

Short communication

Identification of B-cell epitopes of Bet v 1 involved in cross-reactivity with food allergens

Background: The pollen-food syndrome (PFS) is an association of food allergies to fruits, nuts, and vegetables in patients with pollen allergy. Mal d 1, the major apple allergen, is one of the most commonly associated food allergens for birch pollen-allergic patients suffering from PFS. Although the reactions are due to cross-reactive IgE antibodies originally raised against pollen Bet v 1, not every Bet v 1-allergic patient develops clinical reactions towards apple.

Aim of the study: We speculate that distinct IgE epitopes are responsible for the clinical manifestation of PFS. To test this hypothesis we grafted five Mal d 1 stretches onto Bet v 1. The grafted regions were 7- or 8-amino acids long encompassing amino acids residues previously shown to be crucial for IgE recognition of Bet v 1.

Methods: A Bet v 1-Mal d 1 chimeric protein designated BMC was expressed in *Escherichia coli* and purified to homogeneity. IgE reactivity of BMC was tested with patients' sera originating from (i) Bet v 1-allergic patients displaying no clinical symptoms upon ingestion of apples; and (ii) Bet v 1-allergic patients displaying allergic symptoms upon ingestion of apples and other Bet v 1-related foods.

Results and conclusion: Compared to birch pollen-allergic individuals, patients suffering from PFS showed significantly higher IgE reactivity with BMC (chimeric protein). The results suggest that the Mal d 1 regions grafted onto the Bet v 1 sequence comprise important IgE epitopes recognized by Bet v 1-allergic patients suffering from allergy to apples.

**E. Klinglmayr¹, M. Hauser¹,
F. Zimmermann¹, O. Dissertori²,
P. Lackner², N. Wopfner¹, F. Ferreira¹,
M. Wallner¹**

¹Christian Doppler Laboratory for Allergy Diagnosis and Therapy, Department of Molecular Biology, University of Salzburg, Salzburg, Austria;

²Department of Molecular Biology, University of Salzburg, Salzburg, Austria

Key words: Bet v 1 allergen; birch pollen allergy; IgE cross-reactivity; Mal d 1 allergen; pollen-food syndrome.

Dr Michael Wallner
Christian Doppler Laboratory of Allergy Diagnosis and Therapy
Department of Molecular Biology
University of Salzburg
Hellbrunnerstr. 34
A-5020 Salzburg
Austria

Accepted for publication 30 May 2008

Worldwide, 10–20 million people suffer from pollen allergies and among these patients, approximately one-third exhibits allergic reactions towards tree pollen (1). In the temperate climate zone, Bet v 1, the major birch pollen allergen, accounts for most cases of tree pollinosis. Additionally between 50% and 93% of birch pollen-allergic individuals develop hypersensitivity reactions towards certain foods (e.g. apples, carrots, hazelnut, celery), which is mediated by cross-reactive IgE antibodies primarily directed against Bet v 1 (2). This type of hypersensitivity is described as pollen-food syndrome (PFS) (3). Thereby allergen contact with the oral mucosa causes immediate adverse reactions usually restricted to the oral cavity and the pharynx. In case of birch pollen allergy, one of the most frequently implicated allergen with PFS is Mal d 1, the major apple allergen. Mal d 1 shares 64% amino acid sequence similarity with Bet v 1 and cross-inhibition experiments demonstrated the presence of common IgE epitopes (4, 5). Still, there is some discrepancy between serological cross-reactivity between the two allergens and the onset of clinical symptoms caused by the ingestion of Bet v 1-related foods. Indeed,

IgE antibodies of most Bet v 1 allergic patients can bind Mal d 1 regardless of clinical reactivity with apples. Thus, the aim of the present study was to investigate cross-reactive IgE epitopes involved in the clinical manifestation of PFS. Therefore, we generated a chimeric protein (termed BMC) by grafting four short peptide stretches of Mal d 1 onto the Bet v 1 sequence. The transplanted regions were 7-amino acids long encompassing residues previously shown to be crucial for patients' IgE binding towards Bet v 1 as well as Mal d 1 (6, 7). The influence of epitope grafting on IgE binding activity was assessed by ELISA. Our preliminary findings are discussed in the context of IgE cross-reactivity and its clinical significance.

Methods

Patients' sera

Birch pollen-allergic patients with PFS ($n = 11$) and without ($n = 12$) were selected based on typical case history, positive *in vivo*

Table 1. Primer list

Primer	Sequence
Mut10F	5'CGCCATTGTTTCAATTACGAAATGAGttCACCTCTGagATC3'
Mut30F	5'GATGGCGATAATCTCaTTCCAAAGaTTGCACCCCAa3'
Mut30R	5'ATGGCTTGGGGTGCAAtCTTTGGAAtGAGATTATCG3'
Mut57F	5'ATTAAGAAGATCAcCTTTggCGAAGGCTTC3'
Mut57R	5'GAAGCCTTCGccAAAGgTGATCTTCTTAAT3'
Mut112/113F	5'GCAACCCCTagTGGAAgCaCCATCaaGAgTATCAGCAAC3'
Mut112/113R	5'GTTGCTGATacTctGATGGtgCtTCCActAGGGGTTGC 3'
Mut125F	5'CACACCAAAGGTaACatTGAGaTcAAGGCAGAGCAG3'
Mut125R	5'CTGCTCTGCCTTgAtCTCAatGTtACCTTTGGTGTG3'
BetF	5'GGCCCATATGGGTGTTTCAATTACGAA3'
BetR	5'TCGGCTCGAGGTTGTAGGCATCGGAGTG3'

Primers used for construction of Bet v 1.0101-Mal d 1.0108 chimeric proteins. Exchanged bases are shown in lower case letters; restriction sites are underlined.

skin prick test and *in vitro* IgE detection (ImmunoCAP system, Phadia AB, Uppsala, Sweden). Correlation between clinical symptoms and relative IgE binding to Bet v 1 and Mal d 1 was confirmed for both groups.

Cloning of BMC

Chimeric allergens were generated by PCR amplification of mutated fragments of Bet v 1.0101 (X15877). The mutated fragments were created using internal mismatch primers (Table 1). After PCR amplification, DNA fragments were gel-purified, pooled and assembled in a primerless PCR. Full-length cDNAs were amplified with the primers BetF and BetR (Table 1). BMC was cloned into a pHis-Parallel2 vector using *Nde*I and *Xho*I restriction sites (8).

Expression and purification of BMC

Expression plasmids were transformed in *Escherichia coli* BL21 (DE3) pLysS cells (Stratagene, La Jolla, CA, USA) and grown at 37°C to and OD₆₀₀ of 0.8 in LB medium supplemented with 100 mg/l ampicillin. Cultures were cooled to 16°C and protein expression was induced by addition of 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After incubation for 18 h, cells were harvested by low speed centrifugation and resuspended in appropriate lysis buffer. BMC was expressed 6xHis tagged fusion protein and purified from soluble bacterial lysates by immobilized metal affinity chromatography (8). Recombinant proteins were dialyzed against 10 mM sodium phosphate buffer, pH 7.4 and stored at -20°C.

SDS-PAGE and immunoblots

Escherichia coli lysates and purified proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 15% gels. Proteins were visualized by staining with Coomassie Brilliant Blue R-250 (BioRad, Hercules, CA, USA). For immunoblot analysis, proteins separated by SDS-PAGE were electroblotted onto nitrocellulose membrane (Whatman, Brentford, UK). Proteins were detected using the monoclonal anti Bet v 1 antibody BIP-1 (dilution 1 : 10.000) (9). Bound BIP-1 was detected with an AP-conjugated rabbit anti mouse IgG + IgM (Jackson ImmunoResearch Laboratories Inc., Suffolk, UK).

Circular dichroism

Circular dichroism (CD) spectra of proteins were recorded in 5 mM sodium phosphate pH 7.4 with a JASCO J-810 spectropolarimeter (Jasco, Tokyo, Japan) fitted with a Neslab RTE-111M temperature control system (Thermo Fischer Scientific Inc., Waltham, MA, USA). Obtained curves were baseline-corrected. Results are presented as mean residue molar ellipticity [Θ]_{MRW} at a given wavelength.

Molecular modeling

Modeling was performed using the comparative modeling tool MODELLER and evaluated by ProSa2003. All models are based on the PDB structure file 1BV1 (Bet v 1) and figures were prepared using PyMOL 0.98.

ELISA experiments

Maxisorp plates (NUNC, Wiesbaden, Germany) were coated with 50 µl well of allergen titrations (8, 4, 2, 1, 0.5 µg/ml) in PBS, overnight at 4°C to determine optimal coating conditions. Plates were blocked with TBS, pH 7.4, 0.05% (v/v) Tween, 1% (w/v) BSA and incubated with patients' sera diluted 1 : 5 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 1 h at 4°C. 10 mM 4-Nitrophenyl phosphate (Sigma-Aldrich, St Louis, MO, USA) was used as substrate and OD was measured at 405/492 nm. All measurements were performed as triplicates. Results are presented as mean OD values.

Results

Generation of BMC

The aim of the present study was the identification of cross-reactive B cell epitopes correlating with clinical manifestation of Bet v 1-related PFS towards apple. Therefore, mutant allergens were designed by transplanting distinct epitopes of Mal d 1.0108 at corresponding positions of Bet v 1.0101 (Fig. 1). The grafted regions were defined by adding 3 amino acids from Mal d 1 at the N- and C-terminal flanking regions of 6 residues (Thr10, Phe30, Ser57, Ser112/Ile113 and Asp125 in the Bet v 1.0101 sequence), which have been previously identified as crucial for IgE recognition of Bet v 1 and homologues (Fig. 1) (6, 7). Four of these five chimeric proteins (BM10, BM30, BM57 and BM125) were still able to bind patients' serum IgE. However, the chimera BM112/113 displayed no IgE binding activity. So far, only conformational IgE epitopes have been identified on Bet v 1 as well as Mal d 1, therefore, the loss of IgE reactivity of BM112/113 was most likely a result of an alternative fold (7, 10). Thus, this molecule was not further analyzed. Next, we generated a chimeric protein (designated BMC) by combining the mutations of the BM10, BM30, BM57, and BM125 constructs.

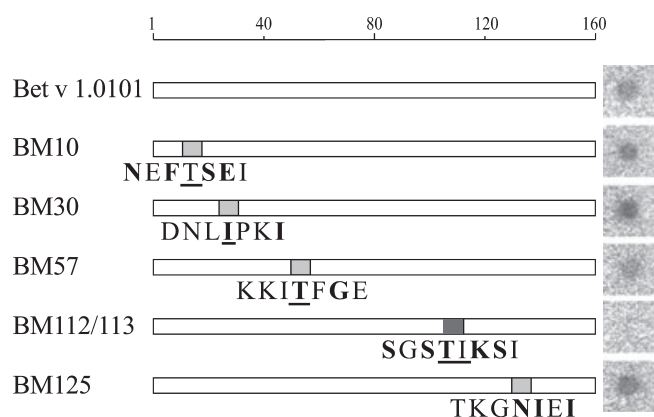


Figure 1. Schematic representation of chimeric allergens. Chimeras were constructed by grafting epitopes of Mal d 1.0108 onto the Bet v 1.0101 template. Stretches matching amino acids which compose the exchanged epitopes are listed in one-letter code. Amino acid residues differing between Bet v 1.0101 and Mal d 1.0108 are in bold, residues previously identified as crucial for IgE binding are underlined. IgE reactivity of the respective proteins was analyzed in a dot blot using a serum pool of birch pollen allergic patients ($n = 11$).

Recombinant production and characterization of BMC

The three-dimensional fold of BMC was first evaluated by calculating a molecular model, which was then compared to the 3-D structure of Bet v 1. The model showed the same conserved shape as the template allergen. All four mutated epitopes were exposed on the protein surface and therefore, available for antibody binding (Fig. 2B). The calculated mutant allergen was cloned, expressed in *E. coli* as a 6xHis tagged fusion protein, and purified to homogeneity. The folding of BMC was analyzed by far UV CD spectroscopy using purified Bet v 1.0101 as reference. The overlay of both spectra indicated almost identical secondary structures (Fig. 2C). Further evidence of similar folding was provided by antibody binding of a monoclonal anti Bet v 1 antibody, which was equivalent for both proteins (Fig. 3A). Together with the data collected from immunoblot experiments using the single mutants, this led to the conclusion that BMC shows the typical Bet v 1-like fold. These results are in agreement with the *in silico* experiments.

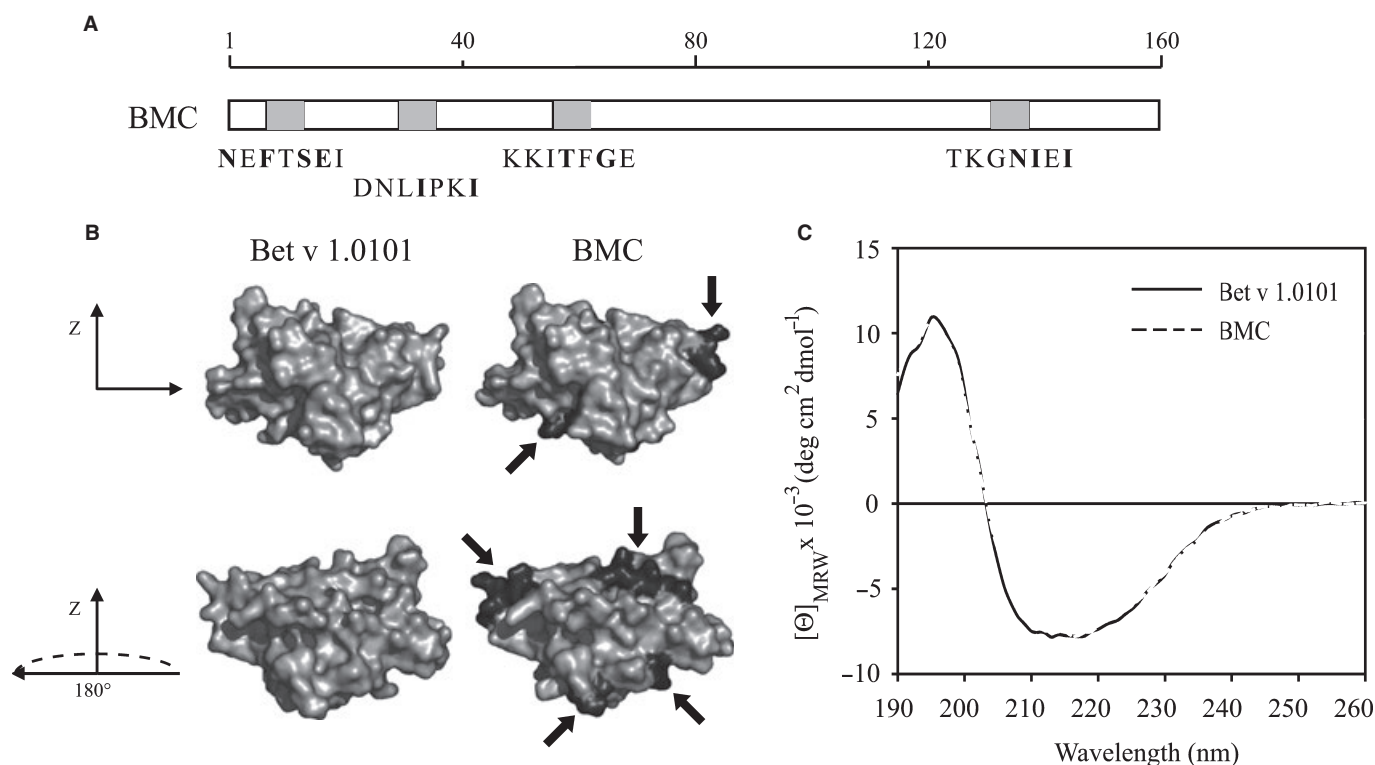


Figure 2. (A) Schematic representation of BMC. The chimeric protein was constructed by combinatorial grafting of four distinct epitopes of Mal d 1.0108 onto the Bet v 1.0101 template. Stretches matching amino acids of Bet v 1.0101 are shown in white, epitopes inserted from Mal d 1.0108 in grey. Amino acids composing the exchanged epitopes are listed in one-letter code. Amino acid residues differing between Bet v 1.0101 and Mal d 1.0108 are in bold. (B) A model of BMC was calculated and the structure was compared to the three-dimensional fold of Bet v 1. Residues identical in BMC and Bet v 1.0101 are shown in grey, epitopes derived from Mal d 1.0108 are presented in black and marked with arrows. The lower panel shows the proteins rotated by 180° around the Z-axis. (C) Secondary structure of BMC was compared to Bet v 1.0101 by circular dichroism (CD). Data is baseline-corrected and presented as mean residue molar ellipticity $[\Theta]_{MRW}$ at a given wavelength.

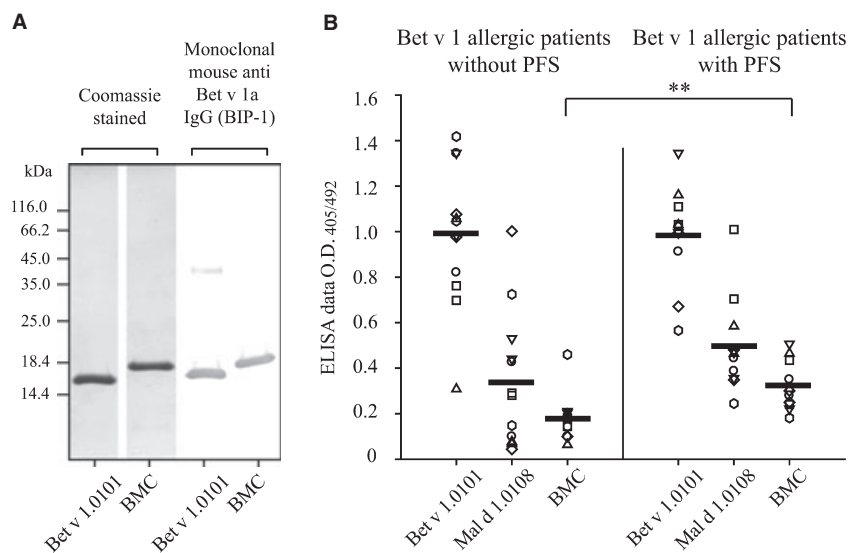


Figure 3. (A) SDS-PAGE and immunoblot analysis of affinity purified BMC (each 5 μ g per lane). Following SDS-PAGE, proteins were visualized by Coomassie Brilliant Blue staining (left panel). For immunoblot analysis, proteins were detected using a monoclonal mouse anti-Bet v 1 antibody. (B) In ELISA, IgE binding activity of Bet v 1.0101, Mal d 1.0108 and BMC was assessed using sera of Bet v 1 allergic patients without clinical symptoms of PFS ($n = 12$) or Bet v 1 allergic patients suffering from PFS to apple and other Bet v 1-related foods ($n = 11$). Proteins were coated in concentration of 2 μ g/ml. Symbols represent individual patients, bars means. P -values were calculated by t -test (** $P < 0.01$).

IgE binding properties of BMC

To investigate the IgE binding activity of BMC, ELISA experiments were performed using sera from 2 groups of patients:

1. Bet v 1-allergic patients without PFS;
2. Bet v 1-allergic patients showing PFS symptoms following apple ingestion.

The allergens Bet v 1.0101 and Mal d 1.0108 were used as reference. No significant differences of patients' IgE binding towards Bet v 1 ($P > 0.99$) was observed between the two groups. IgE binding to Mal d 1.0108 was slightly higher for the PFS group compared to the non-PFS patients. However, IgE binding to BMC was significantly lower in the non-PFS group compared to patients with PFS ($P < 0.01$), suggesting that the grafted epitopes are involved in the Bet v 1/Mal d 1 IgE cross-reactivity (Fig. 3B).

Discussion

In order to investigate antibody cross-reactivity between Bet v 1 and Mal d 1 allergens, we generated five chimeric proteins by grafting short Mal d 1 sequences onto corresponding regions of the Bet v 1 backbone. Analysis of these proteins by immunoblot allowed the identification of four epitopes on Bet v 1.0101, which could directly influence IgE binding to cross-reactive food allergens without destroying the Bet v 1-like fold of the mutated

allergen. These four mutations were combined on the protein BMC, which should serve as tool for further investigation of the distinct epitopes. The protein was heterologously expressed in *E. coli* and purified to homogeneity. Since IgE epitopes of Bet v 1 are conformation-dependent (7, 10), a Bet v 1-like structure of the BMC was considered crucial for proper interpretation of the results. Circular dichroism analysis showed that the BMC chimera is a folded molecule with secondary structural elements comparable to those of wild type Bet v 1. Further, molecular modeling indicated that all grafted epitopes of BMC are exposed on the protein surface and therefore can directly influence antibody binding to the protein. In this context, Bet v 1-allergic patients with no PFS showed marked differences in serum IgE reactivity to BMC when compared to Bet v 1-allergic patients with clinical reactivity to apples. Interestingly, when investigating IgE binding to the different allergens we found that both groups of patients reacted with Mal d 1, though one group did not show clinical symptoms following consumption of fresh apples. We speculate that differences in IgE affinities or avidity of PFS patients might lead to the clinical manifestation of the apple allergy.

ELISA experiments demonstrated that BMC binds more efficiently serum IgE antibodies from patients suffering PFS than IgEs from individuals without PFS. These preliminary results suggest an involvement of the grafted epitopes in the birch PFS. We plan to further investigate the role of the distinct epitopes by grafting single epitopes or epitope combinations of Bet v 1 or Mal

d 1 on structurally related non-allergenic scaffolds. This will also allow us to investigate whether more amino acids than the ones explored in this study are necessary to form the core region of an IgE epitope of Bet v 1 or Mal d 1, respectively. Further, by testing several of these chimeras we will be able to compile IgE epitope maps for individual patients, which will help understanding the antibody-based mechanisms underlying birch pollen PFS.

Acknowledgments

We thank Dr. Thomas Hawranek (Department of Dermatology, Paracelsus Private Medical University, Salzburg, Austria) for serum samples. Further, we would like to thank the Christian Doppler Research Association and the Oesterreichische Nationalbank (Project Nr. 12533) for financial support of this project.

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