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Molecular basis of allergic cross-reactivity between group 1 major allergens from birch and apple

J. Holm[a], G. Bærentzen[a,1], M. Gajhede[b], H. Ipsen[a], J.N. Larsen[a], H. Løwenstein[a], M. Wissenbach[a,1], M.D. Spangfort[a,*]

[a]*Biochemical Allergy Research, ALK-Abelló A/S, Bøge Allé 6–8, DK-2970 Hørsholm, Denmark*
[b]*Protein Structure Group, Department of Chemistry, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen, Denmark*

Abstract

Patients allergic to birch pollen often also react with fruits and vegetables, such as apple. The major cause of cross-reactivity between birch and apple is biochemical and immunological similarity between the major allergens, Bet v 1 and Mal d 1, as demonstrated by serological and cellular immunoassays. In addition, birch pollen-specific therapeutic allergy vaccination has been shown to improve allergic symptoms caused by oral ingestion of apple. Detailed analysis of molecular surface areas based on the crystal structure of Bet v 1, and primary sequence alignment, identify potential epitopes for cross-reactive antibodies. Two or more conserved patches are identified when comparing Bet v 1 and Mal d 1, thus providing a molecular model for serological cross-reactivity involving more than one IgE-binding epitope. A minimum of two epitopes would be necessary for cross-linking of receptor bound IgE in functional histamine release assays and skin test. Individual amino acid substitutions, as occurring in isoallergenic variation, may, however, have a dramatic effect on epitope integrity if critical residues are affected. Thus, one area large enough to accommodate antibody-binding epitopes shared by all known Mal d 1 isoallergens and variants is identified, as well as areas shared by Bet v 1 and individual Mal d 1 isoallergens or variants. The occurrence of limited epitope coincidence between Bet v 1 and Mal d 1 is in agreement with the observation that some, but not all, birch pollen allergic patients react with apple, and that the epitope repertoire recognised by the IgE of the individual patients determines the degree of cross-reactivity. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Food allergy; Cross-reactivity; Birch; Apple

1. Introduction

The observation of an increased frequency of clinical reaction to oral ingestion of various fruits and vegetables, such as apple, among allergic individuals sensitised to birch pollen is well established [1,2]. Cross-reactivity between birch pollen and apple has been demonstrated using allergen extracts in RAST inhibition [3,4], as well as immunoblotting [5], and has been found to involve primarily the group 1 component [6,7]. Cloning and expression of the gene encoding the major allergen from apple, Mal d 1, and analysis of the recombinant protein has confirmed serological cross-reactivity [8,9].

In addition to the serological studies, cross-reactivity has also been reported with respect to allergen-specific T helper cells [10], and finally, the positive

*Corresponding author. Tel.: +45-45-748-156; fax: +45-45-748-942.

E-mail address: msp@dk.alk-abello.com (M.D. Spangfort).

[1]Present address: Pantheco A/S, Symbion Science Park, Fruebjergvej 3, DK-2100 Copenhagen, Denmark.

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clinical effect of birch pollen-specific allergy vaccination on apple sensitivity [11] is a strong indication of immunological relationship.

The resolution of the three-dimensional structure of the major birch allergen, Bet v 1 [12], enables a more detailed molecular analysis of cross-reactivity. The 3-D structure immediately identifies amino acid residues exposed to the solvent, and thus accessible for antibody binding. Combining this information with amino acids conserved among homologous major allergens, identified by primary sequence alignment, reveals areas on the molecular surface that are topographically similar, and, thus, can be assumed to bind cross-reactive antibodies with similar affinities.

Using this approach to analyse the major allergens in the tree pollen allergen family, including alder, birch, hazel and hornbeam, identified three such similar surface areas [12]. These so-called 'conserved patches' not only provide a molecular explanation of cross-reactivity, they are also likely to represent dominating IgE-binding epitopes, since exposure to these epitopes occurs over an extended time period including pollination seasons of not only birch, but also hazel and alder.

In this study the occurrence of surface areas shared by the major allergens of birch and apple, Bet v 1 and Mal d 1, was investigated using the three-dimensional structure model of Bet v 1, primary sequence alignment, and immunochemical methods.

2. Materials and methods

2.1. Cloning of Mal d 1 genes

RNA was purified from a freshly ground Cox Orange apple using a commercially available kit (Qiagen) following the recommendations of the supplier. cDNA was synthesised for 1 h at 42°C using 400 units M-MLV reverse transcriptase, 16 units RNAsin, 5 μ*M* oligo-$(dT)_{15}$ primer and 5–10 μg total RNA in 40 μl standard PCR buffer [13]. Standard PCR was performed immediately following first-strand cDNA synthesis, in 0.1 ml, i.e., 30 cycles of 1 min at 94°C, 1 min at 47°C, and 1 min at 72°C, completed by 10 min at 72°C, using the following degenerate primers: promoter proximal primer 5′-ATG GGT GTY TWC AMW TWY GA, promoter distal primer 5′-TTA GTT GTA KGC RTC SGR GTG. The PCR product was cloned by T/A cloning in pCR 2.1 (Invitrogen) and positive candidate clones were sequenced using a commercial kit (Perkin-Elmer) following the recommendations of the supplier.

2.2. Subcloning of the Mal d 1 genes

Two genes tentatively named *Mal d1* (2619) and *Mal d 1* (2620) were selected for this study, amplified by PCR using primers containing restriction sites for directional cloning (*Kpn*I and *Xba*I), and subcloned into pMAL-c (New England Biolabs). The *Mal d 1* genes were positioned in fusion with the *malE* gene encoding the amino-terminal fusion partner 'maltose binding protein' separated by a factor X_a protease recognition site. Plasmid clones were transformed into *Escherichia coli* strain DH5α [14]. Full sequence was obtained on both DNA strands of all clones following subcloning.

2.3. Expression and purification of rMal d 1

Expression and purification of rMal d 1 was performed using the protocol developed for rBet v 1 [15]. In brief, *E. coli* strain DH5α containing recombinant plasmids was grown in rich medium supplemented with ampicillin, induced and harvested. Cells were broken by ultrasonic treatment and soluble lysates were applied on amylose columns for affinity purification of recombinant fusion protein, which was eluted and cleaved using factor X_a protease. rMal d 1 was finally purified by size exclusion chromatography. The authentic amino-terminal was confirmed by amino-terminal protein sequencing.

2.4. Crossed immunoelectrophoresis

Crossed immunoelectrophoresis was performed as described [16] using a rabbit monospecific polyclonal antibody preparation raised against natural Bet v 1 purified from pollen. The CIE was performed with 1.3 μg rBet v 1.2801 and 1.0 μg each of the rMal d 1 isoallergens using the same concentration of antibodies for all plates.

2.5. SDS–PAGE immunoblotting

SDS–PAGE and immunoblotting was performed as described [17]. One μg of each allergen preparation was separated in the gel, electroblotted to a nitrocellulose membrane, incubated with murine monoclonal antibody BV16, further incubated with biotinylated rabbit anti-mouse Ig (DAKO) and finally developed with alkaline phosphatase-conjugated streptavidin.

2.6. Sequence alignment

Alignment of the sequences of Bet v 1.2801, Mal d 1 (2619) and Mal d 1 (2620) was combined with 15 distinct Mal d 1 sequences exhibiting from 57 to 66% sequence identity retrieved at the Swiss Institute of Bioinformatics (SIB) (http://www.expasy.ch/) using the BLAST network service. Substitutions of amino acids within the following groups were considered conservative: Val–Leu–Ile–Met, Ser–Thr–Cys, Phe–Tyr–Trp, Asp–Glu, Asn–Gln and Lys–Arg–His.

2.7. Molecular surface illustrations

The pdb coordinates (http://www.rcsb.org/pdb/, ID=1BV1) of the three-dimensional structure model of Bet v 1.2801 [12] was used as basis for the depiction of the water accessible surface using WebLab viewerPro ver. 3.5 (www.msi.com). α-Carbon backbone atoms are colored red, side chain atoms of residues identical to Bet v 1.2801 are colored orange, side chain atoms of residues conservatively substituted are colored blue and side chains of all unrelated residues are represented by white color.

3. Results and discussion

RNA was purified from a single Cox Orange apple. Several non-identical Mal d 1 encoding clones were obtained, thus confirming the presence of isoallergenic variation. The expression and purification protocol developed for rBet v 1 [15] was applied in rMal d 1 expression experiments yielding similar results. Net yield after purification, however, was only 2.3 mg/l of culture medium due to inefficient cleavage of the fusion protein.

Deduced amino acid sequences of Mal d 1 may be divided in two groups based on sequence similarity. Fig. 1 shows a sequence alignment comparing Mal d 1 sequences with Bet v 1.2801, with known tertiary structure. One group of sequences has identities with Bet v 1.2801 exceeding 62% ($n=4$), and one group of sequences has identity percentages in the interval 56–58% ($n=13$). The relative high degree of identity/homology between the sequences indicate that the overall folding pattern of Mal d 1 is similar to Bet v 1 [18]. The conservation of the P-loop motif (–G–X–G–G–X–G–), also found in Bet v 1.2801 at position 46–51 [12], in all Mal d 1 sequences but one also indicates that Mal d 1 may have a biological function similar to that of Bet v 1, which unfortunately at present is unknown.

Combining the sequence alignment (Fig. 1) with the three-dimensional structure model of Bet v 1.2801 enables the identification of conserved surface areas, that represent potential epitopes for cross-reacting antibodies (Fig. 2). Not all birch pollen allergic individuals sensitised to Bet v 1 react clinically with apple, it is, thus, anticipated that the sequence differences observed are reflected in a limited area of conserved surface, potentially representing epitopes for cross-reacting IgE. Fig. 2 illustrates the water accessible surface area(s) that are conserved between Bet v 1.2801 and Mal d 1 isoallergens. The relative maximum size of an epitope is indicated on Fig. 2, Panel I.B, showing the residues involved in the binding of the murine monoclonal antibody BV16 identified by X-ray crystallographic analysis of the crystallised Fab-Bet v 1 complex [19]. It should be emphasised that by this methodology all amino acids that are within a distance favourable for binding in the complex are identified. The crystal state is a static condition, and this experimental set-up does, therefore, not address whether or not these amino acids are engaged in binding in the dynamic state in solution. Therefore, the area depicted in Fig. 2, Panel I.B, is the maximum extension of the epitope. Dynamic binding studies in solution are likely to identify a much smaller area as critical for binding.

All the Mal d 1 isoallergen sequences contain amino acid substitutions within the BV16 epitope

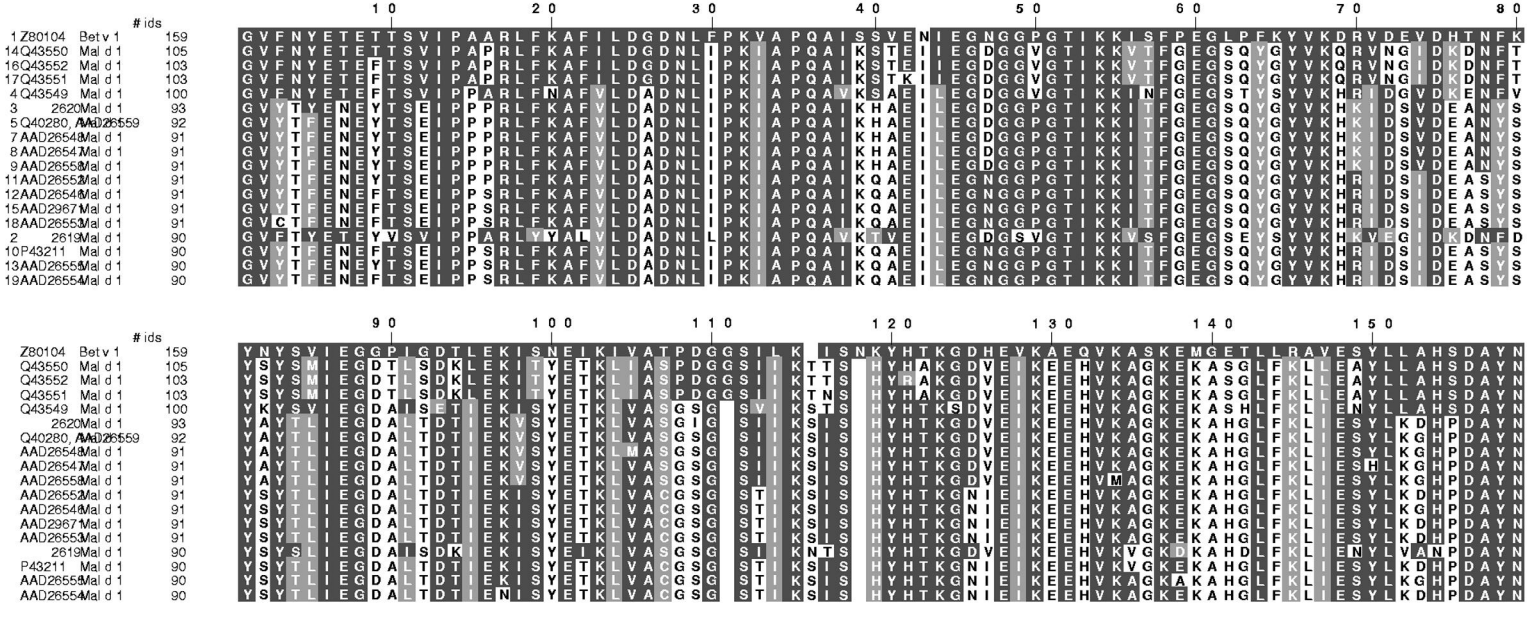
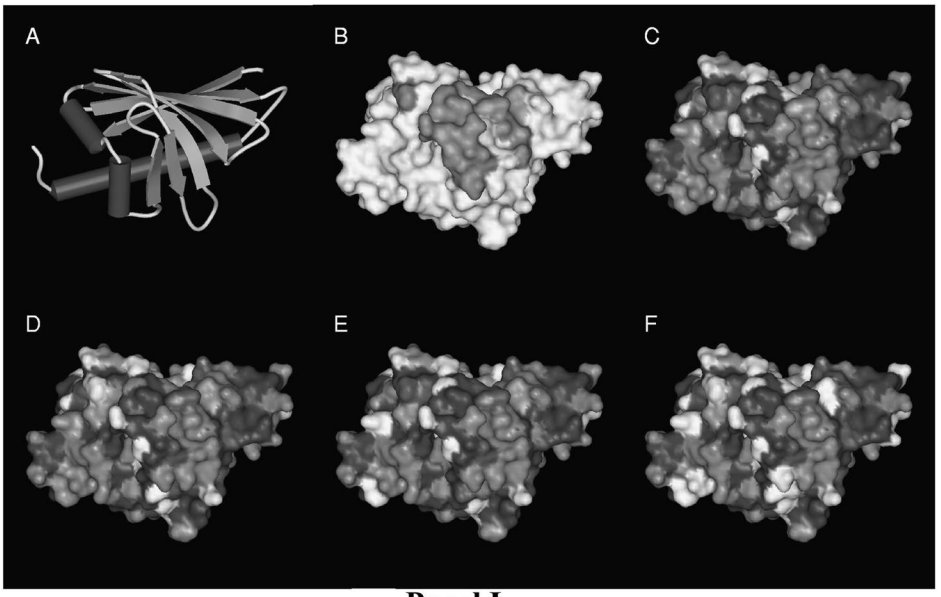
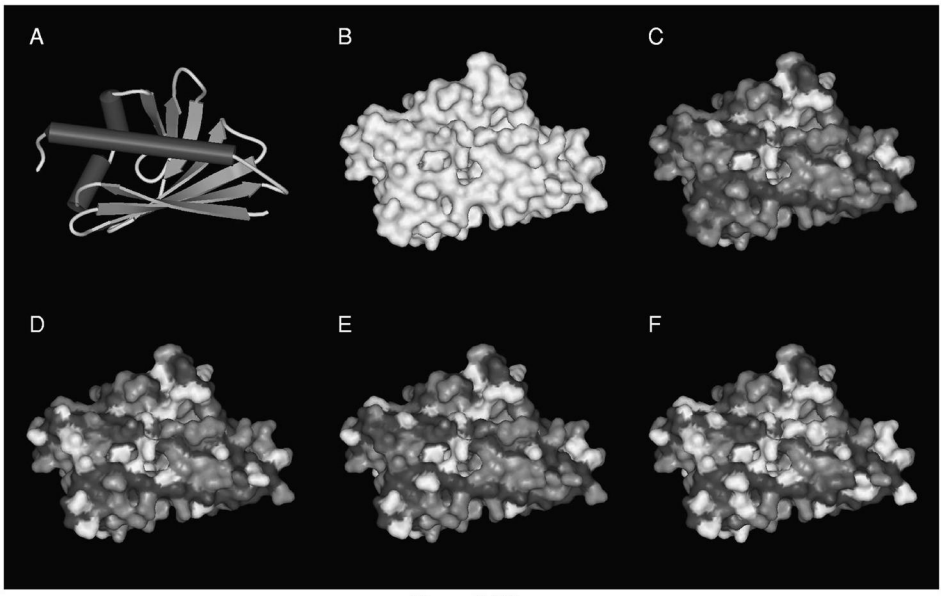


Fig. 1. Sequence alignment. Amino acid sequence of Bet v 1.2801 compared to Mal d 1 isoallergens. Individual amino acid residues identical to Bet v 1.2801 are shown on a red background, conservative substitutions (V–I–L–M, F–Y–W, S–T–C, E–D, N–Q, K–R–H) are shown on cyan background. The number of amino acid residues identical to Bet v 1.2801 is listed between the database accession numbers and the individual amino acid sequences.



Panel I



Panel II

Fig. 2. Surface similarity. Panels I and II: the water accessible surface of Bet v 1.2801 coloured to illustrate the degree of conserved surface area. The α-carbon backbone atoms (always identical) are represented in red, side chains of identical amino acid residues in orange, side chains of conservatively substituted amino acids in blue and non-related amino acids in white. Panel I.A: orientation of secondary structure elements in the angle of view chosen. Panel I.B: amino acid side chains (green) with the potential to participate in the interaction with the murine monoclonal antibody BV16 [19]. The conformational epitope covers a water accessible area of maximum 900 $Å^2$. Panel I.C: illustration of atoms conserved (red, orange and blue) between Bet v 1.2801 and the Mal d 1 isoallergen (Accession number Q43550) with the highest (66%) sequence identity. Panels I.D and E: the surface area conserved between Bet v 1.2801 and Mal d 1 (2620) (D) and (2619) (E). Panel I.F: the surface area conserved between Bet v 1.2801 and all the Mal d 1 isoallergen sequences included in Fig. 1. Panel II represents the same comparisons with the models rotated 180° around the *x*-axis.

(Fig. 2, Panel I.C–F). Only one surface area large enough to harbour an antibody binding epitope is conserved throughout all isoallergens. Thus, IgE raised against Bet v 1 and directed towards this particular epitope is likely to react with all Mal d 1 isoallergens with a comparable affinity. Other coherent surface areas are either smaller or contain substitutions (conservative or non-conservative), that are likely to reduce the affinity of an antibody-Mal d 1 interaction, as compared to that of Bet v 1. Nevertheless, on an individual basis, comparisons with Bet v 1.2801 do identify other shared surface structures; however, the lesser the sequence identity to Bet v 1.2801 the more restricted the surface areas are. The Mal d 1 isoallergens, in general, seem to contain two or more surface epitopes, that are candidates for the binding of cross-reactive antibodies, in agreement with the observation that in some birch pollen allergic individuals Mal d 1 is able to induce mediator release from human leukocytes [7].

The theoretical implications of the sequence alignment (Fig. 1) and the surface visualisations (Fig. 2) are substantiated by circumstantial evidence provided by immunochemical experiments (Fig. 3). In crossed immunoelectrophoresis, using a monospecific rabbit antibody raised against natural Bet v 1 purified from pollen, rBet v 1 forms a well-defined precipitate as expected (Fig. 3A), whereas Mal d 1 forms a blurred precipitate demonstrating a partial identity in epitope structure (Fig. 3B,C). The precipitate of variant Mal d 1 (2620) (Fig. 3B) is less blurred, as compared to the precipitate of variant Mal d 1 (2619) (Fig. 3C), in agreement with the higher amino acid identity percentage of the former. Furthermore, the monoclonal antibody BV16 does not react with Mal d 1 (2620) or (2619) (Fig. 3D), in spite of the fact that only three or, respectively, four amino acids out of 16 residues involved in the BV16-Bet v 1.2801 interaction are substituted to non-related amino acids.

The occurrence of limited epitope coincidence between Bet v 1 and Mal d 1 is in agreement with the observation that some, but not all, birch pollen allergic patients show clinical reactions towards apple. Thus, the epitope repertoire recognised by the IgE of the individual patients determines the degree of cross-reactivity.

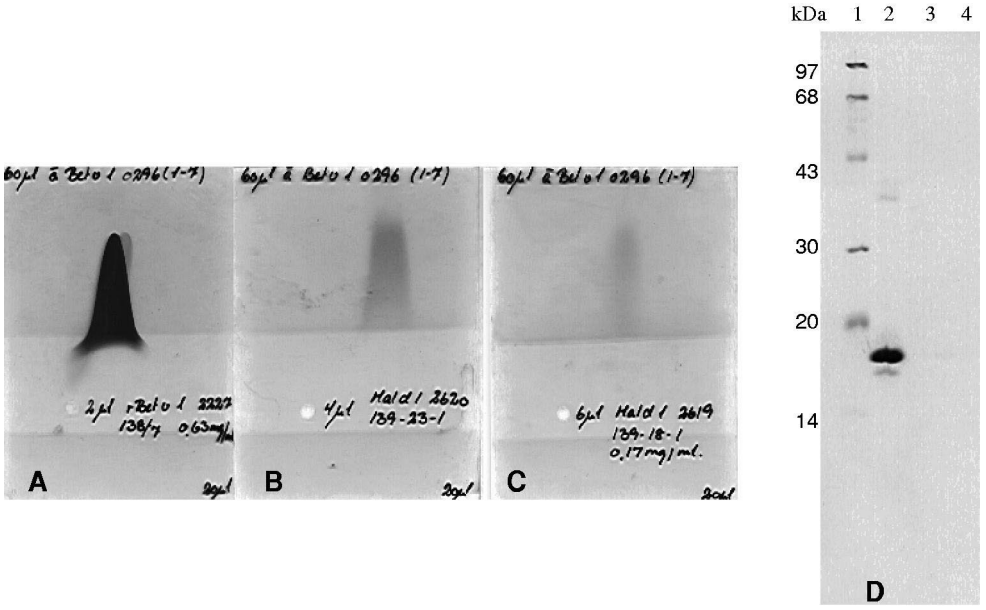


Fig. 3. Immunochemical analyses. Crossed immunoelectrophoresis of Bet v 1.2801 (A), Mal d 1 (2620) (B), and Mal d 1 (2619) (C). (D) Immunoblotting of Bet v 1.2801 (lane 2), Mal d 1 (2620) (lane 3), and Mal d 1 (2619) (lane 4) was performed with murine monoclonal antibody BV16. Lane 1, biotinylated molecular mass markers. Molecular weights are indicated.

Elucidation of the molecular details underlying clinical cross-reactivity is a subject of growing importance in allergy research. Knowledge of the molecular details of the interaction between allergens and the human immune system enables the engineering of vaccines with improved therapeutic potential.

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