Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 314 (2004) 123–130
BBRC
www.elsevier.com/locate/ybbrc

Insights into a conformational epitope of Hev b 6.02 (hevein)

César A. Reyes-López,[a] Alejandra Hernández-Santoyo,[a] Martha Pedraza-Escalona,[a] Guillermo Mendoza,[b] Andrés Hernández-Arana,[c] and Adela Rodríguez-Romero[a,*]

[a] *Instituto de Química, Universidad Nacional Autónoma de México, Circuito Exterior, Cd. Universitaria, Coyoacán México, DF 04510, Mexico*
[b] *Departamento de Bioquímica, Facultad de Medicina, Universidad Nacional Autónoma de México, Coyoacán México, DF 04510, Mexico*
[c] *Departamento de Química, Universidad Autónoma Metropolitana-Iztapalapa, P.O. Box 55-534, Iztapalapa México, DF 09340, Mexico*

Received 27 November 2003

Abstract

Hevein (Hev b 6.02) is a major IgE-binding allergen in natural rubber latex and manufactured products. Both tryptophans (Trp^{21} and Trp^{23}) of the hevein molecule were chemically modified with BNPS-skatole (2-nitrophenylsulfenyl-3-methyl-3′-bromoindolenine); derivatized allergen failed to significantly inhibit binding of serum IgE in ELISA assays. Similarly, skin prick tests showed that hevein-positive patients gave no response with the modified allergen. Dot blot experiments carried out with anti-hevein mono- and polyclonal antibodies confirmed the importance of Trp^{21} and Trp^{23} for antibody-recognition, and demonstrated the specific cross-reactivity of other molecules containing hevein-like domains. We also report the structure of Hev b 6.02 at an extended resolution (1.5 Å) and compare its surface properties around Trp residues with those of similar regions in other allergens. Overall our results indicate that the central part of the protein, which comprises three aromatic and other acidic and polar residues, constitutes a conformational epitope.

Keywords: Allergens; Hev b 6.02; Hevein; Latex; IgE-epitope; X-ray structure

Type I allergic diseases are the result of inappropriate immune responses to certain otherwise innocuous proteins (allergens), in people with a genetic predisposition or atopy. It is well established that the recognition of allergens by immunoglobulin E (IgE) is the key step to the allergic response. The allergen is bound to the IgE high-affinity receptor complex on mast cells and, if this occurs, mediator release is induced leading to the allergic inflammation [1].

Allergenic proteins are structurally and functionally a heterogeneous group, including hydrolytic enzymes, enzyme inhibitors, carbohydrate-binding proteins, actin-binding proteins, and others without a known biological activity [2]. Many structural features of proteins could potentially contribute towards their allergenicity, including availability and identity of the epitopes. However, the stability, the presence of glycosyl groups, and enzymatic or lectin activities can influence allergenicity [3], although in many instances the mechanisms remain unclear. Natural rubber latex (NRL) proteins are a well-recognized cause of type-I allergic reactions that increasingly affect health-care workers and other people using latex products. It is also known that latex allergy is often combined with allergy to certain fruits such as banana, avocado, and kiwi [4], which contain class I chitinases with a hevein-like domain. In addition, cross-reactivity of latex allergens with pollen allergens from ragweed, timothy grass, and mugwort has been described [5]. In general, the presence of similar or common epitopes might explain this cross-reactivity.

Basically two types of IgE epitopes have been identified. Epitopes that consist of a few continuous amino-acid residues are referred to as linear or sequential [6], whereas those composed of at least two non-adjacent regions of the molecule that are brought into close proximity within the folded structure are named discontinuous or conformational. Since most IgE epitopes appear to be conformational [7], it is clear that a proper understanding of the interactions by which allergens are

* Corresponding author. Fax: +52-55-56-16-22-17.
E-mail address: adela@servidor.unam.mx (A. Rodríguez-Romero).

0006-291X/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2003.12.068

recognized by IgE antibodies would benefit greatly from the accurate knowledge of allergen structures.

Hev b 6.02, one of the main allergens from rubber (*Hevea brasiliensis* latex), is a lectin-like protein that also acts as a defense-related protein [8] and whose presence has been demonstrated in NRL products [9]. In addition, nature furnishes several isoforms of hevein, one of them, known as pseudohevein (isoform 1), with six amino acid mutations [10]. Hevein-like domains are also present in agglutinins from wheat germ (four domains) [11] and from *Urtica dioica* (two domains) [12]. In these agglutinins the sugar-binding site is mainly constituted by three aromatic amino acids, which interact with sugar rings through stacking contacts.

To gain new insight into the nature of the IgE epitopes for these proteins, we performed immunological experiments using Hev b 6.02, isoform 2, and the protein modified at positions Trp^{21} and Trp^{23} with BPNS-skatole. We also describe the characterization of the chemical modification by MALDI-TOF experiments. Finally, due to the importance of the carbohydrate-binding region and that some conformational differences were reported between the structure solved by X-ray techniques (2.8 Å) [13] and the one in solution [14,15], we performed a crystallographic study of the allergen at high resolution (1.5 Å). Based on a detailed structural comparison of this protein with allergens from plants such as Bet v 2 (*Betula verrucosa*), Hev b 8 (*H. brasiliensis*), and the agglutinin from wheat germ (WGA), we propose an explanation for the observed cross-reactivity pattern among the allergens under consideration.

Experimental procedures

Protein purification and crystallization. Purification and crystallization of Hev b 6.02 (clon IAN-710) have been described elsewhere [13]. In this work, crystals were grown at 18 °C by the sitting drop method, from a solution containing 60% MPD, 40 mM Tris–HCl, pH 7.1.

Chemical modification of the tryptophan residues of Hev b 6.02. Modification was carried out according to Omenn et al. [16]. Briefly, to a solution of 1 mg Hev b 6.02 in 60% acetic acid, 28 μl of a 2 mg/ml solution of 3-BNPS-Skatole in glacial acetic acid was added (15 min at room temperature). The mixture was then applied to a Sephadex G-10 column (1.0 × 100 cm) equilibrated with 1.5 M acetic acid. The fraction containing modified Hev b 6.02 was dialyzed against distilled water.

Enzymatic digestion of natural and modified Hev b 6.02 with Glu-C and characterization using MALDI-TOF mass spectrometry. Natural (control) and oxidized Hev b 6.02 (350 μg) were treated with 6 M guanidine HCl for 15 min (37 °C). Dithiothreitol (5 mM) in 0.15 mM Tris, pH 8.8, was then added to the samples (37 °C, 20 min). Iodoacetamide (10 mM) in the same buffer was added to the samples (20 min at room temperature). The reaction mixtures were then transferred to a High-Trap desalting column (Pharmacia). Five micrograms of each of the samples was applied to a Superdex-peptide column (16/30) (Pharmacia) equilibrated with the Tris buffer. The rest of the proteins were digested with 5 μg endoproteinase Glu-C (Boehringer–Mannheim), which selectively cleaves peptide bond's C-terminal to glutamic acid residues, for 60 h at 37 °C, in the same Tris buffer. After this, the N-terminal sequence for the peptides obtained was determined by automated Edman degradation on a gas-phase protein sequencer (LF 3000, Beckman Instruments, Irvine, CA).

For the matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) experiments, 270 μg of the digested proteins was applied to a reversed phase column (C-18, 4.6 × 150 mm, Waters). The purified peptides were mixed with α-cyano-4-hydroxycinnamic acid in 30% acetonitrile, 70% water, and 0.1% TFA, and analyzed in a OMNIFLEX MALDI-TOF instrument (Bruker-Daltonics) which has a 337.1 nm nitrogen laser and 19 kV accelerating voltage in the linear mode.

CD analysis of native and chemically modified Hev b 6.02. CD spectra were recorded on a JASCO J-700 spectropolarimeter at 20 °C. The spectral range was 200–260 nm at 10 nm/min and three scans were accumulated. The protein concentration was 0.2 mg/ml in a 10 mM phosphate buffer, pH 7.0.

Enzyme-linked immunosorbent assay inhibition experiments. Briefly, microplates were coated with 5 μg/well of hevein in sodium carbonate buffer (pH 9.6) and blocked. Afterwards, a serum pool of 10 high-risk pediatric patients (Instituto Nacional de Pediatria, Mexico City), diluted 1:10, was co-incubated (2 h at 37 °C) with either Hev b 6.02, isoform 2, or the allergen chemically modified, at five different concentrations (0.1–25 μg/ml). Plates were washed and biotin-labeled mouse anti-human IgE (1:500 dilution) was allowed to react for 1 h at 37 °C. The plates were then washed and incubated with 100 μl/well of streptavidin-peroxidase diluted 1:2000 for 1 h at 37 °C. Produced color was read at 490 nm using an automated Enzyme-linked immunosorbent assay (ELISA) reader (Dynatech MR5000).

Dot blot assays. These experiments were performed to assess the possible cross-reactivity among allergens. Proteins (Hev b 6.02, Hev b 8 (profilin), Hev b 11 (class I chitinase), pseudohevein (isoform 1), isoform 2, chemically modified Hev b 6.02, WGA, and bovine serum albumin (BSA) as a negative control) were applied to nitrocellulose membranes (25 μg/dot), and then washed and blocked. The membranes were incubated with either rabbit polyclonal (PAb) or mouse monoclonal (MAb) IgGs raised against Hev b 6.02. The reaction was revealed by incubation with peroxidase-conjugated anti-rabbit or anti-mouse IgGs and the binding was detected by chemioluminescence.

Skin prick tests. Tests were performed using intact Hev b 6.02 and the protein modified at positions Trp^{21} and Trp^{23} in 100 volunteer health-care workers (50% high-risk individuals). The Ethics Committee of the Instituto Nacional de Pediatria, Mexico City, approved this study. The negative control was allergen diluent (Evans solution) and the positive 1.0 mg/ml histamine solution. Five milliliters of solution containing an allergen (1.0–10.0 μg/ml) was placed on the skin, which was then pricked with the tip of a needle. The presence of a wheal larger than the one produced by histamine (2–4 mm) and no reaction with the Evans solution indicated the presence of IgE against the allergen.

X-ray diffraction analysis. X-ray diffraction data were collected, from a single crystal, at 277 K at the Stanford Synchrotron Radiation Laboratory (Station 7-1) using a MAR 30 image plate detector. Diffraction data were processed with the programs DENZO [17] and CCP4 [18]. The structure was solved by molecular replacement using CNS [19] with the protein solved by NMR methods as the search model (PDB entry 1HEV). Initial crystallographic refinement was done with CNS. Simulated annealing was used and each refinement cycle was followed by fitting of the model into sigma weighted electron density maps using QUANTA2000 (Molecular Simulations Inc., San Diego, CA). Cross-validation [20] was employed throughout and 10% of the data were used for the R_{free} calculation. The initial structure and topology files of MPD were obtained from the website HIC-Up [21]. Solvent molecules were placed using the X-SOLVATE routine in QUANTA2000 [22].

High-resolution refinement with SHELX97 [23] was started using the model from the CNS refinement. Protein bond and angle restraints used were of Engh and Huber [24]. Anisotropic atom *B*-factors were

introduced in a conjugate gradient least-squares (CGLS) minimization, in which the data-to-parameter ratio was 2.1. Anti-bumping distances restraints were added to prevent non-bonded collisions. The refinement of the MPD molecules was carried out against parameters of the SHELXL dictionary [25]. With this model, the R_{free} from the isotropic refinement dropped by 3.1 percentage points to 16.01%. A final step was carried out using a least-squares cycle (including all data) converging at R-factor $= 0.128$ ($F > 4\sigma$). A summary of the data-collection and refinement statistics is given in Table 1.

Structure analysis and comparisons. Analysis of the stereochemistry of the model was done using the program PROCHECK [26]. The molecular superposition of Hev b 6.02 with the two structures obtained by NMR methods was carried out with the program ALIGN [27]. The crystal structures of Bet v 2 (PDB code 1CQA), Hev b 8 (1G5U), and domain C WGA (9WGA) were obtained from the Protein Data Bank.

Table 1
Data collection and refinement statistics

(A) Data collection	
Space group	$P2_12_12$
Unit cell parameters (Å)	
a, *b*, *c*	31.81, 60.95, 22.51
No. of monomers per asymmetric unit	4
Resolution range (Å)	15–1.5
No. of observations	57,822
No. of unique reflections	7113
$I/\sigma(I)$	6.7
Completeness (%)	95.5
Multiplicity	4.7
R_{sym}[a] (%)	5.0
(B) Refinement	
R-factor (%)	12.8
R_{free}[b] (%)	14.5
No. of atoms	
Protein	345
Solvent	39
MPD	32
RMS deviation from ideal values	
Bond length (Å)	0.011
Bond angle (Å)	0.03
Residues in Ramachandran plot (%)	
Most allowed region	88.2
Allowed region	11.8
Average B-factors (Å^2)	
Protein	17.4
Water	33.3
MPD	36.7

[a] $R_{sym} = 100\Sigma_h\Sigma_i|I_i(h) - [I(h)]|/\Sigma_h I(h)$, where $I_i(h)$ is the ith measurement of the h reflection and $[I(h)]$ is the average value of the reflection intensity.

[b] R_{free} is for 10% of the total reflections.

Results and discussion

Characterization of Chemically Modified Hev b 6.02

Hev b 6.02. Hevein, either modified or not by BNPS-Skatole, was purified by reverse-phase HPLC and submitted to reduction with DTT to break its disulfide bridges; sulfhydryl groups so produced were alkylated with iodoacetamide. The alkylated forms gave nearly identical elution volumes on gel filtration, indicating that the polypeptide chain was not cleaved by treatment with BPNS-Skatole. MALDI-TOF mass spectrometry gave for the modified hevein a mass 40 Da larger than that for the untreated protein. The first five residues, as determined by sequence analysis, were the same for both proteins, confirming that the polypeptide chain was not cleaved by the oxidation treatment.

In parallel experiments, samples of both hevein forms were digested with Glu-C after reduction and alkylation. With both samples two peptides were separated by reverse-phase HPLC. The larger peptide obtained from hevein had a mass of 3424.7 Da, corresponding to the mass of segment 1–29 with its five cysteines alkylated. As determined by Edman degradation, the N-terminal residue of this peptide was Glu, which is the N-terminus of the whole protein. Thus, Glu-C cleaved the peptide bond C-terminal of G lu[29] (see Fig. 1). The larger peptide from chemically modified hevein showed a 40-Da increase in mass (3465.5 Da) with respect to the larger peptide from untreated hevein, and Glu was also its N-terminus. In contrast, the mass of the smaller peptide was the same regardless of which sample it came from. This mass corresponds to that of segment 30–43 with its three cysteines alkylated. Tyr was found at the N-terminus of this peptide, confirming the cleavage point of Glu-C. It is clear, therefore, that changes brought about by BNPS-Skatole were restricted to the region where the two Trp residues are located, whereas segment 30–43, which comprises one of the two IgE sequential epitopes of this protein [28] (see Fig. 1), was unaffected. The mass increase of 40 Da suggests that both tryptophans were modified, because derivatives with mass differences of +4 and +20 are known to result from oxidation of tryptophan residues [29,30]. Furthermore, the absorption spectrum of modified hevein increased at 250 nm and decreased at longer wavelengths with respect to that

```
                          10        20        30        40
                  ....|....| ....|....| ....|....| ....|....| ....|
Hev b 6.02        EQCGRQAGGK LCPNNLCCSQ WGWCGSTDEY CSPDHNCQSN CKD
Pseudo-hevein     EQCGRQAGGK LCPNNLCCSQ YGWCGSSDDY CSPSKNCQSN CKGGG
Hev b 11          EQCGRQAGGA LCPGGLCCSQ YGWCANTPEY CGSGCQSQCD GGV
WGA3              IKCGSQAGGK LCPNNLCCSQ WGYCGLGSEF CGEGCQNGAC STD
```

Fig. 1. Sequence comparison of Hev b 6.02, pseudohevein, isoform 3 of WGA (domain C), and class I chitinase (Hev b 11, hevein domain). The linear IgE epitopes of Hev b 6.02 are shadowed.

for the untreated protein, supporting the formation of oxindole moieties (results not shown) [16].

Circular dichroic spectra of Hev b 6.02 and its derivative (not shown) were found to be nearly identical from 250 to 200 nm. It has been reported previously that hevein exhibits an intense positive peak at 221 nm [31], which is also found between 220 and 228 nm in spectra of other small, disulfide-rich proteins [32]. Because this rather peculiar band is thought to be originated from amide groups adjacent to disulfide bridges [33], it is likely that neither disulfide bonds, which are essential for the global stability in proteins of this type, nor other structural characteristics of the polypeptide chain around these bonds were affected by chemical modification of Hev b 6.02.

ELISA inhibition and dot blot assays

ELISA inhibition with a serum pool from pediatric patients is shown in Fig. 2A. It is clear from these experiments that no significant inhibition of IgE binding to solid-phase Hev b 6.02 was observed with the modified allergen; in contrast, the isoform 2 inhibited binding in 75%. These results strongly suggest that the two Trp residues are necessary for the recognition of the molecule by IgE. Besides, it is evident that subtle differences are important for recognition, because the molecular characteristics of the isoform 2, which are currently under study, are likely to be very similar to those of Hev b 6.02, as judged from the small 3-Da mass difference between them (unpublished results). Additionally, dot blot assays were done in an effort to determine if model antibodies could specifically recognize surface epitopes on hevein-like antigens. We prepared a rabbit polyclonal antibody as well as a murine monoclonal antibody (6E7) raised against Hev b 6.02 (results not published). Experiments were carried out to test the reactivity of MAb 6E7 and the polyclonal Abs with several lectins (Hev b 6.02, isoform 2, pseudohevein, and WGA), a profilin (Hev b 8), and the chitinase (Hev b 11) from NRL, chemically modified Hev b 6.02, and bovine serum albumin as a control. The results obtained are shown in Fig. 2B. As can be observed, the MAb raised against Hev b 6.02 only reacted with WGA and the chitinase, indicating that antigen recognition is not due to unspecific lectin–carbohydrate reactions. It is noteworthy that the sequence identity between Hev b 6.02 and pseudohevein is 86%, whereas the chitinase is 60% and WGA domains are only about 50% identical to hevein. Indeed, only residues Trp^{21}, Glu^{29}, and Thr^{27} in Hev b 6.02 are conserved (either one or two of them) at equivalent positions in WGA domains and the chitinase, but not in pseudohevein (Fig. 1). This strongly suggests, therefore, that these residues, which are part of Hev b 6.02 linear epitopes and belong to the carbohydrate-binding region, are essential for the interaction of MAb 6E7 with hevein-like domains. This proposal is supported by the absence of reactivity of the MAb with chemically modified Hev b 6.02. Furthermore, the general relevance that aromatic and acidic side chains may have in IgE recognition by hevein-like domains is made apparent from a primary-structure comparison including 60 domains found in proteins that are recognized as allergens (WGA, Hev b 6.02, and Hev b 11) or potential allergens, belonging to the pathogenesis-related family of plant proteins [34]. In 45 cases (75%), the following motif is conserved (hevein numbering; see Fig. 1): $C^{12}XXX^{15}X$ $CCSX^{20}\varphi^{21}X\varphi CGX\Omega X$ $AcYC^{31}$, where φ represents an aromatic side chain, Ω is Thr, Ser or Gly, and Ac is, in most cases (84%), either Glu or Asp. Positions marked by X are occupied by any type of residue, those of a polar character being much more frequent. The remaining 15 sequences under consideration differ from the mentioned motif only in that either Tyr^{30} is substituted by Phe (six cases) or one of the aromatic residues at positions 21 and 23 is changed for His, Ser or Gly (nine cases).

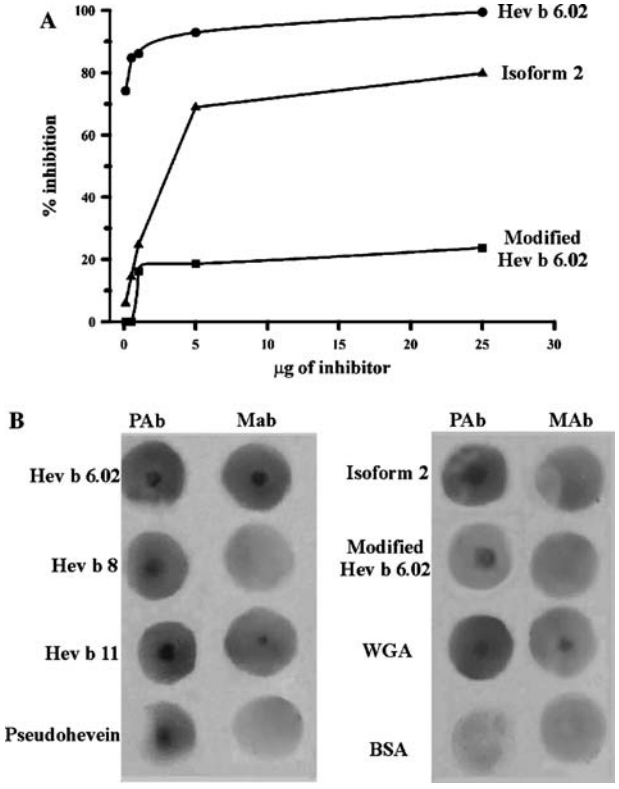

Fig. 2. (A) ELISA inhibition assays. The average inhibition of IgE binding to solid-phase Hev b 6.02 was determined using isoform 2 and the chemically modified allergen with BPNS-skatole in sera from 10 pediatric patients. An average of 75% inhibition was found with isoform 2 and only 20% with the chemically modified allergen at the maximum concentration used. The inhibition curve with Hev b 6.02 as the inhibitor is shown for comparison. (B) Specificity of the polyclonal and monoclonal anti-Hev b 6.02 antibodies in a dot blot assay. The membranes were incubated with the rabbit and mouse IgGs. Proteins used are indicated in the left margin of both figures. BSA was used as negative control.

On the other hand, all the proteins whose structure contain hevein-like domains were recognized by the PAbs anti-Hev b 6.02, and even the modified hevein gave a positive reaction. Interestingly, however, the PAb also reacted with the profilin Hev b8 (Fig 2B). The folding pattern exhibited by the profilin molecule is completely unrelated to, and its sequence shows no evidence of the typical motif present in the structure of, hevein-like domains. However, the actin-binding region in this molecule shares the aromatic and acidic characteristics attributed to antibody-binding sites in hevein-like proteins, and could be responsible, therefore, for the PAb reactivity towards the profilin. Moreover, the observed cross-reactivity among NRL allergens and those from pollen seed, considered in this work, might be explained by the similar features of the binding regions.

Skin prick tests

To assess the relevance that the two Trp residues of Hev b 6.02 may have on defining B cell (conformational) epitopes, SPT were performed using the native protein and the one that had Trp^{21} and Trp^{23} residues chemically modified. Hev b 6.02 gave immediate wheal and flare skin-test responses at concentrations as low as 1.0 μg/ml in 8 of the 50 high-risk volunteers. In contrast, the chemically modified allergen induced no response at all, even at a concentration of 10 μg/ml. These results show that Hev b 6.02 can elicit immediate hypersensitivity responses; however, this capacity is abolished by chemical modification of the Trp residues, thus suggesting that the region responsible for sugar-binding and formed by sequential epitopes (Fig. 4) forms a conformational epitope when the protein folds.

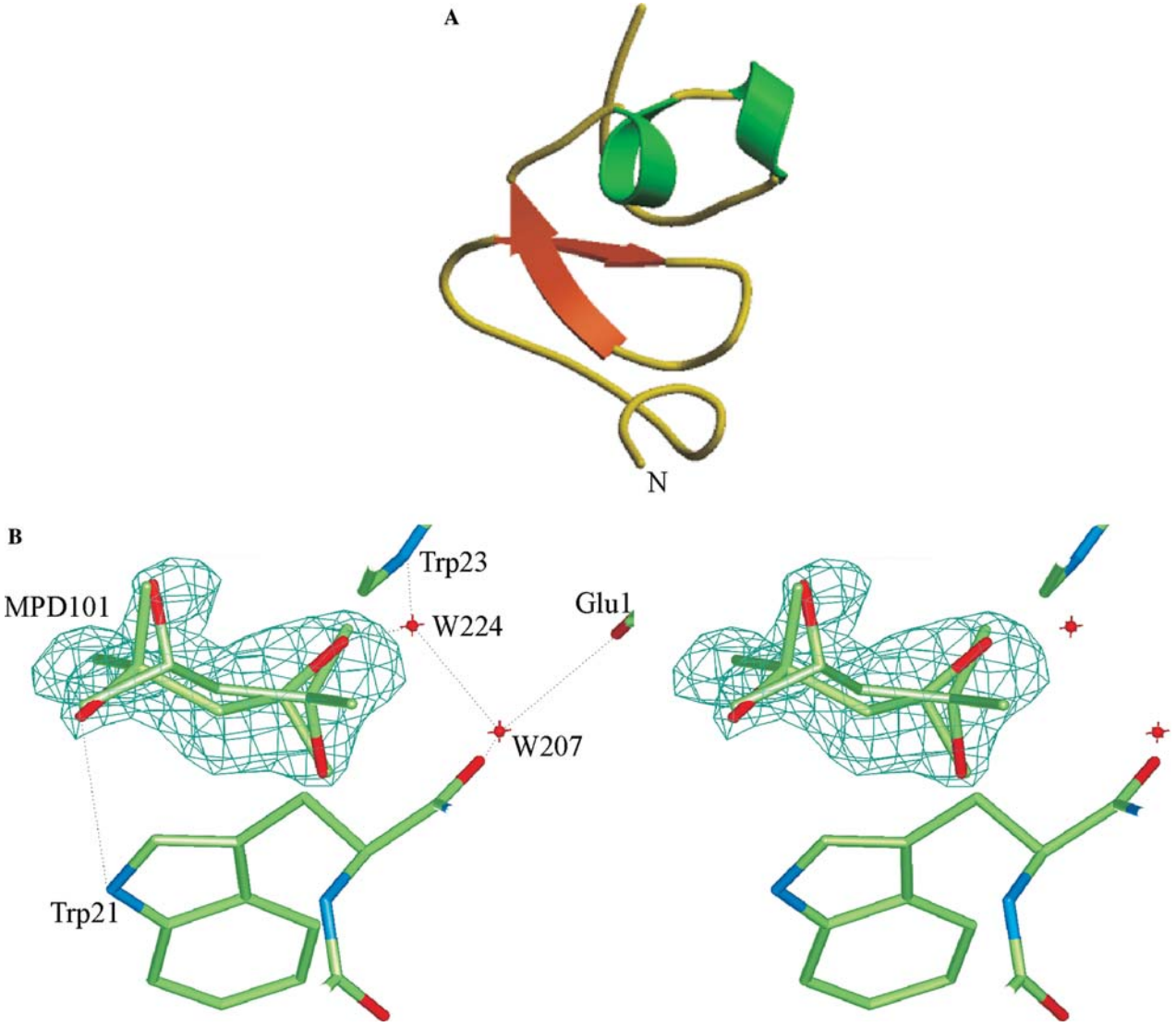

Fig. 3. (A) Overall structure of Hev b 6.02. The figure was generated with MOLSCRIPT [38]. (B) Stereo view of the electron density ($|F_o - F_c|$) corresponding to the MPD^{101} molecule and residues at hydrogen-bond distance. Water (W) mediated interactions are also shown. The contours are drawn at 3.0 σ level and the hydrogen bonds are represented by broken lines.

Molecular structure

The refined model is ovoid in shape and contains 43 residues, 39 water molecules, and two molecules of MPD in two conformations. Four small regions of secondary structure characterize the molecule (Fig. 3A). The most interesting feature of the structure is the carbohydrate-combining site, which shows an aromatic patch (Trp^{21}, Trp^{23}, and Tyr^{30}) encircled by the side chains of Glu^{1}, Gln^{6}, Gln^{20}, and Glu^{29}. It was interesting to find two molecules of MPD in this region, which are making hydrogen bonds to different residues: MPD^{101} (O2 MPD-NHE1 Trp^{21}) (Fig. 3B) and MPD^{102} (O4 MPD-OH Tyr^{30}). On the other hand, the side chains for six residues: Glu^{1}, Leu^{11}, Pro^{13}, Ser^{26} Glu^{29}, and Pro^{33} were built using two conformations.

The overall structure of Hev b 6.02, at this resolution, is very similar to the solution structure obtained by NMR methods [15]. A CA superposition between this model and the two models obtained by NMR methods shows an rmsd of 0.62 Å for the model with bound chitobiose, and 0.83 Å for the one with an empty binding site, while the superposition of the two structures solved by NMR methods shows an rmsd of 1.03 Å. An analysis of the solvent molecules found in the structure shows that the sugar-binding site is full with MPD molecules plus some waters. Thirty-four out of thirty-nine water molecules were found near polar residues exposed to the solvent and five were buried in the structure. Regarding solvent accessibility, the binding-site residues Trp^{21} and Trp^{23}, as well as neighbor residues such as Glu^{1} and Glu^{29}, share high values, as determined with the program NACCESS (S.J. Hubbard, J.M. Thornton, University College, London, 1993), and are not involved in crystal packing interactions. Indeed, the solvent accessibility of Trp^{21} is 63% (when its solvent-accessible surface area is expressed as a percentage of the accessible area of the side chain in the tripeptide Gly–Trp–Gly in extended conformation [35]), which makes this residue one of the most exposed in Hev b 6.02; Trp residues located at equivalent positions in WGA are also highly exposed (about 67% accessibility). In profilins, one Trp also displays high accessibility (63%).

Structural comparison of Hev b 6.02 with different allergens

When we analyzed structures of other plant allergens such as Bet v 2, Hev b 8, and WGA, we noticed a similar motif, which involves a number of solvent-accessible aromatic, polar, and ionizable (mostly acidic) residues on the surface of the protein. Fig. 4 shows these exposed regions on the three-dimensional structures of the four proteins. It should be mentioned that three sequential epitopes have been reported for Bet v 2 (residues 2–19, 28–47, and 108–133; [36]), and two for Hev b 6.02 (residues 13–24 and 29–36; [28]) (Fig. 1). For these allergens the highlighted residues in green belong to their linear epitopes, indicating that those areas of the molecular surface constitute conformational epitopes. On account of the structural resemblances shown in Fig. 4, it is possible that the corresponding regions in the other allergens may play similar roles as superficial epitopes and yet conform to common determinants of allergenicity. Thus, although these proteins possess binding sites widely varying in selectivity (i.e., Hev b 6.02 and WGA bind carbohydrates, whereas Hev b 8 and Bet v 2 bind actin), it is evident that such sites could potentially contribute towards their allergenicity.

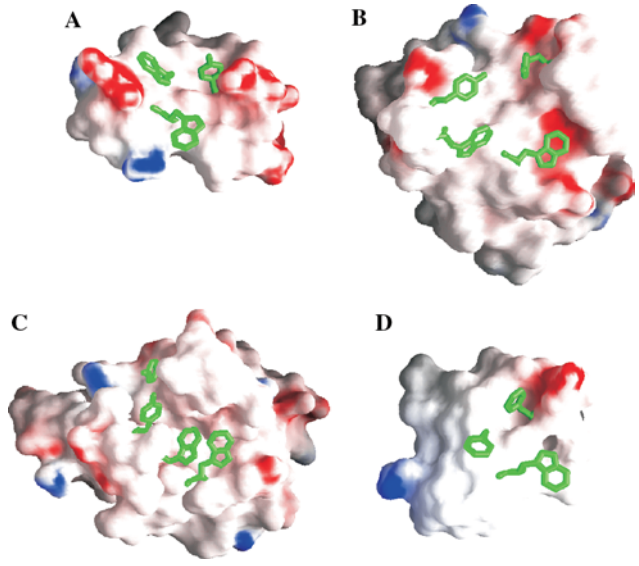

Fig. 4. Electrostatic surface-potential of four allergens. Comparison of Hev b 6.02 (A) with three plant allergens: Bet v 2 (B), Hev b 8 (C), and domain C of WGA (D). In green the aromatic residues comprising binding sites; for Hev b 6.02 and Bet v 2 these residues are also part of the linear epitopes. The protein surfaces have been produced with GRASP [39].

Very recently Karisola et al. [37] described a major conformational IgE binding site epitope of Hev b 6.02 using a chimera-based allergen epitope mapping strategy and molecular modeling. They suggested that its N-terminal and C-terminal regions essentially determined its IgE binding activity. In this study we present evidence supporting that the central part of the polypeptide chain is fundamental for the constitution of a conformational epitope and, therefore, for the interaction with model antibodies. This region includes the binding site, with three aromatic residues and other residues such as Glu, Gln, Asp, and Asn exposed to the solvent. These results support the existence of a main unifying feature as a common critical determinant of allergenicity in plant allergens.

The atomic coordinates and structure factors (code 1Q9B) have been deposited in the Research Collaboratory for Structural Bioinformatics Protein Data Bank.

Acknowledgments

We thank Peter Kuhn for beamline support. This work is based on research conducted at SSRL, which is funded by the Department of Energy, Office of Basic Energy Sciences. The Biotechnology Program is supported by the National Institutes of Health, National Center for Research Resources, Biomedical Technology Program and the Department of Energy. Preliminary X-ray experiments were done at LUEP IQ-UNAM. We thank Dr. Edgar Zenteno and Dra. Concepción Agundis (School of Medicine-UNAM) for their help with the production of antibodies and CONACYT (Grant 32417-E).

References

[1] B.J. Sutton, H.J. Gould, The human IgE network, Nature 366 (1993) 421–428.
[2] A. Bufe, The biological function of allergens: relevant for the induction of allergic diseases? Int. Arch. Allergy Immunol. 117 (1998) 215–219.
[3] D.J.R. Huby, J.R. Dearman, I. Kimber, Why are some proteins allergens? Toxicol. Sci. 55 (2000) 235–246.
[4] R. Brehler, U. Theissen, C. Mohr, T. Luger, Latex-fruit syndrome: frequency of cross-reacting IgE antibodies, Allergy 52 (1997) 404–410.
[5] T. Fuchs, S. Spitzauer, C. Vente, J. Hevler, S. Kapiotis, H. Rumpold, D. Kraft, R. Valenta, Natural latex, grass pollen, and weed pollen share IgE epitopes, J. Allergy Clin. Immunol. 100 (1997) 356–364.
[6] J.A. Berzofsky, Intrinsic and extrinsic factors in protein antigenic structure, Science 229 (1985) 932–940.
[7] R.C. Aalberse, Structural biology of allergens, J. Allergy Clin. Immunol. 106 (2000) 228–238.
[8] J. van Parijs, W.F. Broekaert, I.J. Goldstein, W.J. Peumans, Hevein: an antifungal protein from rubber-tree (*Hevea brasiliensis*) latex, Planta 183 (1991) 258–264.
[9] H. Alenius, N. Kalkkinen, T. Reunala, K. Turjanmaa, T. Palosuo, 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 (1996) 1618–1625.
[10] U.M. Soedjanaatmadja, J. Hofsteenge, C.M. Jeronimus-Stratingh, A.P. Bruins, J.J. Beintema, Demonstration by mass spectrometry that pseudo-hevein and hevein have ragged C-terminal sequences, Biochim. Biophys. Acta 1209 (1994) 144–148.
[11] H.T. Wright, G. Sandrasegaram, C.S. Wright, Evolution of a family of *N*-acetylglucosamine binding proteins containing the disulfide-rich domain of wheat germ agglutinin, J. Mol. Evol. 33 (1991) 283–294.
[12] K. Harata, M. Muraki, Crystal structures of *Urtica dioica* agglutinin and its complex with tri-*N*-acetylchitotriose, J. Mol. Biol. 297 (2000) 673–681.
[13] A. Rodriguez-Romero, K.G. Ravichandran, M. Soriano-Garcia, Crystal structure of hevein at 2.8 Å resolution, FEBS Lett. 291 (1991) 307–309.
[14] N.H. Andersen, B. Cao, A. Rodriguez-Romero, B. Arreguin, Hevein: NMR assignment and assessment of solution-state folding for the agglutinin-toxin motif, Biochemistry 32 (1993) 1407–1422.
[15] J.L. Asensio, F.J. Cañada, M. Bruix, A. Rodriguez-Romero, J. Jimenez-Barbero, The interaction of hevein with *N*-acetylglucosamine-containing oligosaccharides. Solution structure of hevein complexed to chitobiose, Eur. J. Biochem. 230 (1995) 621–633.
[16] G.S. Omenn, A. Fontana, C.B. Anfinsen, Modification of the single tryptophan residue of staphylococcal nuclease by a new mild oxidizing agent, J. Biol. Chem. 254 (1970) 1895–1902.
[17] Z. Otwinowski, W. Minor, Processing of X-ray diffraction data collected in oscillation mode, Methods Enzymol. 276 (1997) 307–326.
[18] The *CCP*4 suite: programs for protein crystallography, Collaborative Computational Project, Number 4, Acta Crystallogr. D 50 (1994) 760–763.
[19] A.T. Brünger, P.D. Adams, G.M. Clore, W.L. DeLano, P. Gros, R.W. Grosse-Kunstleve, J.S. Jiang, J. Kuszewski, M. Nilges, N.S. Pannu, R.J. Read, L.M. Rice, T. Simonson, G.L. Warren, Crystallography & *NMR system*: a new software suite for macromolecular structure determination, Acta Crystallogr. D 54 (1998) 905–921.
[20] A.T. Brünger, Free *R*-value: a novel statistical quantity for assessing the accuracy of crystal structures, Nature 355 (1992) 472–474.
[21] G.J. Kleywegt, T.A. Jones, Databases in protein crystallography, Acta Crystallogr. D 54 (1998) 1119–1131.
[22] T.J. Oldfield, Macromolecular refinement, in: Proceedings of the CCP4 Study Weekend, SRS Daresbury Laboratory, Warringtong, UK.
[23] G.M. Sheldrick, T.A. Schneider, SHELXL: high-resolution refinement, Methods Enzymol. 277 (1997) 319–343.
[24] R.A. Engh, R. Huber, Accurate bond and angle parameters for X-ray protein structure refinement, Acta Crystallogr. A 47 (1991) 392–400.
[25] W.R. Patterson, D.H. Anderson, W.F. DeGrado, D. Cascio, D. Eisenberg, Centrosymmetric bilayers in the 0.75 Å resolution structure of a designed alpha-helical peptide, D,L-alpha-1, Protein Sci. 8 (1999) 1410–1422.
[26] R.A. Laskowski, M.W. MacArthur, S.C. Hutchinson, J.M. Thornton, *PROCHECK*: a program to check the stereochemical quality of protein structures, J. Appl. Crystallogr. 26 (1993) 283–291.
[27] G.H. Cohen, *ALIGN*: a program to superimpose protein coordinates, accounting for insertions and deletions, J. Appl. Crystallogr. 30 (1997) 1160–1161.
[28] D.H. Beezhold, D.A. Kostyal, G.L. Sussman, IgE epitope analysis of the hevein preprotein; a major latex allergen, Clin. Exp. Immunol. 108 (1997) 114–121.
[29] M.M. Vestlin, A.M. Kelly, C. Fenselau, Optimization by mass spectrometry of a tryptophan-specific protein cleavage reaction, Rapid Commun. Mass Spectrom. 8 (1994) 786–790.
[30] V. Argali, J. Gueguen, Chemical cleavage of bovine β-lactoglobulin by BNPS-skatole for preparative purposes: comparative study of hydrolytic procedures and peptide characterization, J. Protein Chem. 18 (1999) 1–12.
[31] A. Rodriguez-Romero, B. Arreguin, A. Hernandez-Arana, Unusual far-ultraviolet circular dichroism of wheat germ agglutinin and hevein originated from cystine residues, Biochim. Biophys. Acta 998 (1989) 21–24.
[32] M.W. Thomas, E.F. Walborg, B. Jirgensons, Circular dichroism and saccharide-induced conformational transitions of wheat germ agglutinin, Arch. Biochem. Biophys. 178 (1977) 625–630.
[33] J.P. Casey, R.B. Martin, Disulfide stereochemistry. Conformations and chiroptical properties of L-cystine derivatives, J. Am. Chem. Soc. 94 (1972) 6141–6151.
[34] H. Breinteneder, C. Ebner, Molecular and biochemical classification of plant-derived food allergens, J. Allergy Clin. Immunol. 106 (2000) 27–36.
[35] S. Miller, J. Janin, A.M. Lesk, C. Chothia, Interior and surface of monomeric proteins, J. Mol. Biol. 196 (1987) 641–656.
[36] A.A. Fedorov, T. Ball, M.N. Mahoney, R. Valenta, C.S. Almo, Crystal structure and IgE-epitope mapping of birch pollen profilin: molecular basis for allergen cross-reactivity, Structure 5 (1997) 33–45.

[37] P. Karisola, H. Alenius, J. Mikkola, N. Kalkkinen, J. Helin, T.O. Pentikäinen, S. Repo, T. Reunala, K. Turjanmaa, S.M. Johnson, T. Palosuo, S.M. Kulomaa, The major conformational IgE-binding epitopes of hevein (Hev b 6.02) are identified by a novel chimera-based allergen epitope mapping strategy, J. Biol. Chem. 277 (2002) 22656–22661.

[38] P.J. Kraulis, MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures, J. Appl. Crystallogr. 24 (1991) 946–950.

[39] A. Nicholls, K.A. Sharp, B. Honig, Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbons, Proteins 11 (1991) 281–296.