Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Crystallization and identification of the glycosylated moieties of two isoforms of the main allergen Hev b 2 and preliminary X-ray analysis of two polymorphs of isoform II

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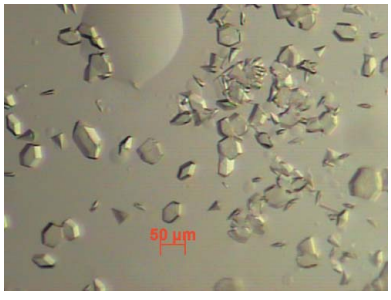
Received 5 July 2007
Accepted 10 August 2007



Latex from *Hevea brasiliensis* contains several allergenic proteins that are involved in type I allergy. One of them is Hev b 2, which is a β-1,3-glucanase enzyme that exists in different isoforms with variable glycosylation content. Two glucanase isoforms were isolated from trees of the GV-42 clone by gel filtration, affinity and ion-exchange chromatography. Isoform I had a carbohydrate content of about 20%, with N-linked *N*-acetyl-glucosamine, *N*-acetyl-galactosamine, fucose and galactose residues as the main sugars, while isoform II showed 6% carbohydrate content constisting of *N*-acetyl-glucosamine, fucose, mannose and xylose. Both isoforms were crystallized by the hanging-drop vapour-diffusion method. Isoform I crystals were grown using 0.2 *M* trisodium citrate dihydrate, 0.1 *M* Na HEPES pH 7.5 and 20%(*v*/*v*) 2-propanol, but these crystals were not appropriate for data collection. Isoform II crystals were obtained under two conditions and X-ray diffraction data were collected from both. In the first condition (0.2 *M* trisodium citrate, 0.1 *M* sodium cacodylate pH 6.5, 30% 2-propanol), crystals belonging to the tetragonal space group $P4_1$ with unit-cell parameters $a = b = 150.17$, $c = 77.41$ Å were obtained. In the second condition [0.2 *M* ammonium acetate, 0.1 *M* trisodium citrate dihydrate pH 5.6, 30%(*w*/*v*) polyethylene glycol 4000] the isoform II crystals belonged to the monoclinic space group $P2_1$, with unit-cell parameters $a = 85.08$, $b = 89.67$, $c = 101.80$ Å, $\beta = 113.6^\circ$. Preliminary analysis suggests that there are four molecules of isoform II in both asymmetric units.

1. Introduction

IgE-mediated allergy to natural rubber latex (NRL) has been recognized as an important health problem in developed countries. Manufactured products contain allergenic proteins that are capable of causing IgE-mediated allergy in sensitized patients (Koh *et al.*, 2005; Asero *et al.*, 2005). *Hevea brasiliensis* latex has been reported to contain several allergenic proteins that cause type I hypersensitivity (Breiteneder & Scheiner, 1998; Poley & Slater, 2000; Yeang *et al.*, 2002). Among them, the 1,3-β-glucanases (EC 3.2.1.39) are a family of hydrolytic enzymes that belong to the pathogenesis-related proteins (PRP), being expressed in response to microbial attack or tissue wounding (van den Bulcke *et al.*, 1989; van Loon *et al.*, 1994). Latex 1,3-β-glucanase is a protein of 36 kDa that has been assigned the allergen name Hev b 2 (Sunderasan *et al.*, 1995; Alenius *et al.*, 1995) and that is recognized by IgEs of latex-allergic patients. This protein has been shown to be one of the most allergenic and both its peptidic and carbohydrate moieties are recognized by IgEs (Kurup *et al.*, 2000, 2005). In general, the allergenic determinants are mainly of peptidic nature and can be linear or conformational epitopes (Reyes-López *et al.*, 2004).

Churngchow *et al.* (1995) detected two isoforms of latex 1,3-β-glucanase from the vacuoles or lutoids of NRL. Both isoforms were endoglucanases; however, isoform I did not have affinity for concanavalin A (Con A) and did not bind to IgEs from the sera of allergic subjects. In 2002, Yagami and coworkers demonstrated that the carbohydrate moiety of Hev b 2 plays a dominant role in the recognition of the allergen by IgEs from latex-allergic patients (Yagami *et al.*, 2002), while Palomares *et al.* (2005) demonstrated the cross-reactivity with pollen and fruit allergens. In recent years, there has

been increasing evidence of IgE antibodies binding to allergenic glycoproteins containing α1,3-fucosylated and β1,2-xylosylated N-glycans of plant and invertebrate origin (van Ree *et al.*, 2000; Wilson *et al.*, 1998; Mahler *et al.*, 2006). Nonetheless, the role of N-glycan-specific IgEs in the clinical manifestations of allergic reactions is still a matter of debate. Batanero *et al.* (1999) and Iacovacci *et al.* (2002) have carried out histamine-release tests and demonstrated that carbohydrates could trigger basophil degranulation. These results suggest that the carbohydrate moieties of the native glycoprotein are important in glucanase allergy.

Knowledge of the three-dimensional structures of native allergens will help in elucidation of the antigenic determinant features of these molecules. However, it is well known that the presence of attached oligosaccharides can negatively influence the crystallizability of proteins. This phenomenon is presumably a consequence of the excess surface conformational entropy exhibited by the sugar chains and of microheterogeneity in glycan chain length and composition. To date, no structures of native 1,3-β-glucanases have been reported, but two three-dimensional structures of recombinant enzymes from plants have been published (Receveur-Bréchot *et al.*, 2006; Varghese *et al.*, 1994). They adopt the $(\beta/\alpha)_8$ topology or TIM-barrel structure. In this paper, we report the purification, glycosylation characterization and crystallization of two glycosylated native isoforms of the Hev b 2 allergen from NRL lutoid particles. Additionally, we report the preliminary X-ray characterization of two isoform II crystals, which crystallized in the tetragonal and monoclinic systems.

2. Materials and methods

2.1. Protein purification and characterization

H. brasilensis (clone GV-42) latex was obtained from the El Palmar plantation (Veracruz state, México). The collected latex was separated into rubber particles, C-serum and lutoids (the bottom fraction) by centrifugation at 70 000*g* for 30 min at 277 K (Rihs & Raulf-Heimsoth, 2003). The lutoid fraction containing the Hev b 2 isoforms was stored at 253 K until use. The following steps were performed at 277 K. Frozen lutoids were homogenized in 0.3 *M* NaCl solution with a Waring blender. The homogenate was cleared by centrifugation and the resultant supernatant was dialyzed against water for 48 h. The precipitate formed was separated by centrifugation and the pellet was resuspended in 20 ml 50 m*M* MES buffer pH 6.5, 0.35 *M* NaCl. The sample was filtered through a 0.22 µm Millipore membrane (Millipore, Billerica, USA) and subjected to gel filtration on a HiLoad 16/20 Superdex 200 column equilibrated and eluted with the same buffer in an Akta FPLC System (Pharmacia-LKB Biotechnology, Sweden) at a flow rate of 1 ml min^{-1}. Fractions containing β-glucanase activity were pooled, concentrated and dialyzed against 50 m*M* Tris–HCl pH 8.0 containing 0.5 *M* NaCl (elution buffer) in Amicon stirred cells through an Amicon membrane with a molecular-weight cutoff of 10 kDa. The activity was measured on laminarin (Miller, 1959). The pooled fraction was loaded onto a Con A Sepharose 4B (Pharmacia) column previously equilibrated with elution buffer and eluted with a linear gradient (0–0.5 *M*) of methyl-α-D-mannopyranoside (Sigma Chemical Co., St Louis, USA). Both the bound and nonbound fractions of Con A showed activity. Each isoform was purified to homogeneity by cation-exchange chromatography using a Mono S 5/50 GL column (Pharmacia-LKB Biotechnology, Sweden) equilibrated in 50 m*M* phosphate pH 7.5 and eluted with a linear gradient from 0 to 1 *M* NaCl. The nonbound fraction was named isoform I, while the bound fraction was named isoform II (Churngchow *et al.*, 1995). The protein concentration was determined by measuring the absorbance at 280 nm using an extinction coefficient of 1.57 g^{-1} ml calculated from the sequence of *H. brasiliensis* endo-β-1,3-glucanase deposited in the National Center for Biotechnology Information data bank (accession No. AAG24921).

2.2. SDS–PAGE and carbohydrate detection

SDS–PAGE was performed according to the method of Laemmli (1970) under reducing conditions. The separating gel contained 12% acrylamide. The isoforms were stained with Coomassie R-250 Brilliant Blue for determination of their relative molecular weight. Low-molecular-weight standards (Bio-Rad, Hercules, USA) consisting of phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa) were run with the samples. Carbohydrate detection of isoforms was performed by staining with fuchsin-sulfite reagent (Sigma Chemical Co., St Louis, USA) and dioxygenin (Roche Molecular Biochemicals, East Sussex, England).

2.3. N-terminal sequencing

Isoform I was digested with trypsin and sequenced by electrospray ionization quadrupole tandem mass spectrometry using a 3200 QTRAP system (Applied Bioscience/MDS Siex, Ontario, Canada) equipped with a nano-electrospray source and nano-flow LC system (Agilent 1100 nano pump, Waldbrow, Germany). Database searching (NCBInr) and protein identification were performed using *Mascot* (http://www.matrixscience.com). The N-terminal sequence of isoform II and its proteolytic fragments generated by trypsin digestion were determined by Edman degradation using an automatic gas-phase protein sequencer (Model LF 3000, Beckman Instruments, Irvine, USA).

2.4. Mass-spectrometric analysis

Spectrometric measurements were performed using a matrix-assisted laser desorption ionization–time of flight MALDI–TOF Omniflex mass spectrometer from Bruker Daltonics (Bremen, Germany) equipped with a pulsed nitrogen laser (λ = 337 nm, 10 ns pulse width). Spectra were acquired in positive-reflection mode with a 19 kV accelerating voltage. The proteins were dissolved in a saturated solution of 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) in 30% acetonitrile, 0.1% trifluoroacetic acid. Carbonic anhydrase (28 kDa) and bovine serum albumin (66 kDa) were used for internal calibration.

2.5. Carbohydrate estimation and monosaccharide composition

The carbohydrate content was determined by means of a Glycoprotein Carbohydrate Estimation kit (Pierce, Rockford, USA) according to the manufacturer's specifications. Additionally, the carbohydrate compositions of both isoforms were determined from the heptafluorobutyrate derivatives of O-methylglycosides obtained after methanolysis with 0.5 *N* methanol–HCl for 24 h at 373 K by gas chromatography with a capillary column (25 × 0.32 mm) of 5% Silicone OV 210 (Applied Science Laboratories, Buffalo, USA). L-Lysine (Sigma Chemical Co., St Louis, USA) was used as an internal standard. Identification of each sugar residue was confirmed by mass spectrometry using a Finnigan Automass II mass spectrometer (Thermo Fisher Scientific Inc., Waltham, USA). The heptafluorobutyrate derivatives of the O-methylglycosides presented relatively high molecular weights: 978 Da for hexoses, 977 Da for hexosamines and 1275 Da for sialic acid (Zanetta *et al.*, 1999).

Table 1
Data-collection statistics of the two Hev b 2 isoform II crystals obtained from *H. brasiliensis*.

Values in parentheses are for the last shell.

Space group	$P4_1$	$P2_1$
Unit-cell parameters (Å, °)	$a = b = 150.17$, $c = 77.41$	$a = 87.08$, $b = 89.67$, $c = 101.40$, $\beta = 113.6$
Resolution range	43.78–2.65 (2.74–2.65)	44.50–2.55 (2.64–2.55)
Total no. of reflections	244012	177127
No. of unique reflections	49848	45830
Average redundancy	4.99	3.86
Completeness	99 (99.2)	98 (86.6)
$R_{\rm merge}$†	0.109 (0.44)	0.085 (0.16)
$\langle I/\sigma(I)\rangle$	8.9 (3.1)	10.6 (4.4)
Solvent content (%)	57	49
Molecules per ASU	4	4

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

Table 2
Sequence of tryptic peptide fragments of Hev b 2 isoforms I and II.

Peptidic fragment	Isoform I	Isoform II
1	G^{79}FWSSVLFR^{87}	G^{210}YKNLFDA^{217}
2	Y^{88}IAVGNEISPVNR^{100}	A^{278}IETYLFAMF^{287}
3	N^{213}LFDATLDVLYSALER^{228}	
4	T^{258}YLSNLIQHVK^{268}	
5	A^{278}IETYLFAMFDENK^{291}	
6	Q^{299}FGLFFPDKWQK^{310}	

2.6. Crystallization

Each isoform was concentrated and dialyzed to 10 mg ml^{-1} in 0.1 *M* sodium citrate pH 5.0 buffer in an Amicon Ultrafree 0.5 centrifugal filter unit (Millipore) with a molecular-weight cutoff of 10 kDa. The hanging-drop vapour-diffusion method and 24-well Linbro crystallization plates were used in conjunction with Hampton Research Crystal Screen I solutions (Hampton Research, Laguna Niguel, USA) to search for suitable crystallization conditions. Solutions with pH values below 8 were selected owing to the basic pI of these proteins. Drops were prepared by mixing 5 µl protein solution and 5 µl precipitant solution at 291 K. Isoform I crystallized in solution No. 27 [0.2 *M* trisodium citrate dihydrate, 0.1 *M* Na HEPES pH 7.5, 20%(*v*/*v*) 2-propanol]. Diffraction-quality crystals of isoform II were obtained using two conditions. In the first condition, 0.2 *M* trisodium citrate dihydrate, 0.1 *M* sodium cacodylate pH 6.5 and 30%(*v*/*v*) 2-propanol (solution No. 8), crystals took six months to grow using either the hanging-drop or the sitting-drop vapour-diffusion method. In the second condition, good-quality crystals grew using 0.2 *M* ammonium acetate, 0.1 *M* trisodium citrate dihydrate pH 5.6 and 30%(*w*/*v*) polyethylene glycol 4000 (solution No. 9) in approximately one year. For X-ray diffraction data collection, isoform II crystals grown in the first condition were cryoprotected by a quick soak in reservoir solution containing 35%(*w*/*v*) trehalose, whereas for crystals grown in the second condition this was not necessary.

2.7. Data collection and processing

Firstly, all protein crystals were characterized in the home laboratory using a rotating-anode generator (Cu $K\alpha$, $\lambda = 1.5416$ Å) with a Rigaku R-AXIS IIC image-plate system (Rigaku, Texas, USA). For isoform II crystals grown in the first condition, X-ray diffraction data were collected to 2.65 Å at 100 K on beamline X6A at the National Synchrotron Light Source (Upton, New York, USA). A total of 180 images with a 1.0° oscillation range per frame were initially integrated and scaled using the *HKL*-2000 suite (Otwinowski & Minor, 1997). A isoform II crystal obtained using the second condition diffracted to 2.55 Å at 100 K on beamline 5-ID at the Dupont Northwestern Dow and Life Science Collaborative Access Team station (DND-CAT) at the Advanced Photon Source, Argonne National Laboratory (Illinois, USA). The raw data from crystals grown under both conditions were finally processed and scaled using the *CrystalClear*/*d***TREK* program suite (Pflugrath, 1999). The data-collection statistics are summarized in Table 1.

3. Results and discussion

Two isoforms of the allergen Hev b 2 from *H. brasiliensis* latex were purified to homogeneity by several chromatographic steps. Purified isoforms were detected as a single band on SDS–PAGE with an apparent molecular weight of 40 kDa (Fig. 1*a*). Mass-spectrometric analysis showed an *m*/*z* ratio of 38 397 for isoform I and of 38 093 for isoform II. Six peptidic fragments from isoform I were sequenced by mass spectrometry and analyzed using the *Mascot* search engine (http://www.matrixscience.com). They were identified in the Hev b 2 sequence of a Malayan clone with GenBank accession No. AAG24921 (Table 2).

Isoform II had its N-terminal end (Gln1) blocked, as shown by Edman degradation. Two reduced-alkylated peptides obtained after trypsin digestion were sequenced. An eight-amino-acid sequence (G^{210}YKNLFDA) and a ten-amino-acid sequence (A^{278}IETYLFAMF) showed identity with the Malayan Hev b 2 sequence and overlap with two other peptidic fragments (3 and 5) of the isoform I sequence (Table 2). Overall, they represent 23% of the whole sequence deposited in the NCBI, which contains two potentially *N*-glycosylated sites at positions Asn27 and Asn65 as predicted with the help of the *NetNGlyc* server (http://www.cbs.dtu.dk/services/NetNGlyc).

SDS–PAGE stained with fuchsin-sulfite reagent showed that both isoforms are glycosylated (Fig. 1*b*). However, dioxygenin did not stain isoform I, which was not bound to the Con A column (results not shown). The carbohydrate contents estimated by the Pierce protocol were 19.8% and 5.7% for isoforms I and II, respectively, which justify the molecular-weight differences shown above.

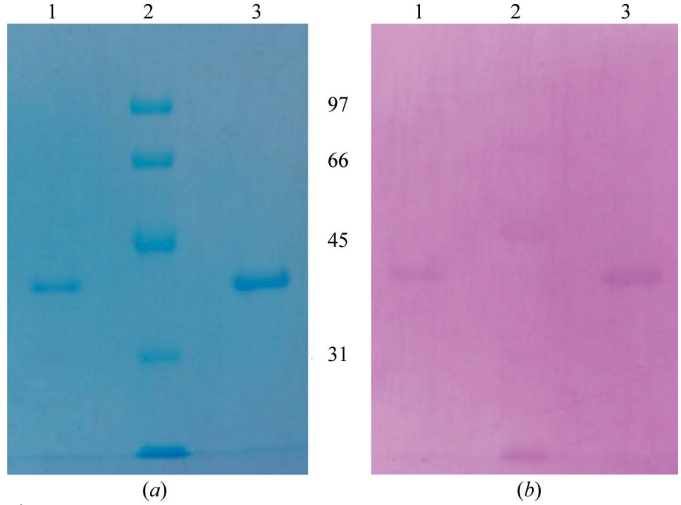


Figure 1
(*a*) Coomassie Brilliant Blue-stained SDS–PAGE of purified Hev b 2 isoforms. Lane 1, isoform II; lane 2, molecular-weight standards (kDa); lane 3, isoform I. (*b*) The same SDS–PAGE stained with fuchsin-sulfite reagent. The band stained in the marker lane is ovalbumin (45 kDa), which has a 3% carbohydrate content.

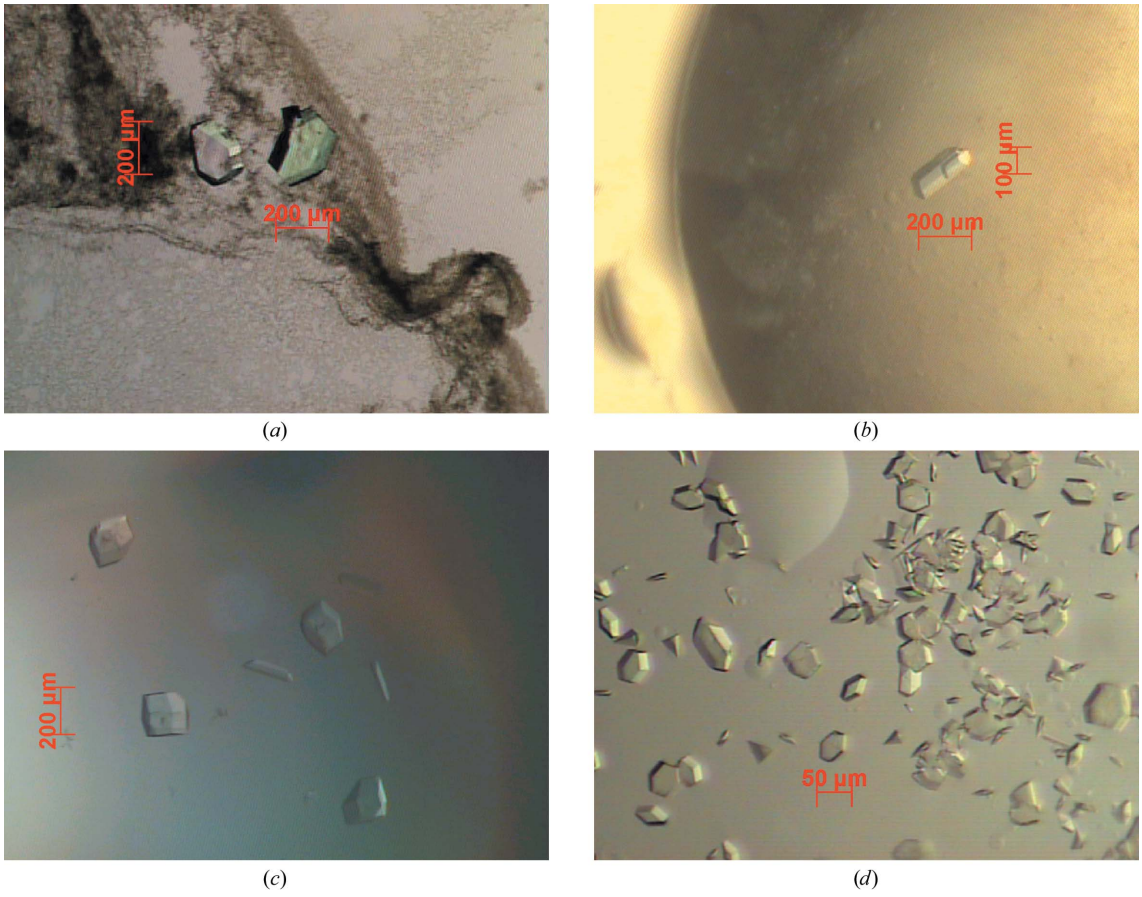


Figure 2
Hev b 2 crystals. (*a*) Isoform II crystals grown in the first condition (see text). (*b*) Isoform II crystals obtained in the second condition (see text). (*c*) Isoform II crystals obtained by the sitting-drop vapour-diffusion method using the first condition. (*d*) Isoform I crystals. The crystals in (*a*), (*b*) and (*d*) were grown by the hanging-drop vapour-diffusion method.

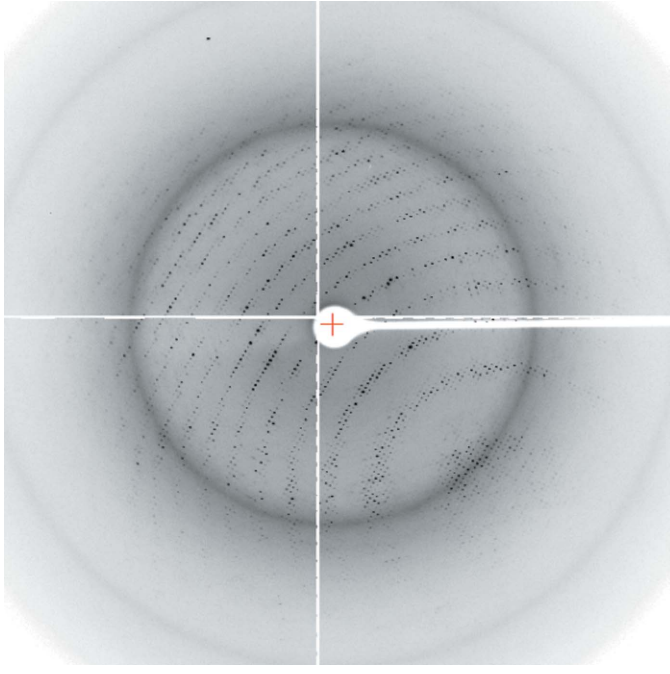


Figure 3
X-ray diffraction pattern of the isoform II crystal (Fig. 2*c*) collected to 2.65 Å resolution on beamline X6A, NSLS.

The monosaccharide compositions of the isoforms also differed. Isoform I contained N-linked *N*-acetyl-glucosamine, *N*-acetyl-galactosamine, fucose and galactose residues, while isoform II contained *N*-acetyl-glucosamine, fucose, mannose and xylose residues. The binding of isoform II to Con A suggests that it contains a trimannosidic core α-(1$\rightarrow$3)-linked and α-(1$\rightarrow$6)-linked to another mannose residue bound to two *N*-acetyl-β-glucosaminyl residues (Debray *et al.*, 1981). These isoforms are the second NRL allergens whose carbohydrate composition has been reported. Previously, Kolarich *et al.* (2006) described another highly glycosylated NRL allergen, Hev b 4, a glycoprotein with glucosidic activity containing mannose, fucose and xylose residues. The Hev b 4 carbohydrate moiety has been implicated in the binding of IgEs from NRL-allergic patients (Sunderasan *et al.*, 2002).

Both glycosylated isoforms of Hev b 2 were crystallized (Fig. 2). The isoform I crystals were small (~25 × ~30 × ~10 µm) and diffracted poorly (Fig. 2*d*). In contrast, two crystallization conditions were found to produce good-quality crystals of isoform II. The crystals grown in the first condition (Figs. 2*a* and 2*c*) diffracted to 2.65 Å (Fig. 3) with 99% completeness. They belonged to the primitive tetragonal space group $P4_1$, with unit-cell parameters $a = b = 150.17$, $c = 77.41$ Å. The Matthews coefficient (Matthews, 1968) of 2.83 Å^3 Da^{-1} suggests the presence of four molecules in the asymmetric unit, corresponding to a solvent content of 57%. Isoform II crystals grown in the second condition (Fig. 2*b*) diffracted to 2.55 Å with 98% completeness. The crystals belong to space group $P2_1$, with

unit-cell parameters a = 87.08, b = 89.67, c = 101.40 Å, β = 113.57°. The Matthews coefficient of 2.63 Å^3 Da^{-1} indicates a solvent content of 49% (Table 1) and again four molecules in the asymmetric unit. In contrast, for other recombinant plant β-1,3-glucanases the asymmetric unit has been reported to be a monomer (Receveur-Bréchot *et al.*, 2006; Varghese *et al.*, 1994). The program *Phaser* (McCoy, 2007) from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994) was employed for molecular-replacement procedures using banana β-1,3-glucanase (PDB code 2cyg) as the search model (Receveur-Bréchot *et al.*, 2006). This model has 59% sequence identity to Hev b 2.

It is interesting to mention that no glycosylated native β-1,3-glucanases have been crystallized to date. In this work, we were able to crystallize two isoforms of native Hev b 2 with different glycosylation patterns, which might be important in IgE recognition from allergenic patients.

The authors thank Dr David W. Krogmman for his valuable comments on the manuscript and MSc Georgina Espinosa Pérez and MSc Carmen Márquez Alonso from the Chemistry Institute and MSc Zoila Vanessa Hernandez from the Biotechnology Institute, UNAM, for their technical assistance. This work was supported by DGAPA-UNAM (IN209506-3) and doctoral scholarship PB/0197 from DGEP-UNAM. The X6A beamline is funded by the NIGMS under agreement GM0080. The NSLS is supported by the US DOE contract No. DE-AC02-98CH10886.

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