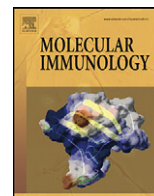




Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm



Review

Mapping of IgE-binding epitopes on the major latex allergen Hev b 2 and the cross-reacting 1,3 β -glucanase fruit allergens as a molecular basis for the latex-fruit syndrome

Annick Barre^{a,b}, Raphaël Culerrier^a, Claude Granier^c, Laetitia Selman^d, Willy J. Peumans^e, Els J.M. Van Damme^e, Françoise Bienvenu^d, Jacques Bienvenu^d, Pierre Rougé^{a,b,*}

^a Surfaces Cellulaires et Signalisation chez les Végétaux, UMR Université Paul Sabatier-CNRS 5546, 24 Chemin de Borde Rouge, 31326 Castanet Tolosan, France

^b Faculté des Sciences Pharmaceutiques, 35 Chemin des Maraîchers, 31062 Toulouse Cedex 4, France

^c CNRS FRE 3009 Bio-Rad, Cap Delta/Parc Euromédecine, 1682 rue de la Valsière, CS 61003, 34184 Montpellier Cedex 4, France

^d Laboratoire d'Immunologie, Hospices Civils de Lyon, Centre Hospitalier Lyon Sud, France

^e Laboratory of Biochemistry and Glycobiology, Department of Molecular Biotechnology, Ghent University, Coupure Links 653, B-9000 Gent, Belgium

ARTICLE INFO

Article history:

Received 7 November 2008

Received in revised form 15 December 2008

Accepted 16 December 2008

Available online xxx

Keywords:

IgE-binding epitope

Hev b 2

1,3 β -glucanase

Latex-fruit syndrome

Allergy

ABSTRACT

Nine distinct IgE-binding epitopes were identified along the entire amino acid sequence of the major latex allergen Hev b 2 (1,3 β -glucanase) using a set of synthetic 15-mer peptides frameshifted by 3 residues immobilized on cellulose membrane (Spot technique). Most of the amino acid residues building these IgE-binding epitopic regions are nicely exposed on the surface and the epitopes usually correspond to charged regions on the molecular surface of the protein. A smaller number of 5 IgE-binding epitopic areas was identified on the banana 1,3 β -glucanase, which exhibits a very similar overall conformation and charge distribution. The latter epitopes might be responsible for the IgE-binding cross-reactivity currently observed in the latex-fruit syndrome. Using rabbit polyclonal IgG anti-BanGluc as a probe instead of IgE from allergic patients the same epitopic regions were identified in both Hev b 2 and BanGluc. Additionally, surface-exposed regions with a very close conformation were predicted to occur on Ole e 9, the 1,3 β -glucanase allergen identified in olive pollen.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

Allergy to natural rubber tree (*Hevea brasiliensis*) latex has been recognized for a long time as a severe allergy in both adults and children (Bernstein et al., 2003a; Niggemann and Breiteneder, 2000). The WHO/IUIS Allergen Nomenclature Committee (<http://www.allergen.org>) listed 13 natural rubber latex (NRL) Hev b allergens characterized at the molecular level. Among these allergens, Hev b 2 (1,3 β -glucanase) (Sunderasan et al., 1996), Hev b 5 (Slater et al., 1996), Hev b 6 (hevein precursor) (Chen et al., 1997), and Hev b 13 (esterase) (Bernstein et al., 2003b) are the

major allergens relevant to latex-sensitized adults (Yeang, 2004). A significant proportion (close to 30%) of individuals experiencing allergy to rubber latex exhibit an associated hypersensitivity to various fresh fruits referred as the “latex-fruit syndrome” (M'Raihi et al., 1991). Documented examples of such fruits are the commonly consumed avocado, banana, kiwi, peach, bell-pepper and tomato (Blanco et al., 1994; Beezhold et al., 1996; Brehler et al., 1997; Gallo et al., 1997). Some of the ubiquitous proteins described as the major allergens of rubber latex, e.g. 1,3 β -glucanase and patatin, showed IgE-binding cross-reactivity with bell-pepper (Wagner et al., 2004) and potato (Schmidt et al., 2002), respectively. According to this IgE-binding cross-reactivity, individuals allergic to rubber latex might display hypersensitivity reactions to fruits containing similar molecules without previous sensitization to the fruit allergens. This cross-allergenicity most probably depends on the occurrence of structurally related B-epitopes on ubiquitous allergens occurring in both rubber latex and food products. To check this hypothesis as a molecular basis for the latex-fruit syndrome, we carried out a conformational analysis of the IgE-binding epitopic regions mapped on the molecular surface of two cross-reacting 1,3 β -glucanase major allergens from rubber latex (Hev b 2) and banana (*Musa acuminata*) fruit (BanGluc) (Peumans et al., 2000). A comparison was also

Abbreviations: BanGluc, 1,3 β -glucanase from banana (*Musa acuminata*); CapsiGluc, 1,3 β -glucanase of bell pepper (*Capsicum annuum*); HCA, Hydrophobic Cluster Analysis; Hev b 2, allergen 2 (1,3 β -glucanase) from *Hevea brasiliensis* latex; LFS, latex-fruit syndrome; NRL, natural rubber (tree) latex; Ole e 9, allergen 9 (1,3 β -glucanase) from olive (*Olea europaea*) pollen; PrupeGluc, 1,3 β -glucanase from peach (*Prunus persica*); TIM-barrel, triose-phosphate isomerase-barrel.

* Corresponding author at: Surfaces Cellulaires et Signalisation chez les Végétaux, UMR Université Paul Sabatier-CNRS 5546, 24 Chemin de Borde Rouge, 31326 Castanet Tolosan, France. Fax: +33 562193502.

E-mail address: rouge@scsv.ups-tlse.fr (P. Rougé).

performed with the structurally related olive (*Olea europaea*) pollen allergen (Ole e 9) (Huecas et al., 2001).

2. Materials and methods

2.1. Sera and chemicals

Blood samples were drawn after informed consent of 6 patients experiencing anaphylaxis to latex-containing gloves (Table 1). All sera interacted with rHev b 2 as previously checked by RAST. Polyclonal IgG against banana glucanase were raised in rabbits in the presence of Freund's complete adjuvant according to a standardized immunization procedure including a first injection of 200 µg of allergen followed by seven booster injections at 10 day intervals. Immune sera were collected 10 days after the last booster injection. After blood clotting, sera were filtered through a 0.2-µm cellulose membrane and stored at –80 °C until used.

2.2. Epitope mapping

Overlapping 15-mer peptides, frameshifted by three residues, corresponding to the entire amino acid sequences of Hev b 2 and banana 1,3β-glucanase were synthesized using the Spot technique (Frank, 1992) as described previously (Laune et al., 2002) except for the use of the MultiPep automatic Spot synthesizer (Intavis). Briefly, peptides were assembled using the Fmoc chemistry on a cellulose membrane carrying an amino polyethylene glycol moiety. The C-terminal residue of each peptide was coupled to the activated membrane. After Fmoc deprotection, the next amino acids were sequentially added. After completing the synthesis, side chain protecting groups were removed by a trifluoroacetic acid treatment leaving the linkage of the peptides to the membrane intact.

The membrane was soaked overnight in 20 mL of Tris-buffered saline (TBS) containing 2 mL blocking buffer (Roche) and 1 g sucrose (pH 7.0), and then washed three times with TBS containing 0.1% (v/v) Tween (TBSTw). A 1:10 (v/v) diluted pool of patient sera was added in the presence of an anti-protease cocktail (Roche) and the membrane was incubated in a moist chamber for 2 h. After three washes with TBSTw (pH 7.0) the membrane was shaken in a 1:4000 dilution of mouse monoclonal anti-human IgE labeled with alkaline phosphatase (Sigma) for 1 h. After three washes with TBSTw (pH 7.0), the interacting peptide spots were stained for 30 min by adding the bromochlorylindolophosphate (BCIP) substrate for alkaline phosphatase (Promega). The membrane was washed three times with deionized water and dried for scanning. The membrane can be used repeatedly after a regeneration step consisting of one wash in dimethylformamide for 1 min, three washes in deionized water, three washes in 8 M urea containing 1% (w/v) SDS and 1% (w/v) β-mercaptoethanol and three subsequent washes in a mixture of C₂H₅OH–CH₃COOH–H₂O (50:10:40, v/v/v). A 1:10 (v/v) diluted polyclonal anti-banana 1,3β-glucanase IgG raised in rabbits was used as the primary unlabeled antibody in similar experiments with immobilized peptides on cellulose membrane.

Four 15-mer peptides corresponding to the core region of the IgE-binding epitope #1 (25NNIARMRLYDPNQA39), epitope #2 (73IRRVVAYWPSVSFR87), epitope #4 (256YNQN-LIRHVGGGTTPR270), and epitope #5 (295QNFGFLYPNKPVYQ309) identified in BanGluc, were synthesized by solid-phase Synthesis (GenScript Corp., Piscataway, New Jersey, USA) and checked for reactivity towards IgE by ELISA in inhibition experiments to assess their IgE-binding activity.

The capacity of synthetic 15-mer peptides to inhibit the BanGluc–IgE interaction was checked by ELISA. The wells of a flat bottomed standard plate (Limbro) were filled with 50 mL of 10 mM

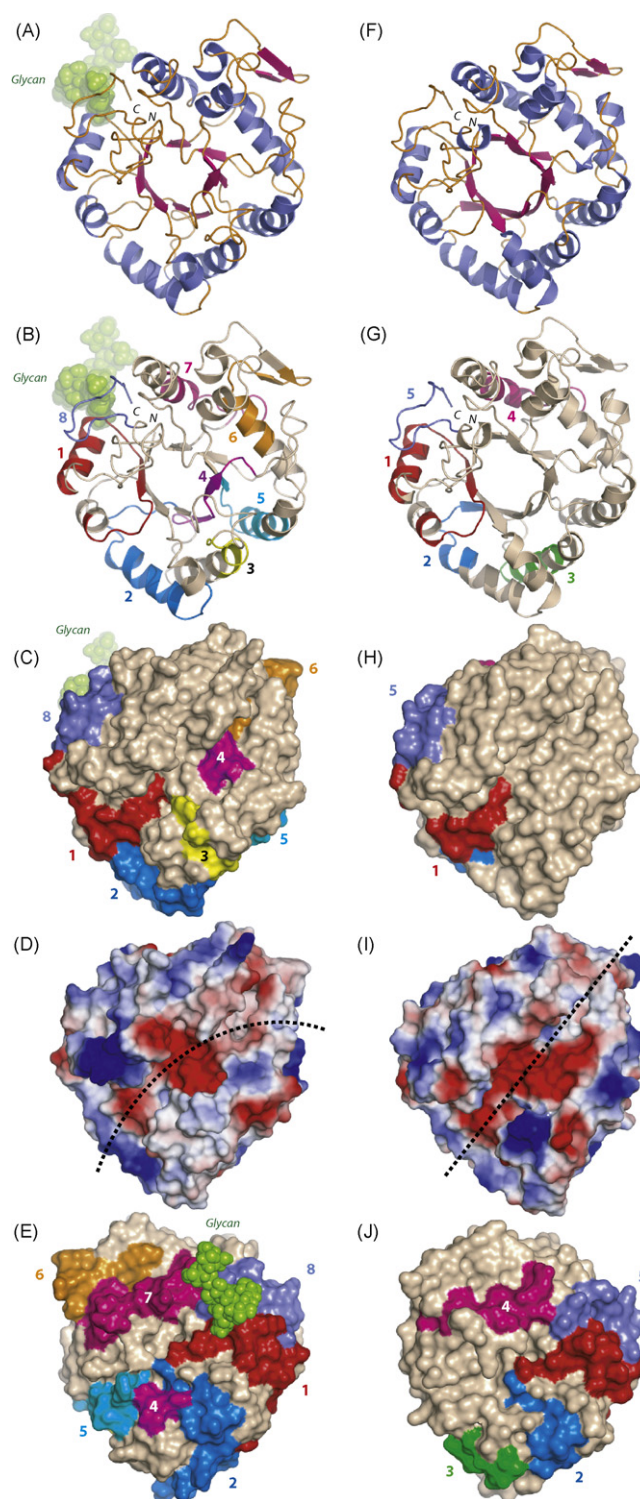


Fig. 1. (A and F): ribbon diagram of Hev b 2 (A) and BanGluc (F). The N-glycan chain of Asn27 is represented in green CPK. (B and G): localization of IgE-binding epitopes on the ribbon diagram of Hev b 2 (B) and BanGluc (G). Epitopes are numbered and distinctly colored. (C and H): surfaces occupied by the IgE-binding epitopes on the molecular surface of the face containing the enzymatic cleft of Hev b 2 (C) and BanGluc. Epitopes are numbered and colored as in B and G. (D and I): mapping of the electrostatic potentials on the molecular surfaces of Hev b 2 (D) and BanGluc (I). The black dashed lines indicate the overall orientation of the catalytic cleft. Electronegatively and electropositively charged areas are colored red and blue, respectively. Neutral surfaces are white. (E and J): surfaces occupied by the IgE-binding epitopes on the molecular surface of the face opposite to the catalytic cleft of Hev b 2 (E) and BanGluc (J). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 1
Listing of patients allergic to latex.

Subjects	Sex/age (years)	Allergic history ^a	Symptoms ^b	Natural latex specific IgE (kU/L) ^c	rHev b 2 specific IgE (kU/L)	Bromelain IgE (kU/L)
ID.SY.	F/36	LA, PA	RH, OED, PRU	1.36	<0.10	<0.10
CA.MA.	M/16	LA, FA(chestnut, pepper)	QOED, UR	33.8	<0.10	<0.10
MO.GA.	M/21	LA	RH, UR	0.58	<0.10	<0.10
RI.SA.	F/20	LA	QOED	12.9	<0.10	<0.10
SC.MA.	M/47	LA	PRU	>100	2.30	0.19
FE.BE.	F/54	LA	ASH	53.10	0.77	<0.10

^a LA: latex allergy, FA: food allergy, PA: pollen allergy.

^b RH: rhinitis, OED: oedema, PRU: pruritis, QOED: Quincke oedema, ASH: anaphylactic shock.

^c Measured using ImmunoCAP Phadia (k82).

Table 2
IgE-binding epitopes identified along the amino acid sequence of Hev b 2 and β 1,3-endoglucanase from banana (BanGluc). Residues exposed on the surface of the allergens are in black boxed white letters. Buried residues are in black letters.

IgE-binding epitopes of Hev b 2	IgE-binding epitopes of BanGluc
#1 16VSEVIALYKKSNI ^{TR} MRITYDPNRA ³⁹	#1 16SEVVS ^{LY} KSN ^{NI} ARM ^{LY} DPNQAA ³⁹
#2 64TNPSNAKSWVQKNVRGFWSSVLFR ⁸⁷	#2 67SAAGDWIRRN ^{VV} AYW ⁸¹
#3 103AWLAQFVLP ¹¹¹	
#4 127DQIKVSTADLT ¹³⁸	#3 109MRNIYNALSSAG ¹²⁰
#5 160DPIIGFLSSIRSP ^{LL} 174	
#6 205WDGQRGYKNLFDATLDAL ²²³	
#7 262NLIQHVKG ^{GT} PKRPN ²⁷⁶	#4 259NLIRHVGGGT ^{PR} 270
#8 301GLFFPD ^{KRP} 309	#5 298GLFY ^{PNKQP} 306
#9 325TEHNATILFLKSDM ³³⁸ (absent on 3D model)	

PBS (pH 7.5) containing 5 mg mL⁻¹ of BanGluc. The plates were incubated overnight at 4 °C and washed 3-times with PBS (pH 7.5). 100 mL of PBS (pH 7.5) containing 0.1% Tween 20 (v/v) and 1% BSA (w/v) (PBSTB) were added and the plates were incubated at room temperature for 2 h. 50 mL of 1:20 and 1:40 diluted patient serum

in PBSTB (pH 7.5) previously incubated with 2 mM of synthetic peptide were added and the plates were incubated for 1 h 30 at room temperature under constant stirring. After 3 washes with PBSTB, 50 mL of 1:500 diluted mouse monoclonal anti-human IgE coupled to alkaline phosphatase (Sigma) were added and, after incubation at

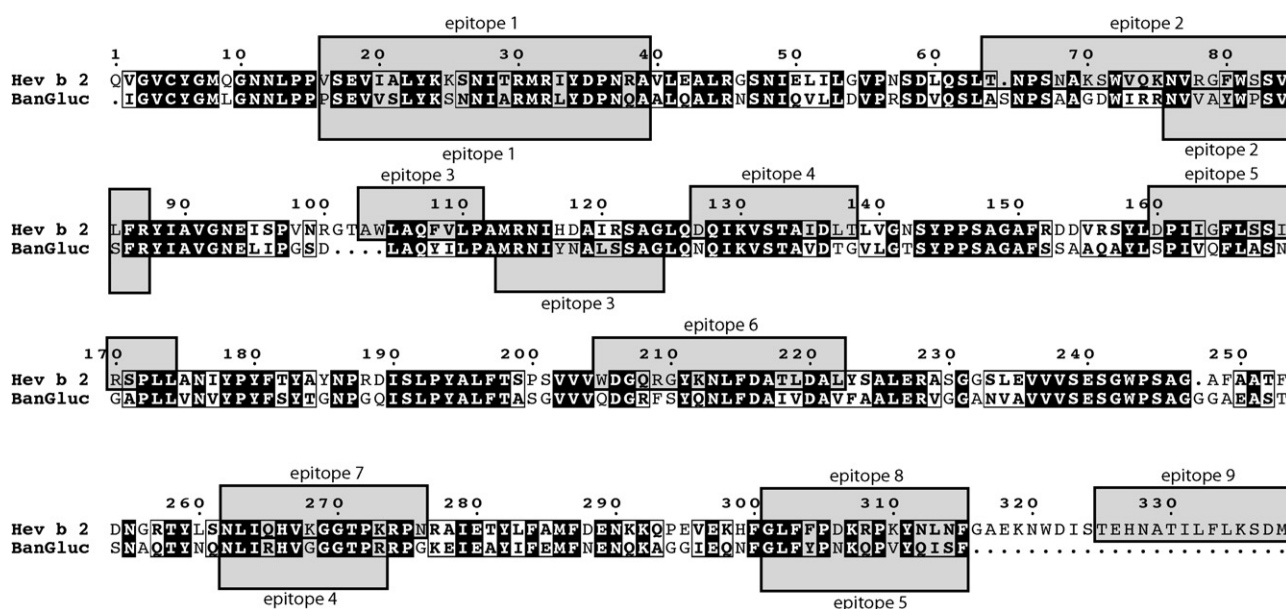


Fig. 2. Amino acid sequence alignment of Hev b 2 and BanGluc. Identical residues are in black boxed white letters and structurally homologous residues are in open boxed black letters. Gaps are indicated by points (.). Upper (epitopes 1–9) and lower (epitopes 1–5) grey boxes correspond to IgE-binding epitopes identified along the amino acid sequences of Hev b 2 and BanGluc, respectively.

room temperature for 1 h, 3 washes were performed with PBSTB. 100 mL of alkaline phosphatase substrate (Sigma) were added and after incubation for 45 min in the dark, 50 mL of 3 M NaOH were added to stop the color reaction. The absorbance at 405 nm was recorded on a Titertek Multiscan spectrophotometer. Each value is the mean of three separate experiments. Appropriate blank and substrate controls were performed under the same conditions.

2.3. Molecular modeling

Multiple amino acid sequence alignments were carried out with CLUSTAL-X (Thompson et al., 1997) and displayed with ESPript (Gouet et al., 1999) using the Risler's structural matrix for homologous amino acid residues (Risler et al., 1988). Hydrophobic Cluster Analysis (HCA) (Gaboriaud et al., 1987) was performed to delineate the conserved secondary structural features (strands of β -sheet and stretches of α -helix) along the amino acid sequence of Hev b 2 using the barley 1,3-1,4 β -glucanase (Muller et al., 1998) as a model. HCA plots were generated using the HCA server (<http://bioserv.rpbs.jussieu.fr>).

Molecular modeling of Fag13S (amino acid sequence stretch 2–224) was carried out on a Silicon Graphics O2 R10000 workstation, using the programs InsightII, Homology and Discover3 (Accelrys, San Diego CA, USA). The atomic coordinates of the banana 1,3 β -glucanase (PDB code 2CYG) (Receveur-Bréchet et al., 2006) were used to build the three-dimensional model of Hev b 2 and Ole e 9, respectively (Berman et al., 2000). Despite the relatively low identity ($\leq 45\%$) between Hev b 2/Ole e 9 and other glucanases, HCA plots indicated a highly conserved three-dimensional fold, which allowed building a fairly accurate three-dimensional model using the X-ray coordinates of 2CYG. Steric conflicts were corrected during the model building procedure using the rotamer library (Ponder and Richards, 1987) and the search algorithm implemented in the Homology program (Mas et al., 1992) to maintain proper side-chain orientation. The geometry of loop regions was corrected using the refine option of TurboFrodo (Roussel and Cambillau, 1989). An

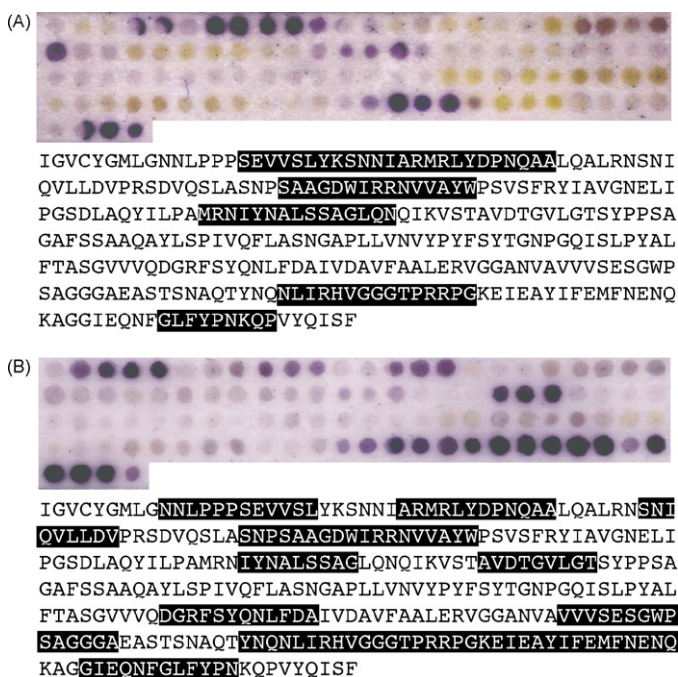


Fig. 3. Examples of Spot results obtained with Hev b 2 (A) and BanGluc (B) using rabbit polyclonal IgG as a probe. IgG-binding epitopes deduced from the stained spots are indicated in black boxed white letters along the amino acid sequences of Hev b 2 and BanGluc.

energy minimization of the final models was carried out by 150 cycles of steepest descent using the cvff forcefield of Discover. The program TurboFrodo was run to draw the Ramachandran plot and perform the superposition of the model with the template protein. PROCHECK (Laskowski et al., 1993) was used to assess the geometric quality of the three-dimensional models. As an example, about 80% of the residues of Hev b 2 were correctly assigned in the best allowed regions of the Ramachandran plot, the remaining residues being located in the generously allowed regions of the plot except for two residues (Gln164 and Glu331) which occur in the non-allowed region (result not shown). Three-dimensional models were similarly built for 1,3 β -glucanases from bell pepper (NCBI accession AAF34761) (Jung and Hwang, 2000) and peach (NCBI accession AAF34761) (Thimmapuran et al., 2001).

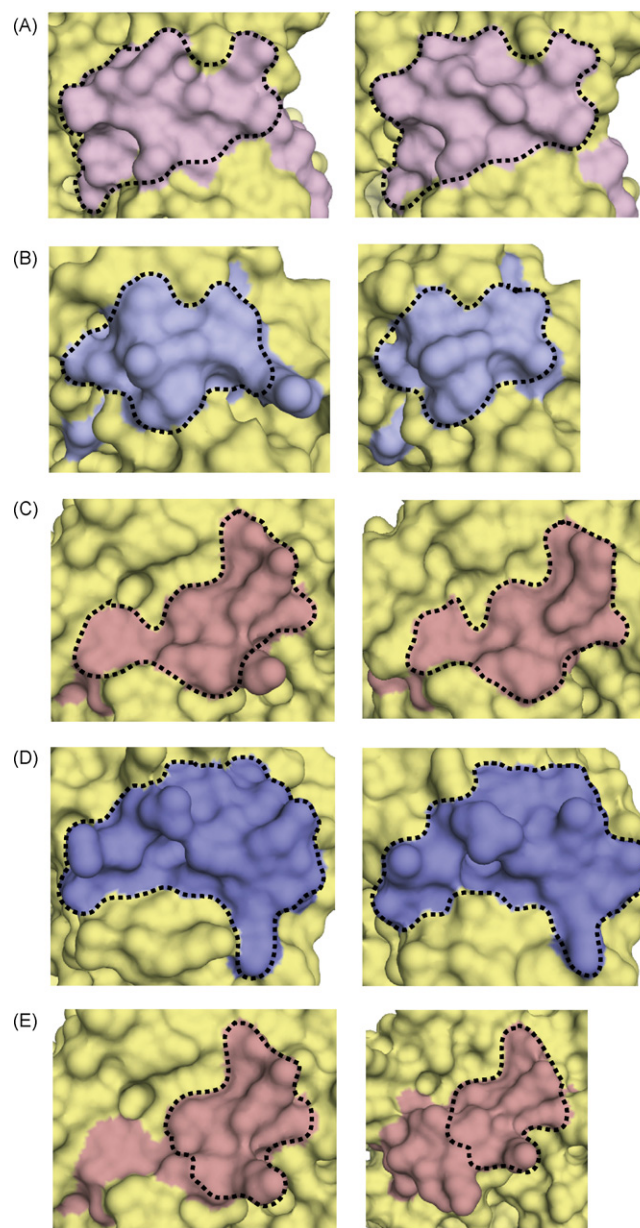


Fig. 4. Left panel: molecular surface of IgE-binding epitopes #1 (A), #2 (B), #7 (C) and #8 (D) of Hev b 2. Regions exhibiting a very similar conformation are shown with dashed line. Right panel: molecular surface of IgE-binding epitopes #1 (A), #2 (B), #4 (C) and #5 (D) of BanGluc. The corresponding epitopic regions of Hev b 2 and BanGluc exhibit a very similar charge distribution. In (E), the partially similar conformation (dashed black lines) of accessible surfaces of IgE-binding epitope #7 of Hev b 2 (left) and IgE-binding epitope #4 of BanGluc (right) are compared.

PyMol (W.L. DeLano (<http://pymol.sourceforge.net>)) was used to draw ribbon diagrams of allergens and delineate the IgE-binding epitopes on their molecular surface. Electrostatic potentials were calculated and displayed with GRASP using the parse3 parameters (Nicholls et al., 1991). The solvent probe radius used for molecular surfaces was 1.4 Å and a standard 2.0 Å-Stern layer was used to exclude ions from the molecular surface (Gilson and Honing, 1987). The inner and outer dielectric constants applied to the protein and the solvent were fixed at 4.0 and 80.0, respectively, and the calculations were performed keeping a salt concentration of 0.145 M.

Assuming that the putative *N*-glycosylation site Asn63–Ile–Thr is actually glycosylated, a classic bi-antennary high-mannose glycan chain with a tri-mannoside core (Man)₂–(Man)₃–(GlcNAc)₂ was modeled using the GlyProt server of Glyco-SCIENCES (<http://www.glycosciences.de/modeling/glyprot/php/main.php>) and represented in CPK on the molecular surface of Hev b 2.

According to the location of the exposed trypsin and chymotrypsin cleavage sites on the three-dimensional models, both the preserved central core structure and the surface-exposed peeled off regions of the proteins upon proteolytic attack were identified.

2.4. Phylogenetic relationship

An unrooted phylogenetic tree was built from the available amino acid sequences of plant 1,3β-glucanases using the parsimony algorithm of the PHYLIP package (available from J. Felsenstein at <http://evolution.genetics.washington.edu/phytip.html>). The tree was rendered with TreeView (Page, 1996).

3. Results

3.1. Hev b 2 exhibits the canonical TIM-barrel structure of 1,3β-glucanases

Like BanGluc and other 1,3β-glucanases, Hev b 2 exhibits the canonical TIM-barrel three-dimensional structure built up of a central crown of eight strands of β-sheet surrounded by an external crown of α-helices (Fig. 1A and F). A long loop containing two strands of anti-parallel β-sheet protrudes from the external α-helical crown. One of the two faces of the protein, referred as the front face, possesses a catalytic cleft which contains two acidic

residues responsible for the hydrolytic attack of the β1,3 osidic bond of β-glucans and thus exhibits a pronounced electronegative character (Fig. 1D and I). In Hev b 2, the catalytic cleft exhibits a rather straight orientation whereas it adopts a curved shape in BanGluc (see Fig. 1D and I). As predicted from the GlyProt server, Hev b 2 might be glycosylated at residue Asn27 of the putative *N*-glycosylation site Asn27–Ile–Thr. Accordingly, an *N*-glycan chain of the high-mannose type (Man)₅–(GlcNAc)₂, which is very common in plant glycoproteins, was tentatively modeled and represented in CPK on both the ribbon diagram and the molecular surface of Hev b

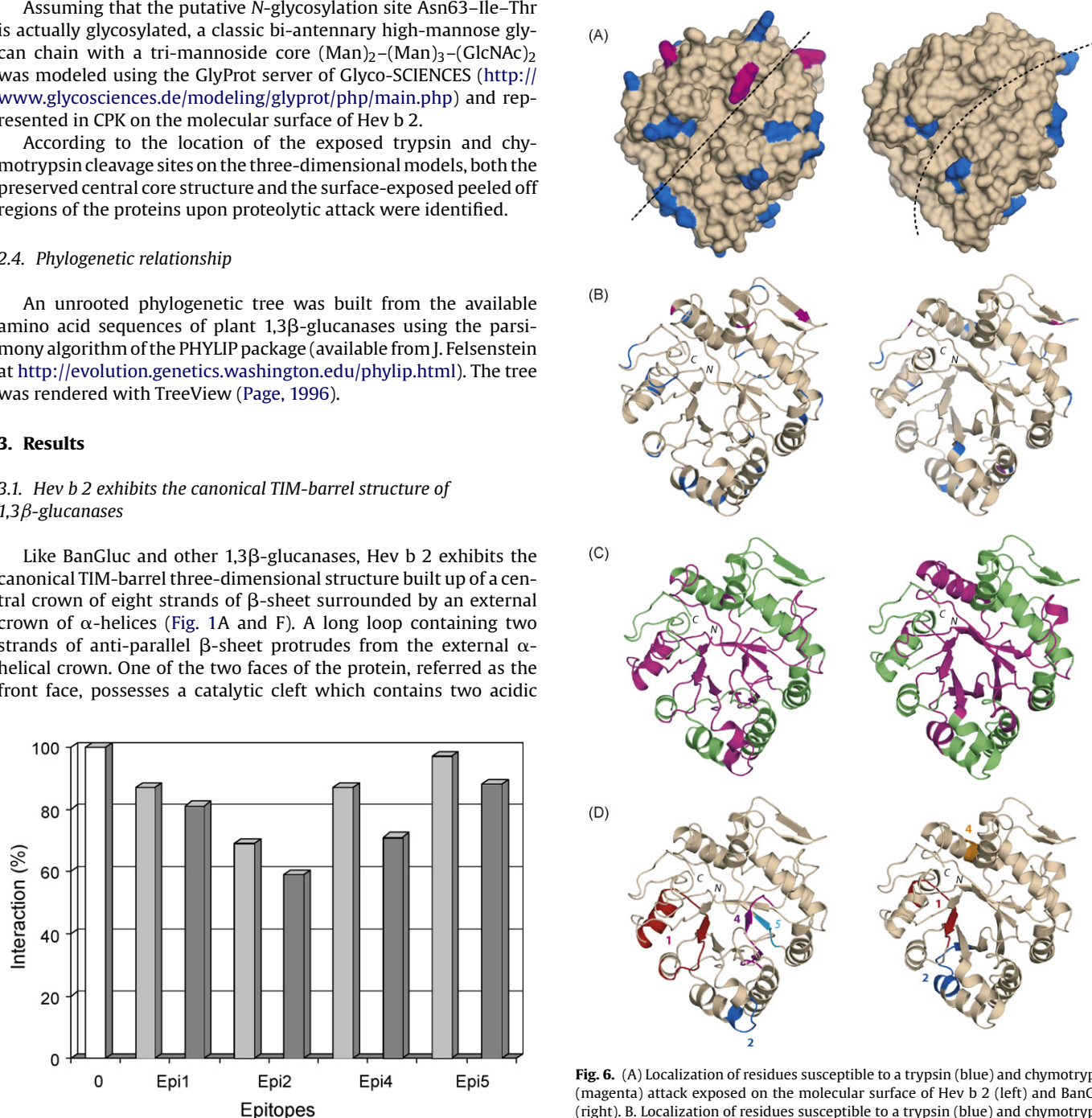


Fig. 5. Interaction of IgE from 1:20 diluted (grey) and 1:40 diluted (pale grey) allergic patient sera with BanGluc in the presence of a 2-M concentration of synthetic epitope #1 (Epi1), epitope #2 (Epi2), epitope #4 (Epi4) and epitope #5 (Epi5). 0 corresponds to the interaction measured in the absence of inhibitory peptides. All values are means of 3 separate measurements.

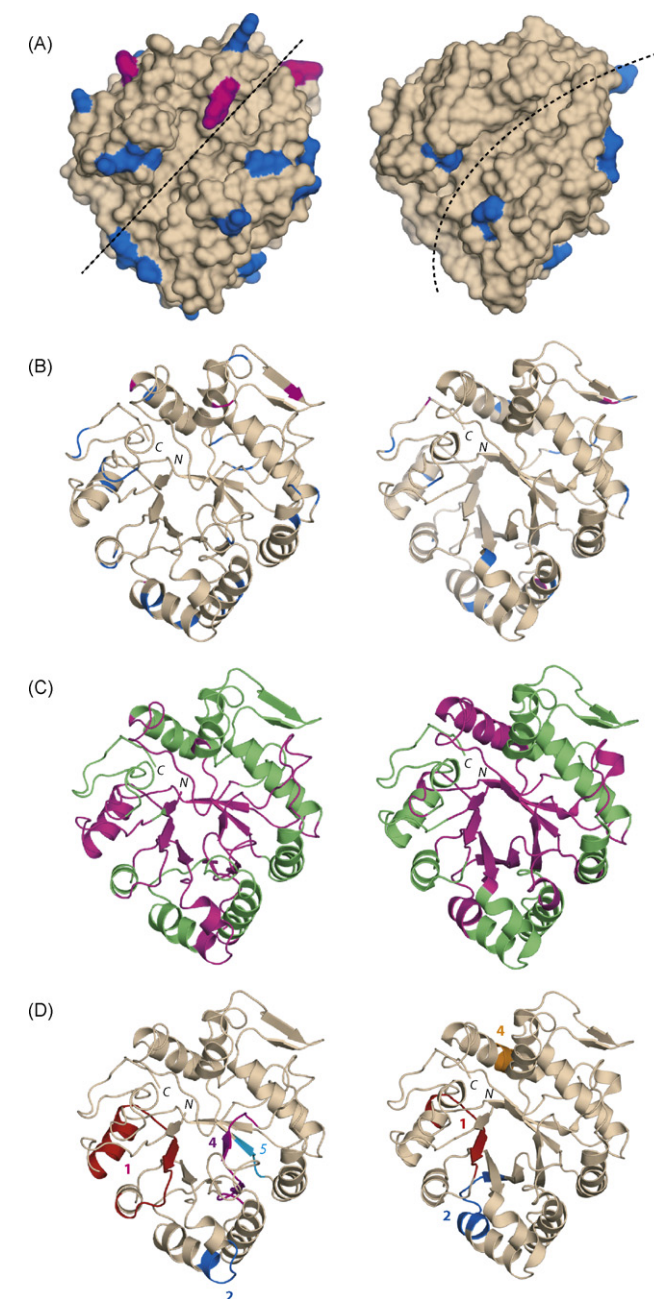


Fig. 6. (A) Localization of residues susceptible to a trypsin (blue) and chymotrypsin (magenta) attack exposed on the molecular surface of Hev b 2 (left) and BanGluc (right). (B) Localization of residues susceptible to a trypsin (blue) and chymotrypsin (magenta) attack on the ribbon diagram of Hev b 2 (left) and BanGluc (right). (C) Ribbon diagram showing the proteolytically peeled off regions (colored green) and the unattacked core structure (colored magenta) of Hev b 2 (left) and BanGluc (right). (D) Ribbon diagram showing the IgE-binding epitopes preserved in the core structure of Hev b 2 (left) and BanGluc (right). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

2 (Fig. 1B,C,E). Previous results from Yagami et al. (2002) indicated that the heterogeneous glycosylation of Asn27 gives rise to different glycoforms of Hev b 2 that could be separated according to their distinct affinities for a column of immobilized Con A.

3.2. Nine different sequential IgE-binding epitopes occur at the surface of Hev b 2

Nine sequential IgE-binding epitopes were mapped along the amino acid sequence of Hev b 2 in Spot experiments using IgE from latex glove allergic patients as a probe (Fig. 2). Mapping with IgE-containing sera from 6 allergic individuals yielded very consistent results that differed only by the staining intensity of the reactive spots. These IgE-binding epitopes correspond to the amino acid sequence stretches 16VSEVIALYKKSNI~~TRM~~RIYDPNRA39 (epitope #1), 103AWLAQFVLP111 (epitope #3), 127DQIKVSTAI~~DLT~~138 (epitope #4), 160DPIIGFLSSIRSP174 (epitope #5), 205WDGQRGYK~~NLFD~~-ATLDAL223 (epitope #6), 262NLIQHVKGGTPK~~RPN~~276 (epitope #7), 301GLFFPD~~KRP~~KYNLNF315 (epitope #8) and 325TEHNATILFLKSDM338 (epitope #9), respectively (Fig. 2). Except for epitope #3, all other epitopes contain charged residues (bold underlined). In addition, many of the residues building the IgE-binding epitopes are readily exposed on the molecular surface of Hev b 2 (Table 2) and, accordingly, the surface area (5840 Å²) occupied by 8 out of the 9 IgE-binding epitopes amounts to 40% of the total surface area (14,370 Å²) of the allergen (Fig. 1B,C,E). The surface occupied by epitope #9 is not available because it is located in the missing C-terminal part of the modeled Hev b

2 protein. The large surface occupied by IgE-binding epitopes most probably accounts for the allergenic potency of Hev b 2. In this respect, epitope #1, which contains an N-glycosylation site, differs from other epitopes since it corresponds to a glycotope (Fig. 1A,B,E). Moreover, some of the identified IgE-binding epitopes are coalescent on the molecular surface, e.g. epitopes #1 and #8 or epitopes #6 and #7, that creates more extended IgE-binding areas on the surface of Hev b 2 which could play a key role in the allergenic potency.

3.3. BanGluc contains IgE-binding epitopes cross-reacting with Hev b 2 epitopes

Using IgE from latex allergic patients as a probe, 5 cross-reacting IgE-binding epitopes were identified in the amino acid sequence of BanGluc in Spot experiments (Fig. 3A). Except for epitope #3 which has no counterpart in Hev b 2, epitopes #1, #2, #4 and #5 overlap IgE-binding epitopes #1, #2, #7 and #8 identified in Hev b 2, respectively (Figs. 1G,H,J and 2; Table 2). They correspond most probably to consensus epitopes that are similarly recognized by the corresponding IgE because they not only share a high sequence identity ranging between 53% (epitope #2 Hev b 2/epitope #2 BanGluc) up to 75% (epitope #7 Hev b 2/epitope #4 BanGluc), but also display very similar conformations on the molecular surface of both allergens (Fig. 4A–D). However, epitope #1 of BanGluc significantly differs from epitope #1 of Hev b 2 by the lack of a putative N-glycosylation site. Ole e 9, the 1,3β-glucanase allergen from olive tree pollen can also readily be aligned with Hev b 2. The regions corresponding to the IgE-binding epitopes of Hev b 2 share only

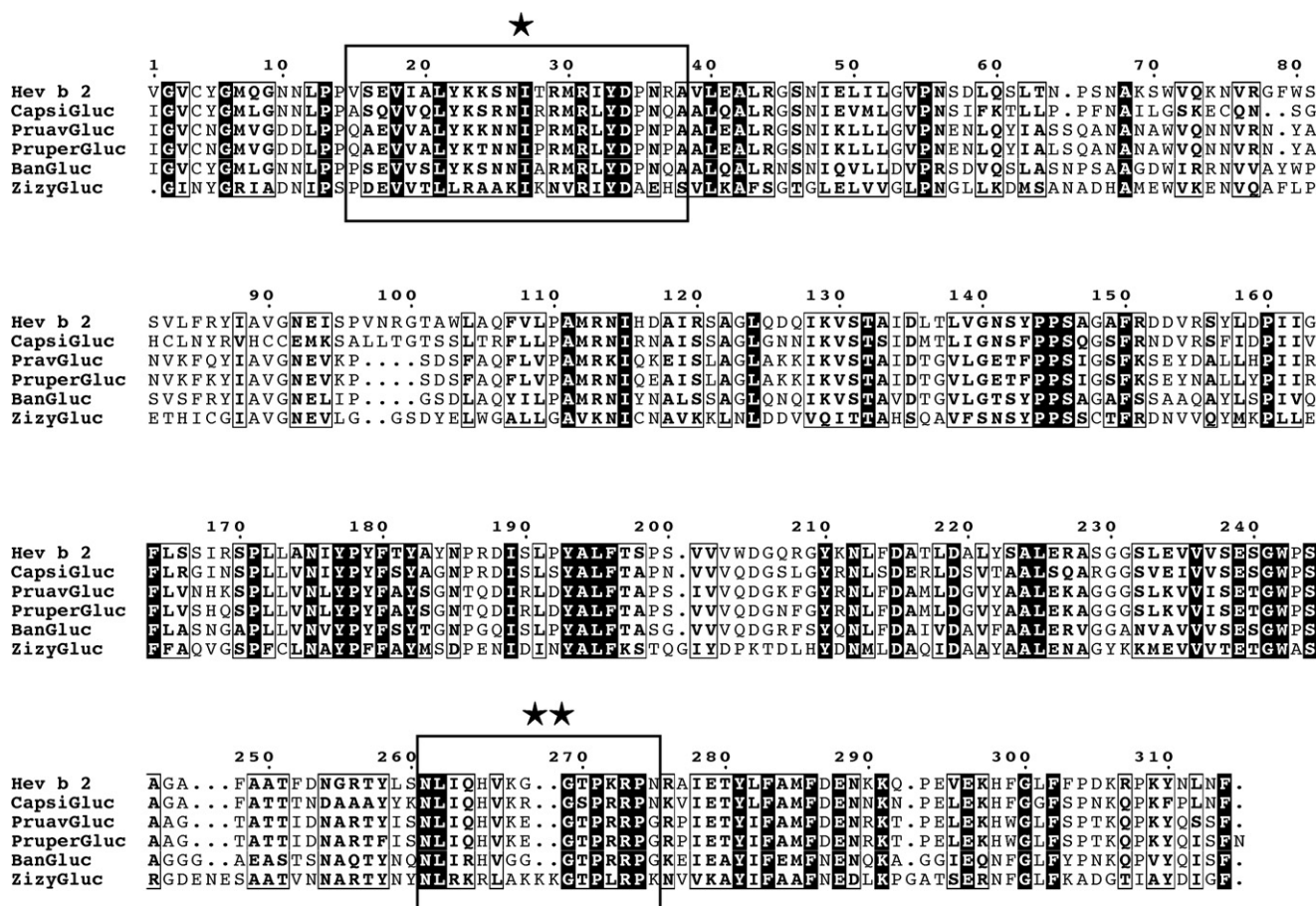


Fig. 7. Multiple amino acid sequence alignment of different 1,3β-glucanases showing the high degree of conservation of regions corresponding to IgE-binding epitope #1 (box *) and #7 (box **) of Hev b 2. Identical residues are in black boxed white letters, structurally homologous residues (according to (Gaboriaud et al., 1987)) are in open boxed black letters.

20–40% sequence identity except for that corresponding to epitope #7. Since this epitope displays 60% sequence identity and in addition exhibits a partially similar conformation (Fig. 4E) it might act as a IgE-binding cross-reactive epitope.

3.4. The identified IgE-binding epitopes account for the allergenicity

The reactivity of the IgE-binding epitopes identified in BanGluc to actually interact with IgE from allergic patients was assessed in ELISA inhibition experiments showing that a 2-mM concentration of synthetic peptides corresponding to epitopes #1 (NNIARMRLYDPNQAA), #2 (IRRNVVAYWPSVSFR), #4 (YNQNLIRHVGGGTPR) and #5 (QNFGFLFYPNKQPVYQ), inhibited the BanGluc/IgE interaction by 19%, 41%, 29% and 12%, respectively (Fig. 5). Using rabbit polyclonal IgG antibodies against BanGluc, up to 10 IgE-binding epitopic regions were identified along the amino acid sequence of BanGluc (Fig. 3B). These epitopic regions partially overlap with the 5 IgE-binding epitopic stretches previously identified using IgE from sensitive human patients. The IgG-binding epitopic regions roughly coincide with most of the 9 IgE-binding epitopic regions characterized along the amino acid sequence of Hev b 2 (IgE-epitopes #1 + #2 BanGluc/IgE-epitope #1 Hev b 2, IgE-epitope #4 BanGluc/IgE-epitope #2 Hev b 2, IgE-epitope #6 BanGluc/IgE-epitope #4 Hev b 2, IgE-epitope #7 BanGluc/IgE-epitope #6 Hev b 2, IgE-epitope #9 BanGluc/IgE-epitope #7 Hev b 2, IgE-epitope #10 BanGluc/IgE-epitope #8 Hev b 2).

3.5. Some of the IgE-binding epitopes of BanGluc are predicted to resist digestive proteolysis

Since as a food allergen, BanGluc is exposed to the digestive enzymes pepsin, trypsin and chymotrypsin some of the IgE-binding epitopes might be altered in such a way that they are no longer recognized by the immunocompetent cells. BanGluc (2CYG) contains 18 and 27 putative trypsin and chymotrypsin cleavage sites, respectively, that are almost randomly distributed along its amino acid sequences (Fig. 6A and B). However, the number of sites that are actually accessible for a proteolytic attack is much lower (12 and 3, respectively) because they are not exposed on the molecular surface of the protein. Hence proteolytic degradation by trypsin and chymotrypsin presumably consists of a peeling off which eliminates only the most exposed regions leaving intact an unattacked core structure corresponding to approximately 45% of the polypeptide chain (Fig. 6C). This core essentially consists of the tightly packed arrangement of β -sheets forming the central crown of the TIM-barrel fold. The occurrence in BanGluc of a tightly packed core that remains poorly attacked by digestive enzymes, should protect most of the IgE-binding epitope #1 and part of epitope #2 (Fig. 6D). Therefore, these epitopes should remain available for a proper recognition by the competent immune cells to trigger some allergenic cross-reaction in latex allergic individuals. A similar analysis performed on Hev b 2 indicates that most of epitopes #1 and #4, and part of epitopes #2 and #5 resist proteolysis by trypsin-like enzymes occurring in inner skin cell layers (Fig. 6A–D). However, the trypsin/chymotrypsin hydrolysis simulation does not take into account the previous pepsin digestion which is also susceptible to drastically reduce the core structure by both a peeling off action on the exposed pepsin sites and the unmasking of previously buried trypsin/chymotrypsin sites.

4. Discussion

Individuals allergic to natural rubber latex (NRL) often display an associated hypersensitivity to freshly consumed fruits described as the so-called “latex-fruit syndrome” (LFS) (Blanco,

2003). Various fruits have been incriminated in LFS, including banana, avocado, chestnut, bell pepper, kiwi, peach and tomato (Wagner and Breiteneder, 2002). As the most prevailing hypothesis for explaining LFS, the occurrence in the incriminated fruits of cross-reacting allergens similar to those found in NRL has been suggested. Pathogenesis-related proteins (PR proteins) corresponding to two major allergens of NRL, hevein (Hev b 6.02) and 1,3 β -glucanase (Hev b 2) and patatin-like protein (Hev b 7), have been identified in fruits. A class I endochitinase of PR-3 family, that contains an N-terminal hevein-like domain, has been identified as the causative allergen in avocado fruits (Seppälä et al., 2000). Endo- β 1,3-glucanases of PR-2 family, have been characterized in bell pepper (Wagner et al., 2004) and banana (Muller et al., 1998). In addition, profilin of bell pepper (Schmidt et al., 2002) and the lipolytic patatin-like protein of potato (Posch et al., 1999), which correspond to the minor allergens Hev b 8 and Hev b 7 of NRL, respectively, have been reported as IgE-binding allergens. More recently, a class III chitinase was identified as a major allergen (Zim m 1) of the latex cross-reacting Indian jujube (*Zizyphus mauritiana*) fruit (Lee et al., 2008). These fruit allergens might contain some IgE-binding epitopes structurally similar to those occurring in the major latex allergens, suspected to trigger an allergenic response in individuals allergic to NRL.

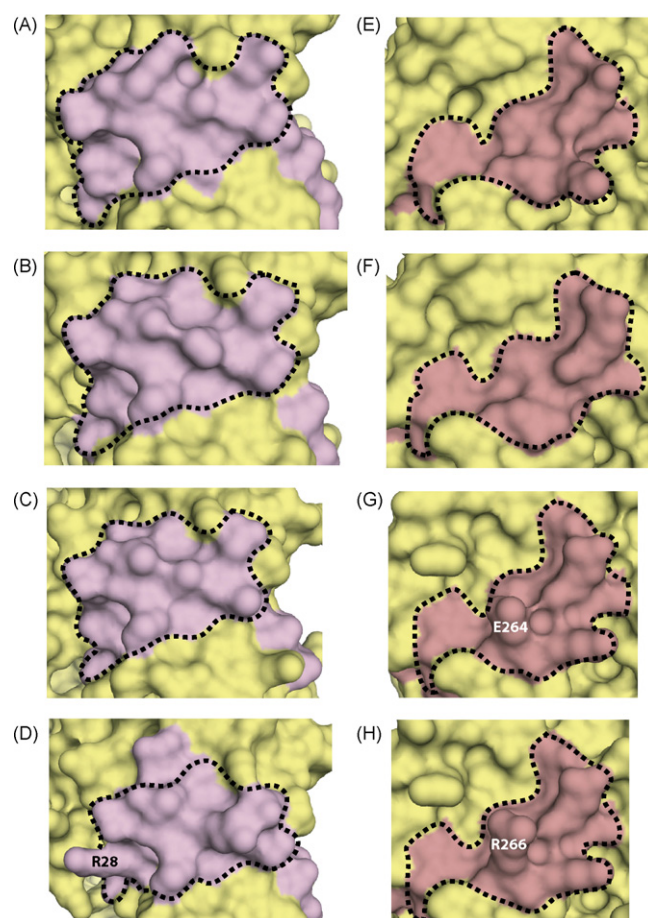


Fig. 8. Left panel: molecular surface of IgE-binding epitopes #1 of Hev b 2 (A) and BanGluc (B), and the corresponding regions on the molecular surface of PrupeGluc (C) and CapsiGluc (D). Right panel: molecular surface of IgE-binding epitopes #7 of Hev b 2 (A) and BanGluc (B), and the corresponding regions on the molecular surface of PrupeGluc (C) and CapsiGluc (D). Regions exhibiting a very similar conformation are shown with dashed line. Some discrepancies exist in the overall conformation of epitopic regions of PrupeGluc and CapsiGluc due to the replacement of tiny residues by the more bulky R28, E264 and R266 residues.

Although a class I chitinase has been recognized as a major banana IgE-binding allergen (Sanchez-Monge et al., 1999), the 1,3 β -glucanase BanGluc also occurs in banana pulp in sufficient amounts (Peumans et al., 2000) to account for another major allergen, like Hev b 2 in latex. In this respect, conserved IgE-binding epitopes were identified along the amino acid sequences of BanGluc and Hev b 2 in Spot experiments using IgE from latex allergic patients as a probe (Fig. 2; Table 2). Moreover, these common IgE-binding epitopes exhibit a very similar conformation on the molecular surface of both allergens (Fig. 4) suggesting that both 1,3 β -glucanases are efficiently recognized by the corresponding IgE from latex allergic patients. Even though they display very similar amino acid sequences, epitope #1 of BanGluc readily differs from the corresponding epitope #1 of Hev b 2 by the absence of an *N*-glycan (linked to Asn27 in Hev b 2) since the Asn26–Ile–Ala stretch corresponding to the *N*-glycosylation site Asn27–Ile–Thr of Hev b 2 can not be post-translationally glycosylated. Inhibition ELISA experiments performed with different glycoforms of Hev b 2 supported the relevance of carbohydrate epitopes in both the *in vitro* IgE recognition and cross-reactivity (Blanco, 2003). However, as shown from the Spot experiments, the IgE-binding epitopic character of the non-glycosylated synthetic epitope #1 of Hev b 2 suggests that the corresponding glycosylated epitope could be deglycosylated in the antigen presenting cells prior to be recognized by the immunocompetent mast cells.

Besides BanGluc, 1,3 β -glucanases from other fruits (bell pepper, peach, cherry, jujuba) that have been incriminated in LFS, share high

percentages of amino acid sequence similarities with Hev b 2, especially in sequence stretches corresponding to IgE-binding epitopes #1 and #7 identified in Hev b 2 (Fig. 7). A surface analysis of the modeled 1,3 β -glucanases revealed a three-dimensional conformation very similar to that found in Hev b 2 for these two epitope regions (except for a few discrepancies due to the replacement of tiny residues by more bulky residues, e.g. E264 and R266 in PrupeGluc and CapsiGluc, respectively) (Fig. 8). Accordingly, they are predicted as consensus IgE-binding epitopes suspected to account for the IgE-binding cross-reactivity observed in the LFS. Other structurally related IgE-binding epitopes apparently occur in Ole e 9, the 1,3 β -glucanase allergen of the olive tree pollen supporting the idea that 1,3 β -glucanases are implicated as major allergens in a broader latex-fruit-vegetable syndrome (Palomares et al., 2005). In addition to IgE-binding epitopes occurring in the TIM-barrel structure of Ole e 9, other epitopic regions were identified in the extended C-terminal domain associated to the TIM-barrel (Trevino et al., 2008).

It is generally believed that 1,3 β -glucanases are involved in the defence of plants against phytopathogenic fungi. Most 1,3 β -glucanases possess antifungal activity, indeed, and are synthesized in response to fungal attack (that is why they are called pathogenesis-related proteins). However, the accumulation of relatively high concentrations of 1,3 β -glucanases in fruits (banana, cherry, bell pepper, peach) seems to be developmentally regulated rather than pathogen induced. To check whether the fruit-specific 1,3 β -glucanases possibly represent a separate subgroup a phyloge-

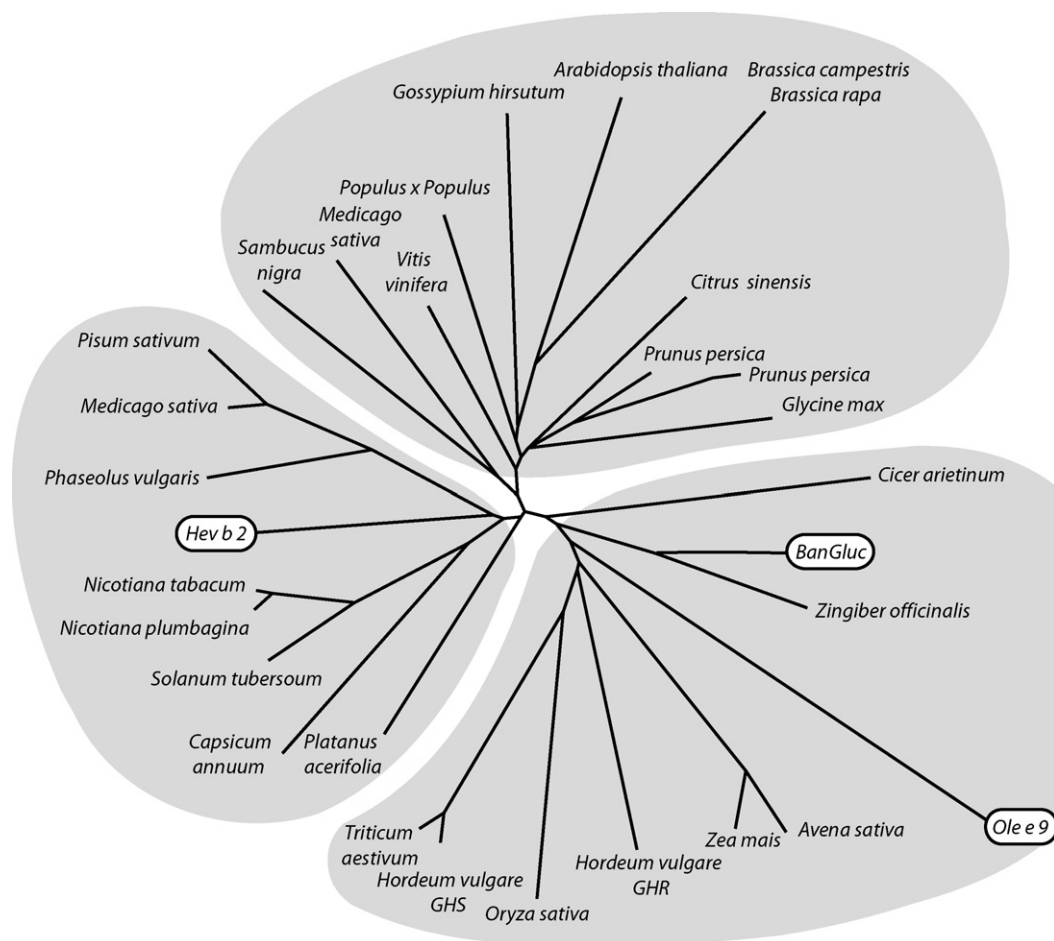


Fig. 9. Parsimony tree showing the grouping of 1,3 β -glucanases in three distinct clades. All of the fruit allergens identified so far occur in each of the three clades. Although occurring in pollen, the olive Ole e 9 allergen is closely related to BanGluc.

netic analysis of the available sequences was performed. As shown in Fig. 9, the parsimony tree consists of three separate branches each of which comprises some of the fruit-specific sequences. Hence, it can be concluded that there is not an evolutionarily distinct clade of fruit enzymes. This is an important observation because allergenicity is apparently not linked to a well-defined phylogenetic group. Hence, it can be concluded that 1,3 β -glucanases in addition to chitinases (Lee et al., 2008; Huecas et al., 2001) are good candidates as cross-reacting allergens responsible for the latex-fruit-vegetable syndrome (Palomares et al., 2005; Huecas et al., 2001).

IgE-binding epitopes of other major allergens from rubber tree latex have already been characterized (Alenius et al., 1996; Beezhold et al., 1997, 1999; Karisola et al., 2002), as a prerequisite for the production of recombinant hypoallergens suitable for application in immunotherapy. The aim is to produce mutated allergens, which display an attenuated B-cell reactivity but retain their T-cell reactivity, to prevent undesired life-threatening reactions in treated allergic patients (Beezhold et al., 2001; Karisola et al., 2004). The characterization of IgE-binding epitopes reported in this work should pave the way for the use of the major latex allergen Hev b 2 as an hypoallergen in latex allergic patients, especially for preventing the latex-fruit-vegetable syndrome.

Acknowledgements

The financial support of CNRS (A.B., R.C., V.S., P.R.) and the Société Française d'Allergologie et d'Immunologie Clinique (A.B., P.R.) is gratefully acknowledged. The authors thank C. Nguyen for expert technical assistance in Spot synthesis.

References

- Alenius, H., Kalkkinen, N., Reunala, T., Turjanmaa, K., Palosuo, T., 1996. The main IgE-binding epitope of a major latex allergen, prohevein, is present in its N-terminal 43-amino acid fragment, hevein. *J. Immunol.* 156, 1618–1625.
- Beezhold, D.H., Sussman, G.L., Liss, G.M., Chang, N.S., 1996. Latex allergy can induce clinical reactions to specific foods. *Clin. Exp. Allergy* 26, 416–422.
- Beezhold, D.H., Kostyal, D.A., Sussman, G.L., 1997. IgE epitope analysis of the hevein preprotein: a major latex allergen. *Clin. Exp. Immunol.* 108, 114–121.
- Beezhold, D.H., Hickey, V.L., Slater, J.E., Sussman, G.L., 1999. Human IgE-binding epitopes of the latex allergen Hev b 5. *J. Allergy Clin. Immunol.* 103, 1166–1172.
- Beezhold, D.H., Hickey, V.L., Sussman, G.L., 2001. Mutational analysis of the IgE epitopes in the latex allergen Hev b 5. *J. Allergy Clin. Immunol.* 107, 1069–1076.
- Berman, H.M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T.N., Weissig, H., Shindyalov, I.N., Bourne, P.E., 2000. The protein data bank. *Nucleic Acid Res.* 28, 235–242.
- Bernstein, D.I., Karnani, R., Biagini, R.E., Bernstein, C.K., Murphy, K., Berends, B., Bernstein, J.A., Bernstein, L., 2003a. Clinical and occupational outcomes in health care workers with natural rubber latex allergy. *Ann. Allergy Asthma Immunol.* 90, 179–180.
- Bernstein, D.I., Biagini, R.E., Karnani, R., Hamilton, R., Murphy, K., Bernstein, C., Arif, S.A., Berends, B., Yeang, H.Y., 2003b. *In vivo* sensitization to purified *Hevea brasiliensis* proteins in health care workers sensitized to natural rubber latex. *J. Allergy Clin. Immunol.* 111, 610–616.
- Blanco, C., 2003. Latex-fruit syndrome. *Curr. Allergy Asthma Rep.* 3, 47–53.
- Blanco, C., Carrillo, T., Castillo, R., Quirarte, J., Cuevas, M., 1994. Latex allergy: clinical features and cross-reactivity with fruits. *Ann. Allergy* 73, 277–281.
- Brehler, R., Theissen, U., Mohr, C., Luger, T., 1997. Latex-fruit syndrome: frequency of cross-reacting IgE antibodies. *Allergy* 52, 404–410.
- Chen, Z., Posch, A., Lohaus, C., Raulf-Heimsoth, M., Meyer, H.E., Baur, X., 1997. Isolation and identification of hevein as a major IgE-binding polypeptide in *Hevea* latex. *J. Allergy Clin. Immunol.* 99, 402–409.
- Frank, R., 1992. Spot-synthesis: an easy technique for the positionally addressable parallel chemical synthesis on a membrane support. *Tetrahedron* 48, 9217–9232.
- Gaboriaud, C., Bissery, V., Benchetrit, T., Mornon, J.P., 1987. Hydrophobic cluster analysis: an efficient new way to compare and analyse amino acid sequences. *FEBS Lett.* 224, 149–155.
- Gallo, R., Cozzani, E., Guarrera, M., 1997. Sensitization to pepper (*Capsicum annuum*) in a latex-allergic patient. *Contact Dermatitis* 37, 36–37.
- Gilson, M.K., Honing, B.H., 1987. Calculation of electrostatic potential in an enzyme active site. *Nature* 330, 84–86.
- Gouet, P., Courcelle, E., Stuart, D.I., Metoz, F., 1999. ESPript: analysis of multiple sequence alignments in PostScript. *Bioinformatics* 15, 305–308.
- Huecas, S., Villalba, M., Rodríguez, R., 2001. Ole e 9, a major olive pollen allergen is a 1,3- β -glucanase. *J. Biol. Chem.* 276, 27959–27966.

- Jung, H.W., Hwang, B.K., 2000. Pepper gene encoding a basic beta-1,3-glucanase is differentially expressed in pepper tissues upon pathogen infection and ethephon or methyl jasmonate treatment. *Plant Sci.* 159, 97–106.
- Karisola, P., Alenius, H., Mikkola, J., Kalkkinen, N., Helin, J., Pentikainen, O.T., Repo, S., Reunala, T., Turjanmaa, K., Johnson, M.S., Palosuo, T., Kulomaa, M.S., 2002. The major conformational IgE binding epitopes of hevein (Hev b 6 02) are identified by a novel chimera-based allergen mapping strategy. *J. Biol. Chem.* 277, 22656–22661.
- Karisola, P., Mikkola, J., Kalkkinen, N., Airenne, K.J., Laitinen, O.H., Repo, S., Pentikainen, O.T., Reunala, T., Turjanmaa, K., Johnson, M.S., Palosuo, T., Kulomaa, M.S., Alenius, H., 2004. Construction of hevein (Hev b 6 02) with reduced allergenicity for immunotherapy of latex allergy by comutation of six amino acid residues on the conformational IgE epitopes. *J. Immunol.* 172, 2621–2628.
- Laskowski, R.A., MacArthur, M.W., Moss, D.S., Thornton, J.M., 1993. PROCHECK: a program to check the stereochemistry of protein structures. *J. Appl. Cryst.* 26, 283–291.
- Laune, D., Molina, F., Ferrieres, G., Villard, S., Bes, C., Rieunier, F., Chardes, T., Granier, C., 2002. Application of the Spot method to the identification of peptides and amino acids from the antibody paratope that contribute to antigen binding. *J. Immunol. Methods* 267, 53–70.
- Lee, M.F., Tsai, J.J., Hwang, G.Y., Lin, S.J., Chen, Y.H., 2008. Identification of immunoglobulin E (IgE)-binding epitopes and recombinant IgE reactivities of a latex cross-reacting Indian jujube Ziz m 1 allergen. *Clin. Exp. Immunol.* 152, 464–471.
- M'Raihi, L., Charpin, D., Pons, A., Bongrand, P., Vervloet, D., 1991. Cross-reactivity between latex and banana. *J. Allergy Clin. Immunol.* 87, 129–130.
- Mas, M.T., Smith, K.C., Yarmush, D.L., Aisaka, K., Fine, R.M., 1992. Modeling the anti-CEA antibody combining site by homology and conformational search. *Proteins Struct. Funct. Genet.* 14, 483–498.
- Muller, J.J., Thomsen, K.K., Heinemann, U., 1998. Crystal structure of barley 1,3-1,4- β -glucanase at 2.0-Å resolution and comparison with *Bacillus* 1,3-1,4- β -glucanase. *J. Biol. Chem.* 273, 3438–3446.
- Nicholls, A., Sharp, K.A., Honing, B., 1991. Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbons. *Proteins Struct. Funct. Genet.* 11, 281–296.
- Niggemann, B., Breiteneder, H., 2000. Latex allergy in children. *Int. Arch. Allergy Immunol.* 121, 98–107.
- Page, R.D.M., 1996. TREEVIEW: an application to display phylogenetic trees on personal computers. *Comput. Appl. BioSci.* 12, 357–358.
- Palomares, O., Villalba, M., Quirarte, J., Polo, F., Rodríguez, R., 2005. 1,3- β -glucanases as candidates in latex-pollen-vegetable food cross-reactivity. *Clin. Exp. Allergy* 35, 345–351.
- Peumans, W.J., Barre, A., Derycke, V., Rougé, P., Zhang, W., May, G.D., Delcour, J.A., Van Leuven, F., Van Damme, E.J.M., 2000. Purification, characterization and structural analysis of an abundant β -1,3-glucanase from banana fruit. *Eur. J. Biochem.* 267, 1188–1195.
- Ponder, J.W., Richards, F.M., 1987. Tertiary templates for proteins. Use of packing criteria in the enumeration of allowed sequences for different structural classes. *J. Mol. Biol.* 193, 775–791.
- Posch, A., Wheeler, C.H., Chen, Z., Flagge, A., Dunn, M.J., Papenfuss, F., Raulf-Heimsoth, M., Baur, X., 1999. Class I endochitinase containing a hevein domain is the causative allergen in latex-associated avocado allergy. *Clin. Exp. Allergy* 29, 667–672.
- Receveur-Bréchet, V., Czjzek, M., Barre, A., Roussel, A., Peumans, W.J., Van Damme, E.J.M., Rougé, P., 2006. Crystal structure at 1.45-Å resolution of the major allergen endo- β -1,3-glucanase of banana as a molecular basis for the latex-fruit syndrome. *Proteins* 63, 235–242.
- Risler, J.-L., Delorme, M.O., Delacroix, H., Henaut, A., 1988. Amino acid substitutions in structurally related proteins. A pattern recognition approach. Determination of a new and efficient scoring matrix. *J. Mol. Biol.* 204, 1019–1029.
- Roussel, A., Cambillau, C., 1989. In: Committee, S.G. (Ed.), TURBO-FRDO. Silicon Graphics Geometry Partners Directory. Silicon Graphics, Mountain View, CA.
- Sanchez-Monge, R., Blanco, C., Díaz-Perales, A., Collada, C., Carrillo, T., Aragoncillo, C., Salcedo, G., 1999. Isolation and characterization of major banana allergens: identification as fruit class I chitinases. *Clin. Exp. Allergy* 29, 673–680.
- Schmidt, M.H., Raulf-Heimsoth, M., Posch, A., 2002. Evaluation of patatin as a major cross-reactive allergen in latex-induced potato allergy. *Ann. Allergy Asthma Immunol.* 89, 613–618.
- Seppälä, U., Palosuo, T., Seppälä, U., Kalkkinen, N., Ylitalo, L., Reunala, T., Turjanmaa, K., Reunala, T., 2000. IgE reactivity to patatin-like latex allergen, Hev b 7, and to patatin of potato tuber, Sol t 1, in adults and children allergic to natural rubber latex. *Allergy* 55, 266–273.
- Slater, J.E., Vedvick, T., Arthur-Smith, A., Trybul, D.E., Kekwick, R.G., 1996. Identification, cloning, and sequence of a major allergen (Hev b 5) from natural rubber latex (*Hevea brasiliensis*). *J. Biol. Chem.* 271, 25394–25399.
- Sunderasan, E., Hamzah, S., Hamid, S., Ward, M.A., Yeang, H.Y., Cardoso, M.J., 1996. Latex B-serum β -1,3-glucanase (Hev b 2) and a component of the microhelix (Hev b 4) are major latex allergens. *J. Nat. Rubber Res.* 10, 82–99.
- Thimmapuran, J., Ko, T.S., Korb, S.S., 2001. Characterization and expression of beta-1,3-glucanase genes in peach. *Mol. Genet. Genomics* 265, 469–479.
- Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL-X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tool. *Nucleic Acids Res.* 25, 4876–4882.
- Trevino, M.A., Palomares, O., Castrillo, I., Villalba, M., Bruin, M., 2008. Solution structure of the C-terminal domain of Ole e 9, a major allergen of olive pollen. *Protein Sci.* 17, 371–376.

- Wagner, S., Breiteneder, H., 2002. The latex-fruit syndrome. *Biochem. Soc. Trans.* 30, 935–940.
- Wagner, S., Radauer, C., Hafner, C., Fuchs, H., Jensen-Jarolim, E., Wüthrich, B., Scheiner, O., Breiteneder, H., 2004. Characterization of cross-reactive bell pepper allergens involved in the latex-fruit syndrome. *Clin. Exp. Allergy* 34, 1739–1746.

- Yagami, T., Osuna, H., Kouno, M., Haishima, Y., Nakamura, A., Ikezawa, Z., 2002. Significance of carbohydrate epitopes in latex allergen with β -1,3-glucanase activity. *Int. Arch. Allergy Immunol.* 129, 27–37.
- Yeang, H.Y., 2004. Natural rubber latex allergens: new developments. *Curr. Opin. Allergy Clin. Immunol.* 4, 99–104.