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RESEARCH LETTER

WILEY

The method makes the extract: Comparative analysis of birch pollen allergen extracts

To the Editor,

All marketed birch pollen allergen products used in allergen-specific immunotherapy (AIT) are based on aqueous extracts prepared from large batches of pollen collected from hundreds of birch trees. So far, little is known how pollen lot selection and processing affect the content and ratio of major and minor allergens in birch pollen extracts. Previously, we have shown that the differences observed between products from several manufacturers were too large to be caused solely by the use of different pollen lots.[1] In a second step, we now investigated the influence of different extraction methods on birch pollen extract composition. Our study included not only quantification of the major birch pollen allergen Bet v 1 (a pathogenesis-related protein, PR-10), but also analysis of several minor birch pollen allergens (Bet v 2, Bet v 4, Bet v 6 and Bet v 7). Like Bet v 1, the profilin Bet v 2 and the polcalcin Bet v 4 are so-called pan allergens, while the cyclophilin Bet v 7 has only been identified in birch pollen and the phenylcoumaran benzylic ether reductase Bet v 6/Cor a 6 in only a few source, including birch and hazelnut pollen.[2] We compared extracts prepared from the same commercial birch pollen lot (Stallergenes Greer, Lenoir, USA, batch P3317516-1). All extractions were performed after defatting in acetone and ether/ethanol solution (3:1, v/v) in a ratio of 1:5 (g pollen/ml) for 1 h at 4°C respectively. The defatted pollen was stored at −20°C. Extracts were prepared from 1g of defatted pollen each, using six extraction protocols, differing in duration, temperature and buffer composition. These three parameters were selected to mirror the extraction conditions used by six European manufacturers in the production of birch pollen allergen products. As respective extraction protocols are confidential, the detailed preparation of the single extracts can unfortunately not be disclosed. All extractions were performed under stirring in aqueous buffers in volumes ranging from 5 to 20 mL per g pollen. Each protocol used a distinct extraction buffer, ranging from different phosphate buffers to phosphate-free salt solutions. Extraction duration ranged from 2 to 24 h and temperatures from 4 to 20°C. Three independent extractions were performed per method, and all extracts were subsequently stored at −80°C. All extracts were analysed for protein content using the Bradford method. Furthermore, allergen content was determined using four sandwich ELISA systems: One commercially available system specific for Bet v 1 (Indoor Biotechnologies, Charlottesville, USA) and three in-house ELISA systems for quantification of Bet v 4,[3] Bet v 6 and Bet v 7[1] respectively. Each extract was measured in each assay in at least two independent runs in duplicates with a coefficient of variation (CV) limit of 15% between assays. SDS-PAGE and Western blot analysis were performed as described previously[3] with minor modifications. To account for the different extraction volumes, extracts were loaded normalized to μg total protein/g extracted pollen. As so far no Bet v 2-specific ELISA system is available, the combination of a specific monoclonal antibody and an anti-mouse IgG-AP (Sigma-Aldrich) was used in Western blot analysis. For generation of the Bet v 2-specific monoclonal antibody, female CBA/J mice (Charles River, Sulzfeld, Germany) were immunized with 10 μg birch pollen extract and 1 μg of recombinant Bet v 2 protein (Biomay, Vienna, Austria) adsorbed to aluminium hydroxide (Sigma-Aldrich, Taufkirchen, Germany) and Montanide ISA Adjuvans (Seppic, Paris, France), respectively, via intraperitoneal injection in 2-week intervals over eight weeks. Generation of hybridoma cells and subsequent clone screening were performed as previously described.[3] Western blot analysis included an approximate densitometric quantification of signals relative to a serial dilution of native purified Bet v 2 protein using the ImageJ software.[4]

Despite the use of the same pollen material, clear differences could be observed between the pollen extracts. Most obviously, the extracts differed considerably in colour (Figure 1A). Also, protein profiles showed differences in band intensities although the overall pattern appeared relatively similar (Figure 1B). Western blot analysis revealed differences in the amount of Bet v 2 extracted from 1g of pollen (Figure 1C). The nature of the weak additional band visible for extracts 3 and 5 could unfortunately not be clarified. Several quantitative assays confirmed the observed heterogeneity between the prepared extracts: While reproducibility of extractions with the same method was satisfactory, major and minor allergen content as well as total protein differed between extracts (Figure 1D). The difference in μg of extracted protein per g pollen was up to eightfold depending on the extraction method. In comparison, the observed range in Bet v 1 and Bet v 4 content was only twofold between the lowest and the highest value respectively. For the minor allergens Bet v 6 and Bet v 7, differences

between extracts were far more pronounced, especially because extracts prepared with extraction method four (E4) did contain only minimal amounts of the two minor allergens. Interestingly, the extraction protocols used for preparation of E3 and E4 were identical with regards to extraction duration and temperature, indicating that the buffer composition is likely to be most critical for allergen composition.

The determining influence of the extraction method has already been described for other allergenic source materials (e.g. mulberry pollen,[5] peanut and tree nuts[6]). However, the next step was to verify the observed link between the choice of extraction parameters and the changes in birch pollen extract composition. Therefore, we compared the minor allergen patterns of the six experimental extracts with the six commercial birch pollen allergen products which provided the basis for the extraction protocols compared in this study (Figure 2). At least three batches of each commercial AIT product from six manufacturers (see acknowledgements) were analysed with the same methods used for the experimental extracts. Notably, in cases of modified and/or adsorbed products, the respective native intermediate was analysed. The minor allergen patterns of the experimental extracts and the commercial products appear surprisingly similar (Figure 2) considering that, on the one hand, only three extraction parameters were replicated in very small scale and, on the other hand, that industrial-scale allergen extracts are subject to additional product-specific manufacturing steps. These findings indicate that changes or variations in the comparatively simple step of extraction may lead to significant changes in the composition of the resulting allergen product. The consequences of such differences can currently not be estimated given the unfortunate lack of knowledge regarding the clinical relevance of single allergen molecules.

Key Messages

- Direct comparison of extraction methods used in commercial manufacturing of allergen products reveals distinct differences.
- Differences in three parameters (extraction buffer, time and temperature) lead to distinct allergen extract compositions.
- Extraction parameters require careful optimization to prevent suboptimal allergen composition or even loss of allergens.

It was postulated more than 20 years ago that 5–20 μg of a major allergen should be contained per AIT maintenance dose in order to be effective.[7] However, subsequent studies have shown that this proposed dose is allergen- and product-dependent, as well as in many cases probably too low.[8,9] Hence, knowledge remains limited even for major allergens, which are regarded to be of the highest clinical relevance in the majority of patients. For minor allergens though, virtually no studies on clinical relevance exist although literature indicates that certain minor allergens are indeed relevant, especially in certain geographic regions.[10] Although unusually high concentrations of minor allergens have even been reported to cause adverse events during AIT, it can be speculated that the more allergens are present in appropriate concentrations in an extract, the more patients are likely to benefit from treatment. Our findings are in line with the common knowledge that allergen products produced by different manufacturers differ reproducibly in their composition,

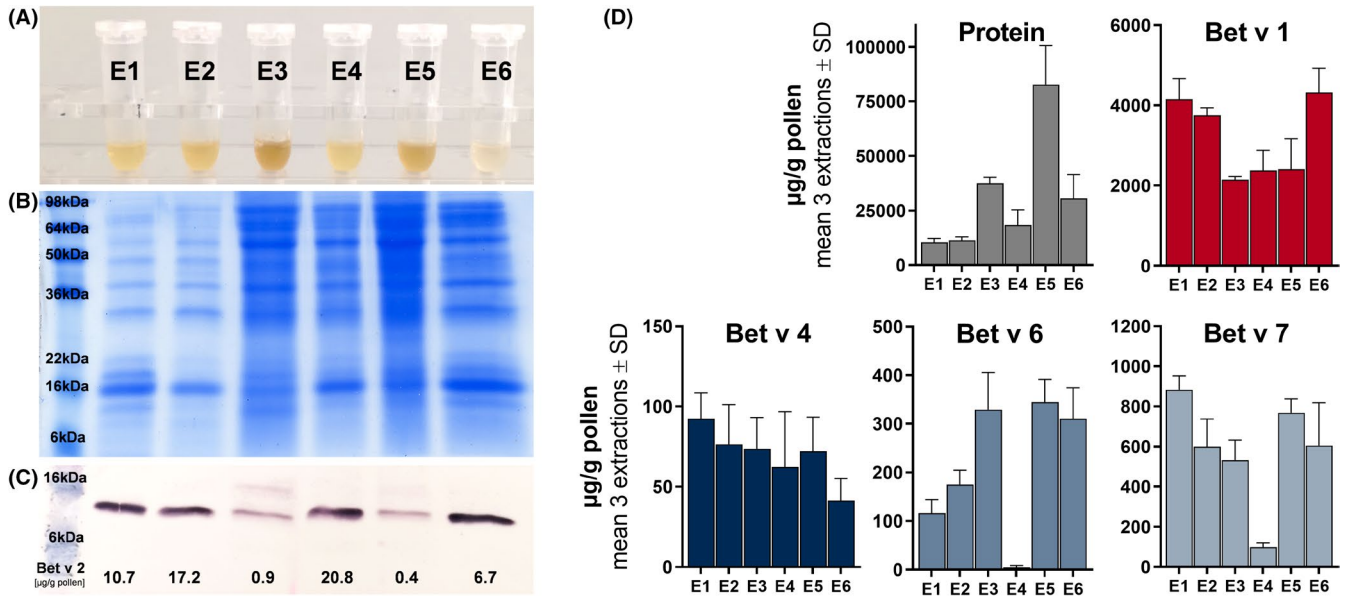

FIGURE 1 Comparison of birch pollen extracts prepared with six different protocols. (A) Colour comparison is shown for six birch pollen extracts (E1–E6). (B) Extracts (E1–E6) were subjected to SDS-PAGE (Brilliant Blue G Staining) or (C) Western blot analysis using a Bet v 2-specific monoclonal antibody. (D) Total protein content and allergen content of birch pollen extracts (E1-E6) are displayed in μg/g extracted pollen as mean value of three independent extractions ±standard deviation (SD)

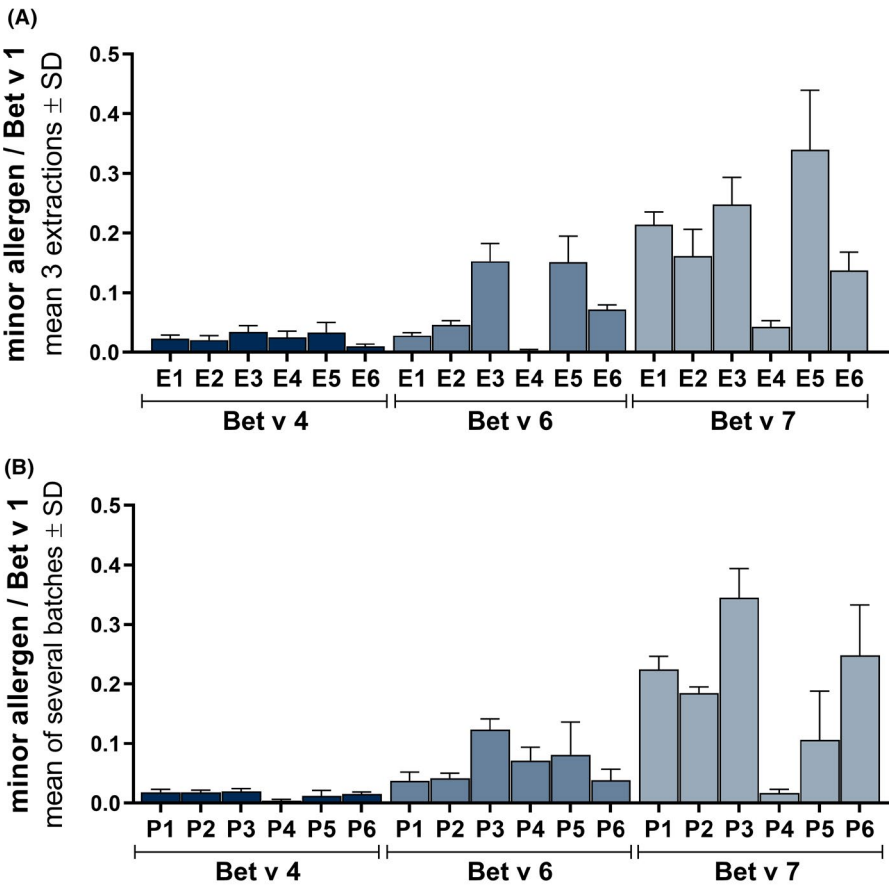

FIGURE 2 Minor allergen pattern of experimental extracts and commercial birch pollen AIT products. (A) The minor allergen content of six experimental birch pollen extracts is displayed normalized to Bet v 1 content as mean of three independent extractions ± standard deviation (SD). (B) The minor allergen content of six commercial birch pollen AIT products is displayed normalized to Bet v 1 content as mean of at least three product batches ±standard deviation (SD)

even if similar allergenic source materials are used.[1] This underlines the current regulatory requirements for therapeutic allergen products: A positive benefit-risk ratio and thus clinical value has to be demonstrated for each product individually, with the exception of products sharing allergenic source materials from the same homologous group and, importantly, are produced in an identical manufacturing process.[11]

Our work has shown that the choice of extraction method is a main determinant of allergen composition in birch pollen allergen products and may even result in the virtual absence of some allergens. Consequently, extraction parameters and especially the extraction buffer should be carefully selected and optimized with regards to single allergen content when developing new extract-based AIT products.

KEYWORDS

allergen extract, allergen-specific immunotherapy, Bet v 1, birch pollen, extraction method, minor allergens

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CONFLICTS OF INTEREST

The authors JZ, SS, JK, SD, DS, SV and SK declare no conflicts of interest in relation to this work.

AUTHORS CONTRIBUTIONS

Zimmer J involved in the design and preparation of study, analysis and interpretation of data, drafting of the article and final approval of the version to be published. Schmidt S involved in analysis and interpretation of data, drafting the article and final approval of the version to be published. Klos J, Döring and Strecker D involved in the performance of experiments, analysis and interpretation of data, critical revision of article draft and final approval of the version to be published. Vieths S involved in analysis and interpretation of data, critical revision of article draft and final approval of the version to be published. Kaul S involved in design and preparation of study, analysis and interpretation of data, critical revision of article draft and final approval of the version to be published.

Julia Zimmer
Sandra Schmidt
Josefin Klos
Sascha Döring

Daniel Strecker
Stefan Vieths
Susanne Kaul

Paul-Ehrlich-Institut, Langen, Germany

Correspondence
Susanne Kaul, Paul-Ehrlich-Institut,
Paul Ehrlich-Straße 51-59,
D-63225 Langen, Germany.
Email: Susanne.Kaul@pei.de

ORCID

Julia Zimmer https://orcid.org/0000-0002-1258-6446

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