

ORIGINAL ARTICLE

EXPERIMENTAL ALLERGY AND IMMUNOLOGY

An IgE epitope of Bet v 1 and fagales PR10 proteins as defined by a human monoclonal IgE

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To cite this article: Hecker J, Diethers A, Schulz D, Sabri A, Plum M, Michel Y, Mempel M, Ollert M, Jakob T, Blank S, Braren I, Spillner E. An IgE epitope of Bet v 1 and fagales PR10 proteins as defined by a human monoclonal IgE. *Allergy* 2012; DOI: 10.1111/all.12045.

Keywords

allergy; Bet v 1; cross-reactivity; IgE epitope; recombinant antibodies.

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Accepted for publication 31 August 2012

DOI:10.1111/all.12045

Edited by: Reto Crameri

Abstract

Background: Analyses of the molecular basis underlying allergenicity and allergen cross-reactivity, as well as improvement of allergy diagnostics and therapeutics, are hampered by the lack of human monoclonal IgE antibodies and knowledge about their epitopes. Here, we addressed the consecutive generation and epitope delineation of a human monoclonal IgE against the prototypic allergen Bet v 1.

Methods: Phage-display scFv hybrid libraries of allergic donor-derived VH epsilon and synthetic VL were established from 107 mononuclear cells. An obtained scFv was converted into human immunoglobulin formats including IgE. Using variants of Bet v 1, the epitope of the antibody was mapped and extrapolated to other PR10 proteins.

Results: The obtained antibodies exhibited pronounced reactivity with Bet v 1, but were not reactive with the homologous PR10 protein Mal d 1. The epitope as defined by the IgE paratope and a set of chimeric Bet v 1 fusion proteins and fragments could be assigned to a C-terminal helix-structured motif comprised by amino acid residues 132–154, including the critical residue E149. Grafting this motif re-established the reactivity of the per se nonreactive Mal d 1 framework. Cross-reactivities predicted by primary structure analyses of different isoforms and PR10 proteins were verified by allergen chip-based analyses.

Conclusions: The obtained results demonstrate that hybrid IgE repertoires represent a source for human antibodies with genuine paratopes. The IgE-derived information about the IgE epitope nature of Bet v 1 and homologues allows for detailed insights into molecular aspects of allergenicity and cross-reactivity within the PR10 protein family.

The development of several immune-mediated diseases is linked to elevated concentrations of IgE (1). In birch pollen allergy, up to 95% of tree pollen-sensitised patients in Northern Europe display IgE binding to the birch pollen allergen Bet v 1, and 60% react exclusively to this allergen (2). The prototype of the PR10 protein superfamily is also considered (3). Pollen of *fagales* species (4) and several foods (5–7) contain homologues sharing epitopes with Bet v 1, and cross-reactive IgE are fundamental for the oral allergy syndrome. Hence, Bet v 1 has been extensively characterised

and its crucial role in sensitisation renders Bet v 1 an outstanding target for development of therapeutic strategies (8).

Apart from phenomenological knowledge, molecular details about the interaction of IgE with allergens remain scarce and the nature of IgE epitopes is discussed controversially (9). The characterisation of allergen–IgE interactions, however, is unequally difficult due to the evanescent number of IgE-producing cells (10). The paucity of successful IgE isolations (11, 12) reflects the fact that an establishment of IgE repertoires is highly ambitious. Other inherent problems are

the vast predominance of IgG- and IgM-derived VL and the random combination of VH and VL, both resulting in shuffled, nonauthentic IgE repertoire libraries.

These difficulties emphasise a common pitfall in allergology. Even though structures of more than 50 allergens are available, the peculiarities of IgE epitopes largely remain enigmatic. In general, the basis of IgE reactivity of allergens can be addressed by a plethora of approaches based on specific antibodies (13, 14) or molecular epitope mimics (14, 15). The most sophisticated strategy relies on the determination of crystal structures of antibody–allergen complexes. The complexity of this approach, however, is impressively illustrated by the fact that only five crystallographic structures of allergen–antibody complexes have been solved so far. The murine nature of the IgG used in three of these complexes (16–18) allows for translational information only. Based on human IgE fragments, only two epitopes have been crystallographically assessed (19, 20). We recently dissected the epitope of a human IgE on Phl p 5a by a set of truncation and grafting experiments (21). For Bet v 1, structural (22) and immunological analyses (23, 24) illustrated the relevance of conformational epitopes. An IgE epitope dissection of Bet v 1 on a molecular level nevertheless remains to be done.

In this study, we report the establishment and use of human IgE with genuine VH paratopes to assign the corresponding IgE epitope on the surface of Bet v 1. Therefore, we generated IgE-derived hybrid libraries gaining access to allergen-specific antibodies and their epitope information. On the basis of a library-derived antibody, the corresponding IgE epitope was assigned to a terminal helical motif of Bet v 1. This motif is conserved throughout Bet v 1 isoforms and *fagales* PR10 proteins and even confers IgE reactivity to nonreactive PR10 proteins. This approach provides insights into IgE reactivity and cross-reactivity of PR10 proteins and may shed further light on the interaction with IgE on a molecular level.

Material and methods

To amplify the VH from gene-specific cDNA, total RNA prepared from human peripheral blood mononuclear cells of three allergic individuals (10^7 cells), an epsilon heavy chain-specific oligonucleotide for reverse transcription and

oligonucleotides covering the entire human VH repertoire were used. Cloning of the VH, establishment of hybrid libraries and selection is described in detail in the Supplementary data methods. Recombinant immunoglobulins were produced in mammalian cells using vectors previously established in our laboratory (25). Assessment of immunoreactivity of the particular proteins was performed by ELISA and immunoblotting. The ImmunoCAP ISAC allergen chip data were obtained according to the instructions of the manufacturer using IgE containing cellular supernatant. The different PR10 proteins, isoforms and mutants were assembled using oligonucleotides in PCR. Detailed descriptions of these procedures are given in the Supplementary data methods.

Standard procedures in protein biochemistry and molecular biology were performed according to the established protocols (26).

Results

Establishment and selection of the hybrid IgE repertoire libraries

The IgE VH repertoires of three allergic donors were amplified from cDNA, and the IgE background of individual VH was confirmed by sequence analysis (Supplementary data Fig. 1). These repertoires subsequently were combined with a human semi-synthetic VL repertoire (27). Resulting hybrid libraries of more than 10^7 independent clones for each donor were pooled for selection by phage display. After three rounds of iterative panning against Bet v 1, ELISA analyses demonstrated significant enrichment of the library (Fig. 1A, B), and reactive clones were subjected to sequence analysis (Supplementary data Fig. 2). Amplification of the VH of one clone within its epsilon context from the cDNA could be performed selectively for one of the three donors and verified the identity and isotype as well as the origin of the VH (Supplementary data Fig. 3).

After prokaryotic expression (Fig. 1C), the monovalent scFv demonstrated robust immunoreactivity (Fig. 1D). These data demonstrate that hybrid IgE repertoires allow for isolation of specific antibodies while circumventing donor-derived VL repertoires.

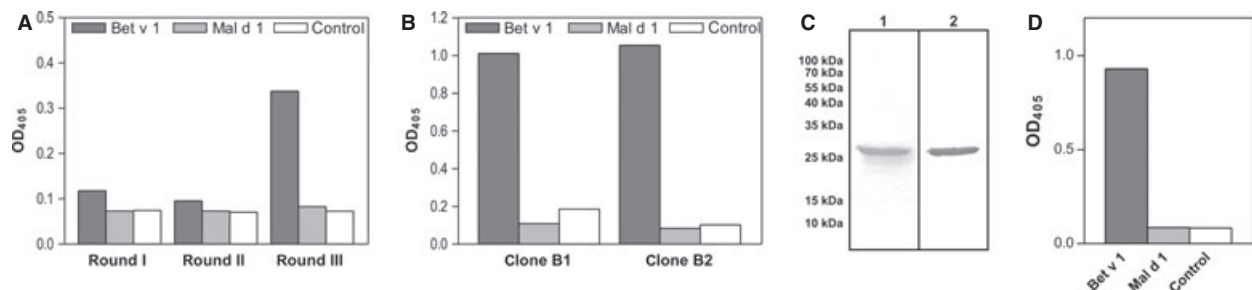


Figure 1 Immunoreactivity and expression of selected antibody fragments. The immunoreactivity of the enriched library (A) as well as selected phage clones (B) against Bet v 1 (filled bars, controls by omission of antigen: open bars) was analysed by ELISA as

described in methods. After expression, the soluble purified scFv was assessed by SDS–PAGE and immunoblot analysis (C1,2), and reactivity was verified by ELISA (D) using anti-myc antibody conjugated to alkaline phosphatase for detection.

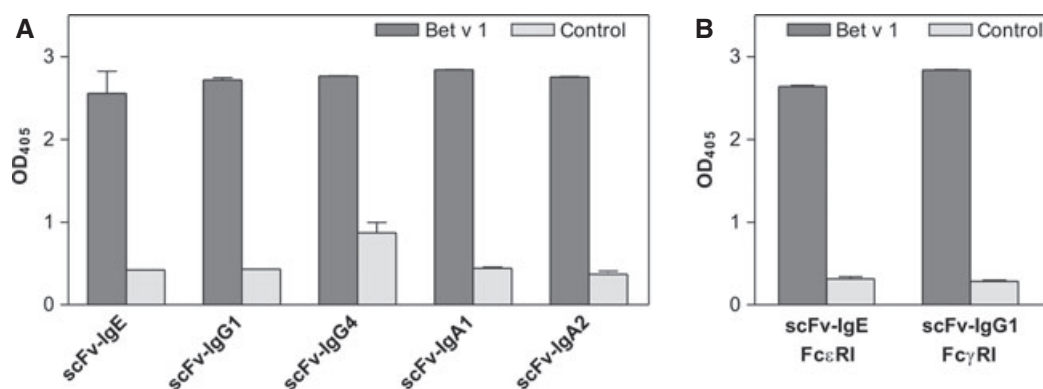


Figure 2 Immunoreactivity in ELISA. Immunoreactivity with allergens and Fc receptors: (A) The immunoreactivity of the different recombinant human antibodies was assessed by ELISA using MBP-Bet v 1- and isotype-specific antibodies conjugated to alkaline

phosphatase. (B) Simultaneous binding to the allergen and Fc receptors was performed as in A, but using the particular high-affinity Fc receptor and anti-chicken IgG conjugated to alkaline phosphatase for detection.

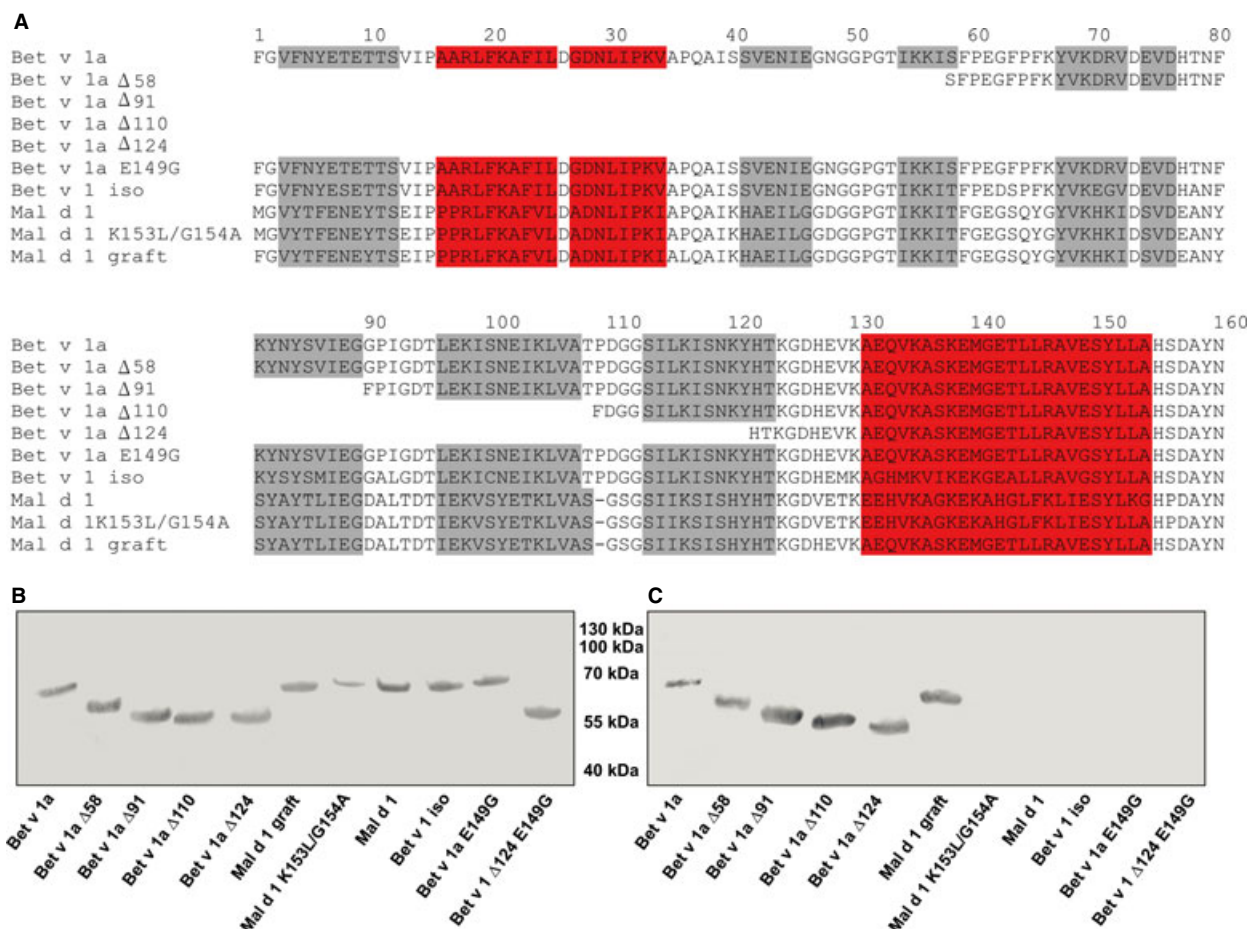


Figure 3 Epitope analyses using fragments and mutants of Bet v 1 in immunoblot. (A) Alignment of the Bet v 1a, isoforms, homologous and derived fragments developed using ClustalW2. Sequences forming predicted beta-sheets or helices are highlighted in grey and red, respectively. (B,C) Immunoreactivities of different

fusion proteins shown in A in immunoblotting using anti-fusion protein antibodies as loading controls (B), and the hIgE (C). Detection was performed using the anti-human IgE conjugated to alkaline phosphatase.

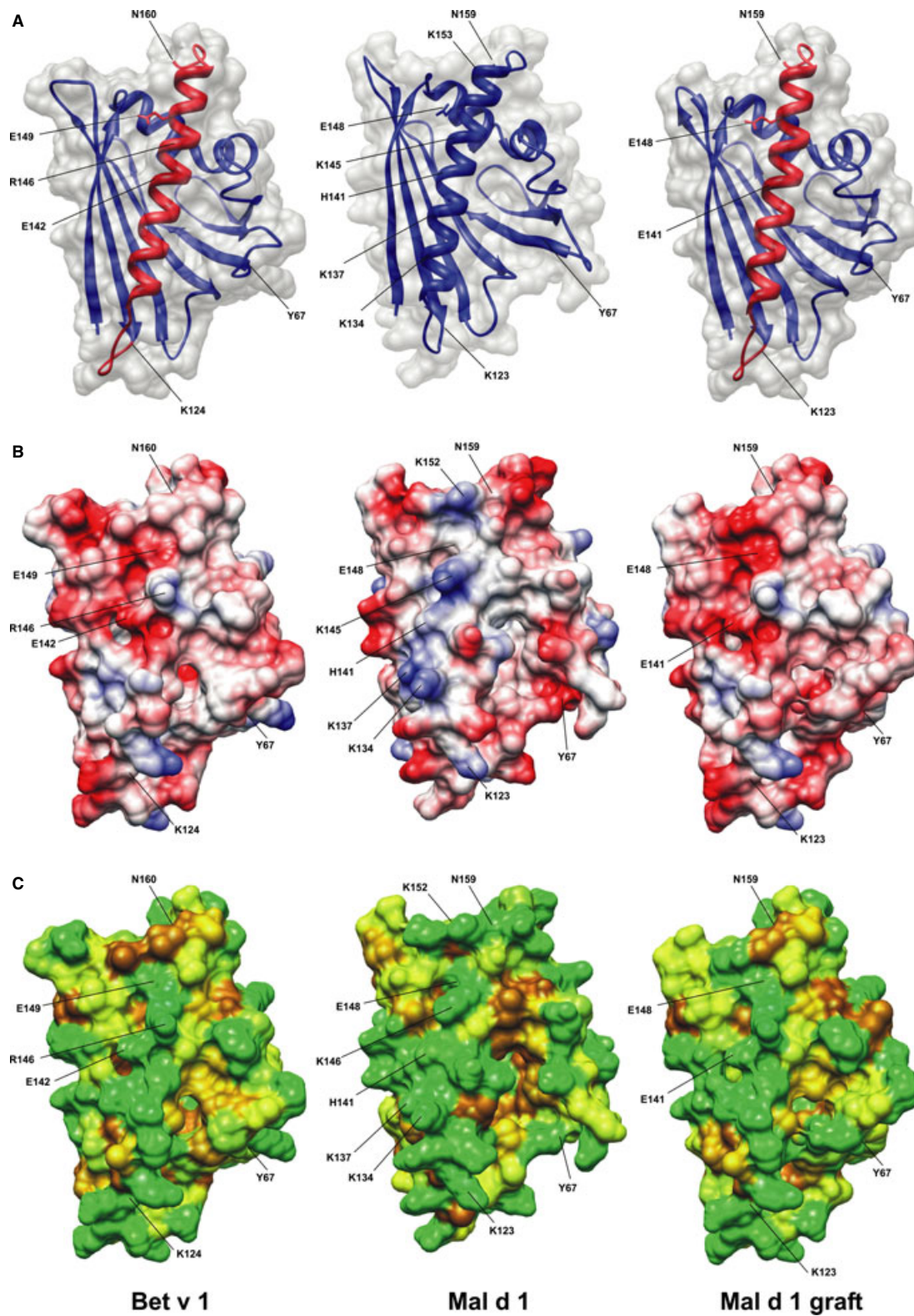


Figure 4 Structures of Bet v 1, Mal d 1 and the epitope grafted Mal d 1. Molecular modelling and IgE epitope mapping on the surface of Bet v 1 (left), Mald d 1 (middle), and the Mal d 1 graft (right). The modelling was performed using the structure of Bet v 1 as

template. Amino acids that correspond to the identified epitope stretch are highlighted in red. The secondary structures and the surface representations according to Coulomb potential (B) as well as hydrophobicity (C) visualise the variations of the molecular topology.

Generation and characterisation of recombinant immunoglobulins

For subsequent analyses, the antibody fragment was converted into homodimeric IgG1, IgG4, IgE, IgA1, and IgA2 immunoglobulin formats. After expression in mammalian cells, immunoblotting analyses (data not shown) verified the molecular masses suggesting proper folding and glycosylation, in particular for the extensively glycosylated IgE and IgA formats. All recombinant antibodies detected their particular target allergen in ELISA (Fig. 2A), commercial diagnostic laboratory analysers (Supplementary data Fig. 4A) and AlaBLOT strips containing a plethora of extracted antigens (Supplementary data Fig. 4B). Furthermore, the antibodies interact with their particular Fc receptors (Fig. 2B), suggesting applicability of the recombinant antibodies in diverse types of downstream approaches.

Epitope analysis of the Bet v 1-specific antibodies

To dissect the molecular interaction of the Bet v 1-specific IgE with its cognate target structure, we took advantage of different Bet v 1 isoforms and the PR10 allergen Mal d 1. Notably, Mal d 1 and a Bet v 1 isoform obtained from birch genomic DNA did not show any reactivity. Immunoblot analyses with N-terminally truncated Bet v 1 fragments and chimeras fused with MBP (Fig. 3A) demonstrated recombinant IgE reactivities with Bet v 1a and the fragments Δ58, Δ91, Δ110 and Δ124 (Fig. 3B). Fragment Δ124 comprises the C-terminal helix, which apparently is sufficient for IgE epitope establishment.

As compared to the nonreactive isoform, only the amino acid residue E149 is substituted. Introduction of the corresponding substitution E149G abolished the IgE reactivity of

the fragment Δ124 E149G, a finding also observed for full length Bet v 1 (Fig. 3C). This inducible loss of IgE reactivity in the fragment and the full length protein proves the critical relevance of this particular residue for the antibody–allergen interaction and strongly suggests that the terminal helix of Bet v 1 constitutes the epitope of the IgE.

To further elucidate the molecular basis of the selective specificity, we grafted the amino acids 128–156 of Bet v 1a into the per se nonreactive framework of Mal d 1. This grafting resulted in de novo establishment of pronounced IgE reactivity of Mal d 1 (Fig. 3C) again emphasising that the terminal helix is crucial for epitope formation.

By modelling approaches, we assessed the surface of Bet v 1, Mal d 1 and the epitope graft. Intriguingly, the residue E149 is conserved in Mal d 1 (E148), and folding of Bet v 1 and Mal d 1 is virtually identical (Fig. 4A). The Mal d 1 E148 nevertheless appears less accessible. Analyses of electrostatic potential and hydrophobicity revealed striking differences (Fig. 4B,C). As evident, the helix of Mal d 1 and a number of residues in proximity is dominated by positively charged residues (H142; K140; K135; K138; K153) forming a linear entity over the length of the molecule and rendering the surface significantly different from that of Bet v 1. K135 and K138 are also present in Bet v 1, but spatially separated by S137.

Epitope evaluation of Bet v 1 isoforms and other PR10 proteins

Primary structure analyses of different Bet v 1 isoforms and PR10 proteins were performed to evaluate the presence of the epitope motif. Expectedly, the 30 Bet v 1 isoforms available from databases show high sequence conservation within the last 37 amino acid residues (Supplementary data Fig. 5).

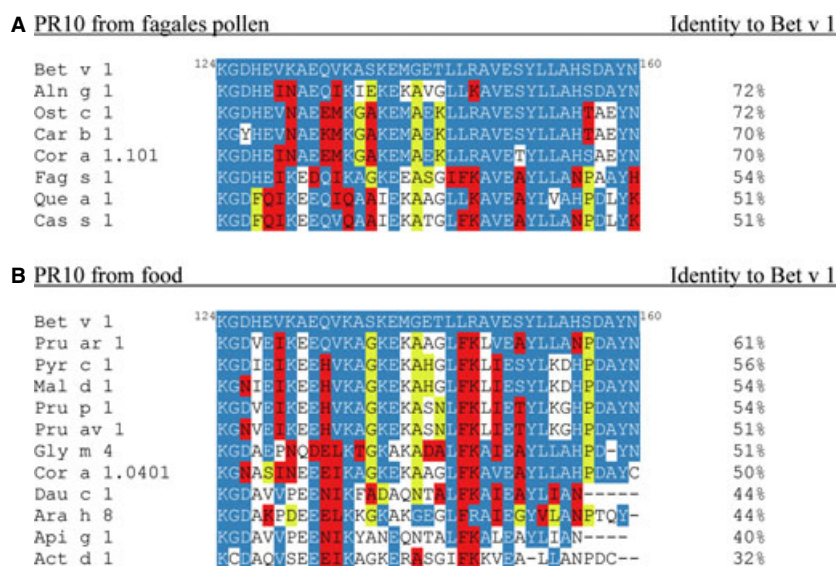


Figure 5 Alignment of PR10 protein C-termini from pollen and food. Alignment of *Fagales* (A) and food-derived (B) PR10 protein C-termini. Residues identical to Bet v 1 are highlighted in blue,

conservative amino acid substitutions in red and semi-conservative substitutions in yellow.

Notably, the residues 144–160 as well as the residues 125–135 show almost full identity in >95% of Bet v 1 isoforms. Non-conservatively replaced residues however include position 137 (S or I), 140 (K or M), and 141 (A, G, or R).

Whereas this motif appears well conserved throughout Bet v 1 isoforms, only particular homologous PR10 proteins show sequence identity rendering a formation of the epitope likely (Fig. 5). Roughly in accordance with overall sequence identities, the 37 C-terminal amino acids of Bet v 1 show an elevated identity to the *Fagales* PR10 proteins *Aln g 1* of 72% and to *Cor a 1.0101* and *Carb b 1* of 70%, to other members such as *Fag s 1* (54%), *Que a 1* (51%) and *Cas s 1* (51%) a more reduced identity (Fig. 5). In PR10 allergens from food, the epitope appears to be absent as terminal sequence identities are in the range of 41% (*Dau c 1*) to 56% (*Pyr c 1*) only (Fig. 5). The nut-specific isoform *Cor a 1.0401* notably exhibits an identity of 50%. Somewhat outstanding (61%) in this context is *Pru ar 1*. Interestingly, the only helix residues conserved throughout all proteins are the E149 and the Y151 pointing to a relevance for PR10 proteins.

To prove the validity of putative cross-reactivities derived from primary structure, the IgE was applied to a panel of PR10 proteins arrayed on the ImmunoCAP ISAC chip. In accordance with the sequence conservation, all three PR10 proteins from tree pollen available on the chip – Bet v 1, *Aln g 1*, and *Cor a 1.0101* – exhibited immunoreactivity (Supplementary data Fig. 6), in stark contrast to all food-derived PR10 proteins including the hazel nut isoform *Cor a 1.0401*.

Together, these findings demonstrate that amino acid substitutions throughout PR10 family members are crucial for IgE cross-reactivity.

Discussion

In this study, we report the isolation of human immunoglobulins with authentic VH paratopes and their subsequent use to assign an authentic IgE epitope on the surface of Bet v 1. Despite the broad knowledge about Bet v 1, potential ligands (28) and their relevance (29), its natural function remains diffuse. Accordingly, structural information including that of singular mutants, isoforms and homologues is available (30, 31) but the localisation of IgE epitopes has been primarily hypothesised based on indirect approaches.

To study human IgE epitopes, it is imperative to establish donor-derived IgE immune repertoires. This approach is labour intensive and inherently difficult for immunological reasons. Interestingly, different allergens seemingly bias the VH usage towards particular families (32). Additionally, the random *in vitro* combination of VH and VL and the high abundance of IgG- and IgM-producing cells favour the loss of the IgE-specific VL repertoire. Despite all limitations, sporadic studies underlining the potential of this approach have reported the generation of allergen-specific antibody fragments (11, 12, 19, 33–35).

We have therefore established hybrid repertoires, and the successful selection of which confirmed the feasibility of our approach. Our strategy might facilitate a broader analysis of IgE repertoires from different donors and characteristics.

With the VH CDR3 as specificity determining and most crucial element, the synthetic VL constitutes a suitable scaffold only. This strategy might favour the identification of paratopes with limited contribution of the light chain. This, however, would also apply for immune VL repertoires. The fact that we isolated VH without having a variety of VL suggests that this bias at least is not dominant.

The obtained antibody is the first Bet v 1-specific IgE of documented origin. The applicability of the set of antibody formats which also includes the novel IgA-based homodimeric format in different assay systems has been shown recently (21, 25).

On the basis of the Bet v 1-specific antibody paratope, we were able to describe the corresponding epitope on the surface of Bet v 1. To the best of our knowledge, this approach represents the first direct assignment of a human IgE epitope on Bet v 1.

Structural data revealed that Bet v 1 essentially consists of a seven-stranded antiparallel beta-sheet that wraps around a C-terminal alpha-helix and a pronounced cavity (22). The structural basis of IgE reactivity of Bet v 1 has been assessed by using monoclonal mouse or polyclonal human antibodies or peptide-specific hybridomas so far (36–38). By mimotope technology, peptide sequences could be assigned to a surface-exposed area (39, 40).

From the structural data, three surface-exposed conserved patches common to all *Fagales* tree pollen major allergens were identified, which might constitute a molecular basis of cross-reactivity (22). One of these surface patches represents the epitope of the murine antibody BV16 used to extrapolate obtained data to human IgE epitopes (17). Its epitope is comprised mainly of a peptidic stretch assembling to a conformational epitope with other proximal residues. Grafting of the BV16 epitope into the *Mal d 1* framework and mutational analyses were used to further define the molecular requirements of BV16 binding (41). The human IgE obtained in our study nevertheless shows in part comparable characteristics regarding reactivity to PR10 homologues and nature of the epitope.

For our epitope dissection, we took advantage of differential reactivities against Bet v 1 isoforms and *Mal d 1*. Truncation and mutagenesis approaches finally led to assignment of the terminal helix of Bet v 1 as IgE epitope and position E149 as relevant for interaction. Although primarily linear, this epitope apparently needs to be exposed in an appropriate context and is not in contrast to the tenet of predominantly conformational Bet v 1 epitopes primarily based on the use of peptides not embedded in any scaffold. Moreover, within the CDR3 specificity of the heavy chain, the light chain in the parental IgE may involve slightly different contact points. Albeit to our knowledge not postulated in the literature, hints for the C-terminal Bet v 1 epitope can be found. Best evidence was provided by the finding that two of three conserved patches common to *fagales* pollen major allergens (22) directly involve the C-terminal helix. Intriguingly, in this study even the residue E149 is highlighted as conserved in >95% of related protein sequences. Studies on T-cell responses also identified the C-terminal helix as

dominant T-cell epitope in Bet v 1 (23, 42) and other PR10 proteins.

A more general view on allergen cross-reactivity is further obtained in the line of the epitope highly conserved in PR10 proteins from tree pollen, but less in homologues from food with lower sequence identity. Although the data obtained by analysing the panel of PR10 proteins arrayed on the allergen chip ISAC represent not an absolute and quantitative measure, the reactivity landscape supports the predictions based on sequence information. The on/off in reactivity is impressively exemplified in the Cor a 1 isoforms from pollen and food.

In summary, we introduced hybrid IgE repertoire libraries for the isolation of allergen-specific antibody fragments. Library-derived immunoglobulins represent attractive tools for elucidation of the interplay of allergens with the immune response. The identification of an IgE epitope of Bet v 1 provides a basis for understanding isoform and allergen cross-reactivity and may open novel prospects and strategies towards generation of hypoallergenic variants.

Acknowledgments

We would like to thank G. Winter, MRC Cambridge, for providing the synthetic scFv library.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Statement of contribution

JH, DS, AD and AS did the cloning, selection and interaction analyses; MP and YM did expression analyses of immunoglobulins; MM and MO isolated patient PBMC; TJ performed the allergen array analyses; SB did the modelling work; IB and ES designed the study, analysed the data and wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Sequence analyses of IgE-derived VH after cloning. The N-terminal residues of the CH1 verify the IgE background of the obtained sequences.

Figure S2. Sequence analyses of Bet v 1-specific scFv. Numbering and determination of the CDR was performed according to Kabat (21). Alignments to V-Base (MRC, Cambridge) revealed usage of a VH3 and a VL1 segment.

Figure S3. Amplification of the VH from cDNA of three individual donors was performed using oligonucleotides specific for the CH1 region of IgE at the 3' end and a framework 3 specific oligonucleotide (A) and a framework 1 specific oligonucleotide (B) at the 5' end.

Figure S4. Diagnostic application of recombinant IgE. (A) Immunoreactivity in diagnostic routine approaches using the laboratory analysers ImmunoCAP 250 and Immulite 2000 was assessed by applying the recombinant IgE to immobilized birch pollen extract (T3) or Bet v 1 (T215). (B) Immunoreactivity in immunoblot-based allergen detection was assessed by applying the recombinant IgE and human serum from a pollen allergic donor to AlaBLOTs according to the manufacturer's recommendations. Detection was performed with anti human IgE conjugated to alkaline phosphatase.

Figure S5. Alignment of the 37 amino acid residue C-termini of Bet v 1 isoforms (position 124–160) as available from the IUIS allergen database (excluding the highly variant isoform 01301).

Figure S6. rIgE reactivity of PR10 proteins from pollen and food arrayed on the ISAC allergen chip.

Data S1. Methods.

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Supplemental data Fig. 1: Sequence analyses of IgE-derived VH after cloning. The N-terminal residues of the CH1 verify the IgE background of the obtained sequences.

[illegible]

Supplemental data Fig. 2: Sequence analyses of Bet v 1-specific scFv

Numbering and determination of the CDR was performed according to Kabat (21).

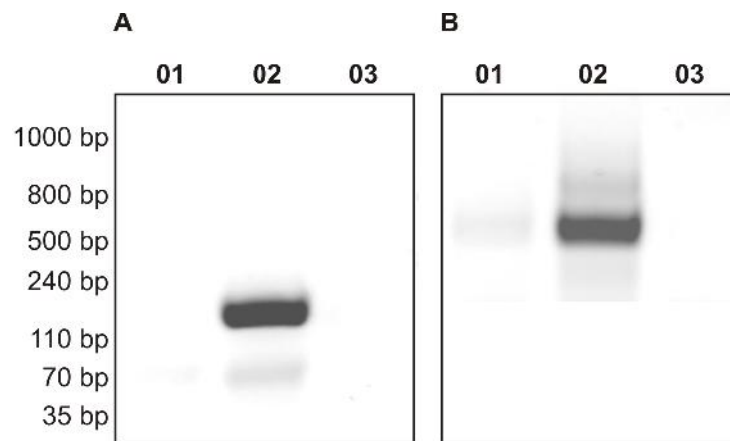
Alignments to V-Base (MRC, Cambridge) revealed usage of a VH3 and a VL1 segment.

```
VH
1      10      20      30      40      50      60      70      80      90      100     110
B1 EVQLVESGGGLVQPGRSLRLSCAASGFTFSSYGMHWVRQAPGKGLEWVAVISYDGSNKYYADSVKGRFTISRDN SKNMLHLQMNSLRAEDTAVYYCAKFGTQSR RFD PWGQGTPVTV
B2 EVQLVESGGGVVQPGRSLRLSCAASGFTFSSYGMHWVRQAPGKGLEWVAVISYDGSNKYYADSVKGRFTISRDN SKNTLYLQMNS.LREDTAVYYCAKFGTQSRKFDFW GQGTPVTV
      CDRH1      CDRH2      CDRH3

linker
SSGGGSGGGGSGGSAL

VL
1      10      20      30      40      50      60      70      80      90      100
B1 QSVLTQPPS.ASGTPGQRVTISCSGSSN.IGSNYVYVYQQLPGTAPKLLIYRNNORPSGVPDRFSGSKSGTSASLAISGLRSEDEADYYCAAWDDSLVL.DVFGGGTKLTVLG
B2 QSVLTQPPS.ASGTPGQRVTISCSGSSN.IGSNYVYVYQQLPGTAPKLLIYRNNORPSGVPDRFSGSKSGTSASLAISGLRSEDEADYYCAAWDDSLGTLFLVFGGGTKLTVLG
      CDRL1      CDRL2      CDRL3
```

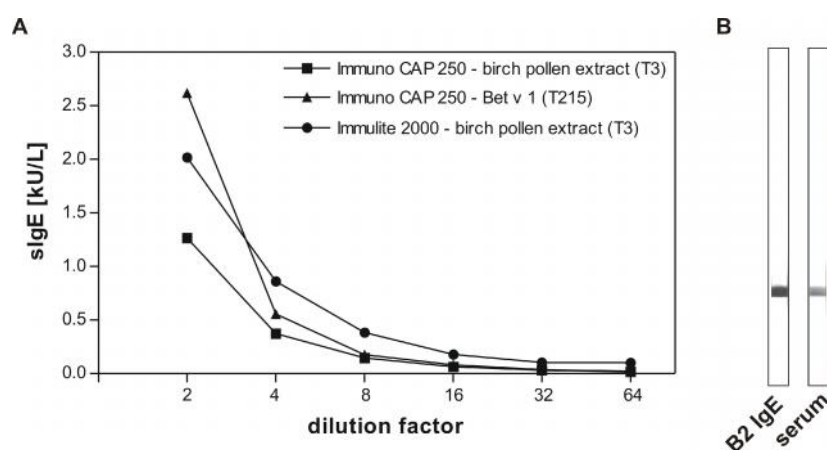
Supplemental data Fig. 3: Amplification of the VH from cDNA of three individual donors was performed using oligonucleotides specific for the CH1 region of IgE at the 3' end and a framework 3 specific oligonucleotide (A) and a framework 1 specific oligonucleotide (B) at the 5' end.



Supplemental data Fig. 4: Diagnostic application of recombinant IgE

A: Immunoreactivity in diagnostic routine approaches using the laboratory analysers ImmunoCAP 250 and Immulite 2000 was assessed by applying the recombinant IgE to immobilized birch pollen extract (T3) or Bet v 1 (T215).

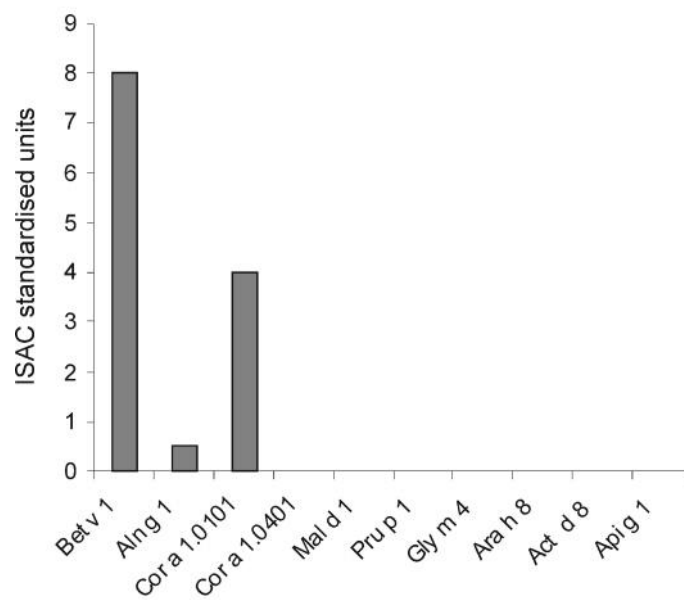
B: Immunoreactivity in immunoblot-based allergen detection was assessed by applying the recombinant IgE and human serum from a pollen allergic donor to AlaBLOTs according to the manufacturer's recommendations. Detection was performed with anti human IgE conjugated to alkaline phosphatase.



Supplemental data Fig. 5: Alignment of the 37 amino acid residue C-termini of Bet v 1 isoforms (position 124-160) as available from the IUIS allergen database (excluding the highly variant isoform 01301).

Bet v 1.0101	KGDHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.0102	KGDHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.0103	KGDHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.0201	KGDHEMKAEHMKAIKEKGEALLRAVESYLLAHSDAYN
Bet v 1.0301	KGDQEMKAEHMKAIKEKGEALLRAVESYLLAHSDAYN
Bet v 1.0401	KGNHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.0402	KGNHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.0501	KGNHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.0601	KGDHEVKAEQIKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.0602	KGDHEVKAEQIKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.0701	KGNHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.0801	KGDHEVKAEQIKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.0901	KGDHEMKAEHMKAIKEKGEALLRAVESYLLAHSDAYN
Bet v 1.1001	KGNHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.1101	KGNHEMKAQIKASKEKAEALFRAVESYLLAHSDAYN
Bet v 1.1201	KGNHEMKAQIKASKEKAEALFRAVESYLLAHSDAYN
Bet v 1.1401	KGDHEMKAEHMKAIKEKGEALLRAVESYLLAHSDAYN
Bet v 1.1402	KGDHEMKAEHMKAIKEKGEALLRAVESYLLAHSDAYN
Bet v 1.1501	KGDHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.1502	KGDHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.1601	KGDHEINAEQIKIEKEKAEGLLKAVESYLLAHSDAYN
Bet v 1.1701	KGDHEVKAEQIKASKEMGETLLRAVERYLLAHSDAYN
Bet v 1.1801	KGDHEMKAEHMKAIKEKAEALLRAVESYLLAHSDAYN
Bet v 1.1901	KGDHEMKAEHMKAIKEKGEALLRAVESYLLAHSDAYN
Bet v 1.2001	KGDHEMKAEHMKAIKEKGEALLRAVESYLLAHSDAYN
Bet v 1.2101	KGDHEINAEQIKIEKEKAVGLLKAVESYLLAHSDAYN
Bet v 1.2201	KGDHEVKAEQIKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.2301	KGDHEVKEEQIKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.2401	KGNHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.2501	KGDHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.2601	KGNHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.2701	KGNHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.2801	KGDHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.2901	KGDHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1.3001	KGDHEVKAEQVKASKEMRETLLRAVESYLLAHSDAYN
	::*: *::* ** *::* * *****

Supplemental data Fig. 6: rIgE reactivity of PR10 proteins from pollen and food arrayed on the ISAC allergen chip.



Supplemental data Methods:

Reconversion into bivalent antibody formats and expression in mammalian cells

Recombinant immunoglobulins were produced using vector systems previously established in our lab (22). The antigen-binding regions were amplified using oligonucleotides containing restriction sites at the 5'- and 3'-termini of the VH (gatcatttaaattgtgtccagtgtgaggtgcagctgggtgg) and VL (gatcgcgatcgcaaccgaggacgggtcagct) by PCR, respectively. Subsequently, the DNA was introduced into the vectors for expression of the particular immunoglobulin isotypes.

Human embryonic kidney cells (HEK-293, ATCC number CRL-1573) were cultivated in Dulbecco's modified Eagle medium (DMEM) supplemented with 100 mL/L fetal calf serum, 10 kIU/L penicillin, and 100 mg/L streptomycin. Tissue culture reagents were obtained from Life technologies. HEK-293 cells were transfected by using 2 µg of the particular expression vector DNA complexed with polyethylene imine (Sigma Aldrich).

Assessment of immunoreactivity in ELISA and immunoblotting

For assessment of immunoreactivity in direct ELISA the particular proteins (10 µg/ml diluted with 20 g/L MPBS) were applied to microtiter plates coated with particular antigens at 4 °C overnight and blocked with 40 g/L MPBS at RT for 1 h. Thereafter, the recombinant immunoglobulins (approx. 1 µg/ml diluted with 20 g/L MPBS) were added to the wells and incubated for 1 h at RT. The ELISA was performed according to established protocols and bound antibodies were detected using human Ig isotype specific antibodies conjugates and para-nitrophenyl phosphate (pNPP) as a substrate at 405 nm. The use of the soluble FcεRI-IgY Fc and FcγRI-IgY Fc in ELISA has been described elsewhere (1, 2). Briefly, allergen and immunoglobulins were applied as above. Bound antibodies then were detected using soluble FcεRI-IgY Fc and FcγRI-IgY Fc (approx. 1 µg/ml diluted with 20 g/L MPBS) and chicken IgG specific antibody antibody conjugate.

For detection of native allergen in immunoblotting procedures the recombinant IgE antibody was diluted using DMEM supplemented with 100 mL/L heat-inactivated fetal calf serum and applied at 1 µg/ml to AlaBLOTs (Siemens Healthcare Diagnostics), providing native allergens after separation by SDS-PAGE and transfer onto nitrocellulose membranes. Visualization of IgE was performed using anti-human IgE conjugated to alkaline phosphatase and nitrotetrazolium blue chloride/5-bromo-4-chloro-3-indoyl phosphate (NBT/BCIP) as substrate. Reactivity with recombinant Bet v 1 isoforms and derived variants in immunoblot was performed after separation of proteins by SDS-PAGE. Recombinant IgE antibodies were applied and visualised as above.

rIgE reactivity of PR10 proteins arrayed on the ISAC allergen chip

The ISAC allergen chip data were obtained according to instructions of the manufacturer using rIgE containing cellular supernatant adjusted to a 200 ng/ml instead of human serum.

Generation of hybrid IgE repertoires

PBMC derived mRNA was prepared from the Trizol-solubilized samples after extraction with chloroform and precipitation with ice-cold isopropanol. cDNA was prepared by using Superscript III reverse transcriptase (Life Technologies) and constant region specific oligonucleotides. PCR was carried out using Pfx DNA Polymerase (Life Technologies) in combination with primers annealing to the 5'-end of the different IGHV subgroups and within the part of the IGHE gene encoding the constant domains of IgE (gtcaaggggaagacggatgggct). PCR-products were cloned and independent colonies subjected to sequencing.

Subsequently, VH-encoding genes were amplified from the PCR product using the 5' primers as above and the 3' primers annealing to the IGHJ genes according to VBASE and carrying restriction sites for SfiI and XhoI. The PCR-products then were used to replace the VH of the synthetic scFv-library Girffin 1 via SfiI and XhoI. Ligated DNA was electroporated

into *E. coli* TG1. Sequencing of randomly picked clones confirmed that the library carried members with a different genetic background.

Phage display selection of repertoire libraries

The selection of repertoires was essentially performed according to established protocols (3). Briefly, immunotubes coated with the particular allergen and blocked with 20 g/L MPBS were incubated with a total volume of 4 mL of 20 g/L MPBS containing 10^{12} to 10^{13} phages for 90 min under continuous rotation at room temperature. The immunotubes were then washed, 1 mL of 100 mmol/L triethylamine was added to elute the bound phages, and neutralization was performed by addition of 0.5 mL of 1 mol/L Tris-HCl, pH 7.4. After reinfection of *Escherichia coli* TG1 and overnight growth on agar plates, the phages were rescued by growth of scraped cells in liquid culture, infection with M13KO7 helper phage, and another period of overnight growth. Phages were then subjected to the next round of selection. Immunoreactivity of polyclonal or monoclonal phages was assessed in ELISA with anti-M13-horseradish peroxidase conjugate (GE Healthcare) and 2,2-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) di-ammonium salt for detection.

Construction of homology models

Homology models of Mal d 1 and grafted Mal d 1 were performed applying the SWISS-MODEL protein modeling server (4-6) and as a template the solved structures of Bet v 1 (PDB entry 1bv1) (7) and of the major cherry allergen Pru av 1 (PDB entry 1e09) (8), respectively. The quality of the models was confirmed by QMEAN4 score (Mal d 1: 0,62; grafted Mal d 1: 0,69) which is a combination of 4 statistical potential terms (9). Hydrophobicity and Coulombic surface coloring were performed using UCSF Chimera (10).

Recombinant production of Bet v 1 isoforms and derived variants

The PR10 protein Bet v 1a was amplified from cDNA using the sense oligonucleotide gatcgaattcgggtgtatttaattatgagactgagaccacc and the antisense oligonucleotide gatcaagctttcaatggtgatgggtggatggtgtaggcacagagtgtg providing an Eco RI and a HindIII restriction site. The truncated fragments $\Delta 58$, $\Delta 91$, $\Delta 110$, and $\Delta 124$ were produced using the sense oligonucleotides gatcgaattcagctttcccagggtcccttcaagtacgtg, gatcgaattcccataggcgacacattggagaag, gatcgaattcgatggaggatccatcttgaagatcagcaac, gatcgaattcaaaggcgaccatgaggtgaaggcag, respectively. The Bet v 1 isoform was obtained from genomic DNA by use of the oligonucleotides gatcgaattcgggtgttttaattacgagagtgagac and gatcaagctttcaatggtgatgggtggatggtgtaggcacagagtgtg.

Site directed mutagenesis of full length protein was performed using the antisense oligonucleotide atcggagtgtgccaagaggtagctccaac in combination with the Bet v 1a oligonucleotides mentioned above. On the basis of this template the corresponding fragment Bet v 1a $\Delta 124$ E 149G was generated as the $\Delta 124$ fragment.

The PR10 homologue from apple Mal d 1 was amplified from cDNA using the sense oligonucleotide gatcgaattcgggtgtctacacattgagaacgagtac and the antisense oligonucleotide gatccgtcgactcagtgatgggtggatggtggtgtatgcgtcgggggtg providing an Eco RI and a HindIII restriction site. Epitope grafting into the C-terminus of Mal d 1 was performed using the antisense oligonucleotide ctttacttgccttaacctgctctgccttcacctcatggtcgcctttgggtg in combination with the Bet v 1a antisense and the Mal d 1 sense oligonucleotides mentioned above. On the basis of this template the corresponding mutant Mal L153/A154 was generated using the antisense oligonucleotide gatccgtcgactcagtgatgggtggatggtggtgtatgcgtcgggggtgtgcaaaaggtagc. The amplicons were inserted into the prokaryotic expression vector pMal-2cX (NEB) and expressed according to the manufacturers' recommendations.

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