



Impact of the vulcanization process on the structural characteristics and IgE recognition of two allergens, Hev b 2 and Hev b 6.02, extracted from latex surgical gloves



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ABSTRACT

Latex allergy is a health problem that mainly affects medical environments, causing anaphylactic shocks in extreme cases. Sensitization and reactions to this material is closely linked to the use of latex gloves. The objective of this study was to purify two of the major allergens from latex surgical gloves to study the biochemical and structural changes that could be generated during the product manufacture and to compare their IgE recognition with the non-processed allergens. Glycosylated allergen Hev b 2 (β-1,3-glucanase) and Hev b 6.02 (hevein) were purified from glove extracts using affinity (Concanavalin A) and reversed-phase chromatographies, respectively. ELISA experiments were performed with both proteins and sera from allergic patients to assess the IgE recognition, which was heterogeneous. Crystallographic methods were used to obtain the 3D structure of Hev b 6.02 from surgical gloves, which did not show evident modification when compared with the protein from the natural non-processed form. Despite having the same crystallographic structure, the IgE from some patients showed different recognition when the glove and the natural allergen were used in ELISA. Furthermore, using electrophoretic techniques, we identified three forms of Hev b 2: one corresponding to the complete polypeptide chain with post-translational modifications, and two glycosylated fragments. The mixture of these three forms showed stronger recognition by IgE from latex-allergic patients than the pure non-processed allergen. In conclusion, IgE from subjects sensitized to latex products showed different recognition between the allergens obtained from a natural source and the processed material, even when the structure was maintained. This demonstrates the importance of using processed allergens in further investigations of diagnosis, prevalence, product allergenicity, and therapies.

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1. Introduction

Allergenic proteins are present in a wide variety of natural and processed products. During the manufacture of processed products, several harsh treatments such as heating or irradiation are used, which can modify the allergenic potential of such proteins. Examples of processed allergenic materials are those produced from different foods, such as egg (Golias et al., 2012), peanuts

(Blanc et al., 2011), fish, among other. Another important group of processed allergens are derived from natural rubber latex (NRL) of the *Hevea brasiliensis* tree, including surgical gloves, catheters, and dental dams, which are mainly employed in healthcare (Cabañes et al., 2012). Important progress in the management of latex allergy has been achieved by the use of non-powdered and low-protein gloves; however, these reports only refer to medical environments in developed countries (Palosuo et al., 2011; Wraggsjö et al., 2012). In general, the prevalence of IgE-mediated NRL allergy among healthcare workers has been estimated to be 3–17%, but it is higher (40–50%) in individuals who have undergone multiple surgeries, such as children with spina bífida (Amarasekera et al., 2010; Cremer et al., 2007).

The glove manufacturing process involves several steps after the harvesting of the latex from the *H. brasiliensis* tree, which is usually preserved in ammonia to prevent its coagulation (Spengler et al., 2001). A number of compounds such as aromatic amines, thiurams,

Abbreviations: HMWF, high molecular weight fraction; LMWF, low molecular weight fraction; ConA, concanavaline A.

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and sulfur are added to the latex to obtain a final product with characteristics of elasticity, excellent tactility, and effectiveness as a barrier to pathogens. The vulcanization process includes a high temperature of usually 100 °C (Long, 2001; Spengler et al., 2001), but methods with higher temperatures of up to 200 °C have also been described (Mcglathlin and Schmid, 2001). Nonetheless, even after these extreme procedures, allergenic proteins are present in NRL products that can affect the susceptible population. In NRL gloves, reports indicate the presence of Hev b 1 (rubber elongation factor), Hev b 2 (β -1,3-glucanase), Hev b 3 (small rubber particle protein), Hev b 5 (acid protein), Hev b 6.01 (hevein precursor), Hev b 6.02 (hevein), and Hev b 14 (hevamine) (Lee et al., 2010; Palosuo et al., 2011; Peixinho et al., 2012), with Hev b 6.02 being the most commonly found.

When a protein undergoes a thermal process, a partial or complete loss of its conformation is usually induced, which is initially reversible and eventually irreversible. After such treatments, some proteins could fold again, but not necessarily with the original conformation. For example, hydrophobic groups may become exposed, inducing aggregation by hydrophobic interactions. Also, disulfide bonds may rearrange either intra- or inter-molecule, which could produce protein multimers or aggregation as well (Boye et al., 1997). The effect of processed proteins in allergic reactions or sensitization has been studied extensively for food allergens. For instance, diminished allergic reaction to egg allergens in a mouse model was observed after allergen heating (Golias et al., 2012). Conversely, the immunogenicity can be enhanced if buried epitopes become exposed or if new epitopes are formed, which is the case for ovalbumin, one of the major allergens in egg (Ma et al., 2013). Also, a 90-fold rise in IgE binding to peanut allergens Ara h 1 and Ara h 2 was observed after roasting the product (Maleki and Chung, 2000). A different study revealed that different thermal treatments for peanuts produce different modifications: when Ara h 1 was boiled, its capability to bind specific IgE was diminished, while when it was extracted from roasted peanuts, it maintained its allergic capabilities (Blanc et al., 2011). The roasted protein also retained more of its secondary structure than the boiled one, suggesting that retaining the original folding after a thermal treatment is a determinant in maintaining allergenicity.

Even though thermal treatment is a constant among processed allergens, a very different treatment is suffered by latex allergens. Besides, these allergens act differently because in most cases they are not exposed to digestive enzymes, since the major routes of exposure are the skin and mucous membranes and therefore latex allergens will not necessarily have a similar behavior. Albeit there are reports on the presence of some allergens in latex gloves, very little is known about their integrity in these products. Therefore, the aim of this work was to investigate how latex allergens are structurally and immunologically affected after the manufacturing process. To assess this, we purified the major allergens Hev b 2 and Hev b 6.02 from latex surgical gloves (gHev b 2 and gHev b 6.02, respectively) to gain insight on how their structure and IgE recognition are affected after the process. We selected these two allergens based on their abundance and because their isolation and purification from the gloves was relatively easier. In addition, we identified the presence of some of the other allergenic proteins from *H. brasiliensis* in the gloves.

2. Materials and methods

2.1. Extraction of proteins from gloves

Protein extracts were obtained from surgical latex gloves used in medical institutions (Mexico City). The gloves were cut into approximately 1 cm² pieces and shaken for 48 h at 4 °C with 5 mL of phosphate buffer saline (PBS) per gram of material. Then, the

mixture was filtered to remove powder and pieces of glove, first through a Whatman no. 2 filter paper and then through a Millipore 0.45- μ m filter. The extract was concentrated using an ultrafiltration membrane with a molecular weight cut-off (MWCO) of 10 kDa on an Amicon stirred cell (Millipore Corporation, Billerica, MA, USA) to separate two fractions: one with proteins of molecular mass above 10 kDa and denominated as the high molecular weight fraction (HMWF), and the other with proteins below 10 kDa and denominated as the low molecular weight fraction (LMWF). The latter was then concentrated using a 3 kDa MWCO membrane. SDS-PAGE analysis of the extracted proteins was done under denaturing and reducing conditions.

2.2. Liquid chromatography/Electro spray ionization tandem mass spectrometry (LC/ESI-MS/MS) analysis

After separation using SDS-PAGE electrophoresis, the bands of interest were cut out from the gel and treated for 5 min with 100 μ L of destaining solution (50% (v/v) methanol, 5% (v/v) acetic acid). After removing the solution, each gel band was incubated with 200 mM ammonium bicarbonate for 20 min, cut into small pieces, completely dehydrated with 100% acetonitrile, and vacuum-dried. The protein was hydrolyzed with 30 μ L of a porcine trypsin solution (20 ng μ L⁻¹; Promega, Madison, WI, USA) in 50 mM ammonium bicarbonate, followed by incubation at room temperature for 12 h. Peptides were extracted with 50% (v/v) acetonitrile and 5% (v/v) formic acid for 30 min and separated using a nanoAcquity Ultra Performance LC System (Waters Corporations, Milford, MA, USA). Mass spectrometry (MS) analysis was carried out on a 3200 Q TRAP hybrid tandem mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada) equipped with a nanoelectrospray ion source (NanoSpray II) and a MicroIonSpray II head. Spectra were acquired in the automated mode using Information-Dependent Acquisition (IDA). Database searching and protein identification were performed with the MS/MS spectra datasets using the MASCOT search algorithm against the taxonomy of Viridiplantae (Green Plants) in the non-redundant NCBI (NCBInr) database (version 1.6b9; Matrix Science, London, UK, available at <http://www.matrixscience.com>).

2.3. Chromatographic methods

Proteins from the two fractions (HMWF and LMWF) were purified using different chromatographic methods. To purify gHev b 6.02, the LMWF was loaded into a reversed-phase (RP) C18 column (Phenomenex® Jupiter 5 μ , 300 Å) using an HPLC system at a flow rate of 3.5 mL min⁻¹. A gradient of 0–60% acetonitrile/0.1% TFA was used to elute the protein. Acetonitrile was then eliminated using a SpeedVac™ system (Thermo Fisher Scientific Inc., Rockford, IL, USA).

For Hev b 2, which is glycosylated, we used a 1-mL Concanavalin A (ConA)-Sephacrose 4B affinity column (GE Healthcare, Piscataway, NJ, USA). The column was equilibrated with 20 mM MES buffer and 0.5 M NaCl buffer, against which the HMWF was previously dialyzed. The retained proteins were eluted with 0.5 M α -D-mannopyranoside in the same buffer.

2.4. Purification of allergens from the natural source

To compare results from processed allergens with the natural ones, Hev b 2 and Hev b 6.02 were purified from fresh latex. Hev b 2 was purified as described previously with slight modifications (Fuentes-Silva et al., 2007). Latex luteoids were homogenized with a 0.3 M NaCl solution and the homogenate was dialyzed against distilled water for 48 h. The precipitate formed was separated by centrifugation and resuspended in 20 mL 50 mM MES buffer pH 6.5, 0.35 M NaCl. The sample was loaded onto a Con A-Sephacrose

4B affinity column (GE Healthcare, Piscataway, NJ, USA) and the protein was eluted with 0.5 M α -D-mannopyranoside in the same buffer. The protein was dialyzed against 50 mM Tris–HCl pH 8 containing 0.1 M NaCl and purified to homogeneity by cation-exchange chromatography using a 1 mL Resource S column (GE Healthcare, Piscataway, NJ, USA), a linear gradient from 0 to 1 M NaCl was used to elute the protein. Hev b 6.02 was purified following previous methodology (Reyes-López et al., 2006). Briefly, luteoids were homogenized with a 0.3 M NaCl solution, the homogenate was precipitated with ammonium sulfate to a 60% saturation. After centrifugation, the supernatant was precipitated at 100% ammonium sulfate saturation. The precipitate was separated by centrifugation and resuspended in distilled water, the sample was loaded onto a reversed-phase (RP) C18 column (Phenomenex® Jupiter 5 μ , 300 Å) using an HPLC system at a flow rate of 3.5 mL min⁻¹. A gradient of 0–60% acetonitrile/0.1% TFA was used to elute the protein. Acetonitrile was then eliminated using a SpeedVac™ system (Thermo Fisher Scientific Inc., Rockford, IL, USA).

2.5. Protein quantification

Hev b 6.02 was quantified by absorption at 280 nm in a Shimadzu spectrophotometer with a 1 cm path length cuvette using the extinction coefficient for a 1% solution ($\epsilon^{1\%}$) of 25.4 (g/100 mL)⁻¹ cm⁻¹ of this protein. Quantification of other proteins, fractions, or extracts was estimated using the bicinoninic acid protein assay following the manufacturer's recommendations (BCA™, Thermo Scientific, Rockford, IL, USA).

2.6. Patients sera

Patients were identified as being allergic to latex through a history of allergic reactions, including anaphylaxis during surgery, allergic asthma and rhinitis, edema in eyelids and mouth labia, and hives in contact sites. Also, skin prick tests and latex-specific IgE in blood were used. The ethics committee of the Instituto Nacional de Pediatría, México approved this protocol. In addition, sera from eight allergic volunteers with a history of fruit allergy were used to test the recognition of Hev b 2. These patients showed positive skin prick test to maize, apple, peach, tomato, pear, carrot, melon, strawberry, and grape. The serum from one non-allergic individual was used as control for both allergens. The volunteers donated serum under informed consent.

2.7. IgE enzyme-linked immunosorbent assay (ELISA) experiments

Each well of a 96-well Nunc MaxiSorp microliter plate was coated with 0.8 μ g of each protein for 12 h at 4 °C. After washing with 0.1% (v/v) Tween-20 in PBS, the plates were blocked with 3.0% (w/v) bovine serum albumin (BSA) in PBS for 2.5 h at 37 °C. All patient sera were diluted to 1:10, and then 50 μ L were added to the wells. The plates were then incubated at 4 °C overnight. HRP-mouse anti-human IgE ϵ -chain specific (Invitrogen, Camarillo, CA, USA) was diluted to 1:1000 with PBS containing 0.1% tween and 0.5% BSA and then applied to the wells. The plates were incubated for 1.5 h at 37 °C.

The color reaction was developed by adding 100 μ L of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Invitrogen, Camarillo, CA, USA) to the wells. After 15 min at room temperature, the plate was read at nm in a BioTek® plate spectrophotometer (Winooski, VT, USA). All absorbances values (Abs405) were calculated as the mean of three independent determinations. The mean Abs405 of the negative control was subtracted from all sample values. Cut-off values for positive reactions were considered by the Abs405 of the non-allergic individual plus three

times the standard deviation. A paired t-test was used to analyze the statistical differences in antigen recognition by IgE of the groups of patients.

For competitive ELISA, pools of three or four patients sera were used. The plates were coated with either nHev b 6.02 or gHev b 6.02 and blocked with 3.0% (w/v) BSA in PBS. The pools of sera were incubated with serial dilutions of the competing allergen (either gHev b 6.02 or nHev b 6.02) and with bovine serum albumin (BSA) as control, for 1 h at 37 °C and 50 μ L of the incubated sera samples were added to the wells. The plate was incubated for 1 h at 37 °C. The rest of the procedure was performed as described above for the direct ELISA. The result is presented as an inhibition curve where the absorbance of the sera incubated with BSA is considered a 0% inhibition and the absorbance of the control serum (from a non-allergic individual) is considered a 100% inhibition.

2.8. Fluorescence experiments of ligand binding to Hev b 6.02

Fluorescence spectroscopy experiments were carried out to record emission curves produced by the two tryptophanes (Trp21 and Trp23) at the carbohydrate-binding site of the gHev b 6.02 in the presence of N-acetyl-chitotriose (GlcNAc)₃. For comparison analysis, we performed the same experiments with nHev b 6.02. The binding affinities for (GlcNAc)₃ of both Hev b 6.02 samples were determined by monitoring the intrinsic tryptophan fluorescence change from 300 nm to 400 nm after addition of the ligand using an Olis® DM45 spectrofluorometer (Olis, Inc., Bogart, GA, USA). The excitation wavelength was set to 290 nm with a slit width of 3 nm, and the temperature was maintained at 25 °C using a Peltier system. Different amounts of the oligosaccharide prepared in 100 mM phosphate buffer (pH 7.4) with 1 mM EDTA were added to the protein solution (600 μ g/mL in the same buffer). Fluorescence was measured 2 min after mixing the solution with the ligand to allow for equilibrium. The changes in fluorescence intensities were used to calculate the dissociation constants for chitotriose by plotting the change in fluorescence at the wavelength of maximum emission ($\lambda_{\text{max}} = 350$ nm) against the ligand concentration. The resulting curve was fitted into the following equation:

$$\Delta F = \frac{\Delta F_{\text{max}}[\text{ligand}]}{K_d + [\text{ligand}]}$$

2.9. Circular dichroism (CD) experiments of gHev b 2

CD measurements were performed at 20 °C using a Jasco-J700 spectropolarimeter (Jasco Inc., Easton, MD, USA) equipped with a Peltier temperature control system. The gHev b 2 sample was dialyzed in 20 mM phosphate buffer at pH 7.5 and filtered through a 0.22 μ m membrane. Far-UV spectra were collected from 200 to 260 nm in a 0.1 cm path-length cell. Three scans were averaged to obtain the final spectra of the sample.

2.10. Protein crystallization and X-ray data collection of gHev b 6.02

Diffraction quality crystals were obtained in 0.1 M sodium citrate, 2 M ammonium sulfate, and a protein solution of 10 mg/mL in Tris buffer at pH 5.5, which was mixed in a 1:1 ratio with the reservoir solution using the vapor diffusion-sitting drop method. X-ray diffraction data were collected using the in-house Rigaku MicroMax007 with a rotating-anode generator (Cu, $\lambda = 1.5416$ Å) under cryogenic conditions (103 K) using 30% glycerol as a cryoprotectant. For sulfur-SAD phasing, we collected highly redundant data (940 frames of 0.5° oscillation) at 1.43 Å resolution with the crystal-to-detector distance set to the minimum possible value of

72 mm using a RAXIS IV++ image plate detector (Rigaku, The Woodlands, TX, USA). Diffraction data were processed and scaled using DENZO and SCALEPACK from the HKL2000 (Otwinowski and Minor, 1997), maintaining symmetry-related reflections separated during scaling.

2.11. Phasing and refinement of collected data

Structure determination and refinement were performed using the crystallographic suite of programs PHENIX (Adams et al., 2010). We used sulfur single-wavelength anomalous diffraction (S-SAD), considering the eight cysteine residues in the four-disulfide bonds present in the protein. Experimental phasing was performed with the AutoSol program (Terwilliger et al., 2009), which is a phasing algorithm that uses HySS (Hybrid Substructure Search) (Grosse-Kunstleve and Adams, 2003) for finding heavy-atom sites and SOLVE and RESOLVE (Terwilliger, 2003) for calculating experimental phases and for density modification. The polypeptide chain was partially built automatically with the AutoBuild program (from residue 6 to 39, 66% of the final structure) (Terwilliger et al., 2008) and completed manually in Coot (Emsley et al., 2010). The resulting model was iteratively refined with phenix.refine (Afonine et al., 2012). TLS refinement was performed in the final cycle at 1.43 Å resolution. In all rounds of refinement, the target function phased maximum-likelihood (MLHL) was used, as well as experimental phase restraints.

3. Results

3.1. Identification of allergens from surgical gloves

Fig. 1A shows the electrophoretic profiles of the HMWF and the proteins identified by the method described above. We detected the presence of the known allergens Hev b 2, Hev b 4, Hev b 5, Hev b 6.01 and Hev b 14, plus thioredoxin (Hev b Trx). Table 1 shows the identified allergens and their fragment scores obtained in the MASCOT database. Notably, more than one allergen was detected in some bands, and some allergens were detected in more than one band. For instance, Hev b 2 was identified in the band close to 38 kDa, which corresponds to its molecular weight, but also in some bands corresponding to lower molecular weights.

From the LMWF, we purified the most abundant peptide and identified it as Hev b 6.02 (gHev b 6.02). The electrophoretic profile of this allergen is compared with the non-processed nHev b 6.02 in Fig. 1B, showing the same migration in the gel for both proteins. Also, a MALDI-TOF spectrum for gHev b 6.02 showed m/z of 4728 Da expected for this protein (Supplementary Fig. S1).

3.2. Protein structure determination of gHev b 6.02 (hevein)

Crystals belonged to the trigonal space group P 31 2 1, as confirmed with POINTLESS (Evans, 2006), with cell unit dimensions of $a=37.33$, $b=37.33$, and $c=48.78$ Å. The crystal contained one molecule in the asymmetric unit, with a Matthews coefficient (VM) of 2.08 Å³/Da and 40.9% solvent content. The experimental map showed nine sulfur sites, eight of which corresponded to the four disulfide bonds. The crystal diffracted at 1.43 Å resolution, which lead to electron density maps of high quality, as shown in Fig. 2A.

Electron density maps 2Fo–Fc and Fo–Fc were interpreted with the 43 amino acids of the protein. The only region that we were not able to interpret was the side chain of the Asp43, probably due to the flexibility of the C-terminus. The overall structure is very similar to the one obtained for the nHev 6.02 (PDB 1Q9B). The final model has the four disulfide bonds Cys3–Cys18, Cys12–Cys24, Cys17–Cys31, and Cys37–Cys41, a small antiparallel β -sheet including residues Cys17–Ser19 and Trp23–Gly25 connected by a β -turn

involving residues Ser19 to Gly22, and a small segment of α -helix comprising residues Asp28–Cys31. A C α superposition of the gHev b 6.02 with the nHev b 6.02 (PDB 1Q9B) (Reyes-López et al., 2004) gave an RMSD of 0.18 Å, indicating that no major changes were detected in the protein from gloves. The carbohydrate-binding site only showed minor differences in the conformation of Trp21. Other residues also exhibit different conformation such as Glu29, which is close to the binding site (Fig. 2B). Data collection and refinement statistics are summarized in Supplementary Table 1.

Interestingly, a high additional density peak was observed near the C-terminal region of the protein, which corresponded to the extra sulfur peak determined in the experimental map (Fig. 2C). We explain this density with a molecule of thiourea (TOU), which is used in the manufacture of latex products as an accelerator for the vulcanization process (Rose et al., 2009), suggesting that the protein carries this compound throughout the purification and crystallization processes. A hydrogen bond is formed between N-Asn40 and N1-TOU with a distance of 3.0 Å. In addition, the other two could be formed in atoms N-Asn40 with S-TOU (3.44 Å) and N-Cys4 with S-TOU (3.20), depending on whether the thiourea is in a thiol or thione form (tautomers). Also, the S-TOU is at a distance of 3.6 Å from the S γ -Cys41 in another probable interaction. No covalent bonds are observed in the electron density maps between the protein and the TOU molecule. After several cycles of phenix.refine, the B-factor for the thiourea molecule was 30 Å², which is the same than the value exhibited for residues Asn40 and Cys41 (the whole protein have an overall average B-factor of 20.9). Besides, its correlation coefficient was 0.9, suggesting that this molecule is very likely to be present in the crystal (Fig. 2D).

3.3. (GlcNAc)₃ (chitotriose) binding to gHev b 6.02 in comparison to nHev b 6.02

We analyzed the carbohydrate-binding site between both proteins using spectroscopic techniques. Hev b 6.02 has two Trp residues in this with a fluorescence emission spectrum centered at 350 nm, indicating an exposed localization in the native folded conformation. As observed in Fig. 3A, both samples produced an increased fluorescence intensity at $\lambda_{\text{max}} = 350$ nm and a slight shift towards shorter wavelength (blue shift), meaning an environment of low polarity after chitotriose binding. The differences in the fluorescence intensity (Δ IF) after the addition of the different ligand concentrations produce similar curves with K_d values of 68.01 and 66.9 μ M for the natural protein and glove protein, respectively (Fig. 3B).

3.4. IgE binding to gHev b 6.02 in comparison to nHev b 6.02

In a direct ELISA assay, from eight latex allergic-patients, IgE from four recognized similarly gHev b 6.02 and nHev b 6.02, while three showed increased recognition to gHev b 6.02. The serum from one patient did not react to either one of the samples (Fig. 4A). A paired t-test indicates that this experiment does not show a significant difference in the recognition of the two forms of the allergen by this group of patients ($p = 0.11$). Since three patients clearly showed an increased recognition for gHev b 6.02 we decided to further investigate the different recognition of the IgE from the patients, to achieve this competitive ELISA assays were performed. The allergen gHev b 6.02 inhibited the recognition of IgE from a pool of patients (2, 3, 4 and 7) sera to nHev b 6.02, the maximum inhibition was obtained when the pool was incubated with a 100 ng/mL solution of gHev b 6.02 (Fig. 4B). The allergen nHev b 6.02 was not able to inhibit the recognition to gHev b 6.02 neither by the IgE from the pool of patients 2, 3, 4 and 7 sera nor by the pool of sera from patients 1, 5 and 6, even at concentrations up to 16 μ g/mL.

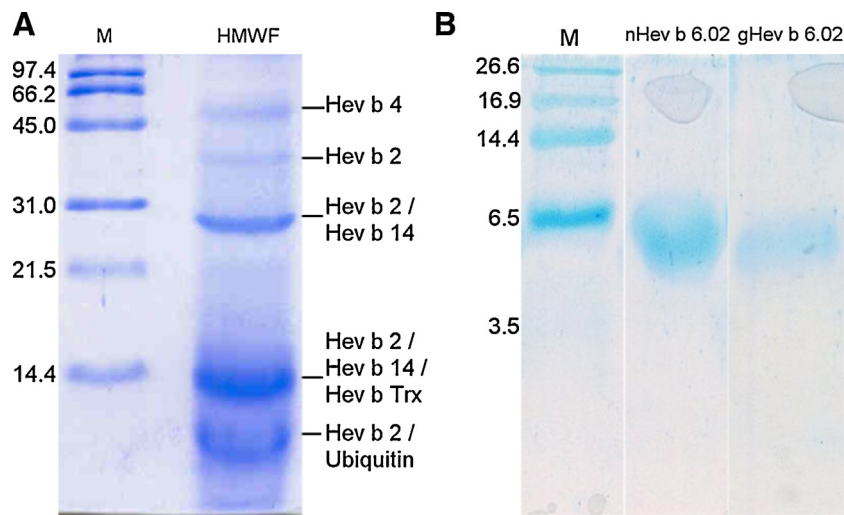


Fig. 1. SDS/PAGE gels showing the identified proteins in the surgical gloves. Lanes M show the molecular weight-size markers (kDa). (A) Proteins present in the HMWF. (B) Shows gHev b 6.02 which was present in the LMWF, and nHev b 6.02 for comparison.

Table 1
Proteins identified by LC/ESI-MS/MS in gloves extracts.

Protein	Molecular mass/pI	Accession number ^a	Coverage (%)	MS/MS peptide sequence (ion scores)
Hev b 4	41.1/4.6	Q5VLJ6	49	SELDEYLFSGDGLYDAGNAK (73), FIYPDKYLPYHHPYGTTFDYPTGR (75) TVVDFVAENVSLPR (104), IPPFK (15), FASEGATASDSNPLIDFR (134), DFGELK (26), GADDYLNIEIPSEASR (52), EQLESIVDVVLGNISDR (102), ELYDFGAR (46), KVVVENVAPLGLIPFIK (67), LPQILEK (48), EIIDAPGEHGFK (60), ESGFISPYGVK (61)
Hev b 2	41.5/9.5	ABN03966	22	NLPPVSEVIALYK (69), IYDPNQAVLEALR (90), GFWSSVR (30), YIAVGNEISPVNR (80), SPILLANIYPYFTYAGNPR (67), TYLSNLQHVK (33), HFGLFFPNK (46)
Hev b 14	22.1/8.3	E7BQV3	63	YSYVNIAFLNK (91), FGNGQTPQINLAGHCNPAAGGCTIVSNGIR (53), VMISLGGGIGSYTLASQADAK (137), NVADYLWSNFLGK (89), SSSRPLGDAVLGDIDFIEHGSLTYWDDLAR (190), YLSAYSKQGK (53), VYLTAAPQCFFPDR (86)
Hev b 5	16.1/4	Q39967	19	TEEPAPEADQTTPEEKPAEPEPVAEEEPK (70)
Hev b 6.01	22.1/5.4	Q6JYR0	27	CLSVTNTGTGAK (60), NGGLDLVDNVFR (67), QLDTDGKGYER (55), GHITVNYQFVD (78)
Thioredoxin	13.8/4.8	Q9XF70	8	LVGANPEEIR (49)

^a UniprotKnowledge database.

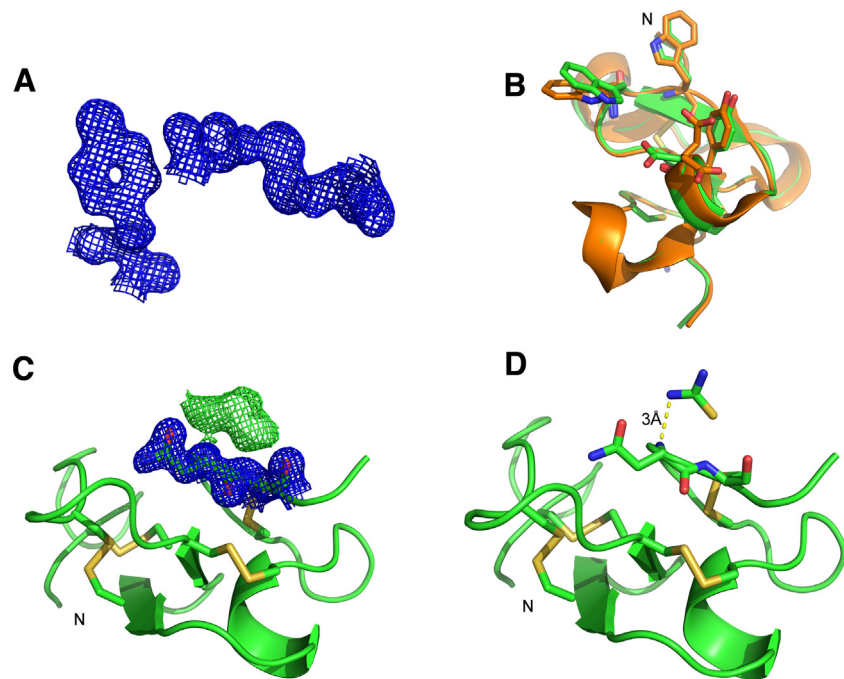


Fig. 2. (A) Two regions of the 2Fo-Fc map ($\sigma = 1$), in the left Tyr30 and in the right a disulfide bridge between residues Cys12 and Cys24. (B) Superposition of gHev b 6.02 (PDB 4WP4) (green) with nHev b 6.02 (PDB 1Q9B) (orange). (C) Model of gHev b 6.02 showing the omission map ($\sigma = 3$) in the region of the TOU molecule and the electron density ($\sigma = 1$) in the amino acids close to it. (D) gHev b 6.02 model showing the TOU molecule.

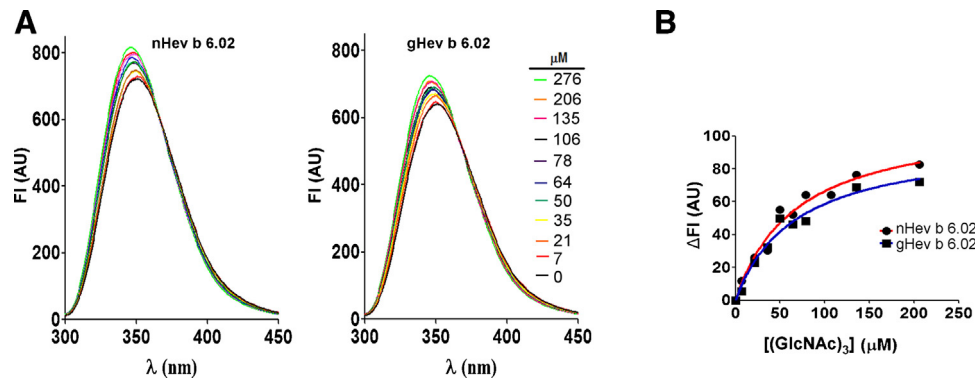


Fig. 3. (A) Fluorescence intensities of gHev b 6.02 and nHev b 6.02 at different ligand (GlcNAc)₃ concentrations. (B) Nonlinear regression analysis of the relative fluorescence intensity changes of the gHev b 6.02 and nHev b 6.02 samples in the presence of various concentrations of (GlcNAc)₃.

3.5. Characterization of Hev b 2 (β -1,3-glucanase) present in gloves

The glycosylated fraction with affinity to ConA showed three bands in SDS–PAGE electrophoresis, which were all identified as Hev b 2 after being excised and identified by the ESI-MS/MS method. The band with higher relative molecular mass (Mr) corresponded to the same electrophoretic profile as the non-processed Hev b 2 (nHev b 2; 38.1 kDa), while the other two had lower

Mr, indicating allergen fragmentation during gloves manufacture (Fig. 5A). The most prominent form of Hev b 2 in gloves has an apparent mass of about 31 kDa. All further experiments were performed using the sample containing these three forms of the allergen (gHev b 2).

The CD spectrum of gHev b 2 is not characteristic of a predominant α -helix structure, as expected for the non-processed protein, considering the 38% α -helix and 17% β -sheet content in its crystal structure (PDB 4HPG) (Rodríguez-Romero et al., 2014). However, the spectrum does not correspond to that of an unstructured protein, indicating that the glove allergen retains some of its secondary structure (Fig. 5B).

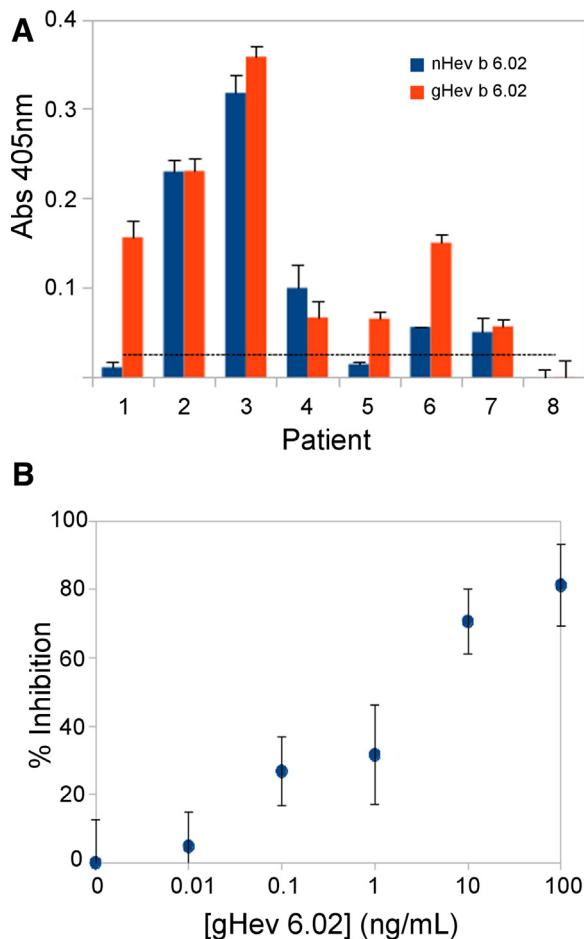


Fig. 4. (A) IgE-ELISA experiments showing recognition of sera from eight latex-allergic patients to gHev b 6.02 and nHev b 6.02. The dotted line indicates the cut-off criteria for a positive reaction. (B) Competitive ELISA experiment. The plate was coated with nHev b 6.02 and the competing allergen was gHev b 6.02.

3.6. IgE binding to gHev b 2 in comparison to nHev b 2

Sera from all eight latex-allergic patients recognized gHev b 2, and only seven of them recognized nHev b 2. All sera from the fruit-allergic patients ($n=8$) were able to recognize both samples. However, six of the latex-allergic patients exhibited increased binding for gHev b 2 (Fig. 6A), while seven of the fruit-allergic patients showed decreased binding to gHev b 2 in comparison to nHev b 2 (Fig. 6B). The statistical analysis shows a significant difference in how these two forms of the allergen were recognized by IgE from the latex-allergic group ($p<0.02$) and the fruit-allergic group ($p<0.001$). The experiments showed that different groups of allergic individuals have IgEs with different specificities.

4. Discussion

We analyzed some structural and biochemical aspects of the major allergens Hev b 6.02 and Hev b 2 present in latex surgical gloves after the vulcanization process and the other treatments involved in their manufacture; such as latex collection and preservation, lubrication, etc. Thermal stability has been recognized as a common characteristic for many allergens (Montserrat et al., 2013; Sharp et al., 2014; Usui et al., 2013). In agreement with these reports, it is remarkable that proteins detected in the studied gloves were almost exclusively known allergenic proteins. In addition to the latex allergens classified by the International Union of Immunological Societies (IUIS), we detected thioredoxin (Hev b Trx) in this material. This protein has been included in the Allergome Database (<http://www.allergome.org>), since it was identified as an IgE-interactive protein in a proteomic report of *H. brasiliensis* (Yagami et al., 2004). Thioredoxin has also been recognized as an allergen in other plants such as corn and wheat, and it has been demonstrated that IgE from allergic individuals cross-react in the presence of thioredoxins from these two sources (Weichel et al., 2006).

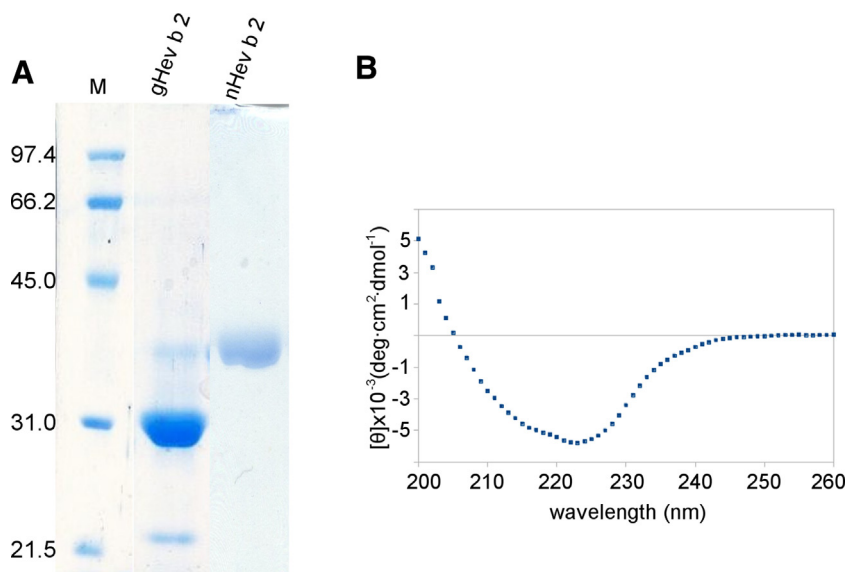


Fig. 5. (A) SDS/PAGE gel: Lane M, molecular weight-size markers (kDa); lane gHev b 2 shows the obtained fraction after purification with ConA, the three bands were identified as Hev b 2; the electrophoretic profile of nHev b 2 is shown for comparison. (B). CD spectrum of gHev b 2 in the far-UV region.

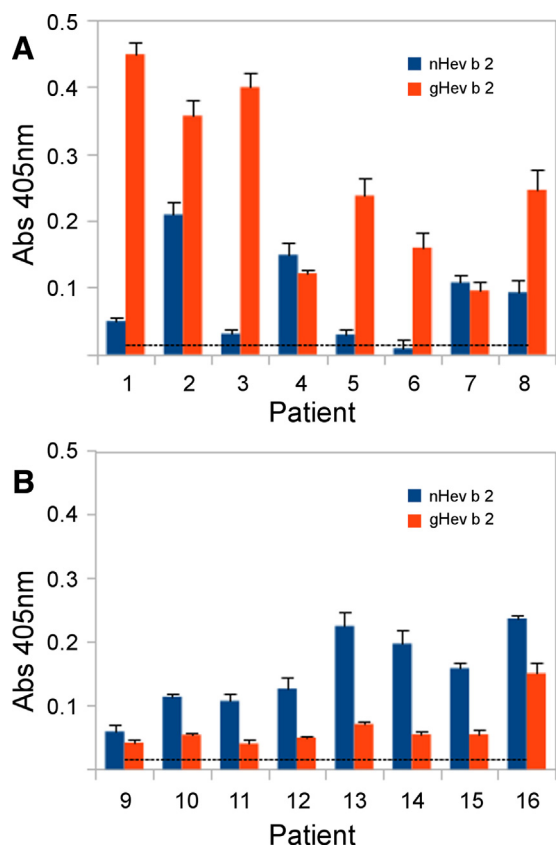


Fig. 6. IgE-ELISA experiments. (A). Recognition of sera from eight latex-allergic patients to gHev b 2 and nHev b 2. (B). Recognition of sera from eight fruit-allergic patients to gHev b 2 and nHev b 2. The dotted line indicates the cut-off criteria for a positive reaction.

Therefore, it is possible that Hev b Trx plays a role in cross-reactivity in people that are sensitized to thioredoxin from other sources.

The high-yield purification of gHev b 6.02 in its complete, properly folded form demonstrates the stability and resistance of the protein due to its compact structure stabilized by four disulfide bonds (Rodríguez-Romero, 1991). Likewise, other allergenic

proteins such as some plant lipid transfer proteins exhibit high thermal stability, which is often conferred by disulfide bonds (Ivanciuc et al., 2009). An example is the resistance to thermal treatment and maintained IgE recognition of the cherry allergen Pru av 3 (Scheurer et al., 2004). In the NRL surgical gloves that we examined, Hev b 6.02 was by far the most abundant protein, with an average yield of 118–196 µg per gram of material, depending on the product batch examined. The resistance of this allergen to high temperatures is consistent with a previous study, where a T_m value of 90 °C at pH 3.7 was reported for this protein (Hernández-Arana et al., 1995). The crystal structure of gHev b 6.02 (PDB entry 4WP4) clearly shows that there are no modifications in the polypeptide chain when compared with the structure of the natural one (PDB 1Q9B).

Interestingly, extra electron density appeared near the C-terminal region in the electron density maps (Fig. 2C), which we interpreted as a thiourea molecule, considering that this organic compound could have been carried by the protein from the manufacture process. Furthermore, we could determine that the protein conserved its biological activity as a lectin, since the K_d values for the ligand (GlcNAc)₃ were very similar between the two proteins. These values are also similar to previously reported results calculated by NMR techniques (86.5 µM) (Asensio et al., 1998). In accordance with the crystallographic structure, these results indicate that the arrangement of the amino acids involved in the carbohydrate-binding site is intact. It is important to mention that some residues of the site are also part of a conformational epitope (Pedraza-Escalona et al., 2009).

Notably, even with highly similar structures, the competitive ELISA experiments showed that gHev b 6.02 is capable of completely inhibit the recognition of nHev b 6.02. Since the only difference appreciated in the crystallographic structure was a molecule interacting with the protein, presumably thiourea bound during manufacture, we suggest that this compound might be the responsible of the different affinity to IgE when compared to nHev b 6.02.

Glycosylation moieties have been reported to play an essential role in the recognition of some allergens by IgE of allergic patients, such as the egg allergen ovomucoid (Benedé et al., 2013) and a variety of allergens from red meat containing galactose-α-1,3-galactose (Apostolovic et al., 2014). This is also the case for Hev b 2 (β-1,3-glucanase), for which two main isoforms have been described (Churngchow et al., 1995); however, the purification

method used allowed us to isolate isoform II from gloves extracts. We have previously demonstrated that the allergenic capability of this isoform of Hev b 2 is diminished when the N-glycosylation moieties are enzymatically removed. We also found that there are three posttranslational modifications (two N-glycosylations and a N-terminal pyroglutamate) that form a patch on the surface of this isoform that are coalescent on the molecular surface with the N and C terminal regions (epitopes 1 and 8 identified by Barre et al., 2009); which we proposed as a conformational epitope (Rodríguez-Romero et al., 2014). These carbohydrates include α -1,3-fucose bound to N-acetylglucosamine that is a common configuration in glycosylated proteins from plants and insects. Considering the possibility that this protein could still be glycosylated in the gloves, we purified it from this material using a ConA affinity column. Interestingly, not only the complete polypeptide chain of Hev b 2 retained the glycan moieties, but also fragments of the allergen, suggesting that these fragments also play a role in the allergenic potential of surgical gloves for Hev b 2-sensitized patients.

β -1,3-glucanase is a very conserved plant defense-related protein that is involved in cross-reactivity reactions with pollen and fruit allergens, sharing epitopes with other allergenic glucanases such as the one from banana (*Musa acuminata*) (Barre et al., 2009; Palomares et al., 2005). Hence, we decided to test the IgE reactivity from fruit-allergic individuals to gHev b 2, in addition to the sera from latex-allergic patients. Both groups of patients were able to recognize gHev b 2. Remarkably, IgE from latex-allergic patients showed increased binding to gHev b 2 in comparison to nHev b 2, while IgE from fruit-allergic patients showed the contrary. These results suggest that these two groups of patients are sensitized to different epitopes, probably the glycosylated epitope is the main recognition site for the latex-allergic patients, while the fruit-allergic group mainly recognize a different epitope; as one of those previously proposed for this allergen (Barre et al., 2009). If the fruit-allergic group recognizes an epitope that was lost after the manufacture process, this group would only react to the complete form of Hev b 2, which is not abundant in the gloves sample.

In summary, our results provide the first structural evidence that some allergens such as Hev b 6.02 maintain their three-dimensional structure, even after harsh procedures such as vulcanization. Others such as Hev b 2 maintain their IgE-binding capacity even after protein fragmentation. This study demonstrates the importance of testing the ultimate conformation of an allergen that undergoes a manufacturing process from the initial raw material. The unprocessed allergen might not always be the most adequate material for diagnosis or even for a therapy design, since we demonstrated here that IgE recognition is different, even when the allergen apparently does not suffer any structural modifications.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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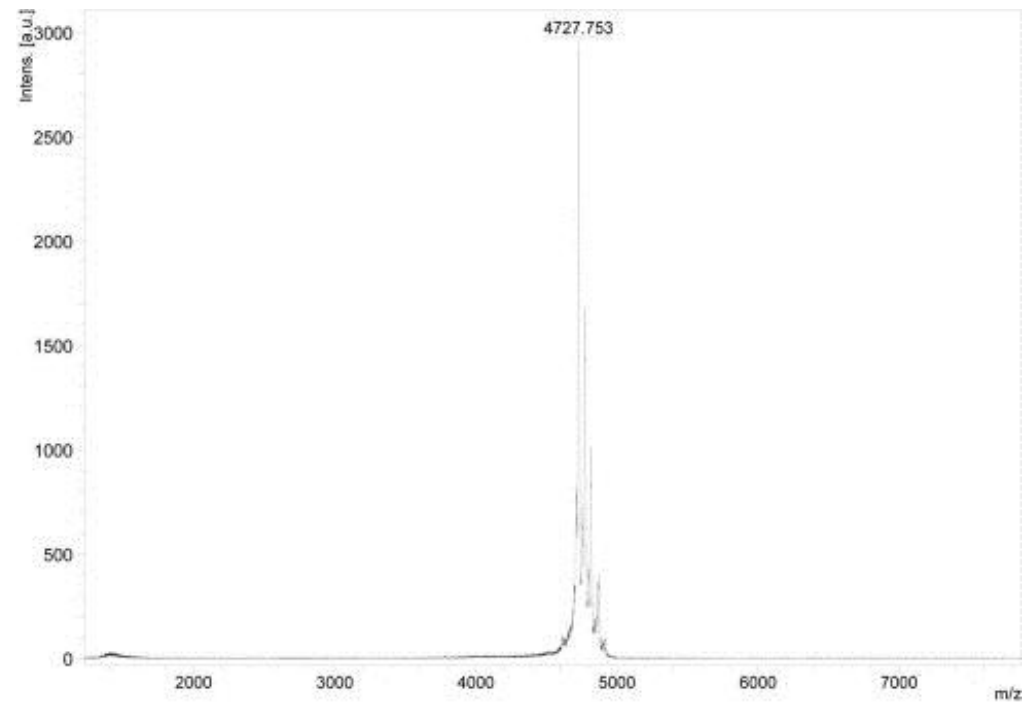
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.molimm.2015.01.018>.

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Impact of the vulcanization process on the structural characteristics and IgE recognition of two allergens, Hev b 2 and Hev b 6.02, extracted from latex surgical gloves

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Positive ion MALDI-TOF mass spectra in the linear mode obtained for Hev b 6.02 from gloves showing the expected mass of the complete protein of 4727 Da. The matrix used was α -Cyano-4-hydroxycinnamic acid.

Table S1. Data collection and refinement statistics.
Values in parentheses are for the highest resolution shell.

<i>Data collection</i>	
Space group	P 3 ₁ 2 1
Resolution (Å)	26.95-1.43 (1.48-1.43)
Unit cell parameters a, b, c (Å)	37.33, 37.33, 48.78
No. of unique reflections	13802 (1330)
Redundancy	11.8 (10.4)
Completeness (%)	99 (96.7)
Average I/σ	64.6 (24.4)
R _{merge} (%)	5.8 (18.6)
R _{pim} (%)	1.7 (5.9)
<i>Refinement</i>	
R _{work} /R _{free} (%) ^a	15.1/16.6
No. of non-hydrogen atoms	386
Average B-factors (Å ²)	20.0
Protein/water atoms	329/53
Ligand atoms	4
Rms deviations:	
Bond lengths (Å)	0.014
Bond angles (°)	1.45
PDB code	4WP4

^a R_{free} test set comprises 9.9% of the randomly selected reflections omitted from a model refinement.