Research Article

Production and characterization of an allergen panel for component-resolved diagnosis of celery allergy

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In celery a relevant food allergen source, three allergens have been identified so far: Api g 1 and Api g 4, and one glycosylated protein, Api g 5. For component-resolved food allergy diagnosis high amounts of well-defined allergens are needed. Depending on the individual celery allergen, protocols for heterologous production and purification from natural source, respectively, were established to obtain homogenous protein batches. Afterwards the purified recombinant allergens, Api g 1, Api g 4 and natural Api g 5 were characterized regarding their structural integrity and immunological activity. Therefore, several methods were applied. Proteins were identified by partial N-terminal sequencing, protein mass was verified by MS and sequence integrity by MALDI-TOF and N-terminal sequencing after tryptic digestion. Presence of isoforms in natural allergen preparations was identified by 2-DE. Secondary and tertiary structures were evaluated by circular dichroism (CD) spectroscopy and NMR analysis. Finally, IgE binding capacity was verified using selected sera from celery allergic patients in IgE immunoblots and IgE ELISA. These well-defined celery allergens will be used to prove the concept of component-resolved diagnosis and will contribute to improve food allergy diagnosis in the future.

Keywords: Allergen / Api g 1 / Api g 4 / Celery / Food allergy

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1 Introduction

Celery represents the most important plant food allergen source among the adult Central European population [1–3]. Sensitization to celery is frequently associated with birch and mugwort pollinosis thus the term ‚birch–mugwort–celery syndrome' has been established [4, 5]. These crossreactivities can be attributed to the three classes of allergenic proteins: Api g 1, in birch-celery allergic patients [6], Api g 4 [7] and crossreactive carbohydrate determinants (CCDs) (also called high molecular weight (HMW) allergens in celery) in both birch-celery and mugwort-celery allergic patients [4, 5, 8].

Api g 1, the major allergen in celery, is a Bet v 1 homologue and belongs to the family of pathogenesis-related plant proteins PR-10 [6, 9, 10]. Api g 1 is recognized by 59% of the celery allergic patients [10]. Other studies found a prevalence of IgE binding to Api g 1 in the range of 74% [4] to 80%, respectively [11]. So far, two different isoforms of the major celery allergen have been cloned, Api g 1.0101

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Abbreviations: CCD, crossreactive carbohydrate determinant; **CD,** circular dichroism; **CRD,** component-resolved diagnosis; **HMW,** high molecular weight; **IMAC,** immobilized metal affinity chromatography; **SEC,** size exclusion chromatography

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[6] and Api g 1.0201 [12]. While Api g 1.0101 was recognized by IgE of 74%, Api g 1.0201 reacted only with 48% of sera from celery allergic patients [13]. These two proteins share approximately 40% sequence identity with Bet v 1. Testing of Api g 1.0101 in skin prick tests revealed that Api g 1.0101 represents a valuable tool for the diagnosis of birch–celery allergy in the group of patients from Central Europe [9]. Recently, the structure of Api g 1.0101 has been resolved and comparison with Bet v 1 structure showed that Api g 1 shares some, but not all epitopes with Bet v 1 [14]. The IgE binding to Api g 1 was completely destroyed after heating of Api g 1 to 100°C for 30 min, showing the heat lability of Api g 1 [15].

Api g 4, the profilin from celery, homologous to the birch pollen allergen Bet v 2, has been cloned and described as a minor food allergen among celery allergic patients (41%, 7/17) from Central Europe with birch and weed pollen allergy [7]. The large extent of crossreactivity among plant profilins is due to their high amino acid sequence identity (71–82%) [7] and similar tertiary structures [16]. Api g 4 was identified as a food allergen in 23% (5/22) of patients whose allergy to celery tuber was confirmed by a positive DBPCFC (double-blind placebo-controlled food challenge) [10, 17] and in 50% (3/6) of patients with a positive DBPCFC to cooked celery [15]. The celery profilin showed a higher heat resistance than the major allergen Api g 1, but still lower compared to CCDs.

These crossreactive HMW glycoproteins from celery have been identified as a major IgE binding component recognized by 55% of sera from DBPCFC patients [10]. The allergenic relevance of crossreactive carbohydrates could be demonstrated by Api g 5, a reference glycoprotein from celery [18]. The biochemical characterization of Api g 5 revealed homology to a family of FAD-containing enzymes which are involved in alkaloid biosynthesis and have been described as allergens so far in Bermuda grass, BG60 [19], Timothy grass, Phl p 4 [20, 21] and in oilseed rape pollen [22]. The allergenic activity of Api g 5 was shown to depend on its carbohydrate moieties. Deglycosylation of the protein completely abolished its capacity to bind IgE from celery and pollen allergic patients and to trigger basophil histamine release [18].

When eaten raw or cooked, celery can induce various IgE-mediated reactions ranging from local symptoms in the oral mucosa, respiratory disorders and gastrointestinal symptoms to life-threatening anaphylactic shock [3, 23]. Therefore, there is a rising need for component-resolved diagnosis (CRD) which facilitates the prediction of symptom severity, cross-sensitization and contributes to improve the quality of life of food allergic patients, especially, avoiding unnecessary exclusion diets. In this study, we present a detailed biochemical and *in vitro* immunological characterization of recombinant rApi g 1, and rApi g 4, and natural Api g 5 celery allergens and discuss their benefits for improved diagnosis of food allergy.

2 Materials and methods

2.1 Expression and purification of recombinant Api g 1.0101

Api g 1 cDNA was subcloned in pMW175, expressed as nonfusion protein and purified as described previously [6]. Protein expression was induced by the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubation was continued for 5 h at 37°C. Cells were harvested and disrupted as described previously [24]. The supernatant, containing rApi g 1, was enriched by ammonium sulphate precipitation to a final concentration of 60%. The precipitate was dissolved in 25 mM imidazole pH 7.4, 0.5 mM β-mercaptoethanol and desalted by dialysis against this buffer. Recombinant Api g 1 solution was applied onto an anion exchange column (DEAE-Sepharose, Amersham Bioscience, Little Chalfont, UK), equilibrated with 25 mM imidazole pH 7.4, 0.5 M β-mercaptoethanol (buffer A). Bound protein was eluted by a linear gradient by increasing buffer B (buffer A, 300 mM NaCl). Fractions containing rApi g 1 were pooled, dialysed against 40 mM Na-phosphate buffer, pH 5.2, containing 1 M ammonium sulphate, 0.5 β-mercaptoethanol (buffer A) and subjected to a Phenyl Sepharose column (hydrophobic interaction chromatography), equilibrated with buffer A. A linear gradient of increasing buffer B (20 mM Tris-HCl, pH 8.0, 7.5% 2-propanol, 0.5 mM β-mercaptoethanol was established to elute bound rApi g 1. Fractions containing rApi g 1 were pooled, dialysed against 5 mM Na-phosphate buffer, pH 7.4, 0.5 mM β-mercaptoethanol. Protein concentration was determined by BCA test (Pierce, Cheshire, UK). Purified rApi g 1 was lyophilized and stored at –20°C.

2.2 Purification of natural Api g 1

Celery tuber protein extract was prepared as previously described [18]. The first purification step was an anion exchange chromatography using 20 mM Tris-HCl, pH 8.0. Bound Api g 1 was eluted by a linear gradient from 0 to 100% using 20 mM Tris-HCl, pH 8.0, 1 M NaCl. Fractions containing natural Api g 1 were pooled and supplied to centricon MWCO 50 kDa (Millipore, Bedford, USA). Purified natural Api g 1 was obtained after centrifugation at 4000 × *g* for 20 min. Protein concentration was determined by BCA test (Pierce). Purified protein was stored at –20°C till use.

2.3 Expression and purification of recombinant Api g 4

The plasmid construct of pET30a-Api g 4 [7] encoding for a fusion protein containing a C-terminal hexahistidine-tag was used for expression and transformation of *Escherichia coli* BL21(DE3)-RIL cells (Stratagene, La Jolla, CA, USA). The expression was performed in LB-medium containing

30 mg/L kanamycin at 37°C using two 2 L bioreactors (MoBiTec, Göttingen, Germany). Protein synthesis was induced with 1 mM IPTG for 5 h at 37°C. Bacteria were harvested by centrifugation (3000 × *g*, 20 min, 4°C) and stored at −80°C. The pellet from a 4 L bacterial culture was resuspended in 100 mL lysis buffer (50 mM Na-phosphate, pH 8.0, 500 mM NaCl and 2 mM imidazole, 6 M urea) and subjected to three freeze–thaw cycles using liquid nitrogen (frozen three times in liquid nitrogen). After centrifugation hexahistidine-tagged rApi g 4 was purified using IMAC (immobilized metal affinity chromatography) and subsequent SEC (size exclusion chromatography). After subjection to IMAC, using Ni^{2+}-charged chelating agarose (Qiagen, Hilden, Germany), the urea concentration was stepwise decreased on the column and the target protein was eluted with increasing imidazole concentrations. Api g 4 containing fractions were screened by Coomassie stained SDS-PAGE under reducing and nonreducing conditions, pooled and concentrated using ultrafiltration units (Vivaspins 15r, 5000 MWCO, Sartorius, Göttingen, Germany). The second purification step was performed on a Superdex 75 prep grade 26/60 column in 20 mM MOPS, pH 7.4, 500 mM NaCl. In a separate run, ovalbumin (43 kDa) and chymotrypsinogen A (25 kDa) were used as molecular weight standards. The purity of final preparation was analysed by SDS-PAGE under reducing conditions, concentrated and stored at −20°C. The protein content was determined using the BCA assay (Pierce).

2.4 Purification of Api g 5 from celery protein extract

Api g 5 was purified as described previously [18]. Briefly, proteins were extracted in 10 mM potassium phosphate buffer, pH 7.0 containing 2% w/v suspended solid polyvinylpolypyrrolidone, by shaking overnight at 4°C. The extract was cleared by centrifugation (40 000 × *g*, 60 min, 4°C) and filtration. Ammonium sulphate precipitation was carried out by addition of the salt to the celery tuber extract solution to achieve 40% saturation. The precipitate was dissolved in 20 mM Tris-HCl, pH 7.4 and desalted by dialysis against this buffer. Protein (10 mg) was loaded onto a concanavalin A sepharose column (Amersham Bioscience), equilibrated with binding buffer (20 mM Tris-HCl, pH 7.4, 500 mM NaCl). Elution of bound proteins was achieved using 400 mM methyl α-D-mannopyranoside in binding buffer. Eluted proteins were dialysed against buffer A (20 mM Tris-HCl, pH 8.0) and loaded onto a Resource Q anion exchange column (Amersham Biosciences). After washing with buffer A, proteins were eluted by a linear gradient from 0 to 100% buffer B (20 mM Tris-HCl, pH 8.0, 500 mM NaCl) over 20 min at a flow rate of 1 mL/min. Concentration of purified Api g 5 was determined by BCA (Pierce) and the protein stored either lyophilized or in solution at −20°C.

2.5 N-terminal sequencing

N-terminal sequencing of purified proteins was performed with an Applied Biosystems Procise 491 sequencer (Applied Biosystems, Foster City, CA, USA). Purified rApi g 1 and rApi g 4 (50 pmol), were adsorbed on a Prosorb cartridge and subjected for sequence analysis. Api g 5 was separated by 12% SDS-PAGE and blotted to a polyvinylidene fluoride membrane (Immobilon-P, Millipore). The membrane was stained with 0.1% w/v CBB R-250 in 50% v/v methanol, 1% v/v acetic acid. Bands were excised and subjected to sequence analysis using the pulsed liquid cleavage program with the manufacturer's chemistry version 1.1.1. Sequence data were compared with the protein databases using the BLAST program.

2.6 SEC

The homogeneity of rApi g 1 and Api g 5 preparation was analysed by HPLC-SEC using a (7.8 × 300 mm) TSKgel $G2000SW_{XL}$ or a (7.5 × 300 mm) TSKgel Super SW3000 column attached to an HPLC.

2.7 ESI-QTOF

For determining molecular mass, purified rApi g 1 were dialysed against water and reduced with 50-fold molar excess of DTT. The proteins were incubated for 2 h at 37°C and directly subjected to mass analysis. Data were acquired using ESI coupled to a Quadrupole TOF MS (ESI-QTOF MS) (Micromass QT of Global Ultima mass spectrometer, Waters, Milford, MA, USA). Protein samples were diluted in aqueous 50% v/v HPLC-grade ACN in 0.1% v/v formic acid at a concentration of approximately 100 fmol/μL and directly infused at a rate of 5 μL/min. The multiply charged spectra for obtaining the molecular mass were deconvoluted with the Micromass MaxEnt1 software. Sequence analysis of Api g 5 was performed by LC-MS/MS after separation of the isoforms by SDS-PAGE and in-gel tryptic digest of the CBB R-250 stained bands, using the Proteoextract trypsin digestion kit (Calbiochem, San Diego, USA). Resulting peptides were separated by capillary RP-HPLC (Waters) directly coupled to the mass spectrometer *via* the Waters Nanoflow spray head (precolumn Waters Nanoease Symmetry300 trap column, separating column Waters Nanoease Atlantis dC18, connected *via* a ten-port stream select valve). The flow rate was adjusted to 300 nL/min by T-splitting. Peptides were eluted with an ACN gradient (solvent A: 0.1% v/v formic acid/5% v/v ACN, solvent B: 0.1% v/v formic acid/95% v/v acetonitril; 5–45% B in 90 min). For sequence analysis, the instrument was calibrated with the fragment ions of [Glu]-Fibrinopeptide B (Sigma, Steinheim, Germany). Data were acquired in the Data Directed Analysis

(DDA) mode. Survey and fragment spectra were analysed using the software PLGS version 2.2.5 (Waters) with automatic and manual data verification. For sequence identification, a combined Swiss-Prot/TrEMBL database was used.

2.8 NMR analysis

All NMR spectra were acquired on a Bruker Avance 700 NMR spectrometer at a protein concentration of 0.37 mM for rApi g 1, 0.06 mM for rApi g 4 and 0.05 mM for Api g 5 in H_2O/D_2O (9:1) solution. Two high resolution NMR experiments were carried out, at a proton resonance frequency of 700 MHz (11.7 T), at 25°C. The zgpr experiment minimizes the water signal, while the zgesgp experiment suppresses the water peak. For each experiment 256 scans were programmed to analyse rApi g 1, 1536 scans for rApi g 4 and 8192 scans were needed for Api g 5. For rApi g 1, a further ^{1}H spectrum with water suppression and a 2-D HSQC spectrum ($^{15}N-^{1}H$, natural abundance) were scanned, by means of a 900 MHz spectrometer equipped with a cryoprobe.

2.9 Circular dichroism (CD) spectroscopy

Secondary structures of purified celery allergens were checked using CD spectroscopy. The proteins were dialysed against 10 mM potassium phosphate, pH 7.4 and measured at concentrations of 0.1 mg/mL (Api g 1, Api g 5) and 0.2 mg/mL (Api g 4), respectively in a 0.1 or 0.2 cm quartz cuvette in a J-810S spectropolarimeter (Jasco, Easten, USA). Far UV spectra were recorded in the range of 190–260 nm at room temperature. Data of five measurements were accumulated.

2.10 2-DE

Gel strips (Immobiline DryStrip pH 3–10, 7 cm, Amersham Biosciences) were rehydrated overnight in 125 µL IEF buffer (7 M urea, 2 M thiourea, 2% 3[(3-Cholamidopropyl)dimethylammonio]-propanesulfonic acid (CHAPS), 0.5% Bio-Lyte 3/10 Ampholyte (BioRad Laboratories, Hercules, CA) and 0.002% bromophenole blue) containing 4 µg of protein sample. Strips were focused for 10 000 V · h, with a maximum of 7000 V at 20°C using an Ettan IPGphor device (Amersham Biosciences). Prior to the second dimension, strips were incubated in equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, 20% glycerol, 0.002% bromophenole blue) with 65 mM DTT for 15 min and then with 250 mM iodoacetamide in the same buffer without DTT. Second dimension was performed by SDS-PAGE on 12% gels and the gels were stained with CBB R-250.

2.11 IgE immunoblotting and inhibition experiment

Detection of IgE binding proteins by imunoblotting was performed as previously described [18, 25]. Briefly, the proteins were separated by SDS-PAGE and blotted to nitrocellulose membrane. After blocking with BSA the membranes were incubated with appropriate diluted sera from celery allergic patients. For inhibition assays serum pool was preincubated with 100 µg/mL purified natural Api g 1. Bound IgE was detected using either alkaline phosphatase (AP)-conjugated antihuman-IgE (BD Pharmingen, San Diego, USA) or ^{125}I-labelled antihuman IgE (MALT Allergy System Isotope Reagent, IBL Hamburg, Germany).

2.12 IgE ELISA

Microtiter plates (Nunc MaxiSorp, Roskilde, Denmark) were coated with 0.1 µg of proteins *per* well. After blocking with Tris-buffered saline (TBS) containing 0.5% v/v Tween 20 and 3% w/v nonfat drymilk, 1:4 diluted sera were applied onto the plates and incubated overnight at 4°C. After washing, the plates were incubated with a 1:1000 diluted AP-conjugated mouse antihuman IgE antibody (BD Pharmingen) for 2 h at room temperature. Colour development was performed using disodium *p*-nitrophenyl phosphate substrate tablets (Sigma) and the OD was measured at 405 nm. Buffer and three sera of nonallergic subjects were used as negative controls. OD values were counted positive if they exceeded the mean OD of the negative controls by more than three SDs.

2.13 Patients' sera

Patients with established celery allergy according to convincing case history and positive DBPCFC to celery [10], and with positive RAST or CAP results were identified and their sera were used as reference to test IgE reactivity of Api g 1 (CAP™ class = 3), Api g 4 (CAP 3) and Api g 5 (CAP 1–5).

3 Results and discussion

3.1 Api g 1

Api g 1.0101 was expressed as a nonfusion protein in *E. coli* BL21. Using ammonium sulphate precipitation, anion exchange and hydrophobic interaction chromatography 80 mg purified rApi g 1.0101 were obtained from one litre *E. coli* culture. Purity of rApi g 1 was checked by SEC, eluting as a single peak and purity was determined as >98%. N-terminal sequencing of rApi g 1 identified the first five correct amino acids GVQTH without the initiating methionine matching the N-terminal sequence of Api g 1.0101 (Swiss-Prot Acc. No. P49372 [6]). Mass analysis of

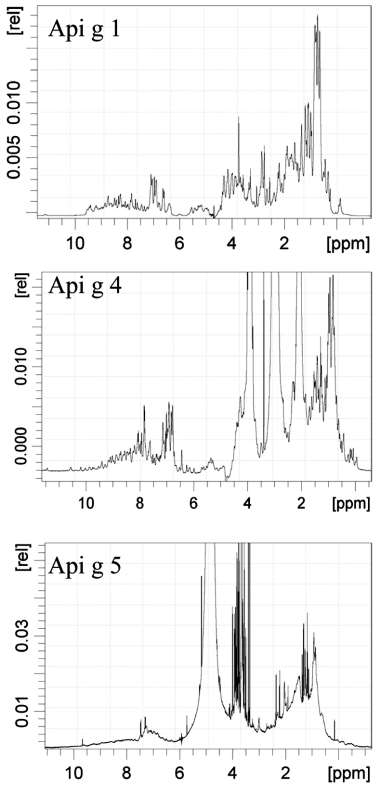

Figure 1. ^{1}H 700 MHz NMR spectra of rApi g 1 (zgesgp experiment 1536 scans), rApi g 4 (zgesgp experiment, 1536 scans) and Api g 5 (zgpr experiment, 8192 scans).

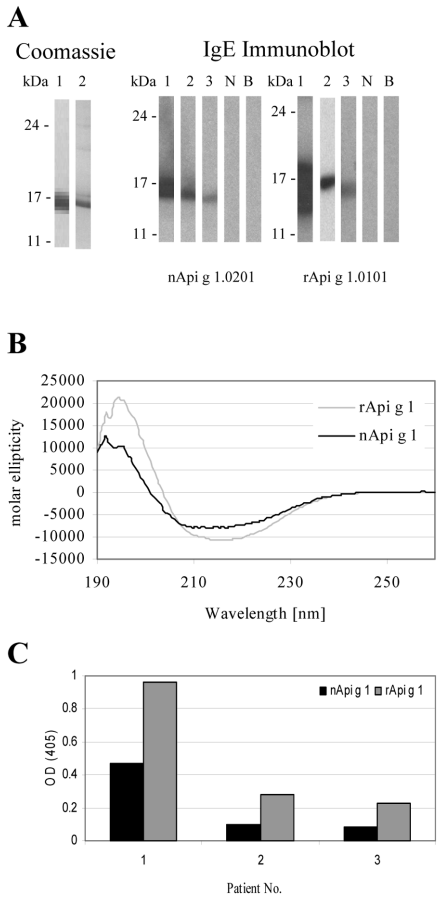

Figure 2. (A) Coomassie-stained SDS-PAGE and IgE immunoblot analysis of purified natural Api g 1 (lane 1) and rApi g 1 (lane 2); IgE immunoblot was performed using sera from celery allergic patients (lanes 1 – 3, N normal human sera pool, B buffer control); (B) CD spectra of natural and rApi g 1; (C) IgE binding of natural and rApi g 1 analysed by ELISA using the same sera as for immunoblotting.

the intact rApi g 1.0101 revealed 16190 Da, which is in agreement with the predicted theoretical mass of 16189 Da. The ^{1}H 700 MHz spectrum of rApi g 1 showed the fingerprint of a well-folded protein (Fig. 1). Within the whole range from −0.5 to 9.5 ppm there are several resolved peaks. For accurate physicochemical and immunological characterization of the recombinant major celery allergen, the natural counterpart was purified from celery tuber extract. Processing 3 kg celery tuber only 10 mg purified natural Api g 1 were obtained after different purification steps. Purified nApi g 1 and rApi g 1 were subjected to SDS-PAGE followed by Coomassie staining (Fig. 2A, lanes 1 and 2). Recombinant Api g 1.0101 migrates as a single band whereas natural Api g 1 appears as a double band at approximately 17 kDa. N-terminal sequencing of natural Api g 1 identified GVQKTVVEAP as the major component within a mixture which matches the N-terminal sequence of Api g 1.0201 (EMBL Acc. No. Z75662 [26]). Therefore, purified natural Api g 1 fraction is considered to consist of isoforms displaying at least two distinct bands in the SDS-PAGE, with Api g 1.0201 being the most abundant isoform. Secondary structure of rApi g 1 determined by CD spectroscopy showed no differences in comparison to natural Api g 1 (Fig. 2B). IgE reactivity of rApi g 1 and nApi g 1 was examined by IgE ELISA, immunoblot and inhibition experiments. Selected celery allergic patients displayed IgE-binding activity to recombinant and natural major celery allergens in ELISA (Fig. 2C) and IgE immunoblot (Fig. 2A). In IgE immunoblot as well as in ELISA lower intensity of IgE binding to nApi g 1 was observed when compared rApi g 1. Api g 1 isoforms display rather low sequence identity and vary also in their IgE-binding capacity as previously shown [12]. Recent data from Wangorsch *et al*. [13] pointed out that individual amino acid residues contribute to the overall IgE binding activity of Api g 1. However, these effects differ in Api g 1.0101 and Api g 1.0201. Api g 1.0101 was crystallized and the structure was determined [14]. On the molecular surface of this protein three conserved patches were identified which represent the molecular basis for crossreactivity with Bet v 1 which in turn identifies Api g 1 not only as a relevant celery allergen but also as a marker allergen for crosssensitization with birch pollen.

3.2 rApi g 4

A plasmid construct designed for cytoplasmic expression of hexahistidine-tagged recombinant Api g 4 was used for the production of celery profilin in a 30 mg scale. A two-step chromatographic procedure was devised for the purification of rApi g 4 from *E. coli* homogenate. From the soluble fraction after culture harvest and cell disruption the recombinant celery allergen was purified by IMAC followed by a second step of SEC. A second step of preparative SEC using a Superdex 75 prep grade high load column was therefore employed, whereby high molecular compounds such as aggregates should separate. In Fig. 3A, the elution profile of preparative SEC and the corresponding fractions analysed by SDS-PAGE are shown. A single peak appeared in the elution profile during SEC corresponding to a well-defined size (15 kDa) based on the elution volumes of molecular weight standards. By contrast, under denaturing and nonreducing conditions different protein bands approximately 35 and 50 kDa are visible in the Coomassie stained gel among the target protein (15 kDa). It seems that these high molecular compounds – probably dimers and trimers of Api g 4 are only formed during SDS-PAGE analysis under nonreducing and denaturing treatment. Unlike, the analysis of the final product under reducing conditions showed a single 15 kDa protein band (Fig. 3B). An explanation for this phenomenon is the formation of intermolecular disulphide bridges *via* two cysteine residues present in the rApi g 4 sequence resulting in the formation of oligomers during SDS-PAGE analysis. In contrast, for the mugwort profilin mainly tetramers and dimers were described after the recombinant production and purification process even under reducing conditions and profilin monomers were detectable only after exposure of multimers to harsh denaturating conditions [27]. These findings are not in accordance with our results whereas we used nearly native conditions during preparation resulting in the formation of properly folded celery profilin monomers. A 22 kDa protein band visible under nonreducing and reducing conditions (data not shown) was coeluted with the target protein using the Superdex 75 column material. Therefore, only fractions 6–11 were pooled in order to obtain pure rApi g 4. Purity of rApi g 4 was >95% as determined by SDS-PAGE (Fig. 3A).

The yield of purified rApi g 4 was 12.5 mg *per* litre *E. coli* culture. Activation of the remaining hexahistidine tag in the plasmid construct by site directed mutagenesis and the simplified purification *via* IMAC led to an enhanced purification yield of Api g 4 (three- to six-fold) using denaturing conditions. In contrast, recombinant nonfusion celery profilin, Api g 4 was purified by poly(L-Proline) affinity chromatography under native conditions with a final yield of 2–4 mg *per* litre [7].

The N-terminal part of the protein was confirmed by amino acid sequencing (SWQAY-5 initial amino acid residues without the initiating methionine) which confirmed

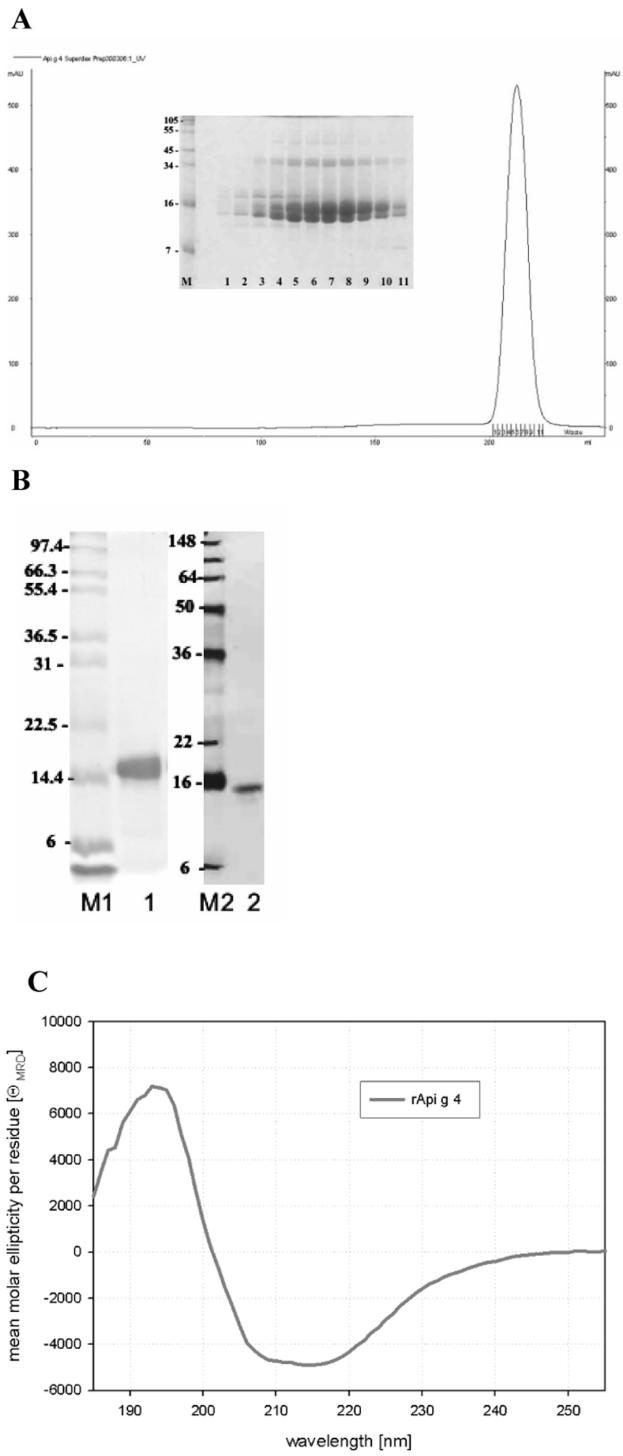

Figure 3. (A) Second purification step of rApi g 4 by SEC (Superdex 75 prep grade 26/60 column): elution profile of pooled IMAC fractions recorded at 280 nm and the corresponding fractions analysed by SDS-PAGE; (B) Coomassie-stained (lane 1) and IgE immunoblot (lane 2) of rApi g 4: IgE immunoblot was performed using a pool of two reference sera from celery allergic patients. (C) CD spectrum of rApi g 4.

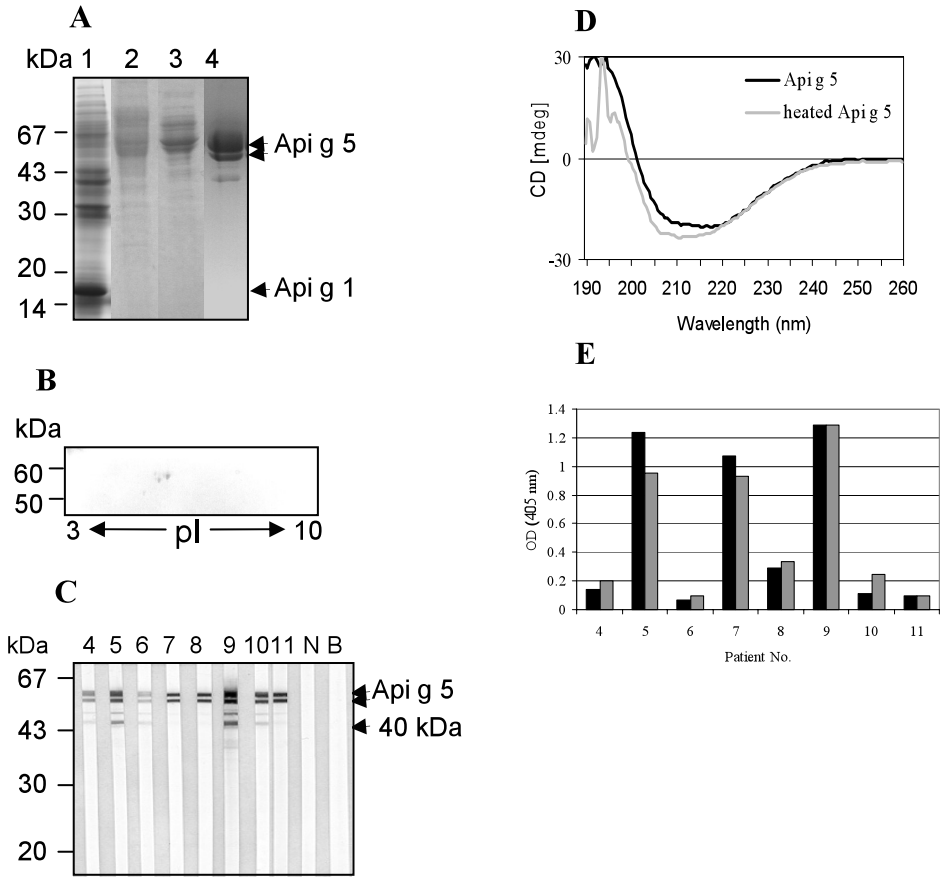

Figure 4. (A) Electrophoretic analysis of purified Api g 5: Coomassie-stained SDS-PAGE of celery tuber protein extract (lane 1), extract after 40% ammonsulphate precipitation (lane 2); eluate from lectin affinity chromatography (lane 3) and purified Api g 5 (lane 4); (B) Coomassie-stained 2D-PAGE showing two isoforms of Api g 5; (C) IgE immunoblot performed with eight sera from celery allergic patients; (D) CD spectra of native Api g 5 and after heating (95°C for 10 min at pH 7); (E) Comparison of IgE binding of eight individual celery allergic patients' sera to native Api g 5 and to heat treated Api g 5.

the published sequence [7]. Furthermore, the secondary structure of rApi g 4 was analysed by CD spectroscopy (Fig. 3C). Recombinant Api g 4 showed a broad minimum around 215 nm that means the protein appeared to be folded with α-helical and β-sheet structures. This result is in accordance with the CD spectrum of its natural counterpart, as previously described [16].

The NMR spectra of Api g 4 are characterized by well-resolved peaks in the whole range of 0.3 – 11.5 ppm (Fig. 1). The presence of tertiary structure is apparent. The peaks around 11.5 ppm probably are the signatures of the two tryptophanes (positions 3 and 36 of the Api g 4 sequence). The amide/aromatic signals are dispersed well above 7 ppm and well below 5 ppm. α-protons signals are dispersed along the range from 3.5 to 5 ppm. Methyl peaks can be observed also in the region around 0 ppm. The protein is folded. Subsequently, IgE-binding capacity was tested by immunoblot using selected reference sera (Fig. 3B) and showed IgE antibody reactivity after the purification process.

3.3 Api g 5

In contrast to the other two celery allergens described in this study, Api g 5 was purified from its natural source, celery tuber. Api g 5 is a glycoprotein with molecular weights of 53 and 57 kDa belonging to a family of FAD containing oxidases. The posttranslational modification (*N*-glycosylation) and the fact that the active enzyme requires covalently bound catalytically essential cofactor FAD explains the difficulties of generation of soluble and active recombinant Api g 5 and achieving high expression levels. In order to purify Api g 5, we enriched it in a first step by 40% ammonium sulphate precipitation of celery protein extract (Fig. 4A, lanes 1 and 2). The precipitate was further purified by lectin affinity chromatography on a concanavalin A column, which binds glycoproteins containing mannosyl residues (Fig. 4A, lane 3). Final purification was achieved by anion exchange chromatography. The fraction contained a mixture of two proteins with slightly different molecular weight of about 53 and 57 kDa, respectively (Fig. 4A, lane 4). Using these procedures, 42 mg Api g 5

were obtained from 12 kg of celery tuber, with an overall yield of about 3.5 mg of pure protein *per* kg celery tuber. Notably, Api g 5 is not an abundant celery tuber protein compared to Api g 1 which represents about 20% of total soluble proteins of celery tuber (Fig. 4, lane 1). Both Api g 5 isoforms gave identical N-terminal sequences (five initial amino acid residues), which matched the N-terminal sequence of Api g 5 (Swiss-Prot Acc. No. P81943), as previously described [18].

Accordingly, ESI-QTOF analysis confirmed both proteins as Api g 5 isoforms. Three peptides were identified after in-gel tryptic digest, comprising the N-terminus (LPNPS GFVTC LSSISK, positions 1 – 16) and two internal sequences (AVIAD PVAK, positions 27 – 35 and LEWIR, positions 67 – 71). 35% of the published sequence of Api g 5 were covered. The NMR Spectrum of nApi g 5 is not well resolved due to the presence of two isoforms (Fig. 1). The peaks in the methyl zone (near 1 ppm) within the aliphatic region are overlapping but the downfield region is not empty. The same accounts for the aromatic region, especially above 9 ppm. Glycosylation peaks can be noticed in the 3 – 4 ppm, region. Therefore, the two proteins can be considered as folded according to NMR analysis. CD spectroscopy analysis of Api g 5 showed the spectrum of a folded protein with α-helical and β-sheet structures. Furthermore, the purity of Api g 5 was studied using SDS-PAGE, 2-DE (Fig. 4B) and SEC (data not shown) and was above 90%. At high concentration (20 μg protein/lane) an additional protein band migrating in SDS-PAGE at 40 kDa was detectable (Fig. 4A, lane 4). 2-DE yielded a mixture of two proteins with molecular weights of 53 and 57 kDa and p*I*s of about 4.5 of the lower isoform and 5.5 of the upper isoform (Fig. 4B).

IgE immunoblotting was performed with eight individual sera of celery allergic patients containing CCD-specific IgE (Fig. 4C). All sera contained Api g 5-specific IgE, and both isoforms displayed equal IgE binding capacity as determined by immunoblots. Five out of eight sera (Fig. 4C lanes 4 – 6, 9 and 10) recognized two additional bands of about 43 and 40 kDa, respectively. The 40 kDa allergen also detectable in the Coomassie stained gel, exhibited an N-terminal sequence with 90% identity to the sequence of an early nodule-specific protein described as Hev b 13 in *Hevea brasiliensis* latex [28]. Hev b 13 has been described as major allergen in the latex B-serum and was shown to display high *in vitro* and *in vivo* reactivity with its *N*-glycans constituting the only IgE epitopes [28]. Our previous data have shown that Api g 5-specific IgE was directed to CCDs [18]. This suggests that the *N*-glycans of 40 kDa proteins are the targets of the IgE response. The 43 kDa proteins with IgE binding capacity was below detection limit in the SDS-PAGE but could be identified by highly sensitive Api g 5-specific sera (Fig. 4C lanes 4 – 6, 9 and 10). N-terminal sequencing failed due to low abundance of the protein. However, IgE cross-reactivity due to *N*-glycans is highly possible.

To determine whether heating to 95°C (10 min) at pH 7.0 affected the secondary structure of Api g 5, CD spectra of native and heated Api g 5 were measured (Fig. 4D). The CD spectrum of heated Api g 5 showed minor irreversible changes in the structure at 200 – 190 nm, but no aggregation of protein was observed. The increase of absorption at 195 – 190 nm suggests that the structural changes of heated Api g 5 may be induced by oxidation of FAD during heating. The heated preparation of Api g 5 was subjected to IgE binding analysis by ELISA using celery allergic patients sera containing IgE specific for Api g 5. Thermal treatment of Api g 5 did not significantly decrease binding of IgE from most of the tested sera (Fig. 4E). This is in accordance with previous studies on thermal stability of celery allergens and is related to IgE reactivity towards HMW celery allergens (multiple bands in the range of 34 – 70 kDa; [11, 15]). The biological activity of Api g 5 was shown to be dependent on the presence of carbohydrate moieties when basophile histamine release experiments were performed.

4 Concluding remarks

Celery is recognized as a relevant food allergen source in Central Europe and one of the most frequent causes of food anaphylaxis [23, 29]. Crosssensitization is established between birch pollen and celery and also with mugwort pollen [4, 9]. So far, three celery allergens have been identified and characterized, two nonglycosylated proteins and one glycosylated protein. Therefore, heterologous production of Api g 1 and Api g 4 was the method of choice, one as a nonfusion protein and the other one as a hexahistidine-tagged protein. For the glycoprotein, Api g 5, purification from natural source seemed to be the appropriate approach.

So far, three individual Api g 1 isoforms have been identified [30] differing in their abundancy and IgE binding capacity, respectively. Api g 1.0201 is the predominant isoform in celery tubers and displays lower IgE binding activity when compared to Api g 1 [13].

Api g 1.0101 was produced as a nonfusion protein in *E. coli.* The overall yield was 80 mg/L *E. coli* culture and and the protein was purified from the soluble bacterial lysate under native conditions. Purified recombinant Api g 1.0101 displayed no posttranslational modifications and showed the characteristics of a well-folded protein according to MS and NMR analysis. Api g 1.0101 is capable to abolish IgE binding to the mixture of natural Api g 1 isoforms and therefore this isoform displays desired characteristics for diagnostic purposes.

Api g 4, celery profilin represents another allergen with crossreactivity to birch pollen and mugwort allergen. A method for high yield production of recombinant celery profilin Api g 4 was developed to obtain the recombinant allergen properly folded and existent in immunological active form. A two-step chromatographic procedure was

performed for the purification of rApi g 4 from *E. coli*. In comparison to former studies, we obtained three- to six-fold yield hexahistidine-tagged Api g 4 (12.5 mg/L) by purification from inclusion bodies using denaturing conditions. Furthermore, the final product was present in a monomeric form showed the presence of secondary and tertiary structures and IgE antibody reactivity.

Api g 5 was the first celery allergen identified from the higher molecular weight region which accounts for a prevalence of IgE reactivity of about 50% in sera from celery allergic patients. We developed a protocol for rapid purification under mild conditions of this glycoprotein from its natural source present as two isoforms. The detailed physicochemical analysis confirmed the presence of *N*-glycans which account for the IgE-binding activity of this protein and probably account for the thermal stability of the protein as shown by heating experiments followed by CD spectroscopy analysis. In the course of the purification procedure two additional celery allergens were identified, the 40 kDa celery allergen and the 43 kDa allergen. The 40 kDa allergen was further characterized and displays sequence similarity to Hev b 13 in the N-terminal sequence.

In conclusion, we present methods for the production of high quality natural and recombinant celery allergens and their detailed characterization which is a prerequisite for the application of future standard CRD of food allergy. This in turn will help to improve the management of the allergic patients' life and reduce the burden of unnecessary exclusion diets.

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5 References

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