

RESEARCH ARTICLE

Allergenic relevance of nonspecific lipid transfer proteins 2: Identification and characterization of Api g 6 from celery tuber as representative of a novel IgE-binding protein family

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Scope: *Apium graveolens* represents a relevant food allergen source linked with severe systemic reactions. We sought to identify an IgE-binding nonspecific lipid transfer protein (nsLTP) in celery tuber.

Methods and results: A low molecular weight protein exclusively present in celery tuber was purified and designated Api g 6. The entire protein sequence was obtained by MS and classified as member of the nsLTP2 family. Api g 6 is monomeric in solution with a molecular mass of 6936 Da. The alpha-helical disulfide bond-stabilized structure confers tremendous thermal stability ($T_m > 90^\circ\text{C}$) and high resistance to gastrointestinal digestion. Endolysosomal degradation demonstrated low susceptibility and the presence of a dominant peptide cluster at the C-terminus. Thirty-eight percent of *A. graveolens* allergic patients demonstrated IgE reactivity to purified natural Api g 6 in ELISA and heat treatment did only partially reduce its allergenic activity. No correlation in IgE binding and limited cross-reactivity was observed with Api g 2 and Art v 3, nsLTP1 from celery stalks and mugwort pollen.

Conclusion: Api g 6, a novel nsLTP2 from celery tuber represents the first well-characterized allergen in this protein family. Despite similar structural and physicochemical features as nsLTP1, immunological properties of Api g 6 are distinct which warrants its inclusion in molecule-based diagnosis of *A. graveolens* allergy.

Keywords:

Apium graveolens / Celery tuber / IgE reactivity / Nonspecific lipid transfer protein 2 / Plant food allergy



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1 Introduction

Apium graveolens represents an important plant allergen source in Europe and IgE-reactive molecules can be found in the tuber (celeriac) as well as in the green aerial parts (celery stalks) [1,2]. The vegetable is consumed raw or cooked and its spicy-tasting dried powder is frequently used as an ingredient in soups and convenient food products. Consumption of *A. graveolens* can lead to allergic reactions, from mild oral allergy syndromes, gastrointestinal, skin, and respiratory symptoms to life-threatening anaphylactic reactions [3–5]. Thus,

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Abbreviations: DLS, dynamic light scattering; HPSEC, high-performance size-exclusion chromatography; nsLTP, nonspecific lipid transfer protein

according to the European declaration, the presence of celery in industrially manufactured food products has to be specified on the packing (European Directive 2007/68/EC, amending Directive 2000/13/EC).

At present, four allergens have been identified in *A. graveolens*. Two isoforms of the major allergen Api g 1, a member of the pathogenesis-related (PR)-10 protein family, have been described which are recognized by 59–75% of patients allergic to *A. graveolens* [6–9]. Due to 38–42% sequence identity with Bet v 1, the major allergen of birch pollen, partial IgE cross-reactivity was reported. Further IgE-reactive molecules are Api g 2, representing a member of the nonspecific lipid transfer protein (nsLTP) 1 family [10], Api g 4, belonging to the family of profilins [11], and Api g 5, a flavin adenine dinucleotide containing oxidase [12].

nsLTPs are classified into the family of PR-14 proteins and are characterized by their stable and compact secondary structure that is highly conserved among members throughout the plant kingdom. The proteins are composed of four α -helices stabilized by four disulfide bonds leading to the formation of a central tunnel [13]. Two subfamilies of nsLTPs with a molecular mass of 9 kDa (nsLTP1) and 7 kDa (nsLTP2) have been described. Members of the nsLTP1 family seem to be solely expressed in the aerial part of the plants, whereas nsLTP2s are predominantly, but not exclusively, found in root tissues [14]. In vitro, nsLTPs were shown to bind hydrophobic molecules such as fatty acids, phospholipids, sterols, and glycolipids, and transport the ligand across biological membranes [15, 16]. Compared to members of the nsLTP1 family, nsLTP2s possess a more flexible central hydrophobic cavity resulting in increased lipid transfer activity [17]. Moreover, nsLTP1s are solely capable to link linear molecules, whereas nsLTP2s are also able to bind rigid, voluminous molecules [15]. In vivo, this type of proteins is considered to be involved in plant defense against bacteria and fungi, however, the mechanism of the antimicrobial function still remains unclear [14, 18, 19].

Allergenic members within the nsLTP family are nearly exclusively confined to the nsLTP1 subfamily and as a result of the high structural conservation, IgE cross-reactivity exists between nsLTPs from different plant food and pollen sources [20, 21]. Many of those allergens have been well characterized at the molecular level, e.g. the major peach allergen Pru p 3, Mal d 3 from apple, Cor a 8 from hazelnut, mugwort pollen Art v 3, and the recently described celery nsLTP1 Api g 2 [10, 22–26]. Api g 2 has a remarkably low susceptibility to degradation by gastrointestinal proteases and a notable resistance to thermal treatment [10]. It was shown that 58% of nsLTP-allergic patients from Italy were sensitized to Api g 2 and IgE cross-reactivity with Art v 3 and Pru p 3 was demonstrated [26]. Despite the fact that several proteins belonging to the nsLTP2 family are identified and characterized to date, little is known about their allergenic potential. So far, IgE-reactive nsLTP2s have been suggested in wheat (*Triticum aestivum*) [27] and rape (*Brassica rapa*) [28], but were not

officially acknowledged by the IUIS Allergen Nomenclature Subcommittee.

In this study, we report the identification of a novel allergen from celery tuber that belongs to the nsLTP2 family and was designated Api g 6. The protein was extracted from celeriac tissue, purified to homogeneity, and characterized at the molecular level regarding its structure and stability to thermal and proteolytic treatment. The allergen's IgE-binding capacity and cross-reactivity with Api g 2 and Art v 3 were evaluated using sera from *A. graveolens* allergic patients.

2 Materials and methods

2.1 Patients and sera

Thirty-two patients from Austria were selected based on positive clinical reactivity upon consumption of celery tuber. Specific IgE reactivity was confirmed by prick-to-prick test and/or in vitro diagnosis using *A. graveolens* extracts. Detailed information on patients, clinical symptoms, and IgE reactivity are given in the Supporting Information Table 1. The study was approved by the local ethics committee (EK-10-033-0310) and informed written consents were obtained from the patients.

2.2 Extract preparation of celeriac and celery stalks

Celeriac tuber and celery stalks were extracted with an equal volume of PBS using a homogenizer. Tissue particles were removed by passing through a fine-mashed sieve and extracts were clarified by centrifugation at $20\,000 \times g$ and filtration through a $0.45\text{-}\mu\text{m}$ syringe filter (Millipore, Billerica, MA, USA). Protein extracts were concentrated using a 3 kDa cut-off ultrafiltration membrane (Millipore). For determination of the protein concentration, a Bradford assay was performed according to the Pierce Coomassie Protein Assay kit (Thermo Scientific, Rockford, IL, USA).

2.3 Purification of natural Api g 6

For lab-scale purification, proteins of the clarified celeriac extract were separated by their molecular weight using a 50 kDa cut-off membrane (Sartorius Stedim Biotech, Aubagne Cedex, France). The flow through was diluted with distilled water and adjusted to 5 mM sodium acetate pH 5.3. Api g 6 was purified by cation exchange chromatography using a 1 mL SP FF column (Äkta prime system, GE Healthcare, Chalfont St. Giles, UK). After gradient elution to 50 mM sodium acetate pH 5.3, 1 M NaCl, fractions containing pure Api g 6 were pooled. Using ultrafiltration, the buffer was changed to 5 mM sodium phosphate pH 7.4 and aliquots of Api g 6 were stored at -20°C until further use.

2.4 MS and N-terminal sequencing

MS-based analysis was performed on a Micromass Q-TOF Global Ultima instrument (Waters, Mildford, MA, USA) as described elsewhere [29]. Briefly, intact masses were determined by direct infusion. Peptides obtained from tryptic digestion (ProteoExtract Trypsin Digestion kit, Calbiochem, Gibbstown, NJ, USA) 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. N-terminal sequencing by Edman degradation was accomplished by the protein sequencing service of the Division of Clinical Biochemistry at the University of Innsbruck, Austria.

2.5 Gel electrophoresis

Protein extracts and purified Api g 6 were analyzed by denaturing SDS-PAGE under reducing conditions using 15% gels. Proteins were visualized by staining with CBB R-250 (Bio-Rad, Hercules, CA, USA).

2.6 Purification of Api g 2 and Art v 3

Recombinant mugwort pollen Art v 3 and celery stalks Api g 2 were produced as previously described [10,22]. Briefly, expression of soluble nonfusion proteins in *E. coli* Rosetta-gami B(DE3) pLysS (Novagen, Gibbstown, NJ, USA) was induced with 0.4 mM isopropyl- β -D-thiogalactopyranoside at OD₆₀₀ = 0.7 and cells were grown at 37°C for 3 h. Soluble extracts were obtained and proteins were purified by cation exchange chromatography at pH 5.3 and gradient eluted with sodium chloride. In a final purification step, the proteins were loaded onto a Superdex 75 10/300 GL column (GE Healthcare) in 5 mM sodium phosphate pH 5.3. Fractions containing pure protein were lyophilized after pooling and stored at –20°C.

2.7 Amino acid analysis and circular dichroism spectroscopy

Amino acid composition and protein concentration were determined using the Pico Tag method (Waters). Briefly, the samples were derivatized with phenyl-iso-thiocyanate after gas phase hydrolysis in 6N HCl and analyzed by RP-HPLC (HP 1100, Agilent Technologies, Palo Alto, CA, USA) [10]. Circular dichroism spectra and thermal denaturation were recorded in 10 mM potassium phosphate pH 7.4 or pH 3.0 with a JASCO J-815 spectropolarimeter (Jasco, Tokyo, Japan). Far UV spectra ranging from 190 to 260 nm were monitored at 20 and 95°C and results are represented as mean residue molar ellipticity. During thermal denaturation from 20 to 95°C ($\Delta T = 1^\circ\text{C}/\text{min}$), spectra were recorded at 222 nm in order

to determine the melting point. Subsequently, Api g 6 was gradually cooled to 20°C to evaluate the protein's refolding capability.

2.8 Structure modeling

Models were created by Swiss-Model (<http://swissmodel.expasy.org>) using Prup 3 (PDB: 2ALG) as template for Api g 2 and rice nsLTP2 (PDB: 1L6H) as template for Api g 6. The three dimensional structure alignment was performed and illustrated with UCSF Chimera (<http://www.cgl.ucsf.edu/chimera>).

2.9 Dynamic light scattering and high-performance size-exclusion chromatography

The aggregation behavior of purified natural Api g 6 in solution was investigated by dynamic light scattering (DLS) and high-performance size-exclusion chromatography (HPSEC), detailed protocols are deposited in the Supporting Information.

2.10 Simulated gastrointestinal digestion assay

Gastrointestinal digestion was simulated in vitro as described elsewhere [30]. Briefly, gastric digestion was performed using 50 ng of pepsin (Sigma-Aldrich, St. Louis, MO, USA) per microgram substrate in 0.1 M HCl pH 2.0. For duodenal digestion, the pH of the 120 min gastric digestion was neutralized by addition of ammonium bicarbonate pH 7.8 and incubated with 2.5 ng trypsin and 10 ng chymotrypsin (Sigma-Aldrich) per microgram substrate, respectively. Reactions were conducted at 37°C and degradation was monitored by gel electrophoresis.

2.11 Endolysosomal degradation assay

Monocyte-derived dendritic cells from nsLTP-allergic donors were used for isolation of microsomes and protein degradation according to established protocols [31]. Detailed protocols are deposited in the Supporting Information.

2.12 ELISA and cross-inhibition assay

Maxisorp ELISA plates (Nalge Nunc, Rochester, NY, USA) were coated overnight with 50 μL purified native or heat-treated Api g 6 (15 min at 95°C in 5 mM sodium phosphate pH 7.4) and celeriac extract at a concentration of 4 $\mu\text{g}/\text{mL}$. Plates were washed with 137 mM NaCl, 10 mM Tris, pH 7.5, 0.05% v/v Tween 20 (TBST) and blocked with TBST, 0.5% w/v BSA, for 1 h at room temperature. Patients' sera were diluted 1:4 in TBST, 0.5% w/v BSA, and incubated overnight at 4°C. After washing, bound IgE was detected with alkaline

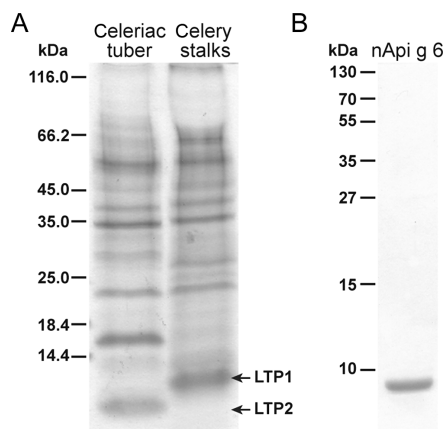


Figure 1. SDS-PAGE of *A. graveolens* extracts and purified Api g 6. Proteins of celery tuber (celeriac) and celery stalks were separated by gel electrophoresis. The migration of the nsLTP1 and nsLTP2 is indicated by arrows at 10 and 7 kDa, respectively (A). Purified natural Api g 6 migrated as single band at 7 kDa (B).

phosphatase-conjugated monoclonal anti-human IgE antibodies (BD Biosciences, Franklin Lakes, NJ, USA). 4-Nitrophenyl dihydrogen phosphate (Sigma-Aldrich) was used as substrate and measurements were performed at OD 405/492 nm. Specific serum IgE levels were calculated based on a quantitative sandwich ELISA using goat anti-human IgE (KPL, Gaithersburg, MD, USA) as catching antibody and different concentrations of purified natural human IgE (Serotec, Düsseldorf, Germany) in TBST. Specific IgE levels were calculated based on a regression curve obtained with the IgE standard. Values were considered positive when exceeding five times SD of controls using TBST, 0.5% w/v BSA instead of serum incubation ($n = 12$). For inhibition assays, sera were preincubated overnight with 100, 1, or 0.01 $\mu\text{g/mL}$ of Api g 2, Api g 6, or Art v 3. OD values of wells using preincubated sera were compared to buffer-treated samples (performed in triplicates) and results are presented as percentage of inhibition.

3 Results

3.1 Identification and purification of a novel nsLTP2 in celeriac tuber

Protein extracts of celeriac tuber and celery stalks were prepared and analyzed by gel electrophoresis. Although the extracts appeared to be rather similar, a striking difference in the lower molecular weight range was observed (Fig. 1A). In contrast to Api g 2, the nsLTP1 from celery stalks, a protein migrating at ~ 7 kDa was detected in celery tuber extracts. In-gel digestion and de novo sequencing of tryptic fragments identified the protein as member of the nsLTP2 family. Notably, MS-based analysis of both extracts demonstrated that expression of Api g 2 was strictly confined to celery stalks,

while the newly identified nsLTP2 was solely present in celeriac tuber.

Celeriac nsLTP2 was purified from the root in sufficient quantities yielding approximately 0.3 mg pure protein per 1000 g raw material. Purity of the protein preparation was demonstrated by SDS-PAGE (Fig. 1B) and MS. The complete amino acid sequence of the mature protein was obtained by de novo and N-terminal sequencing and deposited as P86809 in UniProt (Supporting Information Fig. 1A). Purified celeriac nsLTP2 was detected as single peak presenting 6936.3 Da in mass analysis which is in accordance with the theoretical mass of 6936.7 Da (mass corrected by -8 due to disulfide bond formation). Based on the amino acid sequence, the pI was calculated to be 9.16 (<http://expasy.org/protparam>). The exact concentration was determined by amino acid analysis and amino acid distribution was in agreement with the sequence obtained by MS (data not shown). Since the IgE-binding activity of the celeriac nsLTP2 was unequivocally demonstrated, this molecule was officially acknowledged by the IUIS Allergen Nomenclature Subcommittee and listed as Api g 6.0101.

Api g 6 showed highest sequence identity to an nsLTP2 from *Olea europea* (71%) and sequence alignments to other members of the family are depicted in the Supporting Information Fig. 1B. The nsLTP2s from *B. rapa* [28] and *T. aestivum* [27], representing the only two IgE-reactive members of this family described so far, demonstrated a sequence identity of 49 and 43%, respectively. The novel nsLTP2 showed only 23% sequence identity with Api g 2, the nsLTP1 from celery stalks presenting a conserved region at Api g 6_{21–30} (Fig. 2). Nonetheless, a high degree of similarity was observed at the structural level highlighting that alpha-helical regions are generally arranged in similar positions (Supporting Information Fig. 2).

3.2 Secondary structure and simulated gastrointestinal proteolysis

Circular dichroism spectroscopy under neutral and acidic conditions revealed characteristic spectra for alpha-helical proteins featuring two minima at 209 and 222 nm (Fig. 3A and B). Api g 6 showed a remarkable resistance to heat treatment presenting a melting temperature of $>90^\circ\text{C}$. At physiological pH, far UV spectra suggested a loss of secondary structure upon heating and the original structural elements could not be entirely reconstituted by cooling the protein solution down to 20°C . Under acidic conditions, however, Api g 6 was barely unfolded during thermal denaturation and was able to regain the original spectrum. In simulated gastric digestion, Api g 6 displayed a remarkable resistance to proteolysis, as it was stable up to 48 h of treatment with pepsin (Fig. 3C). Upon duodenal digestion, slight degradation was observed after 2 h, but even after 24-h treatment a weak band was still detectable in gel electrophoresis (Fig. 3D). As a reference, proteolysis of

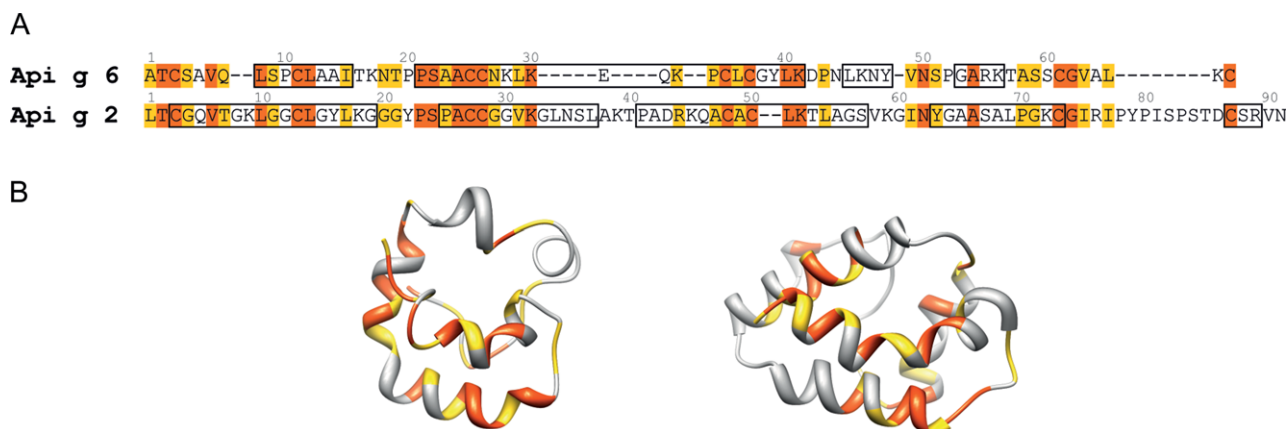


Figure 2. Sequence alignment (A) and three-dimensional models (B) of *A. graveolens* nsLTPs. Api g 6.0101 (UniProt: P86809) and Api g 2.0101 (UniProt: E6Y8S8) were aligned according to Samuel et al. [36] showing a sequence identity of 23%. Identical residues are marked in red, similar residues shown in yellow, and alpha-helical regions are boxed.

Api g 1 was conducted in parallel which was degraded within 5 min of pepsin incubation (data not shown).

3.3 Homogeneity and aggregation behavior in solution

Purified natural Api g 6 appeared as monomeric molecule in solution, as determined by DLS and HPSEC. An R_H

value of 1.2 nm with a polydispersity of 3.6% root square deviation (RSD) corresponding to a molecular mass in the range of 5–6 kDa was determined from the DLS experiments (Fig. 3E). The DLS results were corroborated by an HPSEC analysis, resulting in a single peak eluting at a retention volume typical for proteins of this size (Fig. 3F). The content of aggregates or oligomers as determined by online coupling of HPSEC to light scattering remained <0.5% (data not shown).

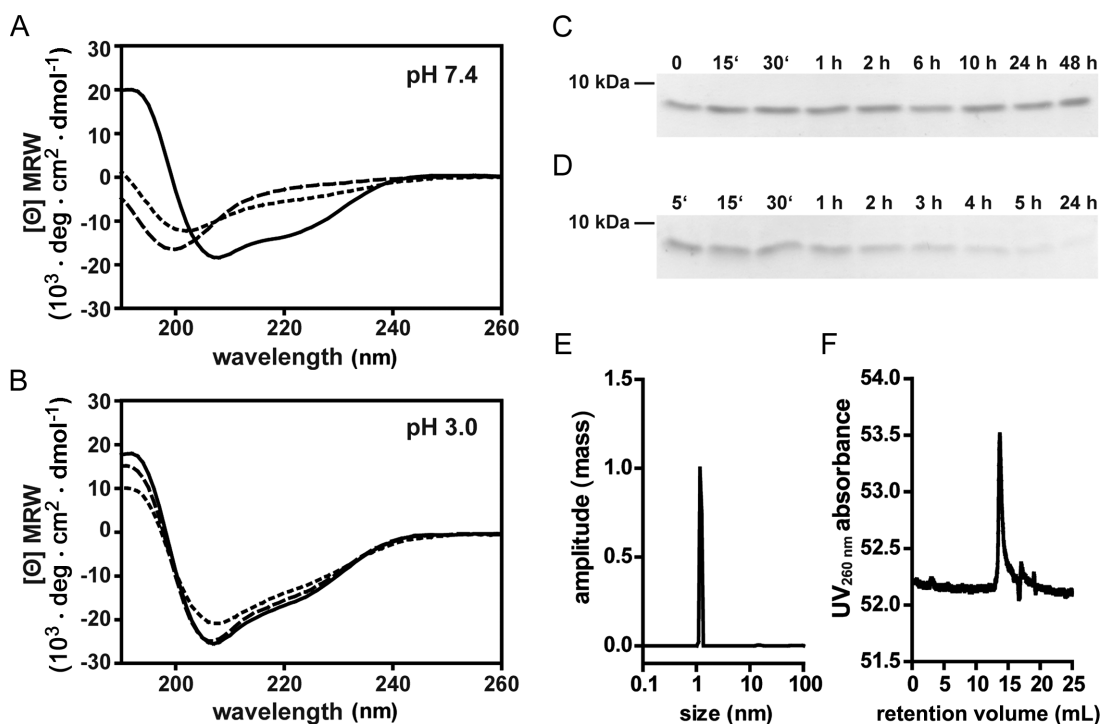


Figure 3. Secondary structure and stability assessment. Far UV spectra of purified natural Api g 6 were recorded at 20°C (solid line), 95°C (dotted line), and 20°C after denaturation (dashed line) at pH 7.4 (A) or pH 3.0 (B). Api g 6 was subjected to simulated gastric digestion with pepsin (C) and duodenal digestion with trypsin and chymotrypsin (D). The aggregation behavior was monitored by DLS (E) and HPSEC (F).

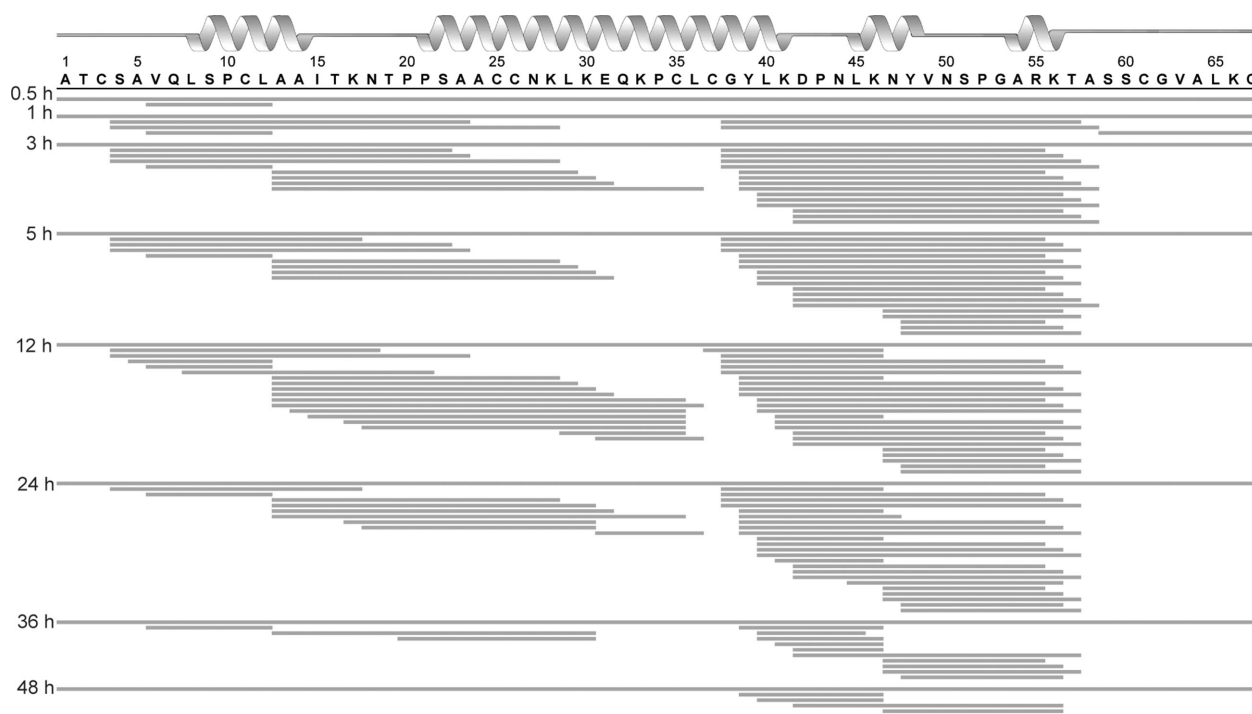


Figure 4. Peptide cluster formation during endolysosomal degradation of Api g 6. The protein was incubated up to 48 h with endolysosomal proteases obtained from dendritic cells and resulting peptides were analyzed by MS. Positions of alpha-helical motifs according to the generated model are shown on top.

3.4 Endolysosomal degradation

Simulated endolysosomal proteolysis with proteases obtained from dendritic microsomes showed that Api g 6 was barely affected by up to 5 h of digestion while it was considerably degraded after 12 h (Supporting Information Fig. 3). Protein degradation was densitometrically evaluated presenting a half-life of 17 h for Api g 6. MS-based analysis of peptide fragments demonstrated the presence of a dominant cluster at Api g 6_{38–58} and two prominent regions around amino acids 4–23 and 13–35 (Fig. 4).

3.5 IgE reactivity to Api g 6 in celery allergic patients

Sera of 32 patients with clinically relevant *A. graveolens* allergy were tested for in vitro IgE reactivity. Twelve patients (37.5%) were sensitized to Api g 6 demonstrating a median IgE level of 18.7 ng/mL to the novel nsLTP2. Heat treatment of Api g 6 resulted in reduced IgE reactivity in sera of nine patients (of those four were negative for heat-treated Api g 6) and a decrease in the median IgE concentration to 13.4 ng/mL was observed (Fig. 5A). Sera of Api g 6 positive patients were additionally analyzed for IgE reactivity to the nsLTP1 from celery stalks and mugwort pollen (Fig. 5B). Notably, only 4 of 12 patients reacted to Api g 2 and Art v 3, and two patients showed additional reactivity solely to Art v 3. Median

IgE levels for Api g 2 and Art v 3 were 7.4 and 10.7 ng/mL, respectively, and thus lower compared to Api g 6. We found no correlation between Api g 6 and Api g 2 ($r = 0.204$) or Art v 3 ($r = 0.123$) specific IgE levels, respectively. In contrast, a statistically significant correlation was found between Api g 2 and Art v 3 ($r = 0.837$; $p < 0.001$).

Cross-inhibition studies using Api g 2 and Art v 3 were performed with sera of four Api g 6 positive individuals (Fig. 5C). One patient was solely sensitized to Api g 6, one showed additionally a positive reaction to Art v 3, and two were sensitized to all three nsLTPs. Api g 6 demonstrated IgE cross-reactivity with Api g 2 and Art v 3 in patients sensitized to all three nsLTPs (P17 and P13). Interestingly, in patient 17, mugwort pollen nsLTP was identified as dominant inhibitor reaching 87.2% at the lowest inhibitor concentration. In contrast, no IgE cross-reactivity was observed in sera of patients reactive to Api g 6 alone or Api g 6 and Art v 3.

4 Discussion

During the past decades, extensive research was conducted regarding the allergenicity of *A. graveolens* as the food is able to elicit severe allergic reactions including anaphylaxis [4, 8, 9, 32]. Although consumption of the raw or cooked root is mainly restricted to Central Europe, celery represents a major ingredient in spice mixtures. The involvement of highly

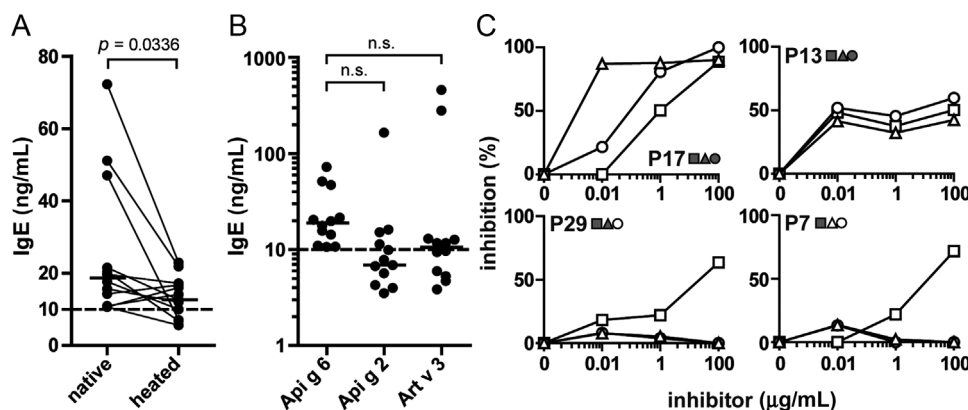


Figure 5. IgE reactivity to native and heated Api g 6 (A), celery stalk, and mugwort pollen nsLTP1 (B) was evaluated in ELISA using sera of 12 Api g 6-sensitized patients. The dashed line represents the cut-off value for positive reactions. Medians are given for each group and statistical evaluation was performed using the Wilcoxon matched pairs signed rank test. IgE cross-inhibition to solid phase coated Api g 6 was determined using purified Api g 6 (square), Api g 2 (circle), and Art v 3 (triangle) (C). Concentration-dependent IgE inhibition is plotted for four individual patients' sera (P7, P13, P17, and P29). Sensitization to the respective nsLTP is given as gray (positive) or open symbols (negative).

stable allergens was suggested to play a role in systemic reactions [8, 33]. So far, the only celery allergen exhibiting considerable resistance to heat treatment and proteolysis is Api g 2 from celery stalks [10], but no linkage between Api g 2 and symptom severity has so far been demonstrated. In this study, the molecular counterpart in celeriac was identified and allocated to the nsLTP2 family. Notably, protein expression of Api g 6 was clearly restricted to celery tuber while Api g 2 was solely found in the aerial stalks, reflecting a tissue localization also found in wheat [14]. The complete amino acid sequence of Api g 6 was obtained by MS and N-terminal sequencing showing a conserved cysteine pattern and sequence identities of up to 71% (olive fruit nsLTP2) with other family members. MS indicated the presence of only one dominant isoallergen. Similarly, only one isoform of nsLTP2 is expressed in common wheat while two different isoforms were identified in an ancient wheat cultivar [34].

The nsLTP2 cysteine pattern and several key amino acids, that is L8, I15, Y48, and V49 shown to be important for stability and lipid binding in rice nsLTP2, are conserved in Api g 6 [35]. The protein contained predominately alpha-helical secondary structures and was found to be monomeric in DLS and HPSEC experiments. Api g 6 showed high resistance to heat treatment, and as judged by the melting temperature, the protein appeared to be even more stable than Api g 2 [10]. Similar observations were made with the nsLTP1 and nsLTP2 from rice and it was suggested to be a result of dissimilar disulfide bond patterns that render family 2 members less vulnerable to denaturation [36]. Acidic conditions favored refolding after exposure to 95°C, a property previously also noticed for nsLTP1 [10, 37]. So far, the three-dimensional structure of nsLTP2 from rice (PDB: 1L6H) and wheat (PDB: 1N89) was resolved by NMR [36, 38]. Previous studies were addressing the structure and in vitro function of nsLTP2, nevertheless little is known about their suscepti-

bility to gastrointestinal proteolysis. Here, we demonstrated a high resistance to gastric and duodenal digestion, similar to results obtained for proteins and allergens of the nsLTP1 family [39, 40]. High thermal and proteolytic stability are typical attributes for class I food allergens and might potentially enable sensitization via the gastrointestinal tract [41, 42].

Simulated endolysosomal digestion can be used to determine protein stability against microsomal proteases, and to identify proteolytic peptides generated by microsomes of antigen presenting cells. Using this approach, it was previously possible to confirm human T-cell epitopes of Pru p 3 [26, 43]. MS revealed a dominant Api g 6 peptide cluster in the C-terminal region, indicating relevant T-cell epitope(s) within this section. The immunogenicity of barley and rape nsLTP2 has previously been demonstrated in rabbits and mice, respectively [34, 44], and the authors suggested that nsLTP2 present strong antigenic structures.

Numerous members of the nsLTP2 family were identified at the amino acid level and some information regarding structure and function were predominately retrieved from rice and wheat nsLTP2 [15, 35, 36, 38]. However, little is known about their potential to bind IgE from allergic individuals. So far, Api g 6 represents the only officially acknowledged allergenic member of the nsLTP2 family. It is noteworthy to mention that Lyc e 3 is also listed as nsLTP2, but based on the length and sequence it might rather be classified as nsLTP1 [45]. In addition to Tri a 14 (nsLTP1) from wheat, skin prick test reactivity to the nsLTP2 was also demonstrated in 20–30% of patients with Baker's rhinitis and/or asthma [27]. Another example can be found in rape since one of three *B. rapa* allergic individuals showed reactivity to a recombinant clone in protein plaque lifts [28]. Conti et al. coeluted an nsLTP2 from apricot pulp while purifying the nsLTP1 but the IgE-binding activity was not evaluated. Since a previous preparation was devoid of the nsLTP2, it was suggested that expression might

be restricted to specific cultivars or selectively turned on in response to environmental stress or pathogens [46].

Generally, there is limited information on nsLTP2 due to the fact that (i) investigations are confined to few members, (ii) studies using purified molecules are scarce, and (iii) in contrast to the family 1 members, they were not investigated for their IgE-binding properties. In this study, we could clearly demonstrate the IgE-binding capacity of Api g 6 using patients' sera reactive to *A. graveolens*. Since we were aware of the fact that minute contaminations with other celeriac allergens could influence the immunological results, only highly pure fractions of natural Api g 6 were used. In addition, a recently obtained recombinant Api g 6 expressed in *E. coli* was shown to have similar IgE-binding properties as the natural allergen. Of note, all Api g 6 positive sera used in this study also displayed IgE reactivity with the recombinant allergen (unpublished data).

Varying performance of commercially available extracts for in vitro diagnosis and skin prick tests is frequently encountered in diagnosis of celery allergies [4]. Notably, Api g 1 is predominately localized in celery roots and nearly absent in stalks (unpublished data), explaining an earlier observation that patients with birch pollen associated celery allergy showed low reactivity to celery stalks [33]. The different tissue distribution of Api g 1, Api g 2, and Api g 6 necessitates the need to standardize the biological sources for extract-based diagnosis. Molecule-based approaches using purified allergens could circumvent this problem and increase diagnostic sensitivity [9].

Based on the sensitization frequency of 37.5%, Api g 6 can be considered a minor allergen. Although the allergen showed lower IgE-binding capacity after thermal treatment, the food matrix might provide a stabilizing effect and thus Api g 6 could play a role in adverse reactions observed to cooked celeriac and/or spice mixtures [8, 33, 47]. Interestingly, the majority of Api g 6 positive patients did not react to Api g 2, suggesting a limited epitope similarity between members of the nsLTP1 and nsLTP2 families [34, 48]. Only 4 of 12 patients were additionally sensitized to Api g 2 and Art v 3, showing partial IgE cross-reactivity in inhibition experiments. Notably, Art v 3 was identified as dominant inhibitor for patient 17 suggesting that this pollen LTP might have acted as primary sensitizer as was previously observed for peach allergic individuals [49]. Allergic reactions to celery are frequently associated with sensitization to birch and mugwort pollen, and hence the terms celery-birch and celery-mugwort-spice syndrome were established [21]. While Api g 1 plays a central role in the celery-birch syndrome, the underlying molecule in the celery-mugwort-spice pollen-food syndrome is not yet unequivocally elucidated. It was noticed previously that patients with confined reactivity to celery and mugwort mainly reacted to celery stalks, which would sustain a possible involvement of Api g 2 and Art v 3 [26, 33]. In contrast, reactivity to Api g 6 seems to be independent of nsLTP1 sensitization and it remains to be clarified if the molecule has the potential to act as type I food aller-

gen or is involved in other, yet unknown cross-reactivity with pollen. Detailed clinical studies with Api g 2 and Api g 6 should elucidate their relevance in a well-characterized cohort of double blind placebo controlled food challenged *A. graveolens* allergic patients [4, 8, 50].

In summary, we identified and characterized Api g 6, a novel allergen from celery tuber. The protein is a member of the nsLTP2 family and its IgE reactivity seemed to be distinct from nsLTP1 frequently found in fruits and pollen. Due to its enormous stability, it exhibited typical features that are characteristic for type I food allergens. The inclusion of Api g 6 in the current panel for component-resolved diagnosis should enhance specificity, sensitivity, and predictive power of *A. graveolens* allergy diagnosis, irrespective of the patients' dietary habits.

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RESEARCH ARTICLE

Allergenic relevance of non-specific lipid transfer proteins 2: Identification and characterization of Api g 6 from celery tuber as representative of a novel IgE-binding protein family

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Supporting Information MATERIALS AND METHODS

Dynamic light scattering and high performance size-exclusion chromatography

The aggregation behaviour of purified natural Api g 6 in solution was investigated by dynamic light scattering (DLS 802, Viscotek Corp., Houston, TX, USA) at a concentration of 0.6 mg/ml. Samples were centrifuged at 14,000 *g* for 10 min and solvent settings for water were used. Data were accumulated for 10 x 10 sec and the correlation function was fitted into the combined data curve, from which the intensity distribution was calculated. The determined intensity distribution was weighted statistically by mass using the mass model for proteins (OmniSize™ software, Viscotek Corp.) displaying molecular size R_H [nm] and polydispersity [% RSD].

High performance-size exclusion chromatography-light scattering (HPSEC) was performed on a HP1100 analytical chromatography system (Hewlett-Packard, San Jose, CA, USA). Chromatography was performed using a dual column setup consisting of 7.8 x 300 mm Ultrahydrogel 250 and 120 columns (Waters, Milford, MA, USA) and samples analyzed at 0.5 ml/min in 100 mM sodium bicarbonate pH 8.3, 150 mM sodium chloride, 0.05% NaN₃. Using a combination of the built-in UV detector measuring at 280 nm and a sequential refractive index (RI), intrinsic viscosity (IV), and right-angle light scattering detection (TDA 302, Viscotek Corp., Houston, TX, USA) the molecular weight (MW) and hydrodynamic radius (R_H) of eluting peaks were determined as previously published [1].

Endolysosomal degradation assay

Monocyte-derived dendritic cells from nsLTP-allergic donors were used for isolation of microsomes and protein degradation according to established protocols [2]. Briefly, cells were harvested by low speed centrifugation, resuspended in 10 mM Tris acetate pH 7.0, 250 mM sucrose and homogenized with a Dounce glass tissue homogenizer. Cellular debris and nuclei were removed by centrifugation at 6000 *g* and microsomes were isolated by ultra-centrifugation at 100,000 *g*. Degradation was performed at 37°C in 100 mM citrate buffer, pH 4.8 and 2 mM dithiothreitol using 7 µg microsomal enzymes for protein

aliquots of 5 µg. Enzymatic reactions were stopped by thermal treatment at 95°C at various time points and analyzed by SDS-PAGE and densitometric quantification of protein bands. Resulting peptides were analyzed by mass spectrometry as previously described [3].

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Supporting Information FIGURE S1

A

ATCSAVQLSPCLAAITKNTPPSAACCNKLKEQKPCLCGYLKDPNLKKNYVNSPGARKTASSCGVALKC

B

	1	10	20	30	40	50	60	%
celeriac	-ATCSAVQLSPCLAAITKNTPPSAACCNKLKEQKPCLCGYLKDPNLKKNYVNSPGARKTASSCGVAL-KC							100
olive	-VTCNPLQLSPCLGAIISGQPPSAACCKLQEQKPCLCGYLKDPNLKKNYVNSPGARKTASSCGVAVPNC							71
birdsfoot trefoil	-VTCSPVALSPCLGAIISSTPPSAACCKLREQKPCLCGYLRNPSLAFYVNSPGARAVASTCGIPYPRC							67
common tobacco	-VTCSPVELSPCAGAITSSPQPPSSACCAKLKEQKPCLCGYLKDPNLKKNYVNSPGARKTASSCGVPTPSC							65
potato	-VTCSPVALSPCLGAIISNSAPSTICCSRIREQKPCLCGYLKDPNLKKNYVNSPGARKTASSCGVPTPSC							65
soybean	-VTCSPVELSPCLGAIISSTPPSTTCQKQVREQPCLCGYLKDPNLKKNYVNSPGARKTASSCGVPTPSC							64
kashgar tamarisk	-VTCSPQLSPCAGAITGSGPPSATCCSRIREQPCLCGYLRDPNLKKNYVNSPGARKTASSCGVPTPSC							62
apricot	-VTCSPVQLSPCLGPIINSAGSPSTTCQKQVREQPCLCGYLKDPNLKKNYVNSPGARKTASSCGVPTPSC							61
black cottonwood	-VTCSPAQLSSCVSAITSSSTPPSKLCCSKIKEQKPCLCGYLKDPNLKKNYVNSPGARKTASSCGVPTPSC							60
common grape wine	-VTCSPLELSSCFAAITSSAPPSMCCSKLREQPCLCGYLRDPNLKKNYVNSPGARKTASSCGVPTPSC							59
sesame	-APCNVVELSPCLAAISGQPPSAACCKLKEQKPCLCGYLKDPNLKKNYVNSPGARKTASSCGVPTPSC							59
barrel medic	-VTCSPVQLSSCVSAITSSSTPPSNLCCSKIKEQKPCLCGYLKDPNLKKNYVNSPGARKTASSCGVPTPSC							58
blue gum	-VTCRPIELSAACVSAITSATPTTILCCSKIKEQKPCLCGYLKDPNLKKNYVNSPGARKTASSCGVPTPSC							56
mouse-ear cress	-VTCSPMQLSPCATAITSSSPPSALCCAKLKEQKPCLCGYLRDPNLKKNYVNSPGARKTASSCGVPTPSC							55
castor oil	-VTCDPVELSACANAITSSSPPSAICCSKILKEQKPCLCGYLKDPNLKKNYVNSPGARKTASSCGVPTPSC							55
cowpea	-VTCNPTLSSCVPAITGGSKPSSTCCSKLKEQKPCLCGYLKDPNLKKNYVNSPGARKTASSCGVPTPSC							55
groundsel	-ATCSVTELMPCSSAFTSSAAPTACCTKLKEQKPCLCGYLKDPNLKKNYVNSPGARKTASSCGVPTPSC							55
Asian rice	--ACDALQLSPCAGAIIGNASPSACCSRMKEQKPCLCGYLRDPNLKKNYVNSPGARKTASSCGVPTPSC							52
maize	VTACDATQLTPCAGAIIGRSPSAACCSRLKEQKPCLCGYLRDPNLKKNYVNSPGARKTASSCGVPTPSC							52
purple false brome	ATTCNALQLSPCAGAIIGNAEPTAGCCSRMKEQKPCLCGYLRDPNLKKNYVNSPGARKTASSCGVPTPSC							52
sorghum	ATACDATQLTPCAGAIIGSSPTAACCSRLKEQKPCLCGYLRDPNLKKNYVNSPGARKTASSCGVPTPSC							52
barley	-ATCNALQLTPCAGAIMGNAAPTASCCSKMKEQKPCLCGYLRDPNLKKNYVNSPGARKTASSCGVPTPSC							50
rape	--ACDPKQLQFCLAAITGGGQPSGDCCAKLKEQKPCLCGYLRDPNLKKNYVNSPGARKTASSCGVPTPSC							49
upland cotton	-VTCNPIELIPCLAAVMSSTPPSETCCSRIREQPCLCGYLRDPNLKKNYVNSPGARKTASSCGVPTPSC							47
common wheat	--ACONSCLAVCASATLSGAKPSGECGCGNTRACOGFCQYAKDETYGQYIRSPHARDTLTSCGLAVPHC							43

Figure S1. Sequence identification and amino acid sequence alignment of Api g 6 and other nsLTP2. Tryptic peptides of Api g 6 were identified by mass spectrometry and N-terminal sequencing and analyzed fragments are represented by solid and dotted lines, respectively (A). The mature sequences of Api g 6 (*Apium graveolens*; P86809) and nsLTP2s from olive (*Olea europea*; fruit; J7FQE3), birdsfoot trefoil (*Lotus japonicus*; tissue not defined; I3SYA6), common tobacco (*Nicotiana tabacum*; leaf trichomes; E3W9R1), potato (*Solanum tuberosum*; fruit; Q8H9B7), soybean (*Glycine max*; fruit; I1N5L5), kashgar tamarisk (*Tamarix hispida*; mixture of roots, stems and leaves; C0KHK5), apricot (*Prunus armeniaca*; fruit; P82353), black cottonwood (*Populus trichocarpa*; leaves; A9P8Y3), common grape wine (*Vitis vinifera*; genomic DNA; D7TSZ9), sesame (*Sesamum indicum*; seeds; A5JU7), barrel medic (*Medicago truncatula*; genomic DNA; B7FM99), blue gum (*Eucalyptus globulus*; tissue not defined; I0IK56), mouse-ear cress (*Arabidopsis thaliana*; tissue not defined; Q9LJQ3), castor oil (*Ricinus communis*; tissue not defined; B9SM06), cowpea (*Vigna unguiculata*; root hairs; Q43681), groundsel (*Senecio odoratus*; tissue not defined; Q41378), Asian rice (*Oryza sativa*; seeds; Q6L4H1), maize (*Zea mays*; tissue not defined; B6UFS4), purple false brome (*Brachypodium distachyon*; genomic DNA; I1HGT0), sorghum (*Sorghum bicolor*; genomic DNA; C5YV13), barley (*Hordeum vulgare*; roots; P93191), rape (*Brassica rapa*; anthers; O64431), upland cotton (*Gossypium hirsutum*; cotton fibers; B6E319) and common wheat (*Triticum aestivum*; leaves and seeds; P82900) were aligned using ClustalW2 on EMBL-EBI (B). Scientific names of plants, the tissues where the proteins have been identified and the accession numbers (UniProt) are given in parentheses. Identity scores for each protein are shown in percent. Amino acids identical to those of Api g 6 are highlighted in red and

similar residues are shown in yellow.

Supporting Information FIGURE S2

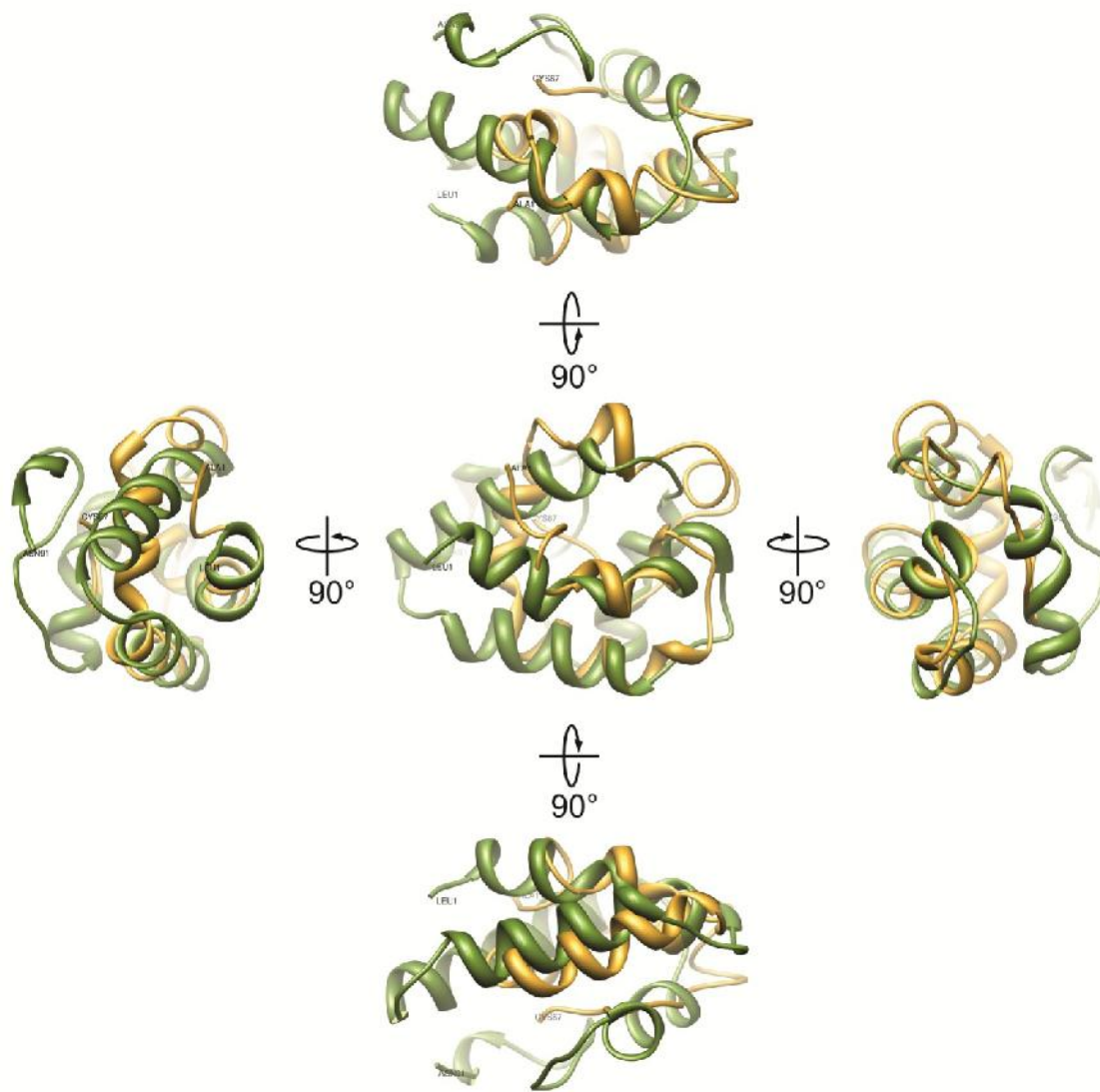


Figure S2. Alignment of three-dimensional structure models of Api g 6 (yellow) and Api g 2 (green). Models were generated using rice nsLTP2 (PDA: 1L6H) as template for Api g 6 and Pru p 3 (PDB: 2ALG) as template for Api g 2. Different orientations of the molecules are depicted to show similar alpha-helical secondary structures.

Supporting Information FIGURE S3

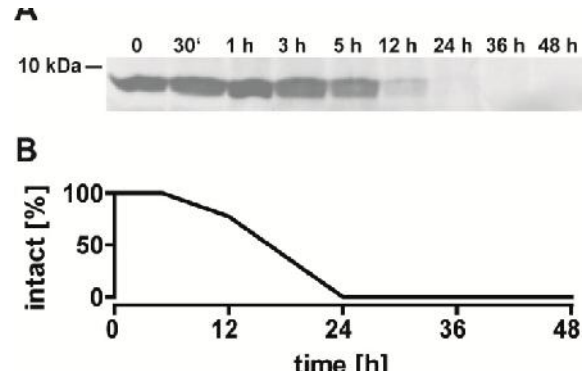


Figure S3. Simulated endolysosomal degradation. Purified natural Api g 6 was incubated with microsomes obtained from monocyte-derived dendritic cells of nsLTP-allergic patients and proteolysis was monitored by gel electrophoresis (A). Degradation of intact Api g 6 was evaluated densitometrically and is given in percent (B).

Supporting Information Table S1.

Patient no.	age	symptoms to celeriac	<i>Apium graveolens</i>			<i>Betula vulgaris</i>			other food allergies
			<i>in vivo</i> IgE reactivity	<i>in vitro</i> IgE reactivity		<i>in vivo</i> reactivity SPT	<i>i n vivo</i> reactivity SPT	<i>in vitro</i> reactivity CAP class	
			SPT (tuber)	S P T (s t a l k s)	SPT (extract)	CAP class	ELISA		
1	28	OAS	++	+	-	nt	-	+	chamomile, mango
2	28	DP, N, OAS, SS **	++	+	-	2	+	-	-
3	41	OAS	nt	n t	+	4	+	-	carrot
4	35	OAS, SA, SS, T	++	+	-	0	-	-	-
5	26	OAS, SA *	++	+	-	0	+	-	honey
6	65	SS	++	n t	nt	nt	++	+	apple
7	43	A, OAS	nt	n t	+	3	+	+	melon, grape, carrot
8	41	OAS, SS	++	+	-	0	+	-	honey
9	37	SS	nt	n t	nt	4	++	-	-
10	56	DP, SS	++	n t	-	nt	-	-	-
11	22	DP, OAS	nt	n t	nt	nt	+	-	-
12	47	OAS, SS	+	-	-	0	+	-	mango
13	21	DP, OAS, RC, SS	++	n t	-	0	+	-	lychee
14	42	OAS	nt	n t	nt	4	+	-	carrot, avocado, banana
15	52	D, GE, HN, N, OAS, SA, SS, V ***	++	n t	-	0	+	-	chamomile, honey, sunflower seeds
16	45	OAS	nt	n t	nt	nt	+	-	carrot
17	29	SS	nt	n t	nt	nt	+	+	apple, peach, hazelnut, carrot, fig, soy, tomato, citrus fruits
18	42	OAS, RC, SS ***	+	-	-	2	++	++	honey, raw potato
19	57	OAS	nt	n t	+	3	++	+	carrot, apple, coconut
20	42	DP, OAS, SS	++	±	-	0	+	-	-
21	35	OAS	++	n t	+	nt	+	++	apple, peach, hazelnut, kiwi, banana, mango, chamomile,

22	38	D, N, SA, SS, V	++	+	-	0	-	-	honey chamomile, honey, mango, pistachio
23	28	DP, HS, OAS, SS **	++	+	-	0	+	-	-
24	62	OAS	nt	n t	+	3	+	+	apple, carrot, kiwi
25	36	DP, OAS, SS **	++	+	-	nt	-	-	honey, mango
26	51	D, DP, OAS, P, T ***	+	±	-	nt	+	-	-
27	49	OAS	nt	n t	+	3	+	+	-
28	41	A, SS, DP, P, V **	++	+	+	2	+	++	apple, peach, hazelnut, fig, chamomile, honey
29	45	SS	++	+	-	0	+	-	honey, cashew nut
30	43	D, HS, OAS, SS, T ***	++	+	-	0	-	-	-
31	32	OAS	nt	n t	-	3	+	-	apple
32	39	OAS, SS	++	+	-	0	+	-	chamomile, honey

SPT, skin prick test; CAP class, ImmunoCAP (Thermo Scientific); ELISA, enzyme-linked immune sorbent assay using in-house prepared extracts; m, male; f, female; +, positive reaction; ++, strong positive reaction; ±, weak positive reaction; nt, not tested; A, anaphylaxis; D, diarrhoea; DP, dispnoea; GE, glossal edema; HN, hypotonia; HS, hypotension; N, nausea; OAS, oral allergy syndrome; RC rhino conjunctivitis; SA, stomach ache; SS, skin symptoms such as angioedema, erythema, exanthema, pruritus or urticaria; P, panic; T, tachycardia; V, vomitus; *, symptoms observed for tuber and stalks; **, patient required hospitalization; ***, patient required emergency treatment.