Published in final edited form as:

Clin Exp Allergy. 2011 December ; 41(12): 1804–1814. doi:10.1111/j.1365-2222.2011.03866.x.

Bet v 1-like pollen allergens of multiple *Fagales* species can sensitize atopic individuals

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Summary

Background—In the temperate climate zone of the Northern hemisphere, *Fagales* pollen allergy represents the main cause of winter/spring pollinosis. Among *Fagales* trees, pollen allergies are strongly associated within the *Betulaceae* and the *Fagaceae* families. It is widely accepted that *Fagales* pollen allergies are initiated by sensitization against Bet v 1, the birch pollen major allergen, although evidence is accumulating that the allergenic activity of some Bet v 1-like molecules has been underestimated.

Objective—To investigate the allergenic potential of the clinically most important *Fagales* pollen allergens from birch, alder, hazel, hornbeam, hop-hornbeam, oak, beech and chestnut.

Methods—To obtain the full spectrum of allergens, the three previously unavailable members of the Bet v 1-family, hop-hornbeam Ost c 1, chestnut Cas s 1 and beech Fag s 1, were identified in the respective pollen extracts, cloned and produced as recombinant proteins in *E. coli*. Together with recombinant Bet v 1, Aln g 1, Car b 1, Cor a 1 and Que a 1, the molecules were characterized physicochemically, mediator release assays were performed and IgE cross-reactivity was evaluated by ELISA and Immuno Solid-phase Allergen Chip (ISAC) IgE inhibition assays.

Results—All allergens showed the typical Bet v 1-like secondary structure elements, and they were all able to bind serum IgE from *Fagales* allergic donors. Strong IgE binding was observed for *Betuloideae* and *Coryloideae* allergens, however, cross-reactivity between the two subfamilies was limited as explored by inhibition experiments. In contrast, IgE binding to members of the *Fagaceae* could be strongly inhibited by serum pre-incubation with allergens of the *Betuloideae* subfamily.

Conclusions and Clinical Relevance—The data suggest that Bet v 1-like allergens of the *Betuloideae* and *Coryloideae* subfamily might have the potential to induce IgE antibodies with different specificities, while allergic reactions towards *Fagaceae* allergens are the result of IgE cross-reactivity.

Keywords

Bet v 1 family; Bet v 1-like allergens; cross-reactivity; *Fagales*; microarray; pollen allergy; recombinant allergens

Introduction

Fagales pollen allergies represent the main cause of spring pollinosis in the temperate climate zone of the Northern hemisphere. The botanical order of *Fagales* is classified into 8 families, 55 genera and 1877 species [1]. These families are *Nothofagaceae* (southern beech family), *Fagaceae* (beech family, including the genera beech, oak and chestnut), *Juglandaceae* (walnut family), *Myricaceae* (bayberry family), *Rhoipteleaceae* (rhoiptelea family), *Ticodendraceae* (ticodendron family), *Betulaceae* (birch family) and *Casuarinaceae* (she-oak family) [2]. The *Betulaceae* family can be further divided into *Betuloideae*, including the genera birch and alder, and *Coryloideae*, including hazel, hornbeam, hop-hornbeam and *Ostryopsis*, the latter describing three species closely related to hazel and hop-hornbeam [3] (Fig. 1). Associations with allergic diseases can be found for pollen of the beech [4], walnut [5], bayberry [6], birch [7] and she-oak [8] families. However, only pollen allergens of the beech and birch families have been officially acknowledged by the WHO/IUIS allergen nomenclature subcommittee (<http://www.allergen.org>). Detailed analysis of the protein content of aqueous pollen exudates revealed that *Fagales* allergies are dominated by cross-reactive allergens belonging to the PR-10 proteins. The best-studied allergenic representative of this family is Bet v 1, the birch pollen major allergen. Depending on the observed population, between 62% and 100% of birch pollen-sensitized individuals show IgE reactivity towards the molecule [9], also in areas where no direct birch pollen exposure is possible [10, 11]. In addition, several allergenic Bet v 1 homologues have been identified in pollen of related *Fagales* trees, and were cloned, produced and characterized immunologically [12–14].

Not all airborne pollen-producing *Fagales* species initiate or elicit allergic reactions to the same extent. These differences might be related to the amount of pollen released by the different species, differences in aerodynamic properties of the pollen, the flowering periods of the respective species or the content of allergenic protein in the pollen. For example, alder pollen represents a rather potent source of allergenic pollen by reaching high numbers of pollen counts in winter [15]. However, the allergenic potential of alder is limited due to the early flowering period where people normally do not spend much time outdoors. Hazel, hornbeam, oak, beech and chestnut produce high pollen counts, especially in the southern regions of Europe, but still their sensitizing potential is considered significantly lower when compared with birch pollen [16]. Thus, it is widely accepted that pollen of these aforementioned species act as supporting factors for allergic sensitization, while birch Bet v 1 is most likely initiating the disease. As acknowledged, within the last years, evidence is

accumulating that also other pollen-derived Bet v 1 homologues might have the potential to sensitize atopic individuals [10, 12]. To further address this question, we took advantage of a large panel of already available recombinant *Fagales* allergens from alder, birch, hazel, hornbeam and oak. In addition, the Bet v 1 homologous allergens from beech, chestnut and hop-hornbeam [4, 17, 18] were identified, produced and characterized. The whole panel of allergens was immobilized on slides for IgE-binding studies in microarray format and IgE inhibition studies with patients' sera from three distinct geographical areas showing different *Fagales* distribution were performed. The sera were tested with Bet v 1, as representative member of the *Betuloideae* and Ost c 1, as representative member of the *Coryloideae* families, to address the question of cross-reactivity vs. co-sensitization for *Fagales* allergies.

Methods

Patients and sera

Fagales pollen allergic patients were selected based on case history, positive skin prick test and *in vitro* IgE detection using Immuno Solid-phase Allergen Chip (ISAC) 103 (Phadia Multiplexing Diagnostics GMBH, Vienna, Austria) [19] according to previously reported protocols [11]. Sera ($n = 25$) were obtained from the Allergy Unit at the University Hospital in Genoa, Italy, from the Center for Molecular Allergology at IDI-IRCCS in Rome, Italy, and from the Allergieambulatorium Reumannplatz, Vienna, Austria (Table 1). According to pollen data, it is suspected that subjects recruited in Genoa were primarily exposed to pollen from hop-hornbeam and other *Fagales* species but not to birch pollen, subjects recruited in Rome to pollen from other *Fagales* species but not to that from hop-hornbeam and birch and subjects recruited in Vienna primarily to birch pollen [15, 20]. The study was approved by the Institutional Review Board (n. 106-CE-2005), and signed informed consents were obtained.

Recombinant allergens

Recombinant allergens (i.e. Bet v 1.0401, Cor a 1.0104, Aln g 1.0101, Car b 1.0109 and Que a 1.0301) used for basophil mediator release assays, ELISA inhibition assays and as inhibiting agents for ISAC inhibition assays were produced according to published methods [12, 21]. Bet v 1.0101 was purchased from Biomay AG, Vienna, Austria. Ost c 1.0101, Cas s 1.0101 and Fag s 1.0101 were produced as described in this article. Ready-to-use allergen chips were obtained from Phadia Multiplexing Diagnostics GMBH.

Cloning of Cas s 1.0101, Fag s 1.0101 and Ost c 1.0101

Beech and chestnut pollen were purchased from Allergon AB, Ängelholm, Sweden. Hop-hornbeam pollen was collected locally by one of the authors (CM) during the peak pollinating season. Pollen was extracted and processed for further analysis as reported for other *Fagales* pollen [12]. The N-terminal sequence of Fag s 1 and Ost c 1 was revealed by mass spectrometric *de novo* sequencing of proteolytically digested natural allergens. Therefore, extracts of beech or hop-hornbeam pollen were separated by SDS-PAGE, and bands in the size range between 17 and 18 kDa were excised, digested (in-gel all-in-one trypsin digestion kit Calbiochem, EMD, Gibbstown, NJ, USA) and analysed with a Global Ultima Q-ToF (Waters Corp., Milford, MA, USA). The N-terminal sequence of Cas s 1 has

been previously reported [17]. Total RNA from chestnut, beech and hop-hornbeam pollen was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The cDNA was generated using SuperScript one-step RT-PCR kit (Invitrogen) using the primer pairs Cas_fwd (5'-CATGCCATGGGTGTTTTYAC-3')/oligo_dT (5'-CCGCTCGAGTTTTTTTTTTTTTTTTTTTTTTT TTTT-3'), Fag_fwd (5'-CATGCCATGGGTGTTTTYAC-3')/oligo_dT, or Ost_fwd (5'-GGCATGCCATGGGTGTTT-TYAAYTAYGARGCMGAAA-3')/oligo_dT. Restriction sites are presented in *italic*, Y represents pyrimidine, R represents purine and M represents amino (adenine or cytosine) deoxyribonucleotides. Forward primers were designed according to the published sequence data of Cas s 1 [17] and *de novo* sequencing results of Fag s 1 or Ost c 1. The resulting cDNA was cloned into a pGem-T Easy vector (Promega, Madison, WI, USA) and sequenced. Thereafter, gene-specific reverse primers were designed and used for amplification of the allergens, i.e. Cas_rev (5'-CGCGAATTCTTATTTGTAGAGATCAGGGTTTGCC-3'), Fag_rev (5'-CGCGAATTCTTAGTGATAGGCAGCAGGATTTGCC-3') and Ost_rev (5'-CGCGAATTCTTAGTTGTATTCAGCAGTGTGTGCC-3'). Full-length Cas s 1, Fag s 1 and Ost c 1 were cloned into the pET 28b vector (EMD Biosciences Inc, San Diego, CA, USA) using *NcoI* and *EcoRI* restriction sites, and several clones were sequenced.

The sequences obtained from PCR cloning were compared with proteolytically digested natural allergens by mass spectrometry (MS).

Protein expression and purification

The Cas s 1, Fag s 1 and Ost c 1 expression constructs were transformed into *E. coli* BL21 Star™ (DE3) cells (Invitrogen) and grown at 37°C in LB medium supplemented with 25 mg l⁻¹ kanamycin to an OD_{600 nm} of 0.8. Protein expression was induced by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and growth was continued at 16°C for 20 h. Harvested cells were dissolved in 1/50 culture volume of 20 mM sodium phosphate buffer containing 0.5 M sodium chloride and 0.5 M urea and cell breakage was performed using liquid nitrogen. After centrifugation at 15 000 g, the solution was filtered through a 0.45-μm filter and protein purification was performed by hydrophobic interaction chromatography using a 5-mL Phenylsepharose column (GE Healthcare Biosciences, Little Chalfont, UK). The protein was eluted with 25 mM Tris/HCl pH 9.3, 8% (v/v) 2-propanol. Final purification was performed by size exclusion chromatography in 10 mM sodium phosphate buffer pH 8 using a Superdex 75 10/300 GL column (GE Healthcare Biosciences). Recombinant proteins were lyophilized and stored at -20°C.

Physicochemical analysis of recombinant proteins

Purity of recombinant allergens was analysed by SDS-PAGE followed by Coomassie Brilliant Blue R-250 (Biorad, Hercules, CA, USA) staining. The stained gels were scanned at 2600 dpi and gel bands were analysed densitometrically using the Photoshop CS5 software (Adobe Systems Incorporated, San Jose, CA, USA). Protein identity was determined by amino acid analysis of allergens using the Pico Tag method (Waters Corp.) and by mass spectrometry using a Quadrupole time-of-flight mass spectrometer with electrospray ionization (ESI-QTOF-MS) (Waters Corp.). Dynamic light scattering (DLS)

was performed on a DLS802 system (Viscotek, Worcestershire, UK) at a protein concentration of 0.75 mg/mL in Tris-buffered saline (TBS) pH 7.4 to determine the hydrodynamic radius (RH) and polydispersity of proteins in solution [22].

Secondary structure analysis

For secondary structure analysis, CD spectra were recorded with a JASCO J-815 spectropolarimeter fitted with a PTC-423S Peltier-type single position cell holder in 5 mM sodium phosphate buffer pH 7.4 (Jasco, Tokyo, Japan). In addition, infra-red spectra of allergens were recorded at a concentration of 1.0 mg/mL, in 5 mM sodium phosphate using the Confocheck™ Fourier transform infra-red (FTIR) system (Bruker Optics, Ettlingen, Germany) equipped with a Mercury Cadmium Telluride detector [23]. Data were accumulated in the temperature-controlled AquaSpec™ flow-through transmission cell with 7 µm path length at 25°C with a spectral resolution of 4 per cm and a data acquisition time of 100 s. Data procession and evaluation were performed with the Opus™ software (Bruker Optics). Secondary structure prediction was performed applying partial least squares calibrations for alpha-helix and beta-sheets.

ELISA experiments

For ELISA experiments, Maxisorp plates (Nalge Nunc, Rochester, NY, USA) were coated with allergen (200 ng/well in 50 µL PBS) overnight at 4°C. Plates were blocked with TBS, pH 7.4, 0.05% (v/v) Tween, 0.5% (w/v) BSA and incubated with patients' sera diluted 1 : 10 overnight at 4°C. Bound IgE was detected with alkaline phosphatase-conjugated monoclonal anti-human IgE antibodies (BD Biosciences, Franklin Lakes, NJ, USA), after incubation for 1 h at 37°C and for 1 h at 4°C. A 10-mm 4-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, MO, USA) was used as substrate and OD was measured at 405/492 nm. Inhibition studies were performed by serum pre-incubation with 1 µg inhibitor overnight at 4°C. All measurements were performed as triplicates; results are presented as mean OD values.

Microarray inhibition studies

Fifteen sera, five each from Genoa, Rome and Vienna, having IgE antibodies to Bet v 1 and other Bet v 1 homologous molecules available on the ISAC 103 microarray test (Phadia Multiplexing Diagnostics GMBH) were used in the inhibition assay. Individual sera were pre-incubated overnight with equal volumes of purified rOst c 1.0101 (1 mg/mL) or rBet v 1.0101 (1.0 mg/mL) solutions, representing the highest inhibitor concentrations available. Buffer was used instead of allergen preparations to obtain the non-inhibited specific binding value. The remaining IgE reactivities to Aln g 1.0101, Bet v 1.0101, Bet v 1.0401, Car b 1.0109, Cor a 1.0104, Fag s 1.0101 and Que a 1.0301 were measured using the ISAC 103 and an experimental microarray version obtained by PMD using the same technology. The assay was run as single-point highest inhibition achievable assay and percentage of inhibition was calculated using a procedure available in the Inter-All software (Allergy Data Laboratories s.c., Latina, Italy). Specificity of the IgE inhibition was assessed by incubation with only the detection antibody. Control IgE values obtained on non-cross-reactive positive allergen available on the same microarrays showed no inhibition and thus are not shown.

Mediator release assays

To determine the allergenic potential of *Fagales* allergens, rat basophil degranulation assays were performed as previously described [24]. Briefly, RBL-2H3 cells transfected with human high-affinity IgE receptor (FcεRI) were passively sensitized with serum IgE from *Fagales* pollen allergic donors. Antigen-dependent β-hexosaminidase release into the supernatant was measured by enzymatic cleavage of the fluorogenic substrate 4-methyl umbelliferyl-N-acetyl-β-glucosaminide and expressed as percentage of total enzyme content of Triton X100-treated cells.

Statistical analysis

Statistical significance was calculated for mediator release assays. Normal distribution of the data was assumed and verified using the Kolmogorov–Smirnov test. Statistical significance was calculated with paired samples *t*-test. A value of $P < 0.05$ was considered statistically significant.

Results

Selection and recombinant production of *Fagales* pollen allergens

For the present study, the nine most relevant allergenic *Fagales* trees have been selected and representative isoforms of the respective Bet v 1-like allergens were either purchased or produced as recombinant proteins according to published protocols [12]. The Bet v 1 homologues of chestnut, beech and hop-hornbeam were cloned by RT-PCR using pollen mRNA. After DNA sequence analysis of several different clones derived from each pollen source, three full-length clones of Cas s 1 (ACJ23861, ACJ23862 and ACJ23863), Fag s 1 (ACJ23864, ACJ23865 and ACJ23866) and one of Ost c 1 (HM366608.1) were identified (Fig. 2). The PCR cloning of the full-length Bet v 1 homologues from chestnut, beech and hop-hornbeam was performed with degenerated primers, which were designed based on the N-terminal amino acid sequences of the respective allergens. Thus, the nucleotide sequence in the 5′ binding region of the primer might not be identical with the original gene sequence. However, the high expression levels of all the three allergens in *E. coli* exceeded 10% of the total soluble protein, and therefore these gene sequences were used for recombinant production of Cas s 1.0101, Fag s 1.0101 and Ost c 1.0101. After purification, the purity of the recombinant non-fusion proteins was determined by SDS-PAGE. Densitometric analysis of Coomassie-stained gels revealed purity grades of >95% for all candidate molecules (Fig. 3a). Differences in the migration pattern of recombinant Cas s 1 compared to other Bet v 1 homologues were observed in the SDS-PAGE. However, a similar migration pattern was detected for natural Cas s 1. Detailed mass spectrometric analyses of the natural chestnut allergen revealed no modifications of the protein (data not shown), and we therefore concluded that the altered migration pattern of Cas s 1 is caused by its primary structure. The sequences of all Bet v 1-like allergens were verified by mass spectrometry of the intact proteins (Fig. 3b).

Bet v 1-like molecules of eight *Fagales* species are characterized by similar secondary structure elements

Circular dichroism (CD) (Fig. 3c) as well as Fourier transformed infra-red (FTIR) (Fig. 3b) spectroscopy were performed to analyse the secondary structure elements of the nine different Bet v 1-like allergens. Both methods revealed very similar secondary structure elements of the investigated proteins. All proteins showed similar CD spectra indicating the presence of related secondary structure elements. Using FTIR spectra, alpha-helical- as well as beta-sheet-contents were calculated for the individual molecules, and again no major differences could be detected.

Recombinant *Fagales* allergens show no tendency to form aggregates

The aggregation behaviour of nine *Fagales* allergens and allergen isoforms was investigated by dynamic light scattering (DLS). The DLS measurements did not detect high molecular weight aggregates in any of the tested allergen preparations (Fig. 3b). The proteins were analysed in TBS buffer to mimic the buffer conditions used for ELISA experiments. The Bet v 1.0101, Car b 1.0109, Que a 1.0301 and Ost c 1.0101 represent allergens with no intrinsic cysteine showing a hydrodynamic radius of approximately 2 nm in DLS, which reflects monomeric fractions of the proteins. In contrast, the allergens Bet v 1.0401, Aln g 1.0101, Cor a 1.0104, Cas s 1.0101 and Fag s 1.0101, have a single intrinsic cysteine, which as reported for Bet v 1.0401 can cause partial dimerization of the molecule indicated by an increased hydrodynamic radius in DLS [25]. At 25°C and TBS buffer pH 7.4, this dimerization of Bet v 1.0401 could not be detected. However, the allergens Aln g 1.0101, Cor a 1.0104, Cas s 1.0101 and Fag s 1.0101 showed partial dimerization via the free cysteine molecules which could be blocked by reducing and alkylating the proteins prior to the DLS measurements. Of note, such dimers occur naturally and were also detected in pollen extracts of the respective allergen sources (data not shown).

Fagales* allergens show different level of allergenicity *in vitro

According to multiple-sequence alignments as well as botanical aspects, the nine allergens were clustered into members of the *Betuloideae* (Bet v 1.0101, Bet v 1.0401, Aln g 1.0101), *Coryloideae* (Cor a 1.0104, Car b 1.0109, Ost c 1.0101) and *Fagaceae* (Que a 1.0301, Cas s 1.0101, Fag s 1.0101) respectively. All molecules were tested in basophil mediator release assays using sera of *Fagales* allergic individuals from Austria (Fig. 4a). There was no significant difference between the allergenic potential of Bet v 1.0101, Car b 1.0109 and Ost c 1.0101 (Fig. 4b). The low IgE-binding isoform Bet v 1.0401, as well as Aln g 1.0101 and Cor a 1.0104 showed significantly lower allergenicity than Bet v 1.0101, and Car b 1.0109, however, higher IgE binding than Que a 1.0301, Cas s 1.0101 and Fag s 1.0101. In summary, the strongest IgE-binding molecules were identified within the *Betuloideae* as well as *Coryloideae*, whereas the allergenic activity of the *Fagaceae* allergens was strikingly lower, with Cas s 1 being the weakest stimulus for basophil activation in this assay setup.

Cross-reactivity profiling of Bet v 1-like allergens by ISAC inhibition assays

Fagales distribution is characterized by a north-south gradient, at which, when focusing on Europe, trees belonging to the *Betuloideae* are mainly found in northern and central parts of

the continent, *Coryloideae* dominate the southern parts, while *Fagaceae* trees are distributed rather equally over Central and Southern Europe [15]. Thus, *Fagales* pollen allergic patients from three distinct areas (Vienna, Genoa, and Rome) with different *Fagales* pollen exposure pattern were selected for the study (Fig. 5a). Vienna represents a birch-dominated area, with moderate exposure to *Alnus*, *Coryloideae* as well as *Fagaceae* pollen. In Genoa, the allergenic pollen landscape in spring is dominated by alder, various *Coryloideae* species, including *Ostria* and *Fagaceae*, while *Betula* species play only a minor role [18]. Rome as third area can be regarded as birch pollen-free. The dominating *Fagales* pollen in Rome are pollen from *Quercus* species, followed not only by *Coryloideae* but also *Alnus* pollen. These different exposure patterns were reflected at the serological level of allergic individuals. In other words, some Italian sera showed higher IgE titres against Car b 1, or Que a 1 as compared to Bet v 1. To investigate this in detail, the whole panel of *Fagales* allergens was immobilized on ISAC microarray slides and inhibition studies were performed selecting one high IgE-binding candidate of the *Betuloideae* (Bet v 1.0101) and one of the *Coryloideae* (Ost c 1.0101) subfamilies (Figs 5b, c and d). For all the three populations, Bet v 1.0101 could fully inhibit IgE binding to members of the *Betuloideae*, however, IgE binding to the *Coryloideae* group was only inhibited by approximately 75%. Despite the fact that different batches of Bet v 1.0101 were used for immobilization on the ISAC chip and for inhibition studies, soluble Bet v 1.0101 inhibited IgE binding to immobilized Bet v 1.0101 to more than 99%. The IgE binding to allergens of the *Fagaceae* family could be inhibited by Bet v 1.0101 by 98% for Viennese patients, 95% for patients from Genoa and 88% for Roman patients, respectively. Inhibition studies performed with Ost c 1.0101 showed full inhibition of IgE binding to members of the *Coryloideae* subfamily, whereas IgE binding to *Betuloideae* was inhibited by 70% for Viennese patients, 83% for patients from Genoa and 84% for Roman patients. The IgE binding to *Fagaceae* was inhibited by 71% for Viennese patients, 64% for patients from Genoa and 67% for Roman patients.

Bet v 1 and Ost c 1 can only inhibit IgE binding to all pollen Bet v 1 homologues in a combinatorial mode

For ELISA inhibition assays, one patient cohort from Austria and one from Italy were selected. The whole panel of nine *Fagales* Bet v 1-like allergens was used for coating and inhibition studies were performed with Bet v 1.0101, Ost c 1.0101, Bet v 1.0101 plus Ost c 1.0101, or Fag s 1.0101 (Figs 6a and b). For the Austrian patients, full inhibition of IgE binding to *Betuloideae* and *Fagaceae* could be achieved with Bet v 1.0101, however, IgE binding to the *Coryloideae* sub-family was only inhibited by 70%. Using Ost c 1.0101 as inhibitor, the IgE binding to *Betuloideae* and *Fagaceae* was reduced by 77% and 70% respectively. Only the combination of both allergens resulted in an almost 100% inhibition of serum IgE binding to any of the *Fagales* allergens. The Fag s 1.0101 could merely inhibit IgE binding to coated Fag s 1.0101 to more than 99%. The picture was very similar for the Italian sera tested. The Bet v 1.0101 could inhibit IgE binding to *Betuloideae* and *Fagaceae* allergens by 95% and 90% respectively, and antibody binding to *Coryloideae* was reduced by 75%. The Ost c 1.0101 alone was only able to block IgE binding to *Coryloideae* allergens to more than 99%, however, the combination of both allergens resulted in over 95% inhibition of serum IgE binding to any of the *Fagales* allergens. Again, Fag s 1.0101 could

only inhibit IgE to coated Fag s 1.0101 to more than 98%, IgE inhibition to other allergens ranged from 45% for Car b 1.0101 to 89% for Que a 1.0301.

Discussion

In the late 1990s, after cloning and characterization of several allergenic Bet v 1 homologues from different *Fagales* species, the question regarding a simplification of *Fagales* allergy diagnosis and therapy emerged [26]. Since then, Bet v 1 was considered as the most potent sensitizer of *Fagales* pollen allergies, however, this view was challenged when more and more members of the *Coryloideae* allergens became available. By testing sera of *Fagales* allergic individuals living in birch-free areas, it could be demonstrated that these patients reacted strongly towards allergens they were actually exposed to (i.e. hornbeam Car b 1) than to Bet v 1 [12]. For the present study, the nine most important allergenic *Fagales* trees were selected, and their pollen major allergens, all belonging to the Bet v 1-family, were produced as recombinant proteins [27], including the herein newly cloned Ost c 1, representing the dominating exposure/sensitizer for one of the included study groups. The secondary structure elements of these molecules, including two isoforms of the major birch pollen allergen, and one additional isoform for every other *Fagales* pollen allergen, were analysed. The secondary structure elements of all these proteins were found to be very similar, thus being indicative for the high quality of the recombinant products. This was considered of high importance for further evaluation of IgE-binding properties of the molecules. First, the allergenic potential of the single molecules was evaluated in basophil mediator release assays. The strongest allergenicity was observed for Bet v 1.0101, Car b 1.0109 and Ost c 1.0101, followed by Bet v 1.0401, Aln g 1.0101 and Cor a 1.0104. The weakest allergenic molecules were identified within the *Fagaceae* family being a first indication that allergic reactions towards *Fagales* pollen could be initiated either by members of the *Betuloideae* or of the *Coryloideae* subfamilies. Previous studies showed similar allergenic activities of different *Fagales* pollen extracts in skin prick tests (i.e. birch, alder, hazel and hornbeam) [28]. However, due to varying allergen contents, extract-based data are difficult to standardize. In contrast, using molecule-based analyses allowed us to evaluate the allergenic activities of Bet v 1-related allergens in detail. In a next set of experiments, cross-reactivity was investigated by ISAC microarray inhibition assays as well as ELISA inhibition experiments. Immobilization of proteins to a solid phase can induce fold changes and thus, leading to alterations in IgE binding. Therefore, ISAC experiments were performed in combination with ELISA to cross-validate the data. In fact, the microarray results correlated significantly with the results obtained in ELISA experiments ($r=0.556$, $P<0.01$). To consider the different exposure patterns of allergic individuals, sera from Austrian as well as Italian patients were collected. According to environmental surveys and published data, Austria is dominated by birch, alder and oak trees. In Italy, hazel, hornbeam, oak and chestnut account for the majority of *Fagales* pollen, while birch seems virtually absent at least in the two areas considered in the present study [29]. Hop-hornbeam pollen exposure is the characteristic for the area of Genoa, Italy included in the present study. Using Bet v 1.0101 and Ost c 1.0101 as representative members of the *Betuloideae* and *Coryloideae*, respectively, full patients' serum IgE inhibition could be achieved with Bet v 1.0101 only within the *Betuloideae* subfamily. The Bet v 1.0101 also inhibited most of the IgE against

Fagaceae, whereas inhibition of IgE binding to *Coryloideae* was around 75%. Interestingly, the picture was inverted when investigating Ost c 1.0101. The molecules could block IgE binding to *Coryloideae*, however, only approximately 65–75% of serum IgE against *Betuloideae* and *Fagaceae* was inhibited. The Bet v 1.0101 in combination with Ost c 1.0101 led to full inhibition of IgE binding towards all nine tested molecules. The Fag s 1.0101, which was used as the strongest IgE-binding molecule of the *Fagaceae* group, could only block IgE towards immobilized Fag s 1.0101. Of note, this was independent of the geographical origin of the analysed serum. In the sera used for inhibition experiments, we were also able to detect allergen specific IgG antibodies, however, these antibodies could not block IgE binding to the immobilized allergens. Based on these data, we speculate that *Fagales* allergies are initiated by independent sensitization against members of the *Betuloideae* as well as *Coryloideae* subfamilies. The three-dimensional structures of Bet v 1-like allergens show great similarities [30, 31]; therefore, antibody cross-reactivity is obvious. This was also observed in the microarray as well as ELISA experiments. When looking at the IgE-binding data, there is clear evidence that 25% of the IgE epitopes of *Betuloideae* and *Coryloideae* allergens are unique for the respective subfamily, whereas epitopes of *Fagaceae* allergens appeared all cross-reactive. Thus, *Fagaceae* allergies are supposedly a result of cross-reactive antibodies primarily directed against allergenic members of the *Betulaceae* family, i.e. Bet v 1 and Ost c 1. For the present study, patients were selected based on positive IgE against Bet v 1. Using those sera, we intended to clarify the IgE epitope recognition on different Bet v 1-like molecules, and indeed, we were able to measure differences in IgE epitopes. Therefore, the study could serve as starting point for future developments in molecule-based immunotherapy. In conclusion, these data suggest that taking advantage of the microarray technology, where several molecules can be tested for IgE binding at once, Bet v 1-like allergy diagnosis should be based on a panel of related molecules. Hence, further studies on larger patient cohorts coming from more geographically distinct areas seem to be of highest interest. Highly homogeneous birch tree areas only exist in very northern parts of Europe, whereas a mix of *Fagales* species is present in large parts of Central and Southern Europe. On the basis of previous studies established with *Fagales* extracts, the question whether a panel of different *Fagales* allergens would truly improve the selection of the correct treatment option for patients remains unanswered. However, tests based on molecules and microtechnology will shed some light on this topic. In consequence, molecule-based IgE epitope profiling of Bet v 1-like allergens will be important for the development of future therapeutic products designed to treat *Fagales* multi-sensitized patients.

Acknowledgements

We thank Dr Christof Ebner (Allergieambulanz Reumannplatz, Vienna, Austria) for serum samples. Further, we thank the Christian Doppler Research Association, Biomay AG, Vienna, Austria, the Oesterreichische Nationalbank (Project Nr. 12533), the FWF – Fonds zur Förderung der wissenschaftlichen Forschung (L688) and the Italian Ministry of Health, Programma Ricerca Corrente 2007–2008, for financial support of this project.

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Fig. 1.

Schematic overview of the botanical order of *Fagales*. *Fagales* trees, which have been reported to be implicated with allergic diseases are indicated with *, allergenic trees with pollen allergens acknowledged by the WHO/IUIS allergen nomenclature subcommittee are indicated with **. The graph is adapted from Li et al. [32].

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Fig. 3.

One microgram of each of purified *Fagales* pollen allergens was separated by SDS-PAGE (15% gel) and stained with Coomassie Brilliant Blue (a). Protein identity was determined by mass spectrometric analysis of intact allergens (b). Circular dichroism spectra of *Fagales* allergens were recorded at 20°C. Spectra are baseline-corrected and presented as mean residue molar ellipticity (c). Secondary structure contents of *Fagales* allergens were calculated from baseline-corrected Fourier transformed infra-red spectra and are presented as % values (d).

Fig. 4.

The allergenic activity of *Fagales* pollen allergens was determined by basophil mediator release assays using *Fagales* pollen allergic patients' sera ($n = 8$) (a). *Betuloideae* allergens are presented as black symbols, *Coryloideae* allergens as grey and *Fagaceae* allergens as white symbols. The protein concentration to obtain half maximal mediator release was calculated. Normal distribution of the data was verified by Kolmogorov–Smirnov test (data not shown), and P -values were determined by paired samples t-test (b). P -values < 0.05 were considered statistically significant.

Fig. 5.

For ISAC inhibition studies, patients ($n = 15$) of three geographically distinct areas were recruited, a schema of seasonal *Fagales* pollen counts is provided for each area (a). *Corylaceae* include the genera *Corylus*, *Carpinus* and *Ostrya* [15]. Serum specific IgE-binding inhibition was performed with either Bet v 1.0101 or Ost c 1.0101 for the respective sera (b, c and d) and is presented as % values of total inhibition. Immobilized *Betuloideae* allergens are indicated in dark grey, *Coryloideae* allergens in grey and *Fagaceae* allergens in light grey.

Fig. 6.

ELISA inhibitions were performed with *Fagales* pollen allergic patients' sera from Vienna, Austria ($n = 5$) and Genoa, Italy ($n = 4$) using either Bet v 1.0101, Ost c 1.0101, Bet v 1.0101 plus Ost c 1.0101, or Fag s 1.0101 as inhibitor. Coated *Betuloideae* allergens are indicated in dark grey, *Coryloideae* allergens in grey and *Fagaceae* allergens in light grey. Data are presented as % values of total inhibition.

Table 1

Total IgE was measured by EIA (enzyme immunoassay) and is presented as kU/L, specific IgE antibodies were determined by ImmunoCAP testing (Phadia AB, Uppsala, Sweden) and are presented as IU/mL. SPTs (skin prick test) were performed with commercially available tree pollen extracts and are presented as mean diameter (mm)

Patient code	Country/region	Gender	Age	Total IgE	sIgE birch	sIgE hazel	SPT birch	SPT hazel	Symptoms	Month
AUSLZ 53770	Austria/Vienna	F	20	ND	>100	ND	ND	ND	ND	ND
AUSLZ 53771	Austria/Vienna	M	26	ND	>100	ND	ND	ND	ND	ND
AUSLZ 53839	Austria/Vienna	F	37	ND	>100	ND	ND	ND	ND	ND
AUSLZ 53840	Austria/Vienna	F	37	ND	>100	ND	ND	ND	ND	ND
AUSLZ 53841	Austria/Vienna	F	25	ND	>100	ND	ND	ND	ND	ND
ITAGE 40157	Italy/Genoa	F	48	157	29	30.7	5	5	R, A	2–4
ITAGE 40158	Italy/Genoa	F	57	280	>100	39	9	7	R	2–4
ITAGE 52737	Italy/Genoa	M	37	323	ND	ND	8	14	R	1–4
ITAGE 52743	Italy/Genoa	F	29	319	ND	ND	8	7	R, A	2–4
ITAGE 52747	Italy/Genoa	F	39	ND	ND	ND	10	8	R, A	3–4
ITARM 52447	Italy/Rome	M	22	257	29.6	ND	ND	ND	C, R	1–3
ITARM 52562	Italy/Rome	M	10	648	38.53	ND	ND	ND	C, R	1–3, 5–6
ITARM 52706	Italy/Rome	M	19	522	20.05	ND	ND	ND	R	5
ITARM 52980	Italy/Rome	M	20	124	10.56	ND	ND	ND	C, R	1–3, 4–5
ITARM 53140	Italy/Rome	M	13	759	65.6	ND	ND	ND	C, R	4–5
ITA 1	Italy/Genoa	M	64	263	35.4	59.5	8	8	ND	ND
ITA 2	Italy/Genoa	M	48	157	29	30.7	5	5	ND	ND
ITA 3	Italy/Genoa	F	57	280	>100	39	9	7	ND	ND
ITA 4	Italy/Genoa	F	65	107	24.4	34.7	3	6	ND	ND
ITA 5	Italy/Genoa	F	32	620	1.91	38.4	neg	6	ND	ND
AUT 1	Austria/Vienna	F	26	321	>100	ND	ND	ND	C, R	3–4
AUT 2	Austria/Vienna	F	33	392	>100	ND	ND	ND	R	3–4
AUT 3	Austria/Vienna	F	40	373	>100	ND	ND	ND	C, R	3–4
AUT 4	Austria/Vienna	F	19	1263	>100	ND	ND	ND	C, R, B	4
AUT 5	Austria/Vienna	M	68	377	>100	ND	ND	ND	C, R, B	3–4

ND, not determined; A, asthma; B, bronchitis; C, conjunctivitis; R, rhinitis; neg, negative.