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A single amino acid substitution on the surface of a natural hevein isoform (Hev b 6.0202), confers different IgE recognition

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Abstract Decreased immune reactivity of isoforms of major allergens has been reported. However, such claims have always been based on experiments with recombinant proteins. This work describes the molecular and physicochemical characterization of a hevein (Hev b 6.0201) natural isoform (Hev b 6.0202), which is present in rubber latex from *Hevea brasiliensis*. The isoallergen has a single substitution Asn14Asp, which gives rise to local differences in the surface potential, as observed from the crystal structure presented here. Besides, ELISA inhibition using serum pools of adult and pediatric patients showed reduced IgE-binding capacity (~27%) with the isoallergen. Overall, these results are relevant to delineate crucial residues involved in this dominant discontinuous epitope.

Keywords: Crystal structure; Isoallergens; Hevein; Hev b 6.0201; IgE-epitope

1. Introduction

Allergic responses to natural rubber latex (NRL) proteins have been recognized as a severe health problem. Unlike many IgE-mediated allergies that are induced by a single or a few allergens, there are hundreds of proteins in NRL that vary in their allergenic potential. Fifty-six different allergens, which are recognized by IgE from people allergic to this material, have been identified [1]. Of those, 13 proteins officially accepted by the International Union of Immunological Societies-WHO are recognized as the main cause of sensitization of susceptible individuals, and most of them consist of a mixture of closely related isoforms, as has been shown by cDNA cloning [2]. The isoforms that have been reported for different allergens are: two for Hev b 7 [3]; six for Hev b 8 [4]; three for Hev b 10 [5] and two for Hev b 11 [6].

Overall, a great variety of isoallergens have been studied, essentially at an immunological level; however, little is known about structural differences between them. For instance, it has been found that Bet v 1 isoforms form *Betula verrucosa* pollen bind with different affinities to the IgEs and that they provoke different inflammatory responses [7]. Isoforms of Der f 2, a major sensitizing allergen present in house dust mites, have also been reported and are known to have different antigenicities [8]. Consequently, in some cases, isoforms can be used in immunotherapy treatments with less side-effects [9]. Nonetheless, in all these cases recombinant proteins were used, natural isoforms have not been studied in this context.

Hevein is one of the major allergens present in NRL. It is a lectin that belongs to the defense related family of proteins [10,11]. This polypeptide chain is present as a chitin-binding domain in several plant proteins, such as multi-hevein domain lectins and class I chitinases [12,13], which have been associated with cross-reactivity between latex and some fruits [14,15]. Recently, information regarding conformational epitopes of this allergen has been described: Karisola et al. [16,17], using, chimeric-hevein, hevein mutants and molecular modeling suggested that the N-terminal and C-terminal regions essentially determined the IgE binding activity. In a parallel work, our group described critical residues, (W^{21}, W^{23}) in an inmunodominat IgE-epitope [18], which were also present in the linear or sequential epitopes described by Beezhold et al. [19] and Banerjee et al. [20].

Several molecular forms of hevein have been identified in NRL, being pseudohevein the only one widely characterized [21]. The importance of natural hevein isoallergens in the sensitization of people allergic to NRL has not been determined. With the aim of gaining insights into these issues, here we report the 3D structure at 1.7 Å resolution of the isoallergen Hev b 6.0202. Additional biochemical and immunological data of this molecule are reported and compared with those for hevein.

2. Materials and methods

Hev b 6.0202 was purified as described in a previous report [22], with some modifications. Briefly, the fresh lutoid fraction from latex was homogenized in 50 mM Tris, 0.15 M NaCl (pH 8.2). The proteins which precipitated with ammonium sulfate (65–100% saturation) were fractionated in a Superdex-75 column (Amersham Biosciences) equilibrated with the same buffer. Fractions containing hevein and other isoforms were applied to a C-18 reverse phase (RP) column (Beckman Co.) in a high performance liquid chromatography (HPLC) system, equilibrated with 0.1% TFA in deionized water. The elution of Hev b 6.0202 was obtained with 0.12% TFA in acetonitrile.

The electrophoretic migration of hevein and its isoform was determined in a 20% PAGE gel at native conditions (pH 8.4). Additionally, capillary zone electrophoresis (CZE) was performed at 18 kV using a Beckman P/ACE System 5500, with diode array detector. The capillary tubing was of uncoated fused silica (75 μm × 57 cm). The samples were introduced into the capillary in a 0.05 M phosphate buffer, pH 2.5, and detection was performed at 200 nm.

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The sequence of the isoallergen was determined by automated Edman degradation on a gas-phase protein sequencer (LF 3000, Beckman Instruments, Irvine, CA) using the purified peptides from enzymatic hydrolysis. Two peptides were obtained from the reduced and alkylated allergen when hydrolyzed with trypsin (Sigma Chemical Co.) (0.15 M Tris–HCl, pH 8.2, for 24 h at 37 °C) and two peptides when Glu-C (Boeringher-Mannheim) was used (0.15 M Tris–HCl, pH 8.8, for 60 h at 37 °C). In both cases, the resulting fragments were separated by RP chromatography and each purified chain was subjected to automated Edman sequencing analysis.

Hev b 6.0202 crystals were grown at 18 °C from hanging drops by mixing 5 μl of isoallergen (6.0 mg/ml) with 5 μl of reservoir solution (60% MPD, 20 mM Tris–HCl, pH 7.5). Diffraction data were collected using a Rigaku R-axis IIC image plate system with a rotating anode generator, at 100 K after soaking a crystal in cryoprotectant solution (70% MPD). The data were processed and scaled with d*TREK [23]. It is interesting to note that, even though the crystallization conditions for both allergens were very similar, they crystallized in different space groups (hevein, orthorhombic and the isoform, trigonal).

The 3D structure was solved by molecular replacement using the program AMoRe [24] and Hev b 6.0201 (PDB code 1Q9B) as the search probe. After finding a clear solution, the structural model was subjected to rigid body refinement using CNS [25] and revised with QUANTA2000 (Accelrys). Further refinement with the simulated annealing protocol of CNS, followed by individual temperature-factor refinement, gave R and R_{free} values of 22.3% and 24.7%, respectively. At the final stage of this process, an anisotropic refinement was made using the REFMAC-TLS program from the CCP4 suite [26], dropping the R_{free} value to 19.2% (R-factor = 15.1%). A summary of the data-collection and refinement statistics is given in Table 1. Coordinates for the isoform have been deposited at the RSCB Protein Data Bank (accession code 1WKX).

Calorimetric assay was performed using a VP-ITC instrument (MicroCal Inc.). The binding reaction was monitored by recording the heat released upon small additions of a *N*-*N*′-*N*″-triacetyl-D-glucosamine (chitotriose) solution to the protein (100 mM phosphate buffer, pH 7.4). In the experiment, 25 aliquots (9 μl each) of an 8 mM chitotriose were titrated into a 0.249 mM protein solution at 25 °C. The heat of dilution of the saccharide was obtained by adding ligand to the buffer solution under identical conditions and injection schedule used with the protein sample. The enthalpy change (ΔH) and binding constant (K_a) were determined from the experimental titration curve using the software ORIGIN.

Table 1
Data collection and refinement statistics

Data collection	
Space group	$P3_121$
Unit cell parameters (Å): *a*, *b*, *c*	37.22, 37.22, 48.79
Resolution range (Å)	16–1.7
Number of observations	17304
Number of unique reflections	4440
$I/\sigma(I)$	25.2
Completeness (%)	97.1
Multiplicity	3.9
R_{sym}[a] (%)	3.7
Refinement	
R-factor (%)/R_{free}[b] (%)	15.2/19.1
Number of atoms: protein/solvent	321/40
RMS deviation from ideal values	
Bond length (Å)	0.014
Bond angle (°)	2.1
Residues in Ramachandran plot (%)	
Most allowed region	91.8
Allowed region	8.2
Average *B*-factors (Å^2): protein/water	15.2/24.9

[a] $R_{sym} = 100\sum_h\sum_i|I_i(h) - [I(h)]|/\sum_h I(h)$, 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.

The ability of Hev b 6.0202 to inhibit the binding of allergic patients' IgE to hevein was investigated by ELISA inhibition assay as described previously [18].

3. Results and discussion

3.1. Purification and characterization of Hev b 6.0202

One main fraction containing hevein (90%) and its isoform (~5%), was obtained from the molecular exclusion chromatography, then the proteins were separated by reverse phase chromatography (results not shown). The presence and proportion of this particular isollergen was constant in all the latex samples used, no matter the clone (GV-42 and IAN-710) or the season of recollection of latex and it was independent on the age of the preparation. At this point it is important to stress that latex was always processed immediately.

A native PAGE gel showed single bands for the purified proteins, being the isoform the more anionic (Fig. 1A). These results were confirmed by CZE, where retention times of 4.66 min and 13.51 min were obtained for hevein and its isoallergen, respectively.

Regarding the primary structure, analysis of the isoallergen peptides obtained after hydrolysis with trypsine and Glu-C, demonstrated the presence of an aspartyl residue in position 14, which corresponds to asparagine in hevein. The origin of this deamidation is uncertain; chemical deamination by aging of the samples or purification procedures is unlikely due to the fact that under the conditions used (Tris, pH 8.2, and 4 °C) this modification has not been described [27,28]. Besides, deamination occurs more readily at sequences where the side chain of the carboxyl adjacent residue does not impose steric hindrance, which is not the case for hevein (Fig. 1B). Enzymatic deamination is not probable, because to date there are no exam-

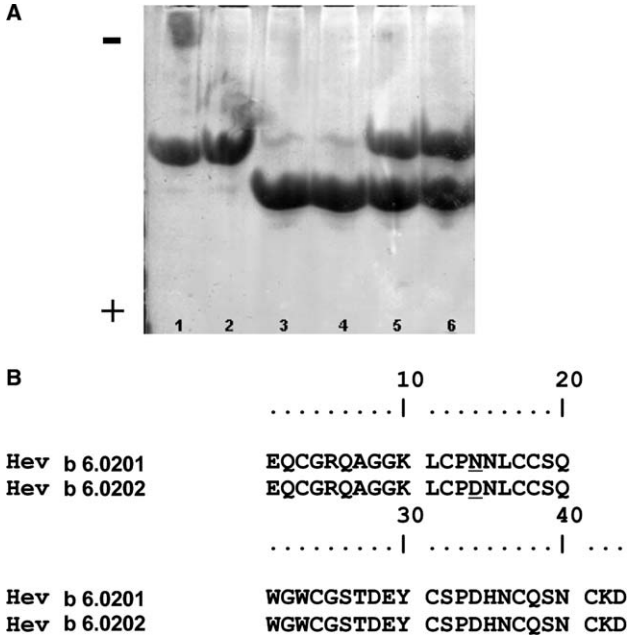

Fig. 1. (A) Electrophoretic profile of hevein and its isoform. Lanes 1 and 2 contain hevein; 3 and 4 the isoform; 5 and 6 have a mixture of both allergens (20 μg each). (B) Sequence comparison between both isoallergens. Residues involved in hevein linear epitopes are 13–24 and 29–36.

ples of naturally occurring asparaginases that deaminate Asn residues within a peptide or protein (XxxAsnYyy). It is then likely that non-enzymatic deamination of Asn14 actually occurs in the plant tissues; however, no isoAsp was detected, probably due to the presence of enzymes that have been found to be widespread in vivo, which convert isoAsp to L-Asp [29].

Finally, a genetic allelic isoform cannot be excluded at this time either. There are reports of hevein isoforms (pseudohevein and others with different C-terminal residues) in the GenBank.

3.2. The overall structure of the isoallergen and comparison with the structure of hevein

The 3D-structure of Hev b 6.0202 at 1.7 Å resolution is comparable with the one of hevein (1.5 Å resolution); with a root mean square deviation (r.m.s.d.) of Cα positions of 0.23 Å. A stereoscopic view of the superposed molecules is shown in Fig. 2A. An insert showing details of the loop Pro13-Leu16 indicates that the side chains of Pro13 and Asn15 have different conformations in both allergens. Most of the residues were clearly modeled, with the exceptions of Lys^{42} and Asp^{43}, since the former lacked density for some atoms of the side chain, while the latter had to be modeled as Ala because no density was observed further the Cβ. An analysis of $F_o - F_c$ and $2F_o - F_c$ electron density maps (Fig. 2B) at position 14 showed no evidence for an isoaspartyl residue, eliminating the possibility of Asn deamination during the purification procedures.

The surface electrostatic potentials of both allergens are slightly different (Fig. 2C), being more negative at the position 14 for the isoform. The effect on the binding of specific serum IgE (Fig. 3) due to the minor change described here suggests that Asn^{14} is an important residue in the human IgE recognition, and consequently as being part of this dominant conformational epitope. It is worth noting that Asn^{14} has been described as being part of one of the two linear epitopes pres-

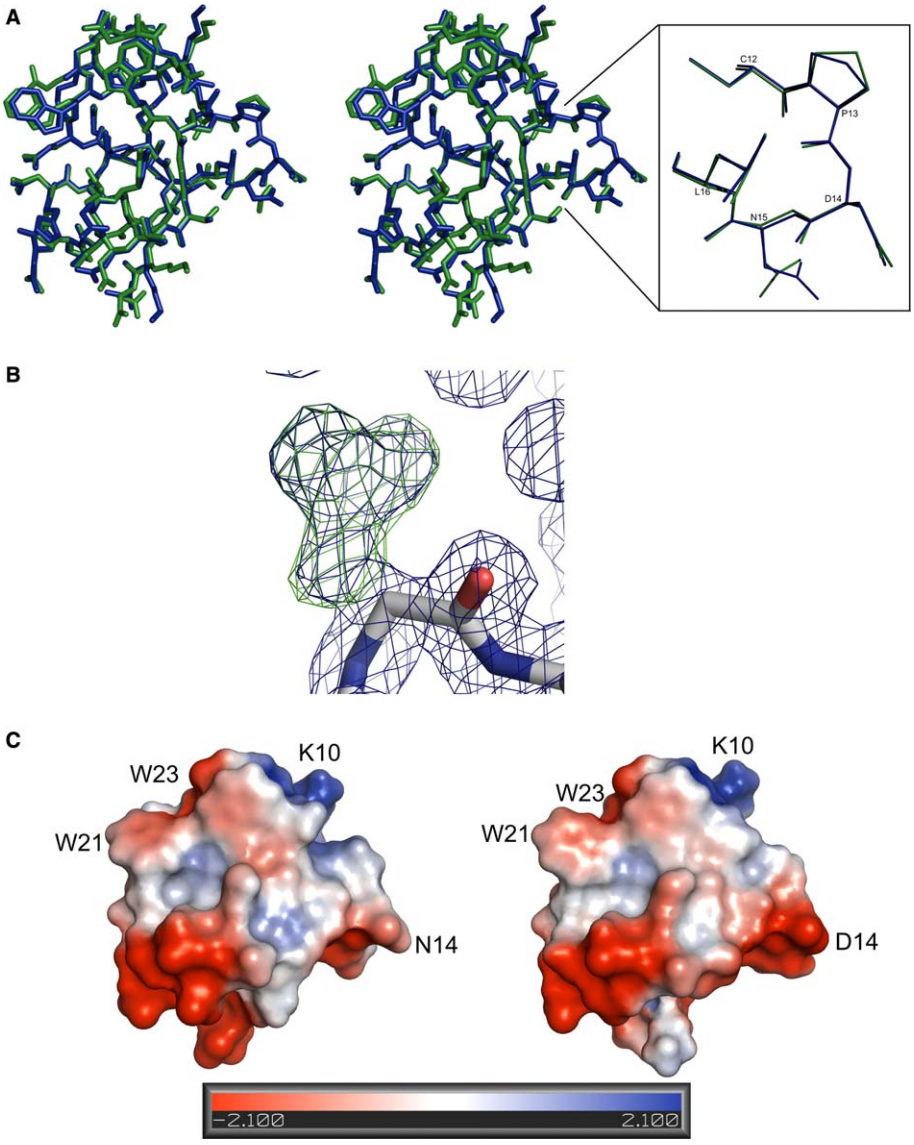

Fig. 2. (A) Stereoscopic view of the superposition of hevein (green) and its isoform (blue). Inset shows details of residues 12–16. (B) Details of the $2F_o - F_c$ (1σ) and the $F_o - F_c$ (3σ) electronic density maps, in blue and green, respectively, in the Asp^{14} region for Hev b 6.0202. (C) Electrostatic potentials for hevein (left) and its isoform (right). The negative potentials are shown in red ($-2.1k_BTle_c$) and the positive ones in blue ($2.1k_BTle_c$). Calculations were done with the program APBS [33] and the figures with PyMOL.

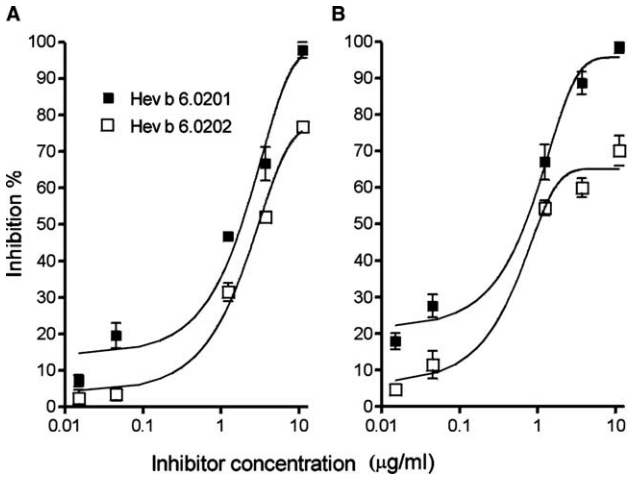

Fig. 3. ELISA inhibition was performed with hevein and its isoform at five different concentrations (0.01–10 μg/ml). Serum pool (diluted 1:27) was obtained from 10 high-risk pediatric patients and another pool from 10 adult high-risk health care workers (Instituto Nacional de Pediatría, México City). The inhibition curve for hevein is shown for comparison. (A) Results for a pool of pediatric sera. (B) Results for an adult sera pool.

ent in hevein [19,20]. It should be mention that previous strategies were not effective in detecting this residue as being part of a conformational epitope [16–18].

3.2.1. Binding energetics of Hev b 6.0202 to chitotriose. There were no significant differences in the K_a or in the ΔH values for both allergens ($14250 \pm 770\ M^{-1}$ and 7.04 ± 0.13 kcal/mol for hevein [30] and $14640 \pm 1300\ M^{-1}$ and 7.2 ± 0.3 kcal/mol for the isoform). The stoichiometry (η) was also nearly one in both cases. Taken together, these results indicate that the residues in the sugar binding site, essentially Trp^{21}, Trp^{23}, Tyr^{30} and Ser^{19}, are spatially arranged in a similar way in both isoallergens [10], giving approximately the same affinity for the chitotriose moiety. Interestingly, Asensio et al. [31] have also demonstrated, using nuclear magnetic resonance (NMR) techniques that the interactions of hevein with $(GlcNAc)_5$ oligomers involve, not only the residues indicated above, but also the loop region (Pro^{13}, Lue^{16}) and Cys^{24}. We could speculate that differences in this loop could also reflect differences in the chitin binding.

3.2.2. Inhibition ELISA. An inhibition of 76% of the IgE-hevein interaction was detected with the isoallergen in the case of pediatric patients and 70% for adults (Fig. 2); thus, one amino acid substitution decreased the binding of both pools of patients' serum IgE to hevein. In our previous work [18], based on results obtained after chemical modification of the Trp residues present in hevein, prompted us to postulate the region on the surface, which comprises the aromatic residues and other polar residues, as one dominant IgE epitope. In this work we found that both allergens are almost identical; however, the net charge and the superficial charge distribution are different, mainly in the substitution site (Asn14Asp) of the isoform (Fig. 2C). Consequently, the change in the immunological activity of the isoallergen can be directly ascribed to the single amino acid substitution, which results in alteration of local molecular surface area and its electrostatic potential that could lead to a reduction in IgE binding affinity. Similar results have been reported for Bet v 1, where the mutation Glu^{45}-Ser inhibited IgE binding between 20% and 50% [32].

Several attempts have been made to delineate conformational epitopes of hevein-like molecules. Karisola et al. reported that Arg^5, Lys^{10}, Glu^{29}, Tyr^{30}, His^{35}, and Gln^{38}, were responsible for IgE interaction using a chimera based epitope mapping, hevein mutants and molecular modeling [16,17]. These results are, however, at variance with previous reports [19,20], which independently demonstrated the presence of the aromatic residues in linear hevein epitopes (Fig. 1B). In contrast, using X-ray crystallography and chemical modification of Trp^{21} and Trp^{23}, we determined that these aromatic residues were relevant for the interaction with model antibodies and IgE [18]. Besides, the relevance that aromatic and polar side chains might have in IgE recognition by hevein-like domains was made apparent from a primary-structure comparison including 60 domains found in proteins that are recognized as allergens (Hev b 6.02, Hev b 11, wheat germ agglutinin, WGA) or potential plant allergens belonging to the pathogenesis-related family. We observed that in 45 cases (75%), the following motif is conserved (hevein numbering; see Fig. 1B): $C^{12}XXX^{15}XCCSX^{20}\varphi^{21}X\varphi CGX\Omega XAcYC^{31}$, where φ represents an aromatic side chain, Ω is Thr, Ser or Gly, and Ac is, in most cases (84%), either Glu or Asp. Analyzing the same data, but now at position 14, Asn was present in 25 cases (41.4%), whereas Asp appeared just in two (3.3%). These results reinforce the experimental evidence that Asn could also be an important residue in IgE recognition and are in accordance with the concept that dominating IgE binding epitopes may be preferentially located in conserved surface areas. Interestingly, it looks as if some hevein residues that are involved in IgE recognition are the same that participate in the binding of large $(GlcNAc)_5$ oligomers [31].

New strategies for type I allergy treatment favor the use of recombinant allergens; however, the results presented in this work show that differential behavior in natural isoform has potentially important immunological implications. Additionally, structural studies can provide critical insights to delineate conformational epitopes.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.03.085.

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