

pH and Heat Resistance of the Major Celery Allergen Api g 1

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Scope: The major celery allergen Api g 1 is a member of the pathogenesis-related 10 class protein family. This study aims to investigate the impact of heat and pH on the native protein conformation required for Immunoglobulin E (IgE) recognition.

Methods and results: Spectroscopic methods, MS and IgE-binding analyses are used to study the effects of pH and thermal treatment on Api g 1.0101. Heat processing results in a loss of the native protein fold via denaturation, oligomerization, and precipitation along with a subsequent reduction of IgE recognition. The induced effects and timescales are strongly pH dependent. While Api g 1 refolds partially into an IgE-binding conformation at physiological pH, acidic pH treatment leads to the formation of structurally heat-resistant, IgE-reactive oligomers. Thermal processing in the presence of a celery matrix or at pH conditions close to the isoelectric point (pI = 4.63) of Api g 1.0101 results in almost instant precipitation.

Conclusion: This study demonstrates that Api g 1.0101 is not intrinsically susceptible to heat treatment in vitro. However, the pH and the celery matrix strongly influence the stability of Api g 1.0101 and might be the main reasons for the observed temperature lability of this important food allergen.

allergy,^[3,4] but its effectiveness has so far not been clearly established, and further studies on side effects and long-term safety are still required.^[5] In addition, several partly successful attempts have been made to deallergize certain foods by applying oxidative stress^[6–8] or combinations of temperature and high-pressure treatment^[9–11] (reviewed by Houska et al.^[12]).

The elicitors of immunoglobulin E (IgE) mediated food allergies can be grouped into two classes based on their pathophysiological mechanism.^[13] Food allergens that act as a primary sensitization agent and subsequently cause symptoms upon secondary contact are termed class I or classical food allergens.^[13,14] The majority of these allergens are members of only a few protein families such as prolamins, cupins, non-specific lipid transfer proteins, lipocalins, or tropomyosins which generally show high stability against heat processing or digestive enzymes.^[14–17]

1. Introduction

The prevalence of food allergies has significantly increased worldwide in the most-recent three to four decades.^[1] In addition to a notable rise in patient numbers, this development is also reflected in the steadily increasing numbers of publications on food allergy (>1500 in 2017, based on PubMed). Although there has been tremendous progress in the understanding and the diagnosis of the disease, the current management system largely relies on the avoidance of the offending food and on rescue medication to counter severe reactions.^[2] Oral immunotherapy has recently been explored as a new therapeutic approach for food

Food allergy classified as class II requires sensitization against an inhalant class I allergen and subsequent IgE cross reactivity against an allergen which is homologous to the primary sensitizer.^[13] Typical class II food allergens are members of the profilin family and the pathogenesis-related 10 (PR-10) class protein family. Class II food allergens elicit mild oral to systemic reactions and are important in terms of clinical relevance and frequency.^[2] For instance, millions of patients are affected by the major birch pollen allergen Bet v 1,^[18,19] and up to 90% further exhibit IgE-mediated cross reactions to homologous PR-10 class allergens from fruits, vegetables, and nuts.^[20] Several studies have reported on the low stability of PR-10 class allergens against proteolytic digestion or heat treatment and the concomitant reduction or loss of IgE recognition,^[21–27] the key event in immediate-type allergic reactions. The abolishment of cross-reactive IgE binding to PR-10 class epitopes is usually explained by a loss of the native protein fold. This has been experimentally demonstrated by the exchange of critical residues to proline yielding completely unstructured, non-IgE-reactive variants of the PR-10 class allergens from birch,^[28] apple,^[29] cherry,^[30,31] celery,^[32] and carrot.^[33] However, studies on the effects of heat processing or pH treatment on the structural properties of PR-10 food allergens are scarce and rely mostly on circular dichroism (CD)

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spectroscopy.^[21,34] Here we were asking if the loss of IgE-binding, if any, induced by heat treatment is simply a result of irreversible denaturation or if there are other mechanisms of epitope modification like amino acid modification, structural reorganization, or aggregation. The answer to this question will improve our current understanding of PR-10 class allergens and might help to develop new strategies for deallergization trials or the treatment and diagnosis of food allergies.

To analyze the influence of heat processing on PR-10 class food allergens, we chose the major celery allergen Api g 1.0101 (for simplicity referred to as Api g 1) as a model since both the recombinant^[35] and the natural form^[22,23,36] have been experimentally found to be susceptible to thermal treatment.

We uncovered that the loss of conformational IgE epitopes of Api g 1 is not just a result of denaturation but also of irreversible precipitation and oligomerization. The timescales of the induced effects can range from minutes up to hours and depend strongly on the pH and the presence of celery matrix components.

2. Experimental Section

2.1. Protein Preparation

The gene coding for Api g 1.0101 (Uniprot: P49372) was cloned into the bacterial expression vector pET-11a (Novagen) as described elsewhere.^[31,32] Gene expression and protein purification were performed as previously described for Bet v 1.0101^[37] with minor modifications.

After anion exchange chromatography, Api g 1.0101 was further purified via size exclusion chromatography (SEC) using a HiLoad 16/600 Superdex 75 pg column (GE Healthcare) at room temperature in 50 mM sodium phosphate buffer, 50 mM NaCl at pH 7.0, and a flow rate of 0.5 mL min⁻¹. Fractions containing pure Api g 1 were pooled, concentrated, and finally stored at -80 °C after shock freezing. Quantification was performed using the BCA Protein Assay Kit (Pierce, Rockford, USA) or ultraviolet/visible (UV/VIS) spectroscopy using the molar extinction coefficient $\epsilon_{280\text{ nm}} = 5960\text{ M}^{-1}\text{ cm}^{-1}$ derived from the ExPASy Server.^[38]

2.2. Mass Spectrometry

Confirmation of the amino acid sequence of the recombinant Api g 1 and presence of Api g 1 in celery extracts after tryptic digestion were carried out by nano ultra-performance liquid chromatography nano electrospray ionization mass spectrometry (nanoUPLC nanoESI-MS) as described elsewhere^[39] with slight modifications: Peptides were separated using an ACQUITY UPLC M-Class Symmetry C18 Trap Column, 100 Å, 5 µm, 180 µm × 20 mm, 2G, V/M; and ACQUITY UPLC Peptide BEH C18 nanoACQUITY Column 10 Kpsi, 130 Å, 1.7 µm, 100 µm × 100 mm. Data analysis was performed with Protein-Lynx Global Server version 2.4 (Waters), searching the Uniprot database (as of January 2016, restricted to reviewed entries). Protein hits were accepted at a false positive rate of less than 4%.

The sequence identity of recombinant Api g 1.0101 (P49372) was confirmed by MS^E. Twelve tryptic peptides were detected with a mean precursor error of 5.1 ppm, covering 95% of the amino acid sequence resulting in a protein score of 12 043.

2.3. Preparation of Root Celery

Untreated root celery was purchased from a local supermarket and stored at 4 °C until usage. For long-term storage, the plant material was diced, immediately shock-frozen in liquid nitrogen and subsequently freeze-dried (Alpha 2-4 LDplus freeze dryer, Christ). Further processing was performed by using a mixer mill (MM400, Retsch) to homogenize the dry plant material which was finally stored at -20 °C.

2.4. pH and Heat Treatment of Api g 1.0101 and Celery Extracts

Aliquots of recombinant Api g 1 were unfrozen before analysis and dialyzed (Spectra/Por7 MWCO 3500 Da, SPECTRUM labs) against the desired buffer (pH 2: 5 mM citric acid; pH 3: 10 mM sodium citrate; pH 4 and 5: 10 mM sodium acetate; pH 6 and 7: 10 mM sodium phosphate) over night at room temperature. To analyze if the formation of oligomers at acidic pH is reversible, 500 µL Api g 1 (1.1 mg mL⁻¹ from stock at pH 7) were dialyzed against 3 L of 10 mM buffers at pH 2, 3, 4, and 7 (control) overnight and transferred back to 5-L 10 mM sodium phosphate buffer at pH 7 for 5 h using a molecular weight cut off of 3500 Da (Spectra/Por7, SPECTRUM labs).

For analysis of heat-treated recombinant Api g 1, samples were incubated at 95 °C in an elite dry bath incubator (major science) for the required time and centrifuged immediately at 25 °C, 16 100 g for 5 min before further analysis.

Heat treatment of celery extracts was performed by resuspending 10 mg celery powder in each 100 µL of 10 mM sodium phosphate at pH 3, 10 mM sodium phosphate at pH 7, or in water. For SDS/PAGE analysis, 30 µL supernatant before and after 15 min of heat treatment was mixed with 10 µL of 4× SDS sample buffer (Roti-Load1, Roth), incubated at 95 °C for 5 min and centrifuged. For analysis 10 µL of each supernatant was applied to SDS/PAGE (19% gels).

2.5. CD Spectroscopy

All CD measurements were recorded on a Jasco J-1100 spectrometer (Japan Spectroscopic) equipped with a 0.1 cm quartz cuvette (Hellma Analytics) using protein concentrations of 5–10 µM at 25 °C, a 0.1 nm step width, and 1.0 nm bandwidth. We used 15 scans in the range between 260 and 190 nm with 50 nm min⁻¹ scanning speed that were averaged for each measurement, and the resulting spectrum was baseline corrected, smoothed, and normalized using the following formula to calculate the mean residue molar ellipticity $[\theta]_{\text{MRW}}$:

$$[\theta]_{\text{MRW}} = (10\theta_{\text{obs}}) / (cdN)$$

where MRW is the mean residue weight, θ_{obs} is the measured ellipticity in degrees, c is the protein concentration in mol L^{-1} , d is the path length in cm and N is the number of amino acids. Secondary structure content was analyzed by the Jasco spectra manager software using the CONTIN method^[40] and the reference set SMP56.

2.6. NMR Spectroscopy

Standard 1D ^1H spectra with WATERGATE solvent suppression^[41] of 20–100 μM Api g 1 after pH and/or heat treatment containing 0.04% sodium azide and 10% D_2O in 10 mM buffers were recorded on a Bruker Avance 600 MHz spectrometer at 25 °C. All data were processed and visualized with the Bruker spectrometer software TOP SPIN.

For 2D experiments ^1H - ^{15}N -HSQC (heteronuclear single quantum coherence) spectra were recorded at 25 °C on a Bruker Avance 700 MHz spectrometer with cryogenically cooled triple resonance probes equipped with pulsed field-gradient capabilities. 110 μM ^{15}N -labeled Api g 1 in buffer or in 10 mg resuspended celery powder in buffer (550 μL , 50 mM sodium phosphate at pH 7, 50 mM NaCl, and 10 % D_2O) were incubated for 0 min or 15 min at 95 °C. All NMR data were processed using in-house software and were visualized with NMR view.^[42]

2.7. Size Exclusion Chromatography

SEC was performed on a Superdex 75 10/300 GL column (24 mL column volume, GE Healthcare) equilibrated with 10 mM sodium phosphate, pH 7, and 300 mM NaCl at RT using a flow rate of 0.5 mL min^{-1} and a sample loop of 100 μL . The column was calibrated with thyroglobulin (670 kDa to determine the void fraction), conalbumin (75 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and ribonuclease A (13.7 kDa).

For analysis 30–100 μg of Api g 1 were loaded and the elution subsequently monitored at 214 nm. Peak integration and quantification of Api g 1 was performed by the Unicorn software version 5.31 (GE Healthcare).

2.8. Dynamic Light Scattering

Dynamic light scattering (DLS) analysis was used to determine hydrodynamic radii (R_{H}) using a Zetasizer Nano ZS (Malvern, Herrenberg, Germany). 10 μM of Api g 1 in the buffers as above were analyzed at 25 °C. Three individual measurements with at least 10 runs per measurement were carried out.

2.9. Sera Used in this Study

Sera of subjects allergic to birch pollen were purchased from DLab Diagnose GmbH, Hamburg, Germany (Sera 1–3) and from PlasmaLab International, Everett, WA, USA (Sera 4–5). Serum from a non-allergic subject was used as negative control for the specific IgE assays (serum control). Sensitization to Bet v 1.0101,

the major allergen of birch pollen was documented by quantitative determination of Bet v 1.0101-specific IgE using ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden). Specific IgE against Bet v 1.0101 were >100, >100, 62.0, >100, 28.2, and $\leq 0.35 \text{ kU}_\text{A} \text{ L}^{-1}$ for sera 1-5 and control serum, respectively. Specific sensitization against Bet v 1-homologous celery allergen Api g 1 was determined in an IgE immunoblot as described below (Figure S4, Supporting Information).

2.10. IgE Immunoblot Analysis

For immunoblot analysis, 1 μg of rApi g 1 was transferred onto 0.2 μm nitrocellulose membranes by semi-dry blotting at 0.8 mA cm^{-2} for 1 h.^[43] After blocking with Tris-buffered saline (TBS) containing 0.3% Tween 20, blots were incubated overnight at room temperature with 10 μL of human serum in 1 mL TBS containing 0.05% Tween 20 and 0.1% BSA. After extensive washing, blotting membranes were incubated for 1 h with horseradish peroxidase-labeled mouse antihuman IgE antibody (Clone B3102E8, Southern biotech via Biozol, Eching, Germany), diluted to 1:100 000. IgE-binding proteins were visualized by chemiluminescence (LumiGLO Reserve diluted 1:3, KPL via Medac, Wedel, Germany).

2.11. pH-Dependent Competitive IgE-Binding of Api g 1.0101

To test the IgE-binding capacity of Api g 1 compared to non-treated Api g 1 under different pH conditions, Nunc Maxiisorp plates (Fisher Scientific, Schwerte, Germany) were coated overnight at room temperature with 75 ng of purified recombinant Api g 1 in 100 μL of 10 mM potassium phosphate-buffered saline (PBS), pH 7.4. After blocking with PBS containing 2% BSA, plates were incubated with pooled human sera (dilution 1:10) and increasing concentrations (0–1000 ng) of Api g 1 in different buffers containing pH values 2–7 over night at room temperature in PBS containing 0.05% Tween 20 and 0.1% BSA. Allergen-specific human IgE was detected with a horseradish peroxidase-conjugated mouse antihuman IgE antibody (Clone B3102E8, Southern biotech via Biozol, Eching, Germany) diluted 1:1000. As substrate, 3,3',5,5'-tetramethylbenzidine (Roth, Karlsruhe) was used for the horseradish peroxidase. The reaction was stopped with 25% H_2SO_4 and the absorbances at 450 nm and 630 nm were measured. For all data points, absorbances at 630 nm were subtracted from absorbances at 450 nm. Data were fitted with a four parameter sigmoidal curve to determine the concentrations of Api g 1 for half-maximal inhibition (EC_{50}) of IgE binding to immobilized rApi g 1.

2.12. Dot Blot Analysis of IgE-Binding

To compare the IgE-binding capacity of Api g 1 at pH 3 and 7 following heat treatment, we incubated Api g 1 at pH 3 and pH 7 at 95 °C. After 0, 15, 30, 45, and 60 min of heat treatment, 1 μg of the protein was transferred to nitrocellulose and binding of

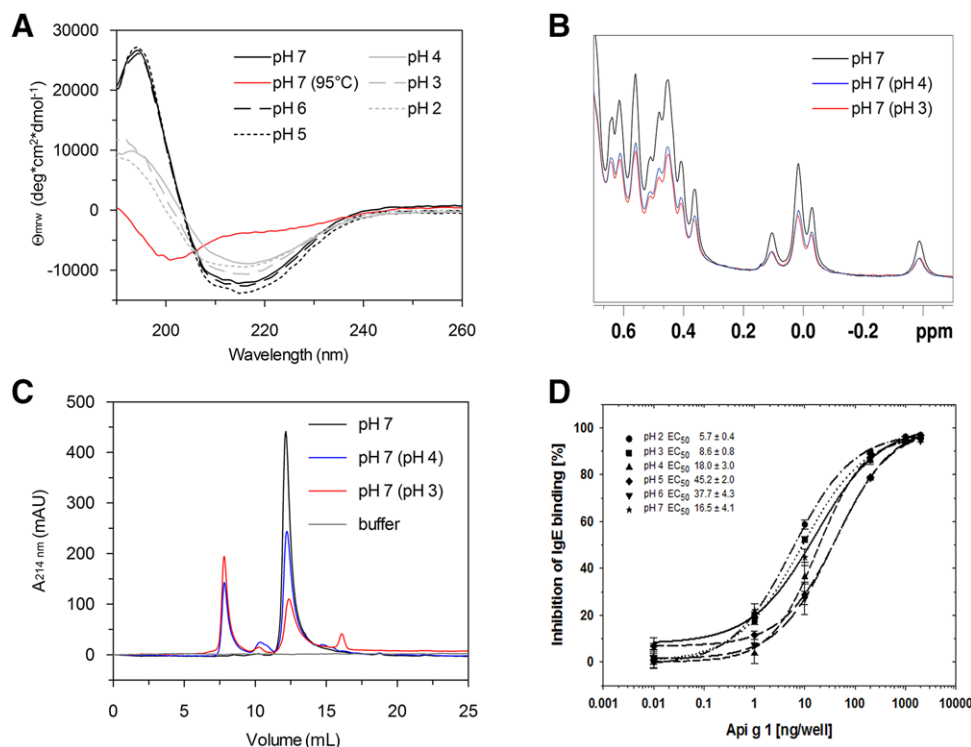


Figure 1. Effect of pH treatment on IgE-binding and structural properties of Api g 1. A) Overlay of the CD spectra of 10 μ M Api g 1 at pH 7, 6, 5 (black continuous, dashed, dotted, respectively), 4, 3, and 2 (gray continuous, dashed, dotted, respectively) at 25 °C and pH 7 at 95 °C (red). B) Api g 1 was incubated at pH 3 (red), 4 (blue), and 7 (black) and transferred back to pH 7 and analyzed by 1D ^1H -NMR spectroscopy and C) analytical size exclusion chromatography. The buffer control is shown in gray. D) Dose-dependent inhibition of IgE-binding to immobilized Api g 1 at pH 2–7. Api g 1 concentrations for half-maximal inhibitions of IgE-binding are indicated.

serum pool IgE was determined as described for the immunoblot analysis before.

3. Results and Discussion

3.1. The Isoelectric Point is a Partly Reversible Structural Switch for Api g 1

Since Api g 1 is exposed to a broad pH range during food processing and in the human digestive system, we initially asked whether the allergen's structure and thus potentially its IgE-binding properties are pH-dependent. In particular, we were interested in the pH 2–7 range to address conditions in the stomach, the duodenum, and the typical celery-containing food matrices like soups, sandwiches, or salad dressings.^[44,45]

At pH 5, 6, and 7, Api g 1 elicits virtually identical NMR spectra with a high degree of signal dispersion and virtually identical CD spectra with an intense positive maximum at 194 nm and a minimum at 216 nm (Figure 1A, Figure S1, Supporting Information) characteristic for proteins with the α -helix and β -sheet secondary structure content typical for proteins with the PR-10 class fold.^[46–48] Hydrodynamic diameters of 3.9 ± 0.2 nm (pH 7) and 4.5 ± 0.1 nm (pH 6) suggest that Api g 1 is monomeric under these pH conditions (Table 1), in agreement with homologous allergens at physiological pH.^[28,48–50] However, in particular

after freezing and thawing at pH 5 the allergen tends to oligomerize (0.8 – 1.2 μ m).

The CD spectra obtained at pH 2, 3, and 4 differ in shape, zero transition, and especially in a reduced positive amplitude compared to the spectra at pH 5–7 (Figure 1A), although the overall shape is conserved. Secondary structure analysis using the CON-TIN method^[40] indicates an increase in β -sheet content and a concomitant loss of α -helical content (Table 1), in line with earlier results with natural Api g 1 and Dau c 1 at acidic pH.^[34] In our hands, the structural rearrangement is accompanied by oligomerization processes as revealed by DLS and corroborated by intense line broadening of resonances in the ^1H -NMR spectra (Table 1 and Figure S1, Supporting Information).

To analyze if the oligomerization process is reversible, Api g 1 was incubated at pH 2, 3, and 4, transferred back to pH 7 and subsequently analyzed by analytical SEC, CD, and NMR spectroscopy. Although soluble at pH 2, Api g 1 completely precipitated after the transfer back to pH 7 and could thus not be analyzed. The 1D ^1H -NMR spectra of all samples are virtually identical concerning the resonance pattern but differ in intensity between the pH 7 control and the samples transferred from pH 3 and 4 (Figure 1B and Table 2) while the CD spectra appear as superposition of the initial spectra, indicating that the secondary structure of monomeric Api g 1 was only partly recovered under the experimental conditions. These data suggest that the oligomers formed at low pH are too large to be observable by NMR spectroscopy and that we only detected the fraction of the

Table 1. Hydrodynamic diameter and secondary structure content of Api g 1.0101 at pH 2–7.

Dynamic light scattering				Circular dichroism			
pH	D _H [nm]	Intensity [%]	Mass [%]	α -helical [%]	β -sheet [%]	β -turn [%]	Unstructured [%]
2	8.5 $\pm$ 2.0	69.3	100	20.0	28.5	21.4	30.2
	>8.5	30.7	0				
3	13.5 $\pm$ 5.2	80.2	100	20.1	30.9	21.8	27.7
	216 $\pm$ 168.8	18.2	0				
	5744 $\pm$ 967.6	1.6	0				
4	54.0 $\pm$ 22.9	22.9	100	18.5	30.9	21.8	28.7
	129.9 $\pm$ 47.9	77.1	0				
5	824.9 $\pm$ 43	33.3	33.3	36.8	18.7	18.0	26.4
	1194 $\pm$ 26.9	66.7	66.7				
6	4.5 $\pm$ 0.1	7.0	99.8	34.0	20.1	18.3	27.7
	393.8 $\pm$ 12.5	30.7	0				
	>393.8	62.3	0				
7	3.9 $\pm$ 0.2	9.5	99.9	31.7	21.6	19.8	27.0
	519.7 $\pm$ 48.7	90.5	0.1				

Table 2. Analysis of Api g 1 at pH 7 after incubation at pH 3 and 4 by SEC and NMR spectroscopy.

Sample	Size exclusion chromatography			NMR spectroscopy
	Elution volume [mL]	Size [kDa]	Peak area [%]	Intensity [%]
Control [pH 7]	12.2	18.7	100	100
Transferred from pH 4	7.83	>75	26	—
	10.3	41.7	6	—
	12.2	18.7	64	$\approx$ 50
Transferred from pH 3	7.8	>75	48	—
	10.2	43.6	2	—
	12.3	17.0	49	$\approx$ 50

recovered monomeric form. To verify those findings, we analyzed the NMR-samples with SEC (Figure 1C). The control elutes at 12.2 mL, which is representative for monomeric Api g 1 (18.7 kDa derived from a standard curve) and in agreement with the hydrodynamic diameter of 3.9 ± 0.2 nm obtained from DLS. The samples transferred from pH 3 and 4 show different elution peaks suggesting molecules with molecular masses >75 kDa, approx. 40 kDa and approx. 18 kDa judged from the standard proteins used for column equilibration (Table 2). The relative fractions of monomeric Api g 1 obtained from SEC and NMR experiments are in good agreement and demonstrate that the oligomerization process at pH 3 and 4 is only partly reversible at physiological pH. It seems that pH conditions around the isoelectric point (pI) of Api g 1 function as a structural switch between the monomeric PR-10 class fold and structured oligomers.

Consequently, we asked if the pH-induced oligomerization of Api g 1 had an effect on IgE recognition of the soluble allergen. We tested competitive IgE-binding between immobilized Api g 1 and soluble Api g 1, which had been incubated in buffers ranging from pH 2 to 7 and transferred back to pH 7 (Figure-1D). Interestingly, pH-treated Api g 1 competed with non-treated

Api g 1 for serum IgE, with only slight differences in the EC₅₀ and up to 98% inhibition of IgE-binding. Thus the pH-treatment per se had no major impact on the IgE-binding capacity of Api g 1.

Only a few studies so far focused on the oligomerization behavior of PR-10 class proteins (reviewed in ref.51). Bet v 1 was proposed to exist as mixture of monomers, dimers, or even higher-order oligomers depending on the buffer composition^[52] and to form concentration dependent, transient dimers.^[50] Furthermore, a dimer-band at approximately 34 kDa was recognized by different groups in IgE-immunoblots for Bet v 1, Api g 1, and Mal d 1.^[53–55] In contrast, several other studies described Bet v 1 as purely monomeric at physiological pH,^[28,49,56,57] in agreement with our findings on Api g 1. However, at pH conditions below the pI, the allergen shows a strong tendency to oligomerize accompanied by changes in the secondary structure composition. Despite the putative loss of α -helices and increase in β -sheets of the oligomer forming Api g 1 units we did not observe a significant reduction in IgE recognition. Oligomerization could even enhance the antibody binding and mediator-release capacities as suggested for variants of Dau c 1^[33] and other allergens.^[50,52]

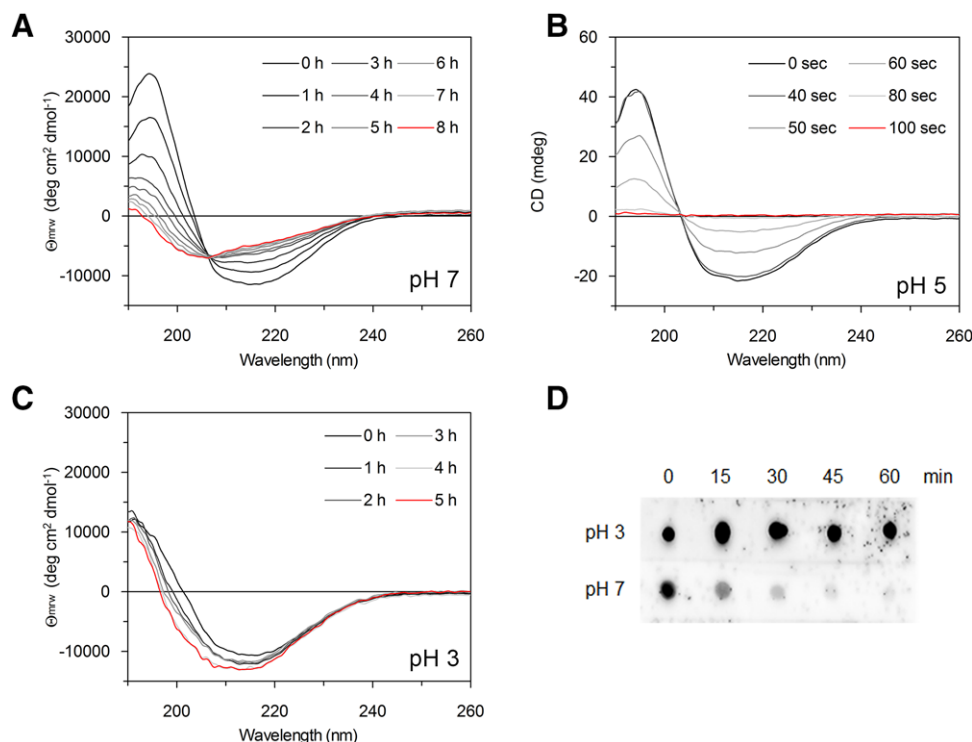


Figure 2. Effect of heat treatment on IgE-binding and secondary structure composition of Api g 1 at different pH values. A) CD spectra of Api g 1 after 0–8 h of cooking recorded at 25 °C at pH 7, at B) pH 5 (0–100 s), and at C) pH 3 (0–5 h). D) IgE binding to immobilized Api g 1 after heat denaturation at pH 3 and 7. Recombinant preparations of Api g 1 were incubated at 95 °C at the times indicated, transferred to nitrocellulose, and analyzed for binding of pooled serum IgE.

Oligomerization capability at acidic pH might be a physiologically important feature of Api g 1. The precise role of PR-10 class proteins is still unknown, but there is evidence that they act as lipid transporters.^[51,58,59] Allergens Fra a 1, 2, and 3 seem to control the flavonoid biosynthesis pathway in strawberry by binding to metabolic intermediates with different affinity and selectivity.^[60] We recently identified the flavonoid quercetin-3-O-sophoroside (Q3OS) as the natural ligand of Bet v 1.0101^[37] in birch pollen. The complexes of Bet v 1.0101:Q3OS and other isoforms with yet unknown ligands^[61] may be involved in UV-protection of the pollen DNA, and Q3OS might stimulate pollen tube formation upon rehydration of the pollen. Binding of celery-containing flavonoids^[62,63] to Api g 1 has so far not been investigated, but it is tempting to speculate that the putative ligand and the corresponding function of the complex is different from those found in pollen or fruits. The structural changes induced by acidic pH are partly reversible and could thus represent an important regulatory mechanism for ligand uptake and release.

3.2. The Stability of Api g 1 During Heat Processing is pH-Dependent

The effect of heat processing on structural properties of Api g 1 was investigated by different groups using mainly thermal denaturation analysis by CD spectroscopy.^[21,32,34] This approach reveals information about the thermal stability of the proteins'

secondary and tertiary structures. The temperature at the mid-point of denaturation determined for Api g 1 at physiological pH was found to be between 70 °C and 73 °C.^[21,32,34,64] Compared to heat-resistant food allergens from other protein families,^[15–17] Api g 1 seems like a heat sensitive protein concerning structural stability. Since heat-processed food is usually consumed or stored at much lower temperature after cooking, we were interested in the refolding properties of Api g 1, in particular, as at least four studies reported the ability of PR-10 class food allergens to refold partially or completely after heating.^[21,53,65,66] Therefore, we incubated the allergen at 95 °C (referred to as heat treatment in the following portions) and performed DLS, CD, NMR measurements and dot blot analysis for IgE-binding after cooling to 25 °C.

Heat treatment at pH 7 and 6 over a period of 8 h and subsequent cooling to 25 °C for 5 min, leads to a continuous reduction of signal intensity in the CD spectra and a shift of maximum and minimum to lower wavelengths (Figure 2A and Figure S2A, Supporting Information), loss of signal dispersion in the NMR-spectra, and oligomerization as indicated by DLS (Figure S3, Supporting Information and Table 3). The CD spectra after 8 h of heat treatment and subsequent cooling to 25 °C are similar to the spectra obtained directly at 95 °C, and the NMR spectra under these conditions are typical for unstructured proteins. Thus, heat processing at physiological pH eventually results in irreversible denaturation of Api g 1.

At pH 4 and 5, the dominant effect of heat treatment on Api g 1 is precipitation due to the low solubility of the protein close to the theoretical pI (Figure 2B, Figure S2B, Supporting

Table 3. Timescales and effects of heat processing on Api g 1.0101 at different pH values in vitro.

pH	Timescale	Dynamic light scattering			CD, NMR, SEC, DLS BCA
		D_H [nm]	Intensity [%]	Mass [%]	
2	4 h	n.a.	n.a.	n.a.	Denaturation
3	5 h	34.0 ± 23.56	97.6	12.4	Oligomerization
		359.1 ± 247.1	2.4	87.5	Precipitation
					Partial denaturation
4	≈60 min	47.0 ± 3.7	1.5	13.1	Oligomerization
		206.2 ± 196	97.5	17.6	Precipitation
		5744 ± 795.7	1	20.5	
5	≈100 s	n.a.	n.a.	n.a.	Precipitation
6	6–8 h	5.9 ± 0.6	1.9	26.6	Oligomerization
		9.3 ± 1.4	7.2	72.5	Precipitation
		118.5 ± 34.1	23.1	0.1	Denaturation
		>118.5	67.8	0.8	
7	6–8 h	5.4 ± 0.9	22.2	34.1	Oligomerization
		9.8 ± 2.1	9.9	65.6	Denaturation
		21.4 ± 3.1	3.2	0.2	
		>21.4	64.7	0	

Information, and Table 3). After 2 min of incubation at 95 °C and subsequent cooling to 25 °C at pH 5 and approximately 60 min at pH 4, the CD spectra are undistinguishable from the buffer control and protein concentrations were below the detection limit of the BCA assay. The observable precipitate could not be significantly resolubilized, thus heating at pH values close to the pI of Api g 1 results in almost complete irreversible precipitation.

Heat resistance of Api g 1 at pH values below 4.4 in HCl, glycine, and sodium acetate buffers has been reported earlier.^[34] We also found that prolonged heat-processing at pH 3 resulted only in slight changes of the CD spectra using sodium citrate buffer (Figure 2C). The CD spectrum after 5 h of heat treatment and subsequent cooling to 25 °C is still clearly different from denatured Api g 1 recorded directly at 95 °C and pH 7 while the hydrodynamic diameter obtained from DLS measurements revealed the formation of larger oligomers (Table 3). The NMR spectrum after 5 h heating and subsequent cooling to 25 °C is typical for a completely unstructured protein suggesting that at least partial denaturation occurred under these conditions (Figure S3, Supporting Information). In contrast, using sodium citrate buffer at pH 2, we observed complete denaturation within 4 h in the CD- and NMR-spectra (Figure S2C, Supporting Information), suggesting that the heat resistance at pH 3 is not a formulation effect of sodium citrate but actually typical for the pH value.

We wondered whether the allergen stability and the structural changes observed by CD spectroscopy at pH 3 would correlate with its capacity to bind serum IgE. Since we observed the highest stability of Api g 1 against heat processing around pH 3, we incubated Api g 1 at 95 °C at either pH 3 or 7 and compared IgE-binding in dot blot analysis after cooling to 25 °C (Figure 2D).

Whereas IgE-binding was abolished in a time-dependent manner after heat treatment at pH 7, no reduction in IgE recognition was observed with heat-treated Api g 1 at pH 3. Thus the remarkable physicochemical heat stability at pH 3 correlated well with the capacity of the allergen to interact with serum IgE. However prolonged heating at pH 3 finally abolished IgE recognition by Api g 1, a phenomenon we also observed for the other pH conditions analyzed herein (data not shown).

Our data demonstrate that Api g 1's structural stability is strongly pH-dependent. Similar characteristics have been previously described for the kiwi allergens Act d 1 and Act d 2.^[67] At physiological pH, Api g 1 unfolds during incubation at 95 °C, but we observed that the allergen partially refolds into the IgE-reactive PR-10 class fold after cooling. Indeed, Ballmer-Weber and coworkers reported on one patient (out of 12 patients) exclusively sensitized to Api g 1 who reacted to a high dose of boiled celery.^[22] The authors suggested that low amounts of Api g 1 were not deactivated by boiling, which would be in line with our present findings.

In contrast to physiological pH conditions, Api g 1 precipitates irreversibly upon heating at a pH range close to the pI on a timescale of minutes. Although we have not addressed the IgE-binding properties of the emerging precipitate, one may assume that the number of accessible epitopes is certainly reduced in the precipitate. Likewise, Urisu et al.^[68] have reported that heated and insoluble aggregated egg white allergens did not elicit allergic symptoms in patients who otherwise had shown reactions to raw egg white. Irreversible precipitation upon heating might be the reason why many of the Bet v 1 homologous food allergens are characterized as heat labile since the pH of many fruits, vegetables, or allergen-containing foods is acidic^[44,69] and the theoretical isoelectric points of PR-10 class allergens cluster in a small pH range from approximately 4.6–6.2, potentially leading to fast precipitation upon heat processing.

An outstanding phenomenon is the heat resistance and IgE reactivity of Api g 1 oligomers formed at pH values below the pI. It seems those oligomers show typical properties of class I food allergens. For instance, oligomers of the major almond allergen amandin are not affected by temperatures below 90 °C while the monomeric units showed lower thermal stability.^[70] Oligomerization might also contribute to the thermal stability and allergenicity of certain parvalbumins.^[71] To our knowledge, the influence of heat in combination with acidic pH treatment on the allergenicity and mediator-release capacity of Api g 1 has never been tested. It would be interesting to analyze how acidic pH-treated, natural Api g 1 would perform in double-blind placebo-controlled food challenge experiments and cell mediator release assays after heat processing of celery.

3.3. The Solubility of Api g 1 is Drastically Reduced during Heat Treatment in Celery Extracts

After the in vitro experiments, we were also interested in the stability of Api g 1 in celery extracts. Previous studies suggest that the allergenicity and in vitro IgE recognition of natural Api g 1 is strongly reduced after several minutes of heat treatment.^[22,23] To monitor the fate of recombinant Api g 1 in celery extracts we

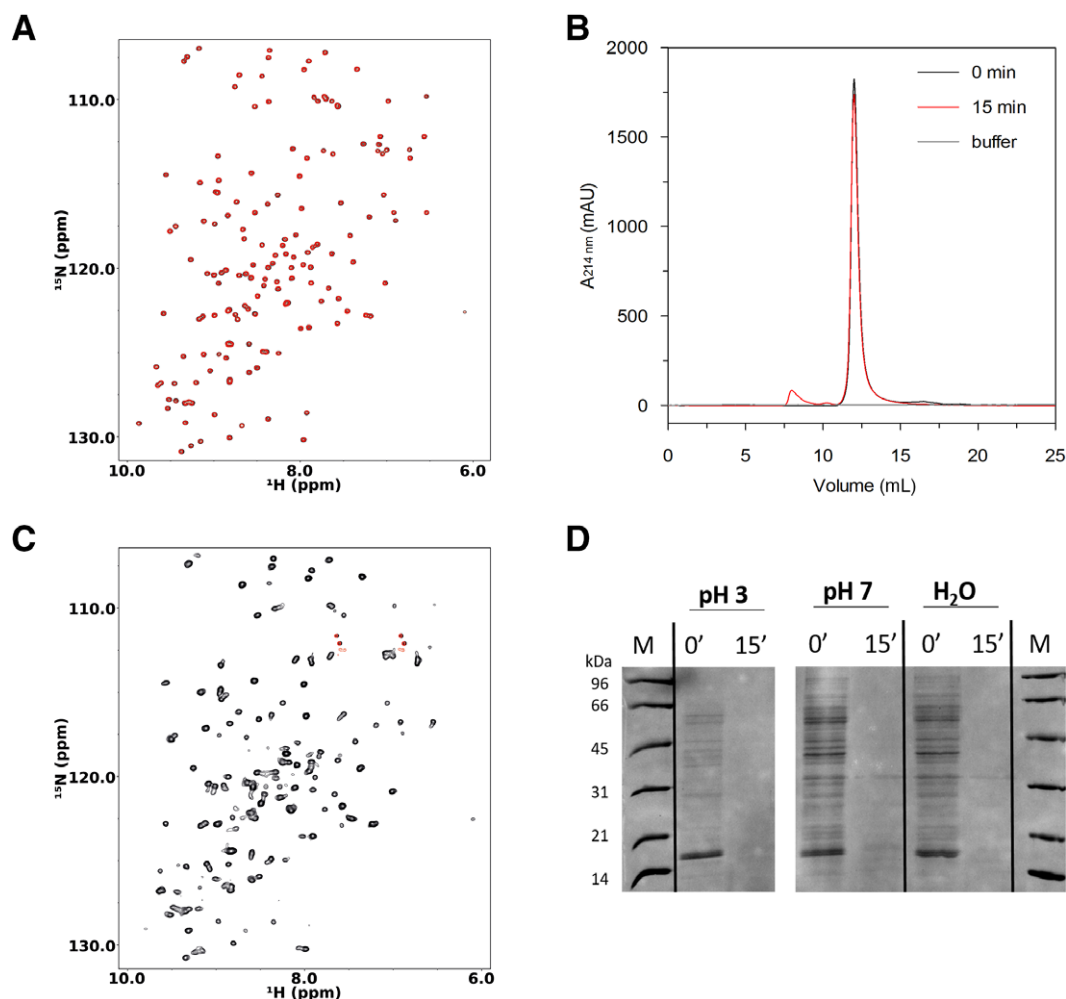


Figure 3. Analysis of heat induced effects on ^{15}N -labeled rApi g 1.0101 or natural Api g 1 in celery extract. A) Overlay of the ^{15}N -HSQC spectra of 100 μM Api g 1.0101 in 50 mM sodium phosphate, pH 7, and 50 mM NaCl before (black) and after 15 min of heat treatment at 95 $^{\circ}\text{C}$ (red). B) Analysis of the NMR samples (each 100 μg) before (black) and after heating (red) by analytical SEC. C) Overlay of the ^{15}N -HSQC spectra of 100 μM Api g 1.0101 in 50 mM sodium phosphate, pH 7, and 50 mM NaCl before (black) and after 15 min of heat treatment at 95 $^{\circ}\text{C}$ (red) in approx. 18.2 mg mL^{-1} celery extract. D) Supernatants of celery extracts at pH 3, pH 7, and in water before (0') and after 15 min of heat treatment (15') analyzed by SDS/PAGE marker (M). The prominent band at approximately 17 kDa at 0 min represents a mixture of natural Api g 1 (Api g 1.0101 and Api g 1.0201), calmodulin, keratin, and superoxide dismutase as identified by mass spectrometry.

recorded ^1H - ^{15}N -HSQC spectra before and after 15 min of heat treatment in sodium phosphate buffer at pH 7 (control) or in buffered celery extracts at pH 7. The spectra of ^{15}N -labeled Api g 1 in this buffer are virtually identical before and after heating (Figure 3A) which demonstrates that Api g 1 refolds successfully into the PR-10 fold after a rather short incubation of 15 min at 95 $^{\circ}\text{C}$ and subsequent cooling. The slight difference in intensity is a result of heat-induced oligomerization as demonstrated by SEC (Figure 3B). The overall spectrum of Api g 1 in celery extract is similar to the control spectrum, but several resonances show reduced intensities or chemical shift perturbations, probably resulting either from modification or interaction with other celery components. Surprisingly, after 15 min of heat treatment in the extract almost all signals were lost (Figure 3C). We thus further analyzed supernatants of untreated and heat-treated (15 min) celery extracts at pH 3, pH 7, or in water ($\approx$ pH 6) using SDS-PAGE and

mass spectrometry. We found that all soluble proteins strongly precipitate upon heating in every condition (Figure 3D), however natural Api g 1.0101 could still be identified in all supernatants after heat processing by mass spectrometry (Table 4). These results demonstrate that the presence of other celery components during heat processing has a strong influence on the solubility of Api g 1 and, although we have not tested all pH conditions from 2 to 7, it seems that this effect is independent of the pH value.

The impact of a food matrix on allergens has been recognized before (reviewed by [14]). The effects are versatile and have been shown to either enhance^[72,73] or reduce^[74,75] the stability or IgE-binding capacity of different food allergens. Api g 1 might bind to other celery proteins or small molecules and coprecipitate during heating. A similar effect has been observed for the egg white allergen ovomucoid when heated together with wheat gluten.^[74]

Table 4. Identification of Api g 1.0101 in supernatants of extracted celery powder (10% w/v) with or without 15 min of heat treatment in 10 mM sodium citrate pH 3, 10 mM sodium phosphate pH 7, or in water.

Condition	S	N	SQ	E
pH 3; 95 °C	4306.4	9	53.9	5.0
pH 3; RT	5390.3	10	88.3	6.0
pH 7; 95 °C	3230.9	7	48.7	4.8
pH 7; RT	9468.9	11	81.2	6.7
H ₂ O; 95 °C	2163.1	7	48.7	7.1
H ₂ O; RT	10 019.0	10	81.2	6.0

S, protein score; N, number of tryptic peptides detected; SQ, sequence coverage in percentage; E, mean precursor mass error in ppm.

4. Concluding Remarks

Heat and pH treatment are probably the most common steps in processing of celery or celery-containing food. Any processing step that influences the structural integrity of an allergen might potentially change its IgE recognition and response by the immune system. Here we indeed observed a strong pH dependence regarding the conformation, solubility, and IgE recognition of the major celery allergen Api g 1.0101 during heat treatment in vitro. Our data are in line with other studies on the impact of heat or pH treatment on food allergens^[67,76] and suggest that the sensitization and IgE-binding profile of an untreated recombinant allergen is not adequate to fully predict its allergenic potency in processed food. Here we further observed that heating in the presence of other celery components leads to immediate precipitation regardless of the pH. While the presence of a celery matrix during heat treatment appears to have a destabilizing effect, which probably explains the previously observed heat lability of Api g 1, recent studies have reported enhancing effects of food matrices regarding sensitization,^[76] degranulation capacity,^[77,78] or T-cell immunogenicity^[79] on other allergens.

We conclude that the impact of a food matrix and of any processing step (pH, heating, freezing, or freeze-drying) should be considered when analyzing the potency of an allergenic protein to further improve the current diagnosis and treatment system of food allergies.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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data, and wrote the manuscript. D.S. initiated and supervised all IgE-binding and dynamic light scattering analyses, analyzed the data, and wrote the manuscript. T.S. analyzed and evaluated mass spectrometry data. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare no conflicts of interest.

Keywords

celery allergen Api g 1, heat treatment, IgE binding, pH treatment, PR-10 class folds

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- [1] H. A. Sampson, *Chem. Immunol. Allergy* **2015**, *101*, 1.
- [2] M. Fernandez-Rivas, *Chem. Immunol. Allergy* **2015**, *101*, 162.
- [3] C. A. Keet, P. A. Frischmeyer-Guerrero, A. Thyagarajan, J. T. Schroeder, R. G. Hamilton, S. Boden, P. Steele, S. Driggers, A. W. Burks, R. A. Wood, *J. Allergy Clin. Immunol.* **2012**, *129*, 448.
- [4] K. Blumchen, H. Ulbricht, U. Staden, K. Dobberstein, J. Beschoner, L. C. de Oliveira, W. G. Shreffler, H. A. Sampson, B. Niggemann, U. Wahn, K. Beyer, *J. Allergy Clin. Immunol.* **2010**, *126*, 83.
- [5] S. Sato, N. Yanagida, M. Ebisawa, *Chem. Immunol. Allergy* **2015**, *101*, 106.
- [6] P. V. Novotna, I. I. Setinova, M. F. Heroldova, M. V. Kminkova, J. V. Pruchova, J. V. Strohalm, V. V. Fiedlerova, R. V. Winterova, P. F. Kucera, M. V. Houska, *Czech J. Food Sci.* **2011**, *29*, 190.
- [7] S. Y. Chung, E. T. Champagne, *J. Agric. Food Chem.* **2007**, *55*, 9054.
- [8] S. Chung, Y. Kato, E. T. Champagne, *J. Sci. Food Agric.* **2005**, *85*, 2631.
- [9] M. Houska, M. Kminkova, J. Strohalm, I. Setinova, M. Heroldova, P. Novotna, S. Honzova, H. Vavrova, P. Kucera, A. Proskova, *High Pressure Research* **2009**, *29*, 686.
- [10] I. Setinova, M. Kminkova, J. Strohalm, M. Heroldova, P. Novotna, S. Honzova, H. Vavrova, P. Kucera, A. Proskova, M. Houska, *High Pressure Research* **2009**, *29*, 680.
- [11] M. Heroldova, M. Houska, H. Vavrova, P. Kucera, I. Setinova, S. Honzova, M. Kminkova, J. Strohalm, P. Novotna, A. Proskova, *High Pressure Research* **2009**, *29*, 695.
- [12] M. Houska, I. Setinova, P. Kucera. In *Advances in Food Process Engineering Research and Applications* (Eds: S. Yanniotis, P. Taoukis, N. G. Stoforos, V. T. Karathanos), Springer, Boston, MA, **2013**, pp. 291–337.
- [13] T. Moriyama, *J. Nutr. Sci. Vitaminol. (Tokyo)* **2015**, *61*(Suppl), S106.
- [14] A. R. Lorenz, S. Scheurer, S. Vieths, *Chem. Immunol. Allergy* **2015**, *101*, 18.
- [15] K. Lehmann, K. Schweimer, G. Reese, S. Randow, M. Suhr, W. M. Becker, S. Vieths, P. Rosch, *Biochem. J.* **2006**, *395*, 463.
- [16] A. Taheri-Kafrani, J. C. Gaudin, H. Rabesona, C. Nioi, D. Agarwal, M. Drouet, J. M. Chobert, A. K. Bordbar, T. Haertle, *J. Agric. Food Chem.* **2009**, *57*, 4974.
- [17] A. I. Sancho, N. M. Rigby, L. Zuidmeer, R. Asero, G. Mistrello, S. Amato, E. Gonzalez-Mancebo, M. Fernandez-Rivas, R. van Ree, E. N. Mills, *Allergy* **2005**, *60*, 1262.
- [18] E. Jarolim, H. Rumpold, A. T. Endler, H. Ebner, M. Breitenbach, O. Scheiner, D. Kraft, *Allergy* **1989**, *44*, 385.

- [19] G. D'Amato, L. Cecchi, S. Bonini, C. Nunes, I. Annesi-Maesano, H. Behrendt, G. Liccardi, T. Popov, P. van Cauwenberge, *Allergy* **2007**, 62, 976.
- [20] M. Geroldinger-Simic, T. Zelniker, W. Aberer, C. Ebner, C. Egger, A. Greiderer, N. Prem, J. Lidholm, B. Ballmer-Weber, S. Vieths, B. Bohle, *J. Allergy Clin. Immunol.* **2011**, 127, 616.e1.
- [21] B. Bohle, B. Zwolfer, A. Heratizadeh, B. Jahn-Schmid, Y. D. Antonia, M. Alter, W. Keller, L. Zuidmeer, R. van Ree, T. Werfel, C. Ebner, *J. Allergy Clin. Immunol.* **2006**, 118, 242.
- [22] B. K. Ballmer-Weber, A. Hoffmann, B. Wuthrich, D. Luttkopf, C. Pompei, A. Wangorsch, M. Kastner, S. Vieths, *Allergy* **2002**, 57, 228.
- [23] A. Jankiewicz, W. Baltes, K. W. Bögl, L. I. Dehne, A. Jamin, A. Hoffmann, D. Hausteine, S. Vieths, *J. Sci. Food Agric.* **1997**, 75, 359.
- [24] D. Mittag, J. Akkerdaas, B. Ballmer-Weber, L. Vogel, M. Wensing, W. Becker, S. J. Koppelman, A. C. Knulst, A. Helbling, S. L. Hefle, R. van Ree, S. Vieths, *J. Allergy Clin. Immunol.* **2004**, 114, 1410.
- [25] E. A. Pastorello, S. Vieths, V. Pravettoni, L. Farioli, C. Trambaioli, D. Fortunato, D. Luttkopf, M. Calamari, R. Ansaloni, J. Scibilia, B. K. Ballmer-Weber, L. K. Poulsen, B. Wutrich, K. S. Hansen, A. M. Robino, C. Ortolani, A. Conti, *J. Allergy Clin. Immunol.* **2002**, 109, 563.
- [26] U. Müller, D. Luttkopf, A. Hoffmann, A. Petersen, W. M. Becker, F. Schocker, B. Niggemann, F. Altmann, D. Kolarich, D. Hausteine, S. Vieths, *Eur. Food Res. Technol.* **2000**, 212, 2.
- [27] M. Venkatachalam, S. S. Teuber, K. H. Roux, S. K. Sathe, *J. Agric. Food Chem.* **2002**, 50, 3544.
- [28] F. Husslik, K. M. Hanschmann, A. Kramer, C. Seutter von Loetzen, K. Schweimer, I. Bellinghausen, R. Treudler, J. C. Simon, L. Vogel, E. Volker, S. Randow, A. Reuter, P. Rosch, S. Vieths, T. Holzhauser, D. Schiller, *PLoS One* **2015**, 10, e0132956.
- [29] D. Y. Son, S. Scheurer, A. Hoffmann, D. Hausteine, S. Vieths *Eur. J. Nutr.* **1999**, 38, 201.
- [30] S. Scheurer, D. Y. Son, M. Boehm, F. Karamloo, S. Franke, A. Hoffmann, D. Hausteine, S. Vieths, *Mol. Immunol.* **1999**, 36, 155.
- [31] P. Neudecker, K. Lehmann, J. Nerkamp, T. Haase, A. Wangorsch, K. Fotisch, S. Hoffmann, P. Rosch, S. Vieths, S. Scheurer, *Biochem. J.* **2003**, 376, 97.
- [32] A. Wangorsch, B. K. Ballmer-Weber, P. Rosch, T. Holzhauser, S. Vieths, *Mol. Immunol.* **2007**, 44, 2518.
- [33] G. Reese, B. K. Ballmer-Weber, A. Wangorsch, S. Randow, S. Vieths, *J. Allergy Clin. Immunol.* **2007**, 119, 944.
- [34] M. A. Bollen, H. J. Wichers, J. P. F. G. Helsper, H. F. J. Savelkoul, M. A. J. S. Boekel, *Food Chem.* **2010**, 119, 241.
- [35] B. Bohle, *Clin. Rev. Allergy Immunol.* **2006**, 30, 97.
- [36] A. Jankiewicz, H. Aulepp, W. Baltes, K. W. Bögl, L. I. Dehne, T. Zuberbier, S. Vieths, *Int. Arch. Allergy Immunol.* **1996**, 111, 268.
- [37] C. Seutter von Loetzen, T. Hoffmann, M. J. Hartl, K. Schweimer, W. Schwab, P. Rosch, O. Hartl-Spiegelhauer, *Biochem. J.* **2014**, 457, 379.
- [38] E. Gasteiger, C. Hoogland, A. Gattiker, S. Duvaud, M. R. Wilkins, R. D. Appel, A. Bairoch. In *The Proteomics Protocols Handbook* (Ed: J. M. Walker), Humana Press, New York, **2005**, pp. 571–607.
- [39] J. Spiric, A. M. Engin, M. Karas, A. Reuter, *PLoS One* **2015**, 10, e0142404.
- [40] S. W. Provencher, J. Glöckner, *Biochemistry* **1981**, 20, 33.
- [41] M. Piatto, V. Saudek, V. Sklenar, *J. Biomol. NMR.* **1992**, 2, 661.
- [42] B. A. Johnson, R. A. Blevins, *J. Biomol. NMR.* **1994**, 4, 603.
- [43] J. Kyhse-Andersen, *J. Biochem. Biophys. Methods.* **1984**, 10, 203.
- [44] M. A. Bridges, M. R. Mattice, *Am. J. Dig. Dis.* **1939**, 6, 440.
- [45] L. Ovesen, F. Bendtsen, U. Tage-Jensen, N. T. Pedersen, B. R. Gram, S. J. Rune, *Gastroenterology* **1986**, 90, 958.
- [46] M. Gajhede, P. Osmark, F. M. Poulsen, H. Ipsen, J. N. Larsen, R. J. Joost van Neerven, C. Schou, H. Lowenstein, M. D. Spangfort, *Nat. Struct. Biol.* **1996**, 3, 1040.
- [47] T. Schirmer, K. Hoffmann-Sommergrube, M. Susani, H. Breiteneder, Z. Markovic-Housley, *J. Mol. Biol.* **2005**, 351, 1101.
- [48] C. Seutter von Loetzen, K. Schweimer, W. Schwab, P. Rösch, O. Hartl-Spiegelhauer *Biosci. Rep.* **2012**, 32, 567.
- [49] E. Nony, J. Bouley, M. Le Mignon, P. Lemoine, K. Jain, S. Horiot, L. Mascarell, M. Pallardy, R. Vincentelli, P. Leone, A. Roussel, T. Batard, K. Abiteboul, B. Robin, O. de Beaumont, M. Arvidsson, S. Rak, P. Moingeon, *Allergy* **2015**, 70, 795.
- [50] J. Rouvinen, J. Jänis, M. Laukkanen, S. Jylhä, M. Niemi, T. Päävinen, S. Mäkinen-Kiljunen, T. Haahtela, H. Söderlund, K. Takkinen, *PLoS One* **2010**, 5, e9037.
- [51] H. Fernandes, K. Michalska, M. Sikorski, M. Jaskolski, *FEBS J.* **2013**, 280, 1169.
- [52] I. Schöll, N. Kalkura, Y. Shedziankova, A. Bergmann, P. Verdino, R. Knittelfelder, T. Kopp, B. Hantusch, C. Betzel, K. Dierks, O. Scheiner, G. Boltz-Nitulescu, W. Keller, E. Jensen-Jarolim, *J. Immunol.* **2005**, 175, 6645.
- [53] Y. Ma, G. Gadermaier, B. Bohle, S. Bolhaar, A. Knulst, Z. Markovic-Housley, H. Breiteneder, P. Briza, K. Hoffmann-Sommergruber, F. Ferreira, *Int. Arch. Allergy Immunol.* **2006**, 139, 53.
- [54] K. Hoffmann-Sommergruber, P. Demoly, R. Crameri, H. Breiteneder, C. Ebner, L. D. Camara, K. Blaser, C. Ismail, O. Scheiner, J. Bousquet, G. Menz, *J. Allergy Clin. Immunol.* **1999**, 104, 478.
- [55] R. Friedl-Hajek, C. Radauer, G. O'Riordain, K. Hoffmann-Sommergruber, K. Leberl, O. Scheiner, H. Breiteneder, *Mol. Immunol.* **1999**, 36, 639.
- [56] C. Faber, A. Lindemann, H. Sticht, A. Ejchart, A. Kungl, M. Susani, R. W. Frank, D. Kraft, M. Breitenbach, P. Rosch, *J. Biol. Chem.* **1996**, 271, 19243.
- [57] N. Groh, C. Seutter von Loetzen, B. Subbarayal, C. Möbs, L. Vogel, A. Hoffmann, K. Fötisch, A. Koutsouridou, S. Randow, E. Völker, A. Reuter, P. Rösch, S. Vieths, W. Pfützner, B. Bohle, D. Schiller, *Clin. Exp. Allergy.* **2016**, 47, 693.
- [58] J. E. Mogensen, R. Wimmer, J. N. Larsen, M. D. Spangfort, D. E. Otzen, *J. Biol. Chem.* **2002**, 277, 23684.
- [59] C. Radauer, P. Lackner, H. Breiteneder, *BMC Evol. Biol.* **2008**, 8, 286.
- [60] A. Casañal, U. Zander, C. Munoz, F. Dupeux, I. Luque, M. A. Botella, W. Schwab, V. Valpuesta, J. A. Marquez, *J. Biol. Chem.* **2013**, 288, 35322.
- [61] C. Seutter von Loetzen, T. Jacob, O. Hartl-Spiegelhauer, L. Vogel, D. Schiller, C. Sporlein-Guttler, R. Schobert, S. Vieths, M. J. Hartl, P. Rosch, *PLoS One* **2015**, 10, e0128677.
- [62] Y. Yao, W. Sang, M. Zhou, G. Ren, *J. Food Sci.* **2010**, 75, C9.
- [63] L. Z. Lin, S. Lu, J. M. Harnly, *J. Agric. Food Chem.* **2007**, 55, 1321.
- [64] M. A. Bollen, A. Garcia, J. H. Cordewener, H. J. Wichers, J. P. Helsper, H. F. Savelkoul, M. A. van Boekel, *Mol. Nutr. Food Res.* **2007**, 51, 1527.
- [65] S. Scheurer, I. Lauer, K. Foetisch, M. San Miguel Moncin, M. Retzek, C. Hartz, E. Enrique, J. Lidholm, A. Cistero-Bahima, S. Vieths, *J. Allergy Clin. Immunol.* **2004**, 114, 900.
- [66] P. Gruber, S. Vieths, A. Wangorsch, J. Nerkamp, T. Hofmann, *J. Agric. Food Chem.* **2004**, 52, 4002.
- [67] M. Bublin, C. Radauer, A. Knulst, S. Wagner, O. Scheiner, A. R. Mackie, E. N. Mills, H. Breiteneder, *Mol. Nutr. Food Res.* **2008**, 52, 1130.
- [68] A. Urisu, H. Ando, Y. Morita, E. Wada, T. Yasaki, K. Yamada, K. Komada, S. Torii, M. Goto, T. Wakamatsu, *J. Allergy Clin. Immunol.* **1997**, 100, 171.
- [69] Anonymous. *Food Eng.* **1962**, 34, 98.
- [70] S. M. Albillos, N. Menhart, T. J. Fu, *J. Agric. Food Chem.* **2009**, 57, 4698.
- [71] Q. F. Cai, G. M. Liu, T. Li, K. Hara, X. C. Wang, W. J. Su, M. J. Cao, *J. Agric. Food Chem.* **2010**, 58, 12964.
- [72] V. Schulten, I. Lauer, S. Scheurer, T. Thalhammer, B. Bohle, *Mol. Nutr. Food Res.* **2011**, 55, 1484.
- [73] A. Petersen, S. Rennert, S. Kull, W. M. Becker, H. Notbohm, T. Goldmann, U. Jappe, *Biol. Chem.* **2014**, 395, 239.

- [74] Y. Kato, H. Watanabe, T. Matsuda, *Biosci. Biotechnol. Biochem.* **2000**, 64, 198.
- [75] K. Thomas, C. Herouet-Guicheney, G. Ladics, G. Bannon, A. Cockburn, R. Crevel, J. Fitzpatrick, C. Mills, L. Privalle, S. Vieths, *Food Chem. Toxicol.* **2007**, 45, 1116.
- [76] B. Guillon, H. Bernard, M. F. Drumare, S. Hazebrouck, K. Adel-Patient, *Mol. Nutr. Food Res.* **2016**, 60, 2722.
- [77] Y. M. Vissers, M. Iwan, K. Adel-Patient, P. Stahl Skov, N. M. Rigby, P. E. Johnson, P. Mandrup Muller, L. Przybylski-Nicaise, M. Schaap, J. Ruinemans-Koerts, A. P. Jansen, E. N. Mills, H. F. Savelkoul, H. J. Wichers, *Clin. Exp. Allergy.* **2011**, 41, 1631.
- [78] M. Iwan, Y. M. Vissers, E. Fiedorowicz, H. Kostyra, E. Kostyra, H. F. Savelkoul, H. J. Wichers, *J. Agric. Food Chem.* **2011**, 59, 7163.
- [79] M. Heilmann, A. Wellner, G. Gadermaier, A. Ilchmann, P. Briza, M. Krause, R. Nagai, S. Burgdorf, S. Scheurer, S. Vieths, T. Henle, M. Toda, *J. Biol. Chem.* **2014**, 289, 7919.

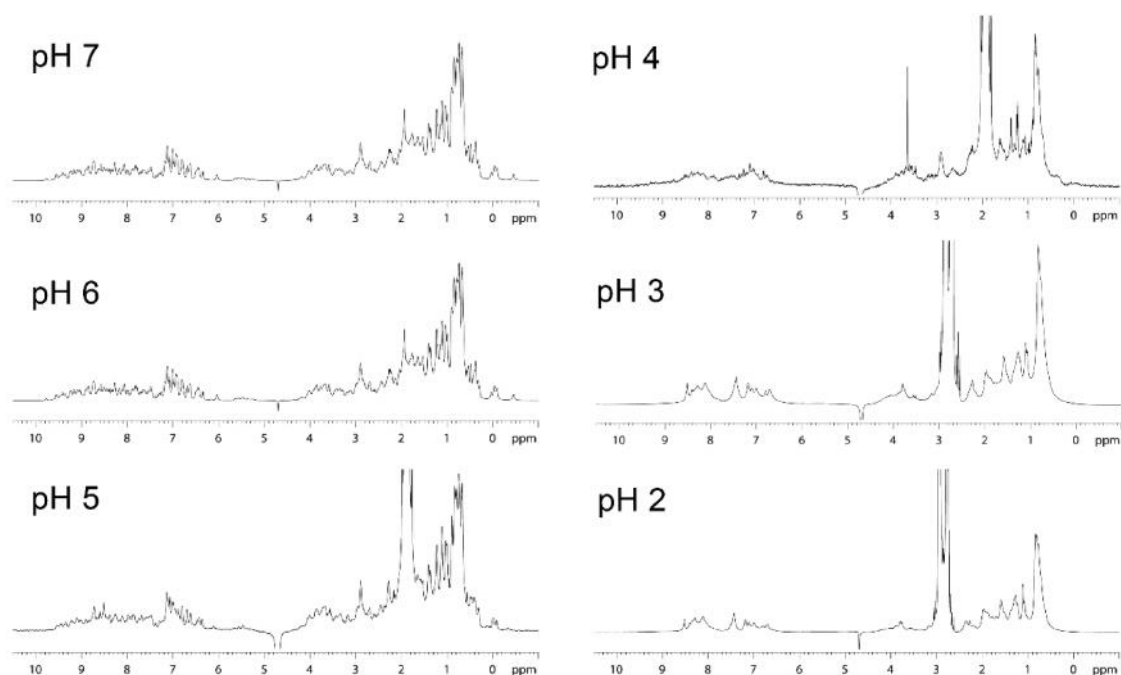


Figure S1: Api g 1 at pH 2 to 7 analysed by ^1H -1D-NMR spectroscopy.

Api g 1 was dialysed against 5 liters of 10 mM buffers (pH 7 and 6: sodium phosphate; pH 5 and 4 sodium acetate; pH 3 and 2: sodium citrate/citric acid) over night. NMR spectra were recorded of 20 to 100 μM Api g 1 containing 0.04% sodium azide and 10% D_2O on a Bruker Avance 600 MHz spectrometer at 25°C

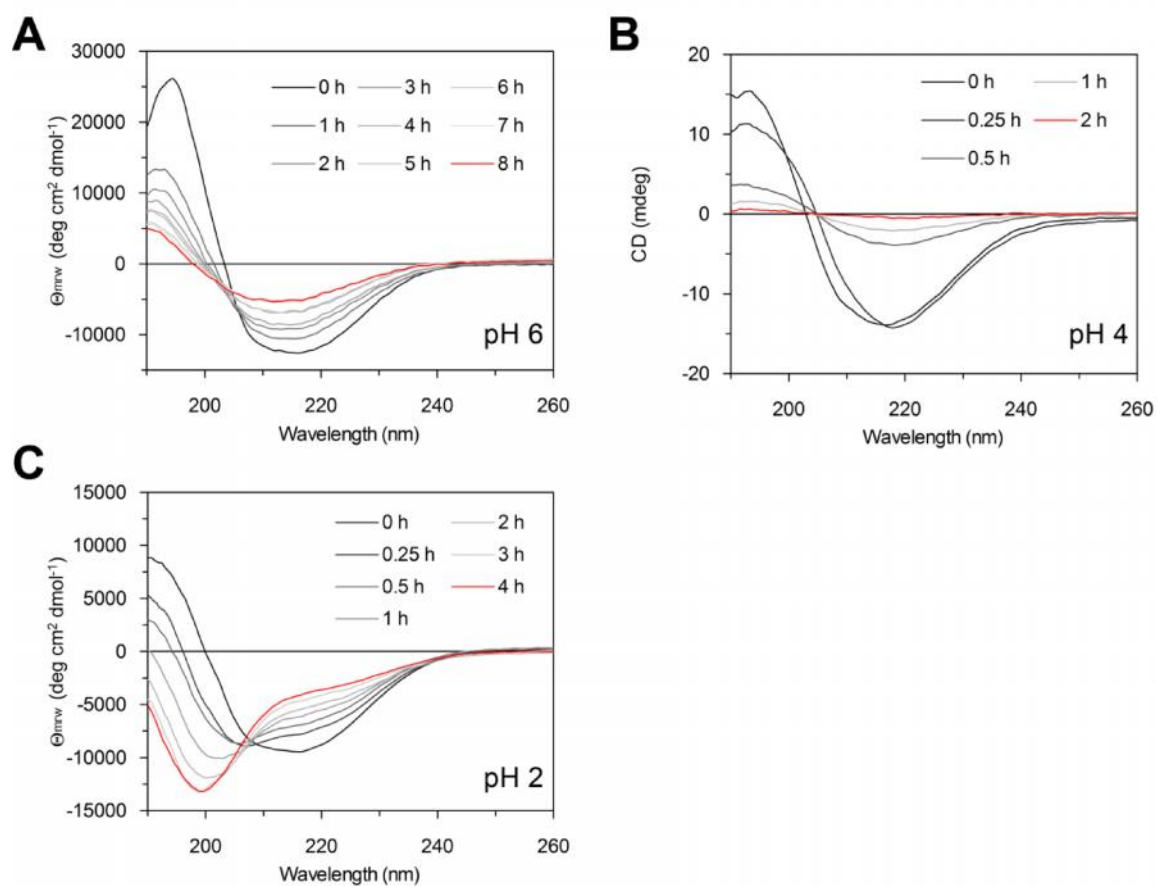


Figure S2: Effect of heat processing on Api g 1.0101 at pH 6, 4 and 2 analysed by CD spectroscopy. (A) CD-spectra of Api g 1 after 0 to 8 hours of cooking recorded at 25°C at pH 6 (B) at pH 4 (0 to 2 hours) and (C) at pH 2 (0 to 4 hours).

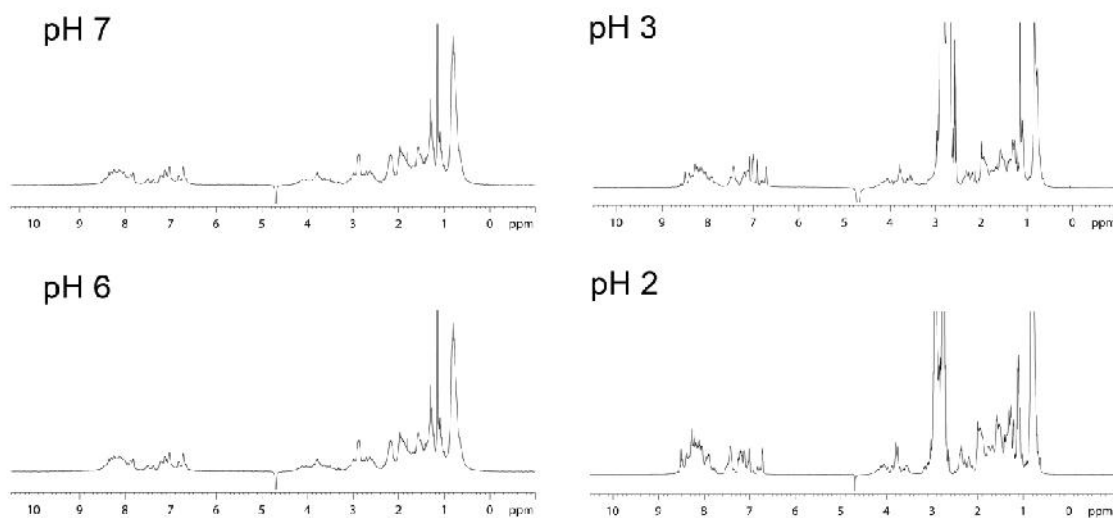


Figure S3: Api g 1 after heat treatment at pH 7 (8 hours), 6 (8 hours), 3 (5 hours) and 2 (4 hours) analysed by ¹H-1D-NMR spectroscopy.

Api g 1 was dialysed against 5 liters of 10 mM buffers (pH 7 and 6: sodium phosphate; pH 3 and 2: sodium citrate/citric acid) over night. After heat treatment NMR spectra were recorded of 20 to 100 μ M Api g 1 containing 0.04% sodium azide and 10% D₂O on a Bruker Avance 600 MHz spectrometer at 25°C

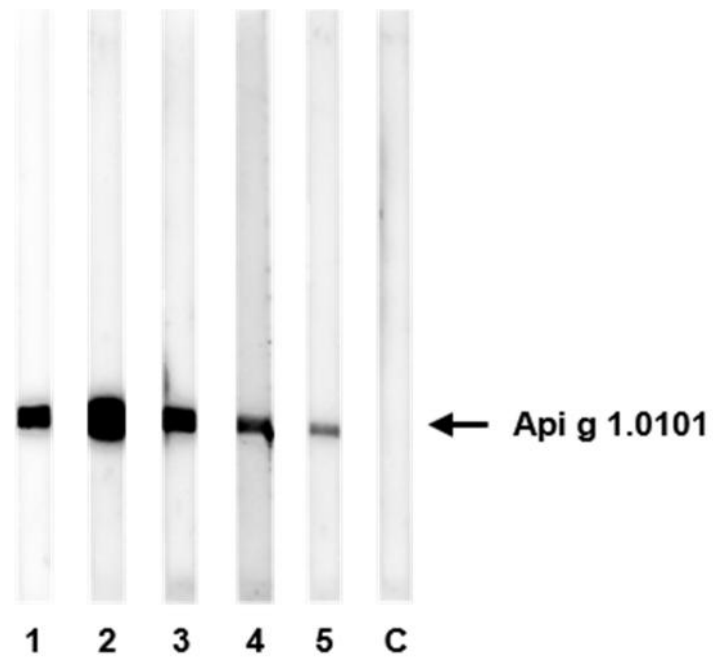


Fig. S4: IgE immunoblot of rApi g 1.0101.

1 μ g of recombinant Api g 1.0101 was transferred onto nitrocellulose. Individual binding of serum IgE of 5 subjects allergic to birch pollen was determined by chemiluminescence (lanes 1-5). C: serum of a non-allergic subject was tested as control.