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Journal of Proteomics

Schenk, M.F.; Cordewener, J.H.G.; America, A.H.P.; Peters, J.; Smulders, M.J.M. et al

https://doi.org/10.1016/j.jprot.2011.03.021

JOURNAL OF PROTEOMICS 74 (2011) 1290–1300

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Proteomic analysis of the major birch allergen Bet v 1 predicts allergenicity for 15 birch species

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ARTICLE INFO

Available online 1 April 2011

Keywords:
Birch pollen allergy
Bet v 1
Isoforms
Betula
Mass spectrometry
Quadrupole-time-of-flight
Quantification
Hypoallergenic variant
IgE-reactivity

ABSTRACT

Pollen of the European and Asian white birch (*Betula pendula* and *B. platyphylla*) causes hay fever in humans. The allergenic potency of other birch species is largely unknown. To identify birch trees with a reduced allergenicity, we assessed the immunochemical characteristics of 15 species and two hybrids, representing four subgenera within the genus *Betula*, while focusing on the major pollen allergen Bet v 1. Antigenic and allergenic profiles of pollen extracts from these species were evaluated by SDS-PAGE and Western blot using pooled sera of birch-allergic individuals. Tryptic digests of the Bet v 1 bands were analyzed by LC-MS^E to determine the abundance of various Bet v 1 isoforms. Bet v 1 was the most abundant pollen protein across all birch species. LC-MS^E confirmed that pollen of all species contained a mixture of multiple Bet v 1 isoforms. Considerable differences in Bet v 1 isoform composition exist between birch species. However, isoforms that are predicted to have a high IgE-reactivity prevailed in pollen of all species. Immunoblotting confirmed that all pollen extracts were similar in immune-reactivity, implying that pollen of all birch species is likely to evoke strong allergic reactions.

1. Introduction

In the boreal and temperate climate zone of the Northern Hemisphere over 30 birch species grow in diverse habitats. Birch trees release large amounts of pollen in spring. This pollen is a major cause of Type I allergies and contains several allergens, including the major allergen Bet v 1. Hay fever caused by birch pollen affects 11–19% of the population in Western European countries (UCB 1997). Bet v 1 belongs to the pathogenesis related-class 10 (PR-10) protein family [1,2]. Pollen from tree species such as alder and hazel also contains PR-10 proteins that share epitopes with Bet v 1, which results in cross-reactivity between these proteins with respect to their antigenic properties [3]. In addition, individuals sensitized to Bet v 1 often become allergic to fruits and vegetables, resulting in the oral allergy syndrome (OAS)[4].

B. pendula occurs in Europe and is currently the only species that has extensively been studied in relation to allergy. Other birch species occur in Europe as well, and sensitization to birch is also reported across Asia and North America, where other *Betula* species predominate [5–8]. Besides, numerous birch species are used as ornamental trees and are planted outside their natural distribution range. These birch species and cultivars might vary in allergenicity. Planting of trees with a reduced allergenicity could potentially reduce the allergenic load. For example, hypoallergenic apple varieties and olive

Abbreviations: LC-MS, liquid chromatography mass spectrometry; LC-MS/MS, liquid chromatography tandem MS; LC-MS^E, alternate scanning LC-MS; MIA, microsphere immuno assay; PR-10, pathogenesis related-class 10; Q-TOF, quadrupole time-of-flight.

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doi:10.1016/j.jprot.2011.03.021

trees are known to exist [9–11]. Reduced allergenicity can result from lowered allergen content [9,11,12] or from the absence of specific isoforms with a high allergenicity [13,14].

Multiple PR-10 genes have been identified in *B. pendula*, of which a subset is expressed in pollen and can therefore be considered true Bet v 1 allergens. Similar genes have been identified in other birch species [15], but expression and, moreover, IgE-reactivity of these allergens is unexplored. Bet v 1 is estimated to account for 10% of the total protein content of *B. pendula* pollen [16]. Various Bet v 1 isoforms exist [17,18], including isoforms with low and high IgE-reactivity [13]. A single *B. pendula* tree expresses a mixture of Bet v 1 isoforms with varying IgE-reactivity [15]. The relative abundance of individual isoforms within this mixture influences total allergenicity of the pollen. LC-MS/MS is a widely used technology to identify proteins in an extract by sequencing the peptides obtained after tryptic digestion. Here, a new variant called LC-MS^E was applied which enables both protein identification and quantification in a single run by alternating scans of low and high collision energy [19]. This label-free method was used for a comparative, quantitative analysis of pollen extracts originating from various birch species, thereby mainly focusing on the relative abundance of different Bet v 1 isoforms [15]. Site-directed mutagenesis has shown that several amino acid residues of Bet v 1 are linked to a IgE-reactivity [20,21] and these can be used as markers for high IgE-reactivity. Zaborsky et al. [22] have shown that a single amino acid change has an effect on antigen aggregation and thereby influences the establishment of a protective antibodies [22]. By determining the abundance of these amino acid markers among pollen-expressed Bet v 1, one can predict birch pollen allergenicity of the other birch species.

The allergenicity of 15 birch species and two hybrids was examined to determine whether birch trees with a reduced allergenicity exist in nature. Based on genomic data on Bet v 1 sequences, we applied LC-MS^E to detect and quantify amino acid residues associated with high and low IgE-reactivity among Bet v 1 in the pollen proteome. These data were linked to IgE-reactivity of the pollen by determining the antigenic and allergenic profiles using SDS-PAGE and Western-blotting, and by determining the amount of Bet v 1 protein in a fluorescent array using polyclonal antibodies. The results indicate that all birch species produce pollen that is likely to evoke allergic responses.

2. Material and methods

2.1. Pollen sampling

Pollen was collected from 24 birch trees belonging to 15 species and two hybrids (Table 1). All trees were previously genotyped using AFLP [25] and we constructed a neighbor-joining tree from the subset that was included in this study to visualize phylogenetic relations using NTSYSpc 2.10j (Applied Biostatistics). Two *Alnus* and *Corylus* accessions were included as outgroup in the analysis. Birch species are grouped into five subgenera/four clusters [23–25] that are all represented here by at least one species, except for subgenus *Betulaster* [23], which belongs to cluster IV-C in the AFLP analysis [25]. Sampled trees grew in the Botanical Garden of Wageningen or as ornamental trees along streets. Pollen was collected in the morning and within a 10 km area around Wageningen (the Netherlands) to minimize effects of environmental factors on pollen content. Timing of collection relative to the ripening stage of the pollen influences the Bet v 1 release from the pollen [26] and, therefore, each tree was sampled 2–3 times during its flowering period and these samples were pooled. However, the onset of flowering varied considerably between different species, and hence pollen was collected over a time span of 4–6 weeks (21 April–20 May 2006; 28 March–7 May 2007). During this period all trees were visited every 2–3 days and flowering catkins were shaken inside 50 ml blue caps to collect pollen. As a consequence, pollen was exposed to different weather conditions (humidity, sunlight, and temperature) before sampling. Samples were then sieved through a 90 μm mesh filter to eliminate extraneous material. Light microscopy observations confirmed that other plant parts accounted for <5% of the pollen samples. Samples were freeze dried before storage at 4 °C.

2.2. Flow cytometry and pollen quality

To deduce pollen characteristics and quality, 25 mg of pollen was suspended in 500 μl of 0.1 M PBS (pH 7.4). Samples were subsequently separated on a DakoCytomation CyAn high-performance flow cytometer and analyzed using Summit 4.0 software (DakoCytomation). Scatter characteristics and autofluorescence were recorded.

2.3. Allergen extracts

Extracts were prepared following a previously described protocol [15,27] in which 50 mg pollen was diluted into 1 ml of 0.05 M Tris–HCl buffer (pH 7.5). Total extraction time was 2 h. The supernatant was stored at −20 °C. The total amount of extracted protein was determined using the Bradford assay. The pellet was analyzed by SDS-PAGE to determine the rigorousness of the extraction procedure. We found no 17 kDa (Bet v 1) band in the resuspended pellet, indicating that all Bet v 1 was indeed extracted.

2.4. Patient sera

Sera from ten birch-allergic Dutch individuals (kindly provided by Y. Vissers and M. Bollen, Wageningen University) were stored at −80 °C until use and pooled prior to the Western blotting. These patients all tested positive for birch pollen. Eight patients cross-reacted to other tree pollen allergens. Two patients also displayed a reaction against grass pollen. Sera were characterized for reactivity against Bet v 1, Bet v 2, and Bet v 4 plus Bet v 6 by determining specific IgE levels using the ImmunoCAP system (Phadia, Uppsala, Sweden). Individuals each had total specific IgE levels to birch exceeding 50 kU/l. Reactivity against individual allergens in these sera ranged from 41 to >100 kU/l for Bet v 1, from <0.10 to 2.52 kU/l for Bet v 2, and was <0.10 kU/l for Bet v 4 plus Bet v 6.

2.5. SDS-PAGE and Western blot

Proteins were separated on 15% (w/v) SDS-PAGE and stained with Coomassie BB R-250 (Bio-Rad) or subjected to immunoblot

Table 1 – Bet v 1 content of birch pollen extracts as determined in a microsphere immune assay (column: Bet v 1 yield). Natural origin (A = Asia; NA = North America; E = Europe; C = cultivar). Total soluble protein yield was determined by the Bradford Assay. Values are means of replicates measured in duplicate at different dilutions. Intensity of the 17 kDa immunostained bands was quantified using densitometry by determining the integrated densities (area × mean gray) with ImageJ 1.38 software [29] *n.d.* = not determined.

Species/ subspecies/ cultivar	Classification (according to [23]; [25])	Natural origin	Total soluble protein yield (μg/mg pollen)	Bet v 1 yield (μg/mg pollen)	Immuno-staining (band density)	Bet v 1 detected by LC-MS^E
B. chichibuensis	Neurobetula; Group I	A	82.3	30.4	3122	Yes
B. nigra	Neurobetula; Group II	NA	81.2	14.8	727	Yes
B. lenta subsp. *lenta*	Betulenta; Group III	NA	75.3	13.1	5039	Yes
B. alleghaniensis	Betulenta; Group III	NA	103.5	36.0	8092	Yes
B. medwedewii	Betulenta; Group III	A	60.1	9.9	3335	Yes
B. pendula 'Youngii'	Betula; Group IV-A	E/A	80.5	23.1	2973	Yes
B. pendula 'Youngii'	Betula; Group IV-A	E/A	98.2	25.9	4040	Yes
B. pendula 'Fastigiata'	Betula; Group IV-A	E/A	107.2	22.7	3798	Yes
B. pendula 'Tristis'	Betula; Group IV-A	E/A	56.8	13.7	1773	Yes
B. platyphylla szechuanica	Betula; Group IV-A	A	30.5	2.6	874	Yes
B. platyphylla mandshurica	Betula; Group IV-A	A	34.1	5.6	1667	*n.d.*
B. pubescens	Betula; Group IV-B	E	123.0	27.4	4389	Yes
B. pubescens tortuosa	Betula; Group IV-B	E	95.1	22.0	3479	*n.d.*
B. papyrifera	Betula; Group IV-B	NA	116.9	26.8	4894	Yes
B. papyrifera cordifolia	Betula; Group IV-B	NA	76.4	23.4	5770	*n.d.*
B. costata (1)/B. costata (2) [a]	Neurobetula; Group IV-D	A	23.1/89	1.8/25.9	0/ *n.d.*	*n.d.*/Yes
B. davurica	Neurobetula; Group IV-D	A	62.2	9.6	1686	Yes
B. humilis	Chamaebetula; Group IV-D	E/A	43.4	2.7	656	*n.d.*
B. albosinensis	Neurobetula; Group IV-E	A	77.0	22.8	4907	*n.d.*
B. utilis	Neurobetula; Group IV-E	A	113.9	15.3	6917	*n.d.*
B. utilis jacquemontii	Neurobetula; Group IV-E	A	51.4	22.4	5058	*n.d.*
B. grossa	Betulenta; Group IV-E	A	50.9	13.5	3861	*n.d.*
B. x 'Edinburgh'	Hybrid	C	52.6	10.4	917	*n.d.*
B. x purpusii	Hybrid	NA	58.2	9.9	1613	*n.d.*

[a] See result section.

analyses. Extracted protein (30 μl of supernatant) was diluted in 10 μl Laemmli loading buffer (4× concentrated), heated for 5 min at 95 °C and subsequently 25 μl of each pollen extract was electrophoresed using a Mini-Protean II system (Bio-Rad), as described before [15]. Relative molecular masses were determined with SDS-PAGE Standards broad range markers (Bio-Rad). For Western blotting proteins were transferred to nitrocellulose membrane (Millipore) using wet blotting and immunological detection was carried out as described by Bollen et al. [28]. Immunoblot images were scanned and the intensity of the bands was quantified by densitometry with ImageJ 1.38 software [29]. Minor differences in the integrated densities (area × mean gray) between blots were corrected by calculating the relative intensity of each band by dividing absolute densities by the density of a control sample that was included on each blot.

2.6. *Microsphere immuno assay*

The polyclonal antibody 5H8, which is raised against Bet v 1 (kindly provided by L. Zuidmeer, Sanquin), was coupled to fluorescent microspheres. 5.0×10^6 carboxylated microspheres (Luminex MAGPlex^{TM}-C; Luminex Corp) were placed in a micro centrifuge tube, washed with distilled H_2O, and resuspended in 80 μl of 0.1 M monobasic sodium phosphate (pH 6.2). The bead suspension was activated by addition of 10 μl of 50 mg/mL sulfo-NHS and 50 μl of 50 mg/mL 1-Ethyl-3-(3-Dimethylaminopropyl)-Carbodiimide hydrochloride (EDC) and incubated for 20 min. Beads were subsequently washed twice with 50 mM (N-morpholino)ethane sulfonic acid buffer (MES; pH 5.0) and then resuspended in 50 mM MES. The 5H8 antibody was added to the bead suspension and incubated while mixing by rotation at room temperature for 2 h. Beads were washed twice in storage buffer (PBS-TBN; pH 7.4) by incubation for 30 min while mixing by rotation, and subsequently stored in the dark at 4 °C.

Pollen extracts were diluted in PBS-TBN (Phosphate-Buffered Saline (pH 7.4) containing 0.02% Tween 20, 0.1% Bovine serum albumin, 0.02% azide) and assayed at two dilutions (1:250; 1:400). 1000 antibody-coupled microspheres were added to each sample. The solution was vortexed to assure homogenous dispersal of microspheres and incubated for 60 min in the dark while mixing gently. After incubation, beads were captured on a Dynal MPC 96S magnet and washed with PBS-TBN. Beads were resuspended in 100 μl PBS-TBN containing biotinylated anti-Bet v 1 as a secondary antibody and incubated for 30 min while mixing gently. Beads were recaptured and washed, and then resuspended in 100 μl PBS-TBN containing 2 μg/ml Streptavidin rPhycoerythrin. After 15 min of incubation, samples were analyzed on the Luminex 100 machine using Applied Cytometry Systems software. We measured 100 beads per sample. Recombinant (r)Bet v 1a (Biomay, Austria) was diluted 1:100 in assay buffer and serially diluted from 1:10 to 1:100,000. These standard dilutions were

measured in triplicate to generate the calibration curve which was fit using a Logistic-5PL curve fit (Brendan Technologies). We measured the fluorescent signal of the beads and calculated median fluorescent intensity for each pollen extract. Using the standard curve, we calculated the corresponding amount of rBet v 1a and expressed this as the Bet v 1 concentration. This assumes that the 5H8 antibody recognizes all Bet v 1 isoforms equally well. Negative control contained assay buffer only.

2.7. LC-MS^E of Bet v 1

LC-MS^E was performed on 14 pollen extracts. These extracts were selected from Table 1 as representative samples covering the genetic diversity in the *Betula* genus. After SDS-PAGE separation and CBB staining of the pollen extracts the 17 kDa protein bands, representing the Bet v 1 isoforms, were cut out from the gels. After destaining, the proteins were digested with trypsin essentially according to Shevchenko [15,30]. The procedure to analyze tryptic digests in high-throughput configuration with the Ettan™ MDLC system (GE Healthcare) directly connected to a Q-TOF-2 Mass Spectrometer (Waters Corporation, UK) using the LC-MS^E method of alternating low and elevated levels of collision energy is described elsewhere [15]. Each sample was injected twice, after which the LC-MS^E data were analyzed following the procedure described by Silva et al. [19] using ProteinLynx Global SERVER (PLGS) v2.3 (Waters Corporation). The search database and procedure described in Schenk et al. [15] was used for peptide/protein identification. Fixed (Cys carbamidomethylation) and variable modifications (Met oxidation) were taken into account in the identification. After processing by PLGS, the Exact Mass and Retention Time (EMRT) table was reclustered using the PACP tool [31] to correct potential misalignment and split peak detection errors, and to normalize retention time.

3. Results

3.1. Antigenic profile and Bet v 1 concentration

To determine whether birch trees with a reduced IgE-reactivity can be identified within the natural variation of the *Betula* genus, we harvested pollen from 24 trees that belonged to 15 birch species and two hybrids. Fig. 1 displays the phylogenetic relationship between these species and hybrids. *B. nigra* and *B. chichibuensis* (cluster I and II) are the most distantly related birch species relative to the well examined *B. pendula*. *B. lenta* and *B. alleghaniensis* also belong to a separate cluster (cluster III). The majority of species are related to *B. pendula* (cluster IV). This cluster is composed of several closely related species, whose relationships are difficult to disentangle, which can be explained by extensive hybridization and introgression.

Pollen extracts were analyzed by SDS-PAGE and were found to have highly similar protein patterns (Fig. 2). Nearly all samples contained clear bands at approximately 17, 29 and 35 kDa. The identities of the bands at 17 and 35 kDa have previously been confirmed for extracts of *B. lenta* and *B. pendula* 'Youngii' by peptide sequencing using LC-MS/MS as Bet v 1 and Bet v 6, respectively [15]. In all pollen extracts except that of *B. costata* the Bet v 1 band was by far the most intense band (Fig. 2). All extracts displayed a band at approx. 29 kDa. We tried to identify this band by peptide sequencing, but the tryptic peptide sequences from this band did not match to any known birch protein or to any protein in related Fagales species. Because the band was not recognized by the pooled serum, we did not pursue its identification further.

The total soluble protein in the 24 pollen extracts as estimated by the Bradford assay is presented in Table 1. The protein yield varied between 50–120 μg/mg pollen, except for the extracts of *B. platyphylla szechuanica*, *B. platyphylla mandshurica* and *B. costata* which showed much lower protein content. Differences in total protein yield between pollen species are also reflected by the intensity of the major Bet v 1 (17 kDa) band, as illustrated by the SDS-PAGE protein profiles of Fig. 2. The reduced protein yield of some pollen species might be associated with poor quality of the harvested pollen grains. We confirmed this by recording the scatter characteristics and autofluorescence in a flow cytometry analysis. Two types of particles were detected in all samples: the first type had a higher forward scatter and higher side scatter, indicating larger size and higher cell complexity. Light microscopy observations of the separated types showed that these types represented intact pollen grain and ruptured pollen grains, respectively. Abundance of both types was indicative of pollen quality, which varied between samples (data not shown). Especially the *B. costata* sample that was used for the SDS-PAGE and Western blot analysis had a low quality due to the high abundance of ruptured pollen grains, which resulted in a low amount of extractable protein (23.1 μg/mg pollen; Table 1) and a very faint band at 17 kDa (lane 8; Fig. 2). We therefore prepared an extract from a batch of *B. costata* pollen with better quality, which was subsequently used in the LC-MS^E analysis.

Given that Bet v 1 is the major birch allergen and the most abundant pollen protein in all investigated species, as demonstrated by the SDS-PAGE protein patterns of Fig. 2, we focused specifically on this allergen in the following analyses. The concentration of Bet v 1 in each extract was determined in a Microsphere Immuno Assay (MIA) using polyclonal Antibody (pAb) raised against rBet v 1. All pollen extracts contained considerable amounts of Bet v 1 with an average concentration of 17.3 μg/mg pollen (Table 1). As expected, the extracts of the 3 species containing the lowest amount of total extractable protein, i.e. *B. platyphylla szechuanica*, *B. platyphylla mandshurica* and *B. costata*, also yielded the lowest amount of Bet v 1 (2.6, 5.6 and 1.8 μg/mg pollen, respectively). Moreover, the extract obtained from the new batch of *B. costata* pollen, containing almost 4 times more total protein, yielded 25 μg Bet v 1/mg pollen as measured by MIA. Differences in total protein yield thus explain a large portion of the variation in Bet v 1 content between different accessions given the correlation between total protein yield and Bet v 1 yield ($r=0.79$; $R^2=0.63$). On average, Bet v 1 accounted for 22% of the total water-soluble protein fraction (Table 1).

3.2. Allergenic profiles

The IgE-binding profiles of the pollen extracts were evaluated using immunoblots. Pooled serum of birch-allergic patients recognized a ~17 kDa band in all extracts except for *B. costata*

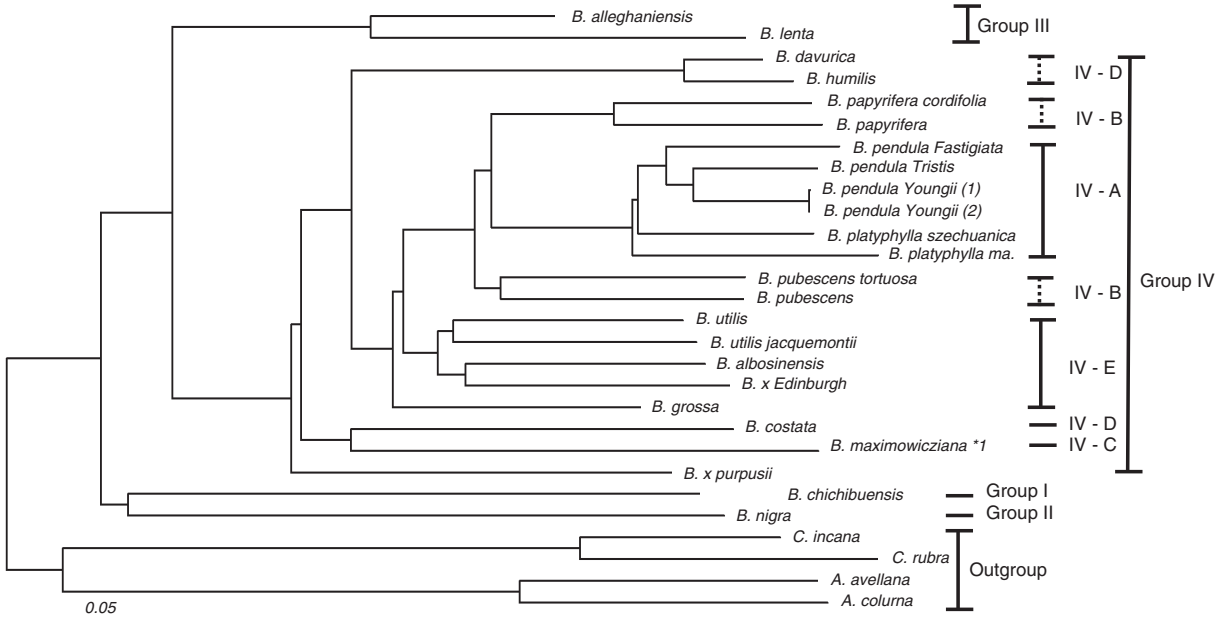

Fig. 1 – Neighbour-Joining tree of Dice similarities among the accessions included in this study based on AFLP genotypes [25]. *1No pollen could be harvested from this accession. *2The relationships within this group are somewhat compromised by the inclusion of the hybrid taxa.

(Fig. 3). As mentioned above, the extract of *B. costata* was excluded from this analysis given the poor pollen quality. The serum pool recognized an additional band at ~35 kDa in a subset of extracts, as well as several faint bands at higher molecular masses. When the intensity of the 17 kDa bands in the Western blots was determined with densitometry, the measured values for the 23 different birch species (Table 1) showed a correlation with the amount of Bet v 1 as determined in the MIA ($r=0.73$; $R^2=0.54$). Some variation in immune-reactivity was observed between pollen extracts from different species. Notably, the extracts of *B. nigra* and *B. chichibuensis* were less reactive than would be expected based on the blotted amount of Bet v 1, while the reactivity of *B. lenta* and of one sample of *B. utilis* were higher than expected.

3.3. *Presence and abundance of Bet v 1 isoforms*

The 17 kDa bands of 14 birch pollen extracts were in-gel digested with trypsin and analyzed with LC-MS^E. These pollen extracts covered the taxonomic variation in the genus *Betula* and represented 11 different species, including one biological replicate of the *B. pendula* cultivar 'Youngii' (Table 1, Fig. 1). Tryptic Bet v 1 peptides were identified and quantified. We focused on peptides that contained amino acid residues which

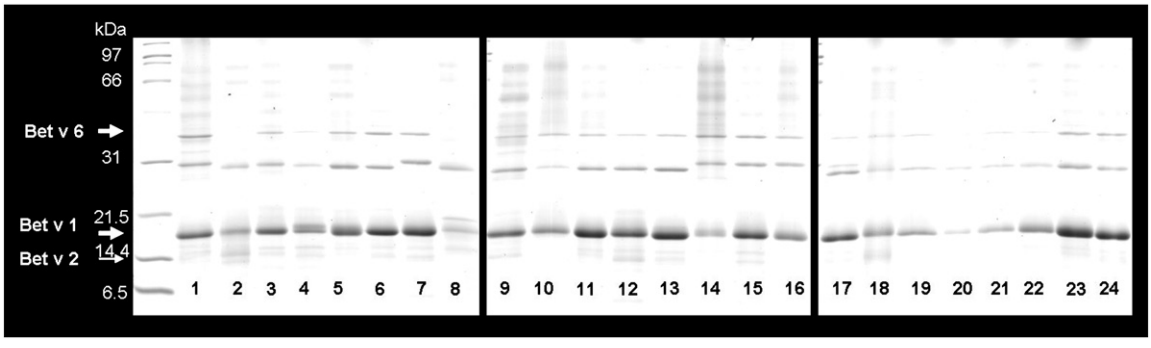

Fig. 2 – Protein profile of pollen extracts of 24 birch trees after SDS-PAGE and CBB staining. Arrows point to bands matching to the expected size of the allergens Bet v 1 and Bet v 6. MW size marker proteins are shown in the first lane on the left. (1) *B. pendula* 'Youngii'; (2) *B. nigra*; (3) *B. chichibuensis*; (4) *B. lenta*; (5) *B. alleghaniensis*; (6) *B. pendula* 'Fastigiata'; (7) *B. pubescens*; (8) *B. costata* *poor quality extract, see main text; (9) *B. medwedewii*; (10) *B. davurica*; (11) *B. papyrifera*; (12) *B. pubescens tortuosa*; (13) *B. utilis*; (14) *B. platyphylla mandshurica*; (15) *B. pendula* 'Youngii'; (16) *B. pendula* 'Tristis'; (17) *B. albosinensis*; (18) *B. grossa*; (19) *B. humilis*; (20) *B. utilis jacquemontii*; (21) *B. platyphylla szechuanica*; (22) *B. x* 'Edinburgh'; (23) *B. x purpusii*; (24) *B. papyrifera cordifolia*.

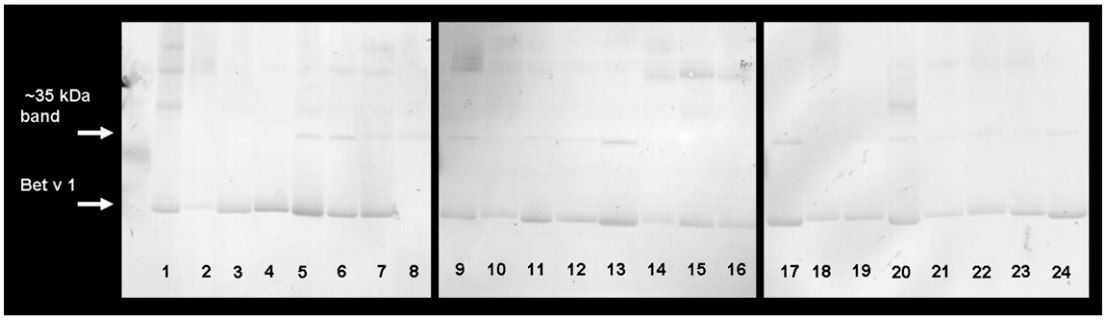

Fig. 3 – Western blots with pollen extracts of 24 birch trees. Bands that are recognized by a serum pool of birch-allergic patients are indicated by arrows. (1) *B. pendula* 'Youngii'; (2) *B. nigra*; (3) *B. chichibuensis*; (4) *B. lenta*; (5) *B. alleghaniensis*; (6) *B. pendula* 'Fastigiata'; (7) *B. pubescens*; (8) *B. costata* *poor quality extract, see main text; (9) *B. medwedewii*; (10) *B. davurica*; (11) *B. papyrifera*; (12) *B. pubescens tortuosa*; (13) *B. utilis*; (14) *B. platyphylla mandshurica*; (15) *B. pendula* 'Youngii'; (16) *B. pendula* 'Tristis'; (17) *B. albosinensis*; (18) *B. grossa*; (19) *B. humilis*; (20) *B. utilis jacquemontii*; (21) *B. platyphylla szechuanica*; (22) *B. x* 'Edinburgh'; (23) *B. x purpusii*; (24) *B. papyrifera cordifolia*.

are associated with either a low or high IgE-reactivity. At least 11 amino acid residues in the sequence of Bet v 1 or its PR-10 homologues are known to affect IgE-reactivity [20,21,32,33]. The presence and abundance of ten of these residues were examined in tryptic peptides by LC-MS^E. The eleventh residue resides in a tryptic peptide that is too small for detection with the used LC system. Five residues were polymorphic, whereas the other residues were identical in the pollen extracts of all examined species.

The polymorphic positions that can be used as markers for high IgE-reactivity were located in the tryptic peptides III, V and X (Fig. 4). The relative amounts of these peptides in the tryptic digests were deduced from peak intensities in the LC-MS^E analysis (Table 2). For example, peptide III harbors one amino acid that is associated with high IgE-reactivity at position 28 [20]. Three to six fragment variants of peptide III are present in all species, indicating that pollen in all species is composed of a mixture of isoforms.

The data on the amounts of peptides can be translated into the relative presence of amino acid residues that are known to influence IgE-reactivity. This shows that the residues which are associated with high IgE-reactivity are abundant in pollen

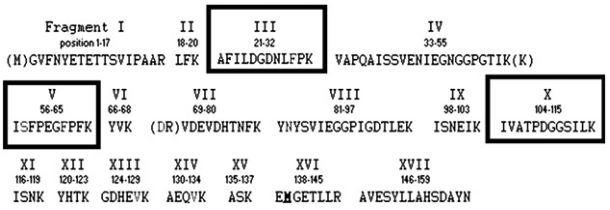

Fig. 4 – Amino acid sequence and predicted tryptic peptides of Bet v 1a (Acc. No. X15877). Fragments are labeled with Roman numbers. Other pollen-expressed Bet v 1 isoforms contain Lysine and Arginine residues at the same positions, and will consequently yield tryptic peptides of the same size. Marked fragments contain amino acid residues which affect IgE-reactivity, and which are polymorphic within the examined species.

of all examined birch species (Table 3). For example, several variants of peptide III contain the amino acid Asn^{28} (namely variants III$_{a, b, e, f, h, k, and m}$), whose presence is associated with high IgE-reactivity. Variants III$_d$ and III$_g$ contain Ile^{28} and Thr^{28}, respectively. The presence of Thr^{28} is associated with a low IgE-reactivity, while the effect of Ile^{28} on IgE-reactivity is unknown. These amino acid variants were observed in pollen of six species, but Asn^{28} was the most abundant amino acid: in each of the species 81–97% of the Bet v 1 protein contains this residue. The residues Ser^{112} and Ile^{113} are also associated with high IgE-reactivity and are even present in 72–100% and 79–100% of the Bet v 1 isoforms, respectively. In contrast, residues that are associated with low IgE-reactivity were rare (Table 3). For example, *B. chichibuensis* did not contain any of these residues. When averaged over all five amino acid positions, residues associated with a high IgE-reactivity were present in 65–84% of the isoforms. In other words, all species contain a mixture of isoforms, but this mixture is always dominated by isoforms that contain amino acids which are associated with high IgE-reactivity.

4. Discussion

Birch pollen is a major cause of Type I allergies in the Northern Hemisphere. In an effort to identify birch trees with a reduced allergenicity, we determined the allergenicity of 15 birch species and two hybrids. We focused mainly on the major allergen Bet v 1. This allergen was identified in all species that were analyzed in this study and turned out to be the most abundant protein in the water-soluble protein fraction of all birch species. On average, Bet v 1 accounted for 22% of the water-soluble fraction of protein, whereas Larsen [16] estimated the Bet v 1 content of *B. pendula* pollen to be 10%. Quantification of the Bet v 1 content is not a straightforward procedure and highly depends on the pollen quality during sampling. This quality may differ greatly among samples as pollen viability is affected by the weather conditions, especially with regard to rain and dew [34]. Pollen from different

Table 2 – Tryptic Bet v 1 peptides which contain amino acids that are associated with high or low IgE-reactivity as quantified using LC-MS^E. Pollen extracts of 14 accessions/11 birch species were examined. Identified peptides are labeled with Roman numbers, while peptide variants are labeled with additional letters. Displayed percentages indicate relative amounts of each variant compared to the total amount of that peptide per species (i.e., per column); percentages are averaged over two replicates.

Peptide sequence	Fragment variant	*B. pendula* 'Youngii' (1)	*B. pendula* 'Youngii' (2)	*B. pendula* 'Tristis'	*B. pendula* 'Fastigiata'	*B. plathyphylla*	*B. pubsecens*	*B. papyrifera*	*B. davurica*	*B. nigra*	*B. chichibuensis*	*B. lenta*	*B. alleghaniensis*	*B. medwediewii*	*B. costata*
AFILDGDNLFPK	IIIa	51	45	44	48	18	47	56	59	39	60	6	32	40	23
AFILDGDNLVPK	IIIb	19	12	11	11	14	14	3	4	–	–	–	–	–	–
AFILDGDILFPK	IIId	–	–	–	–	3	–	–	–	–	–	–	–	–	–
AFILDGDNLIPK	IIIe	13	27	20	41	25	38	39	29	20	23	43	14	28	60
AFILEGDNLIPK	IIIf	17	17	25		22	2	3	–	–	17	10		15	3
AFILEGDTLIPK	IIIg	–	–	–	–	–	–	–	8	–	–	–	6	12	14
AFILDGDNVIPK	IIIh	–	–	–	–	–	–	–	–	–	–	22	48	5	–
AFILDGNNLIPK	IIIk	–	–	–	–	–	–	–	–	42	–	–	–	–	–
AFILGGDNLFPK	IIIm	–	–	–	–	18	–	–	–	–	–	–	–	–	–
(K)ISFPEGFPFK	Va	46	39	45	43	33	31	37	33	46	61		52	13	40
(K)INFPEGFPFK	Vb	23	15	11	14	12	24	12	4	16			20	10	5
(K)INFPEGIPFK	Vc	–	–	–	–	5	–	–	11	–	–	–	–	10	–
(K)ISFPEGFPFR	Vd	–	–	–	–	–	–	–	–	–	–	20	–	24	–
(K)ITFPEGSPFK	Ve	32	46	44	42	49	45	51	52	38	39	80	28	43	56
IVATPDGGSILK LVATPDGGSILK IVATPNGGSILK	Xa+Xg+Xc[a]	82	95	80	87	86	91	95	81	86	100	100	72	89	100
IVATPDGGCVLK	Xb	18	5	20	13	14	9	–	–	–	–	–	21	2	–
IVETPNGGSILK	Xf	–	–	–	–	–	–	–	–	14	–	–	–	–	–
IVATPDGGCILK	Xh	–	–	–	–	–	–	5	19	–	–	–	7	9	–

[a] Fragments Xa and Xg have exactly the same mass and cannot be distinguished. The peak of peptide Xc overlaps with the first isotope peak of peptide Xa = g because they differ exactly 1 Da in size and have the same charge, and as a result Xc cannot be identified separately.

Table 3 – Prevalence of the amino acids which are associated with either a high or a low IgE-reactivity in Bet v 1 isoforms. Based on the abundances of specific peptides (data in Table 2), one can calculate the abundance of amino acid residues that are either associated with high IgE-reactivity, low IgE-reactivity, or with an unknown effect on IgE-reactivity. Relative amounts over 50% are highlighted in grey.

Peptide Sequence	Amino acid	Association with high or low IgE-reactivity	*B. pendula* 'Youngii' (1)	*B. pendula* 'Youngii' (2)	*B. pendula* 'Tristis'	*B. pendula* 'Fastigiata'	*B. platyphylla*	*B. pubsecens*	*B. papyrifera*	*B. davurica*	*B. nigra*	*B. chichibuensis*	*B. lenta*	*B. alleghaniensis*	*B. medwidiewii*	*B. Costata*
Residue 28	N=Asn	High	100	100	100	100	97	100	100	92	100	100	81	94	88	86
	T=THr	Low	—	—	—	—	—	—	—	8	—	—	—	6	12	14
	l=lle	Unknown	—	—	—	—	3	—	—	—	—	—	19	—	—	—
Residue 30	F=Phe	High	51	45	44	48	39	47	56	59	39	60	25	32	40	23
	V=Val	Low	19	12	11	11	14	14	3	4	—	—	—	—	—	—
	l=lle	Unknown	30	44	45	41	47	40	42	37	62	40	75	68	60	77
Residue 57	S=Ser	High	46	39	45	43	33	31	37	33	46	61	20	52	37	40
	N=Asn	Low	23	15	11	14	18	24	12	15	16	—	—	20	20	5
	T=THr	Unknown	32	46	44	42	49	45	51	52	38	39	80	28	43	56
Residue 112	S=Ser	High	82	95	80	87	86	91	95	81	100	100	100	72	89	100
	C=Cys	Low	18	5	20	13	14	9	5	19	—	—	—	28	11	—
Residue 113	l=lle	High	82	95	80	87	86	91	100	100	100	100	100	79	98	100
	V=Val	Low	18	5	20	13	14	9	—	—	—	—	—	21	2	—
Average over all five residues 28,30,57,112,113		High	72	75	70	73	68	72	78	73	77	84	65	66	70	70
		Low	16	7	12	10	12	11	4	9	3	—	—	15	9	9
		Unknown	12	18	18	17	20	17	18	18	20	16	35	19	21	27

species was collected on different days, as the 15 species do not flower simultaneously, but in a period of up to six weeks in spring. Light microscopy and flow cytometry observations confirmed the presence of ruptured pollen in the poor quality samples.

For wind-transported pollen, any protein that is present in such large quantities across a range of species would be expected to serve an essential function. Several hypotheses exist on the function of the Bet v 1 protein in birch pollen. Bet v 1 genes are transcribed during the late stages of anther development [35]. Bet v 1 has initially been reported to act as ribonuclease [36], but a more recent study suggested that the cavity inside the Bet v 1 protein could be related to its function as plant steroid carrier [37]. Bet v 1 is reported to interact with various physiological ligands [38], but which ligands are bound inside the cavity *in vivo* is still unclear. Additional suggestions for the function of PR-10 proteins in general are that they might act as storage proteins [39] or as cryoprotective proteins [40]. In either case, elucidating the function of the pollen-expressed PR-10 proteins remains a target for further study. The examined species were representative for the genetic variation within the *Betula* genus. Therefore, we consider it unlikely that viable pollen with significantly reduced amounts of Bet v 1 will be identified within the natural variation when extending the sampling to other birch species or varieties.

Pollen of *B. pendula* was previously shown to contain a mixture of Bet v 1 isoforms [15] of varying IgE-reactivity [13,41]. Here, we identified and quantified Bet v 1 peptides in pollen of multiple birch species by LC-MS^E. To approximate the overall allergenicity of the pollen, we focused on the eleven amino acid positions that are known to affect IgE-reactivity of Bet v 1 isoforms [20,21,32,33], ten of which could be evaluated. These amino acid positions can be used as markers for high or low IgE-reactivity. The majority of these positions were identified by amino acid sequence comparison between the highly IgE-reactive isoform Bet v 1a and the hypoallergenic isoform Bet v 1d. Five amino acid positions (30, 57, 62, 112 and 113) were polymorphic, whereas the remaining positions were always occupied by residues that were associated with high IgE-reactivity. Residues associated with high IgE-reactivity were present in all birch species and were, on average, the most abundant residues (65–84%) in the Bet v 1 mixture. This strongly suggests that the majority of Bet v 1 in pollen from these birch species has a high IgE-reactivity.

A potential limitation of the validity of our approach is that all amino acid positions were evaluated in the context of the isoforms Bet v 1a and Bet v 1d, which both originate from *B. pendula*. It seems unlikely that individual substitutions fully account for the reduction in IgE-binding. The amino acid substitutions are therefore used as markers for a high/low allergenicity. The serine to cysteine exchange at position 113 (or 112 when excluding the initiating methionine) represents a special case, since this substitution reduces the allergic response because of its effect on antigen aggregation and which thereby influences the establishment of a protective antibodies [22]. However, serine was by far the most abundant residue, being found in 79–100% of the Bet v 1 protein of the various species, again an unfortunate outcome for allergic

patients. A second limitation is the fact that we identified several amino acid substitutions with unknown effects on allergenicity in species other than *B. pendula*. These could, in principle, affect allergenicity as well. However, even when this is true, these substitutions represent a minor fraction given that Bet v 1a is close to the consensus sequence of the Bet v 1 isoforms across the whole range of examined species.

Based on the quantified amino acids and their associated effect on allergenicity, one would expect that birch trees with a reduced allergenicity were not present in the current dataset. Indeed, only limited variation in IgE-reactivity was found between pollen samples of the different birches studied. This variation correlated well with the blotted amount of Bet v 1 as determined using a microsphere immuno assay. A similar method was previously used to quantify six indoor allergens by Earle et al. [42], who found a high correlation (R^2=0.90–0.99) between the results obtained by the fluorescent array and the ELISA. A faint signal was obtained with IgE in the serum pool for a band at approximately 35 kDa in some extracts. We detected peptides of Bet v 6 at this position in the gel. However, the sera that contributed to the serum pool were characterized for IgE-reactivity against Bet v 6 and no specific IgE against this allergen was present in any of the individual sera, excluding this option. Alternatively, Bollen et al. [28] detected a 35 kDa band with a high immune-reactivity in purified natural Bet v 1, even under reducing conditions in the SDS-PAGE. This was shown to be a dimer of Bet v 1.

The blotted amounts of protein were strongly influenced by pollen quality. Taking this into account, *B. nigra* and *B. chichibuensis* showed the lowest reactivity relative to the blotted amount of Bet v 1. These two species take a basal position in the phylogeny of the genus *Betula*, and they are the most distant relatives of *B. pendula*, so it is possible that they are also most different in Bet v 1 reactivity. Regarding the clinical relevance of a reduction in IgE-reactivity, Koppelman et al. [43] indicated that for the major peanut allergen this should be in the order of a 100× to 1000× fold. The largest difference in immune-reactivity that we observed between pollen of different *Betula* species was approximately tenfold, suggesting that the differences we detected are to be too small to have any clinical relevance.

The lack of variation in Bet v 1 content among different birch species facilitates standardization of using recombinant allergens for allergy diagnostics. For diagnostic purposes, recombinant isoforms should be recognized by as many patients as possible. rBet v 1a has already proven to be an excellent choice for diagnosis of birch pollen allergy [44–46]. Bet v 1a was found in high quantities in all examined *B. pendula* cultivars. As a consequence, everyone who is exposed to *B. pendula* pollen during sensitization is exposed to Bet v 1a. We can now conclude that other birch species from North America and Asia express isoforms highly similar to Bet v 1a, which will contain identical or very similar epitopes, suggesting that when sensitization takes place against these species, patients are expected to display sensitization to the same Bet v 1 epitopes and can be diagnosed with rBet v 1a as well. It also suggests that the developed specific immunotherapy for Bet v 1 can be universally applicable. Although the exact mechanism of immunotherapy is not fully understood, the changes to the immune system require action from T cells and consequently rely on the presence of T-cell epitopes [47]. Recombinant allergens can be modified to reduce the risk of IgE-mediated side-effects for use during specific immunotherapy [48]. Application of recombinant allergens introduces the issue of choice: selection of one specific isoform includes a risk for exclusion of particular T-cell epitopes. Current efforts focus on application of recombinant allergens with low IgE-reactivity (Bet v 1d) or of recombinant allergen fragments [41,49]. The high similarity between Bet v 1 isoforms from different species increases the chance of shared T-cell epitopes.

5. Conclusion

In all examined birch species, Bet v 1 was found to be an abundant pollen protein. All pollen contained a mixture of Bet v 1 isoforms that is dominated by isoforms with amino acids which are associated with high IgE-reactivity. The examined species were a representative sample which covered the genetic diversity in the *Betula* genus. Quantitative and qualitative differences of Bet v 1 between birch species are too small to have clinical relevance, which was confirmed by immunoblotting. Pollen from birch species other than the well-studied *B. pendula* is therefore predicted to provoke similar allergic responses.

Acknowledgements

This research was in part funded by the Netherlands Proteomics Centre, an innovative cluster of the Netherlands Genomics Initiative, and by the Dutch Ministry of Economic Affairs, Agriculture, and Innovation through the DLO program 'Plant and Animal for Human Health' (project KB-05-001-019-PRI).

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