

Crystal Structure of the Major Celery Allergen Api g 1: Molecular Analysis of Cross-reactivity

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Many patients who have been sensitised to pollen, display allergic symptoms after ingestion of certain plant food such as fresh fruit, vegetables and nuts. The cause is the cross-reactivity between structurally very similar major plant allergens. In particular, allergy to celery is very frequently associated with birch and mugwort pollen sensitization, known as the birch-mugwort-celery syndrome. The crystal structure of the major celery allergen Api g 1, a homologue of the major birch pollen allergen Bet v 1, has been determined to a resolution of 2.9 Å. The structure of Api g 1 is very similar to that of Bet v 1 with major differences occurring in the segment comprised of residues 23–45, preceding the well conserved glycine-rich P-loop, as well as in loops β3–β4 and β5–β6. In particular, Api g 1 lacks E45, which has been shown to be a crucial residue for antibody recognition in the crystal complex of Bet v 1 with the Fab fragment of a murine monoclonal IgG (BV16) antibody. The absence of E45 and the structural differences in the preceding segment suggest that this region of the Api g 1 surface is probably not responsible for the observed cross-reactivity with Bet v 1. A detailed analysis of the molecular surface in combination with sequence alignment revealed three conserved surface patches which may account for cross-reactivity with Bet v 1. Several residues of Bet v 1 which have been shown by mutagenesis studies to be involved in IgE recognition belong to these conserved surface regions. The structure of Api g 1 and the related epitope analysis provides a molecular basis for a better understanding of allergen cross-reactivity and may lead to the development of hypoallergens which would allow a safer immunotherapy.

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Introduction

Pollen allergic patients often display adverse reactions after ingestion of certain fresh fruit (apples, pears, peach, cherry), vegetables (celery, carrot) or nuts (hazelnut, walnut). Whereas pollen-related allergy to fruit and nuts mainly induces local symptoms affecting the mucosa of the upper aero-digestive tract (oral allergy syndrome, OAS), allergic reactions to celery are often more severe with occasionally life-threatening symptoms.^{1,2}

Abbreviations used: P-loop, residues G46–T52; “P-loop region”, residues E42–T52 of Bet v 1 (residues E43–T52 in Api g 1); rmsd, root mean square deviation.

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Celery tuber is highly allergenic even after prolonged cooking or as a spice,³ in contrast to fresh fruit, such as apples, which lose their allergenicity upon cooking and are quickly degraded by gastric digestion.⁴ In Europe, celery frequently induces adverse reactions in patients allergic to pollens from birch and mugwort (birch-mugwort-celery syndrome)^{1,5}: 70% of pollen-sensitive patients in Germany⁶ and 40% of food allergic patients in Switzerland⁵ are sensitised by celery root. Celery allergy has been unambiguously confirmed by double-blind placebo controlled food challenge (DBPCFC) study on 32 Swiss patients with a history of an allergic reaction to celery.⁷ The cross-reactivity between the major pollen allergen Bet v 1 and related allergens from celery (Api g 1), carrot (Dau c 1) and other fruit and vegetables is well established.^{8–10} Cross-reactivity probably occurs when the IgE

antibodies, originally produced in response to Bet v 1 sensitization, recognise analogous epitopes which are present on the surface of the plant proteins.¹¹ Consequently, re-exposure to pollen or homologous plant allergens will provoke an allergic reaction in already sensitised individuals.^{12,13}

Api g 1 is a Bet v 1 homologue and belongs to the family of pathogenesis-related plant proteins (PR-10), which are produced in a defence-response to various pathogens. It has been suggested that Bet v 1-related proteins function as a general plant-steroid carrier based on the crystal structure of hypoallergenic isoform Bet v 1l in complex with deoxycholate,¹⁴ which is also supported by the binding studies of various phytosteroids to Pru av 1¹⁵ and Bet v 1¹⁶ in solution. Since the two most studied Bet v 1 isoforms, Bet v 1a¹⁷ and its variant Bet v 1.2801, differ only in one amino acid (F62 and L62, respectively), in the following they are referred as to Bet v 1, unless otherwise specified.

Two different isoforms of the major celery allergen have been cloned, Api g 1.0101¹⁰ (subsequently

referred as to Api g 1), an acidic protein composed of 154 amino acid residues, and Api g 1.0201¹⁸ composed of 159 residues. These two proteins share only a 52% sequence identity and approximately 40% identity with Bet v 1. The sequence alignment of Api g 1 and its seven top related sequence homologues with its celery isoform (Api g 1.0201) and three selected sequences of the major plant allergens from apple (Mal d 1), cherry (Pru av 1) and pollen (Bet v 1a) shows two major sequence differences (Figure 1). First, Api g 1.0201, Mal d 1, Pru av 1 and Bet v 1 have a five residue extension at the C terminus (Figure 1). These residues (155–159) were shown to be of no importance for the allergenicity of Pru av 1 based on the mutagenesis data.¹⁹ Second, residue E45 (β 2- β 3 loop) is absent in Api g 1 and carrot allergens. Since E45, preceding the highly conserved P-loop (residues 46–51) of Bet v 1, has been shown to be a critical residue for binding to IgG (BV16) and IgE antibodies,^{20,21} the structural consequences of this absence in Api g 1 are discussed here in relation to reported

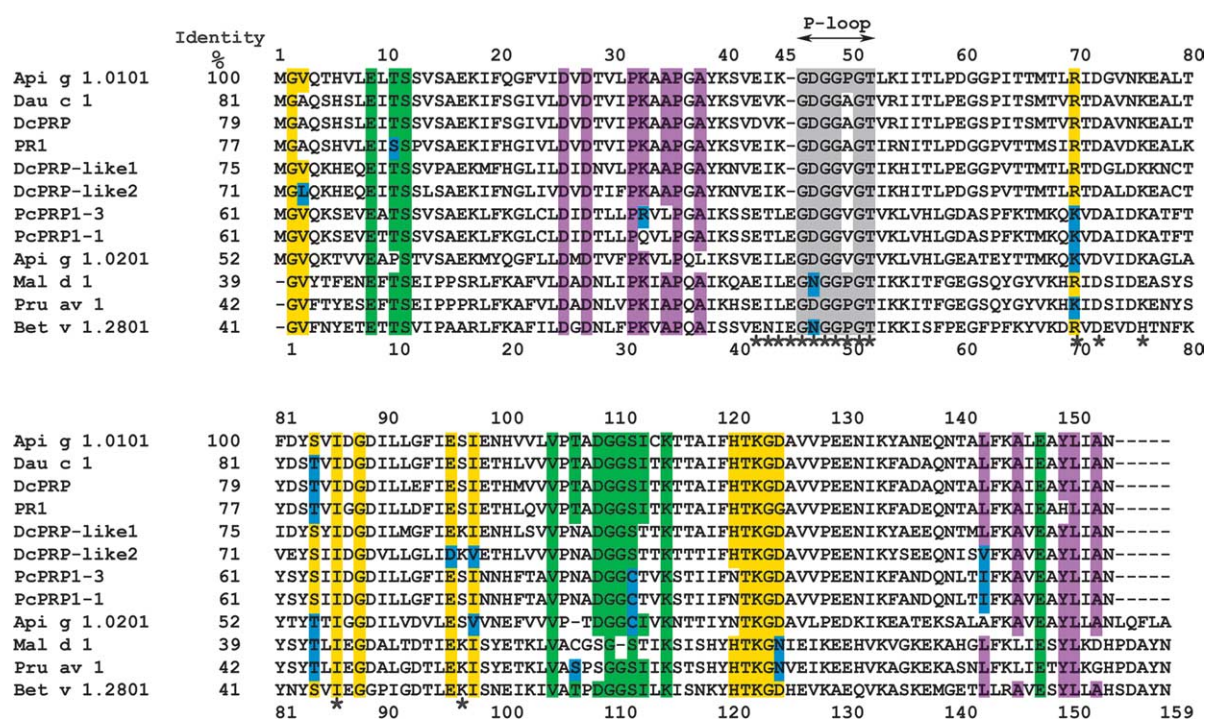


Figure 1. Sequence alignment of Api g 1 and its closest homologues with carrot and parsley (seven top sequences) with Api g 1.0201, Mal d 1, Pru av 1 and Bet v 1a, obtained by BLAST search. The 12 proteins and their accession numbers are (from top to bottom): Api g 1, celery major allergen (*A. graveolens*, P49372); Dau c 1, carrot major allergen (*D. carota*, O04298); DcPRP, carrot pathogenesis-related protein (*D. carota*, Q75T31); PR1, pathogenesis-related protein (*P. brachycarpa*, O81640); DcPRP-like1, carrot pathogenesis-related protein-like 1 (*D. carota*, Q8S903); DcPRP-like2, carrot pathogenesis-related protein-like 2 (*D. carota*, Q75T32); PR1-3, parsley pathogenesis-related protein B (*P. crispum*, P19418); PR1-1, parsley pathogenesis-related protein A (*P. crispum*, P19417); Api g 1.0201, celery major allergen (*A. graveolens*, P92918); Mal d 1, apple major allergen (*M. domestica*, P43211); Pru av 1, cherry major allergen (*P. avium*, O24248) and Bet v 1.2801, birch pollen major allergen (*B. verrucosa*, P15494). The amino acid sequence identities are listed in the second column. The numbering of the amino acid residues is indicated on the top of the alignment for Api g 1 and on the bottom for Bet v 1. Fully conserved amino acid residues of the three proposed epitopes are printed on yellow, green and magenta background, respectively. Residues which are non-conserved or conservatively substituted are printed on white and blue background, respectively. The residues from the glycine-rich P-loop which are highly conserved in all 12 sequences are printed on gray background. Asterisks denotes Bet v 1 residues which interact with monoclonal BV16 antibody fragment in the crystal structure of Bet v 1/Fab complex.²⁰

studies on site-directed mutagenesis and cross-reactivity.

Little is known about the nature of the B cell epitopes of Bet v 1 and related plant allergens. Several amino acid residues of Bet v 1, apple and celery allergens have been identified as being important for IgE recognition based on the site-directed mutagenesis studies.^{22–24} A six point mutant of Bet v 1 showed drastic decrease in IgE reactivity but was still recognised by antigen-specific T cells which are important for successful immunotherapy.²² The production of hypoallergenic variants by site-directed mutagenesis is a promising approach towards the development of safer treatments of immediate type allergies. In another approach based on sequence comparison, three epitopes were proposed for Bet v 1 and homologous tree pollen allergens which may account for their cross-reactivity.²⁵ Later, one of the epitopes was confirmed by the crystal structure of Bet v 1.2801 in complex with the Fab fragment of a murine monoclonal IgG antibody (BV16),²⁰ revealing that the centrally located residue E45 is crucial for antibody recognition. Since BV16 competes with IgEs from human sera in binding to Bet v 1, the elucidated Bet v 1 epitope directed to IgG provides indirect information on the corresponding IgE epitope.

The structure of an antigen plays a crucial role in both stages of a antigen specific type I allergic reaction, namely sensitization and elicitation of symptoms. Apart from the three-dimensional structure of the major birch pollen Bet v 1.2801²⁵ and the natural hypoallergenic isoform Bet v 1L,¹⁴ only a few three-dimensional structures of the Bet v 1-related plant allergens are known to date: the NMR structure of a major cherry allergen Pru av 1¹⁵ and the crystal structures of two proteins from yellow lupine.²⁶ These structures show a similar fold consisting of a seven-stranded β sheet and three α helices. In this work, the crystal structure of a major celery allergen Api g 1 is reported and compared to those of Bet v 1 and Pru av 1. Furthermore, surface patches have been identified which may constitute common epitope(s) responsible for the observed cross-reactivity.

Results and Discussion

Crystal structure of Api g 1 and the comparison to the structures of Bet v 1 and Pru av 1

The three-dimensional structure of the major celery allergen Api g 1 was determined by X-ray crystallography to a resolution of 2.9 Å (Table 1). The protein crystallizes in space group C2 with two monomers in the asymmetric unit. The structure was solved by molecular replacement using the structure of Bet v 1.2801 (PDB code 1BV1²⁵) as the search model, which provided the orientation and location of the two copies in the asymmetric unit. Refinement with strong NCS constraints yielded the

Table 1. X-ray data statistics

A. Data collection	
Space group	C2
Unit cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	105.9, 67.9, 48.0
α , β , γ (deg.)	90.0, 91.2, 90.0
X-ray source	Rot. Anode
Resolution range (Å)	37–2.8 (2.95–2.8)
Unique reflections	7549 (1136)
Intensity (<i>I</i> / σ (<i>I</i>))	8.3 (1.6)
Completeness (%)	90.1 (92.9)
Multiplicity	1.6 (1.6)
<i>R</i> _{symm} (%)	8.8 (39)
B. Refinement statistics	
Resolution range (Å)	30–2.9
Number of reflections	6567
<i>R</i> _{cryst} / <i>R</i> _{free} (%) ^a	22.1/26.8
No. of protein atoms	2197
No. of solvent atoms	0
Average <i>B</i> factor (Å ²)	22
rmsd from ideal values	
Bond lengths (Å)	0.008
Bond angles (deg.)	1.05
Ramachandran plot	
Most favoured regions (%)	92.6
Additional allowed regions (%)	6.2
Generously allowed regions (%)	1.2
Disallowed regions (%)	0.0

Values in parentheses refer to the highest resolution shell.

^a Crystallographic *R* factor: $R_{\text{cryst}} = \Sigma(|F_{\text{obs}}| - |F_{\text{calc}}|) / \Sigma|F_{\text{obs}}|$, where *F*_{obs} and *F*_{calc} are the observed and calculated structure-factor amplitudes, respectively. *R*_{free} was calculated for the 5% of reactions that were not used in refinement.

final model with an *R*(*R*_{free}) value of 22.1%(26.8%). The entire backbone is defined by electron density in both chains. The two chains that form a local 2-fold dimer are held together by isologous contacts involving residues from loop β 6– β 7 and the C terminus of β 6 on one side, and from helix α 1 and the preceding loop β 1– α 1, on the other. Both chains are virtually identical and superimpose with a rmsd of 0.3 Å for all C α positions. The following discussion is restricted to chain A.

The overall fold of Api g 1 (Figures 2 and 4(a)) consists of a seven-stranded antiparallel β -sheet (β 1– β 7) which curves around a long C-terminal helix α 3, while two additional short helices α 1 and α 2 connect β 1 with β 2, the fold being the same as that of the template. Comparison with the structures of Bet v 1 (41% sequence identity) and Pru av 1¹⁵ (42% sequence identity) reveals rmsd values of 1.2 Å (146 C α atoms) and 1.41 Å (141 C α atoms), respectively (Figure 2). Major structural differences between Api g 1 and Bet v 1 involve residues 23–45 (rmsd of 2.15 Å for 23 C α atoms) which belong to helix α 2 and strand β 2 as well as for residues forming loops β 3– β 4 and β 5– β 6. Comparison with the NMR structure of Pru av 1 shows significant differences in loops β 7– α 3 and β 3– β 4 (the latter loop was not well defined experimentally in Pru av 1¹⁵).

Although it has been shown that Api g 1 and Bet v 1 are cross-reactive molecules which compete for IgE binding, the responsible epitope(s) appear(s) to be different from that elucidated by the structure of Bet v 1 in complex with Fab fragment of monoclonal

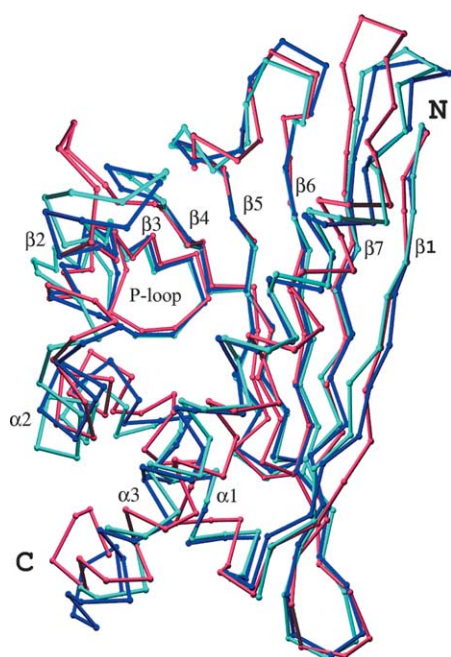


Figure 2. Backbone superposition of the crystal structures of Api g 1 (cyan), Bet v 1 (blue, crystal structure), and cherry Pru av 1 (red, the lowest energy NMR structure). The secondary structure elements and the highly conserved P-loop are labelled.

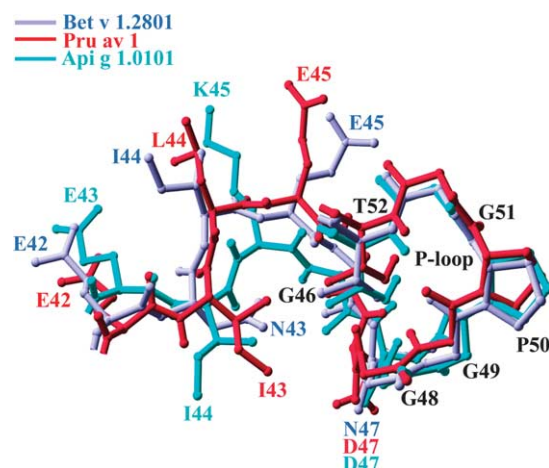


Figure 3. Structural superimposition of "P-loop regions" of Api g 1, Bet v 1 and Pru av 1 (colour code shown in the Figure).

BV16 antibody.²⁰ This can be inferred from the structural differences in the respective surface regions of the two antigens. The Bet v 1 epitope, which binds to BV16 is discontinuous and composed of the linear residue segment E42–T52 plus residues R70, D72, H76, I86 and K97. For antibody binding the segment E42–T52 was shown to be the most relevant, since it constitutes 80% of the contact surface with CDRs. In Api g 1, the P-loop (residues G46–G51) is structurally well conserved, whereas the preceding segment V42–K45 is quite dissimilar, mainly due to the deletion at position 45 (Figure 1). Superimposing residues 42–52 from Api g 1, Bet v 1 and Pru av 1 (Figure 3) shows that the P-loop of Api g 1 (-G46-D-G-G-P-G-) superimposes reasonably well with Bet v 1 (rmsd=0.62 Å) and Pru av 1 (rmsd=1.25 Å). In contrast, the superimposition of the segment -E42-I-K- is worse, showing a rmsd of 2.0 Å with Bet v 1 and 1.88 Å with Pru av 1. In Bet v 1 and Pru av 1, the loop β 2– β 3 begins with E45, whereas in Api g 1 this residue is missing so that the β 2– β 3 loop is one residue shorter and devoid of negative charge. Instead, the residue preceding the P-loop at the C terminus of β 2 in Api g 1 is the positively charged K45 with its side-chain positioned between I44 and E45 of Bet v 1 and Pru av 1 (Figure 3).

In Bet v 1, E45 appears to be a major determinant for antibody recognition as demonstrated by the crystal structure of Bet v 1/BV16 complex²⁰ in which E45 is located at the centre of the conformational epitope and forms two H-bonds with the

heavy-chain variable region of the Fab fragment. The importance of E45 is further confirmed by the observation that the single point mutant E45S of Bet v 1 decreased the binding of human polyclonal IgE for 50% and fully abolished the binding of monoclonal anti-Bet v 1 IgG1 (BV16).²¹ Similarly, in the homologous cherry allergen Pru av 1, replacement of E45 by W resulted in a significantly decreased IgE binding.¹⁹

Api g 1 lacks the negatively charged E45, in contrast to Bet v 1 and Pru av 1. Interestingly, it has been shown that the substitution of the positively charged K45 by E resulted in an increased reactivity to IgE(s) in some of celery allergic patients,¹⁹ which is probably due to the presence of a negative charge as in Bet v 1. Thus, it is likely that the "P-loop region" of Api g 1 is not involved in the recognition of the IgE antibodies due to the absence of E45. Indeed, the BV16 antibodies directed to the P-loop of Bet v 1 are not recognised by Api g 1 and, surprisingly, neither by Pru av 1 despite the structurally well conserved P-loop region.¹⁹ This suggests that the conformation of segment 42–52 of Api g 1 is unfavourable for binding of BV16. In the Bet v 1/BV16 complex, all hydrogen bonds to BV16 are formed with residues 42–52 and none with residues R70, D72, H76, I86, K97 which are part of the same epitope. Thus, it is unlikely that the corresponding residues of Api g 1 (K70, D72, K76, I86, S97), which are not fully conserved, would contribute to BV16 binding. To explore further the hypothesis that segment 42–52 could present a cross-reactive epitope between Bet v 1 and food allergens, eight additional anti-Bet v 1 mAbs, selected for P-loop binding, have been tested.¹⁹ None of them bound to Api g 1 wild-type, whereas two bound strongly to the K45E mutant, reconfirming the indispensability of E45 in the recognition of P-loop directed antibodies.

Structure based prediction of cross-reactive epitopes in Api g 1 and Bet v 1

To identify alternative IgE epitopes which could possibly account for the birch pollen-celery cross-reactivity the molecular surface of Api g 1 was analysed with respect to sequence conservation (Figure 1). The mapping of the conserved residues onto the Api g 1 molecular surface revealed three conserved surface patches, coloured yellow, green and magenta in Figure 4 and being composed of 12, 13 and 11 amino acid residues, respectively. These residues are fully conserved in Api g 1 and Bet v 1 and have an average conservation of 86–90% amongst the 12 investigated sequences aligned in Figure 1. The epitope coloured green (592 Å²) is located at the surface ridge of the antiparallel β sheet, formed by strands β 1, β 7 (N terminus) and β 5, β 6 (C terminus), while the yellow coloured epitope (845 Å²) is clustered on the opposite side of the same ridge and includes residues from strands β 1, β 6 (N terminus) and β 5, β 7 (C terminus). The magenta coloured epitope (690 Å²) contains residues from helix α 2 and neighbouring loops and the C terminus of α 3, with several amino acid residues being located near the two entries to the hydrophobic cavity. Much higher structural conservation is observed for β -strands 1, 7, 6 and 5, which contribute residues to the green and yellow coloured epitope candidates (rmsd of 0.7 Å and 0.8 Å for 12 and 13 C $^{\alpha}$ atoms, respectively) as compared to helices α 2 and α 3 of the candidate (rmsd of 2.14 Å for 11 C $^{\alpha}$ atoms). The magenta coloured epitope contains several amino acid residues from the linear sequence stretch 23–45 which are significantly different in the structures of Api g 1 and Bet v 1, and thus may not be the best candidate for cross-reactive epitope.

The surface area of each proposed epitope is approximately between 8% and 10% of the total solvent accessible molecular surface of Api g 1 (8235 Å²). In fact, if some of the neighbouring non-conserved amino acid residues, which topographically belong to the same surface, were also included this would result in an increase of epitope size and coherence. The non-conserved amino acid residues would contribute to differences of the local molecular surface and the electrostatic potential of the antigen–antibody interface and thus account for the variable degree of cross-reactivity among plant allergens.

Comparison of putative cross-reactive epitopes with Bet v 1 residues involved in IgE recognition, inferred by mutagenesis studies

The three proposed conformational epitopes, deduced by mapping residues which are fully conserved in Api g 1 and Bet v 1 and highly conserved in 12 homologue sequences, contain some amino acid residues which have been shown by previous mutagenesis studies to be involved in Bet v 1-specific allergic response in pollen allergic

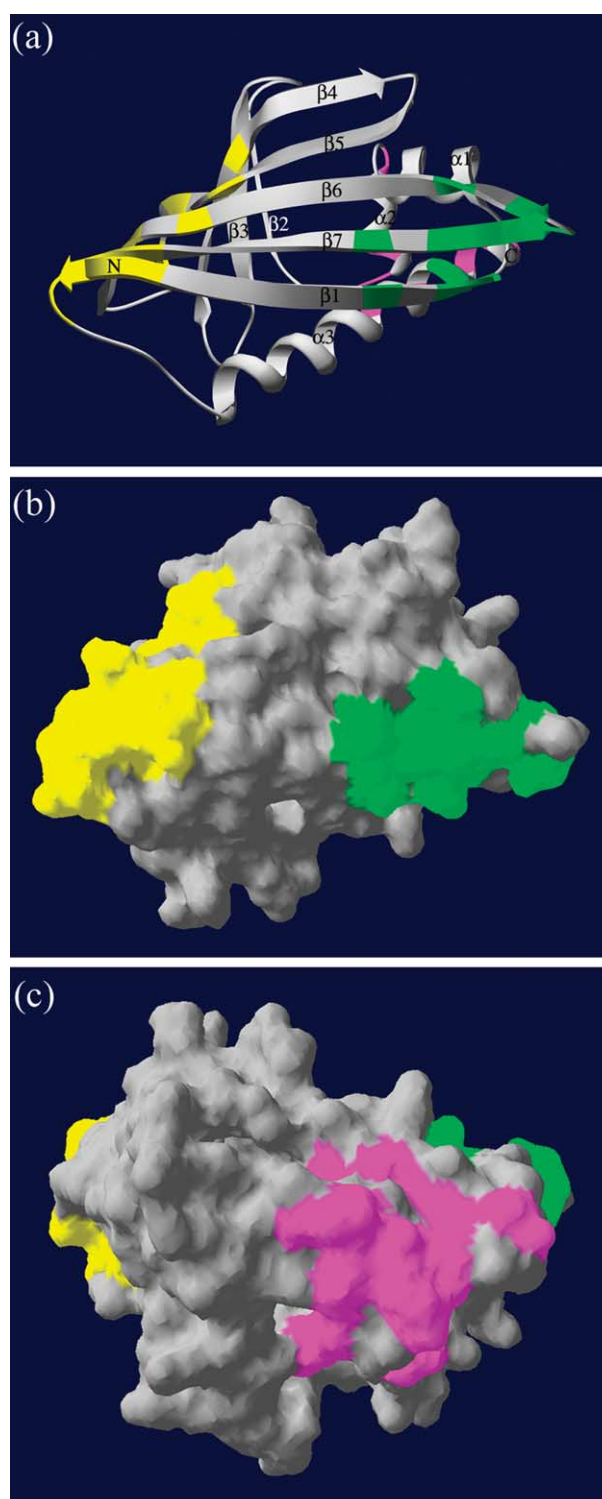


Figure 4. (a) Ribbon presentation of the Api g 1. Residues belonging to one of the three proposed conformational epitopes are coloured yellow, green and magenta. The constituting amino acid residues are highlighted in Figure 1 with the same colour. (b) Surface presentation of Api g 1 with the same viewing direction as (a). The coloured surface patches define three epitopes which are composed of residues that are fully conserved between Api g 1 and Bet v 1 (side-chain and backbone atoms of respective residues are coloured). (c) The rear view of Api g 1 surface, i.e. molecule has been rotated by 180° around the horizontal with respect to the view used in (b).

patients. In fact, each of the three proposed epitopes contains at least one residue whose exchange contributed to the hypoallergenicity of Bet v 1 mutants, either the six-point mutant (T10P,F30V,S57N,S112C,I113V,D125N)²² or the four-point mutant (N28T,K32Q,E45S,P108G).²⁴ Particularly well studied is the hypoallergenic six-point mutant which has been shown to reduce drastically the binding of Bet v 1,²² Api g 1²³ and Mal d 1²³ to their respective IgE antibodies. This demonstrates that the same six amino acid residues, or a subset of them, are crucial for IgE binding in major allergens of pollen, celery and apple, respectively. In view of the fact that most of the Fab fragments bind to relatively contiguous surface patches (600–900 Å²) of antigens,²⁷ composed of surface residues from two or more polypeptide segments, the location of the six mutated residues on the Bet v 1 surface suggests that they must belong to at least three different conformational epitopes. In Bet v 1, the six-point mutant proved to be hypoallergenic in all of the patients tested,²² whereas each of the six single mutations caused reduced IgE binding in some but not all patients, which points to the diversity of the single patient IgE antibodies.

The green coloured epitope which contains three amino acid residues (T11, S112, I113) from the six-point mutant, overlaps, at least partially, with one of the Bet v 1-specific IgE epitopes inferred by Ferreira *et al.*²² The importance of these three Api g 1 residues for IgE binding is supported by several reported observations. In Api g 1, the six-point mutant T11P,L31V,T57N,S112C,I113V,D125N† showed extremely low or no detectable binding to IgE antibodies from all celery-allergic patients.²³ In Bet v 1, the single substitutions T10P²² and S112C (Ferreira, personal communication) affected the largest number of patients compared to other single point mutations. The residues T10 (at the C-terminal end of β1) and S112 (at the N terminus of β7) are spatially close, belonging to a single epitope formed by the selected residues from strands β1 and β7. Interestingly, S112 is completely buried with its side-chain pointing towards the protein core (residue accessible surface area is 1.1 Å²). In Api g 1, the side-chain hydroxyl group of S112 forms a hydrogen bond with the main-chain carbonyl of residue 14 thereby stabilizing the conformations of the loop β1-α1. Additional H-bond, between main-chain atoms of S112 (β7) and S12 (β1), is a part of the H-bonding network of β-sheet. The S112A mutant of Pru av 1 caused no change in IgE binding while S112C mutant of Mal d 1 showed slightly reduced affinity to IgE antibodies.²⁸ In contrast, the S112P

mutant drastically reduced IgE binding in Bet v 1, Pru av 1²⁹ and Mal d 1 (S111P).²⁸ Since proline abolishes most of the H-bonding capacity of residue 112, its presence would presumably destabilize the local secondary and tertiary structure, which in turn would affect allergenicity of the involved epitope. However, the fact that the native structure of the S112P mutant of Pru av 1 is fully disrupted¹⁹ indicates that the drastic decrease of IgE binding affinity of this mutant is caused by the disturbance of the entire molecular surface and not by the local conformational changes.

The yellow and magenta epitopes each contain one charged amino acid, D125 from the six-point mutant (residue accessible surface area of 145 Å², calculated by NACCESS)³⁰ and K33, corresponding to K32 of the four-point mutant (62 Å²), respectively. Aspartic acid 125 is fully exposed to the solvent and is located at the tip of the loop connecting β7 to β3. In particular, the substitution of D125 by N has been found in all hypoallergenic isoforms of Bet v 1,³¹ suggesting that the disturbance of specific electrostatic interactions has a harmful effect on the IgE binding. Although the above comparative analysis does not absolutely validate the proposed cross-reactive Api g 1/Bet v 1 epitopes it shows, nevertheless, that there is at least partial overlap with Bet v 1-specific IgE epitopes which have been inferred by earlier studies.

Concluding remarks

Clinical observations indicate that a high percentage of birch pollen sensitised patients are also sensitised by root celery.⁶ Sensitization by Bet v 1 induces specific IgE antibodies which can cross-react with related proteins from various plant families due to the conserved structure and sequence of the involved epitopes. Clearly, the identification of such cross-reactive epitopes would have a great potential for development of mutant recombinant hypoallergens, which do not bind to IgE-antibodies and could therefore be used for the induction of immune tolerance on the T-cell level,³¹ thus enabling a safer immunotherapy of type I allergy.

In summary, the structure of the recombinant major celery allergen Api g 1 has been solved and this is, together with Pru av 1,¹⁵ the second structure of a pollen-related allergen. Two major conclusions have been reached by structural comparison of Api g 1 with Bet v 1 and Pru av 1 along with consideration of sequence conservation among a selected group of Bet v 1-related food allergens (Figure 1). First, the crystallographically identified Bet v 1 epitope which comprises the “P-loop region” (residues 42–52) that binds to IgE antibodies^{20,21} and is also responsible for cross-reactivity of Pru av 1 with Bet v 1,¹⁹ is probably not important for IgE binding of Api g 1. This is deduced from the fact that both the structure and sequence of the peptide 42–45 are significantly different in Api g 1, mainly due to the absence of E45 which is the

† The discrepancy in residues numbering of proteins aligned in Figure 1, e.g. residues T10 and F30 in Bet v 1 are equivalent to T11 and L31 in Api g 1, stems from the fact that M1 is missing only in sequences of Bet v 1, Pru av 1 and Mal d 1 which all commence with G1. The numbering of the residues 57, 112, 113 and 125 is identical in both proteins because of the insertion introduced after position 45 of Api g 1 during the sequence alignment.

dominant residue of this epitope. This structure-inferred conclusion is supported by the finding that Api g 1 does not bind monoclonal antibodies directed against the "P-loop region" and by the reported increase in IgE binding affinity of the K45E mutant.¹⁹ Second, we have identified three putative epitopes composed of highly conserved residues, which may be responsible for the cross-reactivity between Api g 1 and Bet v 1. Two of the three epitopes (coloured green and yellow) are structurally better conserved and also contain several amino acid residues whose mutation have been shown to affect Bet v 1-specific allergic response in pollen allergic patients.^{22,24} These epitopes are probably responsible for the clinical observation that some, but not all, birch pollen allergic patients react with celery^{5,7,32} together with the results from serological studies with celery extracts⁸ and recombinant Api g 1⁹ which showed that Api g 1 possesses some, but not all of Bet v 1 epitopes. Further studies are required to validate the proposed cross-reactive epitopes, such as site-directed mutagenesis of selected amino acid residues in combination with immunological, serological and skin-prick tests as well as the evaluation of their clinical effects.

Material and Methods

Crystallization

Recombinant Api g 1 was expressed and purified from *Escherichia coli* as described.⁹ Crystals of Api g 1 were grown by the hanging-drop vapour diffusion method at 20 °C, by equilibrating a mixture of 2 µl protein solution dissolved in 5 mM Na-citrate buffer (pH 6.0) (35 mg/ml) with 2 µl reservoir solution containing 10% (v/v) dioxane, 0.1 M Mes (pH 6.5), 1.6 M ammonium sulphate, against 0.7 ml of reservoir solution. Crystals grew within six to eight months to a size suitable for X-ray analysis (0.3 mm × 0.3 mm × 0.2 mm).

Data collection, structure solution and refinement

The X-ray diffraction data were collected in-house to a resolution of 2.9 Å at 4 °C, using a rotating anode generator and a MAR345 image plate area detector. The images were indexed and integrated using MOSFLM.³³ The crystals belong to space group C2 with unit cell dimensions $a=105.9$ Å, $b=67.9$ Å, $c=48.0$ Å, $\beta=91.2^\circ$. There are two molecules in the asymmetric unit, resulting in a solvent content of 53% ($V_m=2.6$ Å³/Da). The data were reduced and scaled using programs of the CCP4 package.³⁴ The structure was solved by molecular replacement with the program AMORE³⁵ using the crystal structure of Bet v 1.2801 (PDB code 1BV1²⁵) as a search model. Side-chains of Bet v 1 were replaced with those from Api g 1 using the program STRIP (A. Philippsen, unpublished software), which preserves identical residues and truncates side-chains of non-identical residues. After the rebuilding of the truncated side-chains and removal of loops with alternative conformation, the side-chains were manually fitted into the electron density using program O.³⁶ Refinement of the

model was carried out using program REFMAC.³⁴ The final model includes two independent full length molecules of Api g 1 (residues G2–N154). No water molecules were discernible at 2.8 Å resolution.

Program SPDBV was used for all structure superposition.³⁷ The solvent accessible surface areas were calculated using the program NACCESS.³⁰ The solvent accessible surface area of a particular epitope includes contributions from the main and side-chains of the involved residues.

Coordinates

Coordinates and structure factors of Api g 1 have been deposited in Protein Data Bank (PDB) with accession code 2BK0.

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References

1. Wüthrich, B., Stäger, J. & Johansson, S. (1990). Celery allergy associated with birch and mugwort pollinosis. *Allergy*, **45**, 566–571.
2. Pauli, G., Bessot, J., Braun, P., Dietemann-Molard, A., Kopferschmitt-Kubler, M. & Thierry, R. (1988). Celery allergy: clinical and biological study of 20 cases. *Ann. Allergy*, **60**, 243–246.
3. Ballmer-Weber, B., Hoffmann, A., Wüthrich, B., Lüttkopf, D., Pompei, C., Wangorsch, A. *et al.* (2002). Influence of food processing on the allergenicity of celery: DBPCFC with celery spice and cooked celery in patients with celery allergy. *Allergy*, **57**, 228–235.
4. Jensen-Jarolim, E., Leitner, A., Kalchauer, H., Znrcher, A., Ganglberger, E., Bohle, B. *et al.* (1998). Peptide mimotopes displayed by phage inhibit antibody binding to bet v 1, the major birch pollen allergen, and induce specific IgG response in mice. *FASEB J.* **12**, 1635–1642.
5. Bauer, L., Ebner, C., Hirschwehr, R., Wüthrich, B., Pichler, C., Fritsch, T. *et al.* (1996). IgE cross-reactivity between birch pollen, mugwort pollen and celery is due to at least three distinct cross-reacting allergens: immunoblot investigation of the birch-mugwort-celery syndrome. *Clin. Exp. Allergy*, **26**, 1161–1170.
6. Jankiewicz, A., Aulepp, H., Baltes, W., Bogl, K., Dehne, L., Zuberbier, T. & Vieths, S. (1996). Allergic sensitization to native and heated celery root in pollen-sensitive patients investigated by skin test and IgE binding. *Int. Arch. Allergy Immunol.* **111**, 268–278.
7. Ballmer-Weber, B., Vieths, S., Lüttkopf, D., Heuschmann, P. & Wüthrich, B. (2000). Celery allergy confirmed by double-blind, placebo-controlled food

- challenge: a clinical study in 32 subjects with a history of adverse reactions to celery root. *J. Allergy Clin. Immunol.* **106**, 373–378.
8. Ebner, C., Hirschwehr, R., Bauer, L., Breiteneder, H., Valenta, R., Ebner, H., Kraft, D. & Scheiner, O. (1995). Identification of allergens in fruits and vegetables: IgE cross-reactivities with the important birch pollen allergens Bet v 1 and Bet v 2 (birch profilin). *J. Allergy Clin. Immunol.* **95**, 962–969.
 9. Breiteneder, H., Hoffmann-Sommergruber, K., O'Riordain, G., Susani, M., Ahorn, H., Ebner, C., Kraft, D. & Scheiner, O. (1995). Molecular characterization of Api g 1, the major allergen of celery (*Apium graveolens*), and its immunological and structural relationships to a group of 17-kDa tree pollen allergens. *Eur. J. Biochem.* **233**, 484–489.
 10. Hoffmann-Sommergruber, K., O'Riordain, G., Ahorn, H., Ebner, C., Laimer Da Camara Machado, M., Pühringer, H. *et al.* (1999). Molecular characterization of Dau c 1, the Bet v 1 homologous protein from carrot and its cross-reactivity with Bet v 1 and Api g 1. *Clin. Exp. Allergy*, **29**, 840–847.
 11. Valenta, R. & Kraft, D. (1996). Type 1 allergic reactions to plant-derived food: a consequence of primary sensitization to pollen allergens. *J. Allergy Clin. Immunol.* **97**, 893–895.
 12. Fritsch, R., Ebner, C. & Kraft, D. (1997). Allergenic crossreactivities. Pollens and vegetable foods. *Clin. Rev. Allergy Immunol.* **15**, 397–404.
 13. Bohle, B., Radakovics, A., Jahn-Schmid, B., Hoffmann-Sommergruber, K., Fischer, G. F. & Ebner, C. (2003). Bet v 1, the major birch pollen allergen, initiates sensitization to Api g 1, the major allergen in celery: evidence at the T cell level. *Eur. J. Immunol.* **33**, 3303–3310.
 14. Marković-Housley, Z., Degano, M., Lamba, D., von Roepenack-Lahaye, E., Clemens, S., Susani, M. *et al.* (2003). Crystal structure of a hypoallergenic isoform of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier. *J. Mol. Biol.* **325**, 123–133.
 15. Neudecker, P., Schweimer, K., Nerkamp, J., Scheurer, S., Vieths, S., Sticht, H. & Rosch, P. (2001). Allergic cross-reactivity made visible: solution structure of the major cherry allergen Pru av 1. *J. Biol. Chem.* **276**, 22756–22763.
 16. Mogensen, J. E., Wimmer, R., Larsen, J. N., Spangfort, M. D. & Otzen, D. E. (2002). The major birch allergen, Bet v 1, shows affinity for a broad spectrum of physiological ligands. *J. Biol. Chem.* **277**, 23684–23692.
 17. Breiteneder, H., Pettenburger, K., Bito, A., Valenta, R., Kraft, D., Rumpold, H. *et al.* (1989). The gene coding for the major birch pollen allergen Bet v 1, is highly homologous to a pea disease resistance response gene. *EMBO J.* **8**, 1935–1938.
 18. Hoffmann-Sommergruber, K., Ferris, R., Pec, M., Radauer, C., O'Riordain, G., Laimer Da Camara Machado, M. *et al.* (2000). Characterization of Api g 1.0201, a new member of the Api g 1 family of celery allergens. *Int. Arch. Allergy Immunol.* **122**, 115–123.
 19. Neudecker, P., Lehmann, K., Nerkamp, J., Haase, T., Wangorsch, A., Fotisch, K. *et al.* (2003). Mutational epitope analysis of Pru av 1 and Api g 1, the major allergens of cherry (*Prunus avium*) and celery (*Apium graveolens*): correlating IgE reactivity with three-dimensional structure. *Biochem. J.* **376**, 97–107.
 20. Mirza, O., Henriksen, A., Ipsen, H., Larsen, J., Wissenbach, M., Spangfort, M. D. & Gajhede, M. (2000). Dominant epitopes and allergic cross-reactivity: complex formation between a Fab fragment of a monoclonal murine IgG antibody and the major allergen from birch pollen Bet v 1. *J. Immunol.* **165**, 331–338.
 21. Spangfort, M., Mirza, O., Ipsen, H., Van Neerven, R. J., Gajhede, M. & Larsen, J. N. (2003). Dominating IgE-binding epitope of bet v 1, the major allergen of birch pollen, characterized by X-ray crystallography and site-directed mutagenesis. *J. Immunol.* **171**, 3084–3090.
 22. Ferreira, F., Ebner, C., Kramer, B., Casari, G., Briza, P., Kungl, A. J. *et al.* (1998). Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. *FASEB J.* **12**, 231–242.
 23. Ferreira, F., Hebenstreit, D., Kramer, B., Himly, M., Breiteneder, H., Scheiner, O. *et al.* (2000). Amino acid positions involved in the formation of IgE-binding epitopes of Api g 1 and Mal d 1 allergens. *J. Allergy Clin. Immunol.* **105**, S137.
 24. Holm, J., Gajhede, M., Ferreras, M., Henriksen, A., Ipsen, H., Larsen, J. N. *et al.* (2004). Allergy vaccine engineering: epitope modulation of recombinant Bet v 1 reduces IgE binding but retains protein folding pattern for induction of protective blocking-antibody responses. *J. Immunol.* **173**, 5258–5267.
 25. Gajhede, M., Osmark, P., Poulsen, F. M., Ipsen, H., Larsen, J. N., Joost van Neerven, R. *et al.* (1996). X-ray and NMR structure of Bet v 1, the origin of birch pollen allergy. *Nature Struct. Biol.* **3**, 1040–1050.
 26. Biesiadka, J., Bujacz, G., Sikorski, M. M. & Jaskolski, M. (2002). Crystal structures of two homologous pathogenesis-related proteins from yellow lupine. *J. Mol. Biol.* **319**, 1223–1234.
 27. Braden, B. C. & Poljak, R. J. (1995). Structural features of the reactions between antibodies and protein antigens. *FASEB J.* **9**, 9–16.
 28. Son, D. Y., Scheurer, S., Hoffmann, A., Haustein, D. & Vieths, S. (1999). Pollen-related food allergy: cloning and immunological analysis of isoforms and mutants of Mal d 1, the major apple allergen, and Bet v 1, the major birch pollen allergen. *Eur. J. Nutr.* **38**, 201–215.
 29. Scheurer, S., Son, D. Y., Boehm, M., Karamloo, F., Franke, S., Hoffmann, A. *et al.* (1999). Cross-reactivity and epitope analysis of Pru a 1, the major cherry allergen. *Mol. Immunol.* **36**, 155–167.
 30. Hubbard, S. J., Campbell, S. F. & Thornton, J. M. (1991). Molecular recognition. conformational analysis of limited proteolytic sites and serine proteinase protein inhibitors. *J. Mol. Biol.* **220**, 507–530.
 31. Ferreira, F., Hirtenlehner, K., Jilek, A., Godnik-Cvar, J., Breiteneder, H., Grimm, R. *et al.* (1996). Dissection of immunoglobulin E and T lymphocyte reactivity of isoforms of the major birch pollen allergen Bet v 1: potential use of hypoallergenic isoforms for immunotherapy. *J. Expt. Med.* **183**, 599–609.
 32. Wüthrich, B. & Straumann, F. (1997). Pollen cross-reactivity Can we establish a link between the *in vitro* results and the clinical situation?. *Allergy*, **52**, 1187–1193.
 33. Leslie, A. G. W. (1992). In joint CCP4 and ESF-EACBM newsletter on protein crystallography no. 26. Technical report SERC Daresbury Laboratory, Warrington, UK.

34. Collaborative Computational Project, Number 4. (1994). The CCP4 suite: programs for protein crystallography. *Acta Crystallog. sect. D*, **50**, 760–763.
35. Navaza, J. (1994). Amore: an automated package for molecular replacement. *Acta Crystallog. sect. A*, **50**, 157–163.
36. Jones, T. A., Zou, J., Cowan, S. & Kjeldgaard, M. (1991). Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallog. sect. A*, **47**, 110–119.
37. Guex, N. & Peitsch, M. C. (1997). Protein modelling for all. *Electrophoresis*, **18**, 2714–2723.

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