

Hevea brasiliensis prohevein possesses a conserved C-terminal domain with amyloid-like properties *in vitro*

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ABSTRACT

Prohevein is a wound-induced protein and a main allergen from latex of *Hevea brasiliensis* (rubber tree). This 187 amino-acid protein is cleaved in two fragments: a N-terminal 43 amino-acids called hevein, a lectin bearing a chitin-binding motif with antifungal properties and a C-terminal domain (C-ter) far less characterized. We provide here new insights on the characteristics of prohevein, hevein and C-terminal domain. Using complementary biochemical (ThT/CR/chitin binding, agglutination) and structural (modeling, ATR-FTIR, TEM, WAXS) approaches, we show that this domain clearly displays all the characteristics of an amyloid-like proteins *in vitro*, that could confer agglutination activity in synergy with its chitin-binding activity. Additionally, this C-ter domain is highly conserved and present in numerous plant prohevein-like proteins or pathogenesis-related (PR and WIN) proteins. This could be the hallmark of the eventual presence of proteins with amyloid properties in plants, that could potentially play a role in defense through aggregation properties.

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1. Introduction

More than 20,000 latex producing plants have been identified [1,2]. The production of latex is conferred by the presence of specialized plant cells called laticifers. Each year more than 10 million tons of natural rubber (NR) are produced [3], and *Hevea brasiliensis* (Willd. Ex A. Juss) Müll. Arg remains the main worldwide source of latex to produce commercial NR. It is a biopolymer made from latex after tree tapping and mainly composed of *cis*-1,4-polyisoprene and non-rubber components. Indeed latex is a white colloidal cytoplasmic system containing rubber particles, non-rubber particles, organelles, proteins, lipids and cytoplasmic C-serum [4]. After centrifugation, it may be separated into three phases: the top cream made of rubber and Frey-Wyssling particles, the intermediate cytoplasmic C-serum and the bottom fraction with lutoids [5]. *Hevea* latex contains more than 1300 proteins [6], from which 15 have been registered as allergens (Hev b 1–15; [7]). At least 186 proteins have been identified as present on rubber particles [8] and 169 proteins were extracted from lutoids [9]. Lutoids proteins mainly play roles in plant defense, stress response, chitin degradation, sugar metabolism, proton transport or signal transduction [9]. Lutoids

are acidic and osmosensitive vacuole-like organelles, which make about 12% of the overall latex [10]. Lutoids are characterized by antipathogen properties [11], role in maintaining ion and pH homeostasis and helping in latex coagulation.

Hevein is a predominant protein of lutoids [12], but is also a well-known latex allergen, called Hev b 6.02 [13]. First isolated from fresh latex in 1960 by Archer, hevein was presented as a small crystalline, non-glycosylated and sulfur-rich protein, forming an apparent 10 kDa dimer [12,13]. Crystals were formed of hexagonal needles that also showed birefringence [12]. In 1975, its complete amino acid sequence was determined and reported as a peptide of 43 amino acids and a molecular weight of 4729 Da [14,15]. 3D-structure was first solved by X-ray crystallography at 2.8 Å and 1.5 Å resolution (PDB 1WKX and 1Q9B; [16–20]) and further analyzed by liquid NMR (PDB 1HEV; [21]). Hevein is heat-stable [17], presents no hydrophobic core [18], but 8 cysteines forming 4 disulfide bridges [18,22].

Broekaert et al., cloned the cDNA encoding the precursor of hevein (HEV1; an open reading frame of 1018 bp encoding 204 amino acids; GenBank accession n° M36986) and predicted an N-terminal translocation signal sequence of 17 amino acid residues [23,24]. Indeed, the larger precursor protein is submitted to post-translational cleavage that may generate the prohevein without the transit sequence (187 amino acid; 18.5 kDa), the N-terminal hevein (43 amino acids; 4.7 kDa), and the C-terminal domain (144 amino acids; 13.3 kDa) [24,25]. In 2005, 5 new hevein precursor genomic sequences were characterized, they appeared to be highly conserved, but with

Abbreviations: Hb, *Hevea brasiliensis* (hevea); ATR-FTIR, attenuated-total reflectance Fourier-transform infra-red.

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different promoter regions [26]. The HEV1 mRNA expression may be stimulated by wounding, abscisic acid and ethylene treatment [23]. Hevein is highly expressed in laticifers. In addition, the promoter *pHEV2.1* in a transgenic rice GUS construct may be activated by a pathogenic fungus or wounding [26]. In transgenic hevea the *pHEV2.1::GUS* construct also showed light stimulation [27]. Prohevein, hevein and C-ter domain have all been shown to be allergenic and registered by WHO-IUIS as Hev b 6.01, 6.02 and 6.03 respectively [28]. Hevein has the main IgE-specific epitopes and is more allergenic than the prohevein or the C-ter domain [15,29,30]. Additionally, hevein is a lectin with chitin-binding specificity, recognizing a motif $\beta(1 \rightarrow 4)$ -linked N-acetyl-D-glucosamine (GlcNAc) [11,31]. Several aromatic residues are involved in this (GlcNAc)₂₋₄ binding domain with a millimolar affinity. They include S19, W21, W23 and Y30, but also the loop region 13–16 [32,33]. Additionally hevein displays hemagglutination properties towards human red blood cells [18] and latex coagulation [34]. When hevein is released after cleavage in the cytosol, it is supposed to bind the GlcNAc moiety of a glycoprotein receptor (24 kDa) located at the rubber particle surface, thereby promoting the agglutination of rubber particles and the plugging after tapping [34,35]. Hevein also showed strong antifungal and antimicrobial activities to protect hevea from plant pathogen invaders, particularly after tapping [11,36]. Hevein-like genes are present in many plants including potato [23,37] or *Arabidopsis thaliana* [38]. They belong to a larger family of chitin-binding proteins involved in plant defenses [39] and usually target cell wall of pathogens, penetrate and modify it, causing aggregation and cell death.

In this work, we focused on prohevein and its C-terminal domain (C-ter). Using biochemical and structural approaches, it was clearly showed that the C-terminus of prohevein is highly conserved in the hevein precursor family and possesses the strong ability to form *in vitro* amyloid-aggregates. In synergy with the hevein chitin-binding domain, this confers to prohevein a strong agglutinating ability through which plant defenses could be exerted.

2. Results and discussion

2.1. Phylogeny of the hevein precursor

The BLAST on the hevein precursor (P02877) identified numerous homologous proteins, many of them being putative proteins but all belonging to the plant kingdom. A phylogenetic analysis clearly shows that the hevein precursor belongs to a larger protein family related to stress and pathogenesis (Fig. 1 and supplementary Fig. S1). In N-ter a short non-conserved sequence is present, corresponding to signal sequence for localization, obviously different from one species to another. The precursor possesses two conserved domains: a chitin binding domain in N-ter corresponding to hevein and a barley-wound-inducible domain (Barwin-like domain) in C-ter (Fig. 1A and supplementary Fig. S1). Both domains are conserved, but the C-terminal domain seems even more conserved than the hevein part. In certain plant species (e.g. *Vitis vinifera* or *Glycine soja*), pathogenesis-related proteins do not present the hevein part, highlighting the role of the C-ter in pathogenesis. Hevein belongs indeed to the carbohydrate binding module family 18 (CBM18) found in the CAZy database (http://www.cazy.org/CBM18_structure.html) [40]. Both domains may be involved in a common defense mechanism in plants. Many homologous proteins are pathogenesis-related (PR proteins) or wound-induced (WIN proteins) as seen on the phylogenetic tree (Fig. 1B). Proteins identified in hevea (red box) are closely related.

2.2. Hevein and its precursor display a strong toxicity in *Escherichia coli*

Hevein is well known for its toxicity towards various fungi or bacteria [11]. Four different constructions for the expression were cloned: the precursor protein, the prohevein, the hevein and the

C-terminal part (Fig. 2A and supplementary Table S1). Expressed in *E. coli* they all showed toxicity towards their host (Fig. 2B). As expected, the strongest toxicity was confirmed for the hevein, but also interestingly for the precursor (even without IPTG induction/due to promoter leakage). It could mean that the precursor is directly processed into hevein in *E. coli*. The signal sequence in N-ter could be responsible of the cleavage. As the expression was not possible for these two constructs, further experiments with hevein were performed with a synthetic peptide of 43 amino acids. To a lesser extent, prohevein and the C-ter domain also showed toxicity after induction by IPTG. Surprisingly, the hevein peptide did not show any toxicity on *E. coli* cells when added *in vitro* to the growth media (Supplementary Fig. S2).

The purifications of recombinant prohevein and C-ter protein were however achieved and showed on SDS-PAGE (Fig. 2C) with the presence of an additional thin band obviously corresponding to dimers. One should note that prohevein possesses 14 cysteines forming potentially 7 disulfide intramolecular bridges, 3 bridges are also formed in the C-ter domain. Extramolecular S–S connections cannot be excluded even in reducing conditions. These two visible dimers were therefore stable to heat and SDS denaturation. Even the presence of DTT in the buffer was not enough to reduce certain cysteine bridges. Indeed the dimer formation of prohevein and hevein was also originally confirmed during their purification from luteoids [11,12,41,42]. Formation of dimers by cysteine rich proteins is classical, and hevein contains 8 cysteines. Dimerization seems also to be a characteristic of numerous allergens [43].

2.3. The C-terminal domain of prohevein has amyloid properties

After the purification of prohevein and C-ter domain, we noticed that the C-ter domain was not stable after thawing and could easily form visible aggregates when left at room temperature. Wondering if these aggregates could be of amyloid nature, prediction of amyloidogenic/aggregation regions in prohevein was first performed with FoldAmyloid [44], Amylpred [45], Waltz [46] and Aggrescan [47] algorithms. All analyses predicted almost 5 hot spots of aggregation and amyloid propensity in the C-terminal domain (Supplementary Fig. S3). The structure of hevein has been previously characterized by crystallography (PDB 1Q9B) and NMR (PDB 1HEV), and was an alternation of small β -sheets, helices, turns and random coils. Fig. 3A visualizes hevein structure by NMR. In addition, a secondary structure prediction model was investigated on the RaptorX server [48,49]. It shows that prohevein could also present β -sheets, helices, turns and random coils. This prediction only modeled 142 (75%) residues from 36 to 187, almost excluding the hevein part. This model suggests the presence of a β -barrel surrounded by helices as seen in Fig. 3B. Fig. 3C shows the annotation of sheets (green) and helices (red) on the modeled sequence. A prediction was also ran on the C-ter domain (Supplementary Fig. S4) and also showed the same kind of structuration. Amyloid prediction and structural modelization were both converging towards the fact that the C-ter domain could be rich in β -sheets and fold as an amyloid.

To confirm structures, ATR-FTIR was used to characterize the soluble prohevein, hevein and the precipitated C-ter domain. ATR-FTIR deconvoluted spectra of the proteins are presented in Fig. 3D and structural results expressed in Table 1. The C-ter aggregates clearly contained higher β -sheet content and less random coils (1645 cm^{-1}) than the prohevein or hevein. The contributions at $1624/1685\text{ cm}^{-1}$ confirm the presence of antiparallel β -sheets, which could indeed fold into a β -barrel. Helices (1657 cm^{-1}) and turns (1670 cm^{-1}) are also present in both structures. Crystallographic study of prohevein is now needed to have further structural informations. ATR-FTIR also shows that hevein contains more β -sheets than helices, this result fits indeed better to the NMR structure than to the crystal one.

Further characterization by WAXS confirmed that the C-terminal domain aggregates obtained after sedimentation or centrifugation (Fig. 4A), were structured in a “ β -sheet core” with a meridional

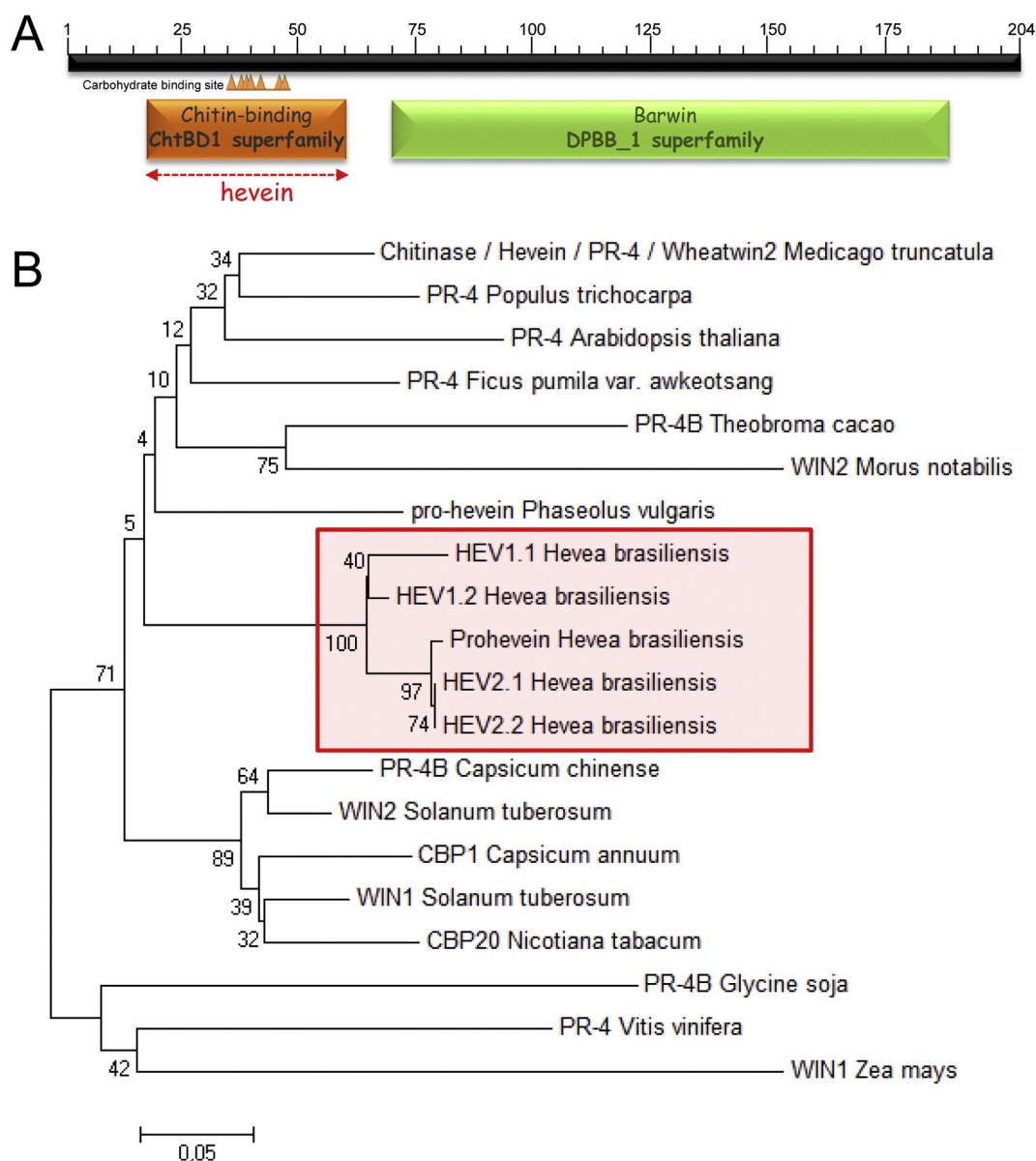


Fig. 1. Phylogenetic analysis of the hevein precursor superfamily. A BLASTp was performed with the sequence of the hevein precursor (P02877). A) Scheme of the hevein precursor family with putative identified conserved domains. B) Phylotree. The accession numbers of each 20 aligned sequences with ClustalW were as follows: *Hevea brasiliensis* HEV1.1 AAO63571, HEV1.2 AAO63572, HEV2.1 AAO63573, HEV2.2 AAO63574, prohevein CAA05978; *Solanum tuberosum* WIN1 P09761, WIN2 P09762; *Theobroma cacao* PR-4B XP_007030564; *Capsicum chinense* PR-4B BAD11073; *Capsicum annuum* CBP1 AAF18934; *Nicotiana tabacum* AAB29959; *Phaseolus vulgaris*, prohevein AGV54773; *Populus trichocarpa* XP_002319077; *Arabidopsis thaliana* NP_187123; *Glycine soja* KHN14699; *Zea mays* AFW60484; *Morus notabilis* XP_010102291; *Vitis vinifera* AAC33732; *Ficus pumila* var. *awkeotsang* PR-4 ADO24163; *Medicago truncatula* KEH24556. *Hevea brasiliensis* protein family is framed by a red dashed box. Abbreviations: WIN: Wound INduced; PR: Pathogenesis Related; CBP: Chitin-Binding Protein. Sequence alignments are presented in Supplementary Fig. S1.

(interstrand spacing) reflection of 4.6 Å, (Fig. 4B). WAXS diffractogram was also showing a pattern of equatorial organization (intersheet spacing) at 9.9 Å. Both reflections are characteristic of amyloid structural organization.

The presence of amyloid fibers was moreover confirmed and visualized by TEM (Fig. 4C). Top left panel already shows quite large assembly of fibrous bundles. Single isolated fiber was barely visible. Larger aggregates were mainly observed. This is the second description of the amyloid propensity *in vitro* of a latex protein. Rubber elongation factor (REF) was also described as having amyloid properties *in vitro*, when lipids were not present to stabilize it on the rubber particles [50,51]. TEM analyses of lutoids also revealed the presence of large microfibrils (~125 Å) formed with acidic proteins, but also microfibrillar protein inclusions [4,52–54]. Their amyloid nature has however never been demonstrated.

In addition, amyloid fibers should be characterized by tinctorial properties. Congo red binding assays were performed and showed that not only C-ter domain aggregates were stained by CR, but also that CR was able to induce the staining and aggregation of prohevein (Fig. 5). In presence of the dye, both proteins displayed a characteristic red shift of their spectra (Fig. 5A). The induction of aggregation by CR has been previously observed in the presence of amyloid-like proteins [55,56]. Interestingly similar results were also observed in the presence of Thioflavin T (ThT), another amyloid fluorescent dye (Fig. 5B). Binding of ThT to prohevein and C-ter was observed along with aggregation, the dye displayed enhanced fluorescence, and a characteristic red shift of its emission spectrum. Furthermore, the C-aggregates stained with CR were also showing birefringence under cross-polarized light (Fig. 5C). All these data therefore confirm the amyloid nature of the auto-assemblies formed by the C-terminal domain. ThT and CR were

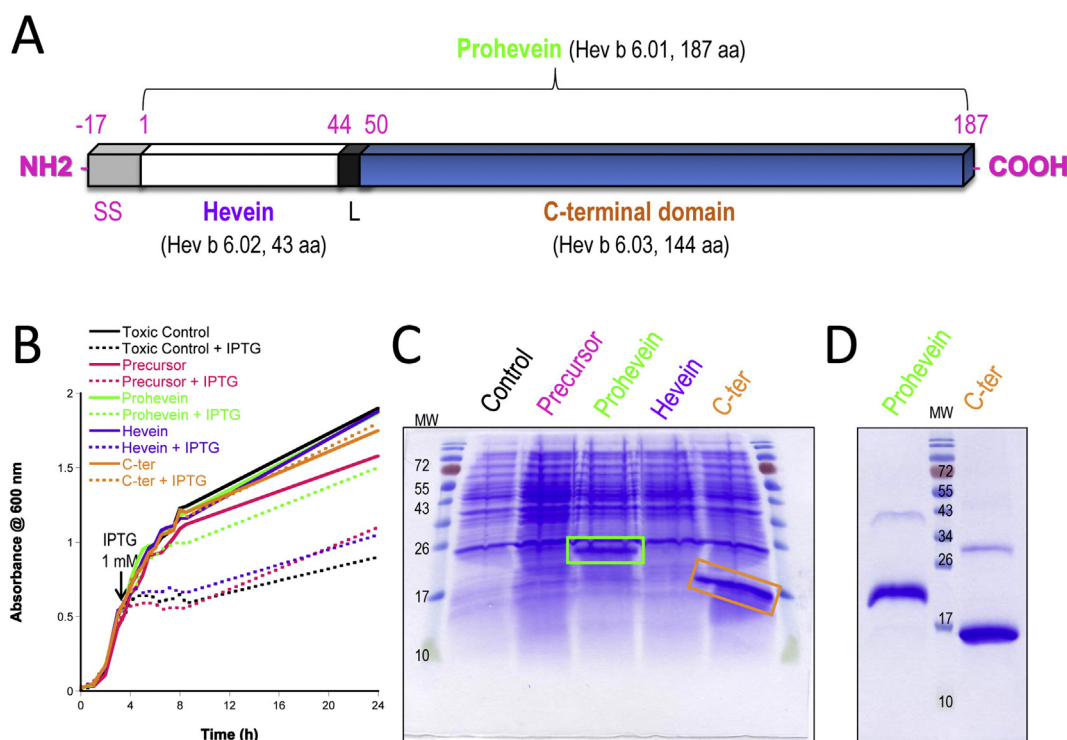


Fig. 2. Toxicity in *Escherichia coli*. A) Composition of the 204 amino acids hevein precursor with an N-terminal signal sequence (SS). Prohevein_(1–187) may then be cleaved in hevein_(1–43) and a C-terminal domain_(50–187) (C-ter). For cloning, a 6His-tag was added in N-ter and the C-ter domain was cloned with the short linker (L) as 44–187 amino acids. B) Growth curves of *E. coli* Gold pLysS after 1 mM IPTG induction at around $A_{600} = 0.6$ in LB medium complemented with kanamycin and chloramphenicol. C) The production of recombinant proteins after overnight induction was controlled by 16% SDS-PAGE. D) Purified prohevein and C-ter domain.

previously shown as able to induce the precipitation of certain amyloid-like proteins [55], as it seems the case of prohevein.

Upon incubation at 37 °C in PBS, the aggregation of prohevein was observed, but was also clearly different from the C-ter aggregation. The C-ter domain neatly precipitated like a kind of white powder, whereas the prohevein was translucent, fluffy, more like a gel. ATR-FTIR also showed that the aggregates of prohevein contained less β -sheets than the C-ter aggregates (Fig. 3D and Table 1). WAXS analysis of prohevein also revealed two weak 4.9 and 10.1 Å peaks (characteristics of amyloids), but the signal was not as strong as the C-ter domain (Supplemental Fig. S5A). TEM however showed that prohevein is also able to auto-assemble into big aggregates, different of the C-ter (Supplemental Fig. S5B). No clear amyloid fibers were observed in the tested samples, but “tissue-like aggregates” were observed locally (right panel). They could explain the amyloid-core signal recorded by WAXS. Interestingly upon incubation at 37 °C, both prohevein and C-ter display a relative resistance to proteolysis (Supplemental Fig. S6). Proteinase K degradation reveals a domain of about 14 kDa that could be the amyloid resistant core of the prohevein. Therefore the prohevein is an amyloid-like protein, but the amyloid moiety is imbedded in the C-terminal part. It could mean that prohevein, after release of hevein (in lutoids), would generate a C-ter domain able to precipitate in the bottom fractions.

2.4. No interactions with hydrophobic interfaces (air/lipids/glycolipids)

Trying to understand the potential function of prohevein and its C-ter domain, their interactions with interface and membranes were investigated. Using ellipsometry and Brewster microscopy at the air-PBS interface, the proteins alone at 1 μ M (prohevein, hevein, C-ter) did not show any surface activity. None of the proteins injected in PBS 1X were able to come to the surface, presenting no increase of surface pressure and thickness. Their non-hydrophobic nature is confirmed by the Kyte–Doolittle hydropathy plot of prohevein, which does not

show any hydrophobic domains (Supplementary Fig. S7). The crystal structure of hevein also revealed a lack of hydrophobic core [18].

In addition the three proteins did not interact with the tested lipids presented as monolayers at the interface (asolectin, DMPC or DMPG). Therefore, various lipopolysaccharides were also tested (lipopolysaccharides from *E. coli*, GM1 and MGDG), but none of these monolayer membranes with integrated glycolipids showed an interaction with hevein or prohevein. We may conclude that prohevein is not a membranous protein and do not have interaction with lipidic moieties. Therefore it seems that this protein could specifically interact with a specific glycoprotein at the surface of the rubber particle as previously suggested [34].

2.5. Prohevein has also agglutination and chitin-binding properties

In 1991, A. Rodriguez-Romero showed that hevein was able to agglutinate human erythrocytes of blood group A [18]. Unable to find this blood group, rabbit and human group AB red blood cells (RBC) were tested on prohevein, hevein and C-ter. Prohevein strongly agglutinates both type of RBC as shown in Fig. 6A. The effect is stronger on human cells. The C-terminal domain also agglutinates RBC but weakly. This may be due to the concomitant aggregation of the amyloid protein. This kind of co-aggregation has been previously observed with HbREF amyloid [57]. No hemagglutination was seen with the hevein peptide, even when the addition of DTT or CaCl₂ was tested. We therefore may suppose that hevein recognizes specifically group A cells. In the 1991 paper, hevein was tested with all AOB human bloodgroups, but only group A apparently showed positive agglutination [18]. In addition the synthetic peptide may fold differently of the native cleaved protein. The circular dichroism spectrum of the synthetic peptide in PBS (Supplementary Fig. S8) was also different than the one recorded with purified and dried proteins in 1989, indicating then more disulfide bridges [58]. Indeed Van Parijs et al., observed that the native hevein purified from the lutoids, probably assembling as a stable unreducible

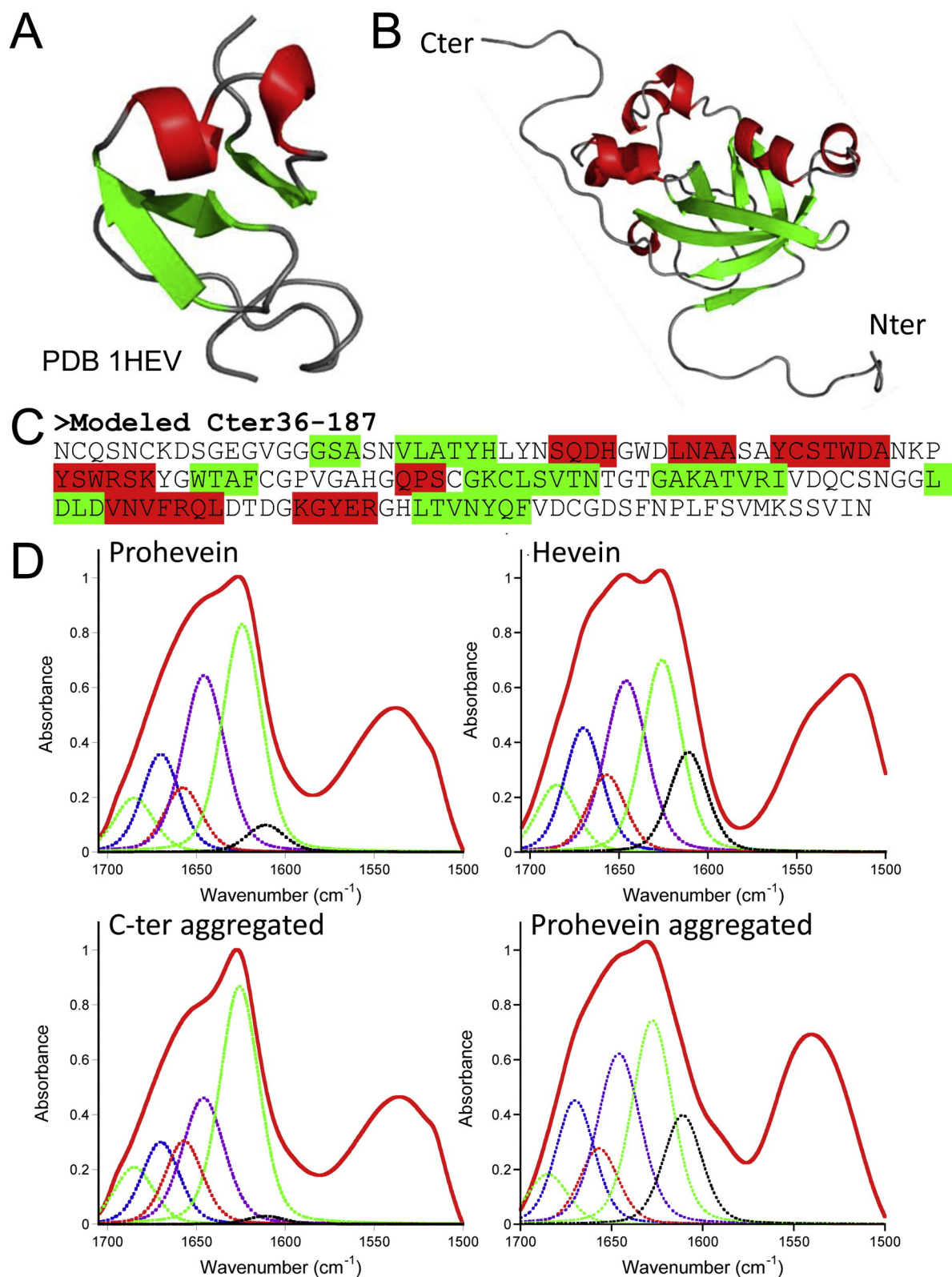


Fig. 3. Structure of hevein, prohevein and aggregated C-ter domain. A) NMR structures of hevein produced using PyMOL with referenced protein database bank PDB HEV1. B) Protein secondary structural prediction model of prohevein. The PDB model was realized using RaptorX. C) Highlights of β -sheets (green) and helices (red) on the predicted model. D) Deconvoluted ATR-FTIR spectra of the three proteins. Each structural contribution is colored: aggregation (black), antiparallel β -sheets (green), random coils (violet), helices (red), turns (blue).

dimer of about 10 kDa, also failed to hemagglutinate rabbit, pigeon and human RBC. Sugar inhibition was also tested on prohevein hemagglutination and N-acetyl glucosamine (GlcNAc) specificity was

confirmed (Fig. 6B), but this inhibitory effect was only seen on one $\frac{1}{2}$ dilution. Prohevein hemagglutination was also inhibited by GlcNAc [18]. Interestingly, prohevein and C-terminal domain also demonstrated

Table 1
ATR-FTIR analyses of prohevein, hevein and aggregated C-terminal domain.

Amide secondary structure element	Wavenumbers (cm ⁻¹)	Relative comparative (%)			
		Prohevein	Aggregated prohevein	Hevein	Aggregated C-ter
Aggregation	1611	4.04	14.55	13.28	1.32
Antiparallel β -sheet	1625	35.17	28.15	26.48	40.27
Random coil	1645	29.20	25.15	25.25	22.84
Helix	1657	9.48	9.97	10.16	13.43
Turn	1670	14.29	16.07	16.18	13.13
Antiparallel β -sheet	1685	7.82	6.11	8.65	8.99

a clear agglutination of the baker's yeast *Saccharomyces cerevisiae* cells (Fig. 6C). In the case of prohevein, the effect was stronger leading to a precipitate but also a kind of coalescence with yeast cells (Fig. 6B, right panel). Interestingly this agglutination was confirmed with another yeast unrelated species, *Candida bombicola* (Fig. 6D).

Prohevein (and dimers) binding to chitin was further confirmed by chitin-binding assays. Indeed, as expected hevein and prohevein presenting a chitin binding domain were able to remain bound on a chitin resin (Fig. 7). Recombinant prohevein showed a higher affinity towards chitin than the synthetic hevein. The presence of hevein was seen before destaining, but is almost invisible on Fig. 7A. Aggregated C-ter domain was also present with the resin as a co-precipitate. The unprecipitated fraction was however

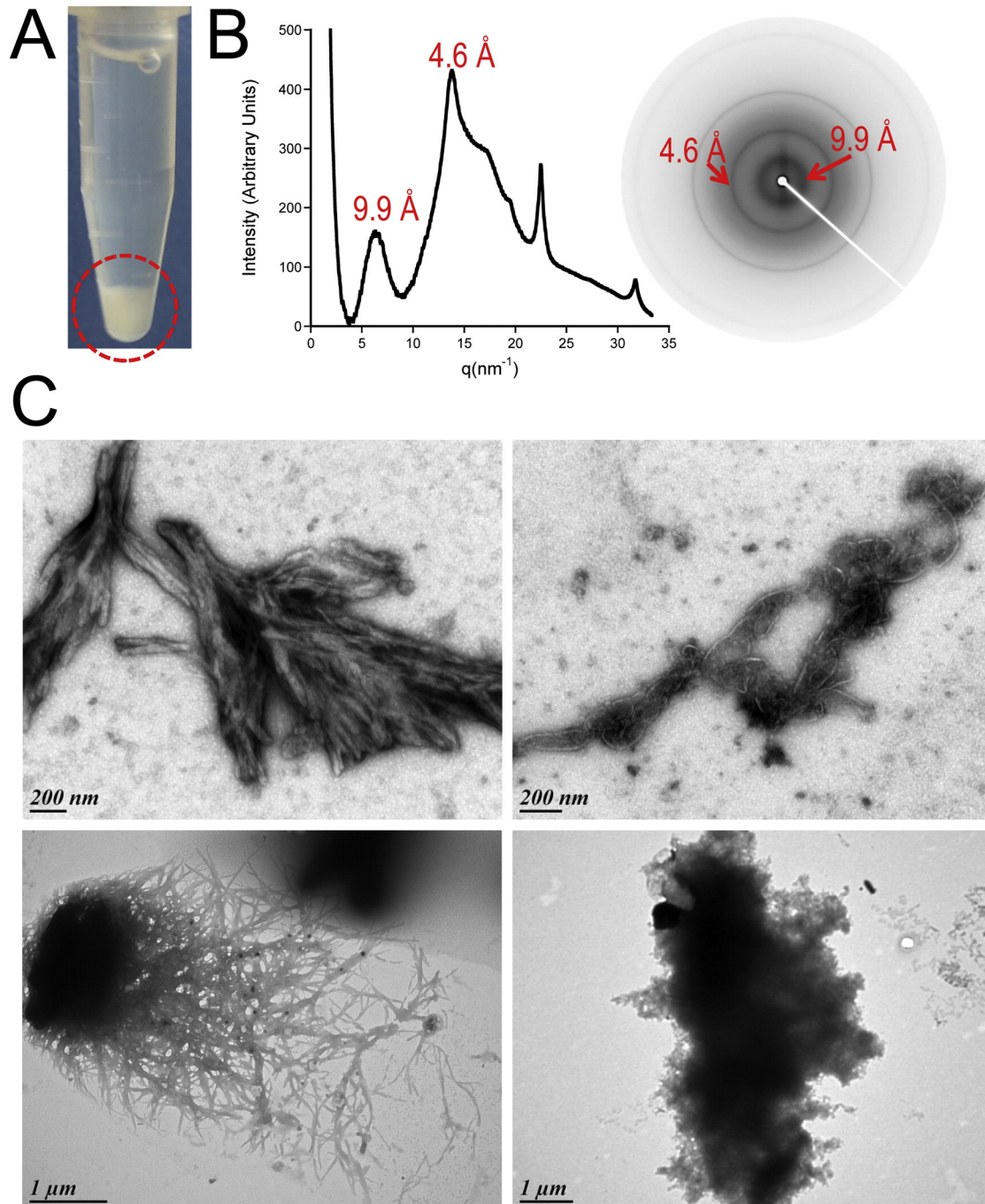


Fig. 4. Aggregated C-terminal domain has amyloid structural characteristics. A) Aggregation and sedimentation of the C-terminal domain in PBS 1X. B) WAXS diffraction pattern of the freeze-dried aggregates after centrifugation. C) TEM observations of C-ter amyloid fibers and aggregates.

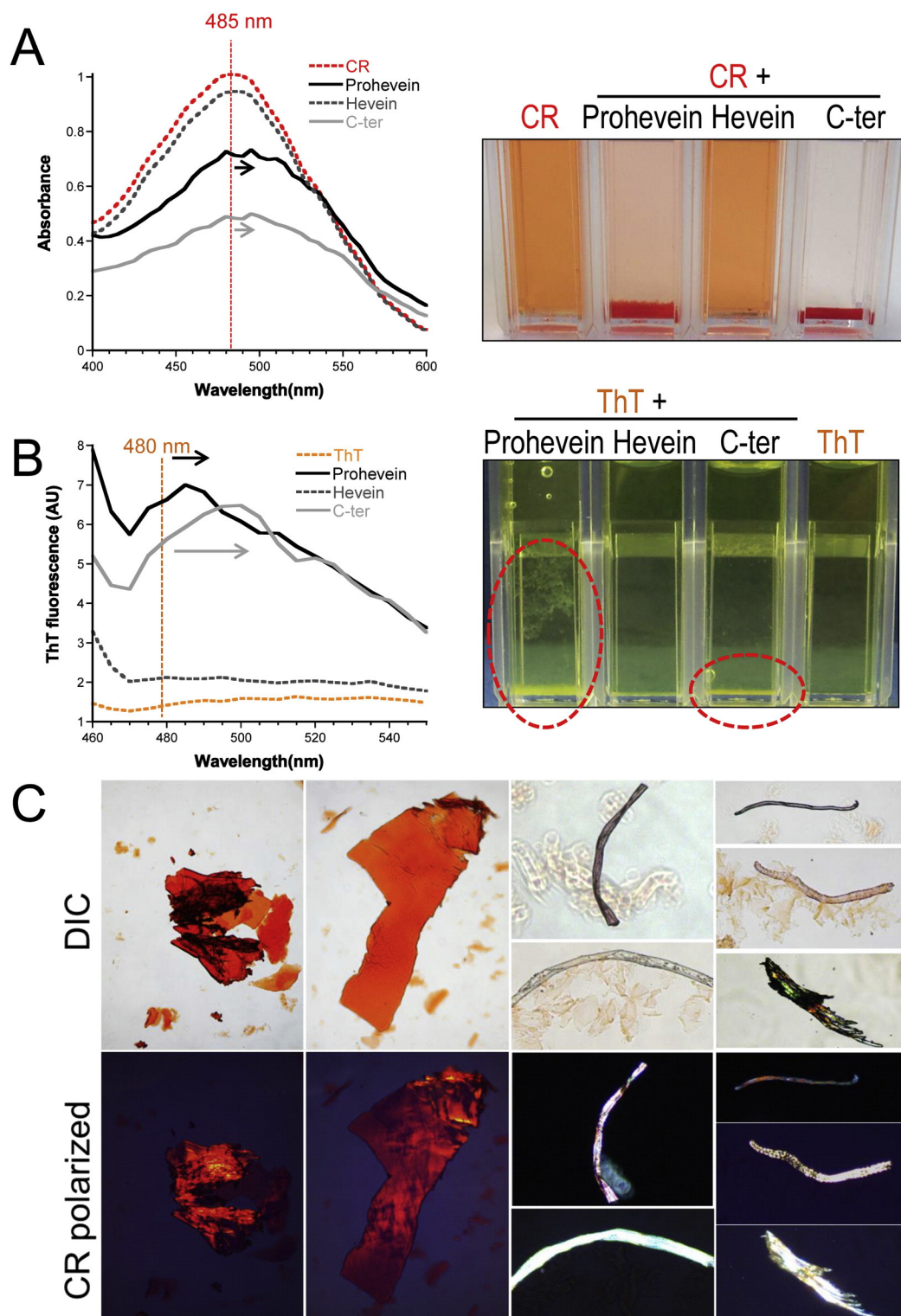


Fig. 5. Congo red and ThT binding assays of hevein proteins. A) Absorbance spectra in the presence of Congo red (CR) with images of respective samples after overnight CR staining. B) Fluorescence in the presence of thioflavine (ThT). C) Microscopic observation ($\times 40$) of Hev b 6.03 aggregates stained with CR. Top panels show DIC images of red aggregates under visible light and bottom panels present the same images in cross-polarized light, showing birefringence.

found in the soluble unbound fraction. In parallel, large amounts of the hevein peptide were first visualized on 16% SDS-PAGE as a large smear around 10 kDa (monomer/dimer) which is barely stained by Coomassie blue and easily destained (Supplementary Fig. S9). This anomalous staining/destaining behavior of hevein on SDS-PAGE was previously reported [14,59]. To further characterize hevein affinity towards chitin, 2–20% Tris–tricine gels were

used, peptide was resuspended in PBS supplemented with 5 mM DTT and 2 mM CaCl_2 and 2 mM MgCl_2 . The detection was then achieved by overloading the gel, hevein monomers were found to bind chitin apparently better than their dimers (Fig. 7A). Hevein synthetic peptide forms spontaneously stable dimers in non-reducing and reducing conditions as observed for the native proteins (Supplementary Fig. S8).

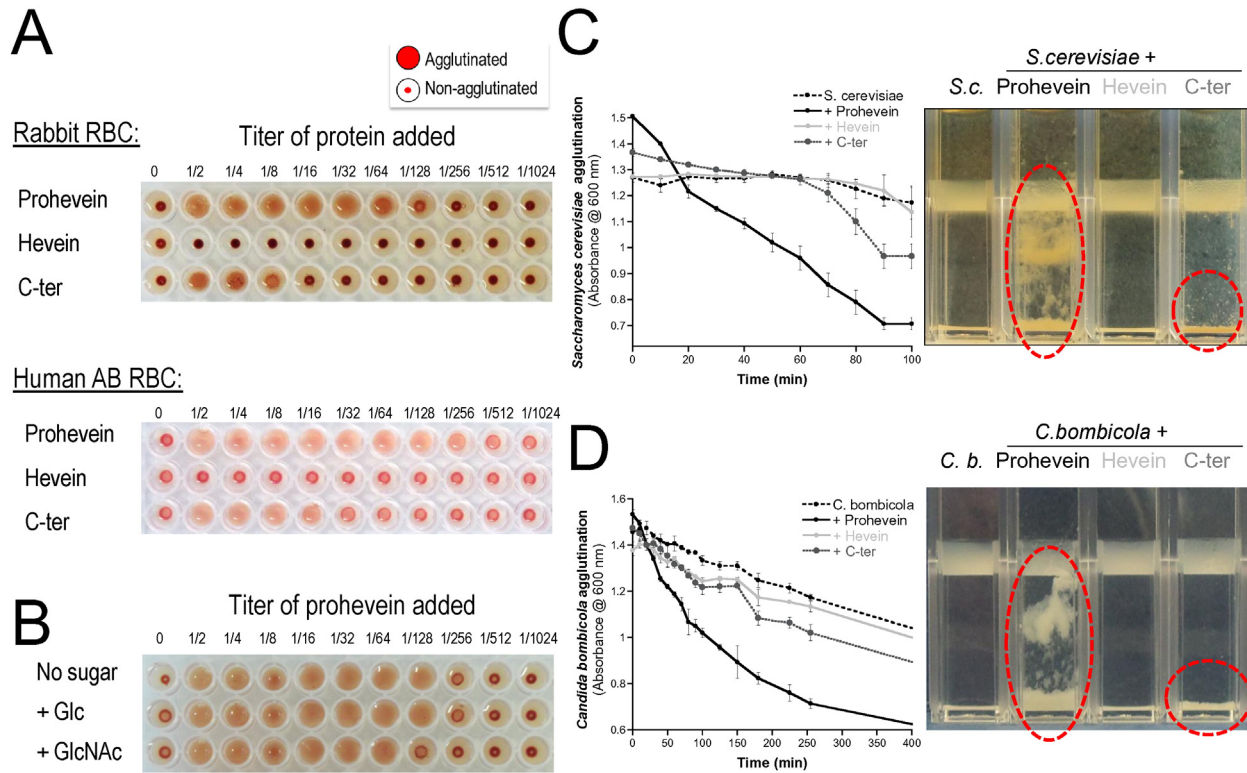


Fig. 6. Hemagglutination and yeast agglutination properties of hevein proteins. A) Hemagglutination of prohevein (183 μ M), hevein (299 μ M) and C-terminal domain (237 μ M) in the presence of rabbit or human AB group red blood cells (RBC). B) Inhibition assay with 0.1 M of glucose or N-acetyl glucosamine. C) *Saccharomyces cerevisiae* BY4742 agglutination. D) *Candida bombicola* ATCC® 22214™ agglutination. Yeast agglutinations with 20 μ M of proteins respectively, were followed by absorbance at 600 nm. Right panels present the final aggregation state after overnight incubation at room temperature.

3. Conclusion

With this work it was clearly shown that prohevein has agglutination properties, with the hevein specificities, but also that the C-terminal domain may confer additional amyloid and aggregative characteristics, at least *in vitro*. In addition, no interaction with lipids was observed, but prohevein possesses a C-terminal domain with amyloid properties that could indeed confer agglutination activity in synergy with its sugar-binding activity. Prohevein is present in the fresh latex C-serum where it can be purified [42], but also interacts with pathogens. In addition, prohevein and C-terminal 14 kDa protein are also found in the bottom fractions [25,60] and we now may suppose in aggregated forms. The mode of antimicrobial

activities, which usually target cell wall of pathogens, penetrate and modify it [61,62], does not seem applicable to hevein toxicity. As a lectin, prohevein should therefore be defined as an agglutinin, causing particles or microbes to coagulate to form a thickened mass that could localize to bottom fractions (with the predominant cleaved hevein) and be degraded by lysozyme enzymes.

In mammals, many amyloids are toxic and linked to diseases [63], however some of them may also be beneficial to their host and fulfill a function. Every year many new functional amyloids are discovered [64], but surprisingly very few amyloid-like proteins were identified in plants [50,65–68]. It is interesting to note that another amyloid-like protein was found in latex, the rubber elongation factor (HbREF; Hev b 1), which is another latex allergen involved in rubber synthesis [50].

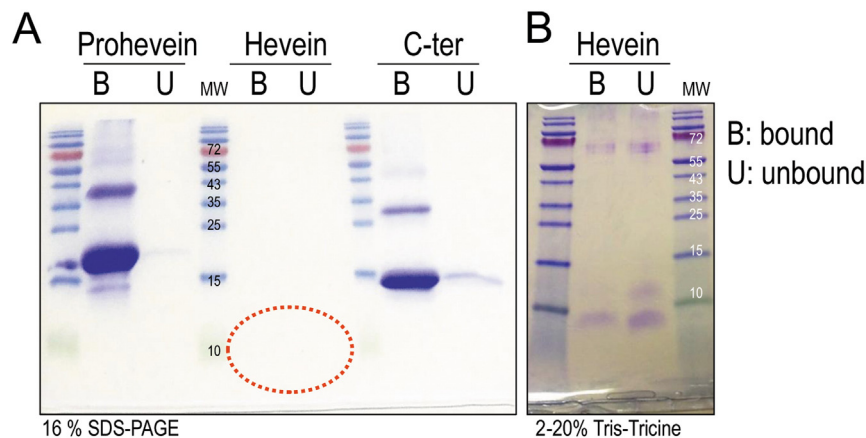


Fig. 7. Chitin-binding properties of prohevein proteins. Pure proteins were incubated with a chitin resin for 90 min and bound (B) and unbound (U) fractions were visualized on 16% SDS-PAGE (A). Hevein was visible as a smear around 10 kDa that almost disappear after destaining. B) More heveins were incubated identically but the fractions were loaded on 2–20% Tris-Tricine SDS-PAGE. Gel was overloaded in order to be able to visualize both hevein monomers and dimers.

Making a prediction of amyloidogenic regions in the hevein precursor superfamily (initially presented in Fig. 1 and Supplementary Fig. S1), using three independent predictive algorithms (FoldAmyloid, AmylPred and Waltz), it can be noted that the N-terminal part of the hevein precursor corresponds to a highly aggregative zone (Fig. 8). These sequences (signal sequences) before the hevein part are however not very conserved (probably corresponding to a species specificity), they are also rapidly cleaved in hevea. The hevein sequence in red only shows a potential hotspot which is certainly not enough to confer aggregative ability and is inside the carbohydrate-binding domain [32]. Interestingly the C-ter homologies show at least 6 amyloid-prone regions, with highly conserved motives.

In conclusion, *in silico* analysis predicts that all tested members of the precursor family may auto-assemble into amyloid-like fibrils via their C-terminal domain. Therefore the presence and role of amyloid proteins in the plant kingdom could be more important than previously thought, and should be watched now with care. Last year, GAPR-1 a mammalian protein rich in cysteines (Golgi-associated plant pathogenesis related protein-1) homologous to other pathogenesis related proteins including PR-1 from *A. thaliana*, was also shown to display amyloid prone hot-spots in their conserved C-terminal parts [69]. GAPR-1 was able to form *in vitro* amyloid fibrils in the presence of lipids [69].

4. Experimental procedures

4.1. Chemicals

Asolectin (AL), Lipopolysaccharides (LPS), Congo red (CR) and Thioflavin T (ThT) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Plant monogalactosyldiacylglycerol (MGDG), 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (DMPG) and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) were provided by

Avanti Polar Lipids (Alabaster, AL, USA). Hevein synthetic peptide (sequence EQCGRQAGGKLCPPNNLCCSQWGWCGSTDEYCSPDHNCQSNCKD, 43 amino acids) was synthesized (in TFA or HCl) by Genosphere Biotechnologies, Paris.

4.2. Phylogenetic analysis

A BlastP was realized on *H. brasiliensis* hevein precursor (GenBank accession n° P02877) at PubMed. Accession numbers of each 20 sequences found are provided in the figure caption. Sequences were aligned with ClustalW (<http://www.genome.jp/tools/clustalw/> program). Evolutionary analyses were conducted in MEGA6 [70], using the Neighbor-Joining method, the bootstrap test (1000 replicates) and the p-distance method. All positions containing gaps and missing data were eliminated.

4.3. Cloning of hevein genes

The *H. brasiliensis* hevein precursor gene (HEV1 GenBank accession n° M36986) was synthesized by GenScript (Piscataway, USA) after codon optimization. Prohevein, hevein and C-ter were respectively amplified from the precursor using following primers: Hevein1(+) GGATCCATGGAACAATCGGTCGCCAAGCGGGT GG; Hevein187(−) CTCGAGTCAGTTGATACAGGAGC; Hevein43(−) GGATCCTCAATCTTTACAATTACTTTGGCAGTTAGTGTCCGGCG and Hevein44(+) GGATCCATGTCCGGTGAAGCGTGGCGGTGGCAG TGC. The PCR products were subcloned into pCR2.1 from TOPO® TA cloning kit (Invitrogen, ThermoScientifique, Illkirch, France). Then the 4 genes were cloned, respectively, in the *Bam*HI-*Xho*I site of pET24a-6His-HbSRPP from a previous work [50] to construct pET24a-6His-HevPrec, pET24a-6His-Hevb6.01 (prohevein, 1–187), pET24a-6His-Hevb6.02 (Hevein, 1–43) and pET24a-6His-Hevb6.03 (Cter domain, 44–187).

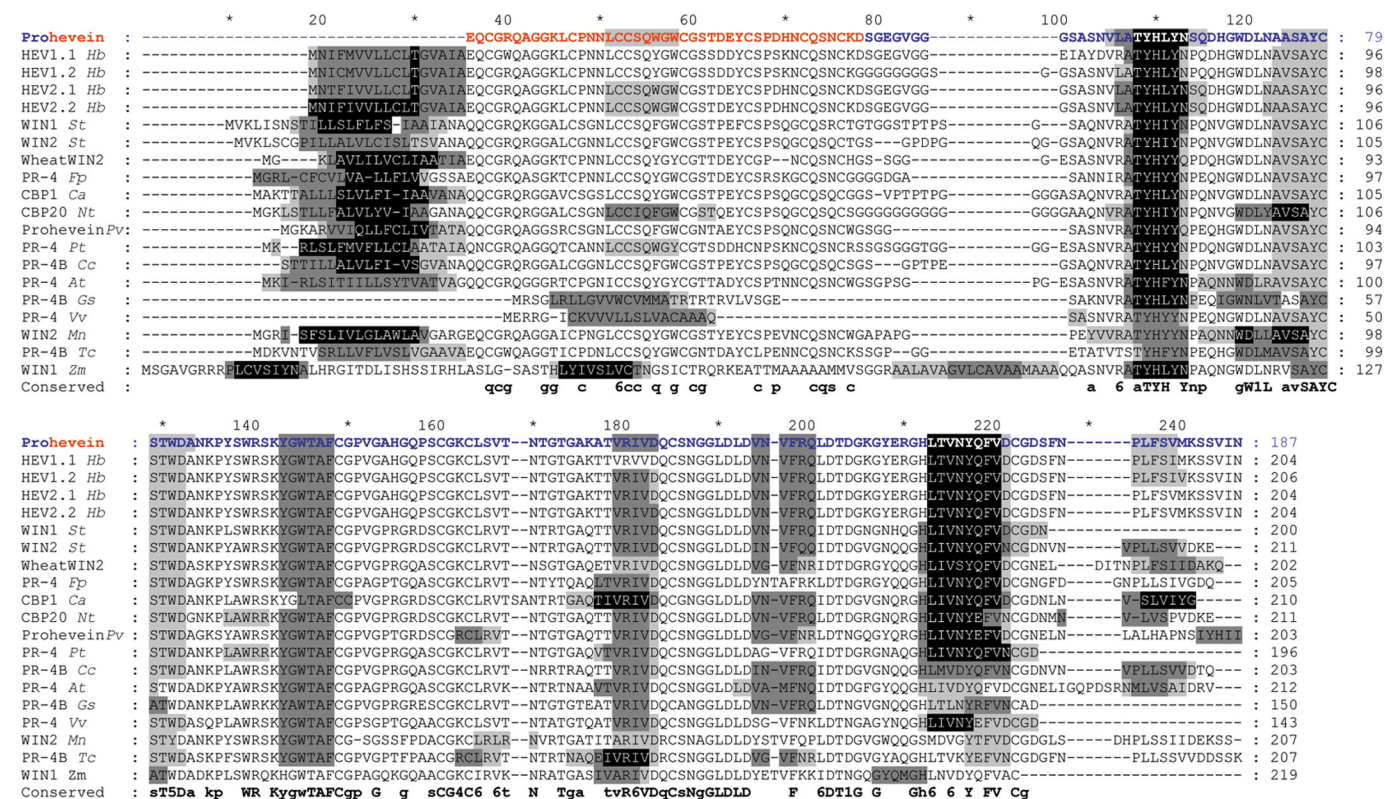


Fig. 8. Prediction of amyloidogenic regions in the hevein precursor superfamily. Sequence alignment was performed as in Fig. 1 and Supplementary Fig. S1. The amyloidogenic regions were predicted from three different amyloid prediction programs (FoldAmyloid, AmylPred and Waltz) and highlighted from gray to black (1, 2, 3 convergences).

Each plasmid was introduced and expressed in *E. coli* BL21(DE3) pLysS Gold cells.

4.4. Expression and purification of recombinant tagged proteins

Bacteria were grown to 0.7 OD in 2xYT medium (16 g/L tryptone, 10 g/L yeast extract, and 5.0 g/L NaCl), and expression was induced by addition of 1 mM isopropyl- β -thiogalactoside (IPTG, Euromedex, Souffelweyersheim, France). After 4 h induction, cells were harvested by centrifugation and frozen at -20°C . Overexpression showed that the proteins were in the inclusion bodies. Cells were sonicated 4×1 min in buffer A (150 mM NaCl and 100 mM Tris/HCl, pH 8.0, 1 mM PMSF). The lysate was centrifuged for 30 min at 20,000 g and the pellet was recovered in buffer A added of 8 M urea. The lysate after centrifugation was incubated with 3 mL Ni-NTA resin (Invitrogen) for 3 h at room temperature. The resin was then washed twice with 35 mL of buffer A/urea, by centrifuging 10 min at 900 g. The proteins were eluted from the resin in the same buffer containing 250 mM imidazole (Euromedex). Protein samples were pooled and dialyzed against Tris/HCl 25 mM pH 8.0 with 5 mM DTT and kept aliquoted at -80°C . This yields ~ 2 – 4 mg of peptide per liter of culture. The peptides were pure as judged by analysis on 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by Coomassie Blue staining. Protein concentrations were determined by quantitative amino-acid analysis. Amino-acid sequences and physico-chemical parameters of the tagged proteins are presented in supplementary Table S1. Data were obtained from the ExPASy proteomics Server, <http://expasy.org/tools/protparam.html>.

4.5. Modeling and amyloidogenic/aggregation region prediction

For molecular modeling, structures were produced using PyMOL 1.3 (PyMOL molecular Graphics system, Schrödinger, LLC) with referenced protein database bank (PDB). Protein secondary structural prediction model of prohevein and PDB model was realized at <http://raptorx.uchicago.edu> using the RaptorX server [48,49].

For amyloid predictions, amino acid sequences of proteins retrieved from the phylogenetic analysis were given as input to the web server-based programs. Prediction of amyloidogenic or aggregation regions in proteins was performed with four different algorithms: FoldAmyloid (<http://bioinfo.protes.ru/fold-amyloid/>) [44], Aggrescan (<http://bioinf.uab.es/aggrescan/>) [47], Waltz (<http://waltz.switchlab.org>) [46] and AmyLPred (<http://biophysics.biol.uoa.gr/AMYPRED>) [45]. The default prediction parameters were used for all programs.

4.6. ThT, Congo red binding assays and birefringence

Proteins were prepared in PBS 1X at 20 μM . The dyes were allowed to bind to the peptides overnight at room temperature. Spectra were then recorded with a SpectraMAX® M2e spectrometer (Molecular Devices, Sunnyvale, CA, USA). A 20 μM solution of Congo red (CR; C.I. 22120; Sigma C6767) in PBS was prepared (extinction coefficient at 498 nm $\sim 3.7 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) and filtered through a polyether sulfone filter with 0.22 μm pores. Thioflavin T (ThT; Sigma T3516) was used at a final concentration of 10 μM in PBS with an λ_{ex} of 450 nm and λ_{em} at 480 nm. For microscopic observations, CR stained aggregates were centrifuged at 16,000 g for 1 min and spread on microscope slides. Staining and birefringence were observed under a Zeiss Axiafold microscope with a $\times 40$ objective (Carl Zeiss, Oberkochen, Germany) equipped with optimally aligned cross-polarizers.

4.7. ATR-FTIR spectroscopy

ATR-FTIR spectra were recorded on a Nicolet 6700 FT-IR spectrometer (Nicolet Instrument, Madison, WI) equipped with a liquid nitrogen cooled mercury–cadmium–telluride detector (ThermoFisher Scientific, San Jose,

CA, USA), with a spectral resolution of 4 cm^{-1} and a one-level zero filling. To determine the secondary structure element of each protein, ATR-FTIR spectra were analyzed with an algorithm based on a second-derivative function and a self-deconvolution procedure (GRAMS and OMNIC softwares, ThermoFisher Scientific) to determine the number and wavenumber of individual bands within the spectral range 1485 – 1750 cm^{-1} , as described in our previous work [50].

4.8. Wide-angle X-ray scattering (WAXS)

Prohevein and C-ter aggregates were obtained by incubation in PBS 1X at 20 μM and 37°C during 3 days. Aggregates were centrifuged and lyophilized with a CHRIST freeze-drier (Osterodeam Harz, Germany). The powder was introduced into 1 mm diameter glass capillaries (Glaskapillaren GLAS, Glas-Technik & Konstruktion, Schönwalde-Glien, Germany). X-ray data were collected at the Centre de Recherche Paul Pascal (CRPP, Pessac, France) on a Rigaku Nanoviewer (XRF microsource generator, MicroMax 007HF), with a 1200-W rotating anode coupled to a confocal Max-FluxH Osmic mirror (Applied Rigaku Technologies, Austin, USA) and a MAR345 image plate detector (MARResearch, Norderstedt, Germany). Spectra were integrated with the FIT2D software (ERSF; <http://www.esrf.eu>). A wide-angle X-ray scattering with transmission geometry setting was chosen, with a sample-detector distance of 152 mm and a 2700 s accumulation, providing access to periodicities in the 1.8 – $38.5 \text{ \AA}$ range. Data were processed by subtracting the scattering pattern due to the air.

4.9. Transmission electron microscopy (TEM)

Proteins were absorbed 10 min onto Formvar-coated, carbon-stabilized copper grids (200 mesh) and washed 3 times with water. Grids were then negatively stained 1 min with 10 μL of freshly prepared 2% uranyl acetate in water, dried with filter paper, and examined with a Hitachi H7650 transmission electron microscope (Hitachi, Krefeld, Germany) at an accelerating voltage of 80 kV. TEM was performed at the Pôle Imagerie Electronique of the Bordeaux Imaging Center.

4.10. Ellipsometry experiments on lipid or glycolipid monolayers at air–water interface

Ellipsometry measurements were performed using a NTElli2000 ellipsometer (Gottingen, Germany) equipped with a doubled frequency Nd:YAG laser at 532 nm with 50 mW power (for more details on ellipsometry see [51,71]). The spatial resolution was about 1 μm and the image size was $450 \times 670 \text{ mm}$, with a $10\times$ magnification lens. Thickness was determined using a mean value of 1.45 for the refractive index.

4.11. Hemagglutinating and yeast agglutinating assays

The red blood cells (RBC) assay was performed in 96-well U-shape microtiter plates (Greiner) in 100 μL . Protein were diluted in 1X PBS and 5–8 μL of rabbit (0.5% final; Fitzgerald Industries International, Acton, MA, USA) or human (group AB; Sigma) red blood cells were added at room temperature. Results were observed after 1 h incubation and are presented as serial dilutions of each pure protein. For inhibition assay, sugars were added at 0.1 M. The agglutination of red blood cells prevents them from settling out of suspension. Negative control is presented as PBS and red cells without proteins.

The yeast agglutinating assay was performed as described previously [57] with the baker's yeast *S. cerevisiae* strain BY4742 (*MAT α his3 Δ 1 leu2 Δ 0 lys2 Δ 0 ura3 Δ 0 can1–100*) and the yeast *C. bombicola* strain ATCC® 22214™ (LGC standards, Molsheim, France). Proteins at 20 μM in PBS 1X were incubated at room temperature with yeast cells at OD about 1.2–1.5 and absorbance was then recorded at 600 nm.

4.12. Chitin-binding assay

After washing in 1X PBS, the chitin resin (100 μ L, New England Biolabs, Evry, France) was incubated with the proteins (100 μ L) for 90 min at room temperature on a rotating wheel. After 5 min sedimentation, unbound supernatant was kept and resin washed twice with 200 μ L of PBS by gravity flow. Bound and unbound fractions were analyzed by 16% SDS-PAGE or 10–20% Tris–Tricine SDS-PAGE (BIO-RAD, Marnes-la-Coquette, France) after denaturation 15 min at 95 °C. Tricine gels were run with the Schagger and von Jagow anode and cathode buffers [72].

Author contributions

KB designed the study, performed the experiments and wrote the paper. SL performed FTIR and ellipsometry. BCS performed TEM. AB carried out the WAXS experiments. KB, SL, BCS and FP analyzed the data and critically read the paper. SL and FP funded and supervised the study. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

The authors declare no conflict of interest.

Transparency Document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbapap.2016.01.006>.

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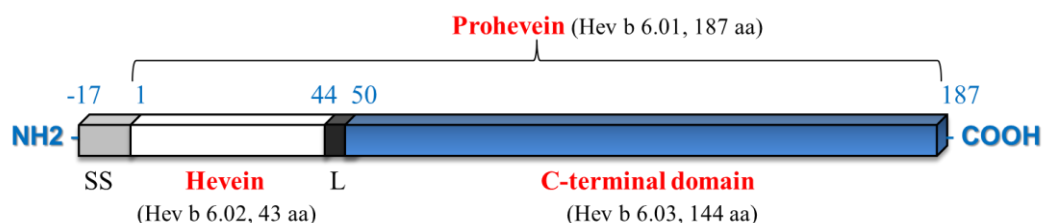
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SUPPORTING MATERIAL

***Hevea brasiliensis* prohevein possesses a conserved
C-terminal domain with amyloid-like properties *in vitro*.**

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and Frédéric Peruch

Supplementary Table S1: Sequences and physico-chemical parameters of N-terminal His-tagged recombinant proteins.



Precursor HEV1

MHHHHHHGSMNIFIVVLLCLTGVAIAEQCGRQAGGKLCPPNNLCCSQWGWCGSTDEYCSPDHNCQSNCK
DSGEGVGGGSASNVLATYHLYNSQDHGWDLNAASAYCSTWDANKPYSWRSKYGTAF CGPVG AHGQSS
CGKCLSVTNTGTGAKTTVRIVDQCSNGGLDLVDNVFRQLD TDGKGYERGHITVNYQFVDCGDSFNPLF
SVMKSSVIN

Prohevein

MHHHHHHGSMEQCGRQAGGKLCPPNNLCCSQWGWCGSTDEYCSPDHNCQSNCKDSGEGVGGGSASNVL
ATYHLYNSQDHGWDLNAASAYCSTWDANKPYSWRSKYGTAF CGPVG AHGQSSCGKCLSVTNTGTGAKT
TVRIVDQCSNGGLDLVDNVFRQLD TDGKGYERGHITVNYQFVDCGDSFNPLFSVMKSSVIN

Hevein

MHHHHHHGSMEQCGRQAGGKLCPPNNLCCSQWGWCGSTDEYCSPDHNCQSNCKD

C-ter domain

MHHHHHHGSMDSGEGVGGGSASNVLATYHLYNSQDHGWDLNAASAYCSTWDANKPYSWRSKYGTAF CG
PVG AHGQSSCGKCLSVTNTGTGAKTTVRIVDQCSNGGLDLVDNVFRQLD TDGKGYERGHITVNYQFVD
CGDSFNPLFSVMKSSVIN

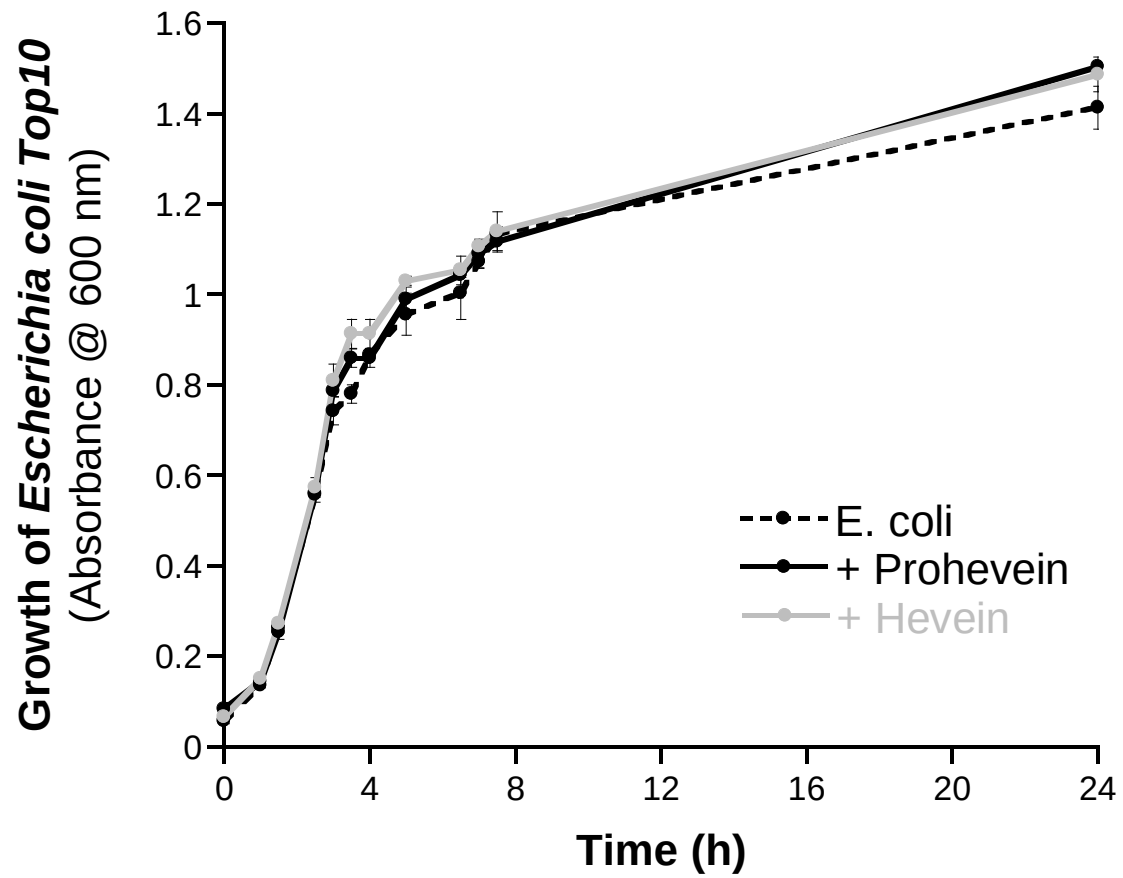
Proteins	Amino acids	MW (kDa)	pI	Ext. Coefficient (L.mol ⁻¹ .cm ⁻¹)	Net charge	Hydropathicity
Precursor	213	22.9	6.22	45795	-4	-0.332
Prohevein	197	21.3	6.22	44920	-4	-0.0572
Hevein	53	5.9	6.33	12490	-2	-1.14
C-ter domain	154	16.6	6.52	32430	-2	-0.447

Data at pH 7.4, molecular weight, extinction coefficient and grand average of hydropathicity (GRAVY) were estimated from the amino-acid sequence of each protein fused to the N- terminal His tag (sequence MHHHHHHGS). Data were obtained from the ExPASy proteomics Server, <http://expasy.org/tools/protparam.html>.

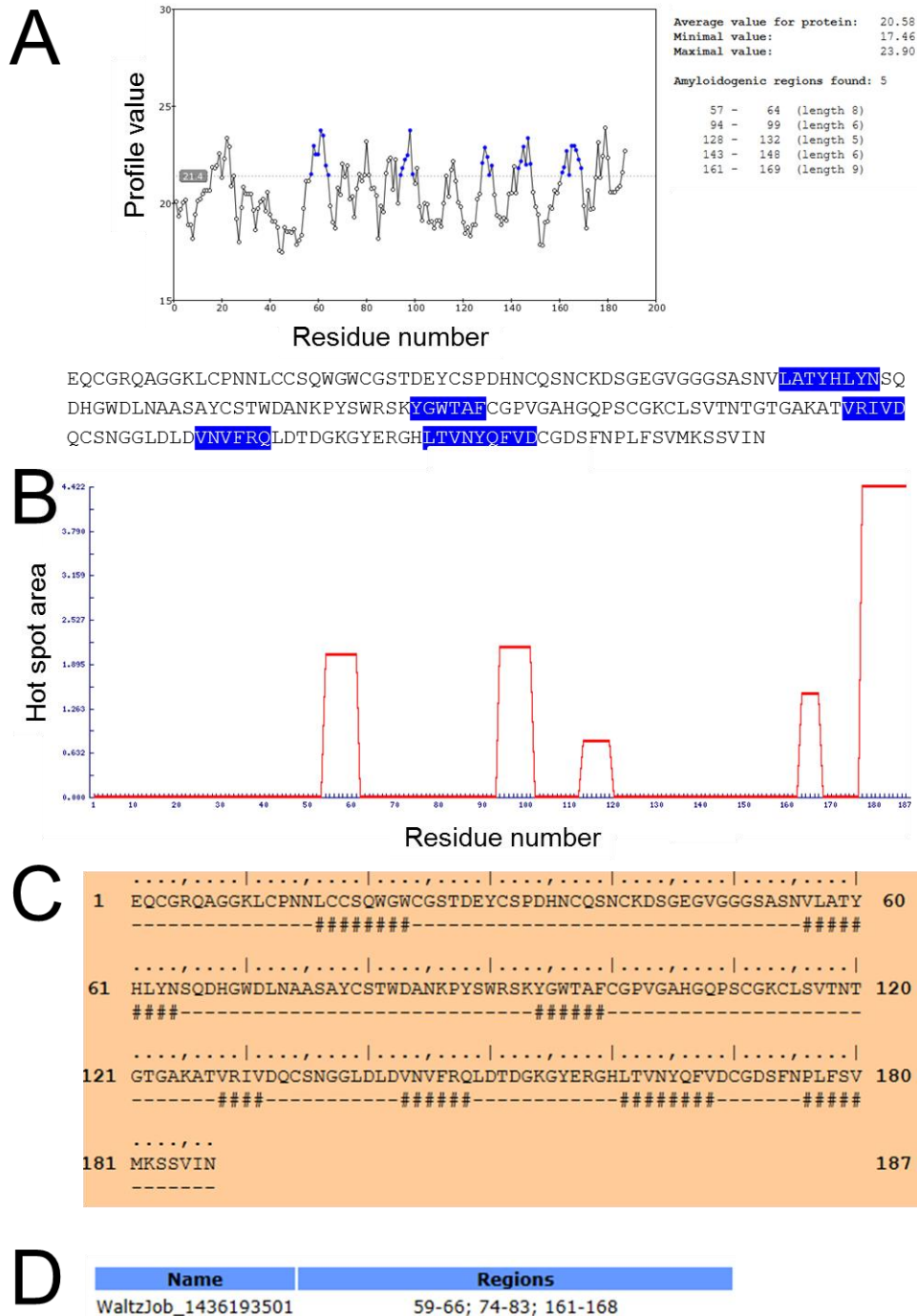
Hevein

[illegible]

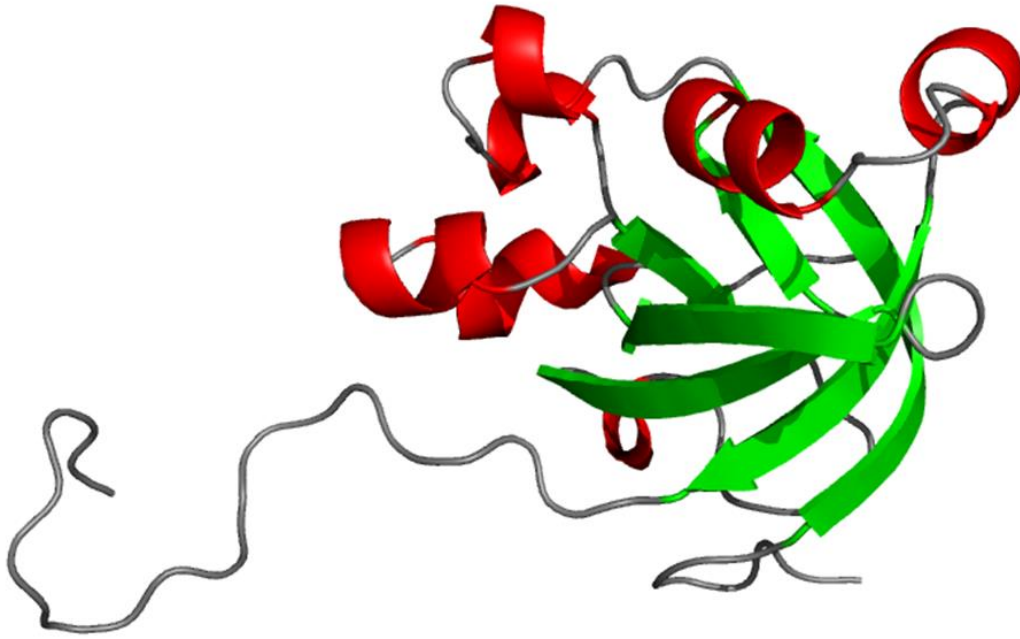
Supplementary Fig. S1. Sequence homology of prohevein-like proteins. Alignment was realized with ClustalW and shading by using GeneDoc 2.7.0 (<http://www.nrbsc.org/>). Corresponding phylogenetic tree is in [Figure 1B](#).



Supplementary Fig. S2. Growth curves of *Escherichia coli* Top10 in presence of prohevein and hevein. Precultured bacteria were diluted at OD=0.06 in LB medium and proteins were added at 3 μ M before incubating at 37°C. Experiments were performed in triplicate (Absorbance at 600 nm $\pm$ SD).



Supplementary Fig. S3. Predictions of amyloidogenic/aggregation regions in prohevein (GeneBank: CAA05978). Amyloid propensity analyses and aggregation analysis were realized with FoldAmyloid (A), Aggrescan (B), AmylPred (C) and Waltz (D).



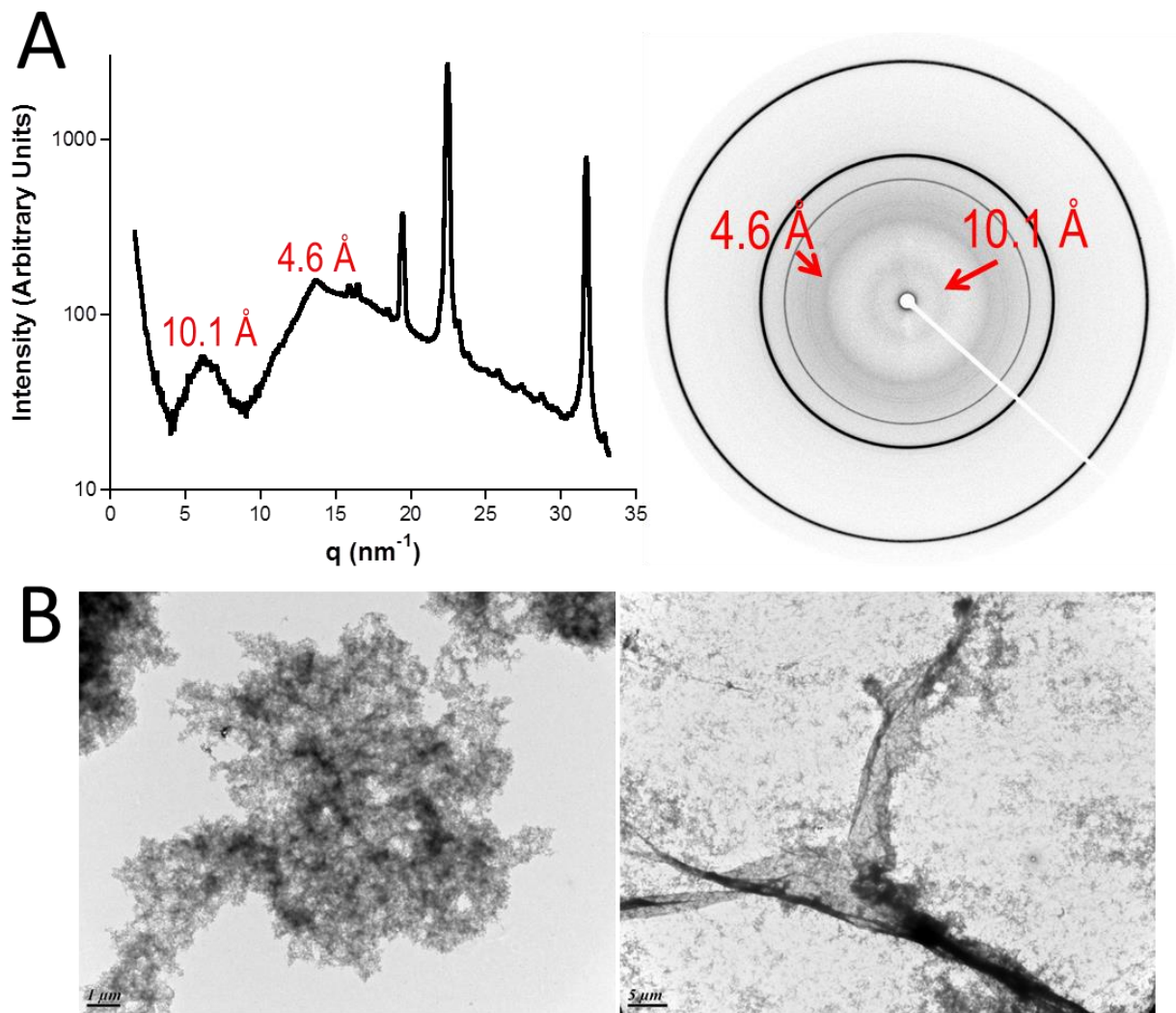
>Modeled Cter44-187

SGEGVGGG SASNVLATYHLYN SQDH GWD LNAA SAYCSTWDANKP YSWRSKYGW
TAF CGPVG AHG QPS CGKCLSVTNTGT GAKATVRIVDQCSNGG LDL DVNVFRQL
DTDG KGYER GH LTVNYQF VDCGDSFNPLFSVMKSSVIN

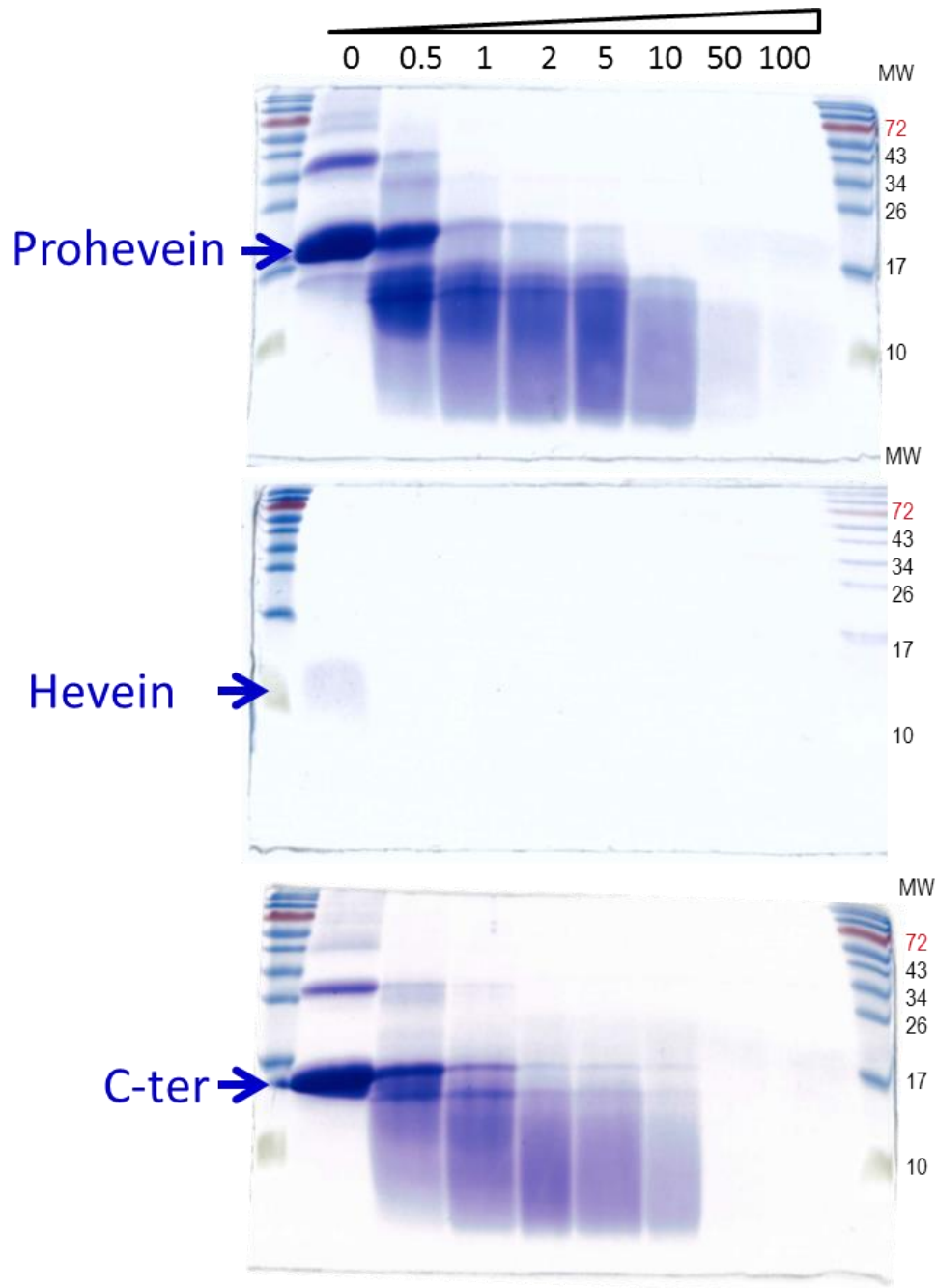
>Modeled Cter36-187

NCQSNCKDSGEGVGG GSASNVLATYHLYN SQDH GWD LNAA SAYCSTWDANKPY
SWRSKYGW TAF CGPVG AHG QPS CGKCLSVTNTGT GAKATVRIVDQCSNGG LDL
DVNVFRQL DTDG KGYER GH LTVNYQF VDCGDSFNPLFSVMKSSVIN

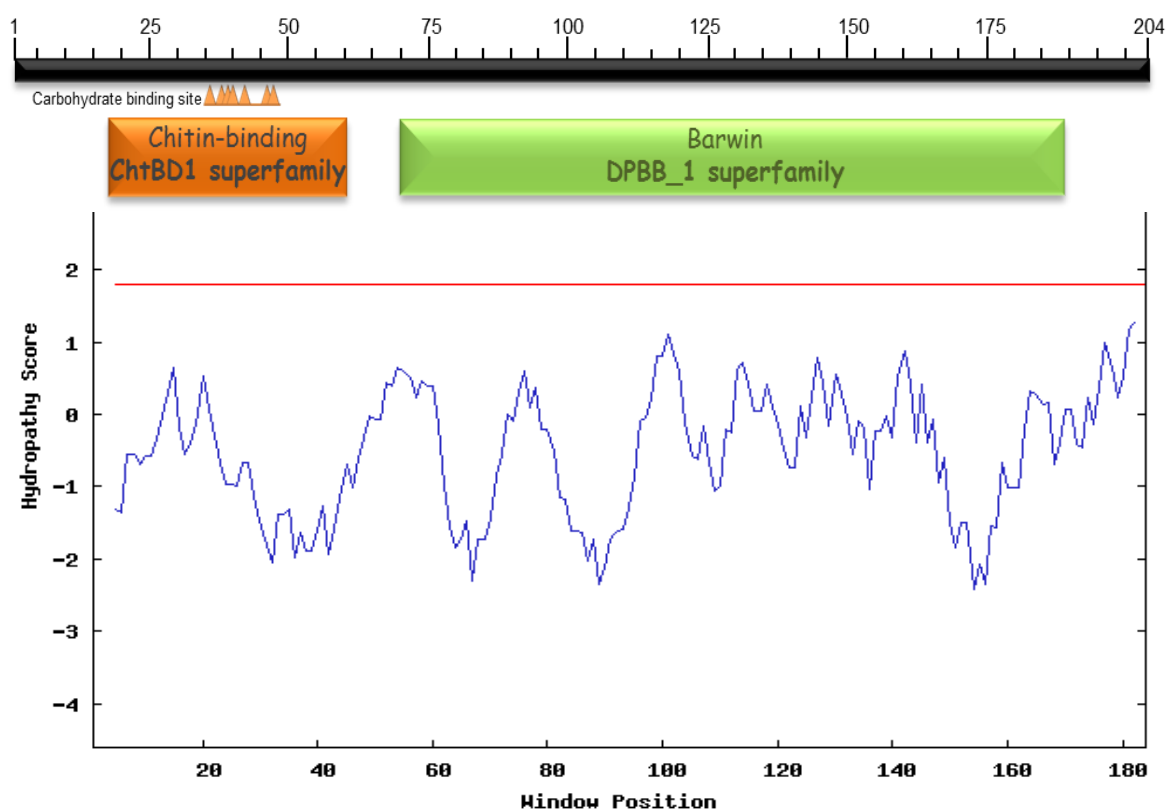
Supplementary Fig. S4. Protein secondary structural prediction model of C-terminal domain of prohevein (44-187). The PDB model was realized using the RaptorX server and visualized with PyMOL.



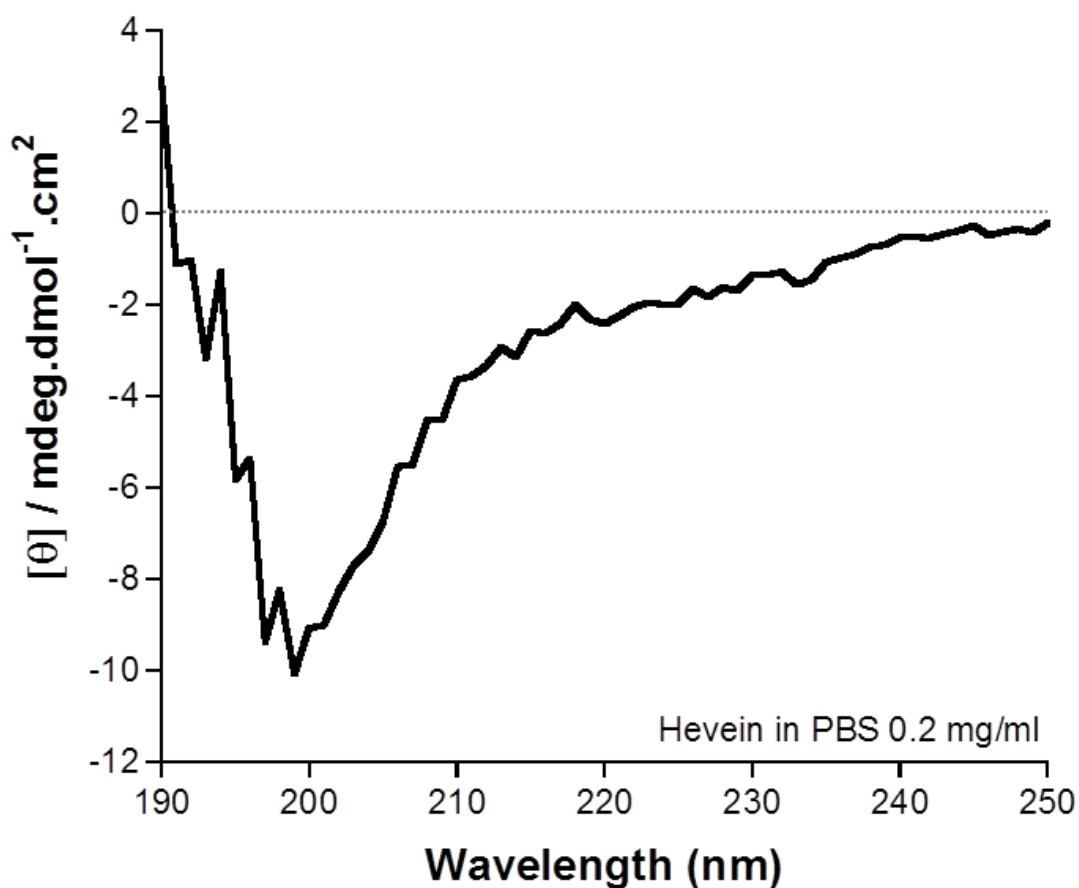
Supplementary Fig. S5. Prohevein presents different aggregation properties, in which amyloid-like structures may contribute. A) WAXS analysis of aggregated prohevein, showing the presence of structures with an amyloid core. B) TEM observation of aggregates formed by prohevein. Tissue-like aggregates were observed (right panel) but were not representative of the samples. No single fiber was observed.



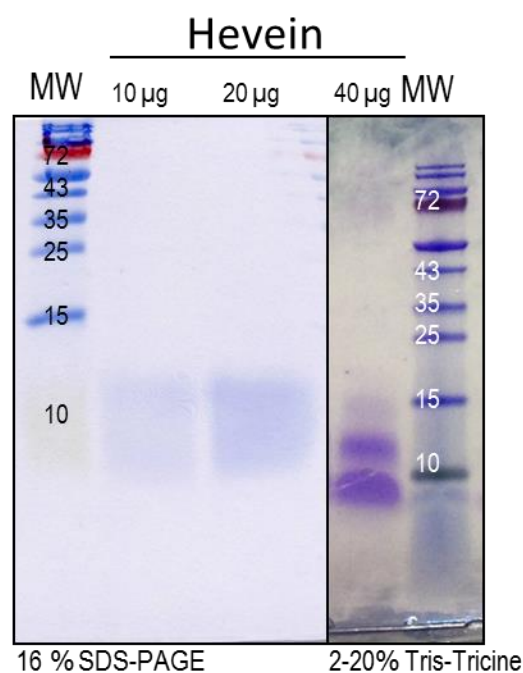
Supplementary Fig. S6. Differential proteinase K resistance of prohevein, hevein and C-ter domain. Proteins were prepared at 100 μ M in 1x PBS. Ten μ L of protein solution were mixed with 10 μ L of an appropriate dilution of proteinase K (Euromedex), and incubated at 37°C for 30 min. Digestion was stopped by adding 6 μ L of electrophoresis sample buffer pH 6.8, containing 4% SDS, 2% mercaptoethanol (v/v), 12% glycerol (w/v), 0.01% Serva Blue G, and phenylmethylsulfonyl fluoride (1 mM). The mixture was then immediately heated at 100°C for 5 min. Samples were analysed by 16% SDS-PAGE and stained with Coomassie blue.



Supplementary Fig. S7. Kyte-Doolittle hydropathy plot of prohevein. Sequence (GenBank N^oCAA05978) was analyzed at <http://gcat.davidson.edu/rakarnik/kyte-doolittle.htm> with a window = 9.



Supplementary Fig. S8. Circular dichroism spectroscopy of hevein. Far-UV CD spectra were collected at 20°C, using a Jasco JS 810 spectropolarimeter (Jasco, Tokyo, Japan) equipped with a JASCO PTC-423 S/15 temperature controller. Measurements were done with a quartz cell with a 0.1 cm path length (Hellma-France). Protein concentration was 0.2 mg/ml in PBS 1x buffer.



Supplementary Fig. S9. Anomalous detection of hevein on SDS-PAGE. Hevein was easily destained after Coomassie blue staining. Very large amounts of proteins were needed to detect hevein monomers and dimers. A better resolution was achieved using 2-20 % Tris-tricine SDS-PAGE. Samples presented were reduced with 5 mM DTT, similar results were observed with unreduced hevein.