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Detection of allergen composition and *in vivo* immunogenicity of depigmented allergoids of *Betula alba*

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Clinical and Experimental Allergy

Summary

Background Chemical modification of allergen vaccines to reduce IgE binding improves safety while maintaining clinical efficacy. However, this also complicates the characterization of allergoids using techniques as for native allergen extracts.

Objectives The objective of this study was to analyse the molecular size of *Betula alba* depigmented allergoids, conservation of major allergens in the allergoids and *in vivo* antibody response to immunization.

Methods The molecular size of depigmented allergoids was evaluated by high performance-size exclusion chromatography and light scattering techniques. Protein composition was compared with native extracts by capillary liquid chromatography–tandem mass spectrometry based peptide mapping. Rabbits were immunized with depigmented allergoid of *Betula* pollen adsorbed onto aluminium hydroxide (Depigoid[®]). IgG antibodies against individual allergens were determined by ELISA and immunoblot.

Results Depigmented allergoids contained a range of high molecular weight particles, approximately 60% of which had a molecular weight of 1–3 MDa. Peptide sequencing confirmed the preservation of five isoforms of Bet v 1, as well as Bet v 2, Bet v 6 and Bet v 7. Sera from immunized rabbits showed high levels of specific IgG to rBet v 1.0101 and rBet v 2.

Conclusions The mean protein content was 544 ± 106 µg per mg of freeze-dried material for depigmented allergoids and 434 ± 71 for native extracts. They retain the capacity to induce specific IgG antibodies against individual allergens present in the native extract. These findings confirm the immunogenicity of depigmented allergoids and may explain why patients treated with these vaccines are protected against the native allergens. Analysis of molecular size and allergen content may be useful techniques for characterization and standardization of allergoid products.

Keywords depigmented-allergoids, immunization, molecular size, peptide sequences, standardization

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Introduction

The modification of allergen extracts to reduce allergenicity (via reduced IgE binding) allows for the administration of higher doses of the allergen extract during immunotherapy, with a decreased risk of systemic reac-

tions [1]. Different studies conducted with depigmented allergoid vaccines (Depigoid[®], Laboratorios LETI S.L., Madrid, Spain) have demonstrated that they are effective in reducing symptoms and are well tolerated in adults [2–5] and paediatric [6] allergic patients with rhinoconjunctivitis and/or asthma. This clinical efficacy suggests that immunogenicity is retained [7]. Allergoids have been shown to have allergen-specific T cell-stimulating capacity dependent on the type of the antigen-presenting cell

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[8]. However, the formation of large complexes reduces the access of allergen-specific antibodies and reduced IgE binding prevents the use of bioassays based on these methods for the assessment of allergen content.

Thus, the characterization and the standardization of polymerized allergen extracts have been difficult and the conservation of major allergens during allergoid preparation and induction of immune responses against individual allergens within extracts has not previously been demonstrated.

The procedure generally used for the modification of allergen extracts to produce allergoids is polymerization with formaldehyde or glutaraldehyde under carefully controlled conditions [9]. This polymerization reaction irreversibly cross-links proteins of allergen extracts at their N-termini and/or lysine residues [10], resulting in covalently linked protein complexes of high molecular weight. The size of the allergoids depends on reaction time, pH and glutaraldehyde concentration. The exact molecular mass of allergoids remains unknown. Some studies have suggested that polymers obtained after glutaraldehyde polymerization are in a range between 200 and 20 000 kDa. It is assumed that allergoids contain all individual proteins/allergens present in the native extract, but this has not been formally confirmed.

Pigments and other substances attached to the proteins can be removed by mild acid treatment followed by dialysis before the polymerization reaction. This results in higher solubility of the material, increased purity and inactivation of enzymatic activities [11]. The resulting depigmented product shows the same IgE-binding characteristics as native extract. After polymerization with glutaraldehyde, depigmented allergoid (Dpg-Pol) extracts are obtained and are adsorbed onto aluminium hydroxide in order to prepare a vaccine for the treatment of allergic diseases (Depigoid®).

The aims of this study were to analyse the molecular size of Dpg-Pol extracts of *Betula alba* after polymerization with glutaraldehyde, to examine the preservation of relevant allergens and to analyse the IgG antibody production against individual allergens *in vivo* after immunizing rabbits with Depigoid® of *B. alba*.

Methods

Extract manufacturing

Two different native extracts (X247LN, 5606LN) and their corresponding Dpg-Pol extracts (X262LP, 7806LP) of *B. alba* were prepared following previously described methods [12]. Briefly, 100 g of two different lots of pollen of *B. alba* (Biopol Laboratory, Spokane, WA, USA) were mixed homogeneously and extracted (1 : 10 w/v) in phosphate-buffered saline buffer (PBS) pH 7.4 for 4 h at 4 °C under continuous magnetic stirring. Then the extracts

were centrifuged at 16 000 g at 4 °C for 30 min and the supernatants collected. The pellets were again dissolved (1 : 10 w/v) in PBS and extracted overnight at 4 °C. Afterwards, the extracts were centrifuged, the supernatants separated and mixed with the first extractions, sterile filtered, and dialysed against highly purified water (Ph. Eur. specification) in a Pellicon® system using membranes of 3 kDa (Millipore, Bedford, MA, USA), filtered, frozen and lyophilized.

Lyophilized native extracts were reconstituted in highly purified water (10 mg/mL) and the pH adjusted to 2 with 2 M HCl. Extracts were dialyzed overnight against highly purified water in dialysis membranes with a cut-off of 3.5 kDa (Cellu Sep Membrane, Seguin, TX, USA). After the pH of the solution was neutralized, the extracts were sterile-filtered, frozen and lyophilized. Depigmented extracts were then reconstituted at 15 mg/mL in PBS and polymerized with glutaraldehyde. The reaction was maintained for 8 h at room temperature under continuous magnetic stirring. Afterwards, the reaction was stopped with glycine and the polymerized extracts were dialyzed in 100 000 Da dialysis membranes (Millipore) and freeze-dried. Protein content of the extracts was measured by the Lowry-Biuret method (BioRad Laboratories, Hercules, CA, USA). The mean protein content values were 434 ± 71 µg per mg of freeze-dried material for native extracts and 544 ± 106 for Dpg-Pol extracts.

Determination of the molecular weight

The native and corresponding Dpg-Pol extracts of *B. alba* were resuspended in water at 1 mg/mL under mild agitation. After 1 h, insoluble particles were removed by centrifugation at 14 000 g for 10 min and the molecular weight distribution was determined by high performance-size exclusion chromatography (HPSEC) using a 7.8 × 300 mm TSKgel G2000SWXL column (Tosoh Bioscience, Stuttgart, Germany) on a HP1100 system (Hewlett-Packard, San Jose, CA, USA). Additionally, the high molecular weight components were resolved by a dual column set-up consisting of a series of identical-sized TSKgel G4000SWXL and G2000SWXL columns coupled in tandem. The HPSEC system was equipped with a built-in UV detector and online coupled with a triple detection array (TDA 302; Viscotek Corp., Houston, TX, USA) of refractive index (RI), intrinsic viscosity (IV) and right-angle light scattering (RALS) detectors [13]. All samples were analysed in duplicates using Omni-Sec™ software version 4.2 (Viscotek). For determination of the protein amount, the RI detector signal was used. Detector calibration was performed using bovine serum albumin from Sigma (A7638, St Louis, MO, USA) weighed in at 1.0 mg/mL. Values for $dn/dc = 0.185$ mL/g, $dA/dc = 0.646$ mL/g, molecular weight (MW) = 66 433 Da and polydispersity = 1 were set.

Mass spectrometry based sequencing

For mass analyses, samples were dissolved/suspended in water at a concentration of 1 mg/mL and digested with trypsin or pepsin. If a sample was not completely soluble in water, a suspension containing both the soluble and the insoluble fractions was used. For the tryptic digest, samples were reduced, alkylated and digested using the Proteoextract trypsin digestion kit (Calbiochem, San Diego, CA, USA) according to the manufacturer's instruction. Treatment with pepsin (Sigma, St Louis, MO, USA; #P6887) was performed, without prior reduction and alkylation, in 10 mM HCl for 2 h at 37 °C at an enzyme to allergoid ratio of 1 : 20.

Resulting peptides were separated by reversed phase-capillary high-performance liquid chromatography (Nanoease Symmetry 300TM trap column and Nanoease Atlantis dC18TM separating column; Waters, Milford, MA, USA; connected via a 10 port stream select valve) directly coupled to an ElectroSpray Ionization-Quadrupole-Time of Flight mass spectrometer (Q-ToF Ultima Global; Waters). The flow rate was adjusted to 300 nL/min by T-splitting. Peptides were eluted with an acetonitrile gradient [Solvent A: 0.1% (v/v) formic acid/5% (v/v) acetonitril; solvent B: 0.1% (v/v) formic acid/95% (v/v) acetonitril; 5–45% B in 90 min). Nitrogen was used as a nebulizing gas. For ionization, the Waters nanoflow spray head was used. Capillary voltage was set to 3.9 kV, Time-

of flight (TOF) voltage was 9.1 kV and Microchannel plate receptor (MCP) voltage was 2 kV. The instrument was operated in positive mode and tuned to maximum sensitivity. Data acquisition and instrument control was done with the MassLynx software V4.1 (Waters). The instrument was calibrated with the fragment ions of [Glu]-Fibrinopeptide B (Sigma). Data were acquired in the data directed analysis (DDA) mode in the mass range from 350 to 1900. Singly, doubly and triply charged ions were selected for fragmentation by collision with argon. Collision energy settings were software controlled and depended on the mass and charge of the ions, typically from 25 to 50 eV. Fragmentation data were acquired in the mass range from 50 to 1900. Fragmentation time was 4 s per ion; four ions were fragmented simultaneously. Survey and fragment spectra were analysed using the software ProteinLynx Global Server (PLGS) version 2.2.5 (Waters) with automatic validation.

For nanoLC-MS/MS (nano liquid chromatography-tandem mass spectrometry) based sequencing, an in-house database consisting of all birch pollen allergen sequences assigned by the SwissProt knowledgebase (Swiss Institute of Bioinformatics, Switzerland) was used. The SwissProt accession numbers of the used sequences are shown in Table 1. The MS/MS datasets for the tryptic and peptic digests were analysed individually using the following parameters: peptide tolerance 100 ppm, fragment tolerance 0.1 Da, estimated calibration error

Table 1. Occurrence of birch pollen allergens in two different native extracts (X247LN, 5606LN) and their corresponding depigmented allergoids (X262LP, 7806LP) as determined by the PLGS analysis software with visual verification

Allergen	SwissProt accession no.	Native extracts		Dgp-Pol extracts	
		Probability of occurrence and sequence coverage		Probability of occurrence and sequence coverage	
		X247LN	5606LN	X262LP	7806LP
Bet v 1.0101	P15494	High (83%)	High (71%)	High (33%)	High (62%)
Bet v 1.0201	P45431	High (80%)	Medium (74%)	High (20%)	Medium (34%)
Bet v 1.0301	P43176	Medium (80%)	Medium (74%)	Medium (20%)	Medium (32%)
Bet v 1.0401/1.0801	P43177	High (84%)	High (81%)	High (32%)	High (43%)
Bet v 1.0501	P43178	Low (60%)	Low (59%)	Medium (22%)	Medium (49%)
Bet v 1.0601/1.0901	P43179	High (79%)	High (78%)	Medium (22%)	High (54%)
Bet v 1.0701	P43180	High (79%)	Medium (74%)	High (28%)	High (36%)
Bet v 1.1001	P43183	Medium (74%)	Medium (73%)	Medium (22%)	Medium (49%)
Bet v 1.1101	P43184	Medium (80%)	Medium (74%)	Medium (20%)	Medium (32%)
Bet v 1.1201	P43185	Low (72%)	Low (62%)	Low (16%)	Low (36%)
Bet v 1.1301/1.1401	P43186	High (84%)	High (78%)	High (25%)	High (36%)
Bet v 2	P25816	High (47%)	High (20%)	High (5%)	High (12%)
Bet v 3	P43187	Low (0%)	Low (0%)	Low (0%)	Low (0%)
Bet v 4	Q39419	High (26%)	High (26%)	Low (0%)	Low (0%)
Bet v 6	P15777	High (64%)	High (40%)	High (12%)	High (20%)
Bet v 7	P81531	High (67%)	High (67%)	High (20%)	High (9%)

Probability values are based on sequence coverage, signal intensities and the quality of the MS/MS data as described in the text (high: 60–100% probability, medium: 5–60% probability, low < 5% probability, as determined by PLGS). The sequence coverage for each allergen is given in brackets. PLGS, ProteinLynx Global Server.

0.005 Da, digest reagent 'non-specific'. For the tryptic digests, 'Carbamidomethyl C' was used as fixed modification. For the analyses of peptic digests, no modifications were considered. Databank searches of tryptic and peptic digests of one sample were performed individually and combined using the 'merge'-function of the PLGS software resulting in the given sequence coverage.

In vivo immunization. After collection of pre-immune sera, two New Zealand White rabbits (Vivotecnia, Madrid, Spain) were immunized with a depigmented allergoid of *B. alba* absorbed onto aluminium hydroxide (Depigoid®). The immunization process consisted of three subcutaneous injections (one immunization and two boosts) containing 126.5 µg protein each. The administered doses were similar to those used in humans. After 21 days, boost 1 was injected. A serum sample was obtained after 31 days. Boost 2 was injected after 51 days. Animals were bled after 58 days and the serum also collected.

Individual sera and two pools of sera consisting of sera from rabbits 1 and 2 of first drawal (pool 1) and of sera from rabbits 1 and 2 after bleeding (pool 2) were stored at -20 °C. Studies followed the local ethical rules for animal experimentation.

Determination of specific immunoglobulin G antibodies by direct ELISA

Specific IgG antibodies in the sera of rabbits being directed against native and Dpg-Pol extracts were detected by direct ELISA. Microtitre plates (Immulon IV; Dynex Technologies, Chantilly, VA, USA) were coated overnight (100 µL/well) with native and Dpg-Pol *B. alba* extracts at a concentration of 10 µg/mL in carbonate/bicarbonate buffer pH 9.6. After washing, the plates were incubated with serial dilutions of rabbit sera in 1% BSA-PBS-Tween (Panreac, Barcelona, Spain) for 2 h at room temperature. Plates were washed and incubated with peroxidase-labelled goat anti-rabbit IgG (Nordic Immunology, Tilburg, the Netherlands) diluted at 1:30 000 in 1% BSA-PBS-Tween for 1 h at room temperature. The reaction was developed and optical densities were measured at 450 nm. Pre-immune sera were used as negative controls.

Rabbit IgG antibodies directed to rBet v 1.0101 and rBet v 2 (Biomay, Vienna, Austria) were detected by direct ELISA. Microtitre plates (Immulon II; Dynex Technologies) were coated overnight at 4 °C (100 µL/well) of 1 µg/mL of recombinant or native allergen in sodium carbonate buffer pH 9.6. Plates were washed three times with PBS-Tween and blocked with 1% BSA-PBS-Tween for 1 h at room temperature. After washing, the plates were incubated with serial dilutions (100 µL/well) of rabbit serum in 1% BSA-PBS-Tween for 2 h at room temperature. Plates were washed five times and incubated with 100 µL/well of peroxidase-labelled goat anti-rabbit IgG (Nordic) diluted

at 1:30 000 in 1% BSA-PBS-Tween for 1 h at room temperature. The colour development was induced with tetramethylbenzidine after an incubation of 30 min. The reaction was stopped with sulphuric acid 1 N, and absorbance was read at 450 nm. Pre-immune sera were used as negative controls.

A titration curve was calculated with each extract in solid phase (Native, Dpg-Pol, rBet v 1 and rBet v 2) in order to determine the optimal dilution point.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis and immunoblots

Native extract (50 µg of freeze-dried material), rBet v 1 (3 µg) and rBet v 2 (10 µg) were subjected to SDS-PAGE and either stained with Coomassie R250 (BioRad Laboratories) or electrotransferred onto a nitrocellulose membrane (Immobilon; Millipore). After transfer, the membrane was blocked for 2 h and incubated overnight with the pool of sera diluted 1:10 000 (native extract and rBet v 1) and 1:2000 (rBet v 2), respectively, in 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% (w/v) BSA, 0.05% (w/v) Tween-20, 0.5% (w/v) NaN₃ at +4 °C. Specific IgG binding was detected using alkaline phosphatase-labelled goat anti-rabbit IgG diluted 1:10 000 (Jackson ImmunoResearch, West Grove, PA, USA). Immunoreactive proteins were visualized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl-phosphate (Applichem, Darmstadt, Germany). Pre-immune sera were used as negative controls. Pre-stained PageRuler was used as molecular weight marker (Fermentas Life Sciences, Burlington, ON, Canada).

Results

Molecular weight distribution

Determination of the molecular weight of two Dpg-Pol extracts by HPSEC resulted in a broad size distribution of molecules. As represented in Fig. 1, approximately 60% of the polymerized molecules had a molecular weight of 1–3 MDa. Approximately 25% of the molecules exceeded 3 MDa in molecular weight. The amount of soluble material (recovery) was determined from the RI signal. The amount of insoluble material was determined as the difference of resuspended material (weighed out) and recovery. Soluble material comprised 86±3% for extract 1 and 87±2% for extract 2, respectively. Low molecular weight material having a molecular weight of <1 MDa represented a very small fraction (2–3%). As control, the native extracts were analysed by HPSEC, as well. More than 90% of the material had a molecular weight <100 kDa. The most prominent peak was found at approximately 17 kDa, most likely corresponding to the Bet v 1, the major allergen of birch pollen (data not shown).

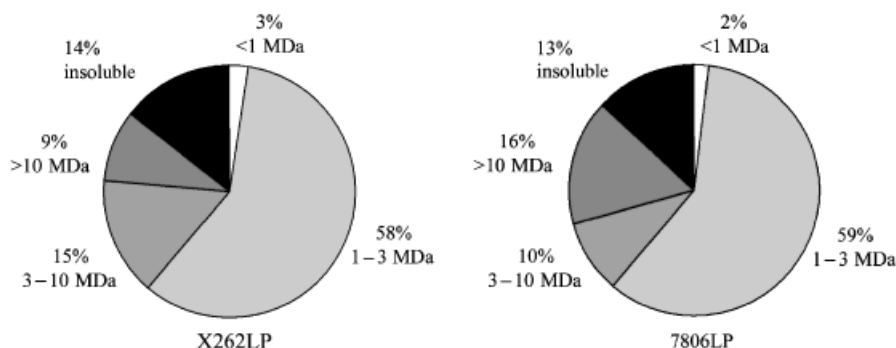


Fig. 1. Molecular weight distributions of two Dpg-Pol extracts X262LP and 7806LP determined by high performance-size exclusion chromatography. Upon reconstitution at 1 mg/mL, insoluble material was centrifuged off (dark grey). Sample recovery and fraction percentages were determined from the refractive index signal.

Allergen content of Dpg-Pol extracts

The presence of birch pollen allergens was determined by mass spectrometry (nanoLC-MS/MS) after proteolytic digests in two different batches of native extracts and their corresponding Dpg-Pol extracts. The PLGS software used for the assignment of fragmentation spectra to birch pollen allergens is based on probability-based matching approach that is commonly used for the analysis of MS/MS data [14]. In short, experimental fragmentation data are compared with *in silico* generated fragmentation data of the database entries. Depending on the degree of matching, a likelihood score is assigned to each peptide. Finally, the software considers all possible combinations of peptides for a given dataset and calculates a probability score for a database protein entry based on the individual peptide scores. It has to be emphasized that this approach does not rely on the identification of diagnostic peptides in order to identify closely related protein species. Nevertheless, both methods (probability-based matching and diagnostic peptide identification) produce comparable results.

Trypsin cleaves proteins at the C-terminus of the basic amino acids lysine and arginine and is commonly used for proteomic analyses by mass spectrometry. As the cross-links of allergoid samples partially block the tryptic cleavage sites, we attempted to increase the sequence coverage by digestion with less specific proteases (pepsin, proteinase K and thermolysin). Only pepsin was able to digest the cross-linked extracts and produced peptides that could be assigned to *Betula* allergens after fragmentation.

Combining the data obtained from the tryptic and peptic digests by PLGS significantly increased the sequence coverage. For instance, sequence coverage of Bet v 1a after tryptic digest in extract X262LP was 16%. After merging the data of the tryptic and peptic digests, we obtained a sequence coverage of 33%. Table 1 and Fig. 2 show the summarized combined results of all extracts. All relevant Bet v 1 isoforms were detected in the native and

Dpg-Pol extracts. Only isoforms Bet v 1.0501 and 1.1201 are not likely to be present in the samples. These results are in excellent agreement with previously published data, obtained by a mass spectrometric approach based on the identification of diagnostic peptides [15]. Presence of the minor *Betula* allergens Bet v 2, Bet v 6 and Bet v 7 was clearly demonstrated in all extracts, as well. Bet v 4 was detectable only in the native, but not in the polymerized extracts. Bet v 3 apparently was not present in any of the samples. This is not entirely surprising, as Bet v 3 has not been detected at the protein level before [16]. In general, the sequence coverage was much lower in Dpg-Pol (5–32%) than in native extract (26–84%), due to a decreased accessibility of cross-linked allergens by proteases and, in the case of trypsin, due to destruction of cleavage sites by the cross-linking reaction.

Immunogenicity of depigmented and polymerized *Betula alba* extract

Immunogenicity of Dpg-Pol extract was tested in rabbits. Specific IgG antibodies were detected in sera after 31 days (after immunization and one boost) and the optimal serum dilution of 1:2000 was determined. At the end of the study (day 58), high levels of specific IgG against Dpg-Pol extract were detected. These antibodies raised with Dpg-Pol extract also efficiently recognized native extract and the major birch pollen allergen rBet v 1. At a lower level, specific IgG for rBet v 2 was detected by direct ELISA (at a dilution of 1:2000) after 31 and 58 days in both rabbit sera. The signal detected in the pre-immune sera was used as baseline (Fig. 3).

The IgG immunoblot experiments were used to demonstrate specificity of the antibody response in rabbits. Sera of immunized rabbits recognized major allergens as well as other proteins of the native extract. When compared with pre-immune sera, a serum pool from two rabbits prepared at day 31 (dilution 1:10 000) recognized two bands with a molecular weight between 14 and 18 kDa

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Bet v 1a  GVENYETETTSVIPAAELFKAFILDGEMLFPKVAPQAISSEVENIEGNGGPGTIKKISFPEGFPFKYVKDRVDEVDHTNFK
Bet v 1b  .....E..T.I.....T...S...E...A...
Bet v 1c  .....S.....E..T.I.....T...S...E...A...
Bet v 1d/h .....K.....V.....N...
Bet v 1e  .....A.....I...G...
Bet v 1f/i .....I..A.....
Bet v 1g  .....S.....E..I.....N...
Bet v 1j  .....A.....
Bet v 1k  .....S.....E..T.I.....T...S...E...A...
Bet v 1l  .....A.....M.....K.V.....N...
Bet v 1m/n .....S.....I.....T...S...E...A...

Bet v 1a  YNYSVIEGGPIGDTLEKISNEIKVATEDGGSSILKISNKYHTKGDHEVKAQVKASKEMCETLLRAVESYLLAHSDAYN
Bet v 1b  .S..M...AL...C...V...M...HM..I..K..A...
Bet v 1c  .S..M...AL...C...V...Q.M...HM..I..K..A...
Bet v 1d/h .....V.....CV.....N...
Bet v 1e  .S.....V.....N...N...I...
Bet v 1f/i .S.....V.....N...N...I...
Bet v 1g  .....V.....CV.....N...
Bet v 1j  .S.....V.....N...N...I...
Bet v 1k  .S..M...AL...C...V...M...HM..I..K..A...
Bet v 1l  .....V.....CV.....N...
Bet v 1m/n .S..M...AL...C...V...M...HM..I..K..A...

Bet v 2  MSWQTYVDEHLMCDIDGQASNSLASAIVGHDGSVWAQSSSFPQFKPQEITGIMKDFEEPGHLAPTGLHLGGIKVMVIQGE
Bet v 2  AGAVIRGKKGSGGITIKKTGQALVFGIYEPPVTPGQCNMVVERLG...YLIDQGL

Bet v 4  MADDHPQDKAERERIFKRFDANGDGKISAAELGEALKTLGSITPDEVKHMMAEIDTDGDGFISFQEFDFARANRGLLKD
Bet v 4  VAKIF

Bet v 6  MAHKSKEITIGGTGIGKFIVEASAKSGHPTFALVRESTVSDPVKGLVEKFKGLGVTLHGDLYDHESLVKAFKQVDVV
Bet v 6  ISTVGHLQLADQVKIIAAIKEAGNIKRFPSEFGNDVRVHAVEPAKTAFATKAEIRKTEAEGIPYTYVSSNFFAGYFL
Bet v 6  PFLAQPGLTSPFEKVVIFGDGNARAVFNKEDDIGTYTIRAVDDPRTLNKIVYIKPAKNIYSFNEIVALWEKKIGKTLEK
Bet v 6  IYVPEEKLLKDIQESPIPINVILAINHSVFVKGDHTNFEIEASFGVEASELYPDVKYTTVEEYLQQFV

Bet v 7  MASNPKVFFDMEVGGQPVGRIVNELYADTTPTAENFALCTGEGKNGRSGKPLHYKKSSFHRVIPGFMCGGDFTAGNG
Bet v 7  TGGESIYGAKFADENFIKKHTGPGILSMANAGPGTNGSQFFICTAKTEWLDGKHVVFGQVVEGLLIVKAIEKVGSSSGRT
Bet v 7  SKPVVVADCGQLS

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Fig. 2. Allergen sequences identified by nanoLC-MS/MS-based peptide mapping. Results from analyses obtained tryptic and peptic samples were combined. Sequences identified in the native extract X247LN are indicated in red; sequences identified in the Dpg-Pol extract X262LP are highlighted in green. Results from extracts 5606LN and 7806LP were nearly identical and are not shown.

putatively corresponding to Bet v 1 and Bet v 2. Another serum pool prepared at day 58 detected different bands over the MW range of 10–100 kDa. Furthermore, recombinant Bet v 1 and Bet v 2 were detected by both serum pools, whereas no bands were detected after incubation with pre-immune sera (Fig. 4).

Discussion

During the manufacture of allergoids such as Depigoid[®], allergen extracts are chemically modified. This type of modification significantly reduces allergenicity of the extracts (IgE-binding capacity) as demonstrated by *in vivo* and *in vitro* experiments [17, 18]. Here, we show Dpg-Pol extracts of *B. alba* to contain a broad range of high molecular weight molecules. We furthermore demonstrate the preservation of major allergens in Dpg-Pol extracts by proteomic methodology and the *in vivo* immunogenicity

of Depigoid[®] with the induction of IgG antibodies specific for the major allergen Bet v 1.

Until present, no studies have been published determining the molecular size of allergoids obtained by glutaraldehyde modification. In our study, two different Dpg-Pol extracts prepared from birch pollen were analysed using HPSEC and light-scattering detection. More than half of the Dpg-Pol extracts adopted a molecular weight in the range of 1–3 MDa, and the molecular weight of one quarter of the material exceeded 3 MDa. No unpolymerized allergen was detected, as the additional dialysis step in the allergoid production ensured the exclusion of molecules with a molecular weight lower than 100 kDa. Previous studies have suggested that the reduced IgE binding of these allergoids could depend on the high molecular size of the modified molecules [19]. Experiments conducted in mice have confirmed reduced IgE interaction without affecting the induction of IgG

responses [20]. High standard quality control is inevitable for therapeutics used in humans. Thus, this method for determination of molecular size represents a useful technique for characterization of allergen vaccines based on chemical modification procedures.

The production of high molecular weight polymers containing the individual allergens reduces direct antibody binding and thereby prevents the detection of allergens by monoclonal or polyclonal antibodies. Thus, the presence of allergens in the allergoid extract cannot be confirmed by conventional methods. The question of allergen composition in Dpg-Pol extracts was addressed

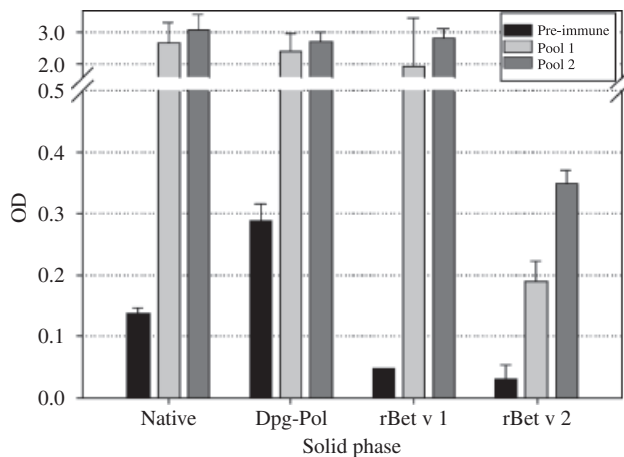


Fig. 3. Specific IgG levels determined in serum pools (dilution 1 : 2000) from two rabbits before (pre-immune), as well as 31 days (pool 1) and 58 days (pool 2) after immunization with *Betula alba* Depigoid®. Native extract, Dpg-Pol extract, rBet v 1.0101 and rBet v 2 were coated on solid phase.

by a proteomic approach involving nanoLC-MS/MS-based protein sequencing of tryptic and peptic fragments. Allergens and allergen isoforms were assigned using the commercial software PLGS. This software uses a probability-based algorithm that makes it possible to identify protein isoform without the need of diagnostic peptides. Our findings confirmed the presence of most relevant allergens in two independent batches of native and Dpg-Pol extracts. In detail, at least five different isoforms of the major birch pollen allergen, Bet v 1, were verified. In addition to Bet v 1, sequences for the minor allergens Bet v 2, Bet v 6 and Bet v 7 were found. Bet v 3-derived sequences were not found in the native or the corresponding Dpg-Pol extracts. This is not surprising, as Bet v 3 has never been reported in pollen extracts at the protein level. In native extracts, two Bet v 4-derived peptides were detected, although at very low intensity. In the Dpg-Pol extracts, Bet v 4 was not detected. Although it cannot be ruled out that Bet v 4 was not conserved in the process of chemical modification, it seems more likely that Bet v 4 was not detected in Dpg-Pol extracts due to low concentration in combination with the destruction of proteolytic cleavage sites by the polymerization reaction. It will be of interest to examine T cell reactivity to different allergens following *in vivo* immunization with native extract compared with allergoid.

We would like to stress that the mass spectrometric method described here produces primarily qualitative results. It is therefore perfectly suitable to determine the allergen composition of natural or polymerized extracts on a batch-to-batch basis if quantitative data are not required. The reason, why mass spectrometry in the described set-up is not capable of delivering quantitative

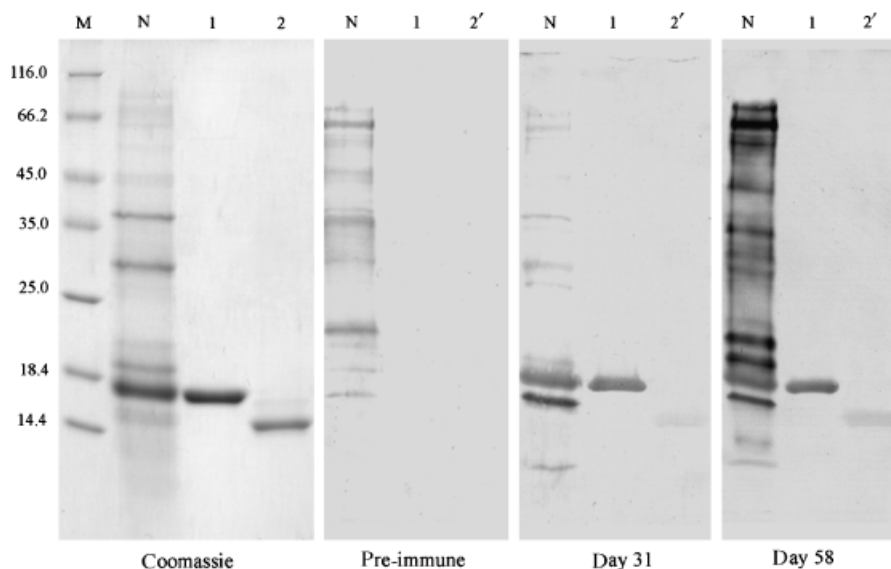


Fig. 4. Coomassie-stained SDS-PAGE and IgG immunoblot of native extract (Lane N; 40 µg), rBet v 1.0101 (Lane 1; 3 µg) and rBet v 2 (Lane 2; 2 µg and 2'; 10 µg). Serum pools of two rabbits before (pre-immune), as well as 31 and 58 days after immunization with *Betula alba* Depigoid® were diluted 1 : 10 000. Fermentas' Unstained Protein Molecular Weight Marker (Lane M).

data, is the fact that ionization of peptides depends largely on their amino acid composition. Two different peptides present in the sample at equimolar amounts will very likely produce MS signals of differing intensities. Some peptides might not produce signals at all. If an exact quantitative comparison of different batches is needed, other approaches have to be used. One example would be labelling of samples with stable isotopes [ICAT (isotope-coded affinity tag)] before mass spectrometry [21].

Immunotherapy with inhalant allergens is associated with an increase of allergen-specific serum IgG [22]. In the same way, different experiments have concluded that depigmented allergoids retain or even increase immunogenicity inducing the synthesis of IgG after immunotherapy [23, 24]. In the present study, the production of IgG was evaluated in rabbits. Animals were injected with a depigmented allergoid adsorbed to aluminium hydroxide and the immune response evaluated by direct ELISA. The results showed that the injection of Depigoid[®] induced significant production of specific IgG, as controlled by pre-immune sera taken before injection. Furthermore, sera recognized the individual major allergens present in native extracts (Bet v 1.0101 and Bet v 2), demonstrating that the immune response after the injection of allergoids was directed against these individual allergens. These results confirm immunogenicity of the Depigoid[®] *in vivo* and suggest that the immune response is elicited against allergenic epitopes present in the polymerized extracts. Furthermore, these antibodies can react with the epitopes present on native allergens. Clinical efficacy has been widely demonstrated in clinical trials [1–5] and this likely depends on preserved immunogenicity of Depigoid[®].

In recent years, interest in the use of recombinant allergens for *in vivo* and *in vitro* diagnosis of allergic diseases has grown considerably [25]. In the present study, rBet v 1.0101 and rBet v 2 were used to detect specific IgG antibodies produced after the injection of Depigoid[®]. A pool of sera prepared from two rabbits recognized the rBet v 1.0101 and rBet v 2 allergens demonstrating specificity of the IgG antibodies induced upon immunization with Depigoid[®]. None of the recombinant allergens were recognized by pre-immune sera confirming that specific IgG was induced by the immunization.

In summary, this study demonstrates that depigmented allergoids of *B. alba* consist of molecules with high molecular weight containing relevant allergens. The techniques described here may significantly improve the spectrum of methods for high standard quality control of allergoids. Allergenic vaccines containing depigmented and polymerized extracts adsorbed to aluminium hydroxide induced specific antibodies against major allergens and the proteins of native extract at doses, which are similar to those used in human treatment. Confirmation of *in vivo* immunogenicity may explain why patients treated with such a modified extract are protected against native

allergens present in nature. Although many of the immunological mechanisms involved in the immune response after immunotherapy have been elucidated, further studies are needed to clarify the role of different cell types in tolerance induction and the generation of antibody subclasses after immunotherapy with allergoids.

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