Supplementary data for article:

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Supporting Information

Sub-pollen particles are rich carriers of major short ragweed allergens and NADH dehydrogenases: quantitative proteomic and allergomic study

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Material and Methods

2D SDS-PAGE

125 μg of short ragweed pollen protein fractions: TOT, SPP and APE were diluted to rehydration buffer (containing also 50 mM DTT, 0.5% IPG buffer ampholytes pH 3-10 NL and 0.002% Bromophenol Blue) under 20 V constant for 14 h. Focusing proceeded through a series of voltage steps (step and hold 100V 1 h; step and hold 250V 0:30 h; step and hold 300V 0:30 h; step and hold 500V 1 h; gradient 1000V 1 h; gradient 4000 1:30 h; gradient 8000V 2 h; step and hold 8000V 1 h) giving 25655 Vh in total. Current was limited to 50 μA per Immobiline Dry Strip gel. Prior to the second dimension, each gel strip was reduced in 5 mL of equilibration buffer (50 mM TRIS-HCl, pH 8.8, 6 M urea, 30% v/v glycerol, and 2% SDS) containing 64 mM DTT for 15 minutes and, subsequently alkylated in the same equilibration buffer but with 135 mM iodoacetamide. Proteins were then separated on the basis of their MW values with 12% SDS-PAGE using the Hoefer SE600 Electrophoresis unit (Amersham Biosciences). The gels were stained with colloidal blue silver Coomassie dye overnight, and de-stained with hot deionized water. Molecular masses were determined by running standard protein markers (Bio-Rad Laboratories, Hercules, CA, USA), covering the range of 10–250 kDa.

Protein spot quantification with Image Platinum version 7.0 software

The 2D SDS polyacrylamide gels with cCBB stain free background were scanned using Typhoon FLA 7000 (GE Healthcare, Piscataway, NJ) in digitization mode with fluorescence stage and 50 pixel size step resolution and latitude. Spot detection, matching and quantification analyses were performed with Image Master 2D Platinum software v7.0 (GE Healthcare). For image analysis, 3 extracts of SPP, TOT and APE from the batch of 2013 season and two extracts

from the batch of season 2014 were compared. Spots with abundance ratio values less than 0.667 and higher than 1.5 compared to aqueous pollen protein extract (APE) set at 1, were selected for downstream nLC-MS/MS analyses.

Sample preparation for shotgun proteomics analysis

In solution digestion of the short ragweed pollen fractions was done according to "urea" protocol https://masspec.scripps.edu/services/proteomics/insol_prot.php (more details in SOM). Briefly, 10 µg of pollen protein samples TOT, SPP and APE were reconstituted in 100 µL of 6M urea dissolved in 25 mM ammonium bicarbonate buffer (ABC) pH 8.5. DTT was added to final concentration of 10 mM as reducing reagent (1 h, at RT with agitation). Iodoacetamide was added as alkylating reagent (1 h, dark). Sample was diluted with 25 mM ABC to 1 mL. Trypsin digestion was performed over night at 37 C in ratio 1:30 to approximate amount of protein by weight. Samples were filtered and cleaned with zip-tips C18 (Thermo Fisher Scientific Inc., Bremen, Germany).

Identification of the short ragweed pollen proteins through protein database search

Identification of the short ragweed pollen proteins was performed by Proteome discoverer 1.3 (Thermo Fisher Scientific Inc., Bremen, Germany) and PEAKS Studio 7.5 version (BSI, Ontario, Canada). Signature MS/MS spectra were searched by SEQUEST and PEAKS DB algorithms against Uniprot derived *Asteraceae* family protein database (downloaded on 05/03/2016 from http://www.uniprot.org/). Database file with size of 10386 Kb contained 30318 sequences, both reviewed (UniProtKB Swissprot) and unreviewed (UniprotKB Trembl), including the common Repository of Adventitious Proteins (the cRAP database).

Carbamidomethylation of Cys was set up as fixed modification, while oxidation (Met) and deamidation (Gln, Asn) were taken into account as potential modifications as well as incomplete cleavage (up to 2 missed cleavage sites). Both peptide and protein filters were applied: positive protein identifications were based on detection of minimum 2 peptide sequences, including at least 1 unique sequence, and a search score up to 1% FDR threshold for peptide sequences (e.g. only high confidence peptides in SEQUEST algorithm). Window for mass precursor error tolerance was set at 15 ppm and error mass fragment tolerance was set at 0.5 Da for SEQUEST, PEAKS DB and PEAKS De Novo search algorithms.

For the gel based protein MS/MS results, spots were analysed as batch samples in duplicates with 3 different biological samples, while in solution trypsin-digested extract of TOT, SPP and APE proteomes, destined for shotgun MS/MS analysis were run in duplicates with 2 different biological batches.

Label free quantification of TOT, SPP and APE proteomes

Label free quantification (LFQ) was done with PEAKS Suite 7.5® (BSI, Ontario, Canada) LFQ algorithm, upon previously identified mass spectrometry shot gun results via PEAKS DB and De Novo algorithms. Filters were set as 20 ppm for precursor mass error tolerance and 0.5 Da for fragment ions, with FDR set at 1%. This algorithm uses raw mass spectrometry files but does rely on previously identified results of PEAKS DB analyses.

To calculate the extent of a pollen fraction allergome within its proteome, firstly sum of area under extracted ion chromatography (XIC) curve for all confidently identified and compared proteins within each pollen fraction (TOT, SPP and APE), was calculated. Next, percentages of allergomes for the respective pollen fractions were calculated by dividing sum of areas under

XIC curve of all officially recognized allergens with the sum of areas under XIC curve of all proteins for each fraction, separately, and then multiplying with 100 to obtain percentage.

Quantitative ELISA measurements

Quantitative IgE ELISA and data calculations, conversion from the absorbance at 450nm to kU_A/L was based from the interpolation of the standard curve with known amounts of kU_A/L of standards, according to Apostolovic et al, Allergy 2016 [2] and Mandhurantakam C et al 2010 [3]. Briefly, duplicates of seven calibrators prepared from an IgE myeloma with 640 kU_A/L of IgE (threefold dilutions, range 0.035–25.5 kU_A/L), two control samples and patient samples were added to a 96-well plate and incubated for 2 h. The wells containing IgE-calibrators and control samples were coated with 50 µL of 2,5 µg/mL monoclonal anti-human IgE (a kind gift from Phadia, Uppsala, Sweden) and wells containing patient sera were coated with 50 µL of 5 µg/mL of the respective ragweed protein extract (TOT, APE and SPP) in 0.1 M carbonate buffer, pH 9.6. Secondary antibody, mouse anti-human IgE conjugated with horseradish peroxidase (Abcam, UK) were incubated for 1 h. TMB was used as substrate with stopping the reaction with 1M H2SO4. The plates were read at 450nm.

Figure S1

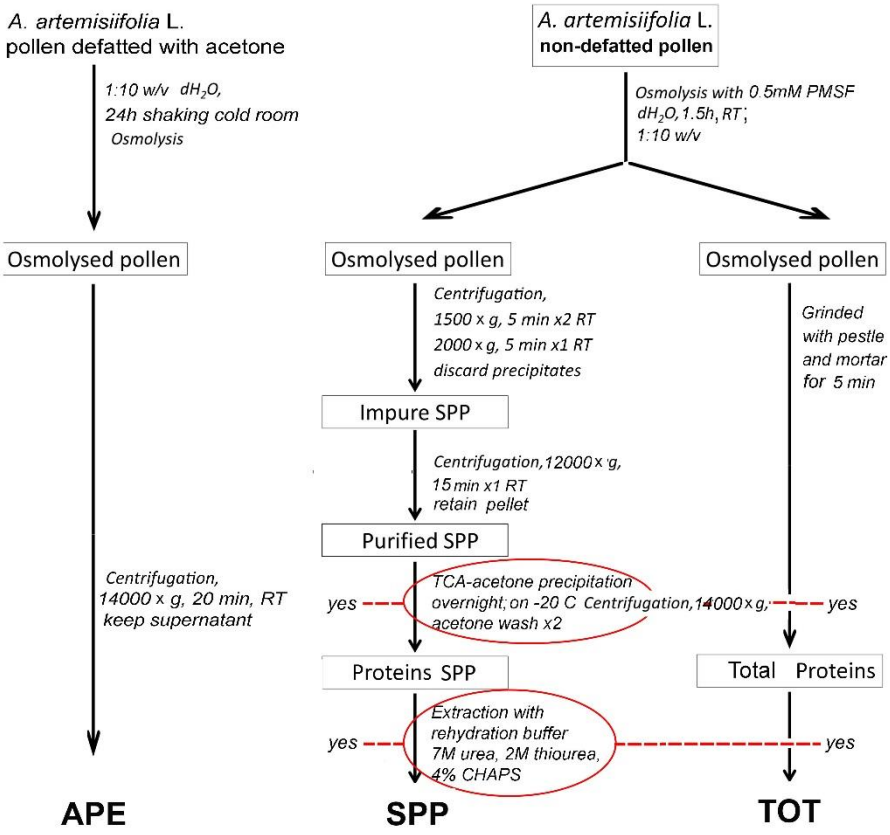

Figure S1. Fractionation and extraction strategy of *Ambrosia artemisiifolia* pollen. Aqueous pollen protein extract (APE), represents aqueous pollen sub-proteome, obtained by the procedure frequently used for preparation of standard allergenic diagnostic skin prick tests; SPP – sub-pollen particles and TOT- total pollen protein extract are further precipitated and cleaned with TCA treatment and immediately solubilized in rehydration buffer for isoelectrofocusing.

Figure S2

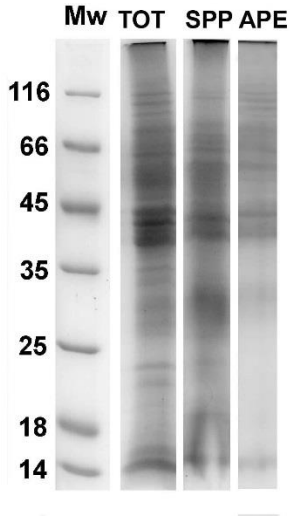

Figure S2 Representative 1D SDS-PAGE profiles of total pollen protein extract (TOT), sub-pollen particles (SPP) and aqueous pollen protein extract (APE) of short ragweed pollen on 12% polyacrylamide gels in reducing conditions. Mw – molecular weight protein markers in kilodaltons.

Figure S3

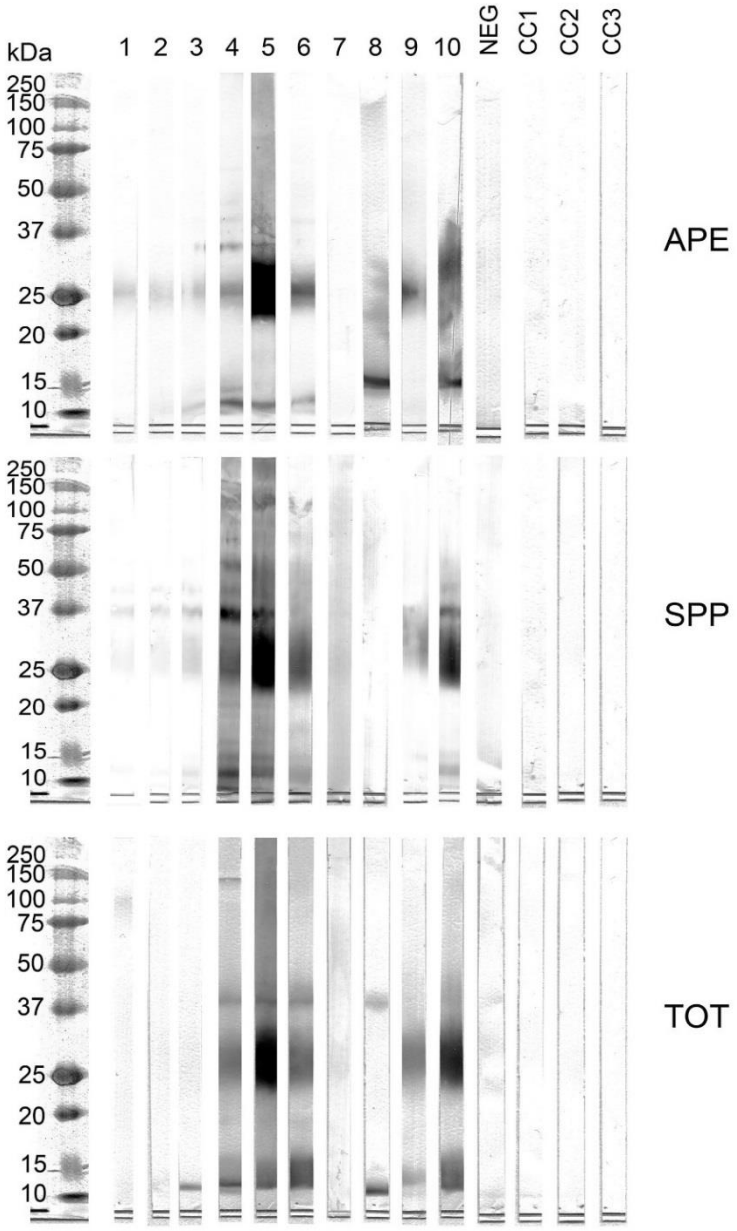

Figure S3. 1D immunoblot with IgE reactivity pattern of the first 10 patients against resolved pollen protein fractions of the short ragweed. Mw – molecular weight protein markers in kilodaltons (kDa). TOT – total pollen protein extract, SPP - sub-pollen particles and APE – aqueous pollen protein extract. NEG – sera of patient who is not allergic to the short ragweed (negative sera); CC1 – conjugate control that does not contain sera at all; CC2 - conjugate control that does not contain secondary antibody (antihuman IgE antibody); CC3 - conjugate control that does not contain tertiary antibody (anti-rabbit IgG coupled to alkaline phosphatase).