

Comparative study of two GH19 chitinase-like proteins from *Hevea brasiliensis*, one exhibiting a novel carbohydrate-binding domain

Siseth Martínez-Caballero¹, Patricia Cano-Sánchez¹, Israel Mares-Mejía¹, Angel G. Díaz-Sánchez¹, Martha L. Macías-Rubalcava¹, Juan A. Hermoso² and Adela Rodríguez-Romero¹

¹ Instituto de Química, Universidad Nacional Autónoma de México, Ciudad Universitaria, México

² Departamento de Cristalografía y Biología Estructural, Instituto de Química-Física 'Rocasolano', CSIC, Madrid, Spain

Keywords

antifungal protein; chitin affinity; plant chitinase-like protein; thermostability; X-ray crystal structure

Correspondence

A. Rodríguez-Romero, Instituto de Química, Universidad Nacional Autónoma de México, Circuito Exterior, Ciudad Universitaria, México D. F. 04510, México
Fax: +52 5556162217
Tel: +52 5556224568
E-mail: adela@unam.mx

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Plants express chitinase and chitinase-like proteins (CLPs) belonging to the glycosyl hydrolases of the GH18 and GH19 families, which exhibit varied functions. CLPs in the GH18 family have been structurally and functionally characterized; however, there are no structures available for any member of the GH19 family. In this study, two CLPs of the GH19 family from the rubber tree *Hevea brasiliensis* (HbCLP1 and HbCLP2) were cloned, expressed and characterized. HbCLP1 was identical to the allergen Hev b 11.0101 previously described by others, while HbCLP2 was a novel isoform exhibiting an unusual half chitin-binding domain before the catalytic domain. Sequence alignments showed that in the two proteins the catalytic residues Glu117 and Glu147 in HbCLP1 and HbCLP2, respectively, were mutated to Ala, accounting for the lack of activity. Nonetheless, both CLPs bound chitin and chitotriose (GlcNAc)₃ with high affinities, as evaluated with chitin-affinity chromatography and tryptophan fluorescence experiments. The chitin-binding domains also bound chitotriose with even higher affinities. The crystal structures of the HbCLP1-isolated domains were determined at high resolution. The analysis of the crystallographic models and docking experiments using (GlcNAc)₆ oligosaccharides provides evidence of the residues involved in sugar binding. Endochitinase activity was restored in both proteins by mutating residues A117E (HbCLP1) and A147E (HbCLP2); the distance between the catalytic proton donor and the catalytic nucleophile in the *in silico* mutated residues was 9.5 Å, as occurs in inverting enzymes. HbCLP1 and HbCLP2 were highly thermostable and exhibited antifungal activity against *Alternaria alternata*, suggesting their participation in plant defense mechanisms.

Database

The atomic coordinates and structure factors have been deposited in the Protein Data Bank with accession numbers [4MST](#) for the catalytic domain (CatD1) and [4MPI](#) for the chitin-binding domain (CBD1). Nucleotide sequence data are available in the GenBank database under the accession numbers [KF648872](#) (chi-L1) and [KF648873](#) (chi-L2).

Abbreviations

CatD1, catalytic domain of HbCLP1; CatD2, catalytic domain of HbCLP2; CatD, catalytic domain; CBD1, chitin-binding domain of HbCLP1; CBD2, chitin-binding domain of HbCLP2; CBD, chitin-binding domain; CLP, chitinase-like protein; GH, glycoside hydrolase; GlcNAc, *N*-acetylglucosamine; HbCLP, chitinase-like protein from *Hevea brasiliensis*; IPTG, isopropyl 1-thio- β -D-galactopyranoside; ITC, isothermal titration calorimetry; MES, 2-(*N*-morpholino)ethanesulfonic acid; NMR, nuclear magnetic resonance; PRP, pathogenesis-related proteins.

Introduction

Chitinases ([EC 3.2.1.14](#)) and chitinase-like proteins (CLPs) are widely distributed among all kingdoms of life, and they participate in several physiological processes, including pathogenesis, nutrition, growth regulation and immunity, among others [1–3]. Chitinases catalyze the hydrolysis of β -1,4-linkages between *N*-acetylglucosamine (GlcNAc) residues in chitin, the second most abundant polysaccharide in nature, whereas CLPs lack enzymatic activity due to mutations in one or more of their amino acids required for catalysis but retain their potential to bind chitin oligosaccharides. Plants express chitinases and CLPs, which belong to the glycosyl hydrolase GH18 and GH19 families, constitutively in tissues vulnerable to pathogen attack and inducibly as pathogenesis-related proteins (PRP) [4–6]. Based on sequence similarity, plant chitinases are classified into seven classes (I–VII) [7–9]. Classes represented in the GH19 family are I, II, IV, VI and VII, while classes III and V are members of the GH18 family. Class I endochitinases are characterized by the presence of an N-terminal, hevein-like, chitin-binding domain (CBD) and a C-terminal catalytic domain (CatD), which are connected by a short linker that varies in length and amino acid composition [10]. Class II chitinases only have a CatD, homologous to that of class I chitinases, while class IV chitinases share sequence homology with the members of class I but are smaller due to four deleted sequences. A heavily truncated CBD along with a proline-rich spacer is dominant in class VI chitinases [11] and class VII members lack the CBD but possess a CatD, which is homologous to that present in class IV chitinases [12]. Furthermore, chitinases are also classified as PRP families 3, 4, 8 and 11 [13].

Plant chitinases are able to degrade fungal cell walls [14] and insect exoskeletons [15], but it is not clear whether their increased expression in response to fungal infection or the presence of insects is a pathogen-specific process or if it only reflects a stress-induced response. These enzymes also exhibit several physiological functions that are not related to their hydrolytic activity. Furthermore, some studies have demonstrated the importance of plant CLPs because they are also involved in different regulatory activities; for instance, in *Arabidopsis thaliana* and rice (*Oryza sativa*), these proteins bind emerging cellulose microfibrils affecting their crystallinity [16,17]. In addition, *A. thaliana* chitinase-like lectins (CLL) affect root architecture [18], while in banana (*Musa acuminata*) some CLPs may serve as storage proteins [19]. More recently, two CLPs in oil palm (*Elaeis guineensis*) [20]

and a CLL in tamarind (*Tamarindus indica*) have been reported. The *T. indica* CLL presents sequence and structural similarities to members of the GH18 family but it has a binding site that differs from the ones observed in members of this family [21], while the oil palm CLPs are PRPs [22]. Plant CLPs also show antifungal and insecticidal activity, probably from their ability to inhibit fungal glycoside hydrolases such as the xylanases or by other mechanisms [6,22].

A high prevalence of catalytically inactive enzyme homologues has been reported in a myriad of genome sequences during the past decade, and it has been shown that these ‘dead’ enzymes give rise to proteins with regulatory functions [23]. Given the great diversity of proteins that hydrolyze or simply bind chitin or its oligomers, it has been proposed that chitinases are good examples of gene evolution, which could be related to gene duplication preceding the acquisition of a new function or to acquisition of minor side activities before the gene duplication [24]. Such non-enzymatic side activities have frequently been documented for plant and mammalian chitinases, where the selective pressure to prevent mutations at the active site of the enzyme could be absent.

Higher plants, such as the rubber tree (*Hevea brasiliensis*), express several chitinases, primarily in the course of defense responses to pathogens [25]. Notably, some of these enzymes are allergens that are responsible for the cross-reactivity in humans that results in pollen-latex-fruit syndrome [26,27], and these include two putative class I chitinase isoforms. These two isoforms were cloned and expressed as fusion proteins with the maltose-binding protein [28,29], and they were classified as the allergens Hev b 11.0101 and Hev b 11.0102 belonging to the pathogenesis-related protein-3 (PRP-3) family and to the class I chitinases of the GH19 family. Nevertheless, in those works, the complete fusion proteins were used for immunological assays, and their activity was only evaluated by limited experiments of fungal growth inhibition. Recently, the draft genome of *H. brasiliensis* was published [25], and it showed the existence of 11 class I chitinase genes related to human allergic responses; however, no CLP gene sequences were analyzed.

This is the first report of a structural and biochemical characterization of two recombinant CLPs of the GH19 family that are present in the rubber tree *H. brasiliensis*. The crystal structures of the individual CBD and the inactive CatD of one isoform, designated HbCLP1, were determined at high resolution. Analysis of our crystallographic models and docking experiments using the structures of the domains and GlcNAc

oligosaccharides provide evidence of the residues involved in sugar binding. Endochitinase activity was restored in both proteins by mutating residues A117E (HbCLP1) and A147E (HbCLP2). Interestingly, we found a new isoform (HbCLP2), with a longer CBD not previously described, which exhibits high affinity for chitotriose. We also demonstrate that HbCLP1 and HbCLP2 exhibit antifungal activity against *Alternaria alternata*.

Results and Discussion

Cloning, expression and purification of the HbCLPs

In previous studies, two recombinant chitinase isoforms from the latex and leaves of *H. brasiliensis* were reported [28,29], yet neither of these sequences was found in the recently published draft genome of this tree [25]. In the course of our experiments, fragments of two sizes, designated chi-L1 and chi-L2 (888 and 978 bp, respectively; Fig. 1A), were isolated from total RNA collected from the leaves and latex of two different clones of *H. brasiliensis* (GV-42 and RIMM-600). We found that these transcripts were present in different proportions depending on the collection season (Fig. 1B).

Rubber trees, which belong to one of the most economically important members of the genus *Hevea*, suffer stress when they are tapped and consequently exposed to pathogens and insects. Therefore they express several defense-related proteins that include chitinases and CLPs, and other proteins that could also be involved in different events such as cell division or flower development [30]. In any case, multiple isoforms of either chitinases or CLPs with different functions and characteristics can be expressed under these conditions [1–3].

As shown in Fig. 1C, HbCLP1 contains one hevein-like CBD and one CatD, which are separated by a linker of 10 residues rich in glycine, as has already been reported for plant class I chitinases [31]. Interestingly, HbCLP2 also contains two domains; however, the CBD is longer, as it exhibits the original CBD (Glu1–Val43), a first linker (Glu44–Gly53) plus a peptide that corresponds to the second half of this domain (Cys54–Val73) and a second linker of the same size (Gly74–Gly83; Fig. 1C). To characterize these proteins, in this work we overexpressed and purified both HbCLPs and their respective domains (Fig. 2).

When we analyzed the amino acid sequence alignment of our recombinant proteins (HbCLPs) with the two sequences previously reported [28,29], we found

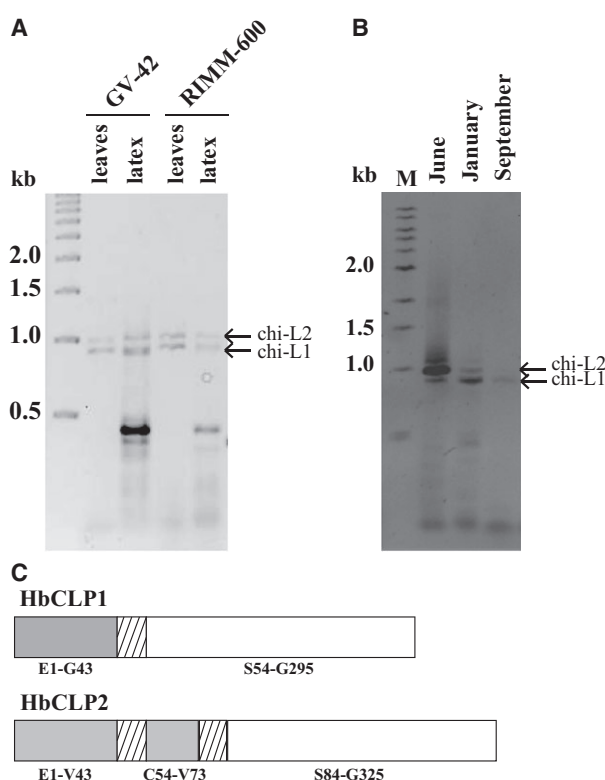


Fig. 1. Agarose gel electrophoresis of the PCR products of HbCLPs using specific oligonucleotides. (A) chi-L1 (888 bp) and chi-L2 (978 bp) from leaves and latex of *H. brasiliensis* trees (clones GV-42 and RIMM600). (B) Amplification of transcripts using total latex RNA collected in different seasons of the year. (C) Diagrams of the different domains present in HbCLP1 and HbCLP2: grey, the chitin-binding domain; diagonal lines, the linkers; white, the catalytic domain. Numbers of residues in each domain are marked.

that they are very similar (Fig. 3). HbCLP1 showed an identical sequence with one of the allergens previously reported as Hev b 11.0101 [28] and displayed 97% identity with Hev b 11.0102 [29]. If only the catalytic domains of the HbCLPs are compared, there are three residues that differ, two along the groove and a third at the end of α -helix 10 (Fig. 3). Intriguingly, no sequences identical to the HbCLP2 or to its longer CBD were found in any database examined; we can therefore say that this type of CBD has not been previously reported. Nevertheless, HbCLP2 exhibits 99% identity with the allergen Hev b 11.0102 when residues Cys54–Gly83 are removed from the analysis (Fig. 3). HbCLP2 is the first protein reported to have one and a half CBDs, a unique characteristic.

The recently published draft genome of *H. brasiliensis* only spans ~ 50% of the estimated 2.15 gigabase haploid genome [25]. Furthermore, the authors focused on the key genes associated with rubber biosynthesis,

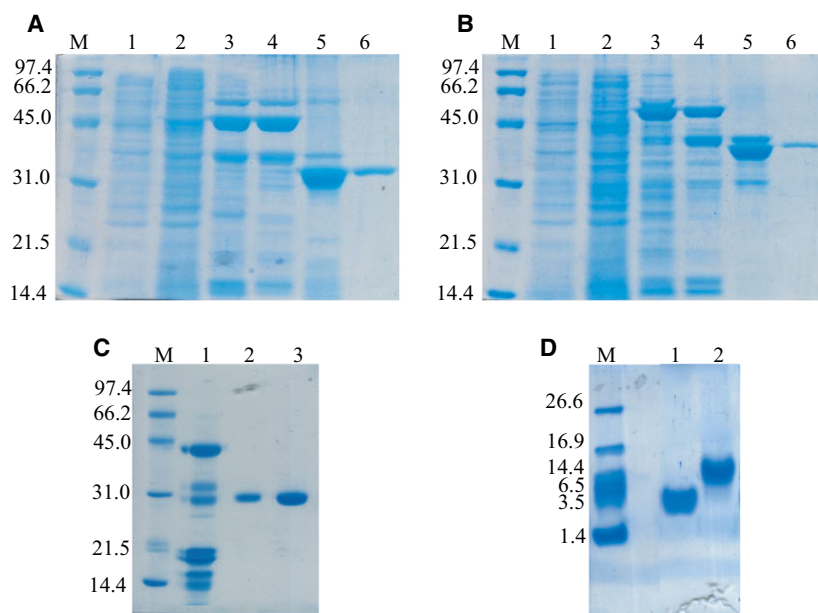


Fig. 2. SDS/PAGE showing the purification steps of HbCLPs and their respective domains. (A) HbCLP1, (B) HbCLP2. Lane M, molecular-weight size markers (kDa); lanes 1 and 2, protein extracts before and after the induction with IPTG, respectively; lanes 3 and 4, bound protein in the affinity chromatographies (nickel); lane 5, elute of the fusion protein from the nickel affinity column after cutting with enterokinase; lane 6, pure proteins after anion exchange chromatography. (C) Catalytic domains: lane 1, fusion protein before cutting with enterokinase; lane 2, CatD1; lane 3, CatD2. (D) Chitin-binding domains after the purification protocol described in Materials and methods: lane 1, CBD1; lane 2, CBD2.

rubberwood formation, disease resistance and allergenicity. Among the genes reported for class I chitinases, we only detected one sequence that was identical to the HbCLP2 sequence, named HBM_contig1331506_s434174c2 gene1 [25]. We therefore cloned the isoforms HbCLP1 and HbCLP2 from genomic DNA and verified that both genes exhibited the two characteristic introns of plant class I chitinases, thus suggesting that different genes encode each isoform in *H. brasiliensis* (Fig. 4). The first intron is localized after the consensus sequence SHATTG, whereas the second intron occurs before the conserved NYNY motif (Fig. 3), as described for class I chitinases from other plants [32].

An interesting result in this work is the inclusion of the N-terminal signal peptide, determined by means of RACE assays (Fig. 4), which had not been previously reported. Comparison of the cDNA-derived amino acid sequence with the N-terminal sequence of the mature CLPs demonstrated that the primary translation product has an amino acid signal peptide, which is removed post-translationally. Furthermore, we found evidence that these proteins are not vacuolar, because the C-terminal sequence did not contain the vacuolar signal peptide or vacuolar sorting determinant characteristic of class I chitinases. This result was confirmed by western blots using lutoids (vacuolysosomal particles) isolated from *H. brasiliensis* latex and specific polyclonal antiCLP-IgG antibodies that gave negative results, in contradiction with previous studies that indicated that one of these isoforms was vacuolar [28].

Overall structure of the HbCLP1 domains

Good quality crystals were obtained only when the individual domains of HbCLP1 were cloned and expressed separately. The complete proteins, alone or in the presence of GlcNAc oligosaccharides, produced crystalline precipitates and microcrystals, which were either not suitable for diffraction experiments or diffracted very poorly.

The overall structure of the CBD1, determined at a resolution of 1.6 Å, comprises 45 amino acids (–1Ala, –2Met, Glu1–Gly43) that exhibit the characteristic folding of hevein-like domains [33]. The crystal contained two molecules in the asymmetric unit with a Matthews coefficient (V_M) of $2.23 \text{ Å}^3 \cdot \text{Da}^{-1}$ and 45% solvent content. The final model has R_{factor} and R_{free} values of 17.86% and 21.78%, respectively (Table 1). This structure includes a short antiparallel β sheet, conformed by two β -strands of four residues each, connected by a β turn (β hairpin), and two short segments of α -helix comprising residues Cys3–Gly8 and Thr27–Gly32 (Fig. 5A). The four disulfide bonds are conserved (Cys3/Cys18, Cys12/Cys24, Cys17/Cys31 and Cys35/Cys39). The residues involved in chitin binding are also conserved and include Ser19 and the three aromatic residues Tyr21, Trp23 and Tyr30 (Fig. 5A), which are identical to those present in other hevein-like domains such as hevein (Trp21, Trp23 and Tyr30), pseudohevein (Tyr21, Trp23 and Tyr30) [34], whose structure has not been reported, and chain B of wheat germ agglutinin, which has three Tyr residues in the binding site (Fig. 5B) [35]. It was interesting to

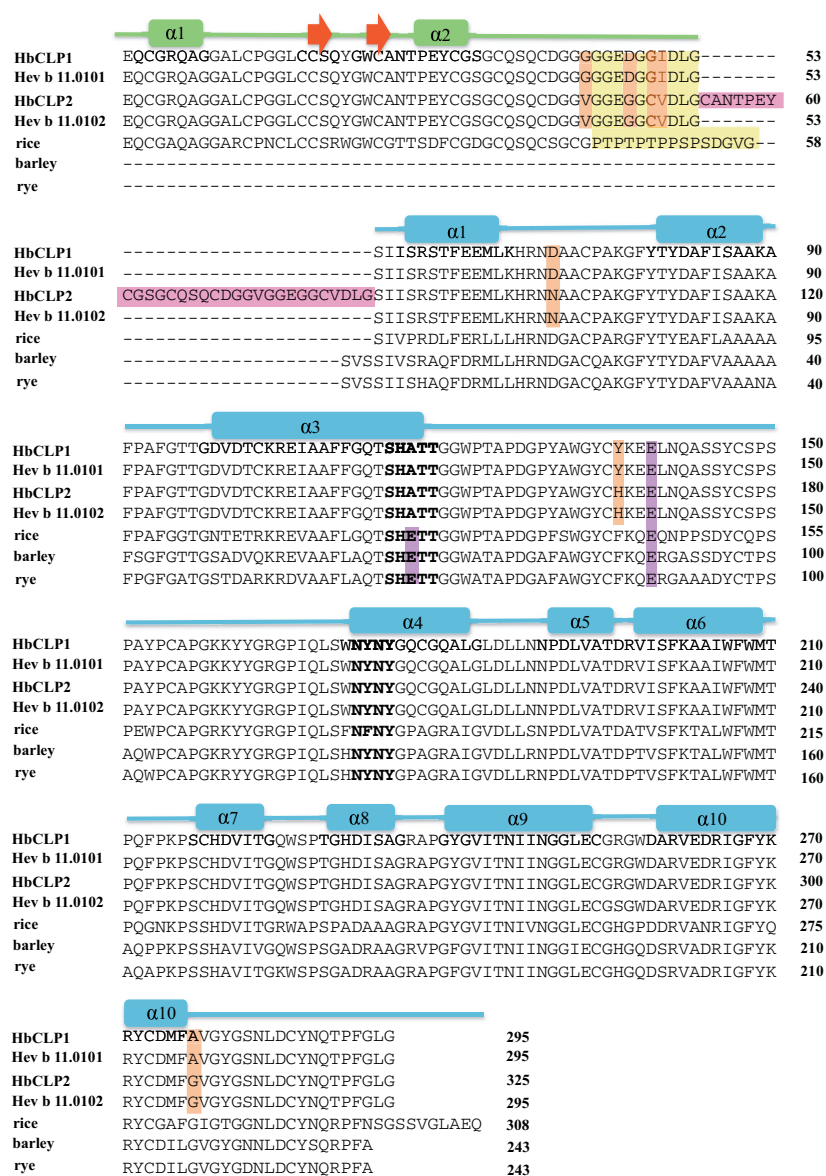


Fig. 3. Multiple-sequence alignment of selected GH19 family chitinases from plants: rice (PDB ID [2DKV](#)), barley (PDB ID [1CNS](#)) and rye (PDB ID [3J0L](#)) are aligned with the HbCLPs. The linkers are shown in yellow, in pink the additional sequence of HbCLP2, in orange the different residues between HbCLP1 and HbCLP2, in purple the catalytic residues and in bold letters two conserved motifs in the GH19 family plant chitinases. The first motif is near the hydrolysis region and includes one of the catalytic residues, and the second motif has been suggested to participate in substrate binding. Secondary structure elements are included for both domains above the sequences (α -helices are shown as green cylinders and β -strands as red arrows for the CBDs). Helices 1–10 are depicted for the catalytic domain.

find a 2-(*N*-morpholino)ethanesulfonic acid (MES) molecule near Trp23 of chain A and the symmetry related B chain in a position that mimics the GlcNAc oligosaccharide interaction with the protein [36]. The MES molecule, located in the carbohydrate-binding site, interacts with the –1Met residue and Trp23. We previously found a 2-methyl-2,4-pentanediol molecule at the carbohydrate-binding site in the hevein structure, which indicates that this domain can bind other molecules in addition to the GlcNAc oligosaccharides [33].

The crystal structure of the 242-residue CatD1 (Ser4–Gly295) was determined at 1.93 Å resolution. Two monomers were found in the asymmetric unit

with a V_M of $2.29 \text{ Å}^3 \cdot \text{Da}^{-1}$, which corresponds to a solvent content of 46.2%. At this point it is important to mention that the interface of the two monomers in both asymmetric units (CBD1 and CatD1) scored 0.0 in complexation significance score in PISA [37], indicating that the interface for dimer formation is not significant in any case (a score of 1 suggests a stable interface and a score of 0 suggests a nonstable interface, i.e. crystal packing). The CatD1 model was refined to final R_{factor} and R_{free} values of 16.77% and 20.69%, respectively. The refinement and final model statistics are summarized in Table 1. The overall CatD1 structure comprises 10 α -helices connected by five main loops (I, II, III, IV and V), which are the

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HbCLP2  MKEIVRALEGYGPPKDKAAEQCGRQAGGALCPGGLCCSQYGCANTPEYCGSGCQSQCDG
HbCLP1  MKEIVRALEGYGPPKDKAAEQCGRQAGGALCPGGLCCSQYGCANTPEYCGSGCQSQCDG

HbCLP2  GVGGEggGCVDLGCANTPEYCGSGCQSQCDGGVGGEggGCVDLGSIISRSTFEEMLKHRNNA
HbCLP1  GGGGEDGGIDLG-----SIISRSTFEEMLKHRNDA

HbCLP2  ACPAKGFYTYDAFISAAKAFPAFGTTGDVDTCKREIAAFFGQTSHTATGctttggtttttattac
HbCLP1  ACPAKGFYTYDAFISAAKAFPAFGTTGDVDTCKREIAAFFGQTSHTATGctttggtttttatcac

HbCLP2  cccttctttttctcatttt--gaggggtttgttttgattaaaactcatttagaactcctaactccaaatctcattgtttta
HbCLP1  cccttctttttttctcatttagaggggtttgttttgattaaaactcatttagaactcctaactccaaatctcattgtttta

HbCLP2  aaggtactcaaatattattgggttaaaactcattaatttaattaattagctttaattaatttaaagattaatcaccttaa
HbCLP1  aaggtactcaaatattattgggttaaaactcattaatttaattaattagctttaattaatttaaagattaatcaccttaa

HbCLP2  ttagtatataaataagtagttaattgagaaattgtgttggtgatgcactgtaggaGWP TAPDGPYAWGYCHKE
HbCLP1  ttagtatataaataagtagttaattgagaaattgtgttggtgatgcactgtaggaGWP TAPDGPYAWGYCYKE

HbCLP2  ELNQASSYCSPPAYPCAPGKKYYGRGPIQLSWTactgttttctttggcagcataattttgattttaagc
HbCLP1  ELNQASSYCSPPAYPCAPGKKYYGRGPIQLSWTactgttttctttggcagcataattttgattttaagc

HbCLP2  acataaattttttattataataaaaaaaattgaattaattacttgcaaatgtaatttaagccatgtctgatataa
HbCLP1  acataaattttattattataataaaaaa-----gttgaattaattacttgcaaatgtaatttaacatgtctgatataa

HbCLP2  tttttttttgtaattattatattatataaaatgatgcaggN YNYGQCGQALGLDLLNPD LVATDRVISF
HbCLP1  tttttttttgtaattattatattatataaaatgatgcaggN YNYGQCGQALGLDLLNPD LVATDRVISF

HbCLP2  KAAIWFWMTPQFPKPSCHDVITGQWSPTGHDISAGRAPGYGVITNIINGGLECGRGWDAR
HbCLP1  KAAIWFWMTPQFPKPSCHDVITGQWSPTGHDISAGRAPGYGVITNIINGGLECGRGWDAR

HbCLP2  VEDRIGFYKRYCDMFGVGYGSNLDLCYNQTFPGLG
HbCLP1  VEDRIGFYKRYCDMFAVGYGSNLDLCYNQTFPGLG

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Fig. 4. Sequence alignment of the two HbCLPs. The amino acid sequences (exons) are represented in capital letters, bold letters indicate the nucleotides corresponding to the introns and the signal peptides are marked in rectangles.

most prominent features of the class I and II GH19 family of chitinases (Fig. 5C). The numbering refers to the complete polypeptide chain, including the CBD1 and the linker at the N-terminal region; therefore the first residue in this domain is numbered Ser54 (Fig. 3). This monomer also exhibits three conserved disulfide bridges: Cys73/Cys135, Cys147/Cys155 and Cys254/Cys286 (Fig. 5C). Finally, the structure shows the characteristic groove responsible for chitin binding that includes loops II, III and IV and helices 3, 4, 6, 9 and 10 [38]. A C α superposition of the two monomers in the asymmetric unit indicates an rmsd of 0.6 Å, with loop III being the one that shows the largest difference.

The C α structural alignments of the different class I and II (GH19) catalytic domains in plant chitinases with the CatD (chain A) of HbCLP1 revealed that these proteins share a relatively high degree of similarity (Fig. 5D). The closest structural models are from barley class II chitinase (PDB ID [1CNS](#)) with an rmsd of 0.9 Å, rice class I chitinase (PDB ID [2DKV](#), rmsd 1.0 Å), mustard (*Brassica juncea*) class I chitinase (PDB ID [2Z38](#), rmsd 1.0 Å), papaya class II chitinase (PDB ID [3CQL](#), rmsd 1.0 Å) and rye class II chitinase (PDB ID [4DWX](#), rmsd 1.2 Å; PDB ID [4DYG](#), rmsd 1.2 Å; and PDB ID [4JOL](#), rmsd 0.9 Å), which was crystallized alone and in complex with one and two molecules of *N*-acetyltetraose (GlcNAc)₄, respectively [39,40].

In all the structures, loops I and III are stabilized by disulfide bonds. The most significant differences were observed in the longest loop (III), which comprises 53

residues (Thr118–Trp171, HbCLP1 numbering) and is located between α -helices 3 and 4 (Fig. 5C). This loop adopts various conformations, as has been observed among the different plant chitinases. In addition, it contains one of the catalytic amino acids (Glu89 in barley chitinase and Glu139 in the CatD1; Figs 3 and 5C), which suggests the direct participation of this region in the binding and catalysis of the substrate. Loop III is generally conserved in chitinases of the GH19 family, while loops I, II, IV and V may be absent in bacterial and plant class IV chitinases [41–43]. Based on structural studies of mustard class I chitinase and rye class II chitinase, it has been proposed that the differences in conformation of some of the loops bordering the active-site groove indicate an opening and closing of the enzyme during the catalytic cycle [40]. Structural alignment of loop III, including 20 residues in each end, demonstrated that even though CatD1 was crystallized in the absence of ligands the loop III is in a closer conformation to the one present in the rye structure with bound ligands in the groove [40] (Fig. 5D) (rmsd 0.69 Å), while the rmsd is 1.0 Å when compared with the structure of the rye chitinase without ligand [39]. Loops IV and V showed similar displacements (Fig. 5D) revealing conformational plasticity in the binding region.

Chitinase activity

Even though HbCLP1 was originally classified as a class I chitinase [28], neither of the two proteins studied in this work showed catalytic activity using

Table 1. Data collection and refinement statistics. Data in parentheses refer to the highest-resolution shell.

	CatD	CBD
Data collection		
Space group	<i>I</i> 121	<i>P</i> 6 ₁
Cell dimensions (Å)	111.11, 40.41, 111.85	38.51, 38.51, 100.93
β (°)	96.73	
Wavelength	1.0	0.979
Resolution (Å)	41.71–1.93 (2.03–1.93)	33.64–1.60 (1.68–1.60)
No. unique reflections	37 433 (5195)	11 080 (1055)
Completeness (%)	99.30 (95.60)	99.47 (96.26)
Redundancy	4.7 (4.0)	3.5 (2.7)
$\langle I/\sigma(I) \rangle$	14.9 (4.4)	21.1 (4.9)
R_{merge}^a (%)	8.8 (51.9)	3.7 (21.6)
Refinement statistics		
Resolution range (Å)	41.66–1.93 (2.0–1.93)	33.35–1.60 (1.66–1.60)
$R_{\text{factor}}^b/R_{\text{free}}^c$ (%)	16.77/20.69	17.86/21.78
No. atoms		
Protein	3749	621
Water	316	86
Ligand molecules		2
Metal ions (Cl [−])	2	
rmsd bond length (Å)	0.011	0.016
rmsd bond angles (°)	1.11	1.78
Mean <i>B</i> -factor (Å ²)		
Protein	22.6	18.9
Ligand/ion	25.7	30.7
Water	28.5	28.9
Ramachandran favoured/outliers (%)	98/0.4	94/0
PDB code	4MST	4MPI

^a $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\langle I(hkl) \rangle$ and $I_i(hkl)$ are the mean and the *i*th measurement of intensity respectively for reflection *hkl*. ^b $R_{\text{work}} = \sum_{hkl} | |F_{\text{obs}}| - |F_{\text{calc}}| | / \sum_{hkl} |F_{\text{obs}}|$. ^c R_{free} is the corresponding *R*-factor value for a randomly chosen 5% of the reflections excluded from the refinement.

substrates such as chitin azure, chitin beads, glycol chitin and shrimp chitin. As previously described for several inverting chitinases belonging to the GH19 family, the catalytic residues are located in a relatively wide groove. This groove contains two conserved residues that are directly responsible for chitin hydrolysis: a proton donor Glu67 and a nucleophile base Glu89 (barley chitinase). These correspond to Ala117 and Glu139 in HbCLP1 and Ala147 and Glu169 in HbCLP2 (Fig. 6A), indicating that one of the catalytic residues is missing in both HbCLPs. Chitinases belonging to the GH19 family exhibit the highly conserved QTSHETGGW motif (Fig. 3); however, it has been reported that a substitution of the Glu residue with an alanine or glutamine, for example, results in

the loss of catalytic activity [44–46]. This lack of activity has been observed in CLPs from plants in which different functions have been detected such as cellulose synthesis [47] or receptor-like kinase activity [48], among others.

Mutating residues A117E and A147E, respectively, restored the enzymatic activity of HbCLP1 and HbCLP2. Using chitin azure as a substrate, the specific activity of HbCLP1-A117E was $17.9 \pm 2.3 \text{ U} \cdot \text{mg}^{-1}$ at pH 4.0, while for HbCLP2-A147E it was $12.9 \pm 1.0 \text{ U} \cdot \text{mg}^{-1}$ at pH 5.0 (Table 2). The specific activity of HbCLP1-A117E at pH 8.0 was approximately three times lower than that at pH 4.0, whereas for HbCLP2-A147E we found no significant differences in the specific activity at the three different pH values examined (Table 2). Residues Asp70 and Tyr136 in HbCLP1-A117E and Asn100 and His166 in HbCLP2-A147E occupy the same positions along the groove in their respective CatDs, if we consider that CatD2 has the same structure as CatD1. Then by using PROPKA [49], which predicts the pK_a value for the ionizable residues and the *in silico* mutation Tyr136 by His166 in our CatD1 structure, we found that the pK_a value for His166 is ~ 6.8 . This means that this residue is very probably protonated at pH values 4 and 5, where we found differences in specific activities, and neutral at pH 8.0, where the activities were similar. In contrast, the Tyr residue that substitutes His in the HbCLP1-A117E does not vary its protonation state with pH. Moreover, when the His166 is present there is an extensive positive charge along the binding groove, probably provoking repulsion between its boundaries, which could explain the differences in activity with HbCLP2-A147E.

Recently, the crystal structure of a class IV chitinase from corn (*Zea mays*) that lacks the CBD provided evidence of the participation of residues Gln62, Arg177 and Glu165 in chitin hydrolysis and of Ser103 and Tyr106 in substrate binding; however, class IV chitinases have evolved to accommodate shorter substrates [42]. Compared with the chitinases of classes I, II and IV [39,41–43], our CatDs exhibit all of the residues that have been implicated in catalysis, thereby allowing our mutants HbCLP1-A117E and HbCLP2-A147E to recover their chitinolytic activity.

Carbohydrate-binding sites of the catalytic domain

Despite the absence of catalytic activity, the volume of the groove present in the CatD1 (1101.2 Å³) is similar to that present in the active barley chitinase (1100.7 Å³; PDB ID [1CNS](#)), which indicates an

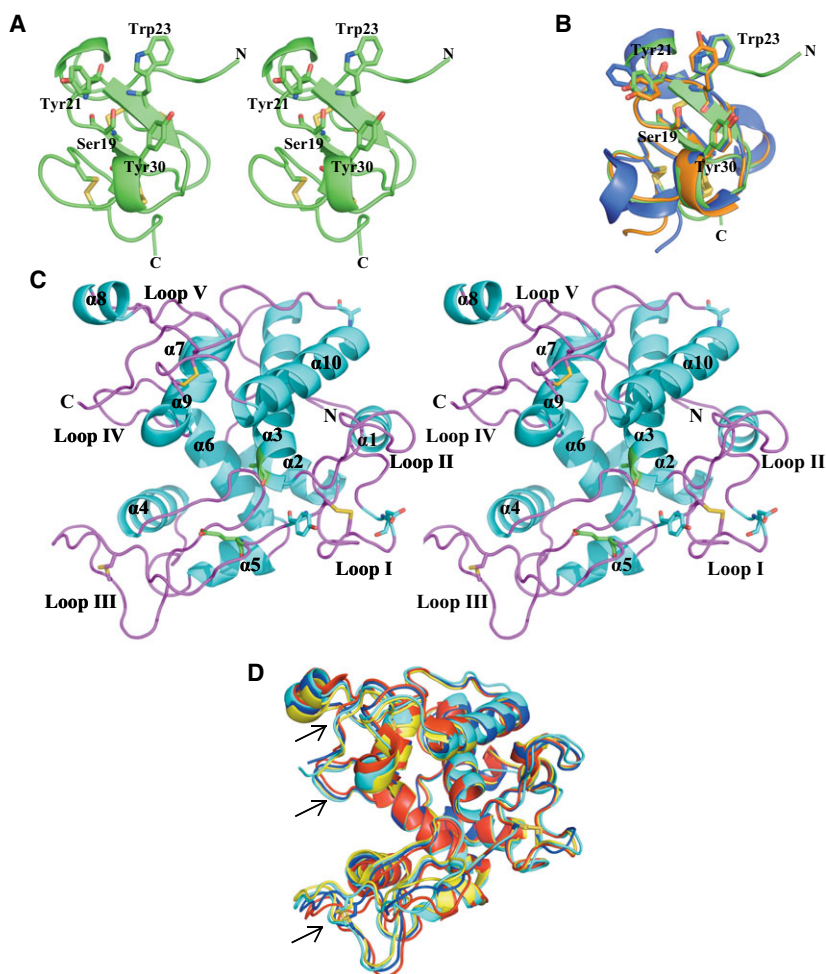


Fig. 5. Overall structures of HbCLP1 domains. (A) Stereo view of the ribbon representation of the CBD1 monomer that shows the conserved residues involved in chitin binding as sticks and disulfide bridges in yellow. (B) C α superposition of CBD1 (green) with hevein (PDB ID [1Q9B](#)) colored in dark blue and wheat germ agglutinin monomer B (orange); also protein residues involved in sugar recognition are represented as sticks. (C) Stereo view of the ribbon diagram showing the CatD1 structure that consists of 10 α -helices, five main loops (I–V) and three conserved disulfide bridges; there are also several β -turns, five of which are located in loop III. The green sticks represent the position of the putative catalytic amino acids and cyan sticks the amino acids that are different between two HbCLPs. (D) C α superposition of CatD (cyan) with active plant chitinases: rye chitinase (red) (PDB ID [4DWX](#)), rye chitinase with two molecules of (GlcNAc)₄ (yellow) (PDB ID [4JOL](#)) and barley chitinase (blue) (PDB ID [1CNS](#)). Major conformational differences are localized in loops III, IV and V, which are indicated with arrows.

appropriate space for the arrangement of a ligand in our protein (Fig. 6B). To provide insight on the ability of the CLPs of *H. brasiliensis* to recognize oligosaccharides, we performed docking simulations to investigate the possible binding interactions of (GlcNAc)₆ with the CatD1. The first five solutions of lower energy were selected and analyzed considering the previously reported experiments on the amino acids involved in interactions with the substrates or products of active chitinases belonging to the GH19 family, as there are no reported structures of CLPs in this family [38,40]. Figure 6C illustrates the network of interactions of the CatD1 with (GlcNAc)₆ when the ligand was positioned throughout the groove in subsites –4 to +2 [40].

Interestingly, one feature observed in our docking model, as well as in other crystallographic reports of chitinases [38,40], is a wide distribution of aromatic residues along the carbohydrate-binding groove that are able to recognize the ligand through different types

of interactions. An interesting one is Trp171, which is located in loop III and interacts with residues –3 and –2 of (GlcNAc)₆, while in other class I and II plant chitinases this residue is replaced by a His or Phe [40,50]. In the CatD1 structure this Trp171 falls outside of the allowed regions of the Ramachandran plot, suggesting its participation in the interaction with the ligand (Fig. 6D) [51].

Another residue that appears to be important for ligand binding is Tyr173 (Fig. 7), whose OH group interacts with the amide group of both the –3 and –2 GlcNAc residues of the hexamer. Other residues involved in carbohydrate recognition are His116, Tyr146 and Phe207, which are strictly conserved and have been described as important for chitin specificity [45,50]. This is not the case for Trp171, which is only present in ~12% of the available sequences. One insight that emerges from our docking analysis is that interactions between the CatD1 and (GlcNAc)₆, primarily at subsites –4 to –2, involve residues located

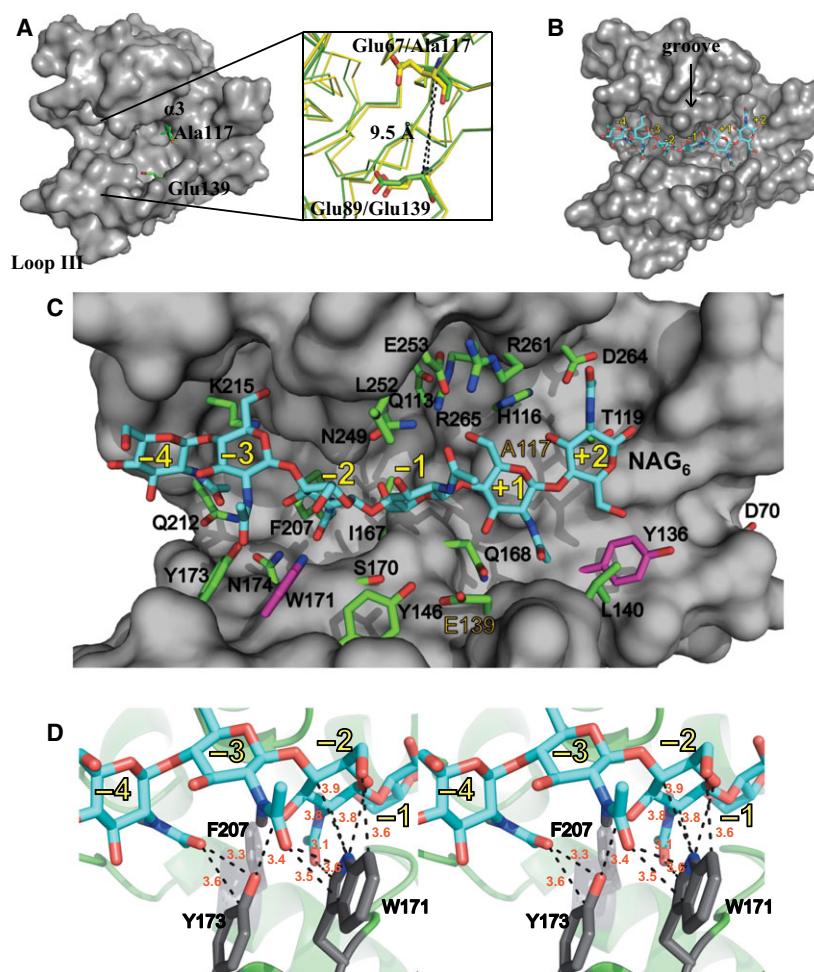


Fig. 6. Surface representation of the CatD1 showing the binding groove. (A) The position of the putative catalytic residues is depicted in green (HbCLP1); the proton donor that should be a Glu residue is an Ala117. In the square is shown a detailed view of the catalytic residues in barley chitinase (yellow) superimposed with the CatD1 structure (green). The distance between the two catalytic residues in active chitinases is 9.5 Å (C α). (B) Docking of (GlcNAc)₆ (blue sticks) in the CatD1 groove. (C) Close-up view of the CatD1 residues involved in the interaction with (GlcNAc)₆. (D) Stereo view of the interactions of the -4 to -2 moieties of (GlcNAc)₆ in the CatD1 binding site. The figure was generated from the docking model. Protein residues making interaction with the ligand are shown as sticks with carbon atoms in grey; the ligand is also depicted as sticks with carbon atoms in cyan, oxygen and nitrogen atoms in red and blue, respectively, for protein and ligand. Interactions are shown as dashed black lines and distances are depicted in Å.

Table 2. Specific activities of HbCLP mutants over chitin azure. Results are the mean value $\pm$ SD from three independent experiments.

	pH 4.0 (U·mg ⁻¹)	pH 5.0 (U·mg ⁻¹)	pH 8.0 (U·mg ⁻¹)
HbCLP1	0.3 $\pm$ 0.2	0.2 $\pm$ 0.1	0.3 $\pm$ 0.1
HbCLP1-A117E	17.9 $\pm$ 2.3	14.6 $\pm$ 1.4	6.7 $\pm$ 0.4
HbCLP2	0.4 $\pm$ 0.2	0.2 $\pm$ 0.1	0.3 $\pm$ 0.2
HbCLP2-A147E	10.6 $\pm$ 1.3	12.9 $\pm$ 1.0	8.0 $\pm$ 0.6

in loop III: Tyr146, Gln168, Ser170, Trp171, Tyr173 and Asn174, the last two being highly conserved in GH19 family members. These residues are conserved between catalytically active chitinases [40,50] and the HbCLPs; nonetheless, there are differences between them, such as the absence of interactions between subsites -1 to +2 with some of the amino acids near Ala117 (His116, Thr119, Gly120 and Trp122) and the polar and apolar interactions of the aromatic residues

(Trp171, Tyr173 and Phe207) present at subsites -4 to -2 (Fig. 6D). Conversely, catalytically active chitinases exhibit weaker interactions in this region or interactions mediated by water molecules [38,43]. Some of the waters in the groove observed in our crystallographic CatD1 structure were included to compare with those present in other chitinase-carbohydrate complexes, and two of them appear to be important, W438 and W511 (Fig. 7). While W438 is conserved in the different chitinases and located in a proper position for the hydrolysis step, W511 occupies a plausible position for the carboxyl group of one of the catalytic Glu residues, which in this case is Ala117, participating in the interaction of Arg265 with position -1 of the carbohydrate. In the (GlcNAc)₆-CatD1 model, GlcNAc moieties in subsites -4 to +1 interact with the protein through multiple polar and non-polar contacts (Fig. 6C). In addition, seven polar contacts mediated by eight different water molecules were observed, as shown in a LIGPLOT+ figure (Fig. 7).

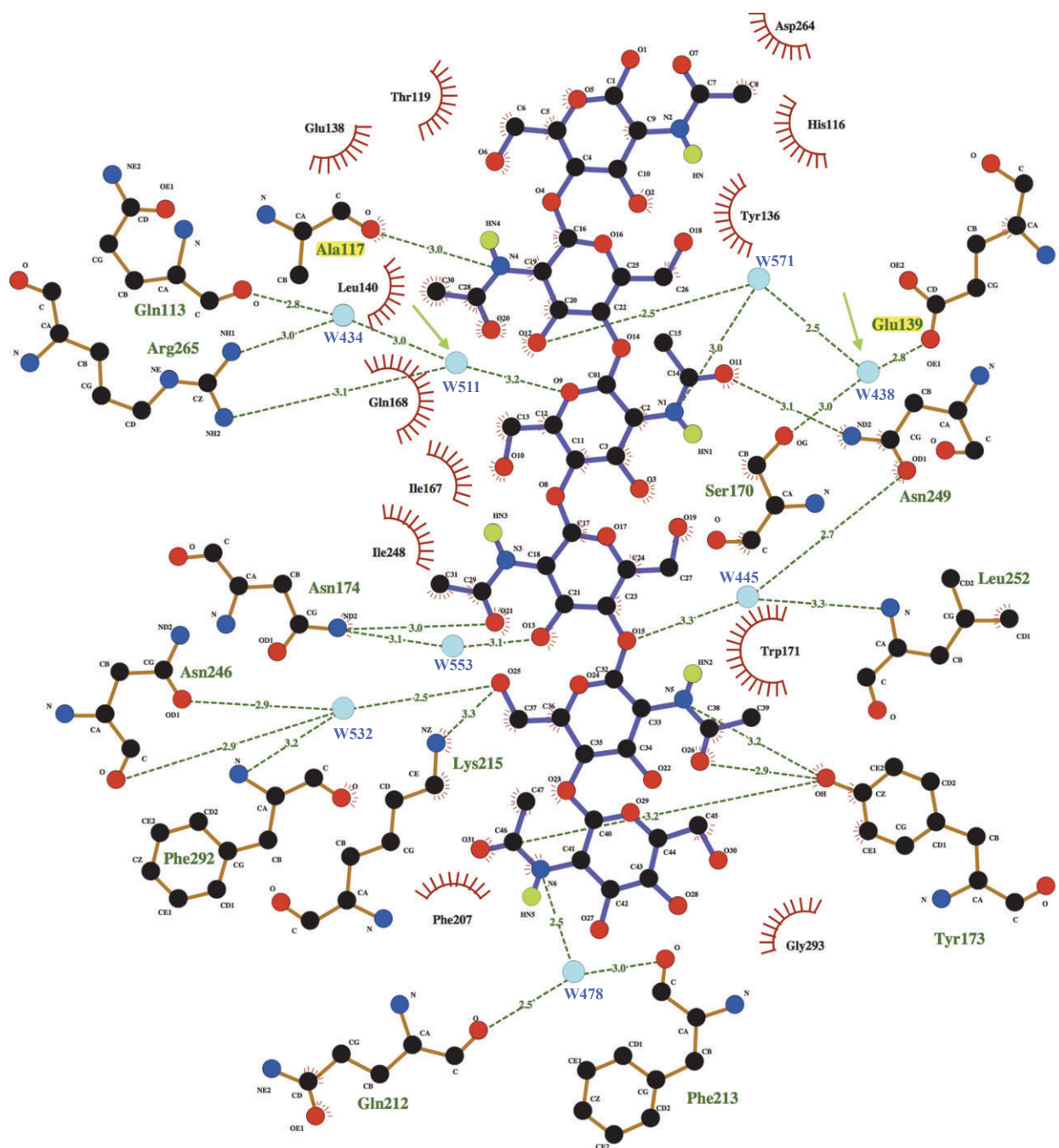


Fig. 7. *In silico* docking results of CatD1 residues interacting with (GlcNAc)₆. The atoms involved in hydrogen bonds (dashed lines) or hydrophobic contacts are depicted. In yellow the two putative catalytic residues are shown, for CLPs the mutated Glu117Ala occupying the position of the Glu proton donor and Glu139, which is the nucleophile base. Arrows show important water molecules that could be involved in the catalytic mechanism (W438 and W511). GlnNAc upper position corresponds to +2. The diagram was generated with the program LIGPLOT+.

It is also noteworthy that a *cis* peptide occurs between Phe213 and Pro214 in loop IV. *Cis* peptides have been associated with structural or functional features, suggesting that Phe213 is also involved in

binding of the substrate (Fig. 7). It has been found that unusual geometry in well-defined regions of electron density most likely signifies an interesting hot spot in the structure (with unusual high-energy

conformations) that could be involved in binding of the substrate [52].

Binding experiments between the HbCLPs and *N*-acetyl-chitotriose [(GlcNAc)₃]

The binding affinities of HbCLP1, HbCLP2 and their respective individual domains (CatDs and CBDs) for (GlcNAc)₃ were determined by fluorescence spectroscopy experiments. Both HbCLPs have one tryptophan

residue (Trp23) located in the CBD and two tryptophan residues in the substrate-binding groove of the CatD, which are Trp122 and Trp171 in HbCLP1 (Fig. 6C) and Trp152 and Trp201 in HbCLP2. For the full-length HbCLPs the ligand produces similar changes after binding, as observed in saturation curves which exhibit an enhanced fluorescence intensity at the λ_{max} of each protein (337.1 nm for HbCLP1 and 335.8 nm for HbCLP2) (Fig. 8A). The same experiments were performed with both CatDs observing that

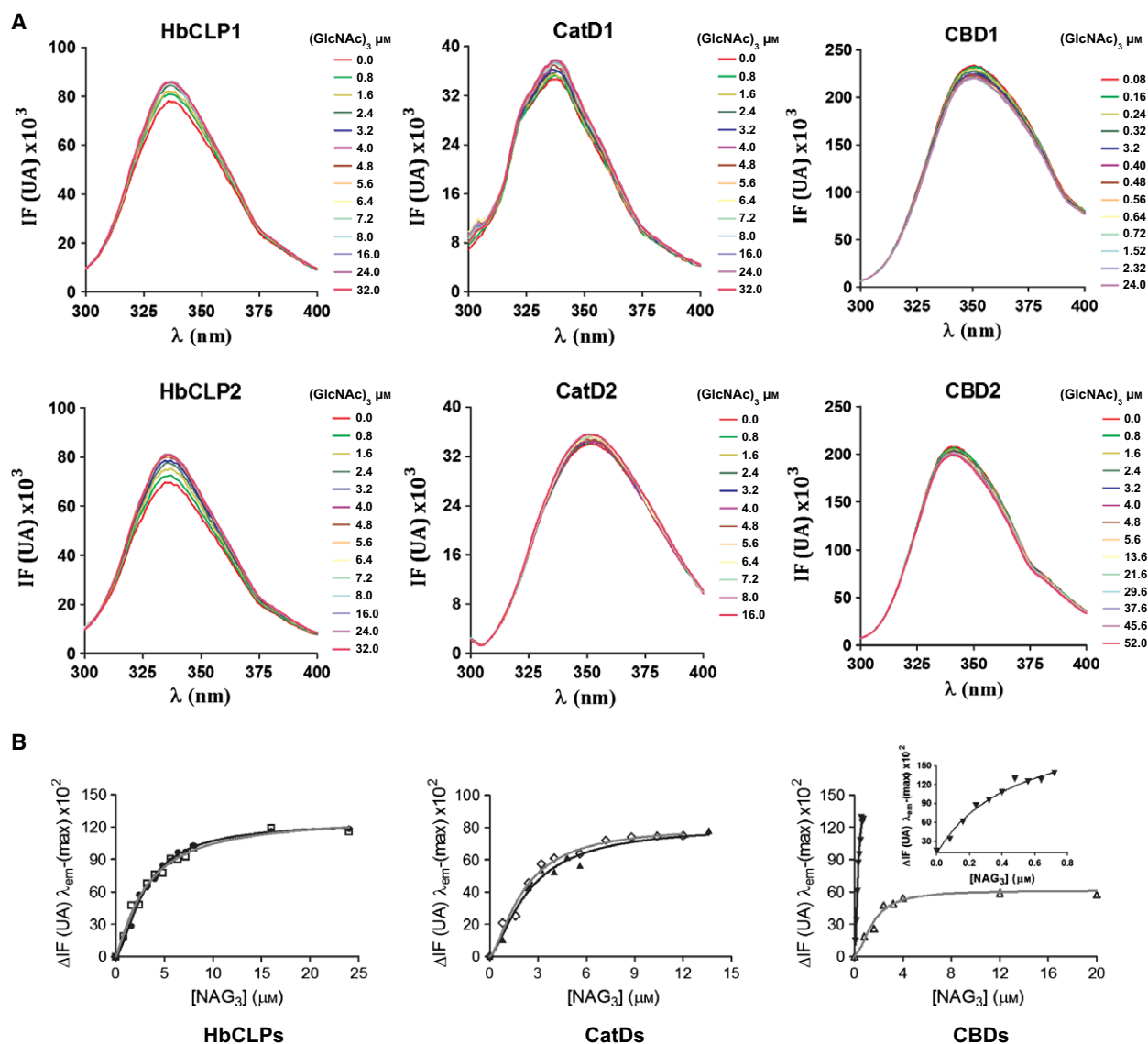


Fig. 8. Chitotriose-binding analysis of the HbCLPs and their respective domains by intrinsic fluorescence. (A) Fluorescence quenching experiments in the titration with (GlcNAc)₃. The concentration used in each experiment (μM) is indicated at the right side of each spectrum. (B) Nonlinear regression analysis of the relative fluorescence intensity changes of the HbCLPs and their domains in the presence of various concentrations of (GlcNAc)₃: HbCLP1 (●) and HbCLP2 (□); CatD1 (▲) and CatD2 (◇); CBD1 (▼) and CBD2 (Δ); the inset corresponds to the CBD1.

the binding of (GlcNAc)₃ produced an increase in the fluorescence intensity in both cases and a slight blue shift in λ_{max} (Fig. 8A). Plots of maximum fluorescence intensity change against the (GlcNAc)₃ concentration are shown in Fig. 8B. The apparent dissociation constants were estimated by a nonlinear regression function analysis of the data using Eqn (1) or (2) (see Materials and methods). The apparent K_d values of HbCLP1 and HbCLP2 for (GlcNAc)₃ were $3.0 \pm 0.13 \mu\text{M}$ and $3.1 \pm 0.33 \mu\text{M}$, respectively, whereas those for CatD1 and CatD2 were $2.4 \pm 0.41 \mu\text{M}$ and $2.1 \pm 0.24 \mu\text{M}$, respectively. Furthermore, we also determined the apparent K_d values for the CBDs that have two extra residues at the N-terminus (–2Ala and –1Met), which were $0.48 \pm 0.14 \mu\text{M}$ and $1.49 \pm 0.14 \mu\text{M}$ for CBD1 and CBD2, respectively. Notably, CBD1 exhibited higher affinity than the complete proteins or their catalytic domains, even higher than the previously reported value for hevein using ITC ($71 \mu\text{M}$) [53], fluorescence methods ($63 \mu\text{M}$ obtained in this work, results not shown) and NMR ($86 \mu\text{M}$) [54] or with pseudohevein that has the same residues in the binding region (Tyr21, Trp23 Tyr 30), which has a K_d of $142 \mu\text{M}$ as determined with NMR [54]. As mentioned above, when we analyzed the CBD1 crystal structure we found that the methionine residue at the N-terminus, which is only present in our molecular biology construction, makes contact with the MES molecule that we found in the carbohydrate-binding site, suggesting that this residue could be involved in protein–carbohydrate interaction increasing the CBD affinities. A docking model of the CBD1 with chitotriose is shown in Fig. 9, which reveals that –1Met establishes several interactions with the GlcNAc moiety at position +1. It is interesting to note that the CBD2 shows a slightly larger K_d suggesting that the 30 additional unfolded

residues could affect the interaction of the oligosaccharide with the hevein-like folded domain. In general, all these results are difficult to interpret, due to presence of two domains that bind (GlcNAc)₃ in each HbCLP. It appears that these proteins and their domains possess high affinity for this oligosaccharide, but the presence of the CBDs, which exhibit the highest value, did not increase the affinity of the complete proteins. This suggests that the two domains do not present a synergistic effect in the binding of this oligosaccharide.

Very recently, the crystal structure of a moss GH19 chitinase was determined in complex with (GlcNAc)₄, and it shows that sugar residues at subsites –2, –1, +1 and +2 interact with several amino acids in the catalytic groove through a number of hydrogen bonds [43]. However, there are no direct interactions with aromatic residues in this structure. This enzyme, which lacks the CBD, has a K_d of $230 \pm 20 \mu\text{M}$ with (GlcNAc)₃ [55], which is two orders of magnitude larger than the K_d values reported here of the complete *H. brasiliensis* proteins or their CatDs. The moss chitinase lacks loop IV, suggesting this loop is also important in the interaction with the carbohydrate.

Binding to chitin beads and antifungal activities

Even though HbCLP1 and HbCLP2 do not exhibit hydrolytic activity, they bound strongly to chitin, as neither of them eluted from the chitin-bead columns using 1 M NaCl or 1 M acetic acid. This result contrasts with data for other class I chitinases, where this type of column is often used for their purification [56]. In this work, we could only disrupt the strong interaction of the HbCLPs with the chitin beads when they were subjected to SDS/PAGE in the presence of 2-mercaptoethanol (Fig. 10A), indicating that the

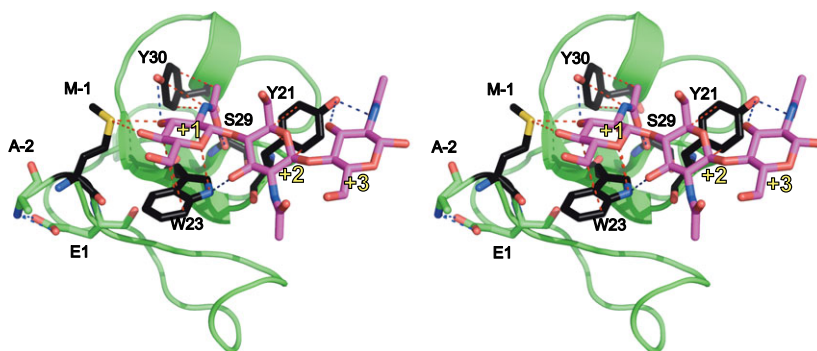


Fig. 9. Stereo view of the CBD1 binding interaction with chitotriose. Protein residues interacting with the ligand are shown as sticks with carbon atoms in black; the ligand is also depicted as stick with carbon atoms in magenta, oxygen and nitrogen atoms in red and blue, respectively, for protein and ligand. Ala-2 and Glu1 are shown as sticks. Apolar interactions are shown as dashed red lines and polar interactions are shown as dashed dark blue lines. (GlcNAc)₃ residues occupy positions +1, +2 and +3.

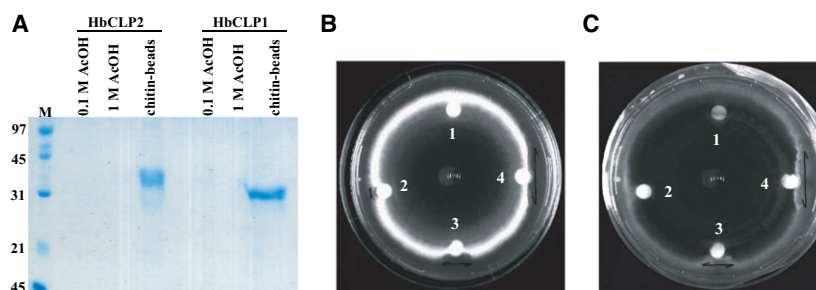


Fig. 10. Chitin-binding and antifungal activities of HbCLPs. (A) SDS/PAGE gel showing the protein bands that were separated from the chitin beads when they were applied to gel after the binding experiments. (B), (C) Plates showing fungal growth inhibition when HbCLP1 and HbCLP2 were used. In both Petri dishes well 1 corresponds to the control (Tris/HCl 50 mM); wells 2, 3 and 4 contained 25, 50 and 75 μ g of each of the HbCLPs, respectively. Double arrows show the fungal growth inhibition.

interaction is broken only after denaturation of the proteins. Additionally, antifungal activity towards *A. alternata* was observed for both of the HbCLP isoforms at different concentrations, suggesting that these HbCLPs are involved in defense through a mechanism that only involves carbohydrate binding. This activity on mycelial growth is observed as crescents of inhibition at the rim of the disks containing the complete proteins (Fig. 10B,C). Plant antifungal proteins can be classified into different groups comprising chitinases and CLPs, chitin-binding proteins, lectins etc. The antifungal action of chitinases is due to the hydrolysis of chitin, a major component of fungal cell wall, which results in an impaired cell wall and subsequently brings about cell lysis [57]. Interestingly, the antifungal activity of a tobacco class I chitinase was substantially augmented in the presence of a CBD [58]. These results indicated that a large proportion of the antifungal activity of chitinases was due to chitin binding and not to chitinase activity. The inhibitory effect of chitin-binding proteins on fungal growth is ascribed to their ability to bind to chitin. Since the nascent chitin of the hyphal apex is easily accessible, chitin-binding proteins bind along fungal cell walls and accumulate at septa and hyphal tips [59]. This binding produces changes in morphology including abnormal branching, hyphal swelling and shorter hyphae [60].

Thermal stability of the HbCLPs

Plant chitinases are generally stable at relatively high temperatures (> 60 °C) and also have a wide range of optimum pH values [56]. Therefore, to better understand the effect of the longer CBD on the stability of the HbCLP2, the unfolding curves of both complete proteins and their individual CatDs and CBDs were obtained by monitoring the CD signal at two different

minima: 222 nm for the complete proteins and their CatDs and 196 nm for the CBDs (Fig. 11A,B). For both HbCLPs and CatDs the unfolding transitions were irreversible. The melting temperature (T_m) for HbCLP1 was 71.4 ± 0.3 °C (Fig. 11C), whereas for HbCLP2 two transitions were observed with T_m values of 65.7 ± 1.08 °C and 73.6 ± 0.22 °C (Fig. 11D). Similar results were observed for the CatDs with a T_m of 68 ± 0.14 °C for CatD1 and also two transitions for CatD2 with T_m values of 60.4 ± 0.7 °C and 73.2 ± 0.2 °C (Fig. 11C,D). For the CBDs of both isoforms, which exhibit a high sequence identity to hevein (68%), no transitions were detected at the temperatures used in the experiment (20–90 °C). Hevein is an allergen (Hev b 6.02) that is extremely resistant to denaturation, exhibiting a T_m value of 90.1 °C at a pH of 3.6 [61]; it is therefore possible that the unfolding transition of the CBDs was not observed under the conditions used in the experiment. Finally, we also determined the T_m values of the mutants HbCLP1-A117E and HbCLP2-A147E, which were 78.3 ± 0.12 °C and 75.6 ± 0.15 °C, respectively (Fig. 11C,D). These values are larger than those for the HbCLPs, thus suggesting that shifting the activity of the CLP to a hydrolase increased the T_m by several degrees. Based on our crystallographic model, Glu117 and Glu147 may be establishing ionic interactions with Arg265 and Arg295, respectively, increasing their T_m values by 7 °C and 2 °C (Fig. 12). The results suggest that the CBDs do not affect the T_m values of the complete proteins; nonetheless, the detection of more than two states in the thermal denaturation experiments is complicated to interpret. To address this we considered that both CatDs have the same structure, since there are only three different residues in CatD2 compared with CatD1 (Fig. 3). Then analyzing the CatD2-A147E *in silico* model we found evidence that Glu147

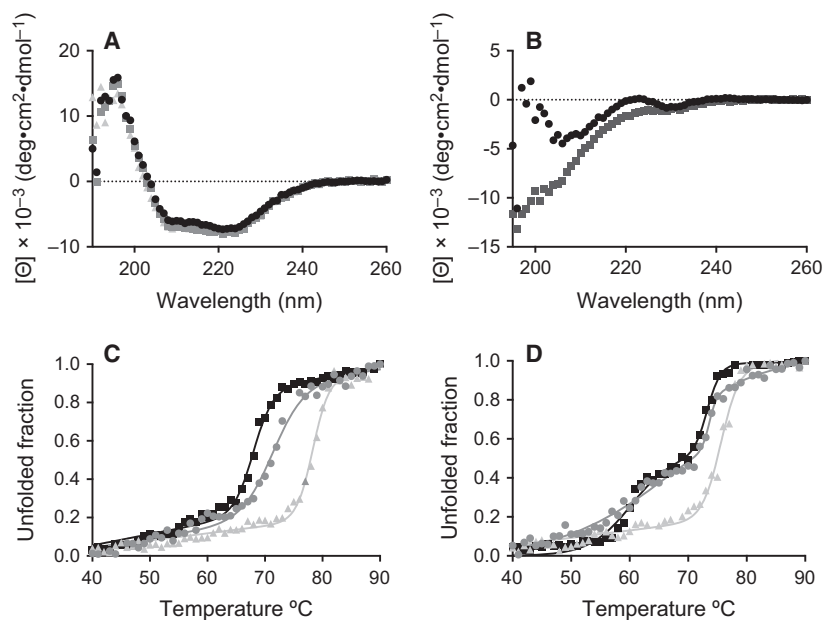


Fig. 11. Thermal denaturation profiles of the HbCLPs, their active mutants and their respective CatDs. CD spectra in the far-UV region for (A) HbCLP1-CatD (●), HbCLP1 (▲), HbCLP2 (■); (B) HbCLP1-CBD (●), HbCLP2-CBD (■). The transitions were followed using the CD signal at 222 nm. Solid lines indicate the theoretical curves obtained using a two-state transition model. (C) CatD1 (■), HbCLP1 (●) and HbCLP1-A117E (▲). (D) CatD2 (■), HbCLP2 (●) and HbCLP2-A147E (▲).

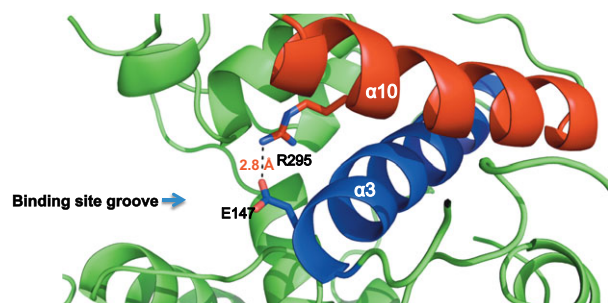


Fig. 12. Ionic interaction between Glu147 and Arg295 present in CatD2-A147E.

that is found at the C-terminus of α -helix 3 may establish an ionic interaction with R295, which is located at the N-terminus of the α -helix 10, therefore stabilizing the domain. In the native HbCLP2 this strong interaction could not exist, suggesting that less stability in the groove and perhaps more conformational states could exist (Fig. 12). Then one possibility for the existence of a three-state transition profile (two T_m values) is the pre-existence of multiple protein conformations with different stabilities and the other possibility is the appearance of an intermediate during thermal denaturation.

In summary, chitinases and CLPs have been considered to play important roles in plant defense responses [1]; nonetheless based on the literature it is far from clear to what extent they participate in these mechanisms. Chitinases and inactive chitinases, such

as CLPs, are produced by a wide variety of plants and are also involved in various physiological processes [24]. Due to their sessile nature, perennial plants such as *H. brasiliensis* are constantly exposed to a multitude of environmental stresses, in addition to the stress produced by the extraction of the latex. Therefore, proteins with diverse functions and evolutionary flexibility are likely to have adaptive value and enable plants to respond to diverse environmental challenges. Adaptive mutations such as those present in chitin-binding proteins can generate beneficial shifts in ligand recognition and/or affinity. For example, in a molecular phylogenetic study of mammalian chitinase-like members of the GH18 family, it was suggested that active chitinases with different specificities resulted from an early duplication event. Then a second duplication event followed by mutations that led to a loss of enzyme activity allowed the evolution of the CLP [24]. Similar adaptive mutations are very likely to occur in plant CLPs, such as those reported for the GH18 family that has been more widely studied [62].

In our study, two recombinant HbCLPs were cloned and expressed using the RNA from *H. brasiliensis* leaves and latex as starting materials. Both proteins are constitutively expressed in varying amounts depending on the season of tissue collection. Interestingly, a point mutation from Glu (GAA) to Ala (GCA) changed their functions from chitinases to CLPs. These HbCLPs are the first examples of naturally occurring plant CLPs belonging to the GH19 family that exhibit strong chitin and chitotriose-binding abilities. HbCLP2

has not been detected before in *H. brasiliensis* and, interestingly, its CBD is longer and exhibits one and a half hevein-like domains plus two linkers. Besides, CBD1 and CBD2 bind chitotriose with higher affinity than hevein or pseudohevein (which has the same residues at the binding site), indicating that the CBD2's N-terminal hevein-like domain is properly folded. Furthermore, the crystal structure of the CBD1 evidenced that –1Met interacts with the MES molecule at the carbohydrate-binding site suggesting that the presence of this residue increases its affinity for chitotriose significantly. Our study further showed that CatD1 has a flexible loop III, which is likely to tolerate moderate structural modifications. On the basis of this observation it was reasoned that adaptive functional diversification of the enzyme or enzyme-like protein families is achieved more often through modifications in the flexible regions than in the rigid structural elements. Modifications in the flexible regions allow for the acquisition of new functions without disrupting the native folded structures. Moreover, HbCLP1 and HbCLP2 are highly thermostable suggesting that during evolution from catalytically active proteins to CLPs they sacrificed activity for stability. Therefore, plants seem to constantly adjust their defensive status to the dynamically changing environment. Although the functions of CLPs in *H. brasiliensis* are still unknown, our studies suggest they are involved in defense mechanisms through their antifungal activity and their high affinity for oligosaccharides; nonetheless, we do not rule out the possibility that they could also be involved in other processes.

Materials and methods

Cloning, expression and purification of HbCLP1, HbCLP2 and their domains

Total RNA was isolated from the latex and young leaves of rubber trees (*H. brasiliensis*) as previously described [63]. Briefly, cDNA was synthesized, and the coding sequences of the chi-L1 (GenBank accession number [KF648872](#)) and chi-L2 (GenBank accession number [KF648873](#)) genes were amplified by PCR using the oligonucleotides CHI-a and CHI-b (all oligonucleotide sequences are detailed in Table S1). Additionally, the coding sequences for the individual CBD and CatD of both proteins (HbCLP1 and HbCLP2) were amplified using the oligonucleotide pairs CBD-a, CBD-b and CatD-a, CatD-b. To improve the cleavage efficiency of the enterokinase EkMax (Invitrogen Corporation, Carlsbad, CA, USA), two residues (Ala and Met) were added at the N-terminus of CBDs. Each of the reaction products were cloned into the pET32a vector (Novagen,

EMD Biosciences, Madison, WI, USA) and sequenced (Laragen, Culver City, CA, USA). *Escherichia coli* Rosetta-gami (DE3) pLysS was transformed with each of these constructs to express the full proteins and their respective individual domains. The cells were grown in Luria–Bertani medium and incubated at 37 °C until an A_{600} of 0.4 was reached, when isopropyl 1-thio- β -D-galactopyranoside (IPTG) was added to a final concentration of 0.25 mM and the incubation temperature was lowered to 18 °C. The cells were harvested by centrifugation at 15 300 g 12 h after the addition of IPTG and resuspended in lysis buffer (50 mM Tris/HCl, pH 7.5; 300 mM NaCl; 0.5 mM dithiothreitol). The cell pellet was lysed by sonication and clarified by centrifugation at 26 000 g for 45 min.

All purification steps were the same for the complete proteins and the individual domains unless otherwise noted. The supernatants were loaded onto HisTrap columns (GE Healthcare, Piscataway, NJ, USA), and the fusion proteins were eluted with gradients of increasing imidazole concentration. The purified fusion proteins were subjected to enterokinase cleavage, and the HbCLPs and their domains were then purified with the HisTrap column. The complete proteins that did not bind to this column were loaded onto a Mono-Q ion exchange column (GE Healthcare) equilibrated with 50 mM Tris/HCl (pH 7.5), and the bound proteins were eluted with an increasing gradient of 1 M NaCl. SDS/PAGE and Coomassie blue staining were used to assess all purification steps. The CBDs and CatDs were pure after the second affinity step using the HisTrap column. In all cases, the protein concentration was determined using the theoretical molar extinction coefficients at 280 nm.

For production of the HbCLP1-A117E and HbCLP2-A147E mutants, site-directed mutagenesis was performed by overlap-extension PCR [64]. The oligonucleotides used to introduce the mutations were CHI/A117E-a and CHI/A117E. The mutants were successfully produced and purified using the same methodology as described for the wild-type proteins.

Genomic DNA extraction and amplification and 5' and 3' RACE

The DNA from the leaves was extracted as described by Kotchoni and Gachomo [65] and used as a PCR template. The CHI-a and CHI-b primers were used to amplify the chi-L1 and chi-L2 DNA sequences, and the PCR products were cloned into a pET32a vector for sequencing. The extracted RNA was subjected to 5' and 3' RACE using the SMART RACE cDNA Amplification Kit (Clontech Laboratories, Mountain View, CA, USA) as described in the manufacturer's instructions. For 5' and 3' RACE, two sets of primers (chi-5'-P1, chi-3'-P1 and chi-5'-P2, chi-3'-P2) were used. The RACE products were cloned into the pGEM-T Easy vector (Promega Corporation, Madison, WI, USA) and subsequently sequenced.

Chitinase and chitin-binding assays

Chitinase activity measurements were performed with the 3,5-dinitrosalicylic acid method [66] using shrimp chitin (Sigma-Aldrich, St Louis, MO, USA), chitin beads (New England BioLabs, Ontario, Canada) and colloidal chitin prepared as described by Khan *et al.* [67] as the substrates. Protein (100 µg) was added to a 250-µL suspension of chitin azure (2 mg·mL⁻¹) in acetate-citrate buffer at two acidic pH values, pH 4.0 and pH 5.0, and in Tris/HCl buffer at pH 8.0. The reaction mixtures were incubated at 37 °C for 72 h with gentle shaking, followed by centrifugation at 14 000 *g* for 5 min; the blue color that developed was measured at 575 nm. One enzyme unit was defined as a change in the optical density at 570 nm (ΔA_{570}) of 1.0 in 72 h at pH 5.0 [68].

All measurements were run in triplicate, with negative controls comprising samples without the enzymes. The chitin-binding abilities of both HbCLPs were examined using chitin-bead columns (1 mL). Each protein (50 µg in buffer A; 50 mM Tris/HCl, pH 7.5) was loaded onto the chitin-bead columns previously equilibrated with buffer A. After thoroughly washing with the buffer, successive elution buffers were used: 1 M NaCl in buffer A, 0.1 M acetic acid and 1 M acetic acid solution.

Protein crystallization

Diffraction quality crystals of the individual domains of the HbCLP1 isoform were grown using the sitting-drop vapor-diffusion method at 18 °C. For the CBD1 (11 mg·mL⁻¹) crystals grew with 0.1 M MES at pH 7.0, 1.6 M (NH₄)₂SO₄ and 4% 1,4-dioxane; for the CatD1 (7 mg·mL⁻¹) crystals grew using 0.1 M Tris/HCl at pH 8.3, 0.2 M MgCl₂ and 30% PEG 4000. CBD1 crystals diffracted to 1.6 Å resolution and belonged to space group *P61*, while CatD1 crystals diffracted to 1.95 Å resolution and belonged to the monoclinic space group *I121*, as confirmed by POINTLESS [69]. Several attempts were made to obtain suitable crystals of the complete proteins in complex with chitin oligosaccharides, without success. Thus, to analyze the interactions between the CatD1 residues and (GlcNAc)₆, docking experiments were performed as described below.

Data collection, structure determination and structure refinement

Diffraction data for the CatD1 crystal were collected on a Pilatus 6M detector at beamline PX06SA of the Swiss Light Source in Villigen, Switzerland. The CBD1 data were collected on a Pilatus 2M detector at beamline ID 29 of the European Synchrotron Radiation Facility in Grenoble, France. This CBD1 crystal was cryoprotected using a saturated Li₂SO₄ solution, whereas the CatD1 crystal required no extra cryoprotectant. All data sets were processed and

scaled using XDS [70] and SCALE, respectively, of the CCP4 suite v.6.1.2 [71]. The CBD1 hexagonal crystals had unit-cell dimensions of *a* = 38.51, *b* = 38.51, *c* = 100.93 Å, while the CatD1 monoclinic crystals had unit-cell parameters of *a* = 111.11, *b* = 40.41, *c* = 111.85 Å with a β angle of 96.73°. The statistics for both data sets are summarized in Table 1.

Both structures were solved by the molecular replacement method with PHASER in the CCP4 suite [71]. For the CBD1, the template was the structure of hevein (PDB ID [1Q9B](#)) [33], which has 68% sequence identity with the domain of interest. In this case, the solution gave a Z-score of 21.2 and a log-likelihood gain of 1334. For the CatD1, the structure of the papaya chitinase (PDB ID [3CQL](#)) [38], which has an 84% sequence identity, served as the search model. The first solution gave a Z-score of 22.8 and a log-likelihood gain of 5410. In both cases, map inspection and subsequent model building were performed with COOT [72], and the resulting models were refined with PHENIX-REFINE [73].

Structural comparisons were performed with the DALI algorithm [74]. The figures were produced using the program PYMOL [75]. The coordinates of the CatD1 and CBD1 have been deposited in the Protein Data Bank under accession codes [4MST](#) and [4MPI](#), respectively.

Docking simulation

We performed *in silico* molecular-docking experiments of the (GlcNAc)₆ oligosaccharide with our X-ray structure of the CatD1, which were generated with SMILES and minimized in CHIMERA using the web-based SWISSDOCK server [76]. The docking was performed using the default parameters without defining a binding region. We selected the best model based on the energy scores. Compatible crystallographic water molecules in the binding groove of the CatD1 were then included in the final model. The monosaccharides in (GlcNAc)₆ were named -4, -3, -2, -1 and +1, +2, beginning from the non-reducing end. We then identified and analyzed the residues potentially involved in ligand binding using the LIGPLOT+ software [77].

For the CBD, the initial complex was obtained using an alignment of the CBD1 coordinates with hevein-(GlcNAc)₃ (PDB ID [1T0W](#)) and subsequently the CBD1-(GlcNAc)₃ model was obtained after exhaustive energy minimization cycles (step-descent/conjugate-gradient) using USFC CHIMERA (<https://www.cgl.ucsf.edu/chimera/>) and the AM1-BCC force field.

Fluorescence assays

The binding affinities for (GlcNAc)₃ of both HbCLPs and each of their domains were measured 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 5 nm, and the temperature was maintained at 25 °C using a Peltier system. Different amounts of the oligosaccharide were added to the protein solution (10 µg·µL⁻¹) in 100 mM phosphate buffer (pH 7.4) with 1 mM EDTA. Fluorescence was measured 2 min after mixing the solution with the ligand to allow for equilibration. The changes in fluorescence intensities were used to calculate the dissociation constants for the ligands by plotting the change in fluorescence intensity at the wavelength of maximum emission ($\lambda_{\text{max}} \sim 330$ nm) against the ligand concentration. The resulting isotherm was fitted with Eqn (1) or (2) as appropriate:

$$\Delta F = \frac{\Delta F_{\text{max}}[\text{ligand}]}{K_d + [\text{ligand}]} \quad (1)$$

$$\Delta F = \frac{\Delta F_{\text{max}}[\text{ligand}]^h}{K_d^h + [\text{ligand}]^h} \quad (2)$$

In the equations, ΔF is the difference in fluorescence intensity of the protein with and without the oligosaccharide, ΔF_{max} is this difference when all of the binding sites of the protein are saturated with the ligand, K_d is the dissociation constant of the ligand from the protein–ligand complexes and h is the Hill coefficient. The data represent two or three independent measurements.

Fungal growth inhibition assay

Bioassays to test the antifungal activity of HbCLP1 and HbCLP2 were conducted using a disk diffusion test. Three different concentrations (25, 50 and 75 µg) of protein in 7 µL of sterile buffer (50 mM Tris/HCl, pH 7.5) were applied to 5-mm sterile paper disks. These disks were placed on potato dextrose agar in a 9-cm diameter Petri dish with a solid inoculum (5 mm³ agar plug) containing the mycelium of a 4-day-old culture of *A. alternata* at the center of the plate. The disks were placed on the agar at a distance of 20 mm from the growing front of the hyphae, and the plates were incubated for 72 h at room temperature and photographed.

Thermal stability measurements using circular dichroism

CD measurements were performed at 20 °C using a Jasco-J700 spectropolarimeter (Jasco Inc., Easton, MD, USA) equipped with a Peltier temperature control system. HbCLP and CatD solutions were dialyzed in 20 mM phosphate buffer at pH 7.5 and filtered. Far-UV spectra were collected for each sample from 190 to 260 nm in a 1-cm path-length cell. Three scans were averaged to obtain the final spectra of the different proteins at a concentration of 20 µg·µL⁻¹. The thermal denaturation profiles were obtained

over a range of 20–90 °C with a temperature rate change of 50 °C·h⁻¹ by monitoring the ellipticity changes at 222 nm for the complete proteins and the CatDs and at 196 nm for the CBDs. Finally, the samples were cooled to the initial temperature and another far-UV spectrum was obtained for each sample. All the spectra were scaled to molar ellipticities Θ (deg·cm²·dmol⁻¹). The fraction of unfolded protein at each temperature was calculated from the CD value by linearly extrapolating the pre- and post-transition baselines into the transition zone and plotting against the temperature. The midpoint temperatures of the unfolding transition (T_m) were obtained from the transition curves between the folded and unfolded protein.

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Author contributions

SMC: Planned experiments, performed experiments, analyzed data and wrote the paper; PCS: Planned experiments and performed experiments; IMM: Performed experiments; AGDS: Performed experiments and analyzed data; MLMR: Planned experiments and contributed reagents or other essential material; JAH: Planned experiments and contributed reagents or other essential material; ARR: Planned experiments, analyzed data, contributed reagents or other essential material and wrote the paper.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Table S1. Oligonucleotide sequences.



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Comparative study of two GH19 chitinase-like proteins from *Hevea brasiliensis*, one exhibiting a novel carbohydrate-binding domain

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Supplementary material to the manuscript:

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Siseth Martínez-Caballero^{1,2}, Patricia Cano-Sánchez¹, Israel Mares-Mejía¹, Angel G. Díaz-Sánchez¹, Martha L. Macías-Rubalcava¹, Juan A. Hermoso² and Adela Rodríguez-Romero¹

1 Instituto de Química, Universidad Nacional Autónoma de México, Circuito Exterior, Ciudad Universitaria, México D. F. 04510, México

2 Departamento de Cristalografía y Biología Estructural, Instituto de Química-Física “Rocasolano”, CSIC, Serrano 119, 28006, Madrid, Spain

Corresponding author:

Adela Rodríguez-Romero, Instituto de Química, Universidad Nacional Autónoma de México, Circuito Exterior, Ciudad Universitaria, México D. F. 04510, México

Tel: +52-5556224568

Fax: +52-5556162217

E-mail: adela@unam.mx

Table 1S. Oligonucleotides used in this study

Oligonucleotide	Sequence
CHI-a	5'-ggccatggagcaatgtggaaggcaagct-3'
CHI-b	5'-aagcgcccgctcaccctaataccgaatggcgt-3'
CBD-a	5'-ggccatggagcaatgggaaggcaagct-3'
CBD-b	5'-cagcgcccgctcaaaactccaccatcacattggctttggcagc-3'
CatD-a	5'-ggccatggacgacgacaagagtatcatctcaagatcaacgtttgaggag-3'
CatD-b	5'-aagcgcccgctcaccctaataccgaatggcgt-3'
CHI/A117E-a	5'-cagacatctcatgaaactacaggaggg-3'
CHI/A117E-b	5'-ccctcctgtagtttcatgagatgtctg-3'
Chi-5'-P1	5'-tgттаagcatctcctcaaacgттга-3'
Chi-5'-P2	5'-cactgccacaatatтcaggtgtgttg-3'
Chi-3'-P1	5'-тctтccagctgaaatatcgтggccgg-3'
Chi-3'-P2	5'-тctтataaaaacccaatacgatctтca-3'