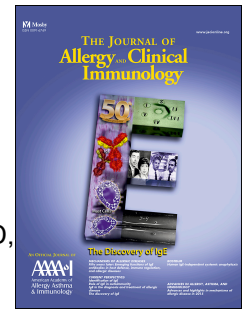


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Endolysosomal protease susceptibility of Amb a 1 as a determinant of allergenicity

Martin Wolf, PhD ^{1,6}, Lorenz Aglas, PhD ¹, Teresa E. Twaroch, PhD ², Markus Steiner, PhD ^{1,3}, Sara Huber, MSc ¹, Michael Hauser, PhD ¹, Maria A. Parigiani, MSc ¹, Heidi Hofer, PhD ¹, Christof Ebner, MD ⁴, Barbara Bohle, PhD ⁵, Peter Briza, PhD ¹, Angela Neubauer, PhD ², Frank Stolz, PhD ², Michael Wallner, PhD ¹, Fatima Ferreira, DDSc, PhD ¹

1) Department of Molecular Biology, University of Salzburg, Salzburg, Austria

2) Biomay AG, Vienna Competence Center, Vienna, Austria

3) Laboratory for Immunological and Molecular Cancer Research, Paracelsus Medical University, Salzburg, Austria

4) Allergy Clinic Reumannplatz, Vienna, Austria

5) Department of Pathophysiology and Allergy Research, Medical University of Vienna, Vienna, Austria

6) Present address: Institute of Experimental and Clinical Cell Therapy, Spinal Cord Injury and Tissue Regeneration Center Salzburg, Paracelsus Medical University, Austria

Corresponding Author:

Fatima Ferreira, PhD

Department of Molecular Biology, University of Salzburg

Hellbrunnerstr. 34, A-5020 Salzburg, Austria

Tel: +43-662-8044-5016, Fax: +43-662-8044-745016

Email: fatima.ferreira@sbg.ac.at

Capsule summary

In the absence of extrinsic adjuvants, purified Amb a 1 induced a strong Th2-polarized immune response in mice. This high allergenic activity seems to be linked to the allergen's susceptibility to endolysosomal proteolysis.

Key words

Ragweed pollen; Amb a 1 allergen; allergenicity; immunogenicity; bacterial pectate lyase

37 **Abbreviations**

38 ALUM: aluminum hydroxide

39 Amb a: *Ambrosia artemisiifolia*

40 CD: circular dichroism

41 DC: *Dickeya* spp

42 DTT: dithiothreitol

43 FTIR: Fourier-transform infrared

44 HPLC: high performance liquid chromatography

45 MS: mass spectrometry

46 MS/MS: tandem mass spectrometry

47 PBS: phosphate buffered saline

48 pelC: pectate lyase C

49 SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

50

To the Editor:

Exposure to pollen from short ragweed (*Ambrosia artemisiifolia*) causes severe respiratory allergies worldwide. Amb a 1, a member of the pectate lyase C family, is a highly allergenic molecule recognized by >90% of ragweed-sensitized individuals and accounts for >90% of the allergenic activity in ragweed pollen¹. However, the factors/features responsible for its high allergenicity (i.e. capacity to induce specific IgE antibodies) remain undefined. Efforts to understand allergenicity have lately focused on extrinsic factors normally accompanying the allergen during the initial interaction with the immune system (e.g. TLR-ligands, lipid mediators, etc.)². However, data from our previous studies suggested that intrinsic features of Amb a 1 might be linked to its high allergenic potential³. Thus, we sought to investigate intrinsic molecular pattern(s) possibly involved in the allergenicity of Amb a 1. As a comparison, we used the non-allergenic pectate lyase C homologue from the phytopathogenic *Dickeya chrysanthemi* (previously *Dickeya dadantii* and *Erwinia chrysanthemi*)⁴, here termed DC-pelC.

Amb a 1.01, the most allergenic isoform, was purified from ragweed pollen extracts whereas the bacterial-derived pectate lyase C (DC-pelC) was produced as a soluble recombinant protein in *E. coli* (**Figure E1A**). After purification, their identity and purity were confirmed by mass spectrometry (**Figure E1B,C**). Despite their relatively low sequence identity (24%), CD and FTIR experiments indicated the presence of the parallel beta-helix structural element typical for polysaccharide lyases from family 1, with a high beta-sheet content (35.6–40.5%) for both molecules (**Figure E2**). These results support the notion that the two proteins share some degree of structural similarity (**Figure E2D**). However, due to low sequence identity, cross reactivity is not expected.

As *Dickeya* spp are known to cause soft root disease on many crops (e.g. bell pepper, tomatoes, potatoes, onions)⁴ and ornamental indoor plants. Contact with this phytopathogen has been recently demonstrated by metagenomic sequencing of the microbiota of human buccal mucosa^{5,6}. These studies unambiguously identified *Dickeya dadantii* as an oral commensal bacterium. Thus, we performed ELISA assays to evaluate immune responses to DC-pelC. None of the 39 ragweed-allergic patients displayed IgE or IgG4 antibodies recognizing DC-pelC. However, 37.5% of the individuals showed IgG antibodies against DC-pelC confirming exposure but no sensitization to DC-pelC (**Figure E3**).

87

88 To further investigate the sensitization capacity of Amb a 1.01 and DC-pelC, we
89 immunized mice in the presence and absence of ALUM as adjuvant (**Figure 1A**). In both
90 immunization protocols, Amb a 1.01 behaved as a very potent sensitizer, inducing high
91 levels of IgG and IgE antibodies (**Figure 1B**). These results clearly demonstrate that the
92 intrinsic allergenic properties of Amb a 1.01 were not significantly altered by adsorption
93 to ALUM as adjuvant. In contrast, in the adjuvant-free model, the non-allergenic pectate
94 lyase DC-pelC induced a moderate immune response with low levels of IgG1 and IgG2a
95 antibodies and failed to initiate B cell class switch for IgE production. When ALUM was
96 co-administered with DC-pelC, a significant increase in the titers of specific IgG1 and
97 IgG2a antibodies was observed. In addition, DC-pelC-specific IgE antibody titers
98 increased slightly, showing mediator release capacity in only 2 out of 5 mice (**Figure**
99 **1C,E**). The amount of DC-pelC needed to induce half-maximal release in basophils
100 sensitized with sera from ALUM-immunized animals and those without ALUM did not
101 differ significantly (**Figure 1D**). Our experiments also showed that DC-pelC-induced
102 murine antibodies did not cross-react with Amb a 1.01 and vice versa (data not shown),
103 further supporting the notion that natural exposure of humans to Amb a 1.01 and DC-
104 pelC or immunization of mice gives rise to antibodies of different isotypes displaying
105 distinct patterns of epitope recognition.

106

107 We next investigated resistance/susceptibility of Amb a 1.01 and DC-pelC to proteolysis.
108 Stability to endolysosomal proteolytic processing has been proposed to correlate with
109 the efficacy of T cell activation and priming, and the resulting immune response *in vivo* ⁷.
110 ⁸. We performed digestions at different pH using proteases from the endolysosomal
111 compartment of a dendritic cell line ⁷. Under the same experimental conditions, Amb a
112 1.01 and DC-pelC differed remarkably in their susceptibility to proteolysis in a pH-
113 dependent manner. At pH 4.8, Amb a 1.01 and DC-pelC showed a half-life of 45h and
114 72h, respectively (**Figure 2A,B**). In contrast, Amb a 1 at pH 4.5 showed a decreased half-
115 life of 12h whereas DC-pelC remained stable. In the presence of ALUM at pH 4.5, we
116 observed stabilizing effects on Amb a 1. Under these conditions, DC-pelC was slightly
117 destabilized at pH 4.5 but was more susceptible to degradation at pH 4.0. Peptides
118 generated by proteolytic degradation of Amb a 1.01 and DC-pelC differed significantly
119 both in their sequences and amounts. (**Figure 2A,B**). These differences in susceptibility
120 to proteolytic degradation might be due to distinct surfaces between the proteins, which
121 in turn could result in distinct accessibility and resistance/susceptibility to proteases

during processing by antigen presenting cells⁸, as recently demonstrated for two Bet v 1 isoforms with different immunogenic/allergenic properties⁹.

Although the route and conditions of exposure remain unclear, the presence of specific serum IgG and no detectable IgE antibodies strongly support the notion that human exposure to DC-pelC does not induce allergic sensitization. Our findings that in an adjuvant-free mouse immunization model DC-pelC failed to induce IgE antibodies further support the view that this pectate lyase lacks intrinsic allergenic activity. It is possible that for some proteins, allergen source-derived or even extrinsic factors are important for their allergenic potential. However, here we showed that highly purified natural Amb a 1.01 is capable of inducing strong Th2 immune responses in mice in the absence of (extrinsic) adjuvants. In contrast, under the same conditions, the non-allergenic bacterial homologue induced a Th1-biased response. Our data on Amb a 1.01 and DC-pelC is in agreement with previous findings for fold-stabilized Bet v 1 mutants⁸. Thus, we propose a model in which the intrinsic stability/susceptibility of a protein to proteolysis can directly influence its processing and determines the intensity and polarization of the ensuing immune response (**Figure 2C**).

Martin Wolf, PhD^{1,6}

Lorenz Aglas, PhD¹

Teresa E. Twaroch, PhD²

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Angela Neubauer, PhD²

Frank Stolz, PhD²

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Fatima Ferreira, DSc, PhD¹

From the ¹ Department of Molecular Biology, University of Salzburg, Salzburg, Austria; ² Biomay AG, Vienna Competence Center, Vienna, Austria; ³ Laboratory for Immunological and Molecular Center Research, Paracelsus Medical University, Salzburg, Austria; ⁴ Allergy Clinic Reumannplatz, Vienna, Austria; and the ⁵ Department of Pathophysiology and Allergy Research, Medical University of Vienna, Vienna, Austria; ⁶ Present address: Institute of Experimental and Clinical Cell Therapy, Spinal Cord Injury and Tissue Regeneration Center Salzburg, Paracelsus Medical University, Austria. Email: fatima.ferreira@sbg.ac.at

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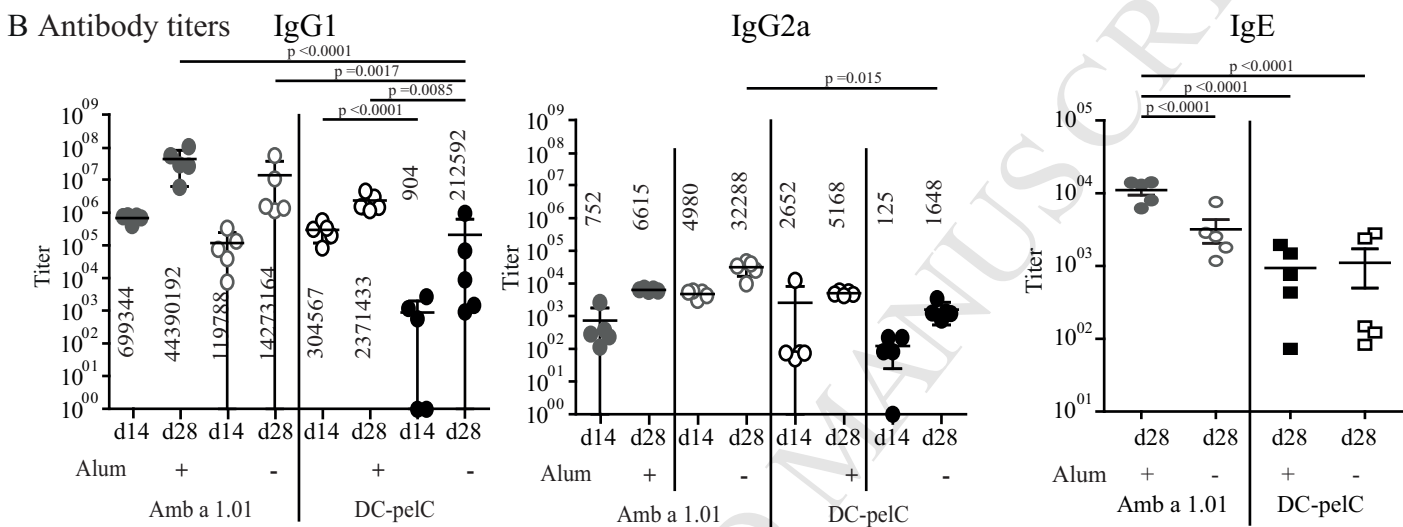
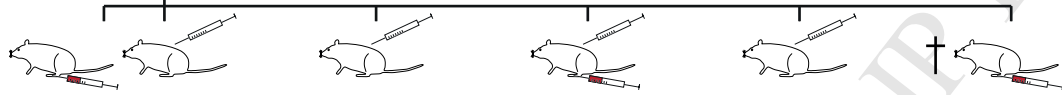
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Figure legends

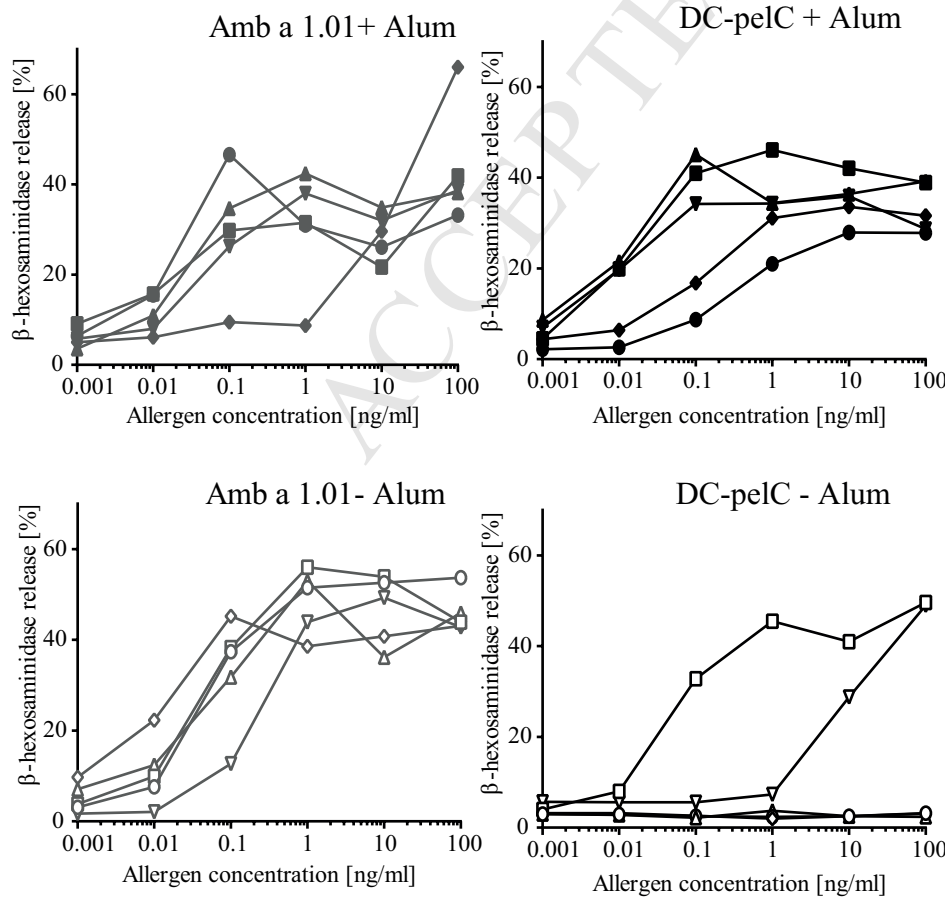
Figure 1. (A) Scheme of mouse immunizations. Mice (5/group) were immunized with antigen adsorbed to ALUM (+) or with antigen alone (-). (B) IgG1, IgG2a, and IgE titers were determined by ELISA. Statistical analyses were performed with ANOVA, $p < 0.05$ was considered significant. Adjusted p values are shown. (C) Functional IgE was measured via mediator release of RBL cells using sera from individual mice collected at day 28. (D) Statistical analysis of the amount of protein needed to induce half-maximal mediator release in RBL cells. (E) Mean titration curves of pooled sera from mice immunized with Amb a 1 and DC-PelC, with and without ALUM.

Figure 2. SDS-PAGE analyses of protein degradation with endolysosomal proteases (upper panels) and mass spectrometric analyses of peptides generated after 48h (lower panels) for (A) Amb a 1.01 and (B) DC-pelC. (C) Model for the influence of antigen proteolytic stability on the intensity (as determined by antibody titers) of the immune responses, and the TH1/TH2 ratios (as determined by IgG2a/IgG1 titers in mice). "+" and "-" indicate ALUM-adsorbed and free antigen, respectively. Data of stability to endolysosomal proteolysis for Bet v 1 and for Bet-mut4, a fold-stability variant of Bet v 1, were previously published by Wallner et al. (*J Allergy Clin Immunol* 2011; 127: 1571-8) and by Machado et al. (*J Allergy Clin Immunol* 2016; 137:1525-34), respectively.

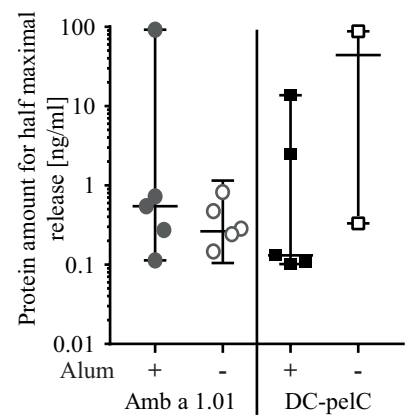
A Immunization schedule -2 0 7 14 21 28 days



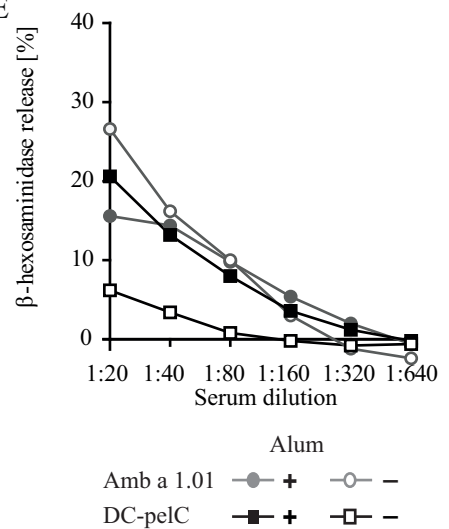
C Mediator release capacity in RBL-cells



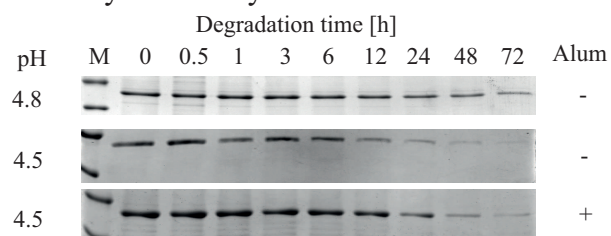
D



E



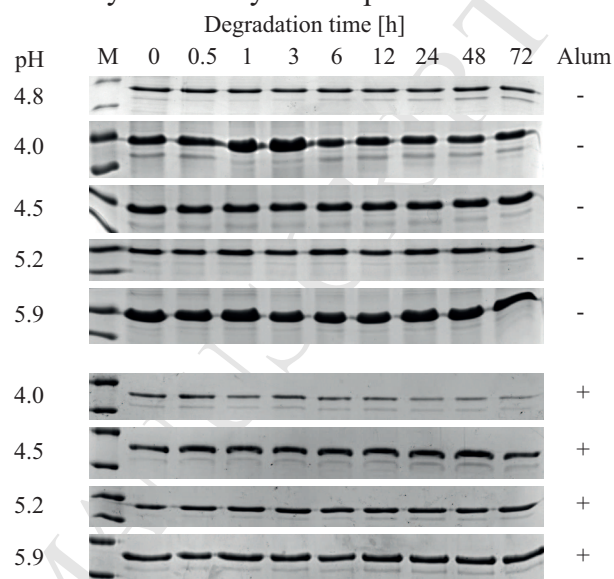
A Proteolytic stability of Amb a 1.01



Peptides of Amb a 1.01 generated after 48h of endolysosomal degradation



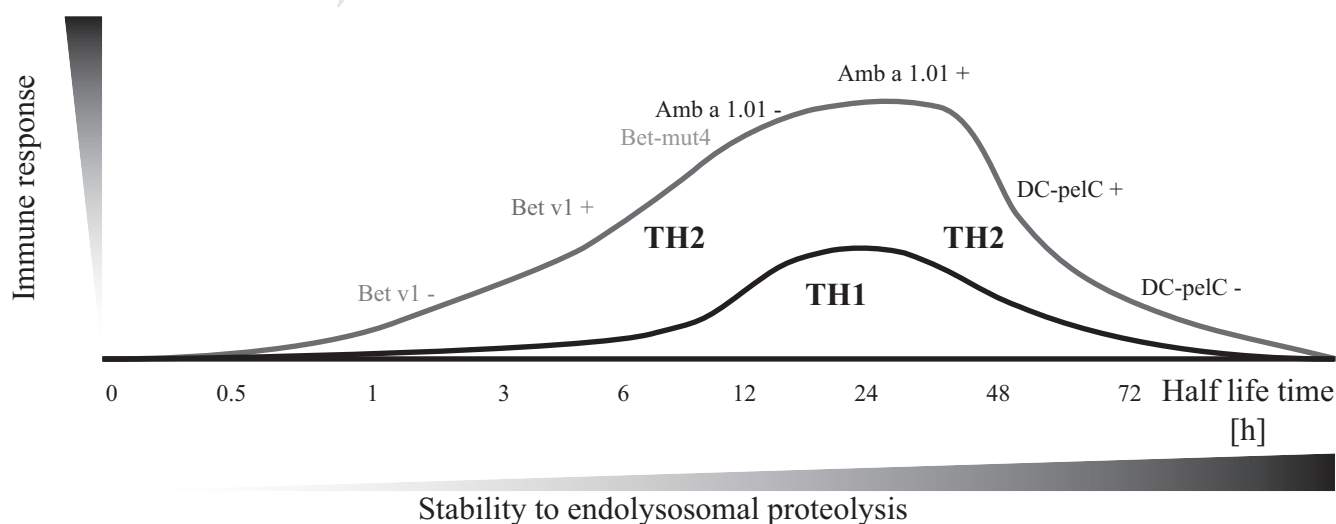
B Proteolytic stability of DC-pelC



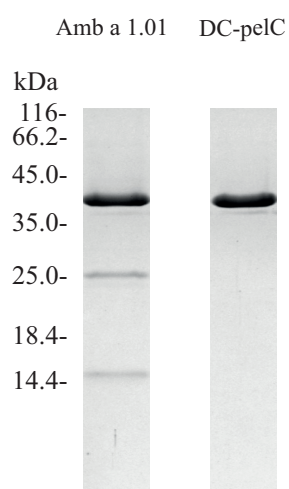
Peptides of DC-pelC generated after 48h of endolysosomal degradation



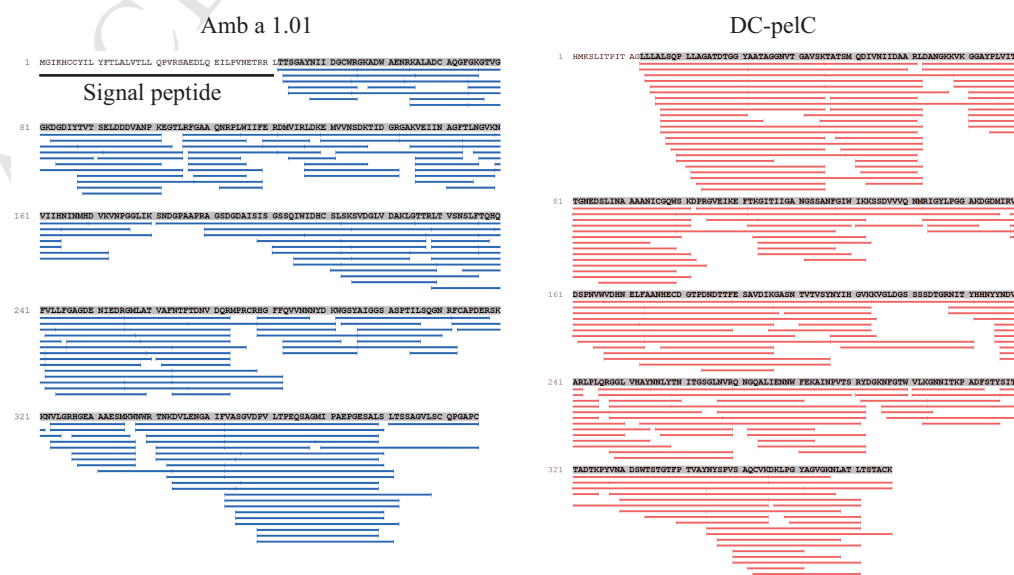
C Relationship between endolysosomal proteolysis and immune response



A SDS-Page



B MS-peptide patterns from Trypsin/Chymotrypsin digests

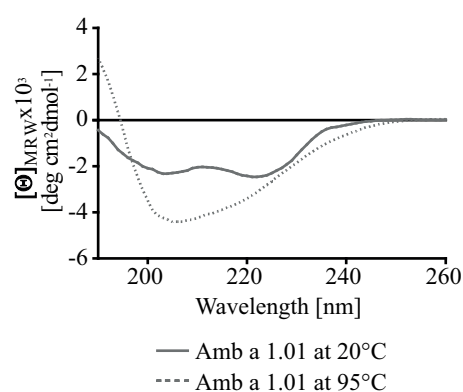


C Protein purity via MS

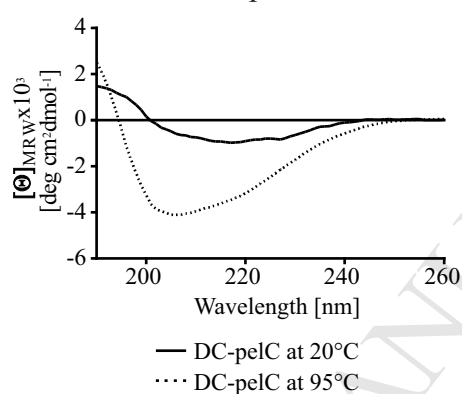
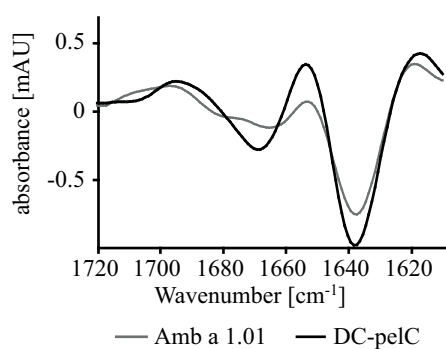
Uniprot	Name	Sequence coverage	Total Peptides	TIC Area	Area %
P11073	DC-pelC	62	20	1,4E+10	100%
P27759	Amb a 1.01	68	25	9,3E+09	98,7%

A CD spectra

Amb a 1.01



DC-pelC

B FTIR 2nd derivative of amide I

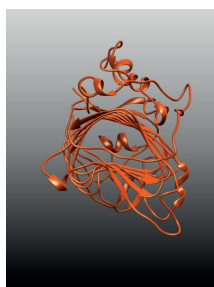
C Secondary structure elements

Secondary structure elements	α -helix	β -sheets
Amb a 1.01	11,6%	33,9%
DC-pelC	12,9%	36,6%

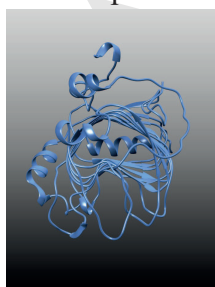
D Structure modeling

Topview

Amb a 1.01

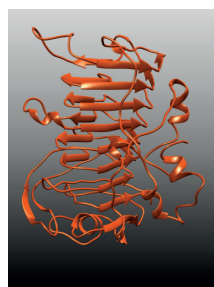


DC-pelC

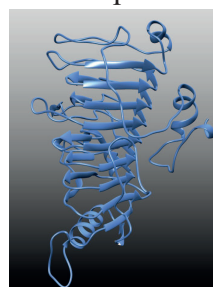


Backview

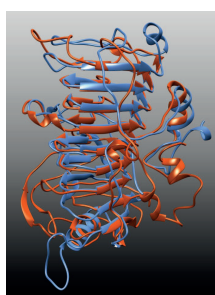
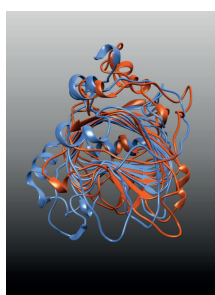
Amb a 1.01



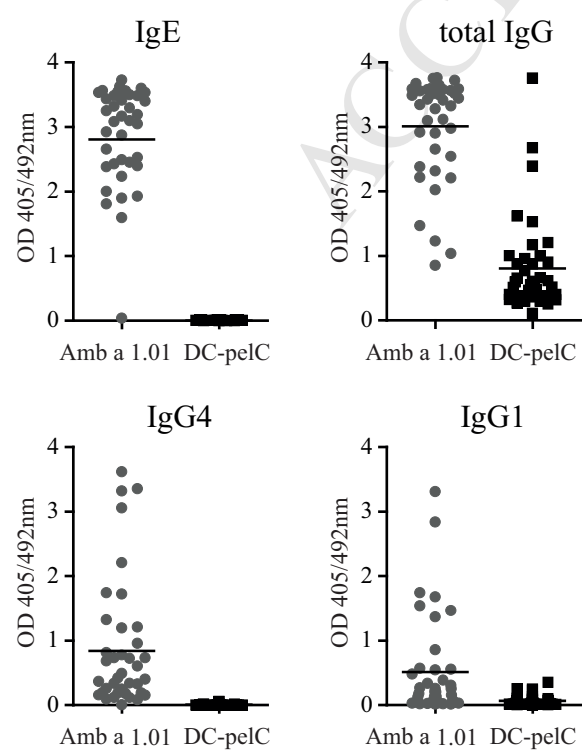
DC-pelC



Structure alignment



A Human antibody reactivity



ONLINE SUPPORT INFORMATION

Endolysosomal protease susceptibility of Amb a 1 as a determinant of allergenicity

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4) Allergy Clinic Reumannplatz, Vienna, Austria

5) Department of Pathophysiology and Allergy Research, Medical University of Vienna, Vienna, Austria

6) Present address: Institute of Experimental and Clinical Cell Therapy, Spinal Cord Injury and Tissue Regeneration Center Salzburg, Paracelsus Medical University, Austria

Corresponding Author:

Fatima Ferreira, PhD

Department of Molecular Biology, University of Salzburg

Hellbrunnerstr. 34, A-5020 Salzburg, Austria

Tel: +43-662-8044-5016, Fax: +43-662-8044-745016

Email: fatima.ferreira@sbg.ac.at

31 Abbreviations

- 32 ACN: acetonitrile
33 ALUM: aluminum hydroxide
34 Amb a: *Ambrosia artemisiifolia*
35 AP: alkaline phosphatase
36 BSA: bovine serum albumin
37 CD: circular dichroism
38 DTT: dithiothreitol
39 FA: formic acid
40 FTIR: Fourier-transform infrared
41 HPLC: high performance liquid chromatography
42 MS: mass spectrometry
43 MS/MS: tandem mass spectrometry
44 PBS: phosphate buffered saline
45 PE: Phycoerythrin
46 RBL: rat basophilic leukemia cells
47 TBS: Tris-buffered saline
48 TFA: trifluoroacetic acid
49 SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis
50

Material and Methods

Protein purification

Pollen (6 g) of *Ambrosia artemisiifolia* (Allergon Healthcare, Ängelholm, Sweden) was extracted with 180 ml of 5 mM NaP buffer pH 6.8, for 16h at 4°C, and clarified by centrifugation at 4,000 x g for 20 min. The supernatant was then treated twice with 8 g of extraction buffer-soaked polyvinylpyrrolidone (Sigma-Aldrich, St. Louis, USA), followed by a 5 min-centrifugation step (1,000 x g) after each addition. Before loading onto a 15 ml column packed with CHT Type I resin (Bio-Rad Laboratories Inc., Hercules, CA, USA), the extract was filtered through a 0.45 µm syringe filter (GE Healthcare, Chicago, IL, USA). The column was eluted with a 4-segment linear gradient (0 to 500 mM sodium phosphate (NaP) buffer pH 6.8 final concentration) and fractions containing Amb a 1.01 were pooled and dialyzed against 10mM NaP pH 8.5. After purification, proteins were stored at -20°C until further use.

The bacterial pectate lyase PLYC_DICCH (Uniprot ID: P11073) from *Dickeya chrysanthemi* (*Pectobacterium chrysanthemi*)(*Erwinia chrysanthemi*)¹ was expressed in *E. coli* SHuffle® T7 Express (New England Biolabs, Ipswich, MA, USA). Bacteria were transformed via electroporation with a plasmid vector pET26b (Novagen, Merck KGaA, Darmstadt, Germany) containing a harmonized DC-pelC construct. Bacteria were cultured in baffled flasks with 2 L LB broth supplemented with 10 ml/L glycerol and 25 mg/L kanamycin at 37°C and 220 rpm. Expression was induced at an OD600 of 0.6 using 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 21 h at 16°C. Bacteria were harvested by centrifugation at 3000 x g for 15 min and dissolved in 100 ml 5 mM NaP pH 6.8/g cell pellet. Cells were disrupted by three freeze/thaw cycles in liquid nitrogen, followed by a sonication step. Soluble protein fractions were collected by centrifugation at 13,000 x g for 20 min and filtered with a 0.45 µm syringe filter (GE Healthcare) before loading onto a 15 ml CHT Type I column (Bio-Rad Laboratories). Proteins were eluted with a 250 ml linear gradient from 0 to 500 mM NaP buffer pH 6.8. Fractions containing DC-pelC were pooled, dialyzed against 5 mM NaP pH 6.8, and reloaded onto the same column. The second elution was performed with a 100 ml gradient from 0 to 1 M NaCl in 5 mM NaP pH 6.8. Fractions containing DC-pelC eluting at 100% B were pooled and dialyzed against 10 mM NaP pH 8.5. Contaminating endotoxins from bacterial cell wall were removed with a Triton wash protocol, as previously described².

The endotoxin content of Amb a 1 and DC-pelC was determined using the HEK-Blue™ mTLR4 reporter cell assay (Invivogen, San Diego, CA, USA), according to manufactures

instructions. The levels of endotoxin in both preparations were determined to be below the detection limit of 6.1 EU/mg.

Physicochemical characterization

Amino acid analysis of the protein preparations was performed according to the PicoTag™ method (Waters, Milford, MA, USA) using a HP1100 HPLC system (Hewlett-Packard, San Jose, CA, USA) equipped with a 3.9 x 150mm Novapak C₁₈ column (Waters). The aggregation behavior and protein dispersity was assayed by dynamic light scattering (DLS) using the DLS 802 system (Viscotek Corp., Houston, TX, US). Data evaluation of 10 accumulated measurements was performed with the OmniSize™ software and displayed as mass weighted distribution of the hydrodynamic radius. For circular dichroism (CD) analysis, samples were measured at 0.1 mg/ml from 190-260nm at resolution of 1 nm with 1 nm bandwidth and a scanning speed of 1 nm/s. Spectra were recorded with a JASCO-J815 spectropolarimeter (Jasco, Tokyo, Japan) fitted with a PTC-423S Peltier type single position cell holder in 10 mM sodium phosphate buffer pH 8 at 20°C or 95°C, respectively. Data from five spectra were averaged, background corrected, and presented as mean residue molar ellipticity³. Infrared spectra were recorded at a constant temperature (25°C) using an AquaSpec transmission cell adapted to a Tensor II Confocheck Fourier transformed infrared (FTIR) system (Bruker Optics Inc., Billerica, MA, USA). Second derivatives were calculated by applying the Savitzky-Golay algorithm with 25 smoothing points. Spectra were vector-normalized and baseline corrected. Secondary structure elements were analyzed using the Quant2 method provided by Confocheck (Bruker Optics Inc.).

Peptide Analysis by nano-LC-MS/MS

Purified proteins (1 µg) were digested with the ProteoExtract All-in-One Trypsin Digestion Kit (EMD Millipore, Billerica, MA, USA). After digestion, samples were desalted using C₁₈ ZipTips (EMD Millipore, Billerica, MA, USA) and resulting peptides separated by reverse-phase nano-HPLC (Dionex Ultimate 3000, Thermo Fisher Scientific, Bremen, Germany, column: PepSwift Monolithic Nano Column, 100 µm x 25 cm, Dionex). The column was developed with an acetonitrile gradient (Solvent A: 0.1% (v/v) FA/0.01% (v/v) TFA/5% (v/v) ACN; solvent B: 0.1% (v/v) FA/0.01% (v/v) TFA/90% (v/v) ACN; 5–45% B in 60 min) at a flow rate of 1 µl/min at 55°C). The HPLC was directly coupled via nano electrospray to a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific). Capillary voltage was 2 kV. For peptide identification, the top 12 method was used with the normalized fragmentation energy at 27%. Survey and fragment spectra

were analyzed with Proteome Discoverer version 1.4 (Thermo Fisher Scientific) or Peaks Studio 7 (Bioinformatics Solutions, Waterloo, Canada), respectively.

Patients' sera

Ragweed allergic patients were selected based on typical case history, positive skin prick test and ImmunoCAP analysis (CAP w1 Common ragweed *Ambrosia artemisiifolia*, Phadia, AB, Uppsala, Sweden). Experiments using anonymized serum samples from allergic patients were approved by the ethics committee of the Medical University of Vienna (712/2010).

ELISA

For ELISA experiments, Maxisorp plates (Nunc, Thermo Fisher, Waltham, MA, USA) were coated with 100 ng protein /well in 50µl PBS overnight at 4°C. After washing and blocking, plates were either incubated with diluted human sera (1:50 for IgG and 1:250 for IgE detection) (buffer: TBS, pH 7.4, 0.05% (v/v) Tween, 1% (w/v) BSA) or with serial dilutions of murine sera (buffer: TBS, pH 7.4, 0.05% (v/v) Tween, 1% (w/v) BSA) overnight at 4°C. Bound human IgE was detected using an AP-conjugated mouse anti-human IgE antibody (Clone G7-26) (BD Biosciences, Franklin Lakes, USA), human IgG1 was detected by an AP-conjugated mouse anti-human IgG1-hinge antibody (Clone 4E6) and human IgG4 was detected using an AP-conjugated mouse anti-human IgG4 pFc' antibody (Clone HP6023). Murine IgG1, IgG2a, and IgE antibodies were detected using AP-conjugated rat anti-mouse IgG1, AP-conjugated rat anti-mouse IgG2a, and rat anti-mouse IgE antibodies (Clones SB77e, SB84a, and 23G3, respectively; Southern Biotech, Birmingham, USA), respectively. Colorimetric detection with 10 mM 4-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, MO, USA) was performed following incubation periods of 1h at 37°C and 1h at 4°C. All measurements were performed in duplicates and the results are presented as mean OD values.

Mediator release assays

For the analysis of murine IgE antibodies, non-transfected RBL-2H3 cells (ATCC® CRL2256™) ⁴ were used after passive sensitization with 100 µl murine serum samples diluted 1:20 in cell culture medium. Following washing steps, serially diluted protein antigens (1 ng/ml - 100 µg/ml) were added to trigger the release of mediators. For serum dilutions, a fixed antigen concentration of 10 ng/ml was used. Antigen-dependent β-hexosaminidase release into the supernatant was measured by enzymatic cleavage of

the fluorogenic substrate 4-methylumbelliferyl-N-acetyl- β -D-glucosaminide and expressed as percentage of total enzyme content of Triton X100-treated cells.

Proteolytic stability

Antigens were digested with a cocktail of endolysosomal proteases as previously described⁵. In brief, microsomal fractions were harvested from JAWSII cells (ATCC® CRL11904™) by differential centrifugation. 5 μ g protein was digested with 7 μ g purified microsome lysates in 100 mM citrate buffer adjusted to different pH values (pH 4.0, 4.5, 4.8, 5.2 and 5.9), 2 mM DTT at 37°C. Degradation assays were also performed with proteins adsorbed to 50% Alu-Gel-S (Serva, Heidelberg, Germany). Reactions were stopped by heating to 100°C. Resulting samples were analyzed by SDS-PAGE and by nano-LC-MS/MS, as described above.

Animal immunizations

Female BALB/c mice (Janvier Labs, Saint-Berthevin, France) at the age of 8 weeks were used for the immunization experiments (5 animals/group). 5 μ g of antigen were either adsorbed to 50% Alu-Gel-S (Serva, Heidelberg, Germany) v/v or formulated without adjuvant in PBS, and administered as two-50 μ l s.c. injections, bilaterally in the lumbar region at days 0, 7, 14, and 21. Sera were taken at days -2, 14, and 28. ELISA was used to analyze specific IgG1 and IgG2a antibodies whereas mediator release assays were performed to detect IgE antibodies. All animal experiments were conducted according to National guidelines approved by the Austrian Ministry of Science (BMWF-66.012/0009-II/3b/2013).

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Supplementary figure legend

Figure E1. (A) SDS-PAGE of purified proteins. Weak bands observed at 24 and 15 kDa correspond to known degradation products of Amb a 1, designated alpha and beta chain, respectively. (B) Mass spectrometry analysis of tryptic digests of purified natural Amb a 1.01 isoform and recombinant DC-pelC. (C) Purity of the protein preparations, as determined by mass spectrometry. Total ion current of diagnostic tryptic peptides were calculated in relation to all identified peptides.

Figure E2. (A) Circular dichroism (CD) spectra of Amb a 1.01 and DC-pelC measured at 20 and 95°C, respectively. (B) Second derivative of FTIR spectra of Amb a 1.01 and DC-pelC in the area of α -helix (1651cm^{-1}) and β -sheet (1637cm^{-1}) signals. (C) Secondary structure elements as calculated from FTIR spectra. (D) Structural model of Amb a 1.01 generated by Swissmodel (template: PDB:1PXZ) and DC-pelC structure (PDB:2EWE). Lower panels, structural alignment of Amb a 1.01 model and DC-pelC structure generated with the UCSF Chimera package

Figure E3. IgE, total IgG, IgG1 and IgG4 ELISA experiments performed with sera from 39 ragweed pollen-allergic patients.