RESEARCH ARTICLE

Molecular characterization of Api g 2, a novel allergenic member of the lipid-transfer protein 1 family from celery stalks

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Scope: Celery represents a relevant cross-reactive food allergen source in the adult population. As the currently known allergens are not typical elicitors of severe symptoms, we aimed to identify and characterize a non-specific lipid transfer protein (nsLTP).

Methods and results: MS and cDNA cloning were applied to obtain the full-length sequence of a novel allergenic nsLTP from celery stalks. The purified natural molecule consisted of a single isoallergen designated as Api g 2.0101, which was recombinantly produced in *Escherichia coli* Rosetta-gami. The natural and recombinant molecules displayed equivalent physicochemical and immunological properties. Circular dichroism revealed a typical α-helical fold and high thermal stability. Moreover, Api g 2 was highly resistant to simulated gastrointestinal digestion. As assessed by ELISA, thermal denaturation did not affect the IgE binding of Api g 2. Natural and recombinant Api g 2 showed similar allergenic activity in mediator release assays. Api g 2-specific IgE antibodies cross-reacted with peach and mugwort pollen nsLTPs.

Conclusion: Based on our results, it can be anticipated that inclusion of recombinant Api g 2 in the current panel of allergens for molecule-based diagnosis will facilitate the evaluation of the clinical relevance of nsLTP sensitization in celery allergy and help clinicians in the management of food allergic patients.

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1 Introduction

Celery (*Apium graveolens*) ranks among the most important plant food allergen sources in the central European adult population [1, 2], whereas celery stalks are worldwide consumed, the use of celery tuber (celeriac) is predominant in the Central European cuisine. Allergic reactions to celery show a broad spectrum of clinical symptoms ranging from mild oral allergy syndromes to life-threatening anaphylactic reactions [3–6]. Thus, the presence of celery or even trace amounts thereof has to be indicated on prepackaged products in the European Union (European Directive 2007/68/EC, amending Directive 2000/13/EC). So far, three allergens of *A. graveolens* have been identified at the molecular level: Api g 1, a PR-10 protein [7], Api g 4 belonging to the pan-allergen family of profilins [8], and Api g 5, a member of the flavoprotein family [9, 10]. Nearly 59 and 23–41% of celery allergic patients are sensitized to Api g 1 and Api g 4, respectively [8, 11]. In addition, cross-reactive carbohydrate determinants of Api g 5 and high-molecular-weight components in celery extracts were

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Abbreviations: nsLTP, non-specific lipid-transfer protein; **SDS-PAGE,** sodium dodecyl sulphate polyacrylamide gel electrophoresis

described as allergenic epitopes [11]. However, the clinical relevance of glycan-specific IgE seems to be limited [12].

Intolerance to celery and other vegetables and spices of the *Apiaceae* or *Umbelliferous* family is commonly observed in patients sensitized to mugwort (*Artemisia vulgaris*) and birch (*Betula verrucosa*) pollen. Hence, the terms celery-mugwort-spice and celery-birch-mugwort syndrome have been established [13]. As the clinical association between birch pollinosis and celery hypersensitivity is mainly attributed to Api g 1, this group of patients tolerated cooked celery [7, 14, 15]. Since this pattern of clinical reactions does not apply to mugwort-sensitized patients with concomitant celery allergy, other molecules that are more resistant to thermal denaturation seem likely to mediate those symptoms [4, 16].

In this study, we identified a novel allergen of celery stalks that belongs to the non-specific lipid-transfer protein (nsLTP) family 1 and was designated as Api g 2. The allergen was purified from its natural source and produced as a recombinant protein in *Escherichia coli*. Both molecules were characterized at the molecular level in regard to structure and stability. In addition, IgE-binding capacity, cross-reactivity, and biological activity of Api g 2 were assessed using sera from nsLTP-sensitized patients.

2 Materials and methods

2.1 Patients and sera

Subjects with positive IgE reactivity to plant food and/or pollen nsLTP, as determined by the ISAC 103, VBC-Genomics, Vienna, Austria [17], were selected among those attending the clinical outpatient department. Diagnostic and clinical features have been recorded for each patient in an electronic allergy record (InterAll, Allergy Data Laboratories s.c., Latina, Italy). The study was approved by the Institutional Review Board (n. 106-CE-2005), and signed informed consents were obtained from all patients.

2.2 Purification of natural celery nsLTP

Celery stalk was obtained from the local grocery stores and ground to fine powder on liquid nitrogen. Proteins were extracted overnight during vigorous shaking at 4°C using different buffers, *i.e.* PBS pH 7.4, 50 mM sodium acetate pH 5.3, and 50 mM ammonium bicarbonate pH 7.8. Extracts were clarified by centrifugation at $20\,000 \times g$ at 4°C and filtration through a 0.45 μm filter. For lab-scale purification, extraction was performed with PBS pH 7.4, 5 mM EDTA and after clarification, the buffer was exchanged to 5 mM sodium acetate pH 5.3 using a 5 kDa cut-off ultrafiltration membrane (Millipore, Billerica, MA). The protein was purified by cation exchange chromatography using a 1 mL SP FF column (Äkta prime system, GE Healthcare, Chalfont St. Giles, UK) at a flow rate of 1 mL/min. After 50 mL gradient elution to 5 mM sodium acetate pH 5.3, 500 mM NaCl, fractions containing pure Api g 2 were pooled, desalted, lyophilized, and stored at −20°C.

2.3 cDNA cloning of celery nsLTP

Celery stalk total RNA was prepared from ground celery stalks using Trizol (Invitrogen, Carlsbad, CA) and subjected to oligo dT-primed reverse transcription using the SuperScript III First-strand Synthesis System (Invitrogen). A degenerated forward primer 5′-STNNNNTGCATGGTKGT-3′ based on the conserved amino acids CMVV in the signal peptide found in a number of nsLTPs (*e.g.* Art v 3 and Pru p 3) was used for amplification and products were cloned into the pGEM-T Easy Vector (Promega, Madison, WI). The 5′-UTR and the entire signal peptide were obtained using the 5′RACE System (Invitrogen) with GSP1 (5′-TCGTGATCATCGAACAA-3′) and GSP2 (5′-AATCAACGAAATCCCTAATCCCTTATTATATAGTAA-3′) primers.

2.4 Production of recombinant celery nsLTP

For recombinant production, the sequence corresponding to the mature protein was cloned into the pHisParallel2 [18] using 5′-GGTGGTCATATGATTACATGTGGACAGG-3′ forward and 5′-GGTGGTCTCGAGTTAATTCACCCT-3′ reverse primers with flanking *Nde*I and *Xho*I sites, respectively. To increase stability during expression, the N-terminal leucine was changed into isoleucine [19, 20]. Recombinant Api g 2 (rApi g 2) was produced as soluble non-fusion protein in *E. coli* Rosetta-gami B(DE3) pLysS (Novagen, Gibbstown, NJ). Briefly, protein expression was induced with 0.4 mM isopropyl-β-D-thiogalactopyranosid at $OD_{600} = 0.7$ and cells were grown at 37°C for 3 h. After centrifugation at $5000 \times g$ for 20 min at 4°C, cells were resuspended in 10 mM ammonium bicarbonate pH 7.8, 5 mM NaCl and lyzed by repeated freezing and thawing. The soluble protein fraction was obtained by centrifugation at $20\,000 \times g$ for 30 min at 4°C. For cation exchange chromatography, the extract was adjusted to 30 mM sodium acetate pH 5.3 and purification was performed as described for the natural molecule. For final purification the protein was loaded onto a Superdex 75 10/300 GL column (GE Healthcare) and eluted with 1.5 column volume 5 mM sodium acetate pH 5.3 at a flow rate of 0.3 mL/min. Fractions containing pure protein were lyophilized and stored at −20°C for further use.

2.5 MS

MS analyses were performed on a Micromass QTof Global Ultima instrument (Waters, Milford, MA) as described [21]. Briefly, intact masses were determined by direct infusion.

Peptides obtained from tryptic digestion (ProteoExtract Trypsin Digestion Kit, Calbiochem, Gibbstown, NJ) were separated by capillary HPLC online coupled to the mass spectrometer. For *de novo* sequencing, the ProteinLynx Global Server, software version 2.2.5 (Waters) was used.

2.6 SDS-PAGE and amino acid analysis

Celery stalk extracts, purified natural and recombinant Api g 2 were analyzed by denaturing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and non-reducing conditions and visualised with Coomassie Brilliant Blue R-250 staining. Amino acid composition and protein concentration were determined using the Pico Tag method (Waters). Approximately, 5–10 μg of proteins were dried and hydrolyzed with constant boiling 6 M HCl (Pierce, Rockford, IL) for 18 h at 121°C under vacuum. After neutralization with aqueous 20% v/v triethylamine and 40% v/v ethanol, samples were redried and derivatized with 10% v/v phenyl isothiocyanate in 70% v/v ethanol and 10% v/v triethylamine for 10 min at room temperature. Dried samples were reconstituted in Pico-Tag Eluent A (prepared according to the manufacturer's instruction) and analyzed by reversed-phase-HPLC using a Nova-Pak C18 column (Waters). Measurements were performed in duplicates and concentrations were calculated based on the analysis of the internal amino acid standard A (Pierce).

2.7 Circular dichroism and modelling

Circular dichroism spectra and thermal denaturation ($\Delta T = 1$°C/min) were recorded in 5 mM potassium phosphate pH 7.4 or alternatively pH 3.0 with a JASCO J-815 spectropolarimeter (Jasco, Tokyo, Japan). Far UV spectra (190–260 nm) were baseline subtracted and results are presented as the mean residue molar ellipticity. Estimation of protein secondary structure from CD spectra (190–240 nm) was performed with the CDPro software (http://lamar.colostate.edu/~sreeram/CDPro/main.html) and the online tool K2D2 (http://www.ogic.ca/projects/k2d2). Molecular modelling of Api g 2 was performed using Modeller 9v2 (http://salilab.org/modeller) and evaluated with ProSa2003 based on the structure of Pru p 3 (PDB: 2B5S) [22].

2.8 Simulated gastrointestinal digestion

Gastrointestinal digestion was simulated *in vitro* as described elsewhere [23]. Briefly, gastric digestion was performed using 50 ng of pepsin (Sigma-Adrich, Saint Louis, MO, USA) *per* μg of substrate in 0.1 M HCl pH 2. After 2 h of pepsin treatment, the pH was neutralized by addition of ammonium bicarbonate pH 7.8, and duodenal protein degradation was performed by incubation with 2.5 ng of trypsin (Sigma-Aldrich) and 10 ng of chymotrypsin (Sigma-Aldrich) *per* μg of substrate, respectively. Reactions were conducted at 37°C and proteolysis was monitored by SDS-PAGE.

2.9 ELISA

For ELISA, Maxisorp plates (Nalge Nunc, Rochester, NY) were coated with 200 ng purified natural and recombinant Api g 2 as well as heat-denatured allergen (15 min at 95°C in 10 mM potassium phosphate pH 3 or pH 7) overnight at 4°C. Unspecific binding was blocked with TBS pH 7.4, 0.05% v/v Tween-20, and 1% w/v BSA. After overnight incubation with 1:4 diluted patients' sera at 4°C, bound IgE was detected with alkaline phosphatase-conjugated monoclonal anti-human IgE (BD Biosciences, Franklin Laker, NJ). Values were considered positive when exceeding three times the standard deviation of the control vials. Statistical evaluation was performed using the Mann–Whitney rank sum test. For inhibition experiments, a serum pool of Api g 2-positive patients ($n = 14$) was preincubated with increasing allergen concentrations. Recombinant Art v 3.0201 and Pru p 3 used for the inhibition assay were produced as previously described [20, 24].

2.10 Mediator release assay

The biological activity of natural and recombinant Api g 2 was investigated using rat basophil degranulation assays as previously described [25]. Briefly, RBL-2H3 cells transfected with the human high-affinity IgE receptor were passively sensitized with patient's serum. Degranulation was triggered by addition of serial protein dilutions (10^{-5} to 10^{1}), and β-hexosaminidase release was measured by enzymatic cleavage of the fluorogenic substrate *p*-nitrophenyl-*N*-acetyl-β-glucosaminide.

3 Results

3.1 Identification of a novel allergenic nsLTP1 in celery stalks

We were able to purify a 10 kDa protein from celery stalks representing a novel member of the nsLTP 1 family. The full-length sequence of the protein was obtained by PCR with degenerated primers on *A. graveolens* cDNA and subsequent RACE protocols (GenBank accession no. FJ643539). Notably, MS-based analyses of the natural preparation revealed a single peak and a primary structure that is in complete accordance with the deduced amino acid sequence. These data indicate the presence of a single


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            1        10        20        30        40        50        60        70        80        90   %
 Api g 2  --LTCGQVTGKLGGCLGYLK-GGGYPSPACCGGVKGLNSLAKTPADRKQACACLKTLAGSVKGINYGAASALPGKCGIRIPY-PISPSTDCSRVN- 100
 Vit v 1  --VTCGQVASALSPCISYLQ-KGGAVPAGCCSGIKSLNSAAKTTGDRQAACKCLKTFSSSVSGINYGLASGLPGKCGVSVPY-KISPSTDCSKVT-  62
 Hel a 3  --LSCGQVSSSLAPCISYLT-KGGAVPPACCSGVKSLNSAAKTTPDRQAACGCLKSAYNSISGVNAGNAASFPGKCGVSIPY-KISPSTDCSKVQ-  60
Pru av 3  --LTCGQVSSNLAPCIAYVR-GGGAVPPACCNGIRNINNLAKTTADRQTACNCLKQLSASVPGVNANNAAALPGKCGVNVPY-KISPSTNCATVK-  59
 Lac s 1  --ITCGQVVSSLSQCASYLR-NGGAVPPACCSGVKSLNSAARTTPDRQTVCGCLKRASG---GVNASNAASLPGKCGVNIPY-KISPSTDCSKVQ-  58
 Pru d 3  --ITCGQVSSNLAPCINYVK-GGGAVPPACCNGIRNVNNLARTTADRRAACNCLKQLSGSIPGVNPNNAAALPGKCGVNVPY-KISASTNCATVK-  57
Pru ar 3  --ITCGQVSSSLAPCIGYVR-GGGAVPPACCNGIRNVNNLARTTPDRRTACNCLKQLSGSISGVNPNNAAALPGKCGVNIPY-KISASTNCATVK-  57
 Pyr c 3  --ITCSQVSANLAPCINYVR-SGGAVPPACCNGIKTINGLAKTTPDRQAACNCLKNLAGSVSGVNPGNAESLPGKCGVNVPY-KISTSTNCATVK-  57
 Mal d 3  --ITCGQVTSSLAPCIGYVR-SGGAVPPACCNGIRTINGLARTTADRQTACNCLKNLAGSISGVNPNNAAGLPGKCGVNVPY-KISTSTNCATVK-  57
 Rub i 3  --ITCGQVTQNVAPCFNYVK-NGGAVPAACCNGVRNLNSQAKTTADRQQTCNCLKNAAGSIPGLNPSLAAGLPGKCGVSVPY-KISTSTNCATVK-  57
 Pru p 3  --ITCGQVSSSLAPCIPYVR-GGGAVPPACCNGIRNVNNLARTTPDRQAACNCLKQLSASVPGVNPNNAAALPGKCGVSIPY-KISASTNCATVK-  56
 Mor n 3  --ITCGQVSSSLAPCINYLR-AGGVVPANCCNGVRSLNNAAKTTADRQAACNCLKSAFNSIKGLNLNLAAGLPGKCGVSVPY-KISPSTDCKSVK-  56
 Cit s 3  --ITCGQVSGSLAPCIGFLR-SGGPIPMPCCNGVRSLNAAARTTPDRQTACNCLKQAAGSIPNLNLNNAAGLPGACGVSIPY-KISTSTDCSKVR-  55
 Art v 3  --LKCSDVSNKISACLSYLK-QGGEVPADCCTGVKGLNDAAKTTPDRQTACNCLKTTFKSNKDFKSDFAASLPSKCGVNIPY-KISLETDCNKVK-  54
 Ara h 9  --ISCGQVNSALAPCIPFLT-KGGAPPPACCSGVRGLLGALRTTADRQAACNCLKAAAGSLRGLNQGNAAALPGRCGVSIPY-KISTSTNCATIKF  54
 Fra a 3  --ITCGQVASSISPCVNYVK-SGGAVPAACCNGIRSLNSAAKTTADRQATCNCLKQASGAIKGLNPSLAAGLPGKCGVSVPY-KISTSTNCAAVK-  53
Zea m 13  -AISCGQVASAIAPCISYARGQGSGPSAGCCSGVRSLNNAARTTADRRAACNCLKNAAAGVSGLNAGNAASIPSKCGVSIPY-TISTSTDCSRVN-  53
 Cor a 8  -SLTCPQIKGNLTPCVLYLK-NGGVLPPSCCKGVRAVNDASRTTSDRQSACNCLKDTAKGIAGLNPNLAAGLPGKCGVNIPY-KISPSTNCNNVK-  52
 Pha v 3  -AISCGQVTSSLASCIPFLT-KGGPVPASCCSGVRSLNAAAKTTPDRQAVCNCLKSAAGAIPGFNANNAGILPGKCGVSIPY-KISTSTNCATIKF  49
 Bra o 3  -ALSCGTVSGNLAACIGYLT-QNGPLPRGCCTGVTNLNNMARTTPDRQQACRCLVGAANAFPTLNAARAAGLPKACGVNIPY-KISKSTNCNSVR-  48
Tri a 14  ---DCGHVDSLVRPCLSYVQ-GGPGPSGQCCDGVKNLHNQARSQSDRQSACNCLKGIARGIHNLNEDNARSIPPKCGVNLPY-TISLNIDCSRV--  47
 Amb a 6  ASPTCDTVQNILAPCAGFLT--GQEPSKACCTGVNNLNNSRKTKADRVAVCNCIKELTKS-IAYDPKRMPLLSTKCGVKPDFPAVDKNLDCSKLPV  36
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Figure 1. Sequence alignment of allergenic members of the nsLTP1 family. The mature sequence of Api g 2.0101 was aligned with Vit v 1 (Q850K5), Hel a 3 (Q39950), Pru av 3 (Q9M5X8), Lac s 1 (A1E2H5), Pru d 3 (P82534), Pru ar 3 (P81651), Pyr c 3 (Q9M5X6), Mal d 3 (Q5J026), Rub i 3 (Q0Z8V0), Pru p 3 (Q5RZZ3), Mor n 3 (P85894), Cit s 3 (Q8L5S8), Art v 3 (C4MGG9), Ara h 9 (B6CEX8), Fra a 3 (Q4PLU0), Zea m 14 (P19656), Cor a 8 (Q9ATH2), Pha v 3 (D3W147), Bra o 3 (Q42614), Tri a 14 (P24296), and Amb a 6 (O04004) using AlignX (Vector NTI, Invitrogen) and SwissProt accession numbers are given in parenthesis. Numbering on top is based on Api g 2 and sequence similarity scores in percent are given for each individual nsLTP. Amino acids identical to Api g 2 are shown in dark grey, similar residues are coloured in light grey.

isoform with an open reading frame of 118 amino acids. The predicted signal peptide cleavage site (SignalP 3.0 Server) between G27 and L28 could be verified by mass-based analysis of the natural molecule and the theoretic isoelectric point of Api g 2 was calculated to be 9.38 (www.expasy.org). BLAST homology searches using the mature sequence demonstrated highest identity to saltbush and clove nsLTPs (67%). Sequence alignments with allergenic members of the nsLTP family are depicted in Fig. 1 with identity scores ranging from 32 to 60%. Since the IgE-binding capacity of the novel nsLTP1 from *A. graveolens* stalks could be verified, it was designated as Api g 2.0101 according to the IUIS allergen nomenclature subcommittee. Although the Arabic number 3 is frequently used for allergens belonging to the nsLTP family, the name Api g 3 could not be assigned as it has previously been allocated to a chlorophyll a–b-binding protein from celery.

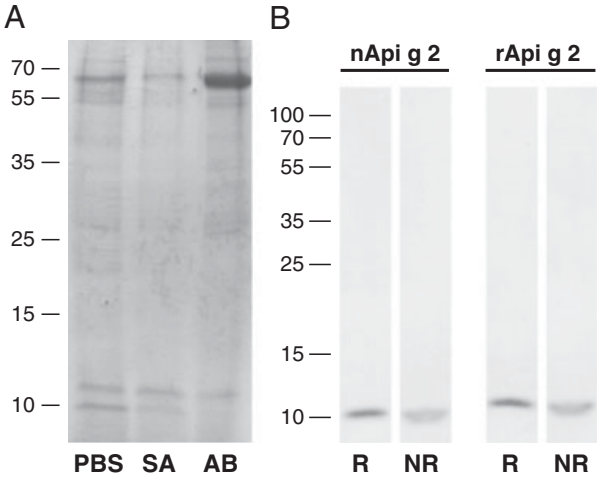

Figure 2. Extraction of Api g 2 from celery stalks using different buffers (A). Purified natural and recombinant Api g 2 evaluated under reducing and non-reducing SDS-PAGE (B). PBS, phosphate buffer saline; SA, sodium acetate; AB, ammonium bicarbonate, R, reducing; NR, non-reducing.

3.2 Protein purification and quality assessment of natural and recombinant Api g 2

Sufficient quantities of natural Api g 2 (nApi g 2) were extractable only in PBS (Fig. 2A) yielding low amounts (0.3 mg/kg raw material) of purified allergen from celery stalks. After recoding the N-terminal amino acid leucine to the more stabilizing residue isoleucine [19, 20], rApi g 2 was expressed as soluble non-fusion protein in *E. coli* Rosetta-gami B(DE3) pLysS with a yield of 2–3 mg/L culture. Exact protein concentrations were assessed by amino acid analysis showing a purity of $>98\%$. In SDS-PAGE, natural and recombinant Api g 2 migrated at 10 kDa under both, reducing and non-reducing conditions (Fig. 2B). Intact molecular masses of purified proteins were determined in MS as follows: (theoretic molecular mass in parenthesis): nApi g 2, 9024.5 (9032.83) and rApi g 2, 9156.4 (9164.02). The difference between the calculated and measured masses of -8.33 and -7.62, respectively, indicates that all eight cysteine residues seem to be involved in disulphide bond formation under non-reducing conditions which cannot be taken into account for the calculation. The difference in the molecular weight ($\Delta = 113$) of natural and recombinant Api g 2 indicates that the N-terminal methionine of the molecule was not cleaved off during heterologous production in

E. coli. Apart from disulphide bond formation, we did not observed any other post-translational modification in MS experiments of the natural molecule.

3.3 Secondary structure and stability analysis

Spectra obtained by circular dichroism showed curves typical for α-helical proteins displaying negative maxima at 222 and 209 nm. To assess the influence of different environment conditions on structural stability, thermal denaturations were performed in neutral and acidic buffers using potassium phosphate at pH 7.4 and 3.0, respectively (Fig. 3). Both natural and recombinant Api g 2 appeared to be extremely heat stable as they had calculated melting temperatures of 84°C at neutral and 89°C at acidic conditions. However, tremendous differences were observed in the degree of unfolding and the refolding capability. Although Api g 2 vastly lost its fold upon denaturation at neutral pH, only a partial loss of structure could be observed at pH 3. Moreover, heat-denatured Api g 2 was able to refold exclusively in the acidic environment and remained entirely unfolded at neural pH. Secondary structure predictions based on the obtained CD data using K2D2 suggested an α-helical content of 87.6% and a negligible fraction of β-sheets (0.48%). Using the CDPro software, Api g 2 was proposed to contain 40.3–48.1% α-helices and 11.0–14.7% β-sheets (varying values were obtained with different calculation softwares *i.e.* SELCON3, CDSSTR, and CONTINLL). Molecular modelling of Api g 2 demonstrated the exclusive presence of helical regions (residues 3–19, 25–37, 41–57, 63–73, and 87–89) that represents 68.6% of the molecule's secondary structure.

Stability assessment during simulated gastrointestinal digestion revealed a remarkably high resistance of both, natural and recombinant Api g 2. Notably, the protein remained unaffected even up to 48 h of gastric pepsin digestion (Fig. 4A). Also, susceptibility to duodenal trypsin/chymotrypsin was extremely low. Up to 4 h of incubation Api g 2 showed only slight proteolytic degradations, and even after extended treatment (24 h), a band corresponding to the intact mass was still detectable (Fig. 4B).

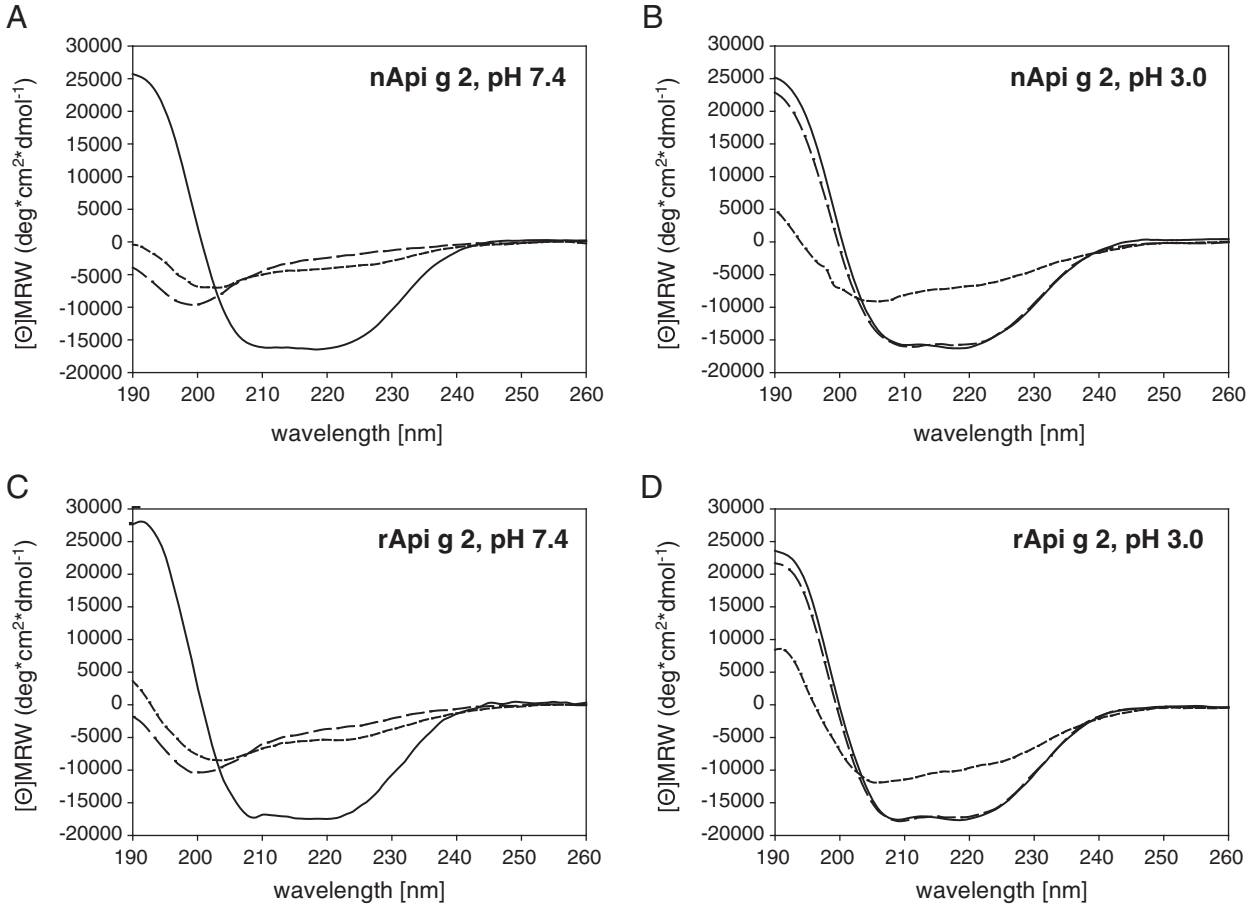

Figure 3. Secondary structure determination using circular dichroism. Far UV-measurements of natural (A, B) and recombinant (C, D) Api g 2 were performed at 20°C (solid line), 95°C (short dashes) and at 20°C after denaturation (long dashes). Different buffer conditions were evaluated using potassium phosphate buffer pH 7.4 (A, C) and pH 3.0 (B, D).

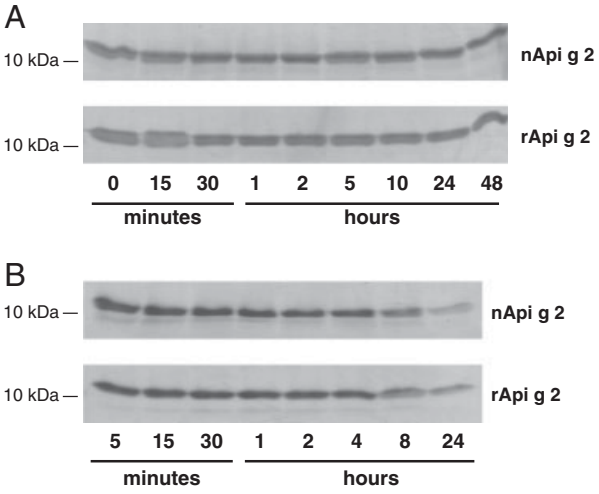

Figure 4. Effect of simulated gastrointestinal digestion. Natural and recombinant Api g 2 were subjected to gastric digestion with pepsin (A) or 2 hours of gastric followed by duodenal digestion with trypsin/chymotrypsin (B) and monitored by SDS-PAGE.

3.4 Immunological characterization and allergological relevance of Api g 2

Sera from 26 Italian patients that were tested positive for any nsLTP spotted on the allergen microarray [17] were used to screen for IgE reactivity to Api g 2 in ELISA. Among the nsLTP-sensitized patients, 15 individuals (57.5%) displayed antibodies specific for the celery nsLTP. Based on the reports of these patients in the electronic allergy record, 5 individuals presented adverse reactions upon ingestion of celery stalks, which mainly involved OAS and angioedema in one case. Five persons were not exposed or actively avoided eating celery because they previously experienced severe reactions upon consumption of nsLTP-containing foods and another five were asymptomatic. Sera reactive to Api g 2 were used to assess the IgE reactivity of natural and recombinant celery nsLTP in ELISA. Both molecules presented highly correlating IgE-binding capacities ($r = 0.992$) with mean 405 nm absorbance values of 0.831 and 0.825, for nApi g 2 and rApi g 2, respectively. To assess the influence of heat treatment on the allergenic capacity of Api g 2, thermal denaturation was performed at neutral as wells as at acidic conditions prior to coating the allergen on the ELISA surface. Notably, heating for 15 min at 95°C did not influence the IgE-binding capacity of the molecule, although a slight but non-significant decrease in reactivity was observed for nApi g 2 at pH 7.4 (Fig. 5). To determine whether Api g 2 shares IgE epitopes with other members of the nsLTP family, cross-inhibition experiments were performed (Fig. 6). Similar inhibitory capacities were observed for natural and recombinant Api g 2. In addition, IgE cross-reactivity with peach and mugwort pollen nsLTP was evidenced, obtaining inhibition values up to 60.6 and 29.8%, respectively (Fig. 6). To assess the biological activity of the nsLTP from celery, RBL assays were performed using sera of three Api g 2-sensitized patients

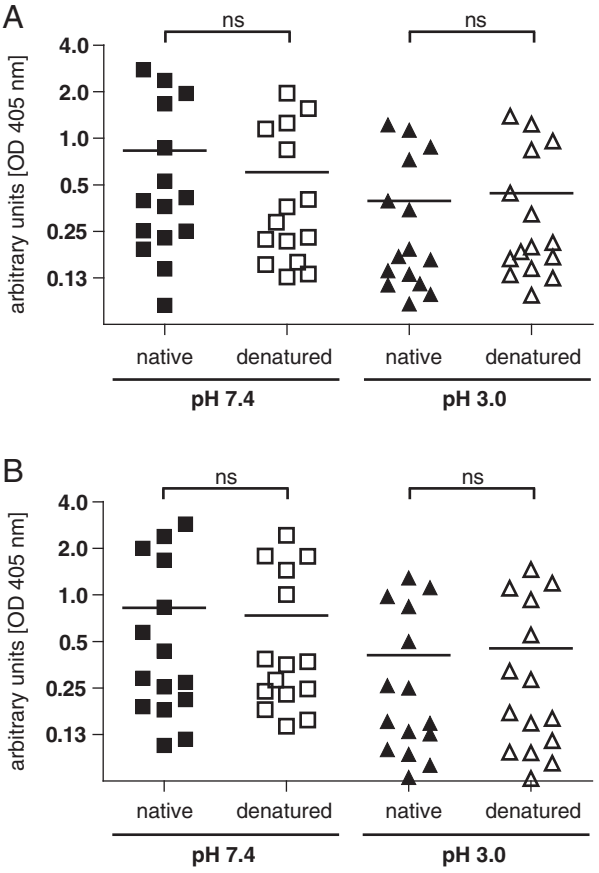

Figure 5. Allergenicity of Api g 2. IgE reactivity of natural (A) and recombinant (B) Api g 2 before (native) and after heat treatment (denatured) was assessed by ELISA using sera from nsLTP-sensitized patients (n=15). Thermal treatment was performed in 10 mM potassium phosphate pH 7.4 or 3.0, respectively and no significant difference in IgE reactivity was observed using the Mann-Whitney rank sum test. ns, not significant.

(Fig. 7). The natural and recombinant allergens triggered comparable ß-hexosaminidase releases, and even slightly higher values were retrieved for rApi g 2 in one patient (Fig. 7C). In general, highest mediator releases were observed in the concentration range of 0.1–10 μg/mL.

4 Discussion

Although researches on allergens from celery tuber (celeriac) have been extensively carried out [5, 12, 26, 27], information on the allergenic potential of celery stalks, a worldwide consumed vegetable and ingredient in several spices is nearly absent. Celery is able to trigger severe allergic reactions in some patients suggesting the involvement of highly stable allergen(s) [4, 5, 16, 26]. Therefore, we investigated *A. graveolens* for the presence of an nsLTP. In this study, we identified a novel allergenic member of the nsLTP1 family in celery stalks that was designated as

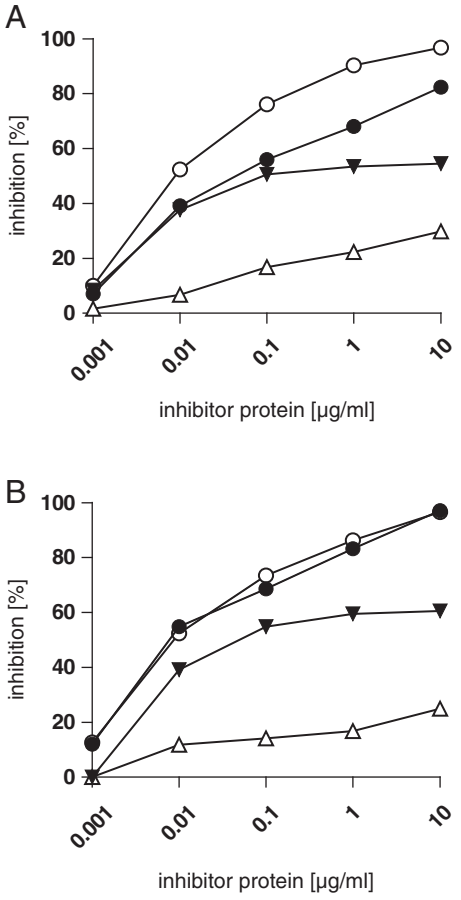

Figure 6. Cross-inhibition ELISA. Reduction of IgE-binding to natural (A) and recombinant (B) Api g 2 was tested using a serum pool of 15 Api g 2-reactive individuals and nApi g 2 (open circles), rApi g 2 (filled circles), rArt v 3 (open triangles), and rPru p 3 as inhibitors (filled triangles).

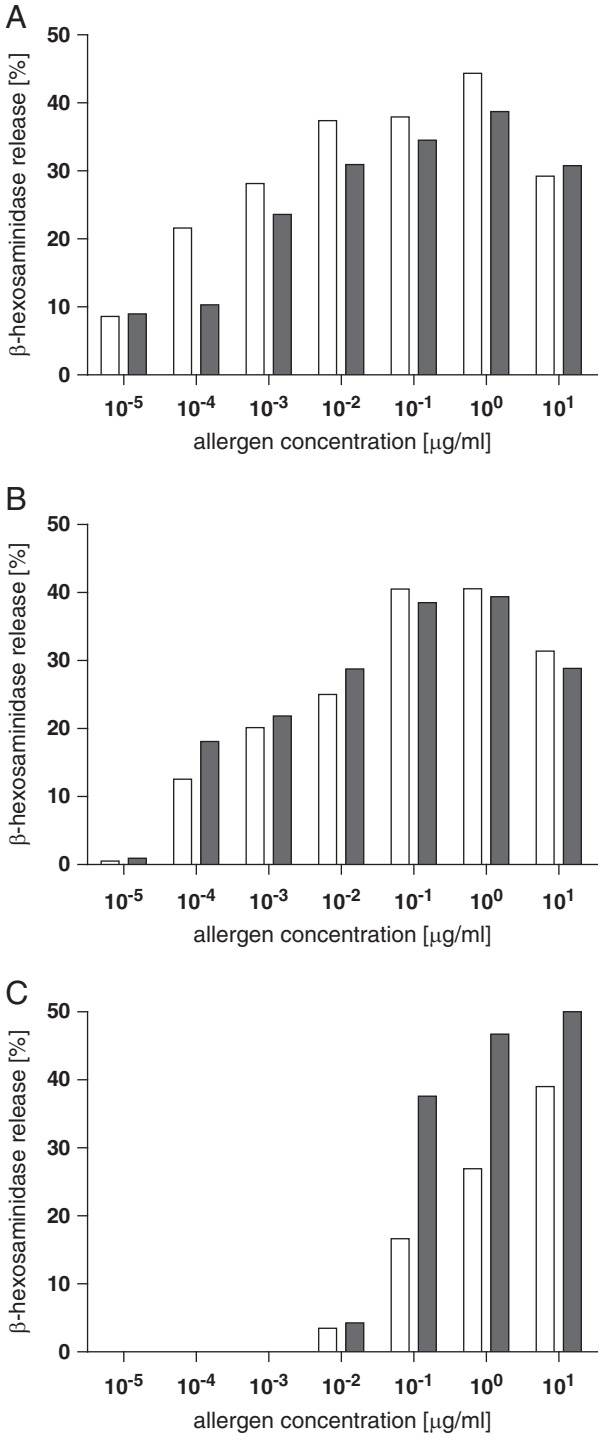

Figure 7. Mediator release values of natural (white bars) and recombinant (grey bars) Api g 2 using transfected RBL-2H3 cells are shown for 3 individual patients (A-C).

Api g 2. Notably, we could not detect Api g 2 in celery tuber and expression seems to be restricted to the aerial part of the plant (unpublished data). Generally, the low performance of commercially available extracts is a frequently encountered difficulty in the diagnosis of celery allergy [12], and might arise from low or absent extractability of certain allergens or from quantitative and qualitative differences in the allergen content between celeriac and stalks. Therefore, molecule-based approaches offer the most reliable alternative for *in vitro* diagnosis of celery allergy [12]. The integration of Api g 2 in the current allergen panel could enhance the diagnostic sensitivity and specificity and thereby encompasses patients independent of their dietary habits. Because extraction and purification of low-abundant allergens from natural sources is a limiting process, we aimed to produce Api g 2 as a recombinant molecule. So far, the majority of nsLTPs has been expressed in *Pichia pastoris*, which has been lately regarded superior to bacterial-based systems [28]. However, utilizing *E. coli* Rosetta-gami B(DE3) pLysS, a strain that allows intracellular disulphide bond formation [20], we were able to produce sufficient amounts of high-quality rApi g 2 in a prokaryotic expression system. Detailed physicochemical and immunological characterization revealed that the recombinant molecule displays similar

properties as its natural counterpart. In cross-inhibition assays rApi g 2 showed a slightly lower inhibitory capacity to coated nApi g 2, although IgE reactivity of natural and recombinant Api g 2 was equivalent and highly correlating in ELISA using 15 individual sera. Therefore, the difference in the inhibition assay was probably due to contaminations with other celery allergens present at trace amounts in the preparation of nApi g 2. The use of a serum pool, which might include a few patients with additional reactivity to other celery stalk allergens in hand with the sensitivity of the assay, would explain the observed difference. Taken together, we conclude that purified rApi g 2 represents an excellent alternative to the natural molecule, as (i) it can be produced at sufficient amounts and (ii) cross-contaminations with other allergenic molecules can be ruled out.

Lipid-transfer proteins are considered the most important food allergens in the Mediterranean area showing sensitization rates up to 60% among fruit/vegetable allergic patients [29]. Currently, 58 allergenic nsLTPs have been identified and characterized to varying degrees [30]. Although nsLTPs from different species demonstrate only moderate sequence identity (45–65%), they show high levels of structural conservation giving rise to IgE cross-reactivity. However, the enormous variety and clinical relevance of this cross-reactivity is controversially discussed and seems to largely depend on the selection of investigated patients [30]. Therefore, molecule-based diagnosis using the growing panel of available nsLTPs would help to avoid unnecessarily diet restrictions that are frequently advised for patients suffering from any nsLTP-mediated allergy. Recently, three linear IgE epitopes have been mapped on Pru p 3 [31] and cross-reactive regions have been identified in nsLTPs originating from other *Rosaceae* fruits [32]. Although Api g 2 displays only 53% sequence identity with Pru p 3, it reaches 70% in the region Pru p $3_{71–80}$, the C-terminal linear IgE epitope. In our experiments, Pru p 3 inhibited Api g 2 reactivity up to 60% suggesting involvement of this conserved region. Besides the common epitopes of Api g 2 and peach nsLTP, we also observed IgE cross-reactivity between Api g 2 and mugwort nsLTP, suggesting a possible involvement of these molecules in the celery-mugwort-spice syndrome. The fact that some patients suffering from mugwort pollinosis-associated celery allergy have encountered symptoms even upon consumption of cooked celery [4, 16] would support this idea. Owing to their rigid disulphide-stabilized structure, nsLTPs are highly stable molecules with the ability to elicit severe reactions and sensitize *via* the gastrointestinal tract [33]. In fact, sensitization to Pru p 3 has been shown to be associated with an increased risk of both, prevalence and severity of food allergies [34]. We found that Api g 2 was highly resistant to simulated gastrointestinal digestion and although the primary sequence contains 19 potential pepsin cleavage sites it displayed an enormous stability comparable to that of Pru p 3 [35, 36]. Even upon prolonged proteolysis (48 h of pepsin and 24 h of trypsin/chymotrypsin treatment) intact Api g 2 could be detected. In addition to proteolytic stability, Api g 2 appeared to be extremely resistant to thermal denaturation, reflected by its high melting temperature. Notably, the refolding capacity of Api g 2 vastly depended on the pH of the environment, an observation that has previously also been reported for Pru p 3 [37]. This phenomenon was attributed to the fact that high temperatures at neutral pH might lead to disulphide bond cleavage, which in turn facilitates generation of inter-molecular cysteine-linkages leading to aggregate formation. By contrast, thermal denaturation of mugwort pollen nsLTP was irreversible and independent of the pH [20]. It remains unclear whether this represents an exception or indicates a general difference regarding the stability properties between plant food and pollen nsLTPs. Molecular modelling of Api g 2 showed a similar α-helical content and localization as Pru p 3 [22] and comparable curves were obtained in the previous circular dichroism experiments [37]. Secondary structure estimation programs calculated considerably higher as well as lower values of α-helices (87.6 and 40.3%, respectively), suggesting a limited applicability of these prediction tools for Api g 2.

Interestingly, we did not observe significant differences of IgE binding to heat-treated Api g 2. These findings might be explained by (i) the involvement of linear epitopes and/or (ii) the low protein concentrations (4 μg/mL) used in heat treatment experiments that prevented aggregation and partially or completely enabled refolding. However, protein denaturation at higher concentrations of $\geq$ 1 mg/mL led to a complete or partial loss of IgE reactivity of several nsLTPs (unpublished data). Of note, concentrations of Api g 2 are also low in its natural source and further, it is unclear how processing as well as the celery food matrix influence allergenicity [38]. Our data clearly indicate that Api g 2-sensitized patients are at risk to develop adverse reactions upon consumption of cooked celery stalks. The remarkable stability to gastrointestinal proteolysis and thermal treatment makes Api g 2 a possible trigger of severe allergic reactions [33]. However, the role of Api g 2 in the celery-mugwort-spice syndrome remains to be determined in clinical studies with *A. graveolens* allergic patients. The fact that Api g 2 expression is clearly restricted to celery stalks and previous studies mainly involved Central European patients that are predominantly exposed to celery tuber [5, 26] suggests that other unidentified cross-reactive molecule(s) could be involved in this pollen-food syndrome.

In summary, we could identify a new allergenic member of the nsLTP1 protein family in *A. graveolens* stalks, termed Api g 2.0101. Because of its low abundance in celery extract, we produced Api g 2 as a recombinant protein, which demonstrated equivalent physicochemical and immunological properties as the natural counterpart. Producible at high amounts and devoid of allergenic contaminants, the recombinant molecule represents the favourable alternative. Including rApi g 2 in the current panel of allergens for component-resolved diagnosis will facilitate to determine the clinical relevance of this nsLTP for patients suffering

from adverse reactions upon celery consumption. It will further help the clinician in the management of food allergic patients thus avoiding unnecessary diet restrictions.

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