

A molecular and *in silico* characterization of Hev b 4, a glycosylated latex allergen

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Received 11 October 2007; received in revised form 30 October 2007; accepted 16 November 2007

Available online 4 December 2007

Abstract

Hev b 4 is a heavily glycosylated latex allergen with seven attached *N*-glycans, comprising of both oligomannose and complex type structures. Treatment with a mixture of *N*-glycosidase A and *N*-glycosidase F resulted in lowering Hev b 4 protein on SDS-gel from 53 to 55 kDa to *circa* 40 kDa, this being comparable to the 38.53 kDa mass predicted by its cDNA. In Western-immunoblots, the enzymatically deglycosylated Hev b 4 showed negligible binding to IgE from latex allergic patients; the results indicated that IgE essentially binds to Hev b 4 via its *N*-glycan moiety. Structural modelling of the Hev b 4 was carried out based on the template protein and carbohydrate crystal coordinates of rhamnogalacturonan acetyltransferase (PDB ID 1DEO). We managed to link four *N*-glycan structures on to the Hev b 4 model; the glycans were scattered over the surface of the model. The structural and functional features of Hev b 4 could prove useful to elucidate its exposed epitopes which are important for IgE binding.

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Keywords: Latex allergen; Enzymatic deglycosylation; Homology-based modelling

1. Introduction

Latex from the rubber tree, *Hevea brasiliensis* contains a multitude of proteins. A small proportion of these proteins causes allergy in sensitized individuals, which is an Immunoglobulin E (IgE) mediated type I hypersensitivity [1]. Hev b 4 (lecithinase homologue) is one of the 13 allergenic latex proteins recognized by The International Union of Immunological Societies (IUIS). In addition to *in vitro* immunoassays, the ability of Hev b 4 to induce allergenic reactions in sensitized individuals has been demonstrated by skin prick tests [2].

On denaturing polyacrylamide gel electrophoresis (SDS-PAGE), Hev b 4 is discerned as a broad diffused band at between 53 and 55 kDa [3]; it was then suspected that the protein could be glycosylated. Subsequently, the complete cDNA, amino acid sequence, and bioinformatics analyses of Hev b 4 indicated the

presence of eight potential *N*-glycosylation and two potential *O*-glycosylation sites. Treatment with trifluoromethanesulfonic acid (TFMS) reduced the molecular weight of Hev b 4 to *circa* 40 kDa. Its glycan moiety was implicated in the binding of Immunoglobulin E (IgE) from blood sera obtained from patients allergic to natural rubber latex [4]. More recently, electrospray ionisation coupled with mass spectrometry (ESI-MS) analysis of the tryptic-digested Hev b 4 peptides revealed that the protein is predominantly *N*-glycosylated with seven out of the eight potential *N*-glycosylation sites occupied, consisting of both high mannose and complex type *N*-glycans [5]. The detailed characterization of Hev b 4 glycans had made it possible to select the appropriate enzymes for effective removal of its *N*-glycan moiety.

In the present study, enzymatic deglycosylation of Hev b 4 and its effect on the recognition to anti-Hev b 4 rabbit polyclonal antibody (IgG), and IgE from sera of latex allergic patients were investigated. Recognizing the significance of *N*-glycan moieties of Hev b 4 in relation to IgE binding, an effort was made to generate a three-dimensional (3D) model of Hev b 4 based on

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the available template crystal structure of rhamnogalacturonan acetyltransferase from protein data bank (PDB) [6] having 1DEO as its PDB code. The structural and evolutionary analysis of Hev b 4 models could prove useful to further elucidate the mechanism by which Hev b 4 interacts with IgE.

2. Materials and methods

2.1. Preparation of B-serum and protein precipitation by dialysis

Latex B-serum was prepared based on a method previously described [7]. Briefly, fresh latex was collected into chilled containers and centrifuged at $43,000 \times g$ for 1 h at 4 °C. The rubber cream and the C-serum were discarded while the bottom fraction was collected and re-suspended in 0.4 M mannitol to aid the removal of remnant C-serum while retaining the precipitated organelles (mainly luteoids) intact. The cleansed bottom fraction was recovered after another centrifugation and subjected to alternate freezing and thawing (four times) to rupture the organelles. The fluid from the organelles, the B-serum was recovered by centrifugation. Dialysis induced protein precipitation, preparation and isolation of the Hev b 4 protein complexes from latex B-serum were carried out essentially as described earlier [3].

2.2. Electrophoresis protocol and Western immunoblot

Analytical SDS-PAGE was performed according to standard procedures described elsewhere [8]. The gels were stained with Coomassie Brilliant Blue R250 (Sigma, USA) and molecular weight markers (14–97 kDa) were obtained from BioRad, USA. Western blotting of the separated proteins on to nitrocellulose membranes, subsequent incubation with rabbit polyclonal antibody, patient serum, and detection of antigen-antibody with a secondary antibody conjugated to alkaline phosphatase were performed essentially as previously described [3]. Anti-Hev b 4 rabbit polyclonal antibody used in this study was a gift from Dr. R.G. Hamilton (Johns Hopkins University School of Medicine, Baltimore, MD, USA). Sera from adult latex allergic patients were obtained from PlasmaLab International, USA. All patients had clinical symptoms to latex allergy, and the serum samples had anti-latex IgE confirmed by RAST or ELISA. Control non-latex-allergic human sera were obtained from Kuala Lumpur General Hospital, Malaysia.

2.3. Enzymatic deglycosylation of Hev b 4

In our preliminary experiments, a consistent removal of Hev b 4 glycan moiety required equal proportions of *N*-glycosidase A and *N*-glycosidase F. Thus in subsequent reactions, 50 µg of lyophilised Hev b 4 was denatured and reacted with the mixture of 0.5 mU *N*-glycosidase A and 5 U *N*-glycosidase F essentially as described in the instruction manual of the Glyco-protein deglycosylation kit (cat. no. 362280, Calbiochem, USA). The digestion reaction was performed in a water bath for 16 h, at 37 °C. After completion of the reaction, an aliquot of the sample was separated on SDS-PAGE to verify success of deg-

lycosylation, and the remainder of the sample was kept frozen until further use.

2.4. Selection of templates and fold recognition for three-dimensional modelling of Hev b 4

The identification of homologues of Hev b 4 (Swiss-Prot code Q6T4P0) was performed using the interactive search mode of SWISS-MODEL template library [9]. Although initial screening gave 14 templates that had similarity to Hev b 4, only those templates which had N-linked glycans attached were selected for homology-based modelling of the protein. The procedures described in FFAS03 [10], SAM-T02 [11], GeneSilico Metaserver [12], 3D-PSSM [13], and FUGUE [14] were used for fold recognition.

The project mode-based modelling using the SWISS-MODEL was adopted. The SWISS-MODEL project files contain the target sequences, the target template alignment, and one or more superposed template structures, *i.e.* the combination of modelled structure and the template structure in appropriate orientations. Project files were prepared using the DeepView program-Swiss-PdbViewer [15]. Four models of Hev b 4 were generated, using four different templates, but only one model was retained for further analysis, selected based on the WHAT IF [16] report.

2.5. Structural and evolutionary analysis

In the structural analysis of the modelled Hev b 4, calculation of secondary structure of the protein was performed using DSSP program [17]. The DSSP program defines secondary structure, geometrical features and solvent exposure of proteins, given atomic coordinates in Protein Data Bank (PDB) format. Functionally important amino acids (responsible for various biological interactions) on the protein surface or domain of known 3D structure were determined, and an evolutionary tree was constructed to relate Hev b 4 and all its homologues using Consurf [18].

2.6. In silico glycosylation of Hev b 4

High mannose carbohydrate content was automatically extracted from the coordinate file of 1DEO using *pdb2lincus* [19] tool. The extracted carbohydrate information was used for the *in silico* glycosylation of Hev b 4. The *in silico* glycosylation was carried out using *glyprot* [20], which is capable of connecting *N*-glycans *in silico* to a given 3D protein structure.

3. Results and discussion

3.1. Immunoglobulin binding to enzymatically deglycosylated Hev b 4

The purified Hev b 4 was treated with a mixture of *N*-glycosidase A and *N*-glycosidase F to effectively strip the α 1,3 fucosylated and high mannose types of *N*-glycans. Treatment with the glycosidases resulted in lowering of the band corre-

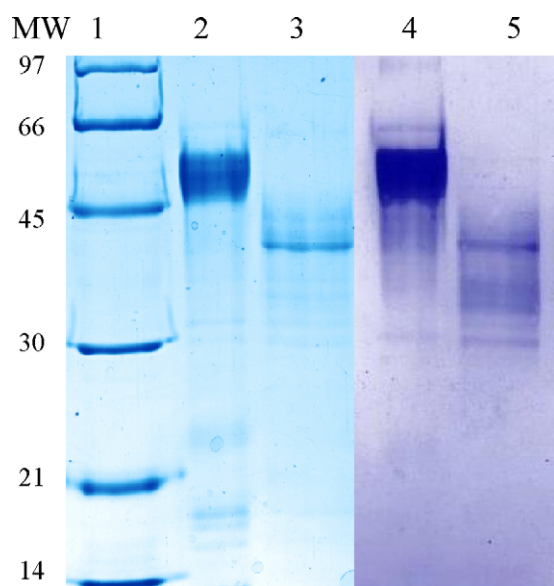


Fig. 1. SDS-PAGE separation and polyclonal IgG binding of Hev b 4. Coomassie blue-stained 12% SDS-PAGE showing molecular weight markers (lane 1), native Hev b 4 (lane 2), enzymatically deglycosylated Hev b 4 (lane 3). Western immunoblot shows binding of native Hev b 4 (lane 4) and enzymatically deglycosylated Hev b 4 (lane 5) to anti-Hev b 4 rabbit polyclonal IgG.

sponding to Hev b 4 from 53 to 55 kDa to *circa* 40 kDa On SDS-PAGE; this is comparable to value 38.53 kDa predicted by its cDNA. A smearing observed below the 40 kDa band is indicative of a slight degradation that could have occurred during the incubation (Fig. 1). The native Hev b 4 and its deglycosylated form were then Western blotted onto nitrocellulose membranes after electrophoresis. Both the native and deglycosylated protein showed binding to anti-Hev b 4 rabbit polyclonal antibody (Fig. 1). Western IgE immunoblots of Hev b 4 tested with sera from three patients with known type I hypersensitivity to the protein revealed that recognition to IgE was retained on the untreated glycosylated Hev b 4 but negligible in the deglycosylated protein; a representative blot of this experiment is shown in Fig. 2. The IgG and IgE binding characteristics of the enzymatically deglycosylated Hev b 4 is also comparable to earlier deglycosylation with TFMS [4]. Thus in agreement to the earlier postulation [5], it could be deduced that the epitopes specific to IgE largely reside in the *N*-glycan moiety of Hev b 4; presumably the α 1,3-fucosylated and β 1,2-xylosylated, the complex type *N*-glycans.

In the recent years, there has been increasing reports on IgE binding to allergenic glycoproteins harbouring complex type *N*-glycans of plant and invertebrate origin [21–24]. The role of *N*-glycan-specific IgE in the clinical manifestation of allergic reactions, however, is still a matter of debate. Although these *N*-glycans do interact with IgE, it is widely assumed that such cross-reactive carbohydrate determinants (CCDs) commonly have no clinical significance in allergies [25,26]. The ability of Hev b 4 to induce allergic reactions, however, has been confirmed by skin prick tests [2]. Likewise, the presence of carbohydrate IgE determinants on Hev b 13, a potent latex allergen, was also conclusively demonstrated [27]. In addition, *N*-glycans of some plant glycoproteins, e.g. Ole e 1 of olive tree pollen [28], Lyc e 2.02 of

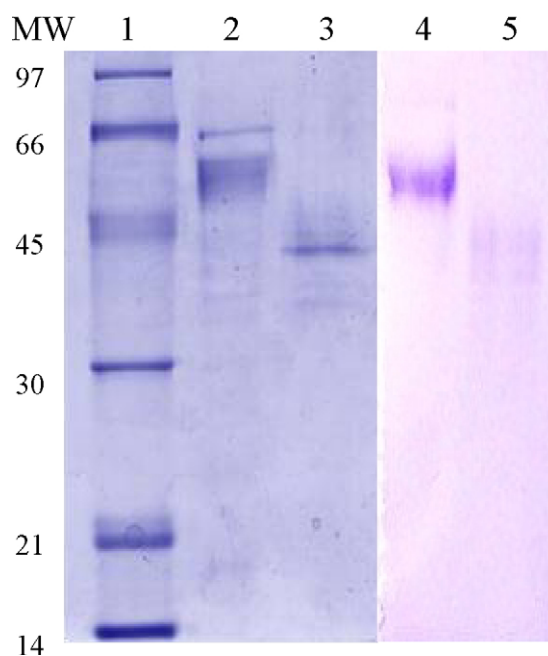


Fig. 2. Western IgE immunoblots of Hev b 4. Coomassie blue-stained 12.5% SDS-gel showing molecular weight marker (lane 1), native Hev b 4 (lane 2), enzymatic deglycosylated Hev b 4 (lane 3). Western immunoblot with sera from a latex allergic patient, IgE binding present in native Hev b 4 (lane 4), negligible IgE binding was detected in the enzymatically deglycosylated Hev b 4 (lane 5).

tomato [29], and Api g 5 of celery [30] were shown capable of eliciting *in vitro* effector release from basophil cells. Thus, examining the histamine release property [28,31,32] of the native and deglycosylated Hev b 4 would further strengthen the role of *N*-glycans, especially the α 1,3-fucosylated and β 1,2-xylosylated *N*-glycans in latex allergy *per se*. In any case, the possibility of conformational IgE epitopes of Hev b 4 is not ruled out.

3.2. Description of the three-dimensional modelling of Hev b 4

The complete coverage of the amino acid sequence and the *N*-glycan structures of Hev b 4 provided the basis for constructing a 3D model of the glyco-allergen. In our attempt to model Hev b 4, a search for suitable templates was performed in the SWISS-MODEL template library [9]. A number of templates from the GDSL-like lipase domain matched Hev b 4, although none of these shared a very good overall homology (sequence identity of ~15%). In addition, analyses of Hev b 4 using fold recognition servers, *i.e.* FFAS03 [10], SAM-T02 [11], Gene-Silico Metaserver [12], 3D-PSSM [13], and FUGUE [14] also derived at GDSL-like lipases. Using the available information, a preliminary model of Hev b 4 containing six out of its seven *N*-glycans on the protein backbone was constructed.

The architecture of Hev b 4 model is very similar to the crystal structure of 1DEO. A major feature of the Hev b 4 (and the template structure) is a way by which alpha helices and beta sheets are arranged (Fig. 4). The Hev b 4 model consists of six alpha helices and three 3–10 helices, whereas the 1DEO comprises of seven alpha helices and three 3–10 helices; both structures consist of seven extended beta sheets.

Table 1
The occurrence of unusual bond angles and bond lengths in some amino acid residues of Hev b 4

Bond lengths				Bond angles			
Residue	Bond length type	Bond length	S.D.	Residue	Bond angle type	Bond angle	S.D.
Pro53	CA C	1.64	4.7	Pro53	N CA C	122.49	4.3
Val85	N CA	1.55	4.7	Pro53	N CA CB	98.41	−4.2
Val85	N−C ^a	1.41	4.7	Asp115	CA CB CG	116.75	4.1

^a Atom names starting with “−” belong to the previous residue in the chain.

3.3. Quality assessment of the model

WHAT IF [16] analysis was preformed to evaluate the stereo chemical quality of the Hev b 4 model. Standard bond lengths and bond angles were determined according to Engh and Huber [33]. The analysis revealed RMS Z-scores for bond lengths and bond angles of 0.781 and 0.906, respectively, which is close to 1, indicating a high quality of structure. Some discrepancies in RMS Z-scores were noted in the form of three unusual bond lengths and bond angles, however, this is the best we could do with the available information (Table 1). The side chain of a number of residues namely, His57, Phe64, Asp115, Tyr66 and Phe204 were found to contain planar groups that deviate from planarity by more than four times of the expected value. The hydroxyl (−OH) group of Tyr197 that is connected to the planar aromatic group in the side chain of the amino acid residue was found to deviate (6.966) from the least square plane. The overall Z-score of −2.565 however, indicated the compliance (not exceeding the expected ranges for well defined structures) of all residues in the backbone conformations corresponding to the known allowed areas in the Ramachandran Plot.

3.4. Structural and evolutionary analysis

Analysis using Consurf showed that 33 amino acid residues were exceptionally conserved while 16 residues were highly variable. Seventeen out of the 33 conserved residues were

present in the loop regions, whereas helix and sheet occupy 10 and 6 residues, respectively. Similarly, Consurf analysis of 1DEO_A reported 45 residues to be highly conserved with 22 residues being mainly present in the loops, while helix and sheets occupy 15 and 8 residues, respectively. The programme calculates the conservation score in every alignment position, taking into account the relationships between the sequences and the physicochemical similarity between the amino acids. The conservation scores are represented on a 10-point scale and can be used to determine functional relevance of a residue in evolution. The phylogenetic tree of Hev b 4 and all of its homologues is presented in Fig. 3.

3.5. Secondary structure and solvent accessibility of Hev b 4 amino acid residues

The DSSP data on the secondary structure depicted that Hev b 4 mainly consists of alpha helix and beta strands, in addition to loops; these values were coherent with their template PDB files. An overall secondary structure pattern of Hev b 4 with the main template (PDB code 1DEO) is shown in Fig. 4.

3.6. In silico glycosylation results

Glyprot [20] recognized all the six N-glycosylation sites (ASN84, ASN99, ASN142, ASN188, ASN224, and ASN256) in the modelled structure of Hev b 4. The software, how-

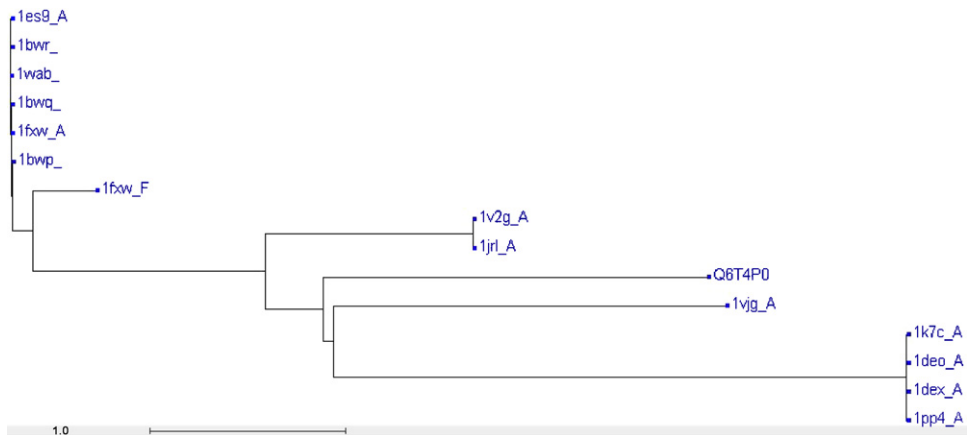


Fig. 3. Phylogenetic tree derived by comparing the amino acid sequences of Hev b 4 and its 14 homologues (represented by their PDB codes) using Consurf program. The Hev b 4 homologues belong to the family of hydrolases and possess flavodoxin-like fold; (1es9_A, 1bwr, 1wab, 1bwq, 1fxw_A, 1bwp and 1fxw_F are platelet-activating factor acetyl hydrolases; 1v2g_A and 1jrl_A are acyl-coA thioesterases; 1vjg_A is a putative GDSL lipase; 1k7c_A, 1DEO_A, 1dex_A and 1pp4_A are rhamnogalacturonan acetyl esterases). The tree revealed that Hev b 4 (Q6T4P0) and the main template (1DEO_A) used for the modelling of the allergen belong to the same group.

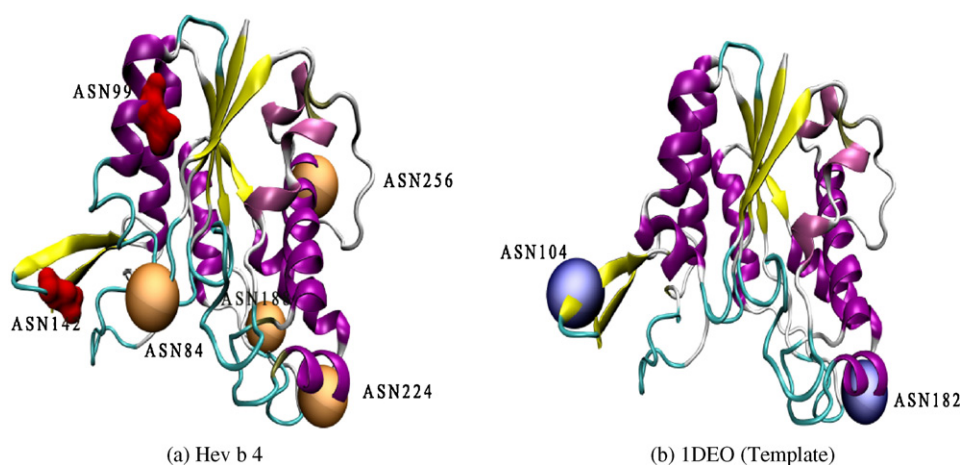


Fig. 4. The preliminary model of Hev b 4 (a) showing the occupied glycosylation sites in orange beads (ASN84, ASN188, ASN224 and ASN256) and unoccupied glycosylation sites in red (ASN142 and ASN99) and the template IDEO (b), showing the two occupied glycosylation sites as blue beads (ASN104 and ASN182). It may be noted that both the figures show the presence of alpha helix and beta strands. (For interpretation of the references to colour in this figure caption, the reader is referred to the web version of the article.)

ever reported only four potential *N*-glycosylation sites (ASN84, ASN188, ASN224, and ASN256) to be accessible for *N*-linked glycosylation. A noteworthy feature of the Hev b 4 model is that all carbohydrate sites were topologically at the surface, similar to the template (Fig. 4a and b).

As mentioned earlier, the *N*-glycosylation sites occupied by Hev b 4 consist of both high mannose and complex type *N*-glycans except ASN224, which was occupied, exclusively by complex type *N*-glycans [5]. In this study we were able to add oligomannosidic *N*-glycans to the modelled structure of Hev b 4 paving way for attachment of more complex glycans as their structure becomes available in the future.

4. Conclusion

As observed from the Western immunoblots, the *N*-glycans of Hev b 4 especially the complex type is deemed important in recognition of IgE from latex allergic patients. The conceptual binding of *N*-glycan moiety of an allergen to IgE has yet to be demonstrated. In the *in silico* part of the study, a suitable template was selected from PDB database, based on homology to a large part of the Hev b 4 sequence. This facilitated the construction of a 3D model of Hev b 4, with structural similarity to a sugar-binding protein, IDEO; the modelled structure of Hev b 4 could pave way for a better understanding of its interactions to antibody, especially IgE. However, the regions of the Hev b 4 sequence that remain outside the similar models (templates) and the quality of the constructed model are among the minor issues that need to be addressed.

Acknowledgement

The excellent technical assistance of Pn. Azlina Azharuddin is greatly acknowledged.

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