Antibodies were obtained as ascitic fluids, purified on protein A coupled to a Sepharose gel (Pharmacia, Uppsala, Sweden), prior to dialysis against phosphate-buffered saline.

SDS-PAGE and Immunoblots

SDS-PAGE was carried out with homogeneous 12.5% SDS Excel-Gel (Amersham Biosciences) for 80 min at 16 °C in a Multiphor II apparatus (Amersham Biosciences) at 50 mA and 600 V. Proteins were diluted in 6% Tris buffer containing 1% SDS, pH 7.5 and incubated 5 min at 95 °C with 5% β-mercaptoethanol. 0.25 µg of Bet v 1 was deposited. After migration, bands were revealed by silver staining (Amersham Biosciences) or immunoblotting. A low-molecular-weight calibration kit (Amersham Biosciences) was used as a reference. Proteins were electroblotted for 1 h onto 0.45 µm nitrocellulose using a Novablot system (Amersham Biosciences). Immunoblots were incubated with IgE (pool of sera from birch pollen-sensitized individuals) or anti-Bet v 1 5B4 or 6H4 mAbs, then with an HRP conjugate, and revealed with a chemiluminescent substrate (Super-Signal West Pico Chemiluminescent Substrate, Pierce).

2-Dimensional Electrophoresis Followed by Immunoblotting

For 2-dimensional electrophoresis experiments, extracts were incubated for 2 h in 8 M urea 2 M thiourea, 2% CHAPS, 10% glycerol, 0.28% dithiothreitol (DTT) and bromophenol blue solution. Prior to IEF, the 11-cm IPG strips (Immobiline DryStrip gel, pH 3–10, Amersham Biosciences, Uppsala, Sweden) were rehydrated for 16 h in 200 µl of sample preparation solution (10 µg deposited). IEF was performed using a Multiphor horizontal electrophoresis apparatus. The experiment was conducted at 150 V for 30 min, 300 V for 1.5 h, then 1,500 V for 1 h, and continued for 4 h 30 min at 3,500 V. After IEF, the IPG strips were equilibrated in 7 ml of 0.05 M Tris-HCl, 6 M urea, 30% glycerol, 3% SDS, pH 8.8 with 1% DTT for 2 × 5 min, and then with 2.5% iodoacetamide (without DTT) and a trace of bromophenol blue for 2 × 5 min. The equilibrated IPG strips were transferred to a horizontal homogeneous 12.5% SDS ExcelGel (Amersham Biosciences) at 20 mA for 30 min. After removing the IPG strip, electrophoresis was performed at 50 mA for 70 min. Immunoblotting after 2-dimensional electrophoresis was performed as described above after SDS-PAGE.

Histamine Release Experiments

Granulocytes were isolated from heparinized blood of two birch pollen-allergic individuals by centrifugation over dextran. Cells were incubated with different concentrations (10 pg/ml to 1 µg/ml) of purified r- and nBet v 1 proteins. Histamine released in the supernatant

was measured by RIA (Immunotech, Marseille, France) as described elsewhere [30]. Results were obtained as mean values from triplicate determinations and expressed as a percentage of total histamine release.

T Lymphocyte Stimulation Assays

Peripheral blood mononuclear cells (PBMC) from 5 birch pollen-allergic patients were obtained from total blood by centrifugation over a Ficoll-Hypaque density gradient. To generate allergen-specific T cell lines (TCL), PBMC (1×10^5 cells/well) were stimulated with rBet v 1a for 5 days. Human recombinant interleukin-2 (rIL-2) was added and at day 7, T cell blasts were enriched by density gradient centrifugation and seeded in 96-well round bottom plates in the presence of irradiated PBMC, 0.5% v/v phytohemagglutinin and rIL-2. T cell blasts were expanded at weekly intervals with irradiated PBMC and rIL-2. Thereafter, these TCL were stimulated with defined concentrations of rBet v 1a and nBet v 1 in the presence of irradiated autologous PBMC as antigen-presenting cells. After 48 h, [³H]-thymidine was added and the incorporated radioactivity was measured after the addition of scintillation fluid. The stimulation index was calculated as the ratio between the counts per minute (cpm) obtained in cultures of TCL plus allergen and the cpm obtained in cultures of TCL without allergen. Statistical significance of the difference between stimulation index obtained with rBet v 1a and nBet v 1 was assessed by Wilcoxon t test, using the SPSS 11.5 computer program for Windows. A probability value above 0.05 was considered nonsignificant.

Results

Biochemical and Structural Characterization of Purified rBet v 1 and nBet v 1

rBet v 1a and nBet v 1 were produced and purified, as described in Materials and Methods as clinical grade products with a low endotoxin content (i.e. <0.02 EU/µg for rBet v 1a and 0.03 EU/µg for nBet v 1a). The yields obtained after purification were 100 mg/l of culture for rBet v 1a, and 400 µg/g pollen for nBet v 1. As expected, the natural extract comprises a mixture of proteins with high amounts of proteins in the 18–20 kDa molecular weight range (fig. 1, lane A). One-dimensional SDS-PAGE analysis showed that rBet v 1a was >95% pure, appearing as a main band at the expected ~18-kDa molecular mass and a very faint ~40-kDa band (see fig. 1, lane B). The latter resolved into the ~18-kDa band in the presence of high SDS concentrations (results not shown), indicating that it corresponds to a noncovalently bound dimer of Bet v 1 molecules.

Purified nBet v 1 (fig. 1, lane C) appears mainly as a triplet of 18- to 20-kDa proteins representing distinct isoforms of Bet v 1 (see below), which are also present in the birch pollen extract (fig. 1, lane A). We estimate nBet v 1 to be pure at 75–80%, with the presence of a 30-kDa main

Table 1. Peptide sequences obtained by MS-MS for the purified nBet v 1 and corresponding published isoforms

Peptide No.	Sequence (location in the Bet v 1 sequence)	Corresponding isoforms
1	EMGETLLR (138–145)	a, j, f/i, d/h, g, e, l
2	ISFPEGFPFK (56–65)	a, j, f/i
3	ASKEMGETLLR (135–145)	a, j, f/i, d/h, g, e, l
4	AFILDGDNLFPPK (21–32)	a, j
5	DRVDEVDHTNFK (69–80)	a, j, f/i, d/h, l
6	AVESYLLAHSDAYN (146–159)	a, j, f/i, d/h, g, e, l, b, k, c, m/n
7	YNYSVIEGGPVGDTLEK (81–97)	d/h, g, l
8	ISNEIKIVATPDGGSILK (98–115)	a, d/h, g, l, b, k, c, m/n
9	VAPQAISSVENIEGNGGPGTIK (33–54)	a, j, f/i, d/h, g, e, l, b, k, c, m/n
10	VAPQAISSVENIEGNGGPGTIKK (33–55)	a, j, f/i, d/h, g, e, l, b, k, c, m/n

The peptide sequences obtained from purified Bet v 1 were matched with published sequences for various isoforms [22]. Only one mismatch was found when combined with the published Bet v 1a sequence (with a valine in nBet v 1 instead of an isoleucine in published sequences at position 91).

contaminant protein shown by mass spectrometry to be birch triose phosphate isomerase (not shown). The identity of purified nBet v 1 was clearly confirmed by mass spectrometry, as the sequences obtained for MS-MS-derived peptides matched sequences submitted by J.N. Larsen (NCBI access No. CAB02156), demonstrating the presence of multiple isoforms (i.e. a, b, c, d/h, e, f/i, g, j, k, l, m/n, table 1) in material purified from a natural source.

The CD spectra obtained in far UV for purified r- and nBet v 1 (fig. 2a), indicative of their secondary structures, were overall similar, showing that both molecules contain α helices and β sheets. Whereas the two molecules exhibit the same proportion of α helices, rBet v 1a appears to have more β sheet content than its natural counterpart: the positive peak obtained for rBet v 1a was shifted to ≥ 195 nm, and the negative band at 217 nm was sharper and more intense. The near-UV CD spectrum of both proteins showed negative bands at 262 and 268 nm, and a broad positive signal between 270 and 295 nm (fig. 2b). We cannot exclude that minor differences in CD spectra could be linked to the presence of some impurities in the nBet v 1 preparation.

The thermodenaturation spectra measured every 5°C (fig. 3a, b) suggested that nBet v 1 tends to adopt a partially folded molten globule-like state [28, 31–34]. These thermodenaturation experiments indicated a strong cooperativity involved in the unfolding of rBet v 1a as opposed to nBet v 1. In denaturation/renaturation experiments (fig. 4), the denaturation observed for both rBet v 1a and nBet v 1 was only partial, because the CD peaks

obtained in far UV between 190 and 200 nm, for example, did not reach negative values. Also, the renaturation of both molecules after heating at 95°C was only partial, since the spectra obtained did not match the ones obtained before denaturation. The isodichroic point observed at about 205 nm in the nBet v 1 spectra (see fig. 4b) suggests that its secondary structure can adopt only two states: folded and unfolded, respectively, while rBet v 1a

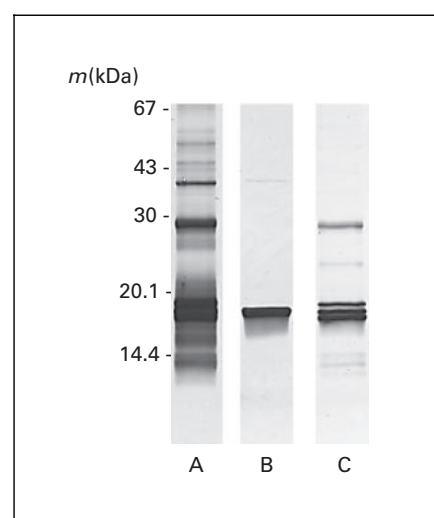
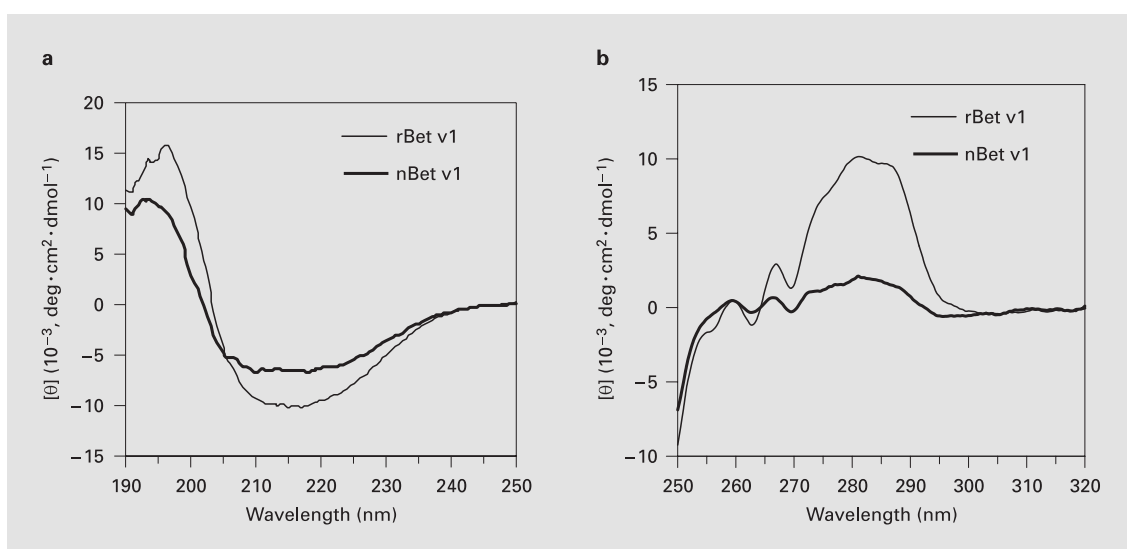


Fig. 1. Electrophoretic patterns of Bet v 1 molecules. SDS-PAGE analysis of birch pollen extract (lane A), purified rBet v 1a (lane B) and nBet v 1 (lane C) was conducted as described in Materials and Methods. For each lane, 0.25 μ g of Bet v 1 was loaded on the gel.



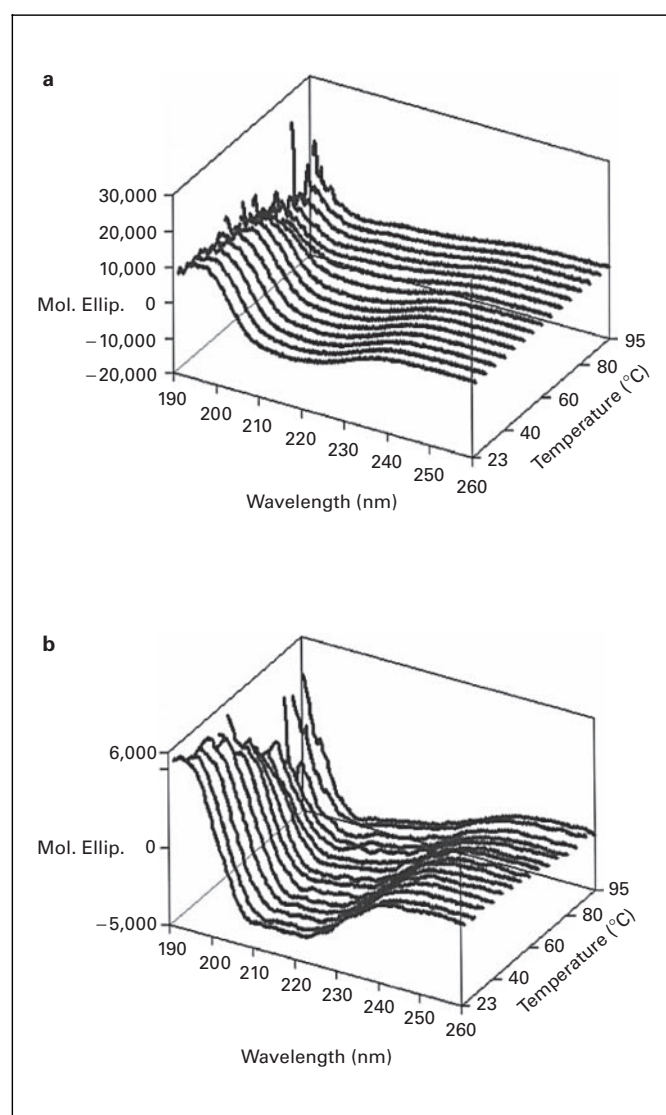
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(for which such an isodichroic point was not observed, see fig. 4a) adopts three different secondary structures: either folded, unfolded, or an intermediate form.

Altogether, these experiments establish that rBet v 1a is properly folded, in a configuration similar to the native molecule, albeit in a more compact and stable form.

Immunological Characterization of Purified rBet v 1 and nBet v 1 Molecules

Purified rBet v 1a was analyzed by SDS-PAGE and immunoblot, using for detection purposes either IgEs from a pool of sera obtained from birch pollen-sensitized individuals or Bet v 1-specific murine mAbs (mAbs 5B4 and 6H4 generated against the native Bet v 1 molecule). Those experiments conducted in parallel with purified nBet v 1 and a birch pollen extract indicate that rBet v 1 is recognized by such specific antibodies in a similar manner in the three samples (fig. 5a, b). It is worth noting that the binding of mAb 6H4 to rBet v 1a was more intense than to nBet v 1. This is likely due to the fact that this antibody recognizes preferentially isoform a of the Bet v 1 molecule (fig. 5c). Alternatively, it could react to a



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Fig. 2. Structural analysis of rBet v 1a and nBet v 1. **a** Far-UV CD spectra. **b** Near-UV CD spectra.

Fig. 3. Thermodenaturation experiments and CD analysis: changes in the secondary structures of rBet v 1a (**a**) and nBet v 1 (**b**) induced by thermal denaturation (using temperatures between 25 and 95 °C) were assessed by CD. Mol. Ellip. = deg·cm²·dmol⁻¹.

Fig. 4. Far-UV CD analysis of rBet v 1a and nBet v 1 following thermodenaturation/renaturation: the mean residue ellipticity (y-axis) at 25 °C, 95 °C, and after cooling to 25 °C (95→25 °C) is shown for different wavelengths (x-axis). Both proteins regain a significant percentage of their initial secondary structure upon cooling to 25 °C. **a** The 25 °C spectrum of rBet v 1 is characterized by a broad minimum at 215 nm and a maximum at 196 nm, which is consistent with a mixed α/β -secondary structure and is in good agreement with secondary structure contents determined by crystallization. Upon heating to 95 °C rBet v 1a changes to random coil conformation. **b** In contrast, the 25 °C spectrum of nBet v 1 shows two minima at 208 and 222 nm, and a maximum below 195 nm. The 95 °C spectrum of nBet v 1 shows a minimum near 200 nm and a maximum below 190 nm, consistent with a shift towards unordered proteins. Mol. Ellip. = $\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$.

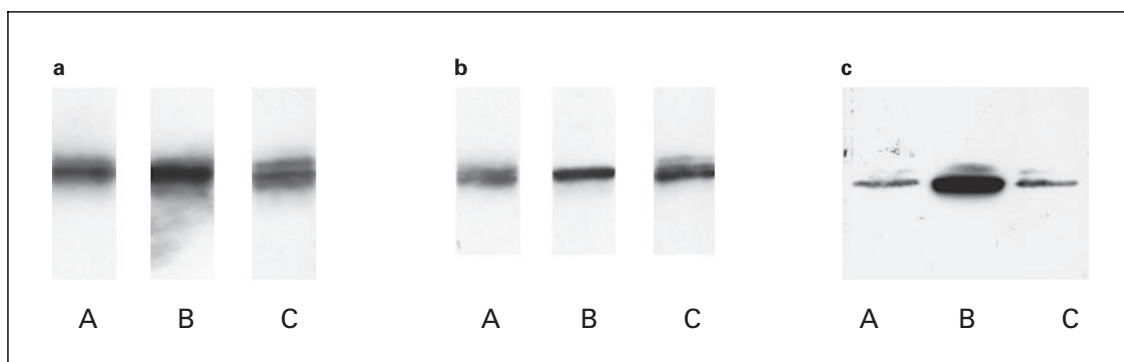
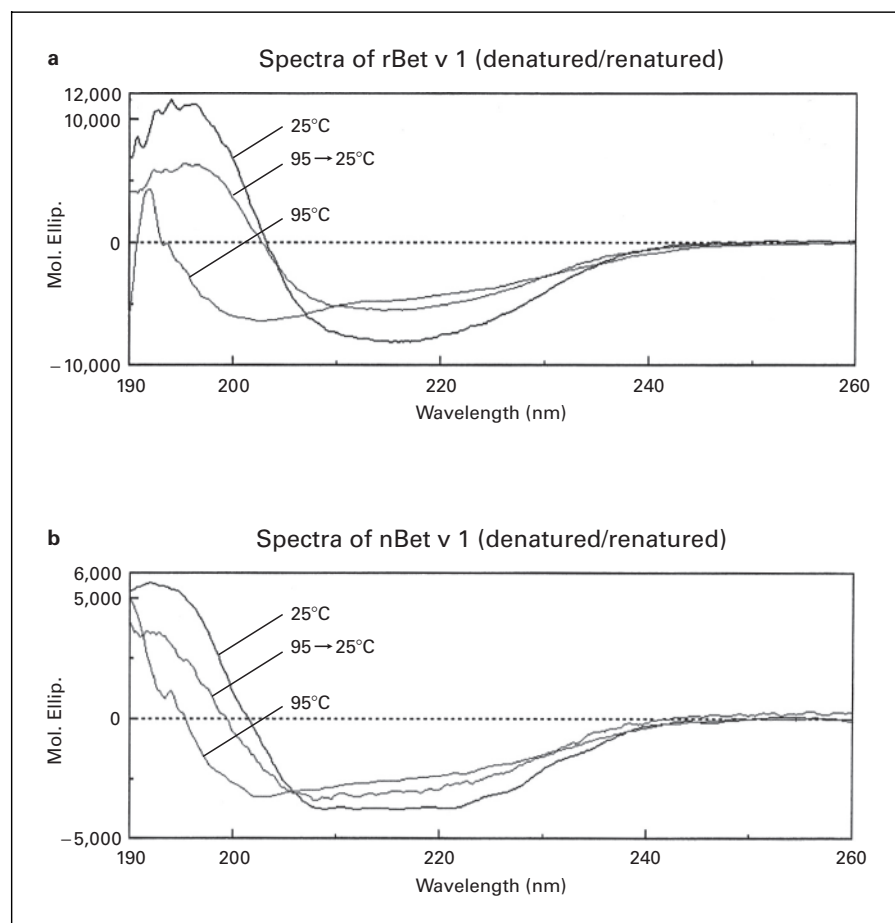


Fig. 5. Immunoreactivity of rBet v 1a and nBet v 1. Immunoprints following 1-dimensional SDS-PAGE of a birch pollen extract (lane A), purified rBet v 1 (lane B), and nBet v 1 (lane C). For each lane, 0.25 μg of Bet v 1 was loaded. Controls performed with sera from a nonsensitized individual or from an individual sensitized to house dust mite but not to birch pollen showed no reactivity, either with the birch pollen extract or with rBet v 1 and nBet v 1 (results not shown). **a** Detection with IgE from a pool of sera obtained from birch pollen-sensitized individuals. **b** Detection with anti-Bet v 1 mAb 5B4. **c** Detection with anti-Bet v 1 mAb 6H4.

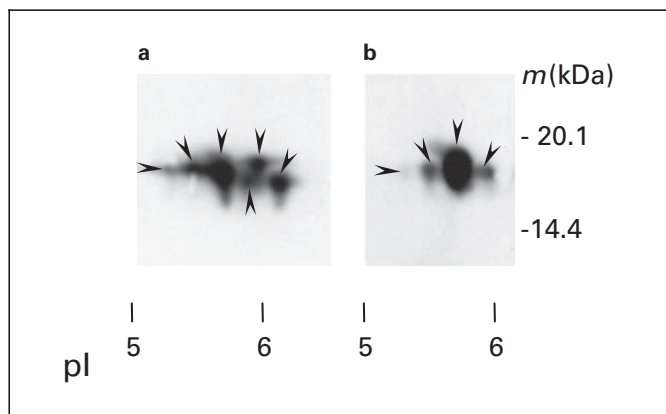


Fig. 6. Two-dimensional gel analysis of rBet v 1a and nBet v 1. Immunoprints following 2-dimensional gel electrophoresis of purified nBet v 1 (**a**) and rBet v 1 (**b**) were revealed with a pool of sera from birch pollen-sensitized individuals.

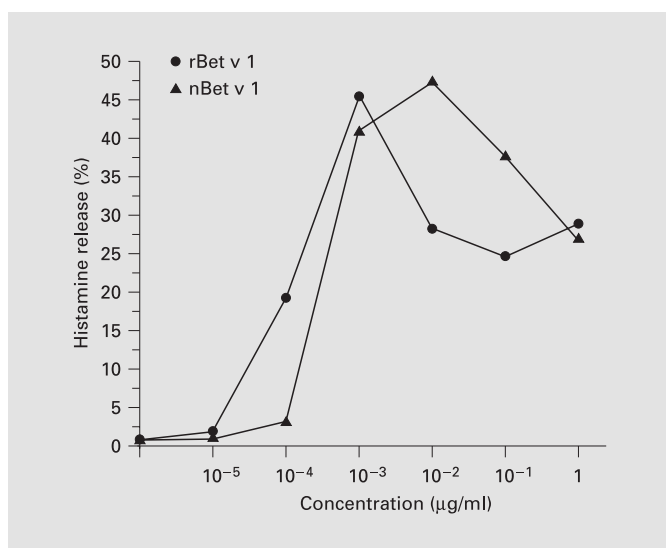


Fig. 7. Induction of histamine release in basophils from birch pollen-allergic patients. Increasing logarithmic concentrations of nBet v 1 and rBet v 1a (10⁻⁵ μg/ml up to 1 μg/ml; x-axis) were incubated with basophils. The percentage of histamine release is displayed on the y-axis. Results are expressed as the mean of triplicate determinations with standard deviation less than 5%.

specific epitope better preserved in the recombinant, as opposed to the natural molecule. In 2-dimensional electrophoresis, nBet v 1 appears as 6 spots, reacting with IgEs from a pool of sera from birch pollen-sensitized individuals (fig. 6, panel a). In contrast, rBet v 1a appeared only as 4 spots, all recognized by antibodies from the same serum

pool (fig. 6, panel b). Collectively, these results suggest that a number of conformational epitopes recognized by patients' IgE and murine mAbs are presented in a native configuration by rBet v 1a.

Histamine Release Experiments

We subsequently analyzed the capacity of rBet v 1a to induce histamine release from sensitized basophils. Figure 7 summarizes the results obtained with basophils from birch pollen-allergic individuals. We found that both the nBet v 1 and the rBet v 1 molecules can efficiently induce histamine release in those assays. When using low concentrations, rBet v 1a was consistently found to be slightly more active than nBet v 1, likely because IgEs in the sera of patients are predominantly directed to isoform a of Bet v 1, i.e. the most abundant isoform in birch pollen.

Proliferative Response of Bet v 1-Specific TCL to nBet v 1 and rBet v 1a

TCL from 5 different birch pollen-allergic individuals (including high and low responders) were established as described in Materials and Methods and stimulated with defined concentrations of both nBet v 1 and rBet v 1. The highest proliferation level was observed when 5 μg/ml of both allergens were used (fig. 8a). The magnitude of the lymphoproliferative response was variable between allergic patients. Nevertheless, proliferative responses obtained with purified rBet v 1a or nBet v 1 were not significantly different at inducing T cell proliferation (using the Wilcoxon t test). Also, as shown with the TCL derived from a representative donor, we did not see any difference in proliferative responses when various doses of natural or rBet v 1 were used (fig. 8b).

Discussion

There is a growing interest in developing allergy vaccines based on recombinant proteins. Such second-generation vaccines would have the advantage over commonly used biological extracts of being molecularly well-defined and easy to standardize [6, 7]. In order to develop a candidate recombinant vaccine against birch pollen, GMP grade wild-type rBet v 1a has been produced in an *E. coli* expression system and purified. rBet v 1a resolves in SDS-PAGE as a single molecule, as expected for a single isoform. In 2-dimensional electrophoresis, rBet v 1a appears as one main and three minor entities with distinct isoelectric points. Those distinct forms are most likely due to

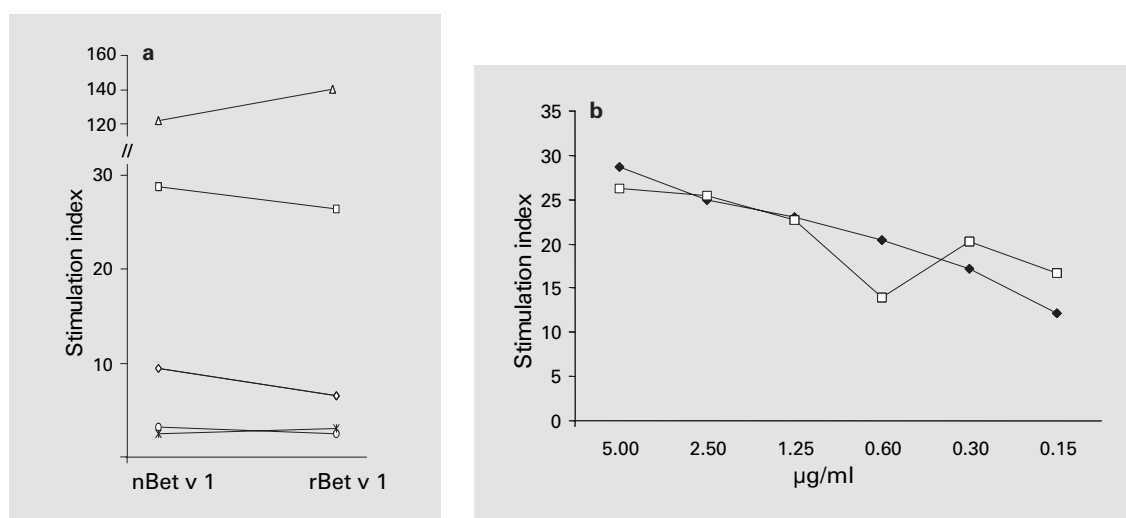


Fig. 8. Stimulation of TCL from birch pollen-allergic patients. **a** TCL from 5 different birch pollen-allergic patients were stimulated with nBet v 1 and rBet v 1 (5 µg/ml). **b** The TCL from one representative donor was stimulated with different concentrations of nBet v 1 (◆) and rBet v 1 (□). The stimulation index obtained as a mean of a triplicate determination is shown for varying allergen concentrations with a standard deviation less than 5%.

posttranslational modifications, such as tyrosine, serine or threonine phosphorylation and/or glutamine or asparagine acetylation. Alternatively, others have reported that rBet v 1a expressed in *E. coli* had no postsynthetic modifications besides N-terminal cleavage of methionine, and proposed that the heterogeneity observed in 2-dimensional gels could be due to distinct folding conformations [35].

In contrast to rBet v 1a, nBet v 1 appears as multiple bands following 2-dimensional electrophoresis, as expected since it is a mixture of several isoforms. This was confirmed by mass spectrometry, with partial sequences obtained matching the isoforms described by Swoboda et al. [22]. On the basis of circular dichroism and thermode-naturation/refolding experiments, rBet v 1 is shown to display a wild-type configuration comparable to nBet v 1. We found, however, that the recombinant molecule adopts a more compact and stable structure than its natural purified counterpart. This could possibly be due to a partial denaturation of nBet v 1 during the purification process, since sulfate ions are used for the salting out of nBet v 1 (instead of chloride ions for rBet v 1a) [36]. Nevertheless, the reactivity of patients' IgE and of specific murine mAbs was similar for both Bet v 1 in crude birch pollen extract, purified nBet v 1 or rBet v 1a, establishing that all these molecules share a number of conformational epitopes in agreement with previous studies [35].

Bet v 1a is known to be the most abundant isoform in birch pollen [22], and thus exposure to birch pollen likely raises antibodies predominantly to this isoform. This probably explains why mAb 6H4 (obtained following immunization of mice with a crude birch pollen extract) was more reactive to rBet v 1a than to nBet v 1. It could also explain why rBet v 1a was slightly more efficient than nBet v 1 in triggering basophil histamine release. Importantly, no significant differences were found in the relative capacity of the two molecules to stimulate specific T cells lines generated from birch pollen-allergic patients.

Altogether, our results indicate that wild-type rBet v 1a and purified nBet v 1 have essentially comparable physicochemical, structural and immunological features. Whereas our strategy is to design a vaccine against birch pollen allergies based on a recombinant allergen in the most native configuration, an alternative approach is based on genetically engineered hypoallergenic forms of Bet v 1 as candidates [6, 18, 20]. This includes for example peptide fragments corresponding to amino acids 1–74 and 75–160, as well as a trimer of Bet v 1. All these molecules exhibit a decreased binding capacity for IgEs, and are shown following intradermal injection to display a lower reactogenicity and a decreased capacity to release proinflammatory mediators and Th2 cytokines when compared to wild-type rBet v 1 [18]. While this approach should lead in theory to safe vaccines, there is as of today

only limited proof of efficacy obtained in small cohorts of patients for nonnative allergens [37]. As a matter of fact, IgE binding might otherwise facilitate allergen capture and presentation by professional antigen-presenting cells such as dendritic cells and activated B lymphocytes, which are known to express both high and low affinity (Fc ϵ RI and CD23) [38, 39] surface receptors for IgE, respectively. Interestingly, besides facilitating cell entry, cross-linking of Fc ϵ RI on antigen-presenting cells could lead to the downregulation of T cell responses to allergens by inducing the tryptophan degradation pathway through the overexpression of indoleamine 2,3-dioxygenase [40–42]. Also a nonmodified form of the molecule is likely to

exhibit a better stability profile when compared with any mutated or truncated form. Altogether, we conclude that wild-type rBet v 1a represents a valid candidate vaccine for the treatment of birch pollen allergy. This rBet v 1a molecule is currently evaluated in birch pollen-allergic patients in a phase I/II clinical study.

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References

- 1 Larché M: Specific immunotherapy. *Br Med Bull* 2000;56:1019–1036.
- 2 Noon L: Prophylactic inoculation against hay fever. *Lancet* 1911;ii:1572–1573.
- 3 Freeman J: Further observations on the treatment of hay fever by hypodermic inoculations of pollen vaccine. *Lancet* 1911;ii:814–817.
- 4 Bousquet J, Van Cauwenberge P, Khaltaev N, Aria Workshop Group, World Health Organization: Allergic rhinitis and its impact on asthma. *J Allergy Clin Immunol* 2001;108:S147–S334.
- 5 Wilson DR, Torres Lima M, Durham SR: Sublingual immunotherapy for allergic rhinitis (Cochrane Review); in *The Cochrane Library*, Issue 2. Oxford, Update Software, 2003.
- 6 Valenta R, Kraft D: From allergen structure to new forms of allergen-specific immunotherapy. *Curr Opin Immunol* 2002;14:718–727.
- 7 Valenta R: The future of antigen-specific immunotherapy of allergy. *Nat Rev Immunol* 2002;2:446–453.
- 8 Akkerdaas JH, van Ree R, Aalbers M, Stapel SO, Aalberse RC: Multiple cross-reactive epitopes on Bet v 1 as detected with monoclonal antibodies and human IgE. *Allergy* 1995;50:215–220.
- 9 Ferreira F, Hirtenlehner K, Jilek A, Godnik-Cvar J, Breiteneder H, Grimm R, Hoffmann-Sommergruber K, Scheiner O, Kraft D, Breitenbach M, Rheinberger HJ, Ebner C: Dissection of immunoglobulin E and T lymphocyte reactivity of isoforms of the major birch pollen allergen Bet v 1: Potential use of hypoallergenic isoforms for immunotherapy. *J Exp Med* 1996;183:599–609.
- 10 Hartl A, Kiesslich J, Weiss R, Bernhaupt A, Mostböck S, Scheiblhofer S, Flöckner H, Sippl M, Ebner C, Ferreira F, Thalhamer J: Isoforms of the major allergen of birch pollen induce different immune responses after genetic immunization. *Int Arch Allergy Immunol* 1999;120:17–29.
- 11 Ebner C, Schenk S, Najafian N, Siemann U, Steiner R, Fischer GW, Hoffmann K, Szepfalusi Z, Scheiner O, Kraft D: Nonallergic individuals recognize the same T cell epitopes of Bet v 1, the major birch pollen allergen, as atopic patients. *J Immunol* 1995;154:1932–1940.
- 12 Bohle B, Radakovics A, Jahn-Schmid B, Hoffmann-Sommergruber K, Fischer GF, Ebner C: Bet v 1, the major birch pollen allergen, initiates sensitization to Api g 1, the major allergen in celery: Evidence at the T cell level. *Eur J Immunol* 2003;33:3303–3310.
- 13 Gajhede M, Osmark P, Poulsen FM, Ipsen H, Larsen JN, van Neerven RJ, Schou C, Löwenstein H, Spangfort MD: X-ray and NMR structure of Bet v 1, the origin of birch pollen allergy. *Nat Struct Biol* 1996;3:1040–1045.
- 14 Markovic-Housley Z, Degano M, Lamba D, von Roepenack-Lahaye E, Clemens S, Susani M, Ferreira F, Scheiner O, Breiteneder H: Crystal structure of a hypoallergenic isoform of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier. *J Mol Biol* 2003;325:123–133.
- 15 Spangfort MD, Gajhede M, Osmark P, Poulsen FM, Ipsen H, Larsen JN, van Neerven RJJ, Schou C, Löwenstein H: Three-dimensional structure and epitopes of Bet v 1. *Int Arch Allergy Immunol* 1997;113:243–245.
- 16 Morgensen JE, Wimmer R, Larsen JN, Spangfort MD, Otzen DE: The major birch allergen, Bet v 1, shows affinity for a broad spectrum of physiological ligands. *J Biol Chem* 2002;277:23684–23692.
- 17 Ferreira F, Ebner C, Kramer B, Casari G, Briza P, Kungl AJ, Grimm R, Jahn-Schmid B, Breiteneder H, Kraft D, Breitenbach M, Rheinberger HJ, Scheiner O: Modulation of IgE reactivity of allergens by site-directed mutagenesis: Potential use of hypoallergenic variants for immunotherapy. *FASEB J* 1998;12:231–242.
- 18 Van Hage-Hamsten M, Kronqvist M, Zetterström O, Johansson E, Niederberger V, Vrtala S, Grönlund H, Grönneberg R, Valenta R: Skin test evaluation of genetically engineered hypoallergenic derivatives of the major birch pollen allergen, Bet v 1: Results obtained with a mix of two recombinant Bet v 1 fragments and recombinant Bet v 1 trimer in a Swedish population before the birch pollen season. *J Allergy Clin Immunol* 1999;104:969–976.
- 19 Nopp A, Halldén G, Lundahl J, Johansson E, Vrtala S, Valenta R, Grönneberg R, van Hage-Hamsten M: Comparison of inflammatory responses to genetically engineered hypoallergenic derivatives of the major birch pollen allergen Bet v 1 and to recombinant Bet v 1 wild type in skin chamber fluids collected from birch pollen-allergic patients. *J Allergy Clin Immunol* 2000;106:101–109.
- 20 Vrtala S, Hirtenlehner K, Vangelista L, Pastore A, Eichler HG, Sperr WR, Valent P, Ebner C, Kraft D, Valenta R: Conversion of the major birch pollen allergen, Bet v 1, into two nonanaphylactic T cell epitope-containing fragments: Candidates for a novel form of specific immunotherapy. *J Clin Invest* 1997;99:1673–1681.
- 21 Khinchi MS, Poulsen LK, Carat F, André C, Hansen AB, Malling H-J: Clinical efficacy of sublingual and subcutaneous birch pollen allergen-specific immunotherapy: A randomized, placebo-controlled, double-blind, double-dummy study. *Allergy* 2004;59:45–53.
- 22 Swoboda I, Jilek A, Ferreira F, Engel E, Hoffmann-Sommergruber K, Scheiner O, Kraft D, Breiteneder H, Pittenauer E, Schmid E, Vicente O, Heberle-Bors E, Ahorn H, Breitenbach M: Isoforms of Bet v 1, the major birch pollen allergen, analyzed by liquid chromatography, mass spectrometry, and cDNA cloning. *J Biol Chem* 1995;270:2607–2613.

- 23 Breiteneder H, Pettenburger K, Bito A, Valenta R, Kraft D, Rumpold H, Scheiner O, Breitenbach M: The gene coding for the major birch pollen allergen Bet v1, is highly homologous to a pea disease resistance response gene. *EMBO J* 1989;8:1935–1938.
- 24 Susani M, Jertschin P, Dolecek C, Sperr WR, Valent P, Ebner C, Kraft D, Valenta R, Scheiner O: High level expression of birch pollen profilin (Bet v2) in *Escherichia coli*: Purification and characterization of the recombinant allergen. *Biochem Biophys Res Commun* 1995; 215:250–263.
- 25 Way M, Pope B, Gooch J, Hawkins M, Weeds AG: Identification of a region in segment 1 of gelsolin critical for actin binding. *EMBO J* 1990;9:4103–4109.
- 26 McLeod M, Stein M, Beach D: The product of the mei3+ gene, expressed under control of the mating-type locus, induces meiosis and sporulation in fission yeast. *EMBO J* 1987;6:729–736.
- 27 Havlis J, Thomas H, Sebela M, Shevchenko A: Fast-response proteomics by accelerated in-gel digestion of proteins. *Anal Chem* 2003;75: 1300–1306.
- 28 Chenal A, Savarin P, Nizard P, Guillaud F, Gillet D, Forge V: Membrane protein insertion regulated by bringing electrostatic and hydrophobic interactions into play. A case study with the translocation domain of diphtheria toxin. *J Biol Chem* 2002;277:43425–43432.
- 29 Kohler G, Milstein C: Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 1975;256:495–497.
- 30 Valenta R, Sperr WR, Ferreira F, Valent P, Sillaber C, Tejkl M, Duchene M, Ebner C, Lechner K, Kraft D, et al: Induction of specific histamine release from basophils with purified natural and recombinant birch pollen allergens. *J Allergy Clin Immunol* 1993;91:88–97.
- 31 Fasman GD: Circular Dichroism and the Conformational Analysis of Biomolecules. New York, Plenum Press, 1996.
- 32 Baldwin RL: Protein folding from 1961 to 1982. *Nat Struct Biol* 1999;6:814–817.
- 33 Wright PE, Dyson HJ: Intrinsically unstructured proteins: Re-assessing the protein structure-function paradigm. *J Mol Biol* 1999;293: 321–331.
- 34 Dunker AK, Lawson JD, Brown CJ, Williams RM, Romero P, Oh JS, Oldfield CJ, Campen AM, Ratliff CM, Hipps KW, Ausio J, Nissen MS, Reeves R, Kang C, Kissinger CR, Bailey RW, Griswold MD, Chiu W, Garner EC, Obradovic Z: Intrinsically disordered protein. *J Mol Graph Model* 2001;19:26–59.
- 35 Ferreira FD, Hoffmann-Sommergruber K, Breiteneder H, Pettenburger K, Ebner C, Sommergruber W, Steiner R, Bohle B, Sperr WR, Valent P, et al: Purification and characterization of recombinant Bet v1, the major birch pollen allergen. Immunological equivalence to natural Bet v I. *J Biol Chem* 1993;268:19574–19580.
- 36 Melander W, Horvath C: Salt effects on hydrophobic interactions in precipitation and chromatography of proteins: An interpretation of the lyotropic series. *Arch Biochem Biophys* 1977;183:200–215.
- 37 Klimek L, Dormann D, Jarman ER, Cromwell O, Riechelmann H, Reske-Kunz AB: Short-term preseasonal birch pollen allergoid immunotherapy influences symptoms, specific nasal provocation and cytokine levels in nasal secretions, but not peripheral T-cell responses, in patients with allergic rhinitis. *Clin Exp Allergy* 1999;29:1326–1335.
- 38 Allam JP, Novak N, Fuchs C, Asen S, Berge S, Appel T, Geiger E, Kochan JP, Bieber T: Characterization of dendritic cells from human oral mucosa: A new Langerhans cell type with high constitutive Fc epsilon RI expression. *J Allergy Clin Immunol* 2003;112:141–148.
- 39 Kikutani H, Suemura M, Owaki H, Nakamura H, Sato R, Yamasaki K, Barsumian EL, Hardy RR, Kishimoto T: Fc epsilon receptor, a specific differentiation marker transiently expressed on mature B cells before isotype switching. *J Exp Med* 1986;164:1455–1469.
- 40 von Bubnoff D, Matz H, Frahnert C, Rao ML, Hanau D, de la Salle H, Bieber T: Fc epsilon RI induces the tryptophan degradation pathway involved in regulating T cell responses. *J Immunol* 2002;169:1810–1816.
- 41 Munn DH, Sharma MD, Lee JR, Jhaveri KG, Johnson TS, Keskin DB, Marshall B, Chandler P, Antonia SJ, Burgess R, Slingluff CL Jr, Mellor AL: Potential regulatory function of human dendritic cells expressing indoleamine 2,3-dioxygenase. *Science* 2002;297:1867–1870.
- 42 von Bubnoff D, Bezold G, Matz H, Hanau D, De La Salle H, Bieber T: Quantification of indoleamine 2,3-dioxygenase gene induction in atopic and non-atopic monocytes after ligation of the high-affinity receptor for IgE, Fc(epsilon)RI and interferon-gamma stimulation. *Clin Exp Immunol* 2003;132:247–253.