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

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Running Title: Short ragweed sub-pollen particles carry complete Amb allergen repertoire.

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Abstract

Background: Short ragweed (*Ambrosia artemisiifolia*) allergies affect more than 36 million people annually. Ragweed pollen grains release sub-pollen particles (SPP) of respirable size upon hydration or a change in air electrical conditions. The aim of this study was to characterise the proteomes and allergomes of short ragweed SPP and total pollen protein extract (TOT), and compare their effects with those of standard aqueous pollen protein extract (APE) using sera from short ragweed pollen-sensitized patients.

Methods: Quantitative 2D gel-based and shotgun proteomics, 1D and 2D immunoblotting, and quantitative ELISA were applied. Novel SPP extraction and preparation protocols enabled appropriate sample preparation and further downstream analysis by quantitative proteomics.

Results: The SPP fraction contained the highest proportion (94%) of the allergome, with the largest quantities of the minor Amb a 4 and major Amb a 1 allergens, and as unique, NADH dehydrogenases. APE was the richest in Amb a 6, Amb 5, and Amb a 3, and TOT fraction was the richest in the Amb a 8 allergens (83% and 89% of allergome, respectively).

Allergenic potency correlated well among the three fractions tested, with 1D immunoblots demonstrating a slight predominance of IgE-reactivity to SPP compared to TOT and APE. However, the strongest IgE binding in ELISA was noted against APE. New allergenic candidates, phosphoglycerate mutase and phosphoglucomutase, were identified in all the three pollen fractions. Enolase, UTP-glucose-1-phosphate uridylyltransferase, and polygalacturonase were observed in SPP and TOT fractions as novel allergens of the short ragweed pollen, as previously described.

Conclusion and Clinical Relevance: We demonstrated that the complete major (Amb a 1 and 11) and almost all minor (Amb a 3, 4, 5, 6, 8, and 9) short ragweed pollen allergen repertoire as well as NADH oxidases are present in SPP, highlighting an important role for SPP in allergic sensitization to short ragweed.

Keywords: *Ambrosia artemisiifolia*, label-free quantification, pollen allergomes, sub-pollen particles, new short ragweed allergens

Abbreviations:

1D – one dimensional

2D – two dimensional

APE – aqueous pollen protein extract

BCIP – 5-bromo-4-chloro-3-indolyl phosphate

CHAPS – 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

cCBB – colloidal Coomassie Brilliant Blue

DTT – dithiothreitol

ELISA – enzyme-linked immunosorbent assay

FDR – false discovery rate

IAA – iodoacetamide

LFQ – label free quantification

MS/MS – tandem mass spectrometry

NADH – nicotinamide adenine dinucleotide dehydrogenase

nLC-MS/MS – nano-liquid chromatography coupled to tandem mass spectrometry

NBT – nitroblue tetrazolium

PMSF – phenyl methyl sulfonyl fluoride

SDS–PAGE – sodium dodecyl sulphate polyacrylamide gel electrophoresis

SPP – sub-pollen particles

RT – room temperature (between 20°C and 25°C)

TOT – total pollen protein extract

TCA – trichloroacetic acid

tPBS – Tween 20 phosphate buffered saline

XIC – extracted ion chromatogram

Introduction

Much effort has been given to understand and alleviate allergic disorders caused by *Ambrosia artemisiifolia* (short or common ragweed), which affect more than 36 million people annually [1]. *A. artemisiifolia* is the most important seasonal aeroallergen in Europe and the USA, triggering rhinitis, conjunctivitis, and asthma, and is an exacerbating factor in atopic dermatitis [2, 3]. Recently, a sublingual tablet based on the major Amb a 1 allergen [4] was released to treat ragweed allergies.

Asthma incidence has long been linked to the presence of pollen, even though pollen grains are too large (15–100 μm) to penetrate into the lower airways where asthmatic responses originate [5]. The aetiology of allergic asthma caused by pollen grains was uncovered when the phenomenon of sub-pollen particles (SPP) release from grass pollen grains upon hydration was discovered [6-9]. Particle expulsion and release from grass pollen grains upon exposure to humid conditions or thunderstorms has been linked to allergic symptom exacerbation and increased incidence of allergic asthma [10, 11].

So far, only two studies have compared the water-extractable proteome and allergome of the pollen grain species and their SPP; these studies were conducted in *Phleum pratense* [12] and *Olea europaea* [13]. The results strongly suggest that in natural conditions, SPP may be the cause of allergic symptoms observed in sensitized patients, and that the allergenic properties of SPP are likely to be due to both their small size, which enables them to penetrate deeper into the bronchial airways, and their allergenic content.

Similar to grass pollen, short ragweed pollen grains, which are generally considered too large to reach alveoli, release SPP of respirable size upon hydration, which contain allergenic proteins and NAD(P)H oxidase activity [14]. NAD(P)H oxidase is an oxidoreductase shown to be involved in initiating adaptive immune responses against innoxious pollen proteins [15].

Recently, allergen characterisation of *Ambrosia artemisiifolia* revealed the novel Amb a 11 allergen group, consisting of a mature cysteine protease of 37 kDa "hidden" between Amb a 1 isoforms, as a new major allergen of short ragweed [16]. All short ragweed allergens have multiple isoforms, complicating proteomics analyses and leading to difficulties when creating compounds like commercial reagents for allergy diagnosis and therapy. Notably, Amb a 1 (pectate lyase) is comprised of more than 10 isoforms (http://www.allergen.org/viewallergen.php?aid=32). Additionally, there are another 8 minor allergen groups (Amb a 3 and Amb a 7, plastocyanines; Amb a 4, defensins; Amb a 5 homologs; Amb a 6, lipid transfer proteins; Amb a 8, profilins; Amb a 9 and Amb a 10, polcalcin and polcalcin-like proteins, respectively) [17, 18]. Even recent comprehensive immunoproteomic studies of short ragweed pollen [16, 18, 19], did not finalize allergen characterisation or determine allergic asthma mechanisms.

Nowadays, diagnostic products for pollen allergies are solely comprised of defatted, aqueous pollen protein extracts (APE) or single components. There has yet been no study of *A. artemisiifolia* pollen proteome fractions that compares the complete set of proteins and allergens and their relative abundance in different parts of pollen: non-defatted total pollen protein extract (TOT), non-defatted SPP proteome, and APE. The aims of our study were to fully characterise short ragweed SPP since they can reach deep into lungs, and to re-assess suitability of standard methodology for allergenic diagnostic preparation by comparing TOT and SPP proteomes and allergomes with those of APE. These aims were accomplished with quantitative gel-based and shotgun proteomics, high resolution 1D and 2D SDS-PAGE, 1D

and 2D immunoblotting, quantitative ELISA, and assays using sera of short ragweed pollen-sensitized patients. Novel SPP extraction and preparation protocols are presented that enable appropriate sample preparation and downstream analysis by quantitative proteomics.

Materials and Methods

Patient cohort and ethics statement

Sera from 16 Serbian ragweed-allergic patients with IgE level in range 4.5–440 kU_A/L (ImmunoCAP, w1; Phadia/Thermo Fisher, Uppsala, Sweden) were collected at the allergy clinic of the Institute for Virology, Vaccines and Sera "Torlak", Belgrade, Serbia (Table 1). Five non-allergic sera (<0.1 kU_A/L) were used as controls. The study was approved by the National Ethics Committee from the University of Belgrade, Serbia (No. 017/6 – 990/66). Written informed consent was obtained from donors prior to blood donation, and their data were processed and stored according to the principles expressed in the Declaration of Helsinki. Sera were either used individually or were pooled. The patient cohort contained a 1:2 female to male ratio, an age range of 15–58 years, median and average age value of 35 years (Table 1).

Pollen samples

Short ragweed pollen was obtained from the Institute for Virology, Vaccines and Sera, "Torlak", Belgrade, Serbia. The pollen was collected during the 2013 and 2014 pollination seasons. Anthers were collected, dried at 27°C, and gently crushed. The pollen released was sieved and stored at 4–8°C before extracting pollen protein fractions. Purity of the non-defatted, short ragweed pollen (99.5%) was checked by the particle count. All proteomic

investigations were run in duplicate, with three isolations of SPP, TOT, and APE fractions in the 2013 season, and two isolations in the 2014 season.

Reagents and material

Spectropor dialysis tubing was purchased from Fisher Scientific (UK). Organic solvents for mass spectrometry were obtained from J.T. Baker (Mallinckrodt Baker, Phillipsburg, USA). Ultra-pure water (18 mΩ) was prepared with a Smart2Pure3 Barnstead aqua purification system (Thermo Fisher Scientific, MA, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Total pollen protein extract and sub-pollen particle isolation

Short ragweed pollen grains (1 g) were osmolysed in deionized (10 mL) water in the presence of 0.5 mM PMSF for 1.5 h at room temperature (RT). In parallel, for the total pollen protein extraction, an extraction protocol by Sheoran et al. [20] was followed with minor modifications. An aqueous pollen grain suspension (1 mL, 1/10 w/v) was ground using a porcelain mortar and pestle for 5 minutes with constant vigorous grinding, and the proteins were precipitated with 4 volumes of cold acetone/10% TCA/25 mM DTT and incubated overnight at -20°C. The pellet was washed two times with pure cold acetone/25 mM DTT. Proteins were extracted from the pellet by direct re-solubilisation in incomplete rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS) for 1 h, followed by centrifugation at 14,000 × *g* for 10 minutes.

SPP were isolated as described Bacsi et al. [14] with several modifications. Intact pollen grains and pollen fragments were removed from suspension by low-speed centrifugation (1500 × *g* for 5 minutes). This step was repeated twice, followed by centrifugation at 2000 × *g* for 5 minutes. Finally, the supernatant suspension containing SPP was pelleted by centrifugation (12,000 × *g* for 15 minutes). SPP proteins and water soluble proteins from the

remaining supernatant were purified by the acetone/TCA method and extracted with rehydration buffer as described for total pollen proteins. The extraction scheme is given in Figure S1.

Preparation of short ragweed aqueous pollen protein extract (APE)

APE from short ragweed was prepared as previously described [21] with minor modifications that reflect the standard procedure for preparing aqueous pollen extracts for diagnostic purposes [22, 23]. Briefly, pollen samples (5 g) were defatted by acetone, suspended in 50 mL of deionized water, and shaken at 4°C overnight. The suspension was centrifuged at 14,000 × *g* for 20 minutes at RT. Protein sample concentrations were determined with the Bradford method [24].

One-dimensional (1D) and two-dimensional (2D) SDS-PAGE

1D electrophoresis was performed on 12% SDS polyacrylamide gels according to the standard Laemmli protocol [25] under reducing conditions. TOT, SPP, and APE samples (15 μg protein per well) were run on the gel (Figure S2). TOT, SPP, and APE protein extracts (125 μg) were isoelectrofocused with an Ettan IPGphor 3 IEF System (GE Healthcare, Uppsala, Sweden) and further separated using 12% SDS-PAGE in a Hoefer SE600 Electrophoresis unit (Amersham Biosciences). The 2D gels were scanned with Typhoon FLA 7000 (GE Healthcare) and spots were quantified and matched with Image Master 2D Platinum software v7.0 (GE Healthcare) (more details in Supp. Info).

1D and 2D immunoblotting

1D and 2D acrylamide gels were transferred to PVDF membranes with a semidry Nova-Blot system (GE Healthcare, Uppsala, Sweden). The membranes were then blocked in 1% BSA dissolved in 0.05% Tween 20 phosphate-buffered saline (tPBS) for 2 h at RT. To identify

allergenic proteins, for 2D blotting, serum was pooled from all 16 patients and samples from the first 10 patients were utilized for individual 1D blot analysis (Table 1). Sera were diluted 1/10 for 1D blotting in 0.2% BSA in tPBS. Membranes were incubated with sera at 4°C overnight with agitation, and washed three times with tPBS. 2D immunoblot detection was carried out by a 1 h incubation with mouse anti-human IgE conjugated with horseradish peroxidase (HRP) (dilution 1:2000, Abcam, UK) and positive signals were developed with a chemiluminescence substrate for HRP detection on a ChemiDoc instrument (BioRad, USA). For 1D blots, membranes were incubated with a rabbit anti-human IgE antibody (dilution 1:2000, MIAB, Sweden) at RT for 2 h. 1D immunoblot detection was carried out by an alkaline phosphatase-conjugated goat anti-rabbit IgG (dilution 1:1000, Jackson Immunoresearch, USA) after 2 h incubation at RT. The membrane strips were than simultaneously developed in 0.165 mg/mL BCIP, 0.33 mg/mL NBT in 100 mM $NaHCO_3$, 5 mM $MgCl_2$, pH 9.5.

IgE-reactivity of A. artemisiifolia pollen sub-allergomes by quantitative ELISA

The rehydration buffer in which TOT and SPP pollen fraction samples were initially dissolved was exchanged with 0.1 M carbonate buffer, pH 9.6, and solutions were filtered with Amicon Ultra-0.5 Centrifugal filters with a cutoff of 3 kDa (Millipore). Analytical 12% 1D SDS-PAGE profiles under reducing conditions before and after this buffer exchange were recorded and the resulting profile showed no major differences (data not shown). Individual serum IgE-reactivity to the commercial short ragweed caps (ImmunoCAP, w1) was determined on the ImmunoCAP System (Phadia AB/Thermo Fisher Scientific) according to the manufacturer's instructions. The results are presented as kU_A/L, where the cut-off for allergen-specific IgE was ≥ 0.10 kU_A/L (Table 1**)**. Quantitative ELISA measurements against 3 pollen fractions were performed using methods described by Apostolovic et al. [26]. After incubation with 50 µL mouse-anti-human IgE-HRP (Abcam, diluted 1/2000) for 1 h at RT,

TMB substrate was added and the reaction was stopped with 1 M H_2SO_4. Results were expressed as kU_A/L and were considered positive when the IgE responses exceeded the mean + 3 SD of the 2 healthy controls ($kU_A/L \geq 0.10$). More details are available in Supplementary Information.

In-gel and in-solution digestion for mass spectrometry and shotgun proteomics analysis

After cCBB staining and scanning, spots were excised and in-gel digested using the method of Shevchenko et al. [27]. The proteins were digested with proteomics-grade porcine trypsin (approximately 150 ng of trypsin in 25 mM ABC per gel spot) as previously described [28]. In-solution digestion of the short ragweed pollen fractions was performed according to the protocol: https://masspec.scripps.edu/services/proteomics/insol_prot.php, presented in detail in Supplementary Information.

Nano-LC-MS/MS

Trypsin-digested peptides were chromatographically separated using an EASY-nLC II system (Thermo Fisher Scientific Inc.) with a 2-column set up: a trap column C18-A1, 2 cm (SC001, Thermo Fisher Scientific Inc.) and an analytical column PepMap C18, 15 cm × 75 µm, 3 µm particles, 100 Å pore size (ES800, Thermo Fisher Scientific Inc., Bremen, Germany). A total of 2 µL of each shotgun sample and 4 µL of each 2D gel sample was loaded and separated as previously described [29].

Identifying short ragweed pollen proteins through a protein database search

Identification of the short ragweed pollen proteins was performed using Proteome discoverer 1.3 (Thermo Fisher Scientific Inc.) and PEAKS Studio 7.5 (BSI, Ontario, Canada) with more details in the Supplementary Information.

Label-free quantification of TOT, SPP, and APE proteomes

Label-free quantification (LFQ) was performed with the PEAKS Suite 7.5 (BSI, Ontario, Canada) LFQ algorithm, upon previously identified mass spectrometry shotgun results through PEAKS DB and De Novo algorithms. Filters were set to 20 ppm for precursor mass error tolerance and 0.5 Da for fragment ions, with a FDR set at 1%. More details are found in the Supplementary Information.

Protein sequence analysis and bioinformatics tools

A homology search and alignment of proteins identified from the amino acid sequences was achieved using UniProt, BLAST, and Align. Further functional subproteome mapping enrichment analyses were performed with the GO ontology consortium, QuickGO software (http://www.ebi.ac.uk/QuickGO), and FunRich software *(*www.funrich.org).

Results

Unique proteins and short ragweed pollen allergens distribution within different pollen fractions

A common obstacle in plant proteome analysis is the lack of sequenced genomes and the very limited number of database protein entries for many plant species and tissues. This problem arose when analysing *A. artemisiifolia* pollen, whose proteins were mostly identified by determining their homology to well-studied model plant species (*Arabidopsis thaliana, Oryza sativa,* etc.,) whose genomes are sequenced, annotated, and the corresponding homologous protein sequences are available in protein databases.

In-solution trypsin-digested TOT, SPP, and APE pollen fractions were subjected to shotgun proteomic analyses. Complete lists of identified proteins in the analysed pollen fractions are presented in Table S1-3. The major qualitative proteome differences observed between these

3 pollen fractions are depicted in Figure 1. It is interesting to note that all three pollen fractions contained a full set of major allergens (Amb a 1 and 11) and almost all minor short ragweed allergens (Amb a 3, 4, 5, 6, and 8).

The TOT fraction also contained unique proteins such as a novel isoform with high sequence homology to the pan-allergen profilin-1 from *Artemisia vulgaris* (mugwort). The APE pollen fraction contained a 10 kDa polcalcin isoallergen (Amb a 9 minor allergen group) (Figure 1, Table S3) as a unique protein entry. In addition to other polcalcins discovered, within all three fractions, a 16 kDa polcalcin from mugwort species was detected (Q2KM81), probably representing an undiscovered, homologous isoform in short ragweed (Tables S1-S3); whether this isoform is an allergen candidate needs to be confirmed, though Q2KM81 is an allergen (Art v 5.0101). Similarly, pectate lyase from mugwort (A0PJ16, minor allergen Art v 6.0101) was detected in SPP, likely representing an undiscovered, homologous pectate lyase isoform in short ragweed (Figure 1, Tables S2 and S5). In addition, NADH dehydrogenase protein was detected, which has a role in the unique allergic response to SPP (Tables S2 and S5).

Gene ontology (GO) cellular component analyses revealed that TOT and SPP fractions contained proteins from many cellular component categories, having almost twice more GO terms than the APE fraction proteins (Figure 2). The APE fraction mostly contained proteins from pollen cytoplasm (50%) and cytoskeleton (37%), with insignificant enrichment in 3 other terms, while SPP contained a substantial share of cytoplasm and cytoskeleton proteins as well as significant enrichment for mitochondrial, respiratory chain, phosphopyruvate-hydratase complex, and endoplasmic reticulum proteins. The TOT fraction also had all these proteins significantly enriched, in addition to microtubules and proton-transporting ATP synthase. Interestingly, SPP appear to possess the richest pollen sub-proteome regarding the number of different GO identifiers (69) as compared to TOT (66) and APE (48) fractions (Table S4).

Quantitative mass spectrometry reveals the most abundant major short ragweed allergens are within SPP among three different pollen fractions

Following shotgun analyses, a label-free quantification of identified proteins provided comparative analysis of proteomes and allergomes from TOT, SPP, and APE fractions. Dominant allergens of the SPP fraction were Amb a 4 (76%), Amb a 6 (10%), and major allergen group Amb a 1 (6%), while for the APE fraction Amb a 6 (42%), Amb a 4 (39%), and Amb a 5 (8%) predominated, and the TOT fraction contained 42% of Amb a 4, 33% of Amb a 8, and 14% of Amb a 1 (Figure 3A). The percentage of shared Amb a allergen groups within each pollen fraction cannot illustrate their actual (absolute) difference in abundance. However, a plot with combined peak areas of all allergen isoforms belonging to a certain Amb a allergen group (Figure 3B) showed the SPP fraction to be the most abundant in Amb a 4, Amb a 1, and Amb a 11, while APE was the richest in Amb a 6, Amb 5, and Amb a 3, and TOT fraction was richest in the Amb a 8 allergen group. The extent of allergens within the total sum of pollen proteins quantified by LFQ approach (e.g. allergome within proteome), showed that 83%, 89%, and 94% of allergens belonged to the Amb pollen allergome of TOT, APE, and SPP, respectively (Table S5).

A heatmap of pollen proteins in TOT, SPP, and APE fractions shown in Figure 4 allowed for effortless visual inspection of differences in protein abundance; green cells with black circles represent totally absent protein isoforms. The TOT fraction had the highest proportion of the most abundant proteins (red-coloured cells) within its proteome, while SPP and APE fractions were almost equal (Figure 4, Table S5). It can be observed that the SPP fraction possessed unique and substantially higher levels of dehydrogenases (NADH) and dismutases (SOD), respectively, while containing fewer missing proteins compared to the APE fraction (Figure 4).

2D SDS-PAGE analysis and MS/MS analysis of selected allergen spots

Spots of interest, such as IgE binding spots and spots with large differences in quantity among the 3 pollen fractions (at least 1.5 times more or less abundant as revealed by Image Master 2D Platinum software v7.0 (GE Healthcare, USA), were analysed by mass spectrometry (Figure 5A, Table 2). All short ragweed allergen groups were identified except for plastocyanine Amb a 7 and polcalcin-like Amb a 10. Additionally, protein spots with the exclusive presence in certain fractions and/or 1.5 times higher abundance compared to the other pollen fractions were analysed, such as spot group 16 and X2 in the SPP fraction (Figure 5A, Table 2).

Allergenic properties of short ragweed-pollen fractions

The allergenic properties of TOT, SPP, and APE fractions were characterised by 2D immunoblotting with pooled serum from all 16 patients (Figure 5B). The TOT, SPP, and APE IgE-binding 2D maps appeared similar with some minor differences (Table 2, Figure 5B). In all three protein fractions, the major allergen Amb a 1 (spots 1, 2, 6, 7, 8, 9 and 10) and Amb a 11 (spots 3, 4 and 5) isoforms, as well as minor acidic allergen isoforms of Amb a 4 (spots 12-15) and Amb a 8 (spot 18), bound IgE from the serum pool, which agrees with our MS/MS data (Figures 5A, B and Table 2). However, not all Amb a 1 group isoforms reacted with the serum pool, such as spot 11 determined to be Amb a 1.0501 (formerly Amb a 2) (Figures 5A, B). In addition, there were some fine differences in Amb a 11 isoform reactivity patterning with enhancement in the SPP fraction (Spot 5 in Figure 5B, Table 2). The minor allergen groups from short ragweed, Amb a 5 (spot 21), 6 (spot 22), and 9 (spot 23) present in TOT, APE, and SPP did not bind IgE from the pooled patient sera, in contrast to the plastocyanine minor Amb a 3 allergens (Spot 20 in Figures 5A, B and Table 2). A protein spot (X3) in the TOT fraction that bound serum IgE at 16 kDa and pI 6.3 was not present in SPP or APE fractions. Similarly, an IgE-reactive protein spot at 16 kDa and pI of

6.7 was present only in TOT and APE fractions (spot X1, Figure 5B). The MS/MS data failed to reveal their identities, including the IgE-reactive spot X4 present in all pollen fractions.

The MS/MS spectra of protein spots at approximately 55 kDa in the acidic region showed that sera from ragweed-allergic patients could bind to new allergen candidates, enolase/UTP-glucose-1-phosphate-uridylyltransferase/polygalacturonases (group of spots 17 in Figures 5A, B, and Table 2). Similar to results observed by Bordas-Le et al. [18], we detected enolase and UTP-Glc at acidic regions at 55 kDa, however, rather we observed separate protein identity spots (see spots 17, 18 within spot group 17 in Table S6). This group of enzymes (spot group 17) reacted strongly in the SPP fraction, faintly in the TOT fraction, and no reactivity is seen on the APE blot fraction, although these proteins were detected using APE 2D SDS-PAGE (Figure 5A). Phosphoglycerate mutase found in the short ragweed pollen proteome by Bordas-Le et al. [18] was not the sole allergen candidate in that particular spot, while in our study this protein reacted together with phosphoglucomutase in all 3 pollen fractions (Spot group 19 in Figures 5A, B and Table 2). In spot group 19 (Table 2), we identified 2,3 phosphoglycerate mutase and phosphoglucomutase as separate spots with single protein hits per protein spot (for more details see spots 32, 33 within spot group 19 in Table S6).

Additionally, the IgE-reactivity of individual patient sera to TOT, SPP, and APE fractions was determined by quantitative ELISA (Figure 6, Table 1) and 1D immunoblotting (Figure S3). The IgE reactivity in ELISA showed a high correlation between three different samples, with the highest correlation between SPP and TOT extracts (rho=0.98; $p<0.0001$) (Figure 6). Interestingly, the IgE potency of the APE fraction was slightly more similar to TOT fraction potency than to SPP fraction potency. Median and average values of IgE binding followed the same decreasing order: APE, TOT, and SPP fractions (1.2, 0.87, and 0.63 kU_A/L for median values, and 2.32, 1.68, and 0.68 kU_A/L for average values, respectively) as calculated from Table 1. With only a few exceptions, the most prominent IgE reactivity was observed

for the APE allergenic extract (Table 1). These data show that individual differences in reactivity exist among patients. These differences are partially supported and better visualized through the individual 1D IgE-reactive immunoblots from the first 10 patients (Table 1, Figure S3), where overall IgE reactivity was similar among 3 fractions, however SPP slightly predominates in terms of different allergenic bands and their frequency.

Discussion

We have, for the first time, comprehensively described the proteome and allergome of short ragweed SPP, and compared these profiles to those from aqueous and total pollen protein fractions. Our aim was to explore how SPP may contribute to the immunopathogenicity of allergenic properties besides their *alveoli*-penetrating size, and also to re-assess the suitability of standard methodology for preparing allergenic diagnostics.

Vrtala et al. [22] previously showed that the pollen proteome pattern depends on the extraction procedure and that majority of allergens emerge with aqueous extraction (e.g. upon pollen grain hydration), while harsher extraction procedures also collect non- and less allergenic membrane and cytoskeletal proteins [22]. Qualitative differences between proteomes in mass spectrometry shotgun analysis revealed that the TOT fraction had the highest number of different protein groups (Figure 1). This can easily be explained due to the grinding step followed by the detergent protein extraction (Figure S1). The grinding step was added to maximize protein qualitative yield in the TOT fraction because proteins not extracted by the aqueous extraction could still be allergenic. It is evident that the TOT fraction contained unique proteins difficult to extract with water only, such as cytoskeletal actins, tubulin alpha and beta, and heat shock proteins (Figure 1). In natural conditions, grass

and tree pollen grains can burst even without heavy rain or extremely high relative humidity (≥80%) [13, 30]. Sometimes, only strong wind or air electric conditions (during thunderstorm) can induce pollen rupture [7, 31], likely exposing membrane proteins, non-soluble cytoskeletal proteins, and other pollen proteins not easily extractable with water. This justifies the use of a detergent to enhance protein extraction in TOT and SPP fractions.

Pollen allergy diagnostic products are currently solely based on defatted, aqueous pollen extracts and/or single protein component from this extract. It has been described that the waxy-lipid coating of *Bermuda* grass pollen contains proteins with IgE-binding capability and protease activity, such as cysteine protease and endoxylanase [32], that are completely removed during defatting. Therefore, we started our isolation of TOT and SPP fractions from un-defatted pollen, to be able to observe any difference stemming from APE fraction, which is normally prepared from de-fatted pollen grain (Figure S1).

Unique protein entries in the SPP fraction that are important from an allergy point of view include NADH dehydrogenase, which acts as a synergizing factor for inducing allergic inflammation via producing reactive oxygen species [33, 34]. Moreover, both shotgun and gel-based proteomic analyses suggested that the SPP fraction contained the full Amb a major (Amb a 1 and Amb a 11) set and the diversified minor (Amb a 3, 4, 5, 6, 8 and 9) set of officially recognized allergen groups, with a total of 22 allergen isoforms, compared to the TOT fraction with 20 and APE with 18 allergen isoforms (Tables S1-S3). However, this diversity cannot be explained by the different repertoire of Amb a allergen functions since in each pollen fraction there is at least one isoform member representing major and minor allergen groups in short ragweed. Rather, the difference is quantitative, where LFQ data supported MS/MS shotgun analyses, pointing to SPP as the fraction with the highest (94%) shared allergome within its proteome (Table S5).

Regarding molecular function, it is worthy to mention that pectate lyase activity (a function of the major short ragweed Amb a 1 allergen) was highest in the SPP fraction, thus confirming previous qualitative shotgun MS/MS data of SPP fraction having the richest repertoire of major Amb a 1 allergens with 10 isoforms (Table S4). The number of different GO terms in biological processes, molecular function, and cellular localization was also highest in the SPP fraction (Table S4), containing the most numerous GO terms in cellular localization (Figure 2). This result can be interpreted as SPP being armed with variety of pollen cellular parts that enhance the allergic response. Additionally, in the cytoplasm and cytosol, where the majority of allergens reside, membrane-associated, extracellular region, and respiratory chain proteins have already been shown to be novel allergenic candidates or allergy response enhancers, such as enolase and NAD(P)H oxidoreductases (Figure 2). This is important in the context of current pollen preparations for allergy diagnostics and treatment, which are mostly based on aqueous pollen extraction [34, 35]. SPP possesses NADH dehydrogenase as a unique entry, suggesting that this enzyme could be associated more firmly with SPP granules, in contrast to the many highly water-soluble proteins in the APE fraction. In addition, it is interesting to observe that there are much more cysteine proteinase inhibitors, cystatins, in the APE fraction than in TOT and SPP fractions. Whether this potential inhibition would influence allergenicity of the APE fraction warrants further investigation, and the relevance of potential cysteine protease inhibition (Amb a 11 major allergen group) in terms of modulating the allergic response also needs further examination.

The results from the 2D gel proteome quantification are in line with the LFQ results, and point to a higher content of acidic Amb a 4 isoforms and basic forms in SPP. Amb a 4 is a minor allergen in short ragweed pollen, but is a major allergen in mugwort (Art v 1). For ragweed pollen, both basic and acidic isoforms of this defensin-like allergen have been described, while the major mugwort pollen allergen, Art v 1, exists only in a variety of basic

isoforms [36]. It is common for mugwort-sensitized patients to react to homologous allergens in *A. artemisiifolia* [37]. In fact, 42% of Art v 1-sensitized patients also react to Amb a 4, while ragweed pollen-allergic patients also react to Art v 6 (a homologous allergen in mugwort, pectate lyase) [37, 38]. Therefore, a high load of both basic and acidic isoforms of Amb a 4 in ragweed SPP may directly contribute to cross-sensitization and cross-reactivity between mugwort and ragweed.

In the IgE immunoblot, we showed that pooled patient sera reacted with most Amb a 1 isoforms, which agrees with previous findings showing that >90% of ragweed allergic patients' IgE-reactivity is directed toward Amb a 1 [39, 40]. However, our patient serum pool did not react with the Amb a 1.05 isoallergen group (former Amb a 2), which is recognized in about 70% of ragweed allergic patients [41, 42]. Additionally, 2D immunoblotting and MS/MS data revealed a strong response to acidic Amb a 4 allergen in all 3 fractions, with the most prominent response in SPP fraction. In addition, as a secondary finding from 1D immunoblots of the first 10 short ragweed allergic patients (Figure S3), 8 out of 10 patients reacted to Amb a 4 in SPP, which is surprising considering that Amb a 4 is a minor allergen. Amb a 11 reactivity is also present in all 3 fractions but was most pronounced in SPP (Table 2). In contrast, Amb a 3 allergens bound IgE in TOT and APE fractions but not in SPP (Table 2, Figures 5A, B). This lack of IgE binding to Amb a 5, 6, and 9 by patient serum pool in the 2D immunoblot may be due to their overall low IgE reactivity to these minor pan-allergens, whose IgE reactivity rate is less than 20% in a population, www.allergen.org), or that these patients were preferably reactive to conformational epitopes of Amb a 5, 6, and 9 that were destroyed in the reducing conditions of SDS-PAGE.

The novel allergen candidates previously reported by Bordas-Le et al. [18], such as the minor enolase and the major polygalacturonases/UTP-glucose 1-phosphate uridylyltransferase (UTP-Glc) were also detected and confirmed in our study. Novel allergenic candidates revealed in this study were phosphoglycerate mutase and phosphoglucomutase.

The IgE-reactivity as determined by quantitative ELISA showed a high correlation between the three different samples, with the highest median and mean values occurring in the APE fraction. These results point to the fact that the APE fraction, due to its highest allergenic potency (approximately double the SPP allergenic potency based on median ELISA IgE binding values), would be suitable for diagnosing short ragweed allergy. In contrast, LFQ and shotgun data from the SPP fraction demonstrated superior Amb a 1 and Amb a 11 abundance, and the SPP fraction IgE reactivity was highest in 1D immunoblots (Figure S3).

In particular, IgE reactivity to the SPP fraction seems to follow the specific sensitization pattern of Amb a 4 allergen. In the future, the SPP fraction should be regarded as an adjuvant component in diagnostics because of its superb potential allergenic properties; this fraction was the richest in the number of distinct officially recognized Amb a allergens, contained the most overlap between allergome and proteome, possessed the highest content of minor Amb a 4 and major Amb a 1, Amb a 11 allergens, and exhibited the unique possession of NADH oxidoreductases. In addition, SPP are the major air allergen carriers beside intact pollen grains, and are especially important when considering the allergen administration route to the lung.

Our study revealed the new allergenic candidates phosphoglycerate mutase and phosphoglucomutase, which were IgE-reactive in all three fractions, and confirmed the presence of the previously described enolase, UTP-glucose-1-phosphate uridylyltransferase, and polygalacturonase as allergens that were primarily reactive in the SPP fraction.

In conclusion, we were able to demonstrate that the full major and minor short ragweed allergen repertoire is present and can reach alveoli through SPP therefore confirming the importance of SPP in the process of allergic sensitization. The very rich content of oxidoreductases, especially NADH oxidoreductase, present in SPP further strengthens the role of these particles in the process of pollen allergic inflammation in the lung.

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Conflict of interests

The authors declare no conflict of interest.

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Table 1. Demographic description of patient cohort with previous clinical history of short ragweed allergy and w1 ImmunoCAP and quantitative ELISA results

No	Sex	Age	short ragweed w1	Amb APE	Amb TOT	Amb SPP
			CAP (kU_A/L) and class	ELISA (kU_A/L)		
1	M	42	18.5 (4)	0.123	**0.231**	0.223
2	M	29	23.1 (4)	**0.262**	0.224	0.227
3	F	30	35.5 (4)	**0.275**	0.231	0.219
4	F	58	>100 (6)	n.d.	n.d.	n.d.
5	M	51	440 (6)	**11.097**	9.377	8.331
6	M	23	82.6 (5)	**1.881**	1.482	1.240
7	M	34	35.4 (4)	**0.309**	0.220	0.225
8	M	48	94.9 (5)	**3.467**	1.798	1.125
9	F	15	65.6 (5)	0.390	**1.702**	1.137
10	F	36	253 (6)	**4.917**	0.867	0.630

11	M	25	54.4 (5)	**1.632**	1.371	1.133
12	M	37	69.3 (5)	**1.198**	0.312	0.233
13	M	29	240 (6)	4.774	4.709	2.880
14	F	35	90.6 (6)	**4.012**	2.385	1.142
15	M	41	29.2 (4)	**0.365**	0.236	0.230
16	M	25	4.5 (3)	**0.121**	0.115	0.110

Legend: w1, ImmunoCAP on *Ambrosia artemisiifolia*; SPP, sub-pollen particles; TOT, total pollen protein extract; APE, aqueous pollen protein extract; n.d., not determined. Bold case values in quantitative ELISA experiments, performed with non-commercial "in-house" prepared TOT, SPP, and APE fractions, denote the highest value of IgE binding among the three pollen protein fractions. The results are presented as kU_A/L, where the cut-off for allergen-specific IgE was ≥ 0.10 kU_A/L, based on the sera assessment of two healthy (non-allergic) patients.

Table 2. IgE reactive protein spots/groups and allergens identified in TOT, SPP, and APE fractions of short ragweed pollen by 2D SDS-PAGE and a 2D immunoblot probed with pooled patient sera

Spot/ Group No	Mw/pI - Gel (average)	Mw/pI database	Accession number	Protein/Allergen Name*	2D Immunoblot		
					TOT	SPP	APE
1	43/5.9	42.7/5.90	P27759	Pollen allergen Amb a 1.0101	●	●	●
2	43/5.8	42.7/5.90	P27759		●	●	●
3	41/6.4	43.1/6.90	V5LU01	Pollen allergen Amb a 11.0101	●	●	●
4	41/6.6	43.1/6.90	V5LU01		●	●	●
5	41/6.9	43.1/6.90	V5LU01		○	●	○
6	38/8.1	43.6/7.20	P27760	Pollen allergen Amb a 1.0201	●	●	●
7	38/8.3	43.6/7.20	P27760		●	●	●
8	38/5.9	42.9/6.10	P27761	Pollen allergen Amb a 1.0301	●	●	●
9	38/6.3	42.9/6.10	P27761		●	●	●
10	42/6.0	42.8/5.97	P28774	Pollen allergen Amb a 1.0401	●	●	●
11	43/6.3-6.8	44.1/6.47	P27762	Pollen allergen Amb a 1.0501	○	○	○
12	26/4.9	9.9/4.82	D4III3	Pollen allergen Amb a 4.0101	●	●	●

13	26/5.1	9.9/4.82	D4III3		●	●	●
14	26/5.4	9.9/4.82	D4III3		●	●	●
15	26/5.9	13.3/5.04	D4IIH1		●	●	●
16	27/9.0	13.4/7.52	Q84ZX5	Major pollen allergen Art v 1, OS=*Art. vulgaris*		○	
17	52-58/5.2-6.0	47.6/5.61	Q43321	Enolase, (Enol) OS=*Alnus glutinosa*	●	●	○
		51.8/5.78	P19595	UTP--glucose-1-phosphate uridylyltransferase, (UTP-Glc) OS=*Solanum tuberosum*			
		43.4/6.86	O22818	Probable polygalacturonase At2g43860, OS=*A. thaliana*			
18	14/4.6-4.9	14.1/4.88	Q2KN23	Pollen allergen Amb a 8.0102	●	●	●
		14.3/5.02	Q64LH0	Pollen allergen Amb a 8			
19	66/6.7	61/5.85	Q9M9K1	Probable 2,3-bisphosphoglyc-phosphoglycerate mutase 2, (PGM), OS=*A. thaliana*	●	●	●
		63/5.71	P93805	Phosphoglucomutase (PglcM), cytoplasmic 2 OS=*Z. mays*			
20	10/6.4	11.4/6.11	P00304	Pollen allergen Amb a 3.0101	●	○	●
21	5/7.9	5.0/8.18	P02878	Pollen allergen Amb a 5.0101	○	○	○
22	11/8.7	12.8/8.93	O04004	Pollen allergen Amb a 6.0101	○	○	○
23	10/4.15	9.3/4.15	Q2KN26	Pollen allergen Amb a 9.0102	○	○	○
X1	16/7.2			n.d.	●		●
X2	16/9.1			n.d.		○	
X3	17/6.5			n.d.	●		
X4	10/5.9			n.d.	●	●	●

* As indicated by www.allergen.org.

● - protein spot or group of spots present in 2D SDS-PAGE and IgE reactive in 2D immunoblot (allergens); ○ - protein spot or group of spots present only in respective 2D SDS-PAGE profile. Lack of any circle indicates complete absence of designated protein/allergen. N.d. not determined. More information on protein identity determination from 2D SDS-PAGE is available in Table S6**.**

Figure Captions

Figure 1. Venn diagram of the short ragweed pollen fractions' protein groups and their unique protein group entries alongside each pollen fraction. Protein group entries appearing as boldface text represent either officially recognized allergens or proteins with an established role in modulating the allergic response, such as NADH dehydrogenase. Numbers in brackets denote the total number of protein groups identified in each pollen fraction. TOT, total pollen protein extract; SPP, sub-pollen particles; APE, aqueous pollen protein extract; HSP70, heat shock protein 70.

Figure 2. Enrichment analysis and comparison of gene ontology (GO) cellular localization of proteins percentages among different short ragweed pollen fractions. Analyses were performed with FunRich software, and the *Asteraceae* database as a background set of proteins (the same database was used for tandem mass spectrometry protein identification), since the short ragweed genome has not been fully sequenced and annotated. The closer the p-value is to zero, the more significant the particular GO term associates with the group of proteins (i.e. less likely that observed annotation of the particular GO term to a group of proteins occurs by chance). TOT, total pollen protein extract; SPP, sub-pollen particles; APE, aqueous pollen protein extract.

Figure 3. Percentage of short ragweed major and minor pollen allergen groups within TOT, SPP, and APE pollen fractions obtained from proteomic shotgun LFQ studies. (A) Percentage of shared Amb allergen groups within different pollen fractions. (B) Plot of combined peak areas under the XIC curve for each Amb a allergen group compared across fractions. Each protein entry within the LFQ peak area analysis was normalized to the TIC of TOT, SPP, and

APE samples at a ratio of 1:0.78:1.06. TOT, total pollen protein extract; SPP, sub-pollen particles; APE, aqueous pollen protein extract; XIC, extracted ion chromatogram; TIC, total ion current; LFQ, label-free quantification of proteins.

Figure 4. Label-free quantification (LFQ) heat map of TOT, SPP, and APE protein fractions of short ragweed pollen. Proteins were clustered when they exhibited a similar expression trend across samples. Protein names in red represent important groups of short ragweed allergens, including allergens identified from mugwort that most likely represent homologous short ragweed allergen isoforms. A heatmap was created by PEAKS LFQ algorithm and cell colour represents the log2 ratio of the sample to the base sample. Due to the best peptide matching features based on retention time, the PEAKS LFQ algorithm chose TOT as the base sample and its cell colour represents the log2 ratio of TOT peak area divided by the smaller value between SPP and APE. Red cell colour denotes highly abundant proteins, while green cell colour marks highly underrepresented proteins or completely missing proteins. Black circles on the green colour boxes denote missing proteins. For normalization, total ion current (TIC) was chosen (experimentally determined 1:0.78:1.06 for TOT:SPP:APE). Underlined text represents substantially higher abundance of dehydrogenases (i.e. NADH) and dismutases (i.e. SOD) in the SPP fraction compared to the APE fraction. TOT, total pollen protein extract; SPP, sub-pollen particles; APE, aqueous pollen protein extract; PEP, phosphoenolpyruvate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SOD, superoxide dismutase; MAPKKK, mitogen-activated protein kinase kinase kinase**.**

Figure 5. (A) Representative cCBB stained polyacrylamide gels for TOT, SPP, and APE short ragweed pollen fractions separated by 2D electrophoresis under reducing conditions and **(B)** Representative 2D immunoblots of TOT, SPP, and APE short ragweed pollen fractions

after transferring proteins to membranes and probing with pooled patients' sera. Rectangles No. 16 and X2 in SPP's SDS-PAGE denote unique protein groups in the SPP fraction, while circled protein groups labelled from 12-15 visually highlight the noticeably increased quantity of acidic Amb a 4 isoforms in the SPP fraction compared to TOT and APE fractions. The remaining rectangles denote two or more protein spots belonging to a certain protein group labelled with the corresponding number. TOT, total pollen protein extract; SPP, sub-pollen particles; APE, aqueous pollen protein extract; Enol, enolase; UTP-Glc, UTP-glucose-1-phosphate uridylyltransferase; PGM, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase; PglcM, phosphoglucomutase.

Figure 6. Correlation of quantitative ELISA assay results with Pearson correlation coefficient rho. TOT, total pollen protein extract; SPP, sub-pollen particles; APE, aqueous pollen protein extract.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. List of protein groups identified in the short ragweed total pollen protein (TOT) fraction *via* proteomic shotgun analysis.

Table S2. List of protein groups identified in the short ragweed sub-pollen particle (SPP) fraction *via* proteomic shotgun analysis.

Table S3. List of protein groups identified in the short ragweed aqueous pollen protein extract (APE) fraction *via* proteomic shotgun analysis.

Table S4. List of cellular component gene ontology (GO) identifiers in TOT, SPP and APE fractions.

Table S5. Label-free quantification results of TOT, SPP, and APE fractions of short ragweed pollen. APE, aqueous pollen protein extract; SPP, sub-pollen particles; TOT, total pollen protein extract.

Table S6. Protein spot identification lists from TOT, SPP, and APE from 2D SDS-PAGE analysed by the SEQUEST algorithm.

Figure S1. Fractionation and extraction strategy of *A. artemisiifolia* pollen. APE, aqueous pollen protein extract; SPP, sub-pollen particles; TOT, total pollen protein extract.

Figure S2. Representative 1D SDS-PAGE profiles of TOT, SPP, and APE fractions of short ragweed pollen under reducing conditions. Mw, molecular weight protein markers in kilodaltons.

Figure S3. 1D immunoblot with IgE reactivity pattern of the first 10 patients against resolved pollen protein fractions of short ragweed. Mw, molecular weight protein markers in kilodaltons (kDa); TOT, total pollen protein extract; SPP, sub-pollen particles; APE, aqueous pollen protein extract; NEG, serum of a patient who is not allergic to the short ragweed (negative, healthy serum); CC1, conjugate control that does not contain serum; CC2,

conjugate control that does not contain secondary antibody (antihuman IgE antibody); CC3, conjugate control that does not contain tertiary antibody.