Research Article

Purification and characterization of natural Bet v 1 from birch pollen and related allergens from carrot and celery

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Birch pollen allergy is predominantly caused by the major allergen Bet v 1 and can lead to crossreactions with homologous proteins in food. Two major cross-reactive food allergens are Dau c 1 from carrot and Api g 1 from celery, which have never been purified from their natural source. Here, we describe a non-denaturing purification method for obtaining natural Bet v 1, Dau c 1 and Api g 1, comprising of ammonium sulfate precipitation, hydrophobic interaction chromatography and size exclusion chromatography. This method resulted in 98 – 99% pure isoform mixtures for each allergen. Characterization of these isoform mixtures with Q-TOF MS/MS clearly showed earlier reported isoforms of Bet v 1, Dau c 1 and Api g 1, but also new isoforms. The presence of secondary structure in the three purified allergens was demonstrated via circular dichroism and showed high similarity. The immune reactivity of the natural allergens was compared with recombinant proteins by Western blot and ELISA and showed similar reactivity.

Keywords: Api g 1 / Bet v 1 / Birch pollen / Dau c 1 / Food allergy

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1 Introduction

The major allergen from birch pollen (*Betula pendula* also *Betula verrucosa)*, Bet v 1, is an important source of airborne allergen in countries of Northern and Central Europe and Northern America during early springtime [1]. Exposure can lead to sensitization characterized by allergen-specific T_H2 cells and IgE antibodies in genetically predisposed individuals. These individuals may develop clinical symptoms of type I hypersensitivity, characterized by rapid and local inflammatory reactions in the upper (allergic rhinitis) and lower airways (allergic asthma) [2]. After birch pollen sensitization, many individuals also develop allergic responses to fresh fruits and vegetables of the *Rosaceae* and *Apiaceae* family, such as apple [3, 4], cherry [5], celery [6] and carrot [7, 8]. This type of food allergy mainly causes local reactions such as oral itching and swelling of the lips and oral mucosa, also known as oral allergy syndrome [9].

Birch pollen-related food allergies are the result of cross-reactivity of IgE antibodies to Bet v 1 homologous proteins, *e. g*. Mal d 1 from apple, Pru av 1 from cherry, Api g 1 from celery and Dau c 1 from carrot. These homologous allergens show a high similarity in primary, secondary and tertiary structure. At the amino acid level, the fruit allergens Mal d 1 and Pru av 1 show 64 – 66% sequence identity with Bet v 1, compared to 44% for the homologous vegetable allergens Api g 1 and Dau c 1 [10]. The secondary and tertiary protein structures also exhibit a high degree of similarity, as shown in X-ray crystallization and NMR studies of

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Abbreviations: CD, circular dichroism; **CE,** cell-free extract; **FF,** FastFlow; **HIC,** hydrophobic interaction chromatography; **SEC,** size exclusion chromatography; **TBS,** tris buffered saline

Bet v 1 and Pru av 1 [11 – 13]. Despite structural similarities, Bet v 1 homologues show different physico-chemical properties and immune reactivities. For example, Bet v 1l is a hypoallergenic isoform, differing in only nine amino acids from the highly allergenic Bet v 1a [11, 14 – 16], despite the high degree of homology and structural similarity between these isoforms.

Current purification methods for natural allergens include steps that may induce conformational changes caused by denaturing agents, *e.g.* acetone used for precipitation [17,] and TFA and ACN, used as components in RP-HPLC protocols [18]. Other methods such as affinity chromatography may be selective for specific epitopes and might thus lead to the loss of isoforms during purification. In addition, elution of the allergen from the affinity column with highly acidic or basic buffers could result in conformational changes [19].

Studies with purified proteins will inevitably approximate *in vivo* situations, because any purification procedure or change in the physical or biochemical environment may lead to changes in functional properties. The use of allergens, isolated from natural sources, avoids possible erroneous chain folding as may occur in heterologous expression systems. In addition, when recombinant proteins are used, patients will be exposed to only one isoform and not to a proportional mixture reflecting their natural presence in food. Avoidance of organic solvents, chaotropic agents, or extreme physico-chemical conditions during isolation from natural sources is more likely to result in purified proteins that maximally resemble their natural equivalents. The aim of this study was to develop a method that not only allows purification of Bet v 1 from pollen, but also cross-reactive allergens, *e.g.* Dau c 1 from carrot root (*Daucus carota*) and Api g 1 from celery tuber (*Apium graveolens*).

2 Materials and methods

2.1 Protein extraction

Carrots from the cultivar *Daucus carota* var. Narbonne (de Wit & Zn., Hoogkarspel, The Netherlands) and celeriac (*Apium graveolens*) purchased from a local supermarket were used for purification of Dau c 1 and Api g 1, respectively. Small pieces of tissue were frozen in liquid nitrogen and ground to a fine powder with a Waring blender. Proteins were extracted from 300 g frozen powder in 300 – 400 mL 10 mM potassium phosphate extraction buffer, pH 7.0, containing 1 mM EDTA, 0.1% w/v ascorbate, 4 mM DTT, 1 mM PMSF, 2% w/v PVP, and 10 mM diethyldithiocarbamate following a combination of the methods of Björksten *et al*. [20], Rudeschko *et al*. [21], and Yamamoto *et al*. [8]. The suspension was blended for at least 5 min, filtered over four layers of cheesecloth and centrifuged for 60 min at 16 000 × *g* at 4°C. The supernatant was used for ammonium sulfate precipitation. For extraction of Bet v 1, 5% w/v pollen from a single tree, *B. pendula* var. Youngii, was stirred overnight at 4°C in the same extraction buffer. The extract was filtered over cheesecloth and centrifuged according to the same procedure.

2.2 Ammonium sulfate precipitation

Dau c 1, Api g 1 and Bet v 1 were recovered from the supernatants by adding gradually increasing concentrations of ammonium sulfate to 50, 60, 70, 80, 90 and 100% saturation followed by stirring for 30 min at 4°C. The protein pellets were collected by centrifugation and redissolved in 10 mM potassium phosphate buffer at pH 7.0 containing 2 mM EDTA. Each fraction was analyzed with SDS-PAGE. The precipitates at 70 – 100% $(NH_4)_2SO_4$ saturation for carrot and birch and at 60 – 80% $(NH_4)_2SO_4$ saturation for celery were collected and redissolved in 10 mM potassium phosphate buffer, pH 7.0, containing 2 mM EDTA and $(NH_4)_2SO_4$ to a final concentration of 1 M for Dau c 1 and 1.5 M for Api g 1 and Bet v 1.

2.3 Hydrophobic interaction chromatography

Protein binding was screened, on an FPLC™ System (Amersham Biosciences, Uppsala, Sweden), with the HiTrap™ hydrophobic interaction chromatography (HIC) selection kit (Amersham Biosciences), containing five different prepacked 1-mL columns with three different alkyl ligands; butyl, phenyl or octyl. The phenyl ligand was coupled to Sepharose in three different columns differing in mean bead size, bead size range and ligand density. Samples were centrifuged at 30 000 × *g* at 4°C for 60 min, followed by filtering on a 0.45-µm ProFill regenerated cellulose filter (Alltech Associates, Deerfield, IL, USA), before loading onto the HIC columns. Buffer solutions of 10 mM potassium phosphate buffer, pH 7.0, containing 2 mM EDTA with and without ammonium sulfate, were degassed and filtered over an OE 66 cellulose acetate 0.2-µm membrane filter (Schleicher & Schuell, Dassel, Germany) before use. The columns were first equilibrated with high salt concentration buffers of $(NH_4)_2SO_4$, 1 M for Dau c 1 and 1.5 M for Api g 1 and Bet v 1. After loading the protein and washing the column, fractions were collected by elution with buffer without $(NH_4)_2SO_4$ at 1 mL/min.

2.4 Size exclusion chromatography

The total protein fraction from HIC was concentrated in a Microsep 10K Omega centrifugal device (Pall Life Sciences, Ann Arbor, MI, USA) to a volume of about 500 µL before SEC. The concentrated samples were loaded on a XK 16/70 column (Amersham Biosciences) packed with 120 mL Superdex® 75 prep grade (Sigma-Aldrich, St. Louis, MO, USA). Proteins were collected upon elution with 10 mM potassium phosphate buffer, pH 7.0, at

0.5 mL/min. Pooled fractions were concentrated and analyzed with SDS-PAGE, followed by quantification with the Micro BCA™ Reagent Protein Assay (Pierce, Rockford, IL, USA).

2.5 SDS-PAGE

Samples from the various purification steps were analyzed by SDS-PAGE for the presence of Bet v 1, Dau c 1 and Api g 1 by monitoring the occurrence of a band with relative masses (Mr) at 16 – 18 kDa. Proteins were separated on a 15% w/v acrylamide SDS-PAGE gel with a 5% w/v stacking gel, using the Mini-Protean II gel system (Bio-Rad Laboratories, Hercules, CA, USA). Gels were stained with CBB R250 and analyzed with Quantity One Bio-Rad scanner software. A Low Molecular Weight calibration Kit (Amersham Biosciences) marker was used to determine relative molecular masses.

2.6 IEF

Homogeneity of the isolated 16 – 18-kDa proteins of Api g 1 and Dau c 1 fractions after SEC was analyzed by IEF on the Phastsystem™ (Amersham Biosciences). Phastgel™ IEF gels (Amersham Biosciences) with a pH range of 4 – 6.5 were used and calibrated with an IEF Kit (Amersham Biosciences). The IEF gels were stained with CBB R350.

2.7 Identification of proteins using Q-TOF MS/MS

The amino acid sequences of the purified allergens from birch, carrot and celery were determined using a Q-TOF 2 mass spectrometer (Waters, Milford, MA, USA). Samples of purified Bet v 1, Dau c 1 and Api g 1, containing 30 µg of protein, were run on a 15% w/v SDS-PAGE gel and subsequently stained with CBB. The Bet v 1 sample showed two protein bands at a relative molecular mass of 16 – 18 kDa and impurities around 29 and 35 kDa. These four bands were cut out of the gel and sliced into 1-mm^3 pieces. In addition, the 16 – 18-kDa protein and minor impurities at 25 and 33 kDa for both Dau c 1 and Api g 1 were all analyzed separately.

Proteins were reduced with DTT and alkylated with iodoacetamide [22]. Gel pieces were dried under vacuum and swollen in 0.1 M $NaHCO_3$, containing 5 mM calcium chloride and sequence grade porcine trypsin (10 ng/µL, Promega, Madison, WO, USA). After overnight incubation at 37°C, peptides were extracted from the gel with 50% v/v ACN, 5% v/v formic acid and dried under vacuum. The peptides were redissolved in 0.5% v/v formic acid in 5% v/v ACN and loaded onto a C18 Atlantis column (15 cm × 75 µm id, Waters, Milford, MA, USA). Peptides were eluted by a linear gradient (30 min) from 0.5% v/v formic acid in 5% v/v ACN to 0.5% v/v formic acid in 50% v/v ACN at approximately 0.2 µL/min (resulting from a 1:20 split of 4 µL/min flow generated by the Waters CapLC pumps). The C18 column was connected to a PicoTip (New Objective, Woburn, Massachusetts), which produced an electrospray to be analyzed by a Q-TOF-2 mass spectrometer (Waters). The Q-TOF mass spectrometer was programmed to determine charge states of the eluting peptides, and to switch from the MS- to the MS/MS mode for $z \geq 2+$ at the appropriate collision energy for Argon gas-mediated CID. The resulting CID MS/MS spectra contained the sequence information for a single peptide per spectrum.

The ProteinLynx GlobalServer package V2.1 software (Waters) was used to process MS/MS data. Raw MS/MS spectra were deconvoluted to produce monoisotopic singly charged spectra with the proprietary MaxEnt3 algorithm. MS/MS spectra containing good quality CID products were automatically searched for sequence matches using the NCBI non-redundant protein database. Unassigned MS/MS spectra were automatically processed using the AutoMod algorithm, developed to identify amino acid substitutions, post-translational modifications and partial or nonspecific cleavages. *De novo* sequences were generated with the MassSeq tool to search for the most likely protein homologues in the database.

2.8 Circular dichroism spectroscopy

CD spectra of Bet v 1, Dau c 1 and Api g 1 were recorded at 20°C on a Jasco J-715 spectropolarimeter (Jasco, Tokyo, Japan) equipped with a quartz cuvette of 1-mm path length. Far-UV spectra were recorded from 185 – 260 nm in 10 mM potassium phosphate buffer pH 7.0 filtered through a 0.2-µm syringe filter (Schleicher & Schuell) at a protein concentration of 2.9 µM Bet v 1, 5,8 µM Dau c 1 and 6.3 µM Api g 1. To increase S/N values, 20 scans were accumulated at a scanning speed of 50 nm/min, a 0.2-nm step width and 2-nm band width. The buffer spectra were subtracted from the protein spectra and the mean residue weight ellipticity $[\theta]_{MRW}$ was calculated from the following equation:

$$[\theta]_{MRW} = \frac{100 \times \theta_{obs}}{C \times l \times n} \quad (1)$$

Here, θ_{obs} is the observed signal in degrees, C is the concentration in mol/L, l is the path length of the cuvette in cm and n is the number of amino acids. The program CDNN was used to deconvolute the secondary structure [23].

2.9 ELISA

Immunodetection was performed by means of indirect ELISA. The 96-well microplates (Greiner Bioone, Frickenhausen, Germany) were coated with 2 µg/mL of natural Dau c 1, Api g 1 and Bet v 1 and the recombinant allergens rDau c 1.2, rApi g 1 and rBet v 1a (Biomay, Vienna, Austria) in coating buffer (40 mM $NaHCO_3$, 9 mM Na_2CO_3, pH 9.6, 100 µL/well), by incubation for 1 h at 37°C. All subse-

quent incubations were performed at room temperature on a microplate shaker. Coating solution was removed and 200 µL/well of blocking buffer (2% w/v BSA in PBS: 1.4 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 mM KCl , 130 mM NaCl, pH 7.4) was added and incubated for 1 h. Microplates were washed after each incubation step in a microplate washer (Anthos Fluido, Anthos Labtec Instruments, Austria) with 4×400 µL/well of washing buffer (0.05% v/v Tween 20, 0.05% w/v BSA in PBS). During incubation, microplates were sealed. Serum in PBS (1:5, 100 µL/well) was added as primary antibody and incubated for 3 h. Human sera of six birch pollen-allergic patients, which were previously shown to cross-react with the carrot allergen Dau c 1 and the celery allergen Api g 1, were obtained from the Laboratory for Primary Health Care (SHO, Velp, The Netherlands). Specific IgE was determined for each serum by the ImmunoCAP method (Pharmacia, Uppsala, Sweden). Sera with birch pollen-specific IgE of > 100 kU/L were used. Wells were subsequently incubated for 1 h with 100 µL of 1:1000 monoclonal mouse anti-human IgE (DakoCytomation, Denmark or Sigma-Aldrich) in dilution buffer (0.1% w/v BSA in PBS pH 7.4) and 100 µL of 1:1000 goat anti-mouse IgG antibodies conjugated with alkaline phosphatase (Sigma-Aldrich) in dilution buffer, both during 1 h. Freshly prepared substrate solution, containing 1 mg/mL 4-nitrophenylphosphate disodium salt in carbonate buffer (35 mM $NaHCO_3$, 15 mM Na_2CO_3, 1 mM $MgCl_2$, pH 9.6), by adding 200 µL/well. Color development was measured at λ = 405 nm using a microplate reader (ThermoLab Systems, Franklin, MA, USA). Sera from non-birch pollen-allergic individuals with birch pollen-specific IgE of < 0.35 kU/L, were included as a negative control. Measurements were performed in triplicate.

2.10 Western blot

After electrophoretic separation of protein sample containing 2 – 3 µg of natural or recombinant protein by 15% w/v SDS-PAGE, proteins were either stained with CBB or transferred to a PVDF Immobilon™–PSQ membrane (Millipore). Semi-dry blotting was performed with a Semi-phor (Hoeffer Scientific Instruments, San Francisco, CA, USA) using a constant current of 100 mA for 75 min. Wet blotting was performed with a Mini Trans-Blot® (Bio-Rad) using a constant voltage of 100 V for 60 min. Membranes were blocked with blocking buffer, containing 2% w/v milk powder in PBS (1.4 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 mM KCl , 130 mM NaCl, pH 7.4) and incubated for 1 h at room temperature. Serum (1:10) in 0.1% w/v milk powder in PBS 7.4 was added as primary antibody and incubated at 4 – 5°C overnight. Membranes were sequentially incubated for 1 h with mouse anti-human IgE (1:1000, DakoCytomation or Sigma-Aldrich) in dilution buffer: 0.1% w/v milk powder in TBS: 20 mM trizma-base, 150 mM NaCl pH 8.2, followed by incubation for 1 h with goat anti-mouse IgG antibodies (1:1000) conjugated with alkaline phosphatase (Sigma-Aldrich) in dilution buffer. After each incubation step, membranes were washed for 2×5 min with washing buffer 1 (0.1% v/v Tween 20, 0.1% w/v milk powder in PBS pH 7.4) and for 3×5 min with washing buffer 2 (0.1% w/v milk powder in TBS pH 8.3). Freshly prepared substrate solution was added, consisting of 5% w/v 5-bromo-4-chloro-3-indolylphosphate and 5% w/v nitroblue tetrazolium in DMF added to 100 mM trizma-HCl, 5 mM $MgCl_2$, 100 mM NaCl, pH 9.6.

3 Results and discussion

3.1 Purification of the natural allergens Dau c 1, Api g 1 and Bet v 1

Birch, carrot and celery extracts were monitored for the presence of proteins, with relative molecular masses at 16 – 18 kDa, by SDS-PAGE during purification. Clear protein bands were visible at 16 – 18 kDa in the cell-free extracts of carrot, celery (Figs. 1A and B) and birch (not shown). The majority of the 16 – 18 kDa protein from carrot was detected in the fractions collected at 80, 90, and 100% ammonium sulfate saturation (Fig. 1A). Similar results were obtained for Bet v 1. The majority of the 16 – 18 kDa Api g 1 protein precipitated at 70 and 80% ammonium sulfate saturation (Fig. 1B).

The precipitated ammonium sulfate saturated fractions from 70 – 100% of Bet v 1 and Dau c 1 and from 60 – 80% of Api g 1 were used for concentration with HIC and further purification using SEC. From five different types of Sepharose HIC columns tested, the best results were obtained with a HiTrap octyl FastFlow column, with purity as selection criterion. The main aim of the HIC step was to concentrate the protein sample and to remove most of the ammonium sulfate. The unbound HIC fractions contained no protein bands at 16 – 18 kDa when analyzed with SDS-PAGE (not shown). This resulted in enrichment by a factor 2 of the three allergenic proteins after elution from the HIC column with low salt. Proteins were not eluted with a gradient, because the protein gradually released the column, resulting in a dilute fraction.

In HIC, proteins bind in their native state to the alkyl ligands of the column by hydrophobic interactions. This type of binding is not likely to be selective for certain isoforms, which can subsequently be recovered by non-denaturing agents. Hydrophobic characteristics of Bet v 1, Dau c 1 and Api g 1 are similar according to the ProtScale tool [24]. Therefore, we conclude that non-selective HIC is a better method to preserve the native state of natural proteins compared to purification methods as Reversed Phase HPLC, in which highly denaturing agents are used.

SEC of HIC-concentrated Bet v 1, Dau c 1 and Api g 1 fractions showed similar results. Two peaks were clearly visible in the elution patterns as shown for the HIC purified

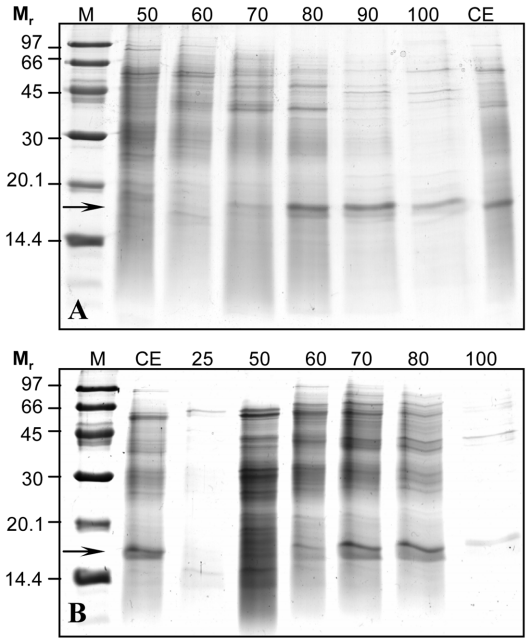

Figure 1. Ammonium sulfate precipitation of carrot and celery proteins. (A) Precipitated protein in each carrot fraction; (B) precipitated protein in each celery fraction. The percentage of ammonium sulfate saturation is displayed on top of the lanes. CE: cell-free extract before ammonium sulfate precipitation. M: molecular marker. The arrow indicates the position of Dau c 1 and Api g 1.

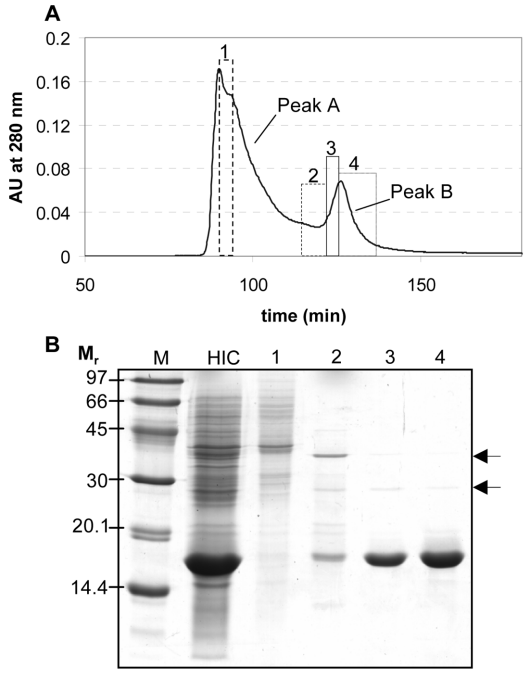

Figure 2. Size exclusion chromatography of carrot proteins, concentrated by HIC. (A) Two major peaks were visible after SEC, peak A and peak B. Boxes 1 through 4 correspond with the numbers of the SDS-PAGE gel of Fig. 2B; (B) SDS-PAGE of the purified Dau c 1 allergen after SEC. M: molecular marker. HIC: sample after HIC. Arrows indicate minor impurities at 25 and 33 kDa.

carrot extract (Fig. 2A). Peak A contained high-molecular mass proteins not related to Dau c 1 (Fig. 2B, lane 1). Peak B contained the 16 – 18 kDa Dau c 1 protein with minor impurities (Fig. 2B lane 3 and 4) of approximately 25 and 35 kDa, which are clearly visible in lane 2 of Fig. 2B. Fraction 4 was used for further experiments and fraction 3 was saved for a second SEC run. Analysis by SDS-PAGE indicated 98 – 99% purity for all purified allergens. Yields for Bet v 1, Dau c 1 and Api g 1 were estimated at 250 mg/kg birch pollen, 3 mg/kg carrot and 10 mg/kg celery, respectively.

An alternative for protein purification of natural Dau c 1 and Api g 1 might be affinity chromatography, but the available mAb used as stationary phases for the purification of Bet v 1 or Mal d 1, do not recognize an epitope of Dau c 1 and Api g 1 (personal communication R. van Ree, AMC, Amsterdam, The Netherlands). Affinity chromatography also results in a mixture of different isoforms, as was reported for Mal d 1 from apple [25]. Another purification method for the natural Mal d 1 allergen is described by Fahlbusch *et al*. [18], who used RP-HPLC and anion exchange chromatography. These methods do not exclude that isoforms are lost by selective extraction, which may lead to an altered immune reactivity as compared to the isoform mixture in the source tissue. As RP-HPLC uses highly denaturing components, *e. g*. TFA and ACN, this may affect protein conformation and therefore immune reactivity.

3.2 Characterization of purified Bet v 1, Api g 1 and Dau c 1 by Q-TOF MS/MS

After tryptic digestion of the 16 – 18 kDa protein bands of purified Bet v 1, Api g 1 and Dau c 1 the resulting peptides were separated by nanoflow RP-LC and on-line sequenced with the Q-TOF operating in MS/MS mode. In Fig. 3, tryptic peptides, from which complete amino acid sequences were obtained in the MS/MS mode, are emboxed. Peptides, only characterized in the MS-mode by their molecular mass, are underlined. Thirteen different peptide sequences were identified in the MS/MS spectra generated for Bet v 1 (Fig. 3A), representing at least three isoforms of Bet v 1 in the purified sample. These isoforms are encoded by different Bet v 1-type genes [26], namely Bet v 1.01A coding for Bet v 1a (Accession no. P15494), Bet v 1.01B coding for Bet v 1d (Accession no. P43177) and Bet v 1.02B coding for Bet v 1m (Accession no. P43186). Six of these peptides could be assigned to tryptic peptides predicted for Bet v 1.01A. Three sequences were specific for the tryptic peptides III, V and X of Bet v 1.01B, while four peptides were specific for the tryptic fragments I, III, V and VII of

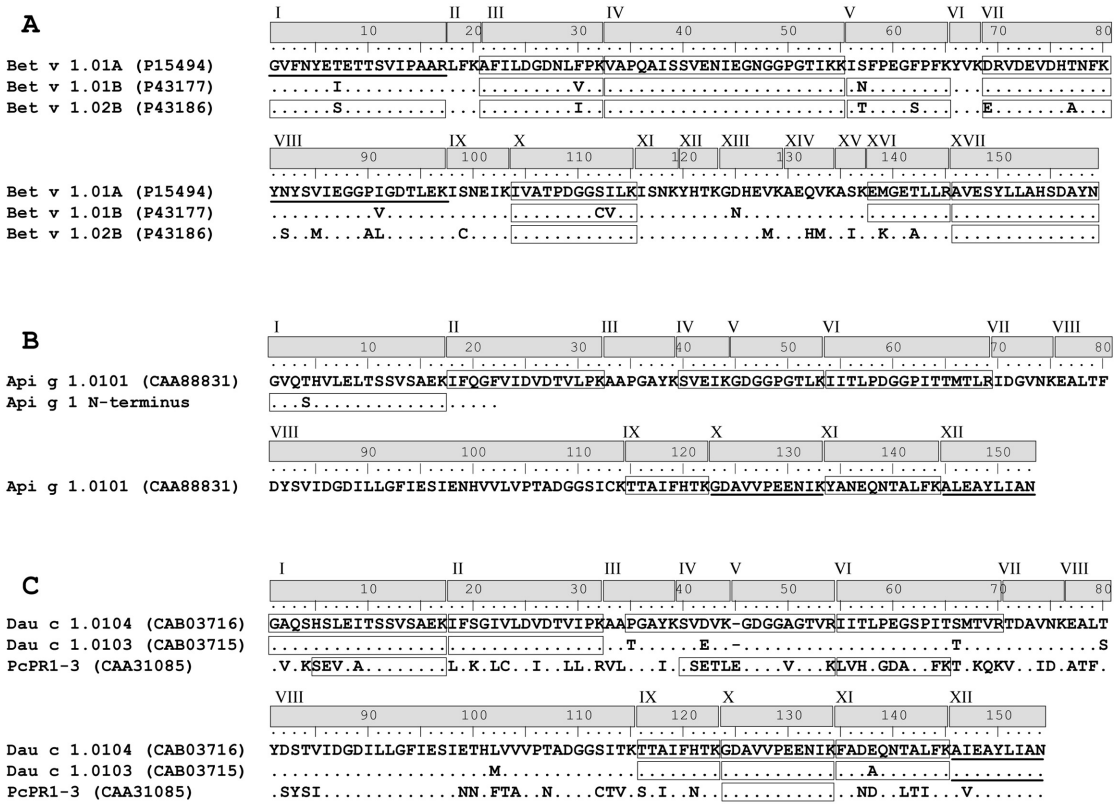

Figure 3. Alignment of Bet v 1, (A), Api g 1 (B) and Dau c 1 (C) with the identified peptides, as revealed by Q-TOF MS/MS analyses. The accession numbers of the different isoforms are given between brackets in the alignments. Theoretical tryptic peptides are displayed with grey-shaded boxes and Roman numbers at the amino acid positions. Peptides identified in the MS-mode and MS/MS-mode are respectively underlined and displayed in a box. Dots indicate identical amino acids with the aligned allergen and dashes indicate gaps.

Bet v 1.02B. From these isoforms, the following proportions of the complete protein were fully sequenced in the MS/MS mode: Bet v 1.01A 50.9%, Bet v 1.01B 57.2% and Bet v 1.02B 62.9%. In total, 13 Bet v 1-type genes are known, but only a subset is predicted to be expressed in pollen based on the detection of mRNA in pollen tissue [16, 26].

In addition to the MS/MS mode, which provides amino acid sequence information, the sample was run in the continuous MS mode (Fig. 4A). The combined MS spectrum reveals the molecular masses after deconvolution from m/z-ratios, where m= molecular peptide mass and z = peptide charge, of all peptides present in the digest. All major peaks could be assigned to tryptic peptides originating from either Bet v 1 a, d or m, using the sequence information as obtained from the MS/MS run. An additional number of peptide masses in the MS spectrum of Fig. 4A could be assigned to tryptic peptides originating from one or more of the three Bet v 1 isoforms based on their exact mass (Fig. 3A, underlined peptides).

The protein band at an estimated relative molecular mass of 35 kDa in the SDS-PAGE gel was identified as dimeric Bet v 1 since it had similar MS and MS/MS spectra of tryptic peptides as the monomeric Bet v 1. A 29 kDa protein in the Bet v 1 sample was also sequenced from the gel, but showed no homology to Bet v 1 and could not be identified on the basis of a search in the NCBI non-redundant protein database.

Breiteneder *et al*. [6] first described the purification and molecular characterization of a major allergen of celery, classified as Api g 1.0101. In our sample, six of the predicted tryptic peptide sequences of Api g 1.0101 were confirmed by MS/MS analysis (Fig. 3B), resulting in a total protein coverage of 41.2%. The predicted peptides IV+V were sequenced as a partial digest (no tryptic cleavage after lysine at position 44). In addition, the homologous N-terminal part of Api g 1 was sequenced. However, we observed an amino acid substitution at position 4 (Ser^4 for Thr^4), which was also found by N-terminal sequencing of the Api g 1 allergen by Schöning *et al*. [27]. This indicates the occurrence of an Api g 1 isoform in our sample, which deviates at the N-terminus from the one present in existing sequence databases.

The sequences obtained after MS/MS analysis of the tryptic peptides present in the digested band of purified Dau c 1 are depicted in Fig. 3C. Sequences of nine tryptic

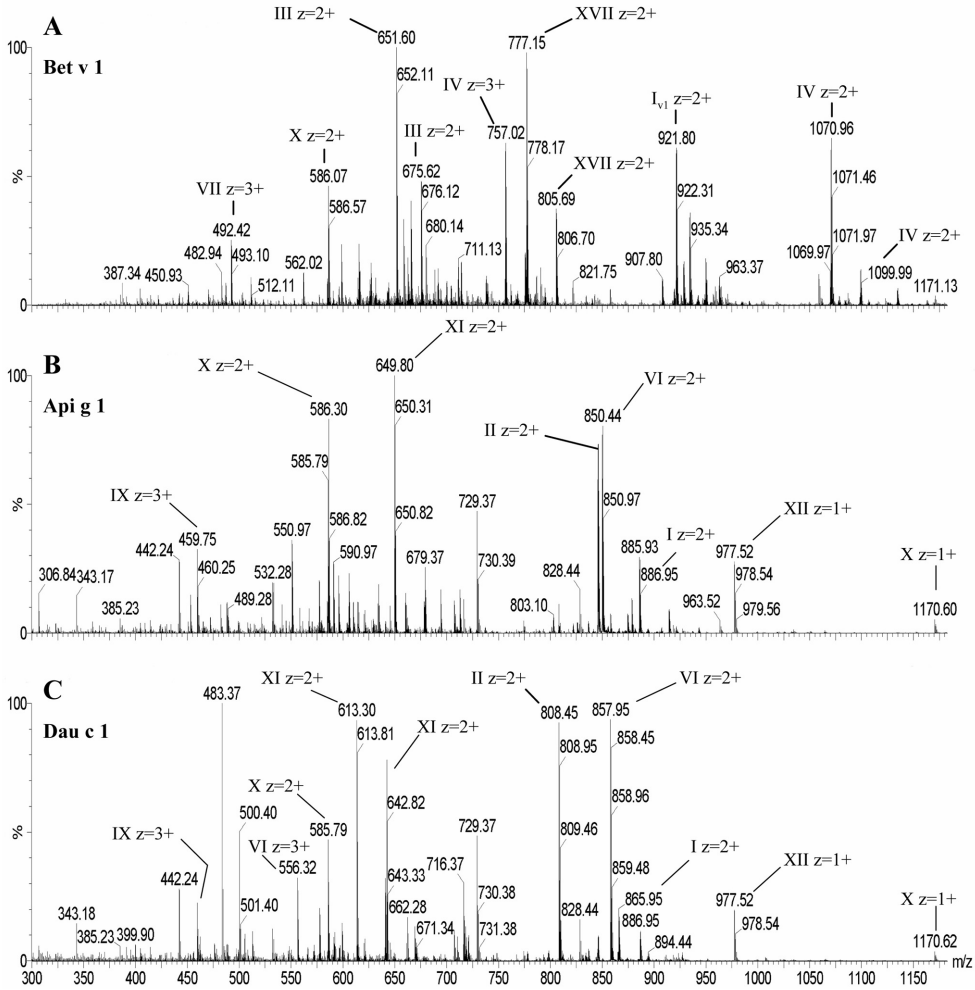

Figure 4. Q-TOF MS spectra of tryptic peptides of Bet v 1, Api g 1 and Dau c 1. The x-axis represents the *m/z*-values of the peptides. Identified peptides are annotated with the tryptic peptide numbers, their charge state (z) and the corresponding *m/z*-values. Peptide numbers refer to Fig. 3.

peptides coincided with the amino acid sequences of the predicted peptides I-VI and IX-XI of Dau c 1.0104 (Accession no. CAB03716) with a protein coverage of 62.7%. The predicted peptides III-V were found as a partial digest, in which trypsin was unable to cleave after lysine residues at positions 39 and 44. In addition, an amino acid substitution was observed in peptide XI (Ala^{137} for Glu^{137}), indicating the presence of one of the isoforms Dau c 1.0101, 1.0102, 1.0103 (Fig. 3C, Accession no. CAB03715), or 1.0105. Four other peptides had sequences identical to the parsley PcPR1-3 Bet v 1 homologue (Accession no. CAA31085) [28]. IEF showed two major bands for Dau c 1 (not shown). This supported the findings with Q-TOF MS/MS for at least two isoforms for carrot, although we were able to identify a third one.

Two additional protein bands in the Dau c 1 sample were visible at 25 and 35 kDa, respectively (Fig. 2B). These minor impurities were identified with Q-TOF MS/MS as actin and isoflavone reductase, respectively. The latter belongs to the group of the Bet v 6 homologous allergens [29]. Therefore, the 35-kDa protein band is not a dimeric form of Dau c 1.

Figures 4B and C show the combined MS spectra of the tryptic peptides present in the purified Api g 1 and Dau c 1 samples. All major peaks could be assigned to sequences as determined by MS/MS analysis of the corresponding tryptic digests of Api g 1 and Dau c 1. The two MS spectra contain several peaks with identical mass peaks. This is in agreement with the observation of identical sequences for these peptides of Api g 1 and Dau c 1 in MS/MS (see also Figs. 3B and C).

3.3 Circular dichroism

The CD spectra of Bet v 1, Dau c 1 and Api g 1 (Fig. 5) showed that the isolated proteins were similarly folded. A broad minimum around 218 nm was observed for these allergens. Similarly, maximum ellipticity was observed at

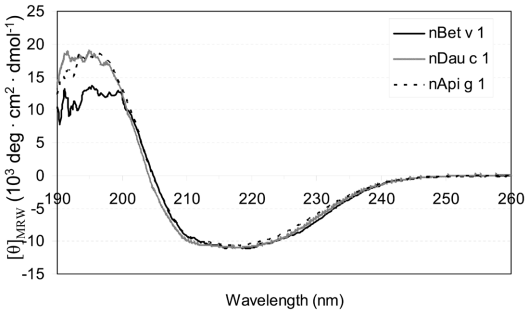

Figure 5. Circular dichroism spectra of natural Bet v 1, Dau c 1 and Api g 1.

Table 1. Secondary structure prediction of Bet v 1, Dau c 1 and Api g 1 as derived from circular dichroism analyses. CDNN was used for secondary structure prediction.

	Protein		
	Bet v 1	Dau c 1	Api g 1
α-helix	29.0%	31.2%	30.3%
β-sheet	19.7%	18.9%	20.5%
β-turn	15.8%	16.3%	16.6%
Random coil	39.2%	33.6%	33.6%

about 196 nm where the peak height for Bet v 1 was lower as compared to that for Dau c 1 and Api g 1. Other small differences were observed at the x-axis intercepts, 205 nm for spectra of Bet v 1 and Api g 1 and 204 nm for Dau c 1. The CD spectra were deconvoluted using the wavelength range from 190–260 nm with the CDNN program of Böhm *et al*. [23]. The distribution of the estimated secondary structure is shown in Table 1. Between the three allergens no significant differences were observed. These CD spectra were also very similar to those of natural and recombinant Bet v 1 [30] and recombinant Pru av 1 [31] reported earlier.

3.4 Immunoblotting

Natural Bet v 1 (Fig. 6A), Dau c 1 and Api g 1 (Fig. 6B) showed immune reactivity in Western blot experiments

Table 2. Results of ELISA with purified natural and recombinant Bet v 1 using several human patient sera A–F of Bet v 1 allergic patients. IgE-binding capacities with SD are given as relative values and compared to the highest score, set to 100%, indicated in bold.

	Bet v 1			
	Nat		Rec	
Serum	%	SD	%	SD
A	44.3	7.24	74.2	8.97
B	59.3	3.54	93.0	15.3
C	84.1	5.00	85.2	10.4
D	81.7	8.36	**100**	6.76
E	20.8	5.88	23.8	4.20
F	33.7	9.99	17.7	5.89

with Bet v 1-specific IgE patient sera cross-reacting with the corresponding recombinant birch, carrot and celery allergens. The IgE-binding capacities of natural and recombinant allergens were similar for carrot and celery. A lower IgE-binding capacity was observed for natural Bet v 1 compared to recombinant Bet v 1. The isoform that is used to produce the recombinant protein is Bet v 1a, which is known to be the most allergenic isoform. Natural Bet v 1 is a mixture of at least three isoforms, including the hypoallergenic isoform Bet v 1d [14]. This mixture is thus likely to have a reduced IgE reactivity compared to the recombinant protein for the majority of patients. Both dimers from natural and recombinant Bet v 1, though not visible in SDS-PAGE, showed clear immune reactivity. Recombinant Dau c 1 showed two antibody-binding proteins, while only one band was visible in SDS-PAGE. The higher molecular mass of recombinant Dau c 1, compared to natural Dau c 1, as visible in SDS-PAGE and Western blot, resulted from a His-tag, fused with the recombinant protein. The second immune reactive protein band in the Western blot of the recombinant Dau c 1 sample, at lower molecular mass, could best be explained as a result of protein degradation, revealing an important epitope for Dau c 1 with higher affinity for IgE (personal communica-

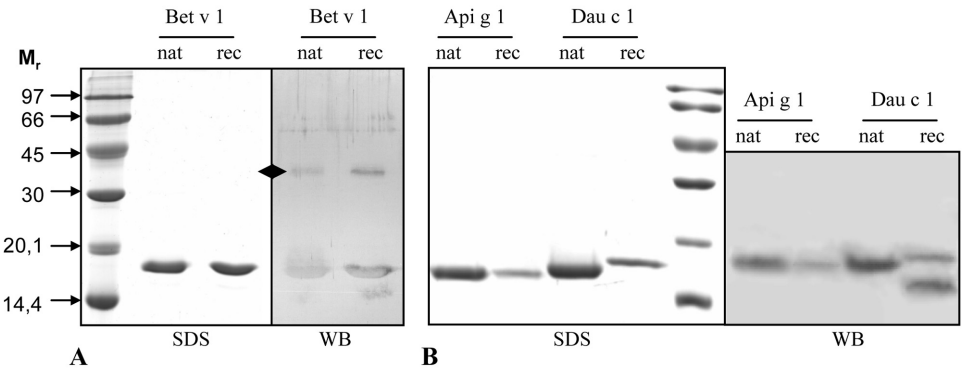

Figure 6. SD-SPAGE and Western blot with natural and recombinant Bet v 1 (A) and Api g 1 and Dau c 1 (B) with human sera from birch pollen-allergic patients. The diamond–shaped marker indicates the location of the Bet v 1 dimer. WB: Western blot, M: molecular marker.

Table 3. Results of ELISA with purified natural and recombinant Dau c 1 and Api g compared to recombinant Bet v 1. Six different human sera were used of Bet v 1 allergic patients cross-reactive to Dau c 1 and Api g 1 (G – L). IgE-binding capacities with SD are given as relative values and compared to the highest score, set to 100%, indicated in bold.

	Bet v 1		Dau c 1				Api g 1			
	Rec		Nat		Rec		Nat		Rec	
Serum	%	SD	%	SD	%	SD	%	SD	%	SD
G	68.5	2.00	29.5	3.68	26.7	0.464	27.4	2.56	25.2	5.66
H	49.7	3.04	25.1	4.25	14.6	0.317	18.5	0.628	15.7	2.14
I	92.2	1.76	21.9	0.912	15.0	0.293	16.5	2.60	23.9	1.07
J	43.4	2.67	21.3	3.03	14.0	1.34	14.2	4.98	11.0	0.518
K	46.6	2.11	17.3	5.58	20.1	0.488	16.7	3.09	23.7	2.62
L	**100**	4.32	18.5	3.69	26.7	1.61	20.6	1.26	28.8	3.25

tion, M. Susani, Biomay, Austria). Possible contaminants in the allergen samples did not exhibit immune reactivity in Western blot experiments with IgE from Bet v 1 allergic patients, demonstrating that immune reactivity in the allergen isolates was caused by Bet v 1, Dau c 1 and Api g 1 only.

The immune reactivity of natural Bet v 1 (Table 2), Dau c 1 and Api g 1 (Table 3) was also confirmed in two different ELISA, where they reacted with six different birch-allergic patient sera. OD-values with SD expressed as relative values with the highest OD-value set to 100, showed that reactivity was not exactly identical for each serum and allergen. However, the natural allergens bind to Bet v 1-recognizing IgE antibodies. Differences in ELISA reactivity between the natural and recombinant proteins could be explained by the presence of isoform mixtures of the natural proteins and possibly by serum specificity. A lower immune reactivity for the natural Bet v 1 allergen was expected due to the presence of a hypoallergenic isoform, as shown by Q-TOF MS/MS. The natural Dau c 1 mixture was shown to be primarily composed of the Dau c 1.0104 isoform, while the recombinant protein consisted of isoform Dau c 1.0103, which may explain the difference in immune reactivity of these two samples.

4 Concluding remarks

So far, no purification method has been described for Dau c 1 and Api g 1 from their natural source. Purification of these allergens was hitherto performed on proteins expressed in recombinant systems. Advantages of recombinant proteins are the high degree of homogeneity, the high yield of protein and a similar reactivity in IgE-binding studies. However, recombinant proteins will not be composed of isoform mixtures and they will not include ligands [12, 15, 32], as present in the natural matrix. Moreover, it is not known whether they are folded into the correct native state [33, 34].

In conclusion, natural Bet v 1, Dau c 1 and Api g 1 allergens can be obtained at 98 – 99% purity using a three-step-method, using ammonium sulfate precipitation, HIC and SEC, which are non-denaturing methods. The Bet v 1 and Dau c 1 samples, contained multiple isoforms as was shown by Q-TOF MS/MS. The characterization of these proteins, based on CD spectra and immune reactivity, indicated a folded state of the protein comparable to that of recombinant proteins. The advantage of these allergen samples from natural sources is that they show the immune reactivity of naturally occurring isoform mixtures.

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