

Homologous *Hevea brasiliensis* REF (Hevb1) and SRPP (Hevb3) present different auto-assembling

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ABSTRACT

HbREF and HbSRPP are two *Hevea brasiliensis* proteins present on rubber particles, and probably involved in the coagulation of latex. Their function is unclear, but we previously discovered that REF had amyloid properties, which could be of particular interest during the coagulation process. First, we confirmed that REF and SRPP, homologous and principal proteins in hevea latex, are not glycoproteins. In this work, we investigated various aspects of protein interactions: aggregation, auto-assembling, yeast and erythrocyte agglutination, co-interactions by various biochemical (PAGE, spectroscopy, microscopy), biophysical (DLS, ellipsometry) and structural (TEM, ATR-FTIR, PM-IRRAS) approaches. We demonstrated that both proteins are auto-assembling into different aggregative states: REF polymerizes as an amyloid rich in β -sheets and forms quickly large aggregates ($> \mu\text{m}$), whereas SRPP auto-assembles in solution into stable nanomultimers of a more globular nature. Both proteins are however able to interact together, and SRPP may inhibit the amyloidogenesis of REF. REF is also able to interact with the membranes of yeasts and erythrocytes, leading to their agglutination. In addition, we also showed that both REF and SRPP did not have antimicrobial activity, whereas their activity on membranes has been clearly evidenced. We may suspect that these aggregative properties, even though they are clearly different, may occur during coagulation, when the membrane is destabilized. The interaction of proteins with membranes could help in the colloidal stability of latex, whereas the protein–protein interactions would contribute to the coagulation process, by bringing rubber particles together or eventually disrupting the particle monomembranes.

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1. Introduction

Laticifers are plant cells producing latex and more than 12,500 plant species have been reported to make lattices [1,2]. But *Hevea brasiliensis* (Willd. Ex A. Juss) Müll. Arg (para rubber tree) is the main worldwide source of latex to produce commercial natural rubber (NR). Its production reaches today 9 million tons per year [3]. This tropical plant belongs to the *Euphorbiaceae* family, and is cultivated worldwide and especially in Southeast Asia. NR is a *cis*-1,4-polyisoprene biopolymer, which can also be produced from alternative sources as guayule (*Parthenium argentatum*) and dandelion (*Taraxacum* sp.), but hevea is still prevalent

and of high economic importance because of its rubber qualities (for review see [4]). Latex is produced after tapping and is a complex white cytoplasmic system. It contains mainly rubber and non-rubber particles, organelles, proteins, lipids and cytoplasmic C-serum [5,6]. Lately, 186 rubber particle proteins have been identified [7], and latex contains far more proteins, from which at least 14 have been assigned as allergens (Hevb1–14; [8]). Hevb3 and Hevb1 are also named small rubber particle protein (SRPP) and the rubber elongation factor (REF) [9–11], as they are associated with rubber particles. They are apparently not localized in the same laticifer layers, SRPP being restricted to the conducting phloem whereas REF could be found in all laticifer layers [12]. At first, REF was detected on large rubber particles (LRP, generally above 0.4 μm in diameter) and SRPP was detected on small rubber particles (SRP, smaller than 0.4 μm in diameter), but it is now established that REF can be found on both types of particles [9,11,13–15]. Interestingly, LRPs enzymatic activity is much lower than the one found in SRPs [14,16].

REF and SRPP are homologous proteins from plants, which belong to a stress-related protein family [2]. Six SRPP homologs have been found in latex of other producing plants: the guayule homolog of SRPP (GHS; [17]) and TbSRPP1–5 in *Taraxacum brevicorniculatum* (dandelion;

Abbreviations: SRPP, small rubber article protein; REF, rubber elongation factor; SRP, stress-related protein; GHS, guayule homologue of SRPP; Hb, *Hevea brasiliensis* (hevea); Pa, *Parthenium argentatum* (guayule); Tb, *Taraxacum brevicorniculatum* (dandelion); PM-IRRAS, polarization-modulation-infrared reflection-adsorption spectroscopy; ATR-FTIR, attenuated-total reflectance Fourier-transform infra-red; DLS, dynamic light scattering; TEM, transmission electronic microscopy.

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[18]). GHS and TbSRPP3–5 are also associated with rubber particles [19–21]. Interestingly, HbREF, HbSRPP, GHS and TbSRPP3 were also described as having a positive effect on rubber elongation and content [9,10,17,19,21–23]. As REF or SRPP are not present in all latex producing plants (e.g. *Ficus* sp. [15]), they are not essential for rubber synthesis. But they could play a role in increasing yield, molecular weight and quality of natural rubber, particularly in hevea, where they are the two main proteins. *HbREF* and *HbSRPP* genes have been cloned [10,24–26] and various isoforms were identified [27,28]. In hevea, their mRNA expression is mainly found in latex and laticifer cells [27,29–31], and may be stimulated by tapping [31,32]. HbREF and HbSRPP proteins are water-insoluble acidic proteins respectively of 14.6 and 24 kDa [11].

We recently provided structures of REF and SRPP as recombinant proteins in bulk solution or in presence of model membranes. In solution, REF demonstrated a capacity to form amyloid fibers and aggregates rich in β -sheets, whereas SRPP displayed the characteristics of a globular protein [2]. Additionally, we also showed that REF and SRPP interacted differentially with different models of membranes, using monolayers or bilayers to mimic the rubber particle biomembrane [2,33]. In order to evaluate the possible functions of REF and SRPP in latex, we purified in this study both recombinant proteins and analyzed various biochemical, biophysical and structural properties. A short 14 kDa variant of SRPP missing the C-terminal part was also generated to resemble REF, but still behaved like SRPP. We went further by studying their protein–protein interactions, showing that as an amyloid, REF is highly aggregative whereas SRPP auto-assembles to form nanomultimers. In addition, both proteins may also interact together. None of them displayed an antimicrobial activity, whereas they are also able to interact with membranes of unicellular models (yeast, erythrocytes) or model membranes [33].

2. Results

Latex-producing plants of interest for natural rubber production (hevea, guayule and dandelion) have all been found to produce proteins from the stress-related REF/SRPP family (Fig. 1). These proteins are homologous, found in latex and have a size ranging from 14 to 27 kDa. Apart TbSRPP2 (pI 8.5, charge +2), they are acidic proteins and negatively charged. With the exception of TbSRPP1 and 2, they are major rubber particle proteins associated with the particles [17,19]. REF is so far only found in hevea. In this study, we will therefore focus on REF

(GenBank accession no P15252) and SRPP (GenBank accession no O82803), the two main proteins of hevea latex framed in grey in Fig. 1.

2.1. REF and SRPP in hevea latex are not glycoproteins

Several glycoproteins have been identified in latex as Hevb 2, 4, 6, 7, 12 and 13 [34]. But until 2008, REF and SRPP were believed to be non-glycosylated [25,35]. In 2008, SRPP was however described as a glycoprotein [22,36], but the glycosylation test was realized on partially purified SRPP from latex. In order to clarify this point, we identified REF (GenBank accession no P15252) and SRPP (GenBank accession no O82803) in latex by mass spectroscopy performed after SDS-PAGE (LC-MS/MS; Supplementary Table S1). As expected, they were the two main proteins in latex with molecular weight of 14.7 and 22.4 kDa, and REF was the predominant protein found in the washed rubber particles (WRP; Fig. 2). Therefore REF is tightly bound to rubber particles, when SRPP can be easily removed by simple washing. Both proteins migrated a bit below the recombinant 6-histidine-tagged proteins we purified from *Escherichia coli*. Coloration of glycoproteins by the periodic acid/Schiff (PAS as in [22,36]) did not show any staining. Compared to the controls, REF and SRPP were not glycosylated in hevea latex, but we could observe few thinner bands above SRPP, and non-proteic bands below 10 kDa (near the front line) that were attributed to glycolipids also present in the monomembrane surrounding the rubber particles [37]. Then, it seems that SRPP is not glycosylated (as REF), and no glycosylation site was detected in SRPP sequence [26]. In addition, it was also previously shown that the recombinant and not glycosylated GST-SRPP is functional and could perfectly help in polyisoprene synthesis [10].

2.2. SRPP Δ Cter has no visible aggregation, no amyloid properties and bind slightly to lipid monolayers as SRPP

In a previous study we showed that REF had aggregative and amyloid properties [2]. As REF and SRPP share significant homology, but differ in their C-terminal part, we generated a short variant of SRPP, deleted of the C-terminal part that we named SRPP Δ Cter (Fig. 3A). Similarly to REF and SRPP we purified it, as a 15.3 kDa recombinant 6-histidine tagged protein (Fig. 3B). The C-ter of SRPP could be responsible of the stability of SRPP, avoiding its folding into amyloid. After one week of incubation at 37 °C in PBS, no aggregates could be visualized by eye with SRPP Δ Cter. But as SRPP, SRPP Δ Cter is very

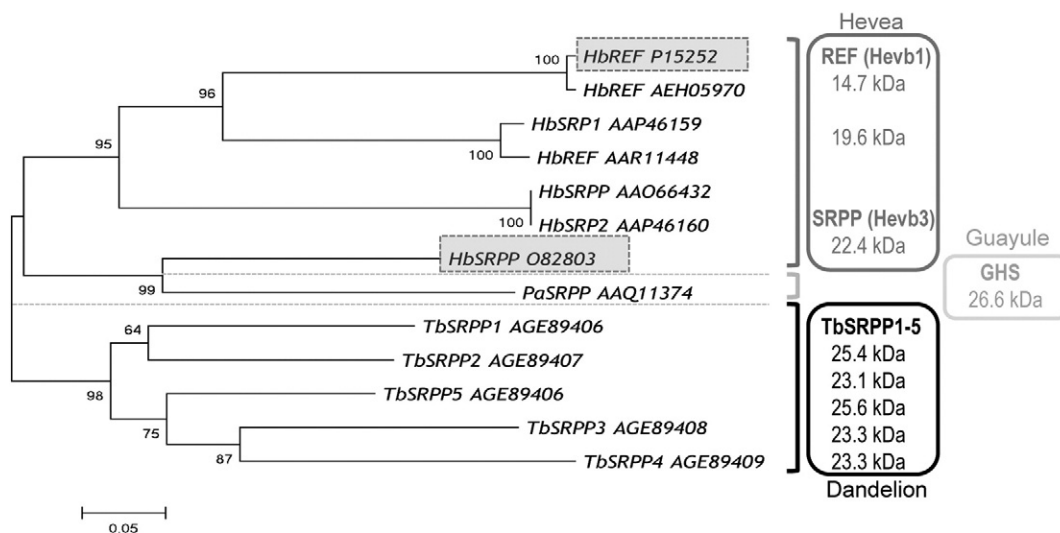


Fig. 1. Phylogenetic analysis of REF and SRPP proteins from latex-producing plants. Hb: *Hevea brasiliensis* (hevea); Pa: *Parthenium argentatum* (guayule); Tb: *Taraxacum brevicorniculatum* (dandelion).

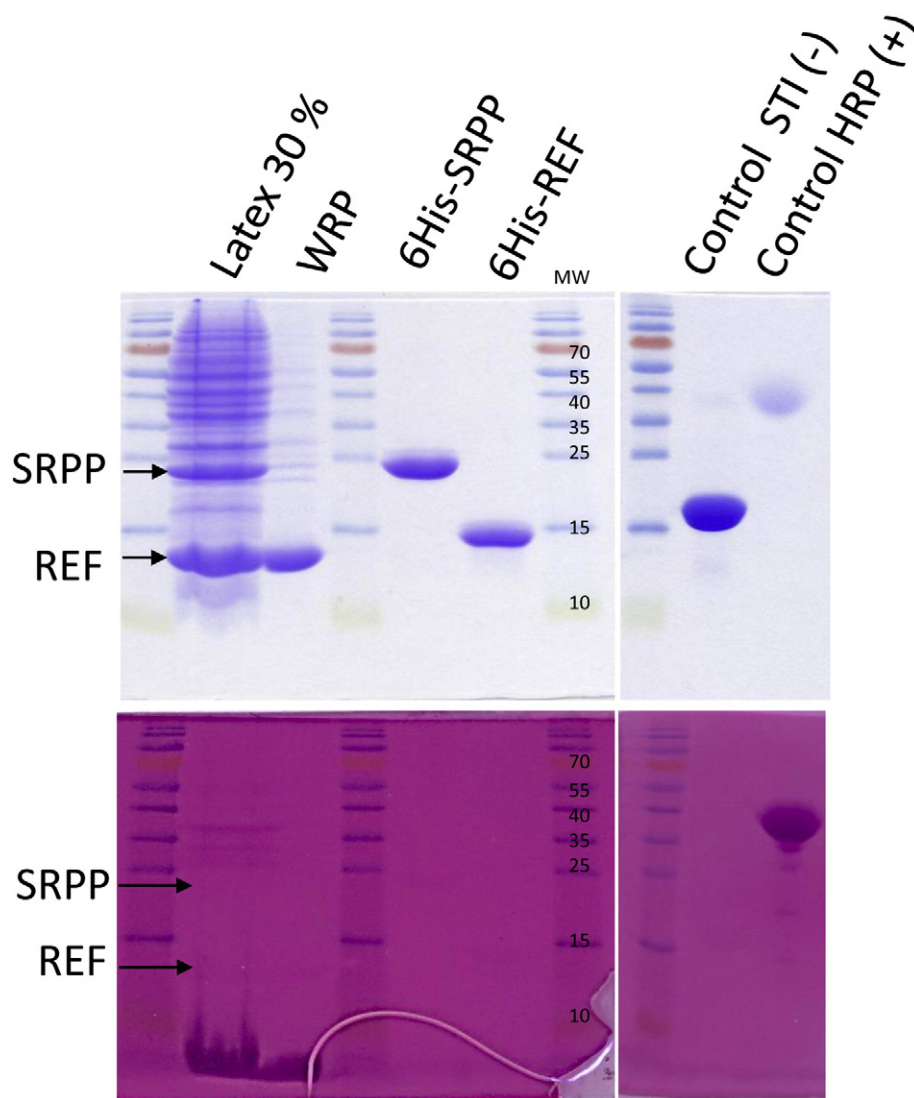


Fig. 2. REF and SRPP from *Hevea brasiliensis* latex are not glycosylated proteins. REF and SRPP were first identified by mass spectroscopy (Supplementary Table S1) as 22.3 and 14.7 kDa proteins (arrows). Top 15% SDS-PAGE gel was stained by Coomassie blue and bottom gel by the PAS method (glycoprotein detection).

susceptible to proteinase K degradation contrarily to REF (Supplementary Fig. S1). This protein was indeed showing the same kind of globular structure observed for SRPP by ATR-FTIR (Fig. 3C and Supplementary Table S2). Alone at the air–water interface, SRPPΔCter was very surface active as SRPP, coming rapidly at the interface. The kinetic of the absorption was similar for both proteins, and the same thickness of the protein film was determined (about 37 nm; see Supplementary Table S3). The presence of the protein at the air–water interface was revealed by the detection of the amide I band by PM-IRRAS and the increase in the thickness of the proteic film (Fig. 3D). We also observed by PM-IRRAS and ellipsometry the same kind of binding to lipid monolayers (DMPC and asolectin) as we observed for SRPP (see Supplementary Table S3 for full comparison and reference [2]). In presence of a lipid monolayer, the thickness of the protein/lipid monolayer was increasing, but we did not observe any large perturbations of the membrane integrity as we observed previously with REF (Fig. 3D right panel). It therefore means that SRPPΔCter come to the contact of the lipid monolayer without inserting in it. No signal of SRPPΔCter with a lipid bilayer was detected by PM-IRRAS, because of the weak interaction. Therefore, the amyloid nature of REF is truly inherent to its sequence, and the C-terminal part of SRPP is not conferring more stability. In addition,

we note that both SRPP and SRPPΔCter present the same net charge (−6; Supplementary Table S4), the same structural behavior and the same kind of interaction with membranes.

2.3. REF has agglutination activities

Considering that REF and SRPP interact with membranes, we may suspect a role in coagulation of latex by bringing latex particles together. We first wanted to know if they were able to agglutinate simple red blood cells (RBC). Indeed we observed that REF and aggregated REF were both able to hemagglutinate rabbit red blood cells, whereas SRPP and SRPPΔCter were not (Fig. 4A). Interestingly, the hemagglutination with REF could be inhibited by SRPP and SRPPΔCter (Fig. 4B). We did not observe hemolysis (release of hemoglobin) but a large aggregation of REF combined with the agglutination of the erythrocytes (Fig. 4C). From a previous experiment, it was shown that REF and SRPP were both able to bind to different model membrane and release calcein from large unilamellar vesicles [33], which means they could destabilize this kind of membranes. Red blood cells, which possess a more complex membrane, were not therefore susceptible to lysis effect and only the binding of REF was observed. The inhibition of this agglutination by

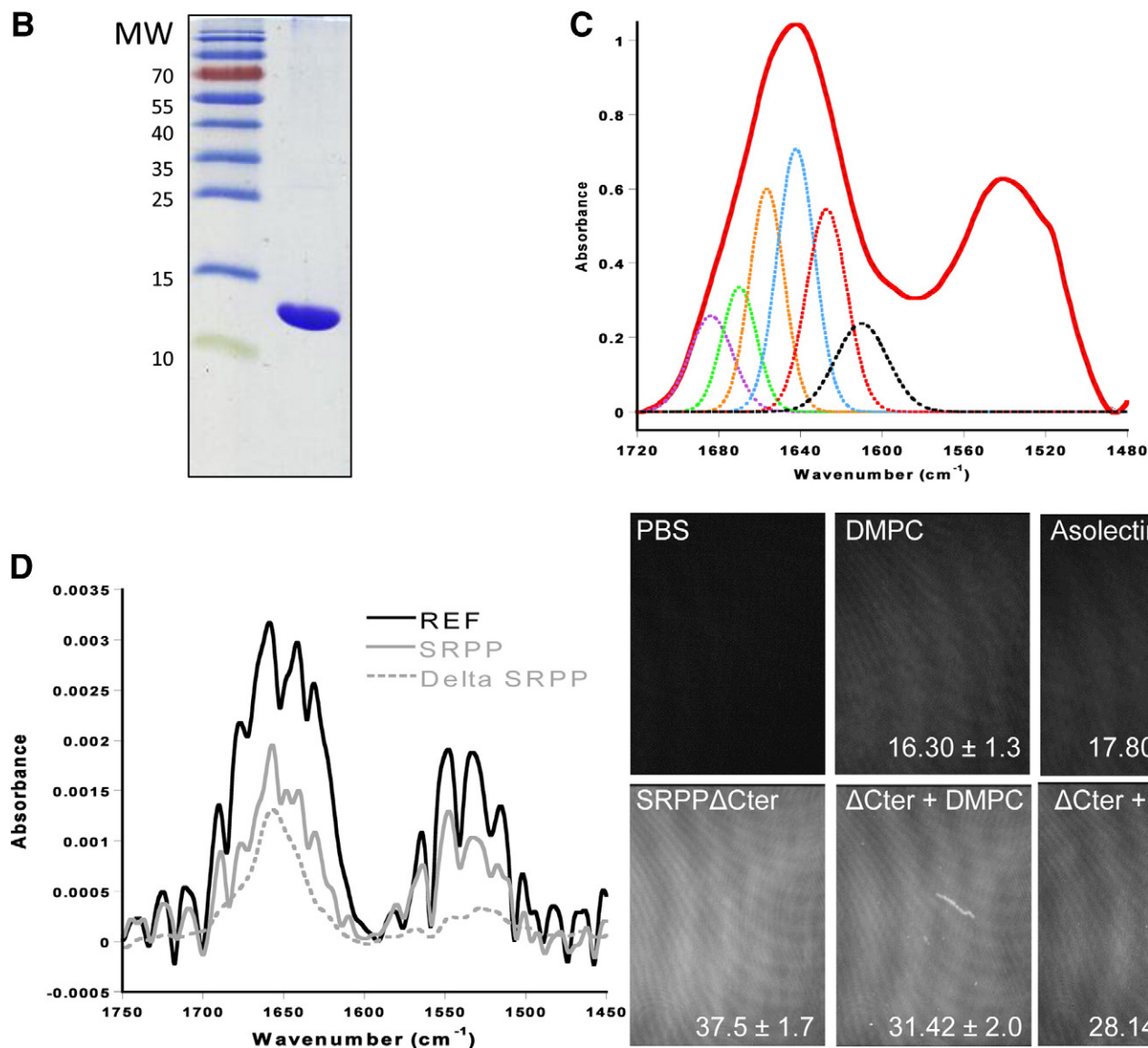
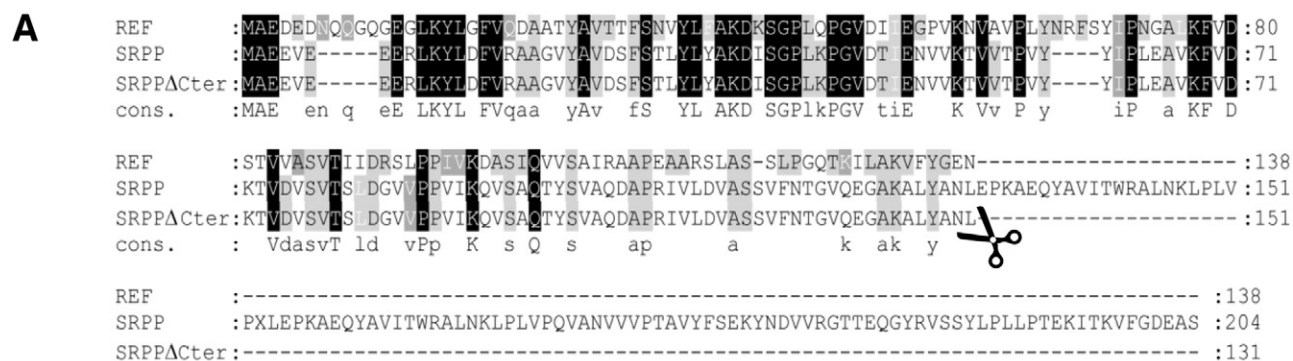


Fig. 3. SRPPΔCter behaves like SRPP. **A)** SRPPΔCter sequence and homology to REF and SRPP. **B)** Purified N-terminal 6-histidines recombinant SRPPΔCter. **C)** ATR-FTIR spectrum of SRPPΔCter. **(d)** SRPPΔCter interaction at the air–water or air–lipid interface. Left panel presents PM-IRRAS spectra of the 3 proteins at the air–water interface. Right panel presents ellipsometric images SRPPΔCter with or without DMPC and asolectin lipid monolayers. Thicknesses in Å of respective layers are indicated in white in the ellipsometric images.

various sugars was tried without success (glucose, galactose, sucrose, fructose, lactose, GlcNAc, maltose, mannose and xylose at 1 mg/mL). What could be seen as a lectin/glycoprotein interaction [22] seems only be due to the auto-assembling properties of REF, and the inhibitory effect of SRPP on REF auto-assembling or hemagglutination.

In 2007, Kanokwiroon [36] showed that SRPP prepared from latex was inducing yeast (*Candida*) cell aggregation. We performed a similar

agglutination test with the yeast *Saccharomyces cerevisiae*. Following the decrease of absorbance at 600 nm, we observed again a clear and rapid agglutination in presence of REF (and aggregated REF) and no effect of SRPP (Fig. 4D). Indeed, we observed a slight stabilizing effect of SRPP, avoiding yeast natural sedimentation. This could simply refer to a “crowding” effect, or a change in the buffer viscosity due to the protein addition. Similarly for RBCs, the massive aggregation of REF

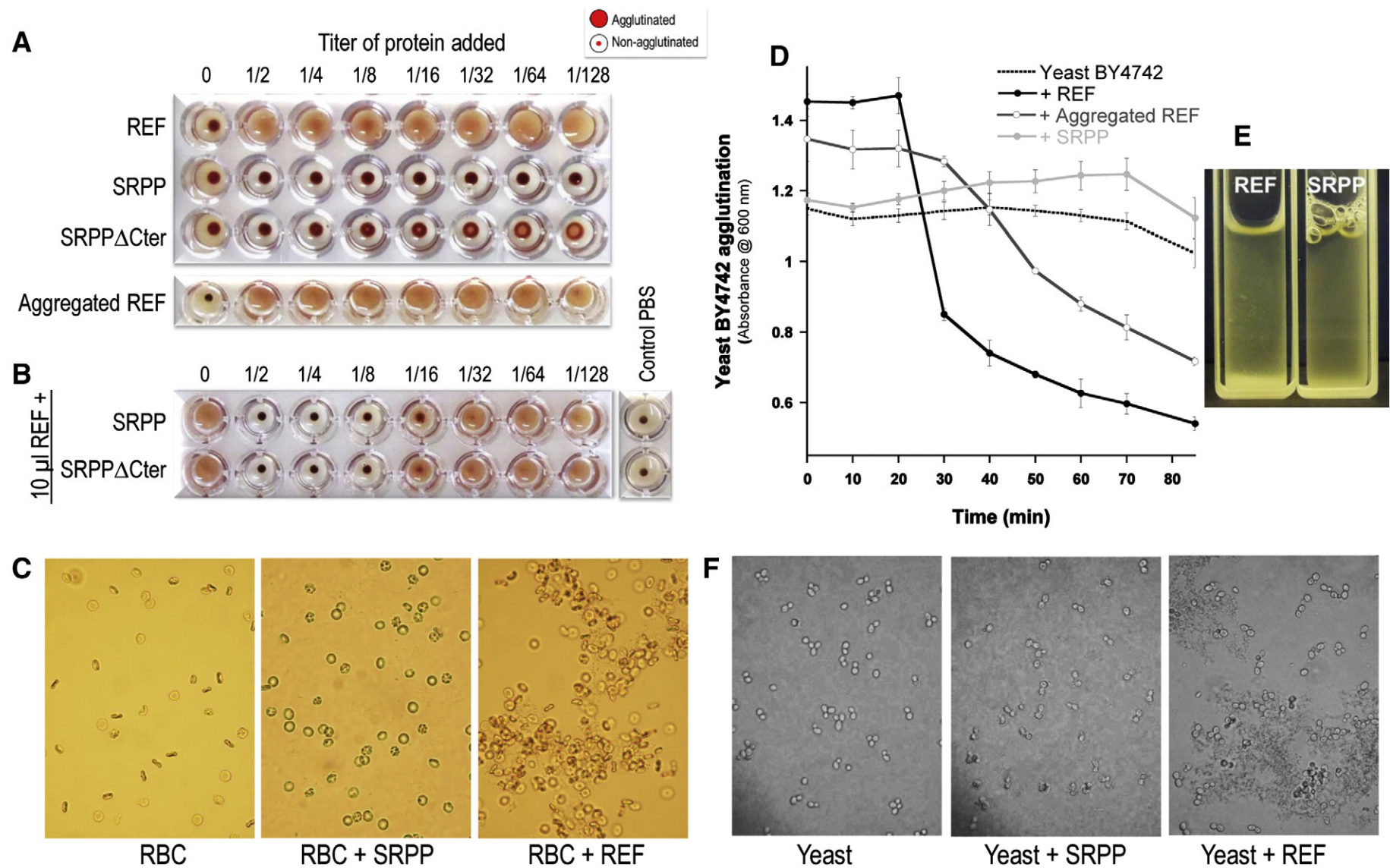


Fig. 4. REF has agglutinating activity and aggregation properties, which can both be inhibited by SRPP or SRPP Δ Cter. A) Hemagglutinating assay with rabbit red blood cells (RBC). B) Inhibition assay was performed with 10 μ l of REF and decreasing concentration of SRPP and SRPP Δ Cter in PBS. C) Microscopic observation ($\times 40$) of RBC. D) Agglutinating assay with *Saccharomyces cerevisiae*. E) Pictures showing the massive aggregation of REF concomitant with yeast agglutination. F) Microscopic observation ($\times 40$) of yeasts in presence of REF and SRPP.

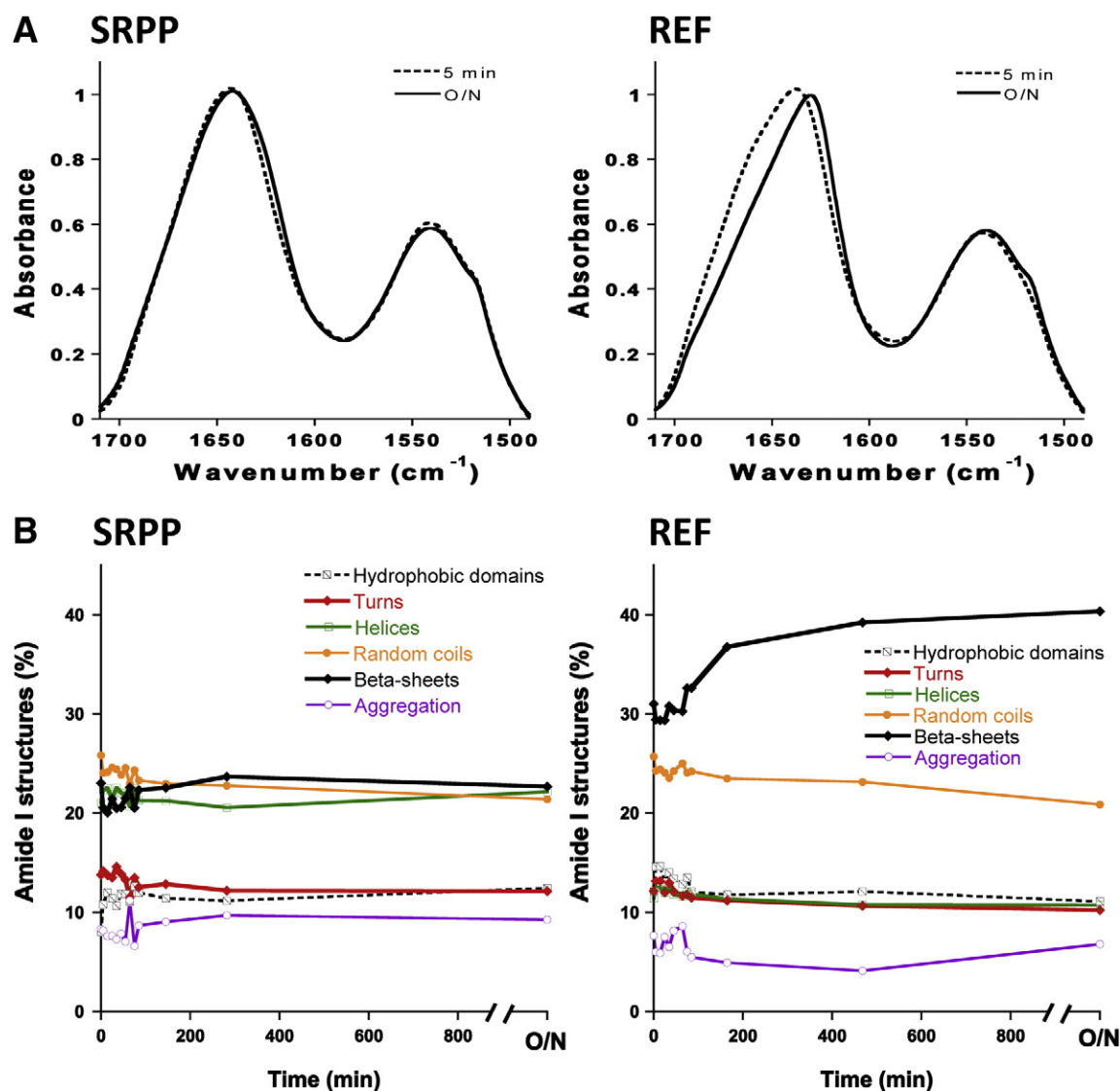


Fig. 5. Kinetics of REF and SRPP structuration followed by ATR-FTIR. **A)** Spectra of REF and SRPP (50 μ M) after 5 min and overnight incubation at 37 °C in PBS. **B)** Kinetic analyses after deconvolutions of respective spectra.

can be seen visually (Fig. 4E) and microscopically (Fig. 4F). Yeast cells seem again captured in REF aggregates, and the effect is amplified when REF auto-assembling occurs during the interaction, as fully aggregated REF has a weaker effect. In a previous study, we showed that REF and SRPP interacted well with various model membranes [33]. Here, we show that REF (but not SRPP) may also interact with eukaryotic and fungal membranes by bringing membranes together as they could do with rubber particles. This is a contradictory result with the Kanokwiroon Thesis, but we may suspect a contamination of their SRPP preparation by REF that could cause agglutination of yeast cells ([36] see p89, two bands of the purified SRPP from the Moir's zone 2).

2.4. SRPP and SRPP Δ Cter inhibit REF amyloidogenesis

We followed the kinetics of REF and SRPP structuration at 50 μ M by ATR-FTIR after incubation at 37 °C (Fig. 5A). Analyzing the amide I bands, we observed that SRPP structure does not vary significantly during time, whereas REF demonstrates a change in its structure leading to a higher β -sheet content and a decrease in random coils and hydrophobic domains (Supplementary Table S2). A slight lag-phase is observed during the structuration of REF in β -sheets, which is a common property of amyloid peptides (Fig. 5B). As we previously demonstrated,

this change in REF structure is correlated to its polymerization into amyloid fibers [2]. In the case of SRPP, the secondary structure remains the same, mainly as globular protein with helices. We also made the same observation for SRPP Δ Cter (data not shown; same as Fig. 3C). With the RBC, we observed that SRPP and SRPP Δ Cter inhibited the hemagglutination of REF, which means that REF could interact directly with SRPP and SRPP Δ Cter.

To verify if SRPP was interacting with REF, we performed aggregation experiments followed by absorbance at 405 nm (37 °C and 20 μ M; Fig. 6A). The aggregation of REF was extremely rapid and the turbid aggregates were quite large to precipitate at the bottom of the tube, whereas SRPP and SRPP Δ Cter show absolutely no turbidity and visible aggregation (Fig. 6B). The important heterogeneity of REF aggregation since the early times explains the great variation in the REF results. SRPP and SRPP Δ Cter were also able to inhibit the aggregation of REF, but this effect was not permanent, and precipitated aggregates could finally be seen.

2.5. SRPP and SRPP Δ Cter also display self-assembling properties but different from REF amyloidogenesis

Native gels were performed with the 3 proteins. Only SRPP Δ Cter was separated as mainly a single band protein, but proteins were also

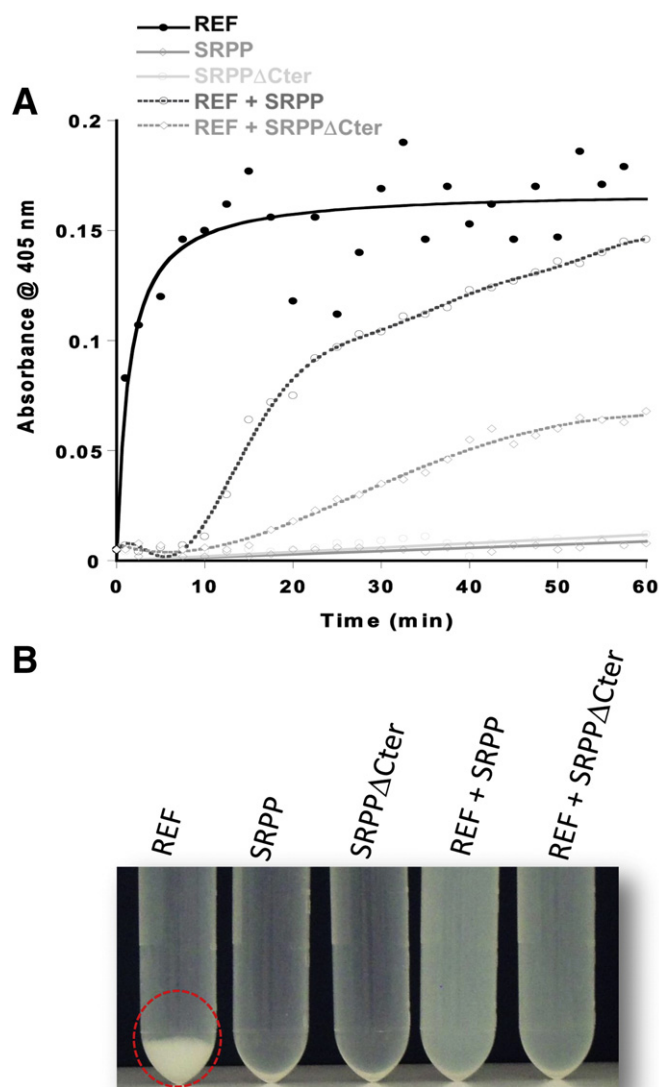


Fig. 6. SRPP and SRPPΔCter inhibit the aggregation of REF. A) Aggregation followed by absorbance at 405 nm. Protein samples were mixed before each measurement and proteins were at 20 μM in PBS and 37 °C. B) Macroscopic observation after 6 h of incubation ($n > 3$).

present in the resolving interface (Fig. 7A, right panel). Compared to denaturing gels where the 3 proteins appeared as single band proteins (left panel), SRPP obviously ran as multimers of several species and REF displayed quite some aggregation (right panel). No fragmentation of REF was observed, but fragmentation and aggregation of REF were previously reported in protein extracts from gloves [38]. In native conditions, none of the proteins separated as single species and polydispersity was observed (Fig. 7A). Some REF proteins were present in the well of the stacking gel, and some others were barely entering the resolving gel. After incubation of these proteins in PBS 1×, at 50 μM, 37 °C and overnight (Fig. 7B), we observed clear aggregation and fragmentation of SRPP on denaturing SDS-PAGE (left panel). We also observed aggregation of SRPP and SRPPΔCter on native gels, but these aggregations were not visualized by eye (Fig. 7B). These fragmentations and aggregations of SRPP purified from field latex, was reported by Yeang et al. after storage in a −20 °C freezer [11]. A slight fragmentation of SRPPΔCter was also observed as a band around 12–13 kDa. We previously showed that SRPP was more susceptible than REF to proteases [2]. This fragmentation could also lead to aggregation of the smaller fragments. In the case of REF (visually completely precipitated), no REF protein could be

seen on the native gel. The addition of REF to SRPP or SRPPΔCter favors auto-assembling, which means that they may interact together as previously seen in Fig. 6.

Transmission electron microscopy (TEM) was carried out to try to identify SRPP multimers. REF amyloid fibers at the early stage of polymerization and finally mainly amorphous super-twisted aggregates were observed (Fig. 8A). For SRPP and SRPPΔCter, numerous polymorphic and small objects (50–200 nm) could be visualized after overnight incubation at 37 °C (Fig. 8B and C). From ATR-FTIR we know that these nanoobjects are not of amyloid nature (e.g. amyloid oligomers), but rather auto-assembling of the original native monomer. Large micrometer-aggregates were not observed as for REF, but small nanosized multimers were observed by TEM. These aggregates remained in a soluble state, and this could explain why no precipitate was observed by eye as for REF.

Dynamic light scattering experiments confirmed also these observations. In this case, pure protein stocks in Tris/HCl 25 mM pH 8.0 were used just after thawing (time 0 h), and then incubated at 37 °C. At first, REF presents mainly himself as monodisperse (Pdl of 0.138) in solution and forming a multimer of about 24 nm in diameter (Fig. 9A). This is coherent with the fact that REF has been described being a native homotetramer of 58 kDa in latex [39]. Rapidly, after 1 h at 37 °C a white trouble is observed by eye, large aggregates appears (>1 μm), the initial 24 nm species disappear towards the formation of micrometer-large aggregates with a high polydispersity of the protein sample (Pdl>1). This corresponds to a largely uncontrolled process of aggregation. For SRPP and SRPPΔCter, the process of auto-assembling is far more controlled. First, both pure proteins principally populate the sample as 14 nm (SRPP) and 9 nm (SRPPΔCter) objects, with a relatively high polydispersity, which means that few other bigger particles may also be present (Fig. 9B and C; see also Sup. Fig. S2 for SRPPΔCter size distribution in numbers). Incubation of SRPP and SRPPΔCter at 37 °C leads to the controlled assembly of nanosized objects (respectively of 31 and 48 nm diameters). Few other bigger particles are also present with the multimers, but interestingly the polydispersity of samples decreases, which could favor the stability of the latest assemblies. These nanometer-objects are clearly seen by TEM, together with few bigger objects. To conclude, REF classically follows the polymerization process of an amyloid (finally precipitating), whereas SRPP auto-assembles into stable nanomultimers, with as similar structure, which are not visible by eye.

2.6. REF and SRPP have no antimicrobial activity

To produce recombinant REF and SRPP, codon-optimized gene for *Escherichia coli* were used. The expression (by 1 mM IPTG induction) produces inclusion bodies for both proteins but does not seem to generate any toxicity towards *E. coli* (Fig. 10A). In 2007, Kanokwiroon [36] showed that SRPP prepared from latex could have antifungal activity. Apart from agglutination, the contact of REF and SRPP with erythrocytes or yeast does not seem to modify membrane integrity (e.g. no hemolysis). We wondered if REF or SRPP were toxic for other systems. To this end we performed a Microtox® standardized toxicity test using *Vibrio fischeri*, a bioluminescent bacterium as reporter of toxicity.

This test is currently used in ecotoxicity evaluation. The bacteria were exposed to increasing concentrations of REF (3.4, 6.8, 13.5, 27 and 54 μM) or SRPP (2.2, 4.4, 8.8, 17.5 and 35 μM) during 30 min. No antimicrobial activity could be reported after 30 min at lower and higher concentrations. A slight, transitory and reversible effect was however visualized at early times of incubation with SRPP at the highest concentration (Fig. 10B). Globally, no antimicrobial activity was detected in *E. coli* or *V. fischeri*.

3. Discussion and conclusions

The REF/SRPP stress-like related family is every year getting larger, but their role is far from understood. This is the first report that both

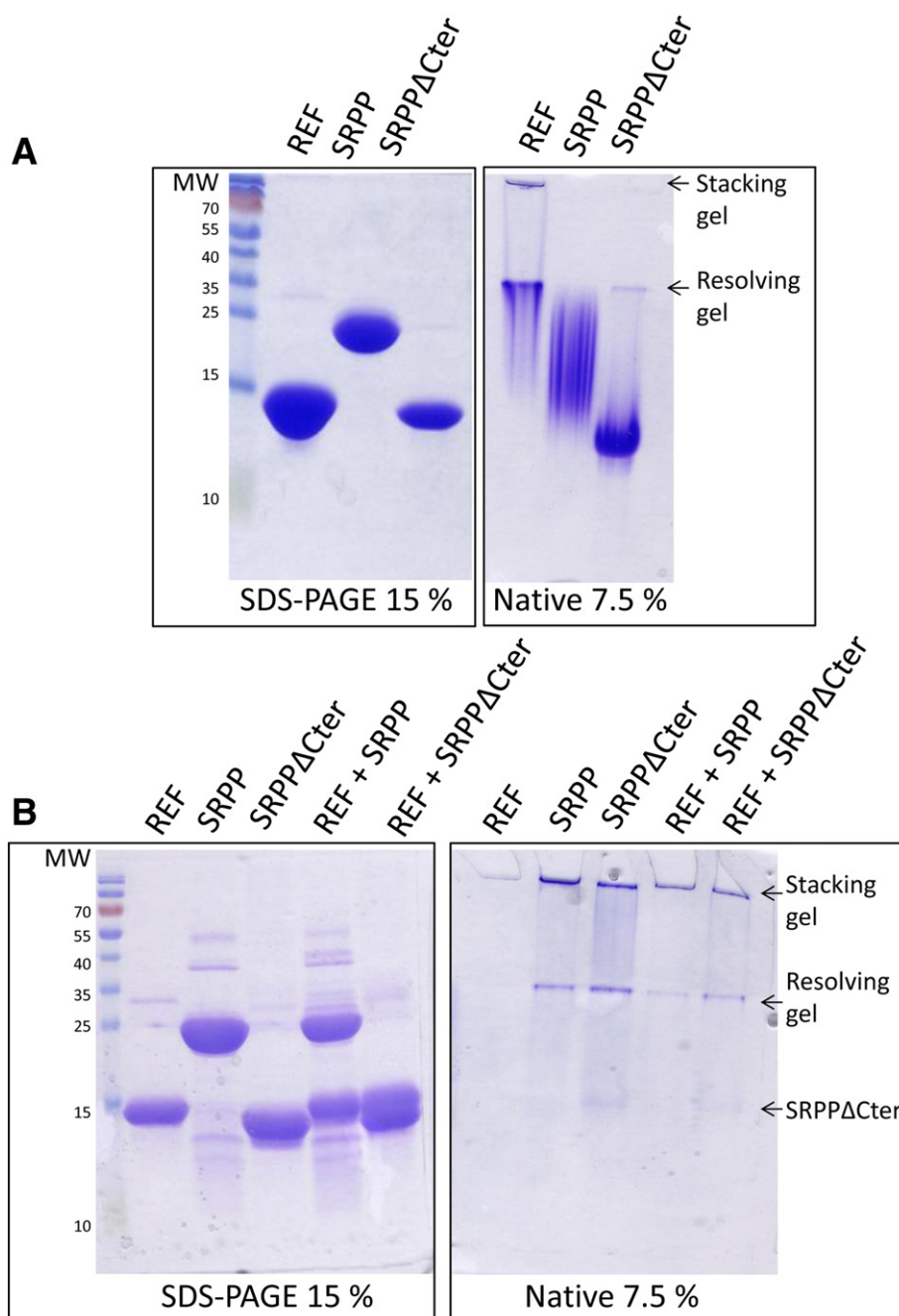


Fig. 7. SRPP auto-assembling and fragmentation. A) Pure REF, SRPP and SRPPΔCter proteins observed on 7.5% native gel (right panel) and compared to the same proteins ran in denaturing conditions on 15% SDS-PAGE (left panel). B) Results after incubation of the proteins at 50 μM, 16 h at 37 °C.

proteins are able of auto-assembling and could also interact together. Latex coagulation is a kind of aggregation process. The presence of REF or SRPPs in various high-molecular-weight rubber plants (hevea, dandelion and guayule) is interesting particularly if they are able to auto-assemble or aggregate, and contribute to the “branching” effect. It is believed that natural rubber during its synthesis is cross-linked by its extremities by interacting with proteins and lipids. The “branching” process with proteins is thought to happen at the ω -terminal (the trans initiator group) of the polyisoprene while the phospholipids could be linked at the α -terminal phosphate group of the linear polymer chains [40,41]. From our various results, we may also suppose multiple interactions (protein–lipid–polymer), these could effectively modulate the gel forming capacity and/or the molecular weight of natural rubber. It is interesting to note that REF and SRPP, if they are homologous, are very different in their aggregative features, but also in their interactive

behaviors with membranes [33]. Protein–protein and membrane–protein interactions are probably driving all the coagulation process. The highly hydrophobic nature of REF and SRPP may certainly prompt their presence on the latex particle and finally in the rubber. At first, they protect and stabilize the latex particles when anchored with the lipid biomembrane. TbSRPPs are also suspected of such role [19,42]. Then, the colloidal nature of latex is preserved by the proteolipidic shell surrounding polyisoprene core. The rubber synthesis platform made of enzymes (e.g. cis-prenyl transferase) is also imbedded in the membrane. REF and SRPP are the two main proteins found on rubber particles, and we showed that they interact strongly but also differently with lipids [2,33]. Interestingly, they are two short proteins (14 and 24 kDa) but with dual properties: as biomembrane proteins they seem to act as stabilizers in normal conditions and destabilizers when environment is changed. In addition, it is known that dehydration or

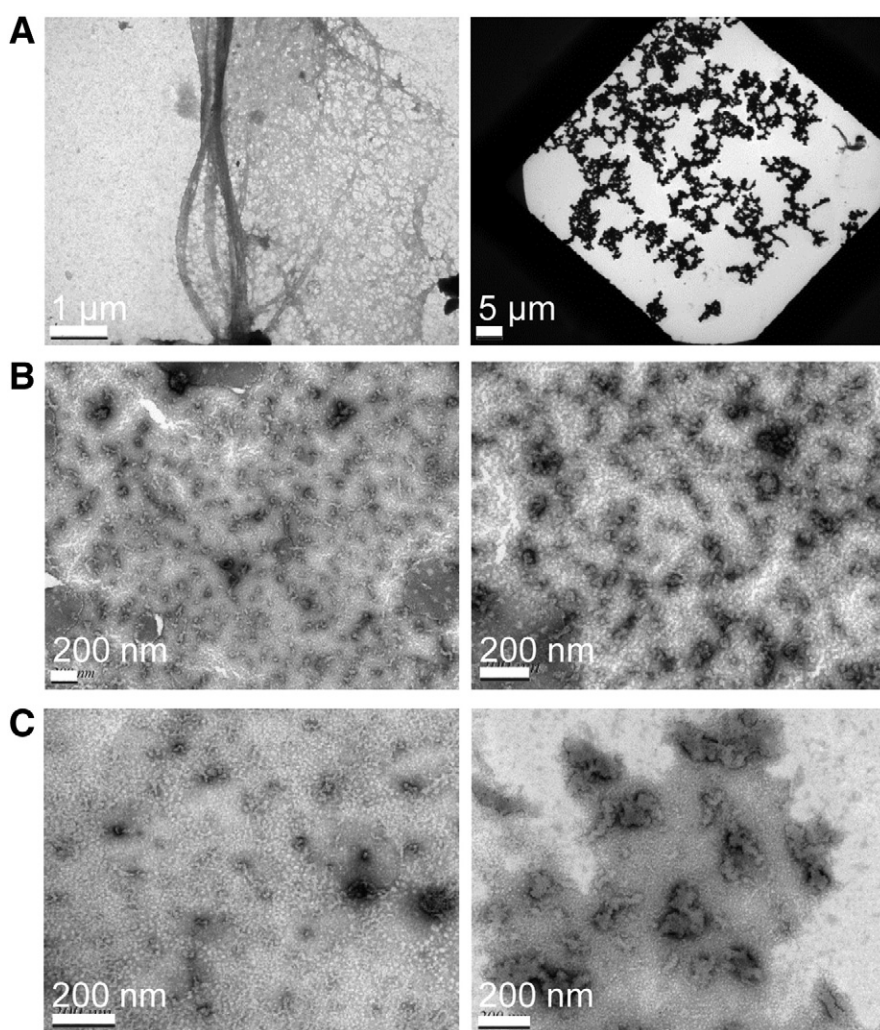


Fig. 8. Transmission electron microscopy of REF (A), SRPP (B) and SRPP Δ Cter (C). Images are presented after incubation of the proteins in PBS at 37 °C overnight, with the exception of the first picture showing REF amyloid fibers, as they can only be seen during early polymerization times (15 min at room temperature).

“crowding” may favor aggregation. Tapping or tapping panel dryness (TPD) syndrome may be assimilated to a plant stress, and REF and SRPP are overexpressed during these particular conditions. An excess of these proteins, not bound to the membrane, would certainly lead to aggregation. When tapping is occurring, latex is not protected anymore in the laticifer cells and evolves to coagulate into rubber. Acidification may occur with the lysis of lutoids, enzymes and proteases are released in the serum [43], oxidations at the air interface take place... various processes that could lead to the destruction of the proteolipidic membrane and generate protein aggregation. For example, during TPD syndrome, the osmotic lysis of lutoids causes the release of REF and SRPP from the rubber particles [32]. Recently, other SRPP-like proteins were proposed to be involved in the binding process to lipid droplets and in the stabilization of lipid-rich particles in the plant cell cytosol [44].

In latex, REF and SRPP are quite cohesive with the rubber particle, and because of their very hydrophobic content, they are contained in surface with the lipids. But we observed that with particular large unilamellar vesicles (LUV) filled with hydrophilic fluorescent calcein, REF and SRPP were able to penetrate these kinds of vesicles and provoked leakage [33]. This type of action may be of particular interest for fighting pests, and numerous amyloids able to interact with membranes have also antimicrobial, antifungal or antiviral activities [45]. Our work does not support the antimicrobial effect of REF or SRPP, and we did not observed any phenotypic effects on yeast or erythrocytes. But other type of membranes may also be targeted by REF

and SRPP. As proteins and phospholipids seem to attract more and more interest to understand rubber synthesis and quality, the development of studies on the impact of non-rubber constituents of latex should open new perspectives. It is already true for vulcanized, unvulcanized or silica reinforced NR, in which the effect of proteins is not trivial [46,47].

To resume our knowledge at this point on their potential functions: a) REF and SRPP are not initially present on the same laticifer layers and on the same rubber particles, but are found in latex and rubber; b) REF and SRPP do not interact in the same way with membrane models, REF show more affinity. But, in the light of a recent work [44], they could play a role as lipid droplet-associated proteins. The stability of the rubber particles and latex could depend on these protective membrane proteins; c) they could take part in rubber synthesis or rubber quality as non-isoprene constituents, their auto-assemblings and/or their mutual interactions *in vivo* could contribute to coagulation; d) they are stress-related proteins, but from our results we can only exclude a potential antimicrobial activity.

4. Experimental procedures

4.1. REF, SRPP and SRPP Δ Cter cloning, expression and purification

The SRPP gene (GenBank accession no [AJ223388](#)) and the REF gene (GenBank accession no [X56535](#)) were previously constructed as codon-optimized pET24a-6His-SRPP and pET24a-6His-REF plasmids

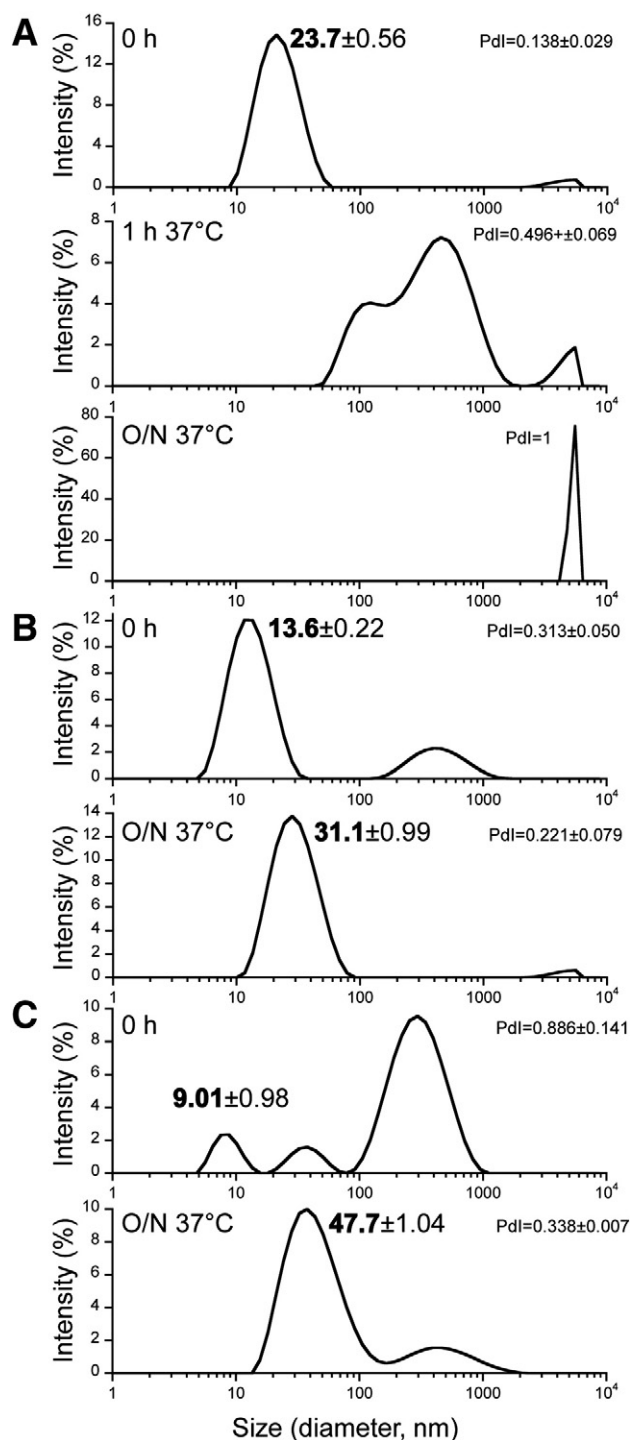


Fig. 9. REF aggregation (A), SRPP (B) and SRPPΔCter (C) auto-assembling followed by dynamic light scattering (DLS). The mean size of the objects ($n = 3-6$) present as the predominant species in the sample is given in bold, and was deduced from the results in numbers (data for SRPPΔCter are presented in Sup. Fig. S2) and compared with the intensity. The sample polydispersity is also reported (Pdl, top right corner).

[2]. SRPPΔCter (amino acids 1–131) was created from pET24a-6His-SRPP by PCR amplification using primers SRPP_Bam(+) 5'-GGATCCAT GGCAGAAGAAGTGG-3' and SRPPd_Xho(-) 5'-CTCGAGTTACAGATTTCGC ATACAGGGCTTT-3'. This construct missing the C-terminal part of SRPP was subcloned into pCR2.1 from TOPO cloning kit (Invitrogen, ThermoScientific, Illkirch, France). pET24a-6His-SRPPΔCter was constructed by insertion into the BamHI/XhoI sites. Protein expression and purification were realized as previously described [2]. Protein

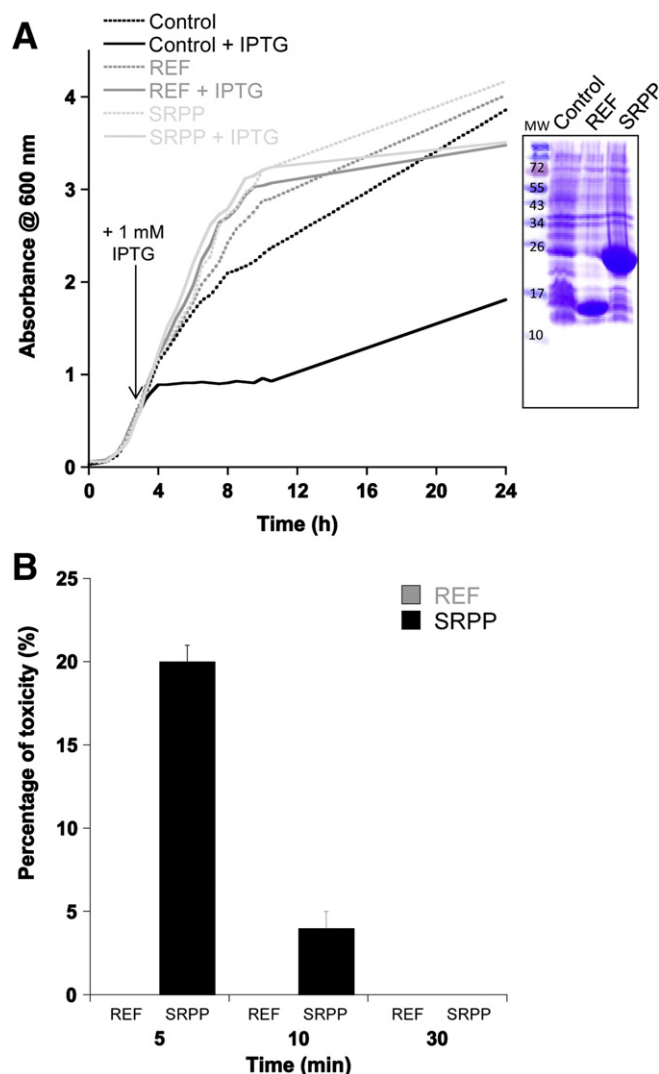


Fig. 10. No antimicrobial activity of REF and SRPP. A) Growth curves of *E. coli* Gold pLysS after 1 mM IPTG induction at $A_{600} = 0.6$. The presence of REF and SRPP after 4 h induction was controlled by 15% SDS-PAGE (right panel). B) Microtox® test of REF (54 μ M) and SRPP (35 μ M) at the maximum tested concentration after 5, 10 and 30 min.

concentrations were determined by quantitative amino-acid analysis. Physico-chemical parameters are presented in Supplementary Table S4. For polymerization experiments, proteins were usually incubated at 20–50 μ M in 1 $\times$ PBS buffer pH 7.4 (Euromedex, Mundolsheim, France) at 37 °C for 3–24 h, and kept at 4 °C.

4.2. Phylogenetic analysis

A BlastP was realized on *Hevea brasiliensis* REF (GeneBank accession no. P15252) at <http://www.phylogeny.fr>. Accession numbers of each 13 sequences found follows the name of the protein in Fig. 1. Sequences were aligned with ClustalW (<http://www.genome.jp/tools/clustalw/program>). Evolutionary analyses were conducted in MEGA5[48], 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. Mass spectrometry LC-MS/MS analysis

Bands from SDS-PAGE stained by Coomassie blue were cut, digested by trypsin, extracted and submitted to a LC-MS/MS analysis. SDS-PAGES with respective analyzed proteins are presented in Supplementary

Table S2. A LTQ Orbitrap XL spectrometer (ThermoFisher Scientific) was used and the experiments were performed at the Proteome CGFB platform, Bordeaux.

4.4. Detection of glycoproteins in latex

Non-ammoniated latex (30 %) was used and diluted 1/2 in gel loading buffer. Crude extracts of proteins from non-ammoniated latex were prepared as followed: 300 μ L of latex was added to 700 μ L of water, 100 μ L SDS 10% (Euromedex) and 100 μ L Triton X100 (Euromedex). After 30 min agitation at room temperature, rubber was eliminated by centrifugation. Protein supernatant was precipitated by TCA 10% final. After centrifugation, the pellet was washed by acetone and directly resuspended in gel loading buffer.

The washed rubber particles (WRP) were purified at 4 °C as previously described [49]. In brief, 1 mL of latex was centrifuged 10 min at 4000g, the top cream was washed with 1 mL of buffer (0.1 M Tris/HCl pH 7.4, 10 mM DTT, 5 mM MgSO_4), and the WRPs were then washed twice by centrifugation 10 min at 2400g and finally resuspended in buffer.

Proteins in loading buffer were denatured 10 min at 100 °C and run on 15% SDS-PAGE before staining by Coomassie Blue and GlycoCode kit (Pierce, ThermoFisher) for glycoproteins. Glycoprotein control (+) was HRP (horse radish peroxidase) and non-glycosylated protein control (–) was STI (soybean trypsin inhibitor).

4.5. PM-IRRAS and ellipsometry experiments on lipid monolayers at air–water interface

DMPC and asolectin were purchased from Aventi Polar Lipids (Alabaster, AL, USA) and Sigma-Aldrich (Saint-Quentin Fallavier, France) respectively. Polarization-modulation-infrared reflection-adsorption spectra (PM-IRRAS) were recorded on a Nicolet (Madison, WI) Nexus 870 spectrometer equipped with an HgCdTe (MCT) detector (SAT Poitiers, France) and cooled at 77 K by nitrogen liquid at a resolution of 8 cm^{-1} by coadding 600 scans. Details of PM-IRRAS experiments were described in previous studies [33,50]. PM-IRRAS spectra were normalized by the subphase spectrum or lipid monolayer spectrum. 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 [2,51]). The spatial resolution was about 2 μ m and the image size was 450 $\times$ 670 mm, with a 10 $\times$ magnification lens. Thickness was determined using a mean value of 1.45 for the refractive index.

4.6. Hemagglutinating and yeast agglutinating assays

The red blood cells (RBC) assay was performed in 96-well microtiter plates in 100 μ L with U or V-shaped wells (Greiner). Protein were diluted in PBS 1 $\times$ as mentioned and 5 μ L of rabbit red blood cells (0.5% final; Fitzgerald Industries International, Acton, MA, USA,) were added at room temperature. Results were observed after 1 h incubation and are presented as serial dilutions of each pure protein: REF (272 μ M), SRPP (146 μ M), SRPP Δ Cter (98 μ M) and REF in its amyloid form (20 μ M). For inhibition assay SRPP and SRPP Δ Cter were first diluted and 10 μ L of pure REF was added before mixing with erythrocytes. 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 with the *Saccharomyces cerevisiae* strain BY4742 (*MAT α his3 Δ 1 leu2 Δ 0 lys2 Δ 0 ura3 Δ 0 can1–100*). Yeast were grown overnight in YPD medium (10 g/L yeast extract; 20 g/L bactopectone, 20 g/L dextrose) and diluted to $A_{600} = 1.1$ –1.2 in PBS 1 $\times$, before adding proteins at 20 μ M and room temperature in 1 mL final volume. Yeast sedimentation and agglutination were measured by following absorbance at 600 nm. Yeast and RBC pictures

were captured with an Axioskop 40 light microscope (Carl Zeiss S.A.S., Pecq, France) equipped with a magnification 40 $\times$ objective. Agglutinating experiments were performed at least 3 times.

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. For kinetics studies, the proteins in 25 mM Tris/HCl pH 8.0 were buffered with 1 $\times$ PBS at pH 7.4 to a final concentration of 50 μ M. Time 0 (T_0) of kinetics corresponded to pure proteins. Incubations were performed at 37 °C without agitation. The first measurement was done after 5 min drying of a 5 μ L drop onto the germanium ATR crystal (Specac, Orpington, UK). All the aliquots generated during the kinetics were analyzed after 5 min drying. One hundred interferograms, representing an acquisition time of 3.5 min, were co-added.

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 [2].

4.8. Transmission electron microscopy (TEM)

Proteins were absorbed 10 min onto Formvar-coated, carbon-stabilized copper grids (400 mesh) and washed 3 times with water. Grids were then negatively stained 1 min with 10 μ L 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.

4.9. Dynamic light scattering (DLS)

Pure proteins after purification and storage at –80 °C (0 h corresponding to thawing) were at 344 μ M (REF), 238 μ M (SRPP) and 179 μ M (SRPP Δ Cter) in Tris/HCl 25 mM pH 8.0. They were then incubated at 37 °C. Measurement to obtain the average size of the protein assemblies were performed by using a Malvern ZetaSizer NanoZS instrument (Malvern Instruments SA, Orsay, France) in a quartz cuvette (Hellma, France). Results were analyzed with the Zetasizer software 6.01.

4.10. Antimicrobial activity

Growth curves of *Escherichia coli* Gold pLysS were followed by absorbance at 600 nm after 1 mM IPTG induction at $A_{600} = 0.6$. pET24a-6His-SRPP and pET24a-6His-REF plasmids were used to express REF and SRPP, and the control was the empty vector pET24a, also known for its toxicity [52]. The presence of the expressed proteins was verified by 15% SDS-PAGE after 4 h induction.

REF and SRPP toxicity was also evaluated with a Microtox® standardized toxicity test (Microtox® Acute Toxicity Reagent, SDI, Newark, USA) using *Vibrio fischeri*, a bioluminescent bacterium as reporter of toxicity. The bacteria were exposed to increasing concentrations of REF (3.4, 6.8, 13.5, 27 and 54 μ M) or SRPP (2.2, 4.4, 8.8, 17.5 and 35 μ M) during 5, 15 or 30 min. This test was realized at EPOC, CNRS UMR 5805/EA-Toxicologie Environnementale, Talence.

Author contributions

KB designed the study, performed the experiments and wrote the paper. SL performed FTIR, PM-IRRAS and ellipsometry. YE prepared

the WRPs. BCS performed TEM. KB, SL, YE, BCS and FP analyzed the data and critically read the paper. 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.

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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.2013.10.017>.

References

- [1] J.M. Hagel, E.C. Yeung, P.J. Facchini, Got milk? The secret life of laticifers, *Trends Plant Sci.* 13 (2008) 631–639.
- [2] K. Berthelot, S. Lecomte, Y. Estevez, B. Coullary-Salin, A. Bentaleb, C. Cullin, A. Deffieux, F. Peruch, Rubber Elongation Factor (REF), a major allergen component in *Hevea brasiliensis* latex has amyloid properties, *PLoS One* 7 (2012) e48065.
- [3] P. Venkatachalam, N. Geetha, P. Sangeetha, A. Thulaseedharan, Natural rubber producing plants: an overview, *Afr. J. Biotechnol.* 12 (2013) 1297–1310.
- [4] J.B. van Beilen, Y. Poirier, Establishment of new crops for the production of natural rubber, *Trends Biotechnol.* 25 (2007) 522–529.
- [5] J. d'Auzac, J.L. Jacob, H. Chrestin, The composition of latex from *Hevea brasiliensis* as laticiferous cytoplasm, in: J. d'Auzac, J.L. Jacob (Eds.), *Physiology of the rubber tree latex*, CRC Press, Boca Raton, Florida, 1989.
- [6] L. Vaysse, F. Bonfils, J. Sainte-Beuve, M. Cartault, 10.17 - Natural Rubber, in: M. Editors-in-Chief: Krzysztosf, M. Martin (Eds.), *Polymer Science: A Comprehensive Reference*, vol. 10, Elsevier, Amsterdam, 2012, pp. 281–293.
- [7] L. Dai, G. Kang, Y. Li, Z. Nie, C. Duan, R. Zeng, In-depth proteome analysis of the rubber particle of *Hevea brasiliensis* (para rubber tree), *Plant Mol. Biol.* (2013) 82.
- [8] WHO-IUIS, <http://www.allergen.org/search.php?allergen=hevea&search=Search>, in: Allergen Nomenclature Sub-Committee, 2013.
- [9] M.S. Dennis, D.R. Light, Rubber elongation factor from *Hevea brasiliensis*. Identification, characterization, and role in rubber biosynthesis, *J. Biol. Chem.* 264 (1989) 18608–18617.
- [10] S.K. Oh, H. Kang, D.H. Shin, J. Yang, K.S. Chow, H.Y. Yeang, B. Wagner, H. Breiteneder, K.H. Han, Isolation, characterization, and functional analysis of a novel cDNA clone encoding a small rubber particle protein from *Hevea brasiliensis*, *J. Biol. Chem.* 274 (1999) 17132–17138.
- [11] H.Y. Yeang, K.F. Cheong, E. Sunderasan, S. Hamzah, N.P. Chew, S. Hamid, R.G. Hamilton, M.J. Cardoso, The 14.6 kd rubber elongation factor (Hev b 1) and 24 kd (Hev b 3) rubber particle proteins are recognized by IgE from patients with *spina bifida* and latex allergy, *J. Allergy Clin. Immunol.* 98 (1996) 628–639.
- [12] T. Sando, T. Hayashi, T. Takeda, Y. Akiyama, Y. Nakazawa, E. Fukusaki, A. Kobayashi, Histochemical study of detailed laticifer structure and rubber biosynthesis-related protein localization in *Hevea brasiliensis* using spectral confocal laser scanning microscopy, *Planta* 230 (2009) 215–225.
- [13] A.R. Bahri, S. Hamzah, Immunocytochemical localization of rubber membrane protein in hevea latex, *J. Nat. Rubber Res.* 11 (1996) 88–95.
- [14] B.L. Archer, B.G. Audley, E.G. Cockbain, G.P. McSweeney, The biosynthesis of rubber. Incorporation of mevalonate and isopentenyl pyrophosphate into rubber by *Hevea brasiliensis* latex fractions, *Biochem. J.* 89 (1963) 565–574.
- [15] A.P. Singh, S.G. Wi, G.C. Chung, Y.S. Kim, H. Kang, The micromorphology and protein characterization of rubber particles in *Ficus carica*, *Ficus benghalensis* and *Hevea brasiliensis*, *J. Exp. Bot.* 54 (2003) 985–992.
- [16] N. Ohya, Y. Tanaka, R. Witsuwannakul, T. Koyama, Activity of rubber transferase and rubber particle size in *Hevea* latex, *J. Rubber Res.* 3 (2000) 214–221.
- [17] I.J. Kim, S.B. Ryu, Y.S. Kwak, H. Kang, A novel cDNA from *Parthenium argentatum* Gray enhances the rubber biosynthetic activity *in vitro*, *J. Exp. Bot.* 55 (2004) 377–385.
- [18] T. Schmidt, A. Hillebrand, D. Wurbs, D. Wahler, M. Lenders, C. Schulze Gronover, D. Prüfer, Molecular cloning and characterization of rubber biosynthetic genes from *Taraxacum kok-saghyz*, *Plant Mol. Biol. Rep.* 28 (2010) 277–284.
- [19] A. Hillebrand, J.J. Post, D. Wurbs, D. Wahler, M. Lenders, V. Krzyzanek, D. Prüfer, C.S. Gronover, Down-regulation of small rubber particle protein expression affects integrity of rubber particles and rubber content in *Taraxacum brevicorniculatum*, *PLoS One* 7 (2012) e41874.
- [20] D. Wahler, T. Colby, N.A. Kowalski, A. Harzen, S.Y. Wotzka, A. Hillebrand, R. Fischer, J. Helsper, J. Schmidt, C. Schulze Gronover, D. Prüfer, Proteomic analysis of latex from the rubber-producing plant *Taraxacum brevicorniculatum*, *Proteomics* 12 (2012) 901–905.
- [21] J. Collins-Silva, A.T. Nural, A. Skaggs, D. Scott, U. Hathwaik, R. Woolsey, K. Schegg, C. McMahan, M. Whalen, K. Cornish, D. Shintani, Altered levels of the *Taraxacum kok-saghyz* (Russian dandelion) small rubber particle protein, TKSPP3, result in qualitative and quantitative changes in rubber metabolism, *Phytochemistry* 79 (2012) 46–56.
- [22] R. Witsuwannakul, K. Rukseeree, K. Kanokwiroon, D. Witsuwannakul, A rubber particle protein specific for *Hevea* latex lectin binding involved in latex coagulation, *Phytochemistry* 69 (2008) 1111–1118.
- [23] G. Ponciano, C.M. McMahan, W. Xie, G.R. Lazo, T.A. Coffelt, J. Collins-Silva, A. Nural-Taban, M. Gollery, D.K. Shintani, M.C. Whalen, Transcriptome and gene expression analysis in cold-acclimated guayule (*Parthenium argentatum*) rubber-producing tissue, *Phytochemistry* 79 (2012) 57–66.
- [24] D.P. Attanyaka, R.G. Kekwick, F.C. Franklin, Molecular cloning and nucleotide sequencing of the rubber elongation factor gene from *Hevea brasiliensis*, *Plant Mol. Biol.* 16 (1991) 1079–1081.
- [25] E. Goyvaerts, M. Dennis, D. Light, N.H. Chua, Cloning and sequencing of the cDNA encoding the rubber elongation factor of *Hevea brasiliensis*, *Plant Physiol.* 97 (1991) 317–321.
- [26] B. Wagner, M. Krebitz, D. Buck, B. Niggemann, H.Y. Yeang, K.H. Han, O. Scheiner, H. Breiteneder, Cloning, expression, and characterization of recombinant Hev b 3, a *Hevea brasiliensis* protein associated with latex allergy in patients with *spina bifida*, *J. Allergy Clin. Immunol.* 104 (1999) 1084–1092.
- [27] K.S. Chow, K.L. Wan, M.N. Isa, A. Bahari, S.H. Tan, K. Harikrishna, H.Y. Yeang, Insights into rubber biosynthesis from transcriptome analysis of *Hevea brasiliensis* latex, *J. Exp. Bot.* 58 (2007) 2429–2440.
- [28] P. Priya, P. Venkatachalam, A. Thulaseedharan, Molecular cloning and characterization of the rubber elongation factor gene and its promoter sequence from rubber tree (*Hevea brasiliensis*): a gene involved in rubber biosynthesis, *Plant Sci.* 171 (2006) 470–480.
- [29] J.H. Ko, K.S. Chow, K.H. Han, Transcriptome analysis reveals novel features of the molecular events occurring in the laticifers of *Hevea brasiliensis* (para rubber tree), *Plant Mol. Biol.* 53 (2003) 479–492.
- [30] K.H. Han, D.H. Shin, J. Yang, I.J. Kim, S.K. Oh, K.S. Chow, Genes expressed in the latex of *Hevea brasiliensis*, *Tree Physiol.* 20 (2000) 503–510.
- [31] P. Priya, P. Venkatachalam, A. Thulaseedharan, Differential expression pattern of rubber elongation factor (REF) mRNA transcripts from high and low yielding clones of rubber tree (*Hevea brasiliensis* Muell. Arg.), *Plant Cell Rep.* 26 (2007) 1833–1838.
- [32] U. Sookmark, V. Pujade-Renaud, H. Chrestin, R. Lacote, C. Naiyanetr, M. Seguin, P. Romruensukharom, J. Narangajavana, Characterization of polypeptides accumulated in the latex cytosol of rubber trees affected by the tapping panel dryness syndrome, *Plant Cell Physiol.* 43 (2002) 1323–1333.
- [33] K. Berthelot, S. Lecomte, Y. Estevez, V. Zhendre, S. Henry, J. Thévenot, E.J. Dufourc, I.D. Alves, F. Peruch, Rubber particle proteins, HbREF and HbSRPP, show different interactions with model membranes, *Biochim. Biophys. Acta Biomembr.* (2013), <http://dx.doi.org/10.1016/j.bbamem.2013.08.025> (in press).
- [34] M.J. Dochniak, Multiple sclerosis and glycoprotein immunotherapy, <http://dochniak.info/>, in: Michael J. Dochniak (Ed.), *Medical hypothesis*, 2013.
- [35] S.A.M. Arif, R.G. Hamilton, F. Yusof, N.-P. Chew, Y.-H. Loke, S. Nimkar, J.J. Beintema, H.-Y. Yeang, Isolation and characterization of the early nodule-specific protein homologue (Hev b 13), an allergenic lipolytic esterase from *Hevea brasiliensis* latex, *J. Biol. Chem.* 279 (2004) 23933–23941.
- [36] K. Kanokwiroon, Antimicrobials From Latex of *Hevea brasiliensis*, Biochemistry, PhD thesis, Prince of Songkla University, Songkhla, Thailand, 2007.
- [37] S. Liengprayoon, K. Siroth, E. Dubreucq, L. Vaysse, Glycolipid composition of *Hevea brasiliensis* latex, *Phytochemistry* 72 (2011) 1902–1913.
- [38] Z. Chen, R. Cremer, A. Posch, M. Raulf-Heimsoth, H.P. Rihs, X. Baur, On the allergenicity of Hev b 1 among health care workers and patients with *spina bifida* allergic to natural rubber latex, *J. Allergy Clin. Immunol.* 100 (1997) 684–693.
- [39] A.B. Czuppon, Z. Chen, S. Rennert, T. Engelke, H.E. Meyer, M. Heber, X. Baur, The rubber elongation factor of rubber trees (*Hevea brasiliensis*) is the major allergen in latex, *J. Allergy Clin. Immunol.* 92 (1993) 690–697.
- [40] L. Tarachiwin, J. Sakdapipanch, K. Ute, T. Kitayama, T. Bamba, E. Fukusaki, A. Kobayashi, Y. Tanaka, Structural characterization of alpha-terminal group of natural rubber. 1. Decomposition of branch-points by lipase and phosphatase treatments, *Biomacromolecules* 6 (2005) 1851–1857.
- [41] L. Tarachiwin, J. Sakdapipanch, K. Ute, T. Kitayama, Y. Tanaka, Structural characterization of alpha-terminal group of natural rubber. 2. Decomposition of branch-points by phospholipase and chemical treatments, *Biomacromolecules* 6 (2005) 1858–1863.
- [42] J. Fricke, A. Hillebrand, R.M. Twyman, D. Prüfer, C. Schulze Gronover, Abscise acid-dependent regulation of small rubber particle protein gene expression in *Taraxacum brevicorniculatum* is mediated by TbbZIP1, *Plant Cell Physiol.* 54 (2013) 448–464.
- [43] J. d'Auzac, H. Crétin, B. Marin, C. Lioret, A plant vacuolar system: the lutoids from *Hevea brasiliensis* latex, *Physiol. Vég.* 20 (1982) 311–331.
- [44] P.J. Horn, C.N. James, S.K. Gidda, A. Kilaru, J.M. Dyer, R.T. Mullen, J.B. Ohlrogge, K.D. Chapman, Identification of a new class of lipid droplet-associated proteins in plants, *Plant Physiol.* 162 (2013) 1926–1936.

- [45] B.L. Kagan, H. Jang, R. Capone, F. Teran Arce, S. Ramachandran, R. Lal, R. Nussinov, Antimicrobial properties of amyloid peptides, *Mol. Pharm.* 9 (2011) 708–717.
- [46] S. Amnuaypornsi, J. Sakdapipanich, S. Toki, B.S. Hsiao, N. Ichikawa, Y. Tanaka, Strain-induced crystallization of natural rubber: effect of proteins and phospholipids, *Rubber Chem. Tech.* 81 (2008) 753–766.
- [47] S.S. Sarkawi, W.K. Dierkes, J.W.M. Noordermeer, The effect of protein content in natural rubber on performance of silica filled compounds as influenced by processing temperature, *KGK-Kautsch. Gummi Kunstst.* 66 (2013) 27–33.
- [48] K. Tamura, D. Peterson, N. Peterson, G. Stecher, M. Nei, S. Kumar, MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods, *Mol. Biol. Evol.* 28 (2011) 2731–2739.
- [49] K. Cornish, R.A. Backhaus, Rubber transferase activity in rubber particles of guayule, *Phytochemistry* 29 (1990) 3809–3813.
- [50] D. Blaudez, J.-M. Turlet, J. Dufourcq, D. Bard, T. Buffeteau, B. Desbat, Investigations at the air/water interface using polarization modulation IR spectroscopy, *J. Chem. Soc. Faraday Trans.* 92 (1996) 525–530.
- [51] H.P. Ta, K. Berthelot, B. Couлары-Salin, B. Desbat, J. Géan, L. Servant, C. Cullin, S. Lecomte, Comparative studies of nontoxic and toxic amyloids interacting with membrane models at the air–water interface, *Langmuir* 27 (2011) 4797–4807.
- [52] Y. Liao, J. Lescar, J.P. Tam, D.X. Liu, Expression of SARS-coronavirus envelope protein in *Escherichia coli* cells alters membrane permeability, *Biochem. Biophys. Res. Commun.* 325 (2004) 374–380.

SUPPLEMENTARY INFORMATION

Homologous *Hevea brasiliensis* REF (Hevb1) and SRPP (Hevb2) present different auto-assembling

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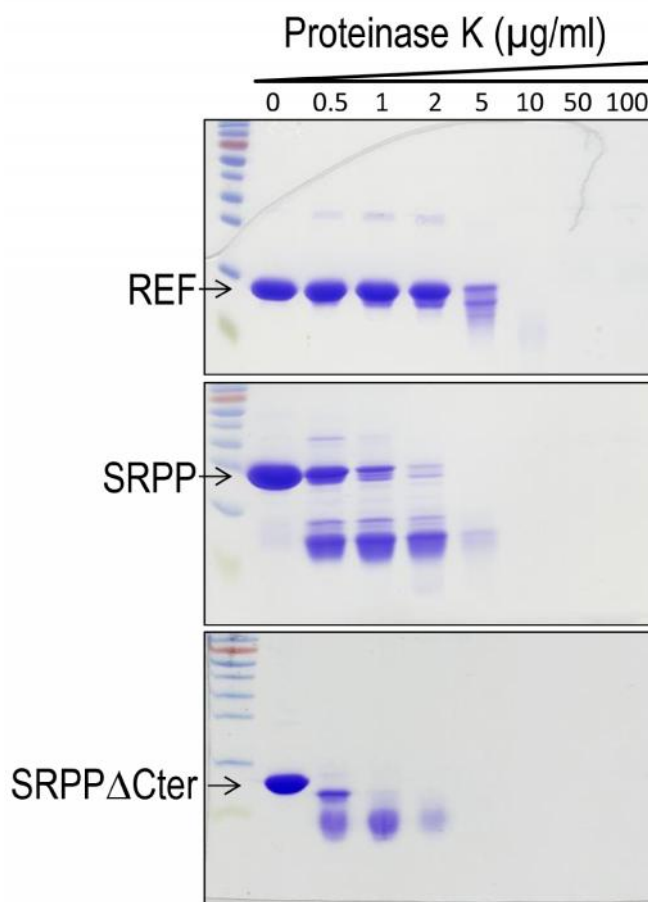
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SUPPLEMENTARY MATERIAL & METHODS

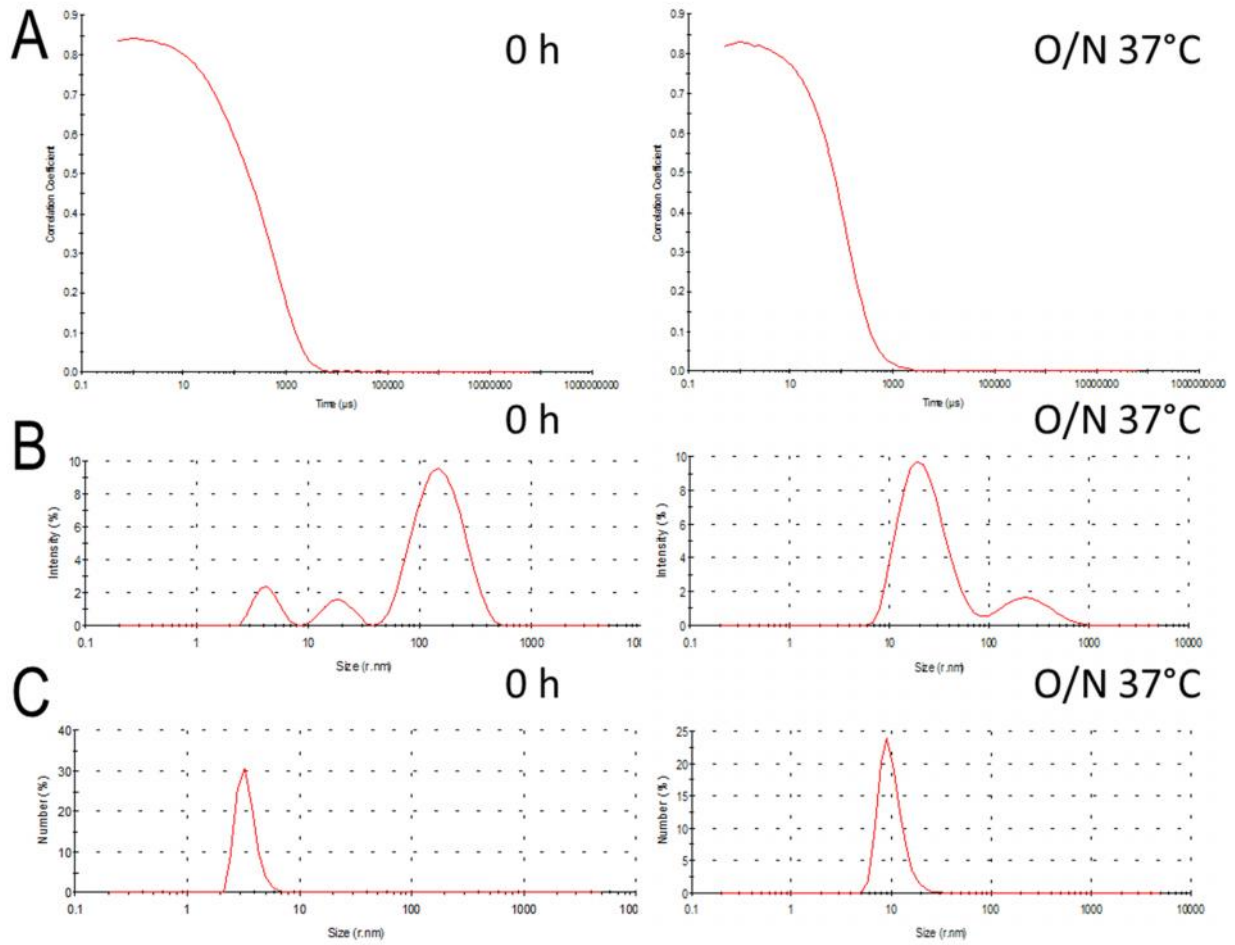
Limited proteolysis with proteinase K

Proteins were incubated at 100 μ M in 1X PBS for 3 h at 37°C. Eight μ l of each 100 μ M protein solution were mixed with 8 μ l of an appropriate dilution of proteinase K (Euromedex), and incubated at 37°C for 30 min. Digestion was stopped by adding 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 analyzed by 15% SDS-PAGE and stained with Coomassie blue.

SUPPLEMENTARY FIGURE AND TABLES

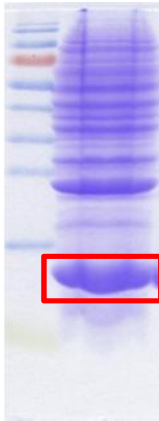


Supplementary Fig. S1. Differential proteinase K resistance of REF, SRPP and SRPP Δ Cter after incubation with increasing concentration of proteinase K, 30 min at 37°C and visualization on 15% SDS-PAGE. REF and SRPP results were previously published in Berthelot *et al.*, 2012. *PLoS One*, 7, e48065. Note: the fragmentation of SRPP seems to generate auto-assembling of SRPP, also visible for REF.



Supplementary Fig. S2. Raw data of SRPP Δ Cter auto-assembling followed by DLS. A) Correlograms of SRPP Δ Cter at 0 h (left panels) and after overnight incubation at 37°C (right panels). B) Size distribution in intensity. C) Size distribution in numbers. Note that the sizes are expressed as half diameters (r).

SUPPLEMENTARY TABLE S1: Identification of REF and SRPP on SDS-PAGE^a by mass spectrometry LC-MS/MS analysis^b.

Protein Name	Accession n°	Peptide sequences	Score coverage	AAs	MW [kDa]
	[REF_HEVBR] P15252	1# Proteins ; 9# Unique Peptides ; 23# Peptides DASIQVVS AIR DKSGPLQPGVDIIEGPVK DKSGPLQPGVDIIEGPVKNVAVPLYNR FSYIPNGALK FSYIPNGALKFVDSTVVASVTIHDR FSYIPNGALKFVDSTVVASVTIHDRSLPPIVK FVDSTVVASVTIHDR FVDSTVVASVTIHDRSLPPIVK FVDSTVVASVTIHDRSLPPIVKDASIQVVS AIR ILAKVIFYGEN MAEDEDNQGGQGEGLK NVAVPLYNR NVAVPLYNRFSYIPNGALK NVAVPLYNRFSYIPNGALKFVDSTVVASVTIHDR SGPLQPGVDIIEGPVK SGPLQPGVDIIEGPVKNVAVPLYNR SLASSLPGQTK SLASSLPGQTKILAK SLASSLPGQTKILAKVIFYGEN SLPPIVKDASIQVVS AIR SLPPIVKDASIQVVS AIRAAPEAAR YLG FVQDAATYAVTTFSNVYLFAK YLG FVQDAATYAVTTFSNVYLFAKDK	100.00	138	14.7
	[SRPP_HEVBR] O82803	1# Proteins ; 19# Unique Peptides ; 19# Peptides AAGVYAVDSFSTLYLYAK AEQYAVITWR ALNKLPLVPQVANVVVPTAVYFSEK ALYANLEPK ALYANLEPKAEQYAVITWR DISGPLKPGVDTIENVVK DISGPLKPGVDTIENVVKTVVTPVYYIPLEAVK FVDKTVDSVTS LDGVVPPVIK IVLDVASSVENTGVQEGAK LKYLD FVR LPLVPQVANVVVPTAVYFSEK LPLVPQVANVVVPTAVYFSEKYN DVVR QVSAQTYSVAQDAPR TVDVSVTS LDGVVPPVIK TVDVSVTS LDGVVPPVIKQVSAQTYSVAQDAPR TVVTPVYYIPLEAVK TVVTPVYYIPLEAVK FVDK VSSYLPLL PTEK YLDFVR	86.76	204	22.3

^a Bands from SDS-PAGE were cut, digested by trypsin, extracted and submitted to a LC-MS/MS analysis. Parameters for chromatography were as followed: chromatography solvent A, 95/05/0.1 H₂O/ACN/HCOOH v/v/v, B, 20/80/0.1 H₂O/ACN/HCOOH v/v/v; gradient 5-40 % B in 35 minutes; injected volume 10 µL; precolumn 300-µm ID x 5-mm C18 PepMapTM; precolumn flow rate 20 µl/min; column 75-µm ID x 15-cm C18 PepMapTM; column flow rate 200 nl/min.

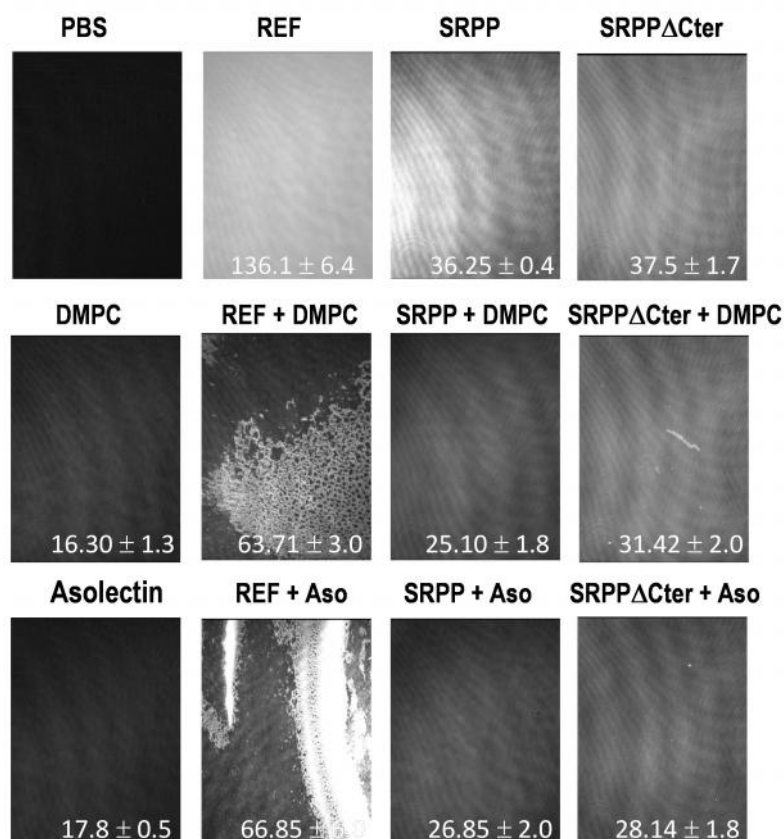
^b Parameters for mass spectrometry were as followed: needle voltage 1.8 V; capillary voltage 39 V; µscan MS 1; µscan MS² 1; MSrange m/z 300-1700; M/MS strategy, MS + 6 CID MS/MS; min. signal required 500; ion isolation window 3 m/z units; normalized collision energy 35%; default charge state 3; activation Q 0.25; activation time 30; dynamic exclusion ON; dynamic exclusion params, Repeat Count 1, Repeat Duration 30s, Exclusion Duration 30s; charge state screening ON; charge state rejection ON; charge state rejection params +1, +4 and unassigned rejected.

SUPPLEMENTARY TABLE S2. ATR-FTIR analyses of REF, SRPP^a and SRPPΔCter.

Amide Secondary structure Element	Wavenumbers (cm ⁻¹)	Relative comparative (%)		
	REF, SRPP, SRPPΔCter	REF	SRPP	SRPPΔCter
Aggregation	1614, 1614, 1610	9.12	6.52	11.77
β-sheet	1630, 1629, 1628	37.81	20.39	21.31
Random coil	1646, 1642, 1642	15.20	24.59	25.37
Helix	1657, 1656, 1656	14.25	21.04	19.98
Turn	1670, 1670, 1670	11.37	13.61	11.13
Hydrophobic domain	1685, 1685, 1684	12.25	13.85	10.44

^a Results of REF and SRPP analyses were obtained previously (Berthelot *et al.*, 2012).

SUPPLEMENTARY TABLE S3. Comparison of surface layer thicknesses and ellipsometric angles of REF, SRPP and SRPP Δ Cter with lipid monolayers on PBS pH 7.4 as subphase.



Ellipsometric angle ($\delta\Delta$, Ψ) and layer thickness						
	Protein at air/water interface		Lipid monolayer + protein ^a			
			Asolectin		DMPC	
	$ \delta\Delta ^\circ$ Ψ°	Thickness ^c Å	$ \delta\Delta ^\circ$ Ψ°	Thickness Å	$ \delta\Delta ^\circ$ Ψ°	Thickness Å
No Protein	N/A N/A	N/A	6.36 ± 0.15 2.40 ± 0.12	17.80 ± 0.50	4.57 ± 0.37 2.40 ± 0.12	16.30 ± 1.32
REF	38.12 ± 1.80 2.40 ± 0.12	136.1 ± 6.40	18.72 ± 1.69 2.40 ± 0.12	66.85 ± 6.00	17.84 ± 0.84 2.40 ± 0.12	63.71 ± 3.00
SRPP	10.15 ± 0.12 2.40 ± 0.12	36.25 ± 0.40	7.52 ± 0.52 2.40 ± 0.12	26.85 ± 2.00	7.01 ± 0.52 2.40 ± 0.12	25.10 ± 1.85
SRPP Δ Cter	10.50 ± 0.50 2.45 ± 0.12	37.50 ± 1.79	7.88 ± 0.51 2.45 ± 0.12	28.14 ± 1.82	8.8 ± 0.52 2.45 ± 0.12	31.42 ± 2.00

N/A, not applicable

^a Lipid monolayers were formed at a constant pressure of 28 mN/m and at 25°C. REF and SRPP were injected at 1 μ M in a PBS 1X buffer subphase.

^b Ellipsometry angles $|\delta\Delta|$ and Ψ ; $|\delta\Delta| = |\Delta - \Delta_0|$. Pressure was at the equilibrium state (less than 1h), when the pressure values were at plateau.

^c The thickness was determined using a mean value of 1.45 for the refractive index. Results are presented as mean $\pm$ SD. Ellipsometric images (top panel) are presented with their respective thickness.

Results of REF and SRPP were obtained from Berthelot *et al.*, 2012.

SUPPLEMENTARY TABLE S4: Physico-chemical parameters of His-tagged REF, SRPP and SRPP Δ Cter proteins

Proteins	Amino acids	MW (KDa)	pI	Ext. Coefficient (L.mol ⁻¹ .cm ⁻¹)	Net charge	Hydropathicity
REF	147	15.8	6.3	8940	-2	-0.084
SRPP	213	23.4	5.5	24870	-6	0.003
SRPP Δ Cter	140	15.3	5.5	11920	-6	0.076

Data at pH 7.4, 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 ExPASy proteomics Server, <http://expasy.org/tools/protparam.html>.