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Identification of a defensin as novel allergen in celery root: Api g 7 as a missing link in the diagnosis of celery allergy?

To the Editor:

Allergy to celery (*Apium graveolens*) root (celeriac) is one of the most common food allergies in Northern Europe, with a prevalence of up to 0.45% in the adult population.¹ It mostly leads to local oral symptoms, but even severe anaphylactic reactions are reported², with a prevalence of up to 6% in Europe among subjects with recorded anaphylactic reactions.³ Six celery allergens are listed by the WHO/IUIS Allergen Nomenclature Sub-Committee⁴: Api g 1 (PR-10 protein), Api g 2 and Api g 6 (two non-specific lipid transfer proteins), Api g 3 (chlorophyll a/b binding protein), Api g 4 (profilin) and Api g 5 (FAD-containing oxidase).

Celeriac allergy is often accompanied by respiratory allergy to mugwort (*Artemisia vulgaris*) pollen⁵, which cannot be explained by IgE-cross-reactivity to known homologous celeriac allergens. However, celeriac allergic patients often show sensitization to mugwort defensin Art v 1 (Table S1).⁶ This prompted us to investigate the potential presence and role of a defensin-related allergen in allergy to celeriac. A celeriac defensin cDNA was cloned (accession# MT380846) and a recombinant (r) Api g defensin was produced. In parallel, mass spectrometric analysis demonstrated the presence of a corresponding defensin-like protein in celeriac extract and confirmed its entire mature amino acid sequence (Figure S1). An alignment with

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other defensin-like sequences is shown in Figure S2A. IgE-binding capacity and allergenicity of celeriac defensin (officially named Api g 7, www.allergen.org⁴) was investigated by immunoblotting and ImmunoCAP testing in eight celeriac allergic patients, two celeriac tolerant controls with mugwort pollen allergy and a nonatopic control. Six of the celeriac allergic patients underwent DBPCFC, showing symptoms ranging from mild subjective reactions to stronger objective symptoms (Table S1), while two patients with a history of anaphylactic reactions to celeriac did not undergo DBPCFC. All celeriac allergic patients were sensitized to rApi g 7 (Figure 1A, lanes 1-8). Levels of specific (s) IgE determined by ImmunoCAP showed no clear correlation between rApi g 7 and celeriac extract, whereas sIgE levels to rApi g 7 and rArt v 1 correlated strongly (Figure 1B, Table S1). Allergenic activity of rApi g 7 was assessed by mediator release (MR) assay, using a humanized rat basophil leukemia cell line. rApi g 7 was able to cross-link Fc ϵ RI bound IgE and induced MR_{max} of around 60% (Figure 1C), a slightly lower level than to rArt v 1 (MR_{max}=70%). Api g 7 and Art v 1 share 60% sequence identity (Figure S2A, B) and display common IgE-binding epitopes as demonstrated by IgE cross-inhibition experiments. rArt v 1 was able to completely abolish IgE-binding to rApi g 7 in all of three studied sera (Figure 2A) while rApi g 7 could only partially outcompete IgE-binding to rArt v 1 (Figure 2B), suggesting that Art v 1 may act as primary sensitizer of a cross-reactive response to Api g 7. The observation that 5 of the 8 allergic patients studied here had a negative ImmunoCAP test to celeriac, but were clearly sensitized to rApi g 7 (Table S1), suggested underrepresentation of Api g 7 in that allergen extract. To compensate for a scarcity of Api g 7 in allergen reagents for routine testing, extract spiking or complementary testing with Api g 7 could serve to improve diagnostic sensitivity.

In conclusion, our data show that celeriac contains an IgE reactive defensin, that likely could explain the previously known association between celeriac allergy and mugwort pollen sensitization. Furthermore the novel allergen Api g 7 may be useful for enhancing the sensitivity of *in vitro* diagnostic testing. Further studies in larger patient cohorts will show if an association with more severe reactions to celeriac can be established.

Author contributions

BBW, SV, JL, AW, SS and GG contributed to the design of the study. The clinical work was conducted by PB and BBW. The experiments were performed by AW, AR, LM, HL and MG. The manuscript was written by AW, SS, JL and SV. All authors reviewed and amended the manuscript.

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Conflict of interest

JL, LM and HL are employees of Thermo Fisher Scientific. The other authors declare no conflict of interest in relation to this work.

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Figure Legends:

Figure 1: Api g 7 shows IgE-binding and allergenic potency. IgE binding to rApi g 7 as determined by (A) immunoblotting using sera from celeriac allergic patients (lanes 1-8), atopic controls (9, 10) and a nonatopic control (D), M: molecular weight marker, P: PonceauS stain, N: secondary antibody control; (B) IgE levels determined by ImmunoCAP, blue: celeriac allergic patients (1-8), grey: atopic controls (9, 10), r and p values calculated with serum #1-8; (C) stimulation of humanized RBL cells with rApi g 7 in comparison to rArt v 1 and celeriac extract. BSA and milk extract served as negative controls.

Figure 2: Api g 7 shows partial IgE cross-reactivity with Art v 1. ELISA cross-inhibition experiments using sera of celeriac allergic patients and rApi g 7 (A) or rArt v 1 (B) on the solid phase; BSA and milk extract served as negative controls.

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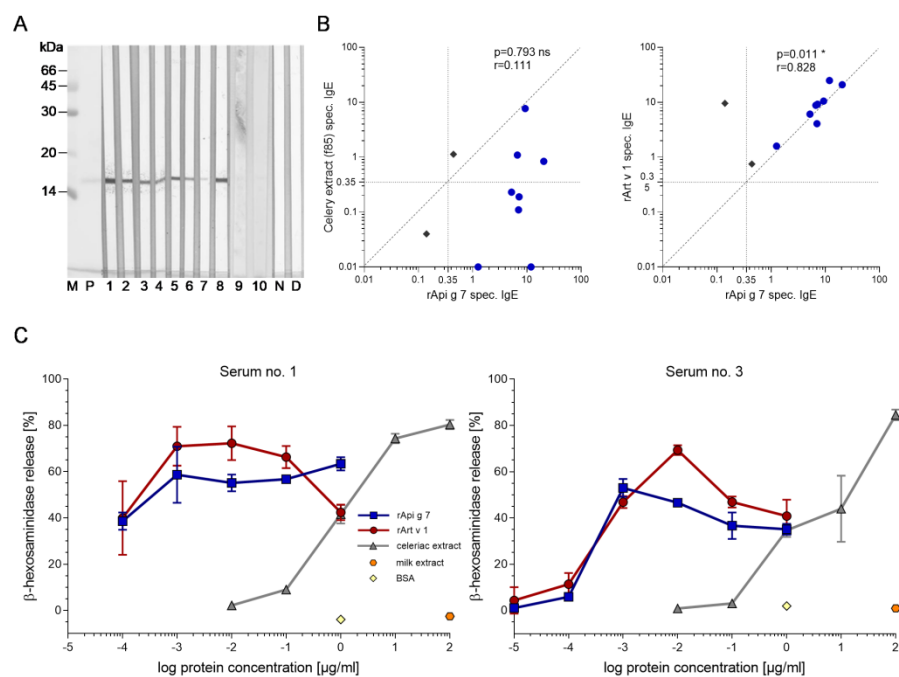


Figure 1_Wangorsch et al.

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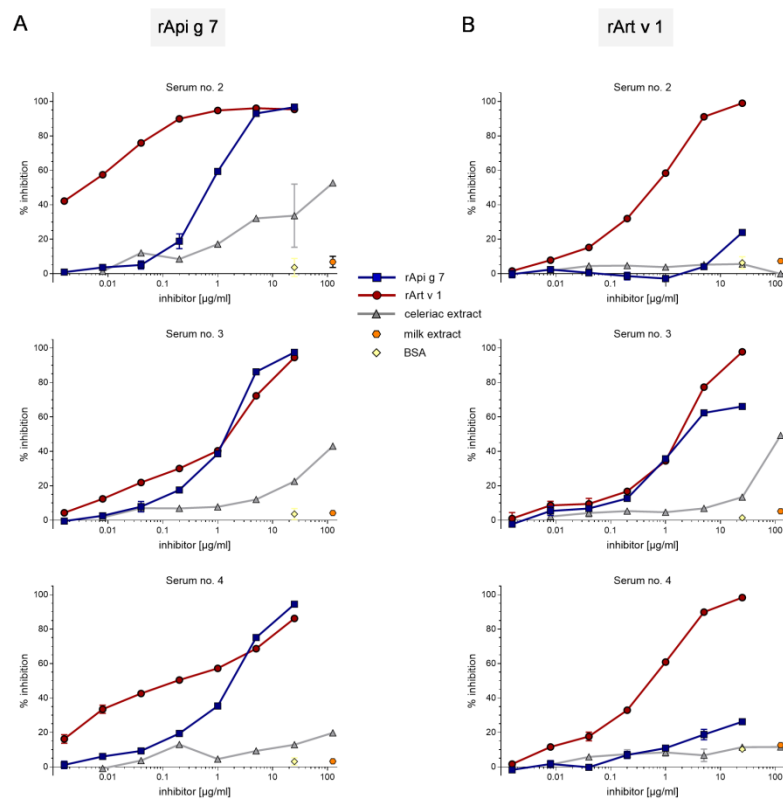


Figure 2_Wangorsch et al.

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1 Supplementary data Api g 7 letter

2 **Patients**

3 Eight celeriac allergic patients, preselected by concomitant mugwort pollen sensitization, two
4 celeriac tolerant controls with mugwort pollen allergy and one nonatopic control were included in
5 the study. Of the eight celeriac allergic patients, six underwent a double-blind placebo-controlled
6 food challenge (DBPCFC) with celeriac, all showing either mild subjective reactions such as oral
7 allergy syndrome (OAS) or strong objective symptoms like urticaria and dyspnea (Tab. S1). Two
8 patients (nos. 5 and 6) who had suffered previous anaphylactic reactions after ingestion of celeriac
9 were not subjected to celeriac DBPCFC. Tolerance to celeriac in the atopic controls was confirmed by
10 a negative open oral food challenge. Serological investigation of sensitization profiles was performed
11 by ImmunoCAPTM (Thermo Fisher Scientific, Uppsala, Sweden) measurements, using celeriac extract
12 (f85), mugwort extract (w6), and experimental ImmunoCAP tests carrying recombinant (r) mugwort
13 defensin (rArt v 1) and rApi g 7, prepared as previously described.¹ Specific IgE levels ≥ 0.35 kU_A/L
14 were considered positive and the results are depicted in Table S1. The study was approved by the
15 local ethics committee (approval no. SPUK/KEK-207 and SPUK/KEK-597) and written informed
16 consent was obtained from all participants.

17 **Purification of a defensin-like protein from celery root for mass spectrometric analysis**

18 Fresh celery root obtained from a local grocery store was ground in a food processor (Bosch, Munich,
19 Germany) and extracted in 2.7 mL 50 mM Na₂CO₃ pH 11.3, 20% DMSO per gram of raw material with
20 end-over-end agitation for 2 h at room temperature. Following clarification by centrifugation and
21 filtration through a 0.7 μ m glass microfiber filter (Cytiva, Uppsala Sweden), the extract was subjected
22 to sequential steps of chromatographic purification, with selection of fractions guided by binding of a
23 polyclonal rabbit anti-Art v 1 IgG antibody. After 1:1 dilution with 50 mM Na₂CO₃ pH 11.3, 5%
24 isopropanol, the extract was first applied on a Workbeads 40Q anion exchange column (Bioworks,
25 Uppsala, Sweden) equilibrated with the same buffer. The column flow-through was collected and

applied to a reversed phase column (RPC) (Source 15RPC, Cytiva) and eluted with a linear 0-54% gradient of acetonitrile in 0.065% TFA buffer. The RPC fractions containing the highest anti-Art v 1 IgG binding activity were applied to a Sephadex 30 column (Cytiva) equilibrated with 50 mM Na₂CO₃ pH 11.3, 0.15 M NaCl, 5% isopropanol. Elution was performed with the same buffer and fractions with high anti-Art v 1 IgG binding activity were analyzed by mass spectrometry. Throughout the purification process, measurement of rabbit anti-Art v 1 IgG binding to fractions of interest were performed using the ImmunoCAP assay platform (Thermo Fisher Scientific), as previously described.^{1,}

²

Mass spectrometry

To establish the identity and primary structure of the protein recognised by rabbit anti-Art v 1 IgG antibodies, relevant chromatographic fractions were analyzed by LC-MS/MS on an Orbitrap Fusion Tribrid instrument (Thermo Fisher Scientific, San Jose, CA). Prior to the analysis, the sample was reduced by DTT, alkylated with acrylamide and enzymatically cleaved with either trypsin, chymotrypsin, Lys-C or Glu-C. Data analysis was made on a combination of spectra obtained from the four protein digests. De novo sequencing confirmed each amino acid position in the mature protein covered by identified peptides. PEAKS Studio software (Bioinformatics Solutions Inc., Ontario, Canada) was used for analysis of MS/MS spectra against the NCBI non-redundant Viridiplantae ("other") database section (www.ncbi.nlm.nih.gov).

Experimental determination of the intact molecular mass of natural Api g 7 was performed by MS analysis of the uncleaved proteins, in a reduced or unreduced state. The analysis revealed a main peak corresponding to a mass of 5898.56 Da in the reduced state and 5890.50 Da in the non-reduced state. These determined masses are in exact agreement with the monoisotopic mass calculated for the sequence from position 43 (ALCEK---) of the sequence deduced from the cDNA (Fig. S1) in a reduced or non-reduced state. Further, the mass increment on reduction corroborate the assumption that all eight cysteine residues in the protein are engaged in disulfide bonds. In addition to the dominant form of the protein, a minor form with a mass of 6287.72 Da in the reduced state and

6279.65 Da in the non-reduced state was detected, consistent with an N-terminus at position 40 of the sequence (YPEALCEK---). No mass consistent with a larger form of the protein was detected. We conclude that residues 30-42 comprise a propeptide of which the last three residues are incompletely removed.

Identification, cDNA cloning and recombinant expression of a defensin-like protein from celeriac

For the preparation of total RNA from celery (*Apium graveolens*), small pieces of celery root were ground to a fine powder under liquid nitrogen and applied to the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). RNA was then transcribed to cDNA using the First Strand cDNA Synthesis Kit (Cytiva, Freiburg, Germany) and Not I-(dT)₁₈ as primer. For PCR amplification of a defensin-like cDNA from celeriac, degenerated forward and reverse primers 5'-AGR ACM TGY GAS TCH CAG AGY MAY MRG TTC-3' and 5'-TYA ACA ATG TYK DRT GCA RAA RCA DCK RCG-3' were designed based on an alignment of cDNA sequences from several known defensin-like proteins (Fig. S2A). The PCR product obtained was cloned into vector pCRTMII-TOPO (Fisher Scientific, Schwerte, Germany) and sequenced (Eurofins, Ebersberg, Germany). To obtain the entire cDNA sequence, the 5' and 3' untranslated regions were determined using the InvitrogenTM 5' RACE System (Fisher Scientific), a PCR with a poly (dT)₁₈ primer for 3' RACE and a subsequent PCR amplification with primers in the 5' and 3' untranslated regions (5'-ACA TAC ACA TAT ATA AAA TC-3' (forward) and 5'-GAT CGA ACA CAC TCG AT-3' (reverse)), followed by cloning and sequencing as described above. The full-length sequence of Api g defensin-like protein was submitted to the NCBI GenBank database and accepted as a new entry with the accession number MT380846. The protein has been officially recognized as an allergen by the IUIS/WHO Allergen Nomenclature Sub-Committee (www.allergen.org) and named Api g 7.0101. The newly established Api g 7 sequence, without its signal peptide (amino acids 1-29), was used to generate an *E. coli* optimized synthetic gene (Eurofins) which was cloned to the expression vector pET52b (VWR, Darmstadt, Germany) and transformed into *E. coli* strain SHuffle T7 Express LysY (New England Biolabs, Frankfurt, Germany). Translation of the Api g 7 cDNA displaying the sequence of the mature protein (including the pro-peptide), a thrombin cleavage site and a

10 x histidine-tag at the C-terminus will lead to a protein with a theoretical molecular weight of 11 kDa. Subsequent protein expression was carried out for 24 h at 30°C. After induction of protein expression by adding 0.4 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG) at an optical density (OD_{600}) of 0.6, the cells were harvested by centrifugation and lysed in 20 mM Na-phosphate, 500 mM NaCl, 20 mM imidazole pH 7.4 containing 8 M urea for 30 min at room temperature (RT) on an end-over-end rotator. rApi g 7, carrying a 10 x histidine-tag at the C-terminus of the protein, was purified from the supernatant by two steps of chromatography. First a HisTrap Crude FF column (Cytiva) using on-column refolding with a 8-0 M urea gradient, followed by a 0-1 M imidazole gradient for elution, both in 500 mM NaCl, 20 mM imidazole pH7.4, performed on an ÄKTA Pure protein purification system (Cytiva). Eluted fractions containing rApi g 7 were pooled and applied to size exclusion chromatography (HiPrep 26/60 Sephacryl S-100 High Resolution, Cytiva) using 20 mM MOPS, 150 mM NaCl, 1 mM EDTA pH7.4 as running buffer. Fractions containing the pure protein were pooled and total protein content was determined using the BCA assay (Fisher Scientific). In contrast to the calculated molecular weight of 11 kDa the purified rApi g 7 always appeared with an apparent molecular mass of around 16 kDa, as shown in Figure 1, independently if applying under reducing or non-reducing conditions.

Preparation of celeriac and milk extract for ELISA inhibition and allergenic activity testing

Celeriac and milk extract were prepared by adding 30 g of ground and freeze dried celeriac powder (Kräuterhaus Wolf, Radebeul, Germany) and skimmed milk (Carl Roth, Karlsruhe, Germany), respectively to 240 ml 50 mM MES buffer pH 6.0. To precipitate interfering celeriac pectin $CaCl_2$ was added to the celeriac mixture to a final concentration of 40 mM. Both mixtures were incubated overnight at 4°C with end-over-end rotation, followed by a two times centrifugation step (first time 30 min, 10.000xg and second time 30 min, 20.000xg). Supernatants of the last centrifugation step were dialyzed against 20 mM MOPS, 150 mM NaCl, 1 mM EDTA pH7.4, passed through a 0.45 μ m filter and the whole protein content was determined by applying the respective extracts of celeriac and milk to the Roti-Nanoquant 5x Bradford reagent (Carl Roth).

IgE reactivity of rApi g 7 and cross-reactivity with rArt v 1

IgE binding to Api g 7 was determined using an experimental ImmunoCAP assay as described above and by IgE-immunoblotting as published elsewhere.³ Cross-reactivity between the allergens was investigated by competitive IgE enzyme linked immunosorbent assay (ELISA) inhibition. Experiments were performed using high binding ELISA plates (Sarstedt, Nümbrecht, Germany) coated with 1 µg/mL rApi g 7 or rArt v 1, respectively and serum dilutions of 1:80 for serum #2 and #3 and 1:50 for serum #4, as previously described.⁴ Recombinant Art v 1 used in this assays was prepared as described elsewhere.⁵

Rat basophil leukemia cell assay

The allergenic activity of Api g 7 and Art v 1 was assessed using a humanized rat basophilic leukemia cell line as published by Vogel et al..⁶ Briefly, cells were passively sensitized with human sera diluted 1:20 (serum #1) or 1:10 (serum #3). After overnight incubation, cells were washed and allergen was added to assess activation through cross-linking of Fc_εRI bound IgE. Allergens were added in a 1:10 dilution series starting at a concentration of 1 µg/mL for rApi g 7, rArt v 1 and BSA and 100 µg/mL for celeriac and milk extract. Mediator release was determined by quantitative measurement of β-hexosaminidase in the supernatant, in relation to the total release induced by 10% Triton X-100.

In silico and physicochemical characterization of recombinant Api g 7

Multiple sequence alignment of different defensin-like proteins was done using the Clustal Omega⁷ and BoxShade (https://embnet.vital-it.ch/software/BOX_form.html) online tools. A three dimensional model of Api g 7 was generated with the SWISS-MODEL server for protein structure homology-modelling⁸ using the Art v 1 structure as template (pdb: 2kpy.1.A) and PyMOL (Molecular Graphics System, Version 2.0 Schrödinger, LLC) for the superimposition of the two proteins. All other analyses of sequences were done using the VectorNTI software (Thermo Fisher). Secondary structure elements of the recombinant proteins were analyzed by circular dichroism (CD) spectroscopy as described elsewhere⁹, the secondary structure estimation using Yang's reference¹⁰ and the melting

temperature was assessed by nanoscale differential scanning fluorimetry (nanoDSF) measurements using a Prometheus instrument (NanoTemper Technologies, Munich, Germany) as described by Jacob et al.¹¹

CD spectroscopy and nano DSF measurements revealed that rArt v 1 showed no intact secondary structure (Fig. S3). This is in line with former findings, and can be explained by the high proportion of prolines at the C-terminus of the protein (Fig. S1).¹² rApi g 7 does not contain a proline rich part at the C-terminus and the secondary structure elements are therefore different in comparison to rArt v 1. This can be seen as a shift of the negative peak from 204 nm for rArt v 1 to 209 nm for rApi g 7 (Fig S3A). The CD-spectroscopy results lead to the conclusion that the secondary structure of rApi g 7 was only partial intact, as it displayed around 31% of random coil elements. By using nano DSF measurements we could show that rApi g 7 was very stable as it started to unfold at around 74°C and had a melting temperature of 100°C (Fig S3B). In contrast, the rArt v 1 curve did not show any transition point, meaning the protein had no intact structure. Both proteins were unable to refold after heat denaturation and neither of them displayed any scattering (aggregation) (data not shown).

Supplementary Table S1: Clinical and serological data of celeriac allergic patients, preselected by concomitant mugwort pollen sensitization (#1-8) and atopic controls (#9, 10).

#						ImmunoCAP _{TM} [kU _A /L] (CAP-class)			
	sex	age	Symptoms by History	Symptoms by DBPCFC	outcome	celery extract	mugwort extract	rApi g 7.01	rArt v 1.01
1	f	20	OAS,U,AE,R,D,C	OAS,U,Lpru	obj	0.84 (2)	34.81 (4)	20.50 (4)	20.84 (4)
2	f	22	OAS,U,AE	OAS,Erythema	obj	0.19 (0)	13.60 (3)	7.16 (3)	9.21 (3)
3	f	27	U,N	OAS,G	subj	7.69 (3)	19.14 (4)	9.28 (3)	10.49 (3)
4	m	32	OAS,Dizziness	OAS	subj	0.11 (0)	5.53 (3)	7.00 (3)	4.08 (3)
5	f	22	U,N,D,C,V,BP, V	not challenged	NA	0.23 (0)	4.45 (3)	5.20 (3)	6.08 (3)
6	f	19	OAS,AE,U,N,V,BP	not challenged	NA	1.09 (2)	7.37 (3)	6.65 (3)	8.80 (3)
7	f	30	AE,F,P	OAS,RC,F	obj	0.00 (0)	6.28 (3)	1.26 (2)	1.59 (2)
8	f	42	U, GIT-pain	OAS	subj	0.00 (0)	39.20 (4)	11.90 (3)	24.80 (4)
9	f	31				0.04 (0)	9.94 (3)	0.14 (0)	9.55 (3)
10	m	15				1.13 (2)	1.26 (2)	0.44 (1)	0.75 (2)

Symptoms of celeriac allergy by history or DBPCFC; abbreviations: DBPCFC, double-blind placebo-controlled food challenge; AE, angioedema; BP, drop of blood pressure with loss of consciousness; C,

149 conjunctivitis; D, dyspnea; Dph, dysphagia; F, flush; GIT, gastro-intestinal tract; Lpru, local pruritus; N,
150 nausea; OAS, oral allergy syndrome; P, pruritus of the skin; R, rhinitis; RC, rhino-conjunctivitis; U,
151 urticaria; V, vomiting; NA, not applicable.

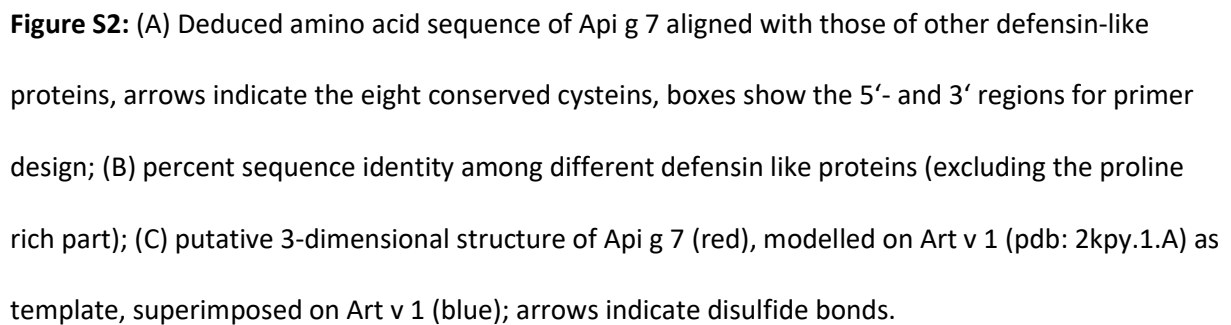
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153 **Supplementary Figures**

154

155 **Figure S1: Sequence confirmation of natural Api g 7 by means of LC-MS/MS analysis.** Blue bars
 156 indicate all identified unique peptides. A predicted signal peptide (position 1-29) is underlined in red.
 157 Residues 30-42 comprise a propeptide of which the last three residues are incompletely removed
 158 during protein maturation.

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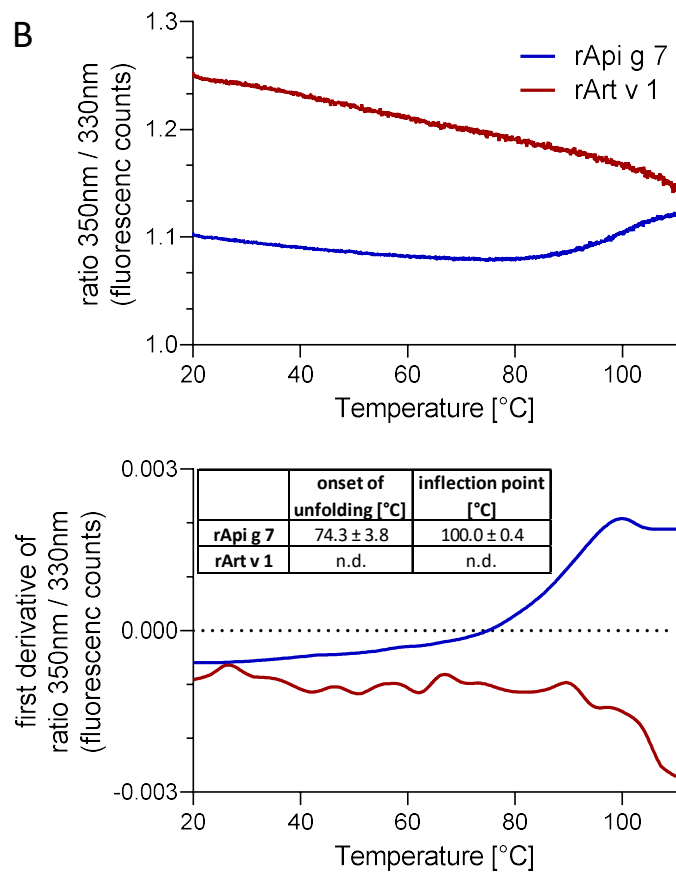
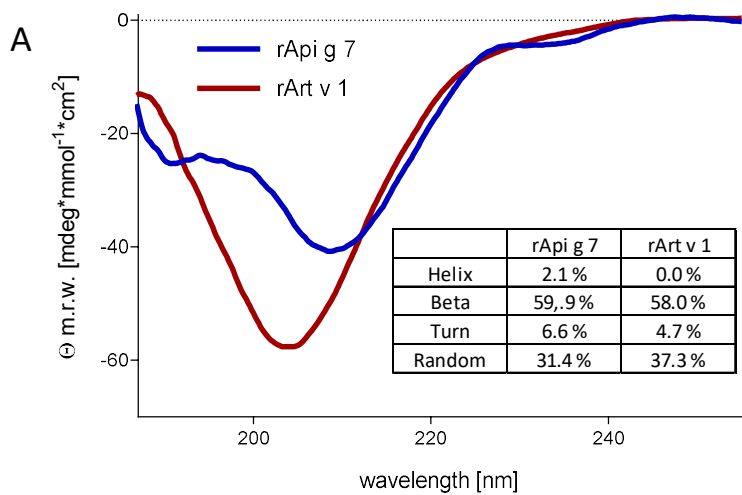


Figure S3: Physicochemical characterization of rApi g 7 in comparison to rArt v 1. (A) CD-spectra of rApi g 7 (blue line) and rArt v 1 (red line), including secondary structure estimation; (B) nanoDSF measurement of rApi g 7 and rArt v 1, showing the 350/330 nm ratio and the first derivative thereof; n.d.: not detectable.

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