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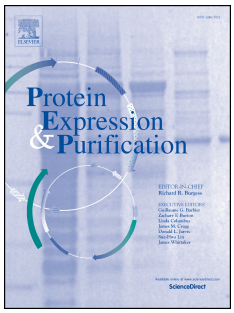

Integrated process for the purification and immobilization of the envelope protein domain III of dengue virus type 2 expressed in *Rachiplusia nu* larvae and its potential application in a diagnostic assay

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Abstract

Dengue incidence has grown dramatically in the last years, with about 40% of the world population at risk of infection. Recently, a vaccine developed by Sanofi Pasteur has been registered, but only in a few countries. Moreover, specific antiviral drugs are not available. Thus, an efficient and accurate diagnosis is important for disease management. To develop a low-cost immunoassay for dengue diagnosis, in the present study we expressed the envelope protein domain III of dengue virus type 2 in *Rachiplusia nu* larvae by infection with a recombinant baculovirus. The antigen was expressed as a fusion to hydrophobin I (DomIIIHFBI) to easily purify it by an aqueous two-phase system (ATPS) and to efficiently immobilize it in immunoassay plates. A high level of recombinant DomIIIHFBI was obtained in *R. nu*, where yields reached 4.5 mg per g of larva. Also, we were able to purify DomIIIHFBI by an ATPS with 2% of Triton X-114, reaching a yield of 73% and purity higher than 80% in a single purification step. The recombinant DomIIIHFBI was efficiently immobilized in hydrophobic surface plates. The immunoassay we developed with the immobilized antigen was able to detect IgG specific for dengue virus type 2 in serum samples and not for other serotypes.

Keywords: *Rachiplusia nu* larvae; Recombinant fusion protein; Hydrophobin; Domain III; Dengue; Diagnostic immunoassay.

44 **Abbreviations**

45 *Ac*MNPV: *Autographa californica* multiple nucleopolyhedrovirus

46 ATPS: aqueous two-phase system

47 DomIII: envelope protein domain III

48 dpi: day post-infection

49 DENV: dengue virus

50 DENV-2: dengue virus type 2

51 E: envelope protein of dengue virus

52 HFBI: hydrophobin I of *Trichoderma reesei*

53 MOI: multiplicity of infection

54

Introduction

Dengue is a viral disease transmitted to humans by female mosquitoes mainly of the species *Aedes aegypti* [1]. The incidence of dengue has grown dramatically in the last years and around 390 million infections occur each year [2]. Recently, a vaccine developed by Sanofi Pasteur has become available, but it has been approved only in a few countries (Mexico, The Philippines, Brazil, El Salvador and Costa Rica), while specific antiviral drugs are not available yet [3, 4]. Thus, an efficient and accurate diagnosis is very important for the management of the disease and the prevention of epidemics. For dengue diagnosis, serological assays are routinely used because of their simplicity and availability [5, 6]. Currently, most of the available serological assays use the whole virus as a source of antigens, which implies high costs associated with virus cultivation and potential biohazard associated with the exposure to infectious viral particles [7]. Moreover, the use of the whole virus decreases diagnosis specificity because of cross reaction with other flaviviruses. On the other hand, the commercially available kits that use recombinant antigens are expensive. Thus, the production of a recombinant antigen in a low-cost platform that avoids whole virus manipulation and enhances specificity becomes an attractive alternative [8, 9]. Among the structural proteins of dengue virus (DENV), the domain III (DomIII) of the envelope (E) protein induces serotype-specific antibodies [10] and thus represents an interesting antigen for the development of a specific diagnostic assay [11-16].

The baculovirus-insect cell system is a versatile eukaryotic expression system for the production of recombinant proteins for biotechnological or pharmaceutical applications [17, 18]. Sf9, Sf21 and HiFive insect cell lines are widely used as hosts because of their susceptibility to *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV), the

most commonly used baculovirus expression vector. However, the main disadvantages of this system at industrial scale are its high cost associated with the use of reactors and the need of tissue-culture facilities [19], and the high risk of contamination. A low-cost alternative is the production of recombinant proteins directly in live insect larvae as "biofactories" [20, 21]. Particularly, lepidopteran pest insects, such as *Rachiplusia nu*, which is widely distributed in tropical and subtropical regions of the Americas, are susceptible hosts to *Ac*MNPV infection [22, 23]. Previous studies in our lab showed that *R. nu* was an excellent host for the production of horseradish peroxidase, when comparing to three other lepidopteran species (*Spodoptera frugiperda*, *Helicoverpa zea* and *Heliothis virescens*), since it was susceptible to intrahemocele and oral infection with *Ac*MNPV, reaching high yields in both systems [21, 23]. *R. nu* larvae are a destructive plague affecting several economically important crops such as soy and corn. In general, the use of larvae as biofactories yields higher amounts of the recombinant protein than cultured insect cells [24]. However, this technology lacks well-established downstream processes.

Aqueous two-phase systems (ATPS) are an attractive technology that integrates clarification, concentration, and partial purification in one step [25]. Hydrophobins (HFBs) –small amphiphilic proteins of around 7-10 kDa produced by filamentous fungi [26]- can be used as fusion tags for the purification of recombinant proteins by surfactant-based ATPS [27-32]. In these systems, HFB-fused proteins partition to the surfactant-rich phase while the majority of the proteins remain in the aqueous phase. Thus, in a simple purification step, the fusion protein is obtained with high purity and yield. On the other hand, HFBs interact strongly and spontaneously with hydrophobic surfaces, allowing the

immobilization of HFB-fused antigens to solid supports commonly used in serological assays (such as enzyme-linked immunosorbent assays-ELISAs) [33, 34].

The aim of this work was to produce a recombinant antigen of dengue virus in a low-cost platform for the development of a serological diagnostic assay. Particularly, we assessed the expression of dengue virus type 2 domain III fused to HFBI (DomIIIHFBI) in Sf9 insect cells and *R. nu* larvae. We also studied the purification of DomIIIHFBI by surfactant-based ATPS and showed the application of the recombinant antigen in a specific diagnostic immunoassay.

Materials and methods

Materials

Sf900II insect tissue culture media, the *Spodoptera frugiperda* Sf9 cell line, Cellfectin® and penicillin/streptomycin (ATB/ATM) were from Invitrogen Life Technologies (Gaithersburg, MD, USA). *Rachiplusia nu* larvae were obtained from AgIdea S.A. (Pergamino, Buenos Aires, Argentina). Fetal bovine serum (FBS) was from Internegocios (Buenos Aires, Argentina). Agarplaque Plus and BaculoGold Bright were from BD Biosciences Pharmingen (San Diego, CA, USA). Disposable materials and multiwell plates were from Nunc International (Naperville, IL, USA). Triton X-114 was from Sigma-Aldrich (St. Louis, MO, USA). Dengue Virus Subtype 2 Envelope15kDa, C-Terminal (Domain III) Recombinant was from Prospec Tany TechnoGene Ltd. (Ness Ziona, Israel). The PageRuler™ Prestained Protein Ladder (10 – 170 kDa), Cat. number SM0671 used in SDS-PAGEs and Western blots was from Fermentas.

121 Expression cassette

122 The cDNA of DENV-2 E protein (strain 16681, GenBank accession no. **U87411.1**) was
123 kindly provided by Dr. A. V. Gamarnik (Fundación Instituto Leloir, Argentina). Using this
124 cDNA as a template, the DomIII sequence was amplified by PCR1 with the specific
125 primers 5'CGCGGATCCATGGACAAGCTACAGCTC3' (Primer n°1, sense, BamHI site
126 underlined) and 5'AGAGCCTCCACCTTGGCCGATAGAACTTTCCTT3' (Primer n°2,
127 antisense, HFBI specific sequence underlined). The cDNA of HFBI of *Trichoderma reesei*
128 was kindly provided by Dr. R. Menassa (Agriculture and Agri-Food Canada, Canada), in a
129 plasmid called pjjj161 which contains the gene GFPHFBI [30]. Using this cDNA as a
130 template, the HFBI sequence was amplified by PCR2 with the specific primers
131 5'TCTATCGGCCAAGGTGGAGGCTCTGGTGGA3' (Primer n°3, sense, DomIII specific
132 sequence underlined) and 5'GGAATTC**CTATCA**CTTCTCAAATTGAGGATG3' (Primer
133 n°4, antisense, EcoRI site underlined, stop codons in bold). The fusion DomIIIHFBI was
134 obtained by overlapping PCR using the products of PCR1 and PCR2 as templates and the
135 specific primers n°1 and n° 4. DomIIIHFBI gene was then cloned using BamHI and EcoRI
136 sites into the pAcGP67-B vector (BD Biosciences Pharmingen), which contains a sequence
137 for the glycoprotein 67 (GP67) leader peptide that targets the recombinant protein for
138 secretion (pAcDIIIHFBI).

139 Another vector containing the expression cassette for green fluorescent protein (GFP) fused
140 to HFBI (GFPHFBI) was constructed to use as a control of the process. The GFPHFBI
141 gene was amplified by PCR using the plasmid pjjj161 as a template and the specific
142 primers 5´CGCGGATCCGTGAGCAACGGCGACGAG (sense, BamHI site underlined)

and Primer n°4. GFPHFBI gene was then cloned using BamHI and EcoRI sites into the pAcGP67-B vector (pAcGFPHFBI).

Virus production

One million Sf9 cells were co-transfected with 2 µg pAcDIIIHFBI and 1 µg linearized BaculoGold Bright DNA (BD Biosciences Pharmingen) in the presence of Cellfectin®. BaculoGold Bright DNA contains the gene for GFP. After a 4-day incubation at 27 °C, the cell culture supernatant was collected and centrifuged at 3000 ×g for 10 min. Co-transfection efficiency was determined by measuring GFP expression by fluorescence under UV light. The recombinant baculovirus polyhedrin-minus vector containing the DomIIIHFBI expression cassette was named AcDIIIHFBI. Following three amplification steps, the virus titer was determined by a plaque assay (1.1×10^8 pfu/ml) [35]. This amplified virus stock was used for the production of the recombinant protein in further experiments.

A recombinant baculovirus vector containing the GFPHFBI expression cassette (AcGFPHFBI) was also produced, following the same protocol and using pAcGFPHFBI vector.

Insect cell infection

Sf9 suspension cultures (2×10^6 Sf9 cells/ml) grown in Sf900II medium supplemented with 1% of FBS were infected with AcDIIIHFBI at a multiplicity of infection (MOI) of 0.5 or 2 and then incubated in the dark at 27 °C for 6 days or until the day indicated for sample collection. To study the expression among the different day post-infection (dpi), samples of

1 mL were collected each day. The culture supernatant was separated from the cells by centrifugation at 10,000 × g for 10 min. The pellet and the supernatant were stored at -20°C until further experiments. For SDS-PAGE and Western blot analysis, the culture supernatant was assessed without any extra treatment while the cell pellet was treated as it is indicated in the next section to obtain total protein extracts. For control purposes, Sf9 suspension cultures infected with AcGFPHFBI were used.

Total protein extraction from infected cells

The cell pellet was resuspended in lysis buffer containing 3% sodium dodecyl sulfate (SDS), 1 mM dithiothreitol (DTT) and protease inhibitor cocktail (Sigma Aldrich) by adding 100 µl of lysis buffer per ml of original culture. After incubation at 4°C for 40 min, the cellular debris was separated by centrifugation at 12,000 × g for 30 min at 4°C. The pellet was discarded and the supernatant was stored at -20°C until further experiments.

Larvae infection

R. nu larvae were obtained from a laboratory colony fed on a high-wheat germ diet [36]. Second-instar larvae were reared in six-well dishes (one per well) at 23‑25 °C in a 70% humidified chamber, with a 16:8 photoperiod (L:D). Fifth-instar larvae (14 days old, approximately 100 mg) were infected with 50 µl AcDIIIHFBI recombinant baculovirus (1 × 10^7 pfu/ml) by intrahemocele injection. Control larvae were infected with AcGFPHFBI baculovirus. At 3 dpi, larvae were harvested and stored at −20°C until further experiments.

Total protein extraction from *R. nu* larvae

Infected larvae were homogenized in the presence of 1 ml of lysis buffer per larva using a mortar and pestle. Phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.0) with and without 3% SDS and 1 mM DTT was tested as lysis buffer. Protease inhibitor cocktail (Sigma Aldrich) and glutathione crystals were added in all cases and three cycles of sonication were assessed when indicated. Then, samples were centrifuged at 14,000 × g for 10 min at 4°C and the supernatants were collected obtaining the larval crude extracts.

DomIIIHFBI purification by ATPS with Triton X-114

ATPS purification was performed according to the procedure described by Joensuu *et al.* [30], with minor modifications. Briefly, Triton X-114 surfactant was added directly to ice cold cell culture supernatants and larval crude extracts at final concentrations of 2%, 5% and 8% v/v, mixed with a vortex for 5 min and incubated for 15 min in an ice bath. The phases were then allowed to separate for 20 min at 30°C in a water bath. After a centrifugation step (3000 × g, 5 min, 30°C), the lower surfactant phase was recovered. To wash the detergent, one volume of isobutanol was added to this phase, mixed with a vortex for 5 min and incubated for 10 min at room temperature. The phases were allowed to separate for 30 min at 30°C in a water bath and then samples were centrifuged as mentioned above. The upper isobutanol rich phase was discarded and the interphase and the lower phase were recovered for further analysis. Samples of each step of the purification process were analyzed by SDS-PAGE and Western blot. Twenty µg of total proteins was loaded into the gels for comparison between conditions, except for the samples named Aq2 which contain less concentration of total proteins, so 10 µg was loaded. Samples of DENV-

206 2 DomIII –without HFBI tag– (DomIII, Prospec) were also assessed, following the same
207 protocol.

208 Total protein measurement

209 Protein concentration was measured using the Bradford assay [37] with bovine serum
210 albumin as the standard.

211 Electrophoretic analyses

212 Laemmli sample buffer was added to protein samples and after boiling for 5 minutes the
213 samples were loaded and separated by SDS-PAGE on 12% polyacrylamide gels following
214 the protocol described by Laemmli [38] and stained with Coomassie Blue [39]. For
215 Western blot analysis, gels were transferred onto a nitrocellulose membrane and
216 DomIIIHFBI was detected using a rabbit polyclonal anti-DENV 1+2+3+4 antibody
217 (Abcam) in a dilution 1/833 as the primary antibody and Peroxidase-conjugated AffiniPure
218 Mouse anti-rabbit IgG (Jackson ImmunoResearch) in a dilution 1/4000 as the secondary
219 antibody [40]. Development was carried out with an enhanced chemiluminescent substrate
220 (Thermo Fisher Scientific) and high performance chemiluminescence films (GE
221 Healthcare). For image processing, gels were scanned and then analyzed with the ImageJ
222 software (National Institutes of Health, USA). The amount of DomIIIHFBI per gram of
223 larva, the purity of the recombinant protein and the purification yields were assessed by
224 densitometric analysis of band intensities from SDS-PAGE.

225 ESI-Orbitrap mass spectrometry

A purified protein sample was separated by SDS-PAGE on 12% polyacrylamide gels and stained with colloidal Coomassie Blue. The band corresponding to the molecular weight of DomIIIHFBI was cut off, in-gel digested with trypsin and the peptides obtained were separated by HPLC (EASY-nLC 1000, Thermo Scientific) using a reverse phase column (EASY-Spray Column P/N ES801, Thermo Scientific) and analyzed by ESI-Orbitrap mass spectrometry (Q-Exactive, Thermo Scientific) with the software Proteome Discoverer version 1.4 (Thermo Scientific).

Protein immobilization

Purified DomIIIHFBI samples were diluted with PBS at concentrations ranging from 0.1 µg/ml to 12.5 µg/ml (68-540 nM) and 50 µl of each dlution was loaded in multiwell plates and incubated overnight at 4°C for protein immobilization. Two different plates were tested: polystyrene multiwell plates (Nunc) and *PolySorp* surface-treated –enhanced affinity to hydrophobic proteins– multiwell plates (Nunc). The immobilized protein was detected using the antibodies mentioned above, tetramethyl benzidine (TMB) chromogen solution (Life Technologies) and H_2SO_4 to stop the reaction. Plates were read using an ELISA EZ Read 400 Microplate Reader from Biochrom (Milton Road, Cambridge, UK) at a wavelength of 450 nm, using 620 nm as reference wavelength. All determinations were performed in triplicate and results are expressed as the average ± standard deviation (SD). Samples of DENV-2 DomIII –without HFBI tag– (DomIII, Prospec) were also assessed, following the same protocol. GFPHFBI-coated wells were included as a control.

Enzyme-linked immunosorbent assays (ELISAs)

Serum samples were tested for the detection of anti-DENV IgG antibodies by using polystyrene plates coated with DomIIIHFBI in a concentration of 6.25 µg/ml. Briefly, sera were diluted 1/100 in PBS-0.05% Tween 20 (PBS-T) and 2% non-fat dry milk and 50 µl of this dilution was incubated in the polystyrene plate for 1 h at 37°C. Plates were next washed with PBS-T and incubated with 50 µl of goat anti-human IgG horseradish peroxidase-conjugated (EMD Millipore Corporation) diluted 1/1000 for 1 h at 37 °C. Plates were washed again and the colorimetric reaction was developed by adding 100 µl of TMB as a substrate solution. After a 20-min incubation period at room temperature in the dark, color development was stopped by adding 100 µl of 0.18 M H_2SO_4. Optical densities (OD) were measured at 450 nm, using 620 nm as reference wavelength. All determinations were performed in triplicate and results are expressed as the average ± SD. Positive and negative sera were determined previously by a 90% plaque reduction neutralization test (PRNT90) with a flavivirus panel composed by West Nile virus (WNV, strain ChimeriVax TM WNV), Saint Louis encephalitis virus (SLEV, strain ChimeriVax TM SLEV), DENV-1 (strain Hawaii), DENV-2 (strain NGC), DENV-3 (strain H87), DENV-4 (strain H241) and Yellow fever virus (YFV, vaccine strain 17D-YEL). A selection of paired sera from patients showing monotypic neutralizing antibody pattern was employed [41]. Cut off was determined as the mean + 3 SD of negative samples.

Results and discussion

Expression and purification of recombinant DomIIIHFBI.

We cloned the DomIIIHFBI expression cassette into the pAcGP67-B vector (BD Biosciences Pharmingen) as a fusion with gp67 signal peptide under the control of the

strong baculovirus polyhedrin promoter and we obtained the vector pAcDIIIHFBI. The gp67 signal peptide targets the recombinant protein to the secretion pathway. Then, by co-transfection of this vector with linearized BaculoGold Bright DNA (BD Biosciences Pharmingen) and after amplification in Sf9 insect cells, we obtained AcDIIIHFBI recombinant baculovirus stock for the expression of DomIIIHFBI in insect cells and larvae. We also produced a recombinant baculovirus for the expression of GFPHFBI to use as a control in the expression and purification processes (Fig. 1).

To determine DomIIIHFBI expression and localization in insect cell cultures, we infected Sf9 cells with AcDIIIHFBI at a MOI of 0.5 and analyzed culture supernatants and cell pellets at different dpi by Western blot (Fig. 2). Also, a MOI of 2 was assessed and no significant differences were found (data not shown). In order to compare among the different dpi, 20 µl of the culture supernatant of each day and 40 µg of total proteins from cell extracts of each day was analyzed for the extracellular and intracellular expression, respectively.

Sf9-infected cells expressed DomIIIHFBI with the expected molecular weight of 22 kDa. Despite having the gp67 signal peptide, part of the expressed protein was efficiently exported to the supernatant while part remained in the intracellular compartment. As the purification process is generally easier from culture supernatant and secreted proteins are in a soluble form and well processed, thus yielding a homogeneous product, we continued working with the secreted protein. Therefore, for further purification experiments, we collected the 2 dpi supernatant of the Sf9 cell line infected at a MOI of 0.5.

To develop a simple and inexpensive purification process, we assessed recombinant DomIIIHFBI recovery by surfactant-based ATPS. Thus, Triton X-114 was added directly to culture supernatants at three concentrations: 2%, 5% and 8% v/v. Figure 3 shows the SDS-PAGE and Western blot analysis of the purification process. In the surfactant-based ATPS, DomIIIHFBI partitioned to the surfactant lower phase. This behavior is due to the presence of HFBI in the fusion protein, since DomIII without the HFBI tag (Prospec) partitioned mainly to the aqueous upper phase of the surfactant-based ATPS (data not shown). When isobutanol was added to the surfactant phase for the recovery of the recombinant protein in an aqueous phase depleted of surfactant, the protein was found in an interphase that developed between the aqueous lower phase and the isobutanol upper phase. Linder *et al*. reported that, among the solvents they tested, isobutanol was the most efficient for the back-extraction of HFBI from the surfactant phase [27]. Joensuu *et al.* also reported similar results for the recovery of GFPHFBI with isobutanol from the surfactant phase [30], without observing denaturation of the protein. Jacquet *et al*. partially purified Influenza virus hemagglutinin fused to HFBI by ATPS with Triton X-114 and isobutanol, without observing protein denaturation [42]. In contrast, we observed protein precipitation due to isobutanol when we purified DomIIIHFBI and also GFPHFBI. Despite this, we took advantage of this interphase formation to further concentrate the protein and recover it in an isobutanol-free buffer.

We found that the purity of the recombinant protein decreased with increasing Triton X-114 concentrations. When the purification process was performed with 2% of Triton X-114, we recovered 16.7 µg of DomIIIHFBI per ml of culture supernatant with a purity of 20%.

312 Although the purity reached was low, in a single purification step it was possible to
313 concentrate and partially purify the recombinant protein.

314 Based on these results obtained using the insect cell line, we assessed the expression of
315 DomIIIHFBI directly in insect larvae to develop a low-cost platform suitable to scale up the
316 process. Thus, we infected *R. nu* larvae with AcDIIIHFBI baculovirus by intrahemocele
317 injection. We found that *R. nu* larvae were able to express DomIIIHFBI with the expected
318 molecular weight of 22 kDa and observed a single band in the Western blot analysis, which
319 indicates that the protein is obtained as a homogenous product. We efficiently extracted
320 DomIIIHFBI from larvae with PBS without adding SDS and sonication disruption (Fig. 4),
321 in contrast with the results obtained in insect cells where the extraction was not possible
322 using only PBS and more drastic extraction conditions were needed (data not shown).
323 DomIIIHFBI expression level in *R. nu* larvae was 4.5 mg per g of larva according to the gel
324 densitometry analysis, higher than that reached for other recombinant proteins previously
325 produced in our laboratory with the same expression system: wheat germ agglutinin (346.6
326 ± 88.5 µg per g of larva) [43], feline interferon alpha (116 ± 6.3 µg per g of larva) [44] and
327 Influenza A H1N1 neuraminidase (1.2 mg per g of larva)[45].

328 For DomIIIHFBI purification by ATPS, we added Triton X-114 directly to larval crude
329 extracts at three concentrations: 2%, 5% and 8% v/v. As observed for culture supernatants,
330 DomIIIHFBI partitioned to the surfactant lower phase and then to the interphase of the
331 isobutanol aqueous system. Also, the purity decreased when the concentration of Triton X-
332 114 increased (Fig. 5). In this case, the fact that the protein remained in the interphase of
333 the isobutanol aqueous system increased the purity of the recombinant protein as many
334 contaminant proteins partitioned to the aqueous phase of this system. In a single

purification step, when using 2% surfactant, the purity reached more than 80% and we recovered 3.3 mg of DomIIIHFBI per g of infected larvae, with a yield of 73% and a purification factor of 13. Although larval crude extracts contain much more contaminants than culture supernatants, the purity obtained from larval extracts was higher than that obtained from supernatants when using the same purification process. Moreover, the use of larval crude extracts without the need of conditioning made this purification strategy a promising approach for recombinant protein purification from insect larvae, one of the main difficulties of this system.

Lahtinen et al. previously expressed chicken avidin fused to the HFBI of *T. reseei* (avidin-HFBI) in baculovirus infected insect cells. The authors showed that, despite having the authentic signal sequence of avidin, the recombinant protein was not detected in the extracellular space. Avidin-HFBI was purified from cell extracts by ATPS with the non-ionic surfactant Agrimul NRE 1205 at a final concentration of 10%. This surfactant is an ester of a linear fatty alcohol (whereas Triton X-114 contains an aromatic structure) and a short poly ethoxy chain and thus, the surfactant phase forms the upper layer of the ATPS. Avidin-HFBI was concentrated in the upper surfactant phase with a purity of 97%. Although the purity reached was very high, it seems that a great amount of the recombinant protein was lost in the lower aqueous phase of the ATPS [29]. In contrast, we did not detect DomIIIHFBI in the aqueous phase of the Triton X-114 based ATPS neither when purifying the protein from the culture supernatant (Fig. 3), nor when purifying the protein from larval extracts (Fig. 5). The authors did not study the expression and purification of avidin-HFBI in insect larvae.

We performed a mass spectrometry analysis of the purified protein after a trypsin treatment and found that the peptides obtained for our sample perfectly matched with those predicted for DomIIIHFBI, thus confirming the identity of the recombinant protein (data not shown). This analysis also allowed confirming that the signal peptide for gp67 glycoprotein was successfully processed during protein expression and was absent in the purified recombinant protein.

Table 1 shows a comparative analysis for the production of 1 mg of DomIIIHFBI in *R. nu* larvae and in the supernatant of Sf9 cells. In both cases, we considered the purification process using 2% of Triton X-114, the best condition among the assessed. Since we obtained 16.7 µg of DomIIIHFBI from 1 ml of culture supernatant, it is necessary to process 60 ml of culture supernatant to obtain 1 mg of the recombinant protein with a purity of 20%. On the other hand, we obtained 3.3 mg of DomIIIHFBI per gram of larva, so considering a weight of 0.2 g per larva, it is necessary to process only 2 larvae to obtain 1 mg of the recombinant protein with a purity higher than 80%. The amount of recombinant baculovirus needed for the production in insect larvae is up to 60 times lower than the amount needed for the production in culture supernatant and also the purification volume is 30 times lower. Thus, for further experiments, we used *R. nu* larvae as expression host and purified the recombinant protein by an ATPS, by adding 2% of Triton X-114 to larval crude extracts.

Development of an ELISA for the detection of anti-DENV IgG in serum samples

To develop an indirect ELISA for the detection of anti-DENV IgG, we tested the immobilization of DomIIIHFBI to multiwell plates. It has been reported that HFBs can

379 self-assemble at hydrophilic-hydrophobic interfaces [46], allowing the efficient
380 immobilization of fusion proteins to hydrophobic supports [47]. Thus, we selected two
381 hydrophobic supports for DomIIIHFBI immobilization: polystyrene multiwell plates
382 without any surface treatment and *PolySorp* surface-treated multiwell plates (Nunc), with
383 enhanced affinity for hydrophobic proteins. We compared the immobilization of
384 DomIIIHFBI with that of DomIII to study the effect of HFBI on the immobilization. We
385 found that DomIIIHFBI efficiently bound to both hydrophobic supports, while unfused
386 DomIII did not (Fig. 6). These results suggest that HFBI may be involved in DomIIIHFBI
387 immobilization on hydrophobic plates. As the results obtained with both plates were similar
388 and the cost of the polystyrene plates is half that of the *PolySorp* plates, we used
389 polystyrene plates for further experiments.

390 To optimize protein immobilization, we coated plates with DomIIIHFBI in concentrations
391 between 0.1 and 12.5 µg/ml. We found that 6.25 µg/ml was the lowest concentration of
392 antigen that gives the highest OD value, so we selected this concentration to coat plates for
393 further experiments. Considering that we used 50 µl per well of the diluted antigen, with
394 the amount of recombinant DomIIIHFBI recovered from a single larva, we were able to
395 coat 22 plates of 96 wells each.

396 Once the immobilization of the antigen was fixed, we used DomIIIHFBI-coated plates to
397 detect anti-DENV IgG in serum samples. We found that the ELISA we developed was able
398 to detect IgG specific for DENV-2 and was not able to detect IgG for the other serotypes
399 (DENV-1, DENV-3 and DENV-4) (Fig.7). These preliminary results are consistent with the
400 known characteristic of DomIII: it induces serotype-specific antibodies [48, 49]. Cardoso et
401 al. developed an indirect immunoassay for the detection of IgG and IgM anti-DENV by

using a recombinant DENV-1 DomIII produced in *Pichia pastoris* that showed a high degree of sensitivity and specificity for dengue diagnosis. However, the authors did not analyze the performance of the immunoassay for the detection of serotype specific IgG [16]. Tripathi et al. reported an excellent agreement among the results obtained with the in-house dipstick ELISA developed with DENV-4 DomIII antigen and commercial rapid Immunochromatography test and capture ELISA for the immunodiagnostic of dengue infections. The authors affirmed that the recombinant antigen obtained from *E. coli* was recognized by the antibodies present in the sera from all four serotypes of dengue virus infected samples [13]. We need to test a larger number of positive sera in order to confirm the serotype specificity of the immunoassay.

Currently, laborious and high-cost plaque reduction neutralization tests are required for the serotyping of dengue viruses in convalescent serum samples. Thus, the extension of this technology to produce the domain III of the other dengue virus serotypes would allow determining the serotype of the virus in a simple and cost-effective way, if the serotype specificity of the immunoassay is confirmed. On the other hand, one of the main problems of the serological assays commercially available is that they cross react with the immunoglobulins for other flaviviruses. The use of DomIII instead of the whole virus antigen may reduce this cross reactivity among flaviviruses. We tested IgG-positive sera for YFV and SLEV with the immunoassay we developed and found that it did not cross react with the immunoglobulins present in these samples (Fig. 7). More exhaustive tests should be performed with patient samples to accurately determine the immunoassay specificity.

Conclusions

We expressed the envelope protein domain III of dengue virus type 2 fused to hydrophobin I in *Rachiplusia nu* larvae with a high yield and efficiently purified it from larval crude extracts by a surfactant-based aqueous two-phase system. The use of live larvae as biofactories combined with ATPS for purification resulted in a low-cost platform for the production of DomIIIHFBI at large scale. To our knowledge, this is the first time that HFBI is used as a tag for the purification of a recombinant protein from larval extracts. This purification technology arises as a promising alternative for protein purification from larval extracts, one of the main disadvantages of this expression system. Moreover, HFBI enabled DomIII immobilization to polystyrene plates and the immobilized antigen was recognized by anti-DENV-2 IgG from serum samples.

Finally, this expression and purification platform could be applied for the production of antigens of other viruses or bacteria to develop diagnostic assays and subunit vaccines without the need to manipulate microorganisms.

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References

1. WHO. *Dengue and severe dengue: Fact sheet N° 117*. 2015 [cited 2015 May]; Available from: http://www.who.int/mediacentre/factsheets/fs117/en/.
2. Bhatt, S., et al., *The global distribution and burden of dengue.* Nature, 2013. **496**(7446): p. 504-507.
3. Heinz, F.X. and K. Stiasny, *Flaviviruses and their antigenic structure.* Journal of Clinical Virology, 2012. **55**(4): p. 289-295.
4. Lim, S.P., et al., *Ten years of dengue drug discovery: Progress and prospects.* Antiviral Research, 2013. **100**(2): p. 500-519.
5. Guzmán, M.G. and G. Kourí, *Dengue diagnosis, advances and challenges.* International Journal of Infectious Diseases, 2004. **8**(2): p. 69-80.
6. Darwish, N.T., Y.B. Alias, and S.M. Khor, *An introduction to dengue-disease diagnostics.* TrAC Trends in Analytical Chemistry, 2015. **67**: p. 45-55.
7. Anandarao, R., et al., *Recombinant multiepitope protein for early detection of dengue infections.* Clin Vaccine Immunol, 2006. **13**(1): p. 59-67.
8. Cuzzubbo, A.J., et al., *Use of recombinant envelope proteins for serological diagnosis of Dengue virus infection in an immunochromatographic assay.* Clin Diagn Lab Immunol, 2001. **8**(6): p. 1150-5.
9. Maldaner, F., et al., *Dengue virus tetra-epitope peptide expressed in lettuce chloroplasts for potential use in dengue diagnosis.* Applied Microbiology and Biotechnology, 2013. **97**(13): p. 5721-5729.
10. Crill, W.D. and J.T. Roehrig, *Monoclonal antibodies that bind to domain III of dengue virus E glycoprotein are the most efficient blockers of virus adsorption to Vero cells.* J Virol, 2001. **75**(16): p. 7769-73.
11. Jaiswal, S., N. Khanna, and S. Swaminathan, *High-level expression and one-step purification of recombinant dengue virus type 2 envelope domain III protein in Escherichia coli.* Protein Expr Purif, 2004. **33**(1): p. 80-91.
12. Zulueta, A., et al., *Amino acid changes in the recombinant Dengue 3 Envelope domain III determine its antigenicity and immunogenicity in mice.* Virus Res, 2006. **121**(1): p. 65-73.
13. Tripathi, N.K., et al., *Production and characterization of recombinant dengue virus type 4 envelope domain III protein.* Journal of Biotechnology, 2008. **134**(3-4): p. 278-286.
14. Chavez, J.H., et al., *Domain III peptides from flavivirus envelope protein are useful antigens for serologic diagnosis and targets for immunization.* Biologicals, 2010. **38**(6): p. 613-8.

15. Reddy, P.B., et al., *Expression, purification and evaluation of diagnostic potential and immunogenicity of dengue virus type 3 domain III protein.* Protein Pept Lett, 2012. **19**(5): p. 509-19.
16. Cardoso, S.A., et al., *Dengue-1 envelope protein domain III produced in Pichia pastoris: potential use for serological diagnosis.* Protein Expr Purif, 2013. **92**(1): p. 9-13.
17. Miller, L.K., *Baculoviruses for foreign gene expression in insect cells.* Biotechnology, 1988. **10**: p. 457-65.
18. Miller, L.K., *Baculoviruses: high-level expression in insect cells.* Curr Opin Genet Dev, 1993. **3**(1): p. 97-101.
19. Ikonomou, L., Y.J. Schneider, and S.N. Agathos, *Insect cell culture for industrial production of recombinant proteins.* Applied Microbiology and Biotechnology, 2003. **62**(1): p. 1-20.
20. Targovnik, A.M., et al., *Horseradish peroxidase production from Spodoptera frugiperda larvae: A simple and inexpensive method.* Process Biochemistry, 2010. **45**(6): p. 835-840.
21. Romero, L., et al., *Recombinant peroxidase production in species of lepidoptera frequently found in Argentina.* New Biotechnology, 2010. **27**(6): p. 857-861.
22. Ferrer, F., et al., *Induction of virus-neutralizing antibodies by immunization with Rachiplusia nu per os infected with a recombinant baculovirus expressing the E2 glycoprotein of bovine viral diarrhea virus.* Journal of Virological Methods, 2007. **146**(1-2): p. 424-427.
23. Romero, L., et al., *Rachiplusia nu larva as a biofactory to achieve high level expression of horseradish peroxidase.* Biotechnology Letters, 2011. **33**(5): p. 947-956.
24. Gomez-Casado, E., et al., *Insect larvae biofactories as a platform for influenza vaccine production.* Protein Expression and Purification, 2011. **79**(1): p. 35-43.
25. Conley, A.J., et al., *Protein body-inducing fusions for high-level production and purification of recombinant proteins in plants.* Plant Biotechnology Journal, 2011. **9**(4): p. 419-433.
26. Linder, M.B., et al., *Hydrophobins: the protein-amphiphiles of filamentous fungi.* FEMS Microbiology Reviews, 2005. **29**(5): p. 877-896.
27. Linder, M., et al., *The Hydrophobins HFBI and HFBII from Trichoderma reesei Showing Efficient Interactions with Nonionic Surfactants in Aqueous Two-Phase Systems.* Biomacromolecules, 2001. **2**(2): p. 511-517.
28. Linder, M.B., et al., *Efficient Purification of Recombinant Proteins Using Hydrophobins as Tags in Surfactant-Based Two-Phase Systems.* Biochemistry, 2004. **43**(37): p. 11873-11882.
29. Lahtinen, T., et al., *Hydrophobin (HFBI): A potential fusion partner for one-step purification of recombinant proteins from insect cells.* Protein Expression and Purification, 2008. **59**(1): p. 18-24.
30. Joensuu, J.J., et al., *Hydrophobin Fusions for High-Level Transient Protein Expression and Purification in Nicotiana benthamiana.* Plant Physiology, 2009. **152**(2): p. 622-633.
31. Lorence, A., et al., *Bioseparation of Recombinant Proteins from Plant Extract with Hydrophobin Fusion Technology*, in *Recombinant Gene Expression*. 2012, Humana Press. p. 527-534.

32. Mustalahti, E., M. Saloheimo, and J.J. Joensuu, *Intracellular protein production in Trichoderma reesei (Hypocrea jecorina) with hydrophobin fusion technology.* New Biotechnology, 2013. **30**(2): p. 262-268.
33. Linder, M., et al., *Surface adhesion of fusion proteins containing the hydrophobins HFBI and HFBII from Trichoderma reesei.* Protein Science : A Publication of the Protein Society, 2002. **11**(9): p. 2257-2266.
34. Takatsuji, Y., et al., *Solid-support immobilization of a "swing" fusion protein for enhanced glucose oxidase catalytic activity.* Colloids and Surfaces B: Biointerfaces, 2013. **112**: p. 186-191.
35. Reed, L.J. and H. Muench, *A simple method of estimating fifty per cent endpoints.* American Journal of Epidemiology, 1938. **27**(3): p. 493-497.
36. Greene, G.L., N.C. Leppla, and W.A. Dickerson, *Velvetbean Caterpillar: A Rearing Procedure and Artificial Medium.* Journal of Economic Entomology, 1976. **69**(4): p. 487-488.
37. Bradford, M.M., *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.* Analytical Biochemistry, 1976. **72**(1): p. 248-254.
38. Laemmli, U.K., *Cleavage of structural proteins during the assembly of the head of bacteriophage T4.* Nature, 1970. **227**(5259): p. 680-5.
39. Schagger, H., *Tricine-SDS-PAGE.* Nat. Protocols, 2006. **1**(1): p. 16-22.
40. Burnette, W., *"Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A.* Analytical Biochemistry, 1981. **112**(2): p. 195-203.
41. Russell, P.K., et al., *A Plaque Reduction Test for Dengue Virus Neutralizing Antibodies.* The Journal of Immunology, 1967. **99**(2): p. 285-290.
42. Jacquet, N., et al., *Hydrophobin Fusion of an Influenza Virus Hemagglutinin Allows High Transient Expression in Nicotiana benthamiana, Easy Purification and Immune Response with Neutralizing Activity.* PLoS ONE, 2014. **9**(12): p. e115944.
43. Urtasun, N., et al., *High-level expression and purification of recombinant wheat germ agglutinin in Rachiplusia nu larvae.* Process Biochemistry, 2015. **50**(1): p. 40-47.
44. Targovnik, A.M., et al., *Expression and purification of recombinant feline interferon in the baculovirus-insect larvae system.* Process Biochemistry, 2014. **49**(6): p. 917-926.
45. Faletti, L.E., et al., *Expression of recombinant Influenza A H1N1 neuraminidase in Rachiplusia nu larvae.* Current Topics in Virology, 2014. **12**: p. 65 - 75.
46. Zhang, M., et al., *Immobilization of anti-CD31 antibody on electrospun poly(É›-caprolactone) scaffolds through hydrophobins for specific adhesion of endothelial cells.* Colloids and Surfaces B: Biointerfaces, 2011. **85**(1): p. 32-39.
47. Qin, M., et al., *Bioactive Surface Modification of Mica and Poly(dimethylsiloxane) with Hydrophobins for Protein Immobilization.* Langmuir, 2007. **23**(8): p. 4465-4471.
48. Tripathi, L., et al., *Pichia pastoris-expressed dengue 3 envelope-based virus-like particles elicit predominantly domain III-focused high titer neutralizing antibodies.* Frontiers in Microbiology, 2015. **6**: p. 1005.

49. Emmerich, P., A. Mika, and H. Schmitz, *Detection of Serotype-Specific Antibodies to the Four Dengue Viruses Using an Immune Complex Binding (ICB) ELISA.* PLoS Neglected Tropical Diseases, 2013. **7**(12): p. e2580.

582 **Figure and table legends**

583

584 **Figure 1.** Recombinant baculovirus for the expression of DomIIIHFBI (AcDIIIHFBI) and
585 GFPHFBI (AcGFPHFBI).

586 The expression cassettes for DomIIIHFBI and GFPHFBI were fused in-frame to the viral
587 secretion signal GP67 and under control of the polyhedrin promoter. GP67: glycoprotein 67
588 leader peptide.

589

590 **Figure 2.** DomIIIHFBI expression in Sf9 cells.

591 Analysis of localization and expression kinetics by Western blot at 0, 1, 2, 3, 4, 5 and 6
592 days post-infection (dpi) with the baculovirus AcDIIIHFBI using a multiplicity of infection
593 (MOI) of 0.5. C+: Sample of DomIIIHFBI expressed in Sf9 cells in a previous experiment
594 used as a positive control. C-: Sf9 cells or supernatant infected with AcGFPHFBI with a
595 MOI of 0.5 at 6 dpi.

596

597 **Figure 3.** DomIIIHFBI purification by aqueous two-phase systems (ATPS) from culture
598 supernatants.

599 Analysis of the purification process with 2%, 5% and 8% of Triton X-114 by SDS-PAGE
600 (A) and Western blot (B). T: total proteins from supernatant starting material. Aq1: upper
601 aqueous phase of the surfactant-based ATPS. Aq2: aqueous phase of the isobutanol
602 extraction. I: solubilized proteins from the interphase of the isobutanol extraction. CNEG:
603 Purification process with 5% of Triton X-114 of the supernatant obtained from cells
604 infected with AcGFPHFBI. Arrows point out DomIIIHFBI. MK: PageRuler™ Prestained
605 Protein Ladder (10 – 170 kDa), #SM0671, Fermentas.

606

607 **Figure 4.** DomIIIHFBI expression in Rachiplusia nu larvae.

608 A: SDS-PAGE of PBS crude extracts from larvae infected with AcDIIIHFBI (Lane 1) and
609 AcGFPHFBI (Lane 2). Lane 3: PageRuler™ Prestained Protein Ladder (10 – 170 kDa),
610 #SM0671, Fermentas. B: Western blot analysis of crude extracts from larvae infected with
611 AcGFPHFBI (Lane 1) and AcDIIIHFBI (Lane 2-4). Lane 2: PBS extract; lane 3: PBS +
612 sonication extract; lane 1 and 4: PBS + SDS + sonication extract.

613

614 **Figure 5.** DomIIIHFBI purification by aqueous two-phase systems (ATPS) from larval
615 extracts.

Analysis of the purification process with 2%, 5% and 8% of Triton X-114 by SDS-PAGE (A) and Western blot (B). T: total proteins from supernatant starting material. Aq1: upper aqueous phase of the surfactant-based ATPS. Aq2: aqueous phase of the isobutanol extraction. I: solubilized proteins from the interphase of the isobutanol extraction. CNEG: Purification process with 5% of Triton X-114 of a crude extract obtained from larvae infected with AcGFPHFBI. Arrows point out DomIIIHFBI. MK: PageRuler™ Prestained Protein Ladder (10 – 170 kDa), #SM0671, Fermentas.

Table 1: Comparative analysis for the production (expression and purification with 2% Triton X-114) of 1 mg of DomIIIHFBI in *R. nu* larvae and in the supernatant of Sf9 cells.

Figure 6. DomIIIHFBI immobilization.

Purified DomIIIHFBI samples at concentration ranging from 68 to 540 nM (0.1 to 12.5 µg/ml) were loaded in multiwell plates and incubated overnight at 4°C for protein immobilization. Two different plates were tested: polystyrene multiwell plates (Nunc) and PolySorp surface treated multiwell plates with enhanced affinity to hydrophobic proteins (Nunc). The immobilized protein was detected using a rabbit polyclonal anti-DENV 1+2+3+4 antibody (Abcam) in a dilution 1/50 and Peroxidase-conjugated AffiniPure Mouse anti-rabbit IgG (Jackson ImmunoResearch) in a dilution 1/2000. Samples of DENV-2 DomIII –without HFBI tag– (DomIII, Prospec) were also assessed at the same concentrations. All determinations were performed in triplicate and results are expressed as the average ± standard deviation. OD: Optical density. Ag: antigen, DomIIIHFBI or DomIII according to the experiment.

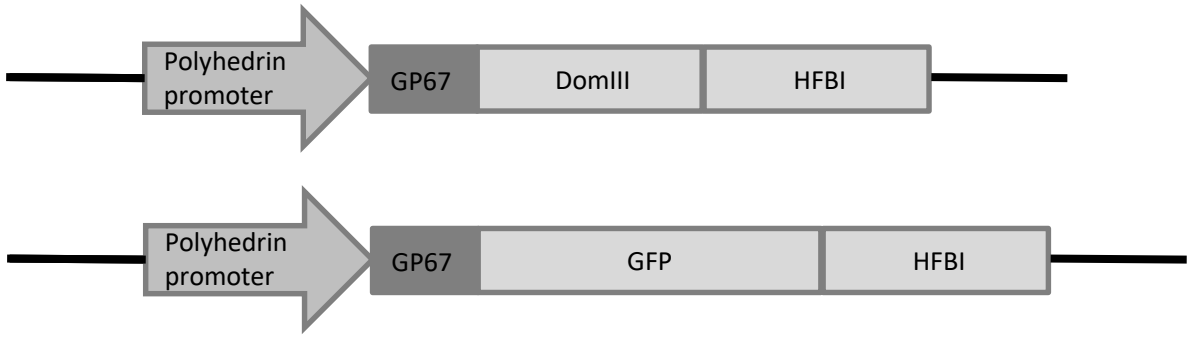
Figure 7. Immunoassay performance with patient´s serum samples.

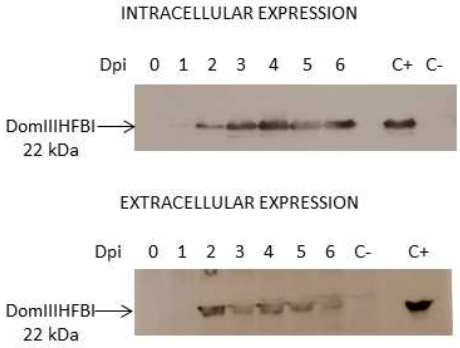
Serum samples diluted 1/100 were tested for the detection of anti-DENV IgG antibodies by using polystyrene plates coated with DomIIIHFBI in a concentration of 6.25 µg/ml. Two positive samples for each Dengue serotype were tested. Cross reactivity with Yellow fever and Saint Louis encephalitis viruses antibodies was also tested, by analyzing 2 positive samples for each virus. All determinations were performed in triplicate and results are expressed as the average ± standard deviation. Positive and negative sera were determined previously by a plaque reduction neutralization test. The cut off was determined as the mean + 3 standard deviation of 30 negative samples. DV1/2/3/4: Serum sample containing IgG specific for DENV-1/2/3/4. YFV: Serum sample containing IgG specific for Yellow fever virus. SLEV: Serum sample containing IgG specific for Saint Louis encephalitis virus.

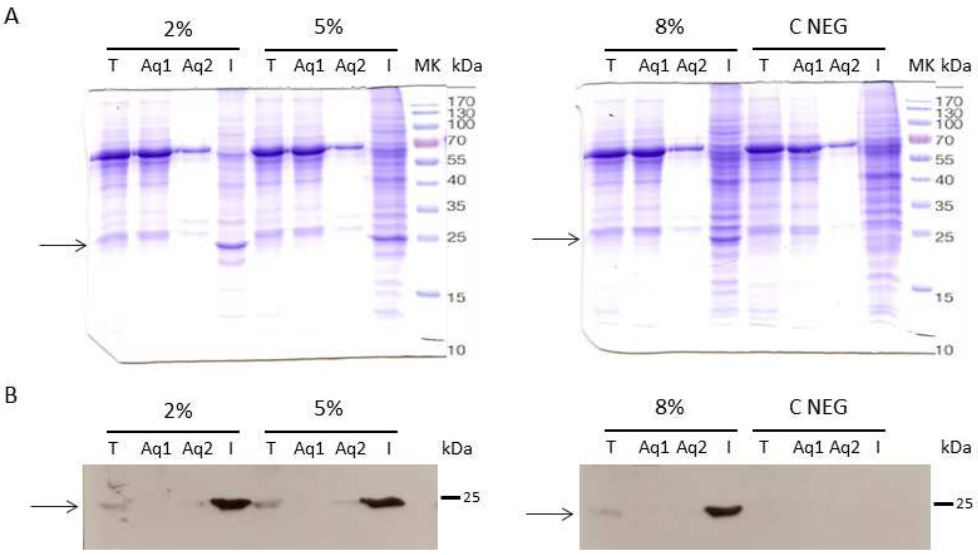
	***R. nu* larvae**	**Sf9 cells**
Yield (Purified DomIIIHFBI)	3.3 mg/g of larvae*	16.7 µg/ml of culture supernatant**
N° larvae/ cell suspension volume needed	2 larvae	60 ml cell suspension
Virus needed from a stock 1.1 x 10^8 pfu/ml (ml)	0.009	0.545
Starting volume for purification (ml)	2	60
Purity (%)	> 80	20

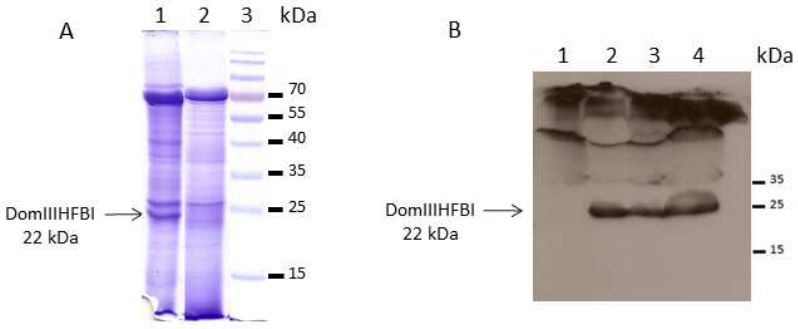
* 1 g of larva is equivalent to 5 larvae approximately.

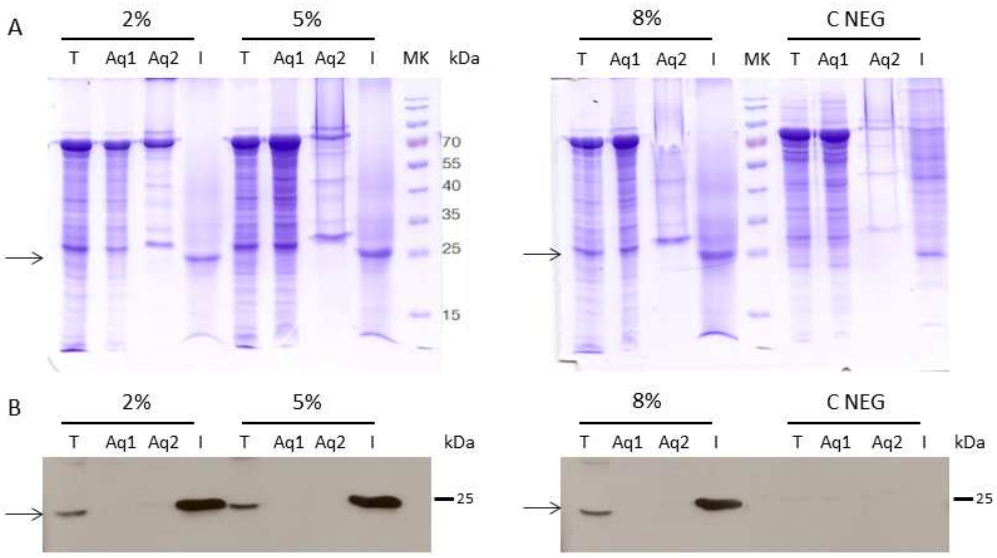
** 1 ml of culture supernatant from a 2 x 10^6 Sf9 cells/ml suspension.

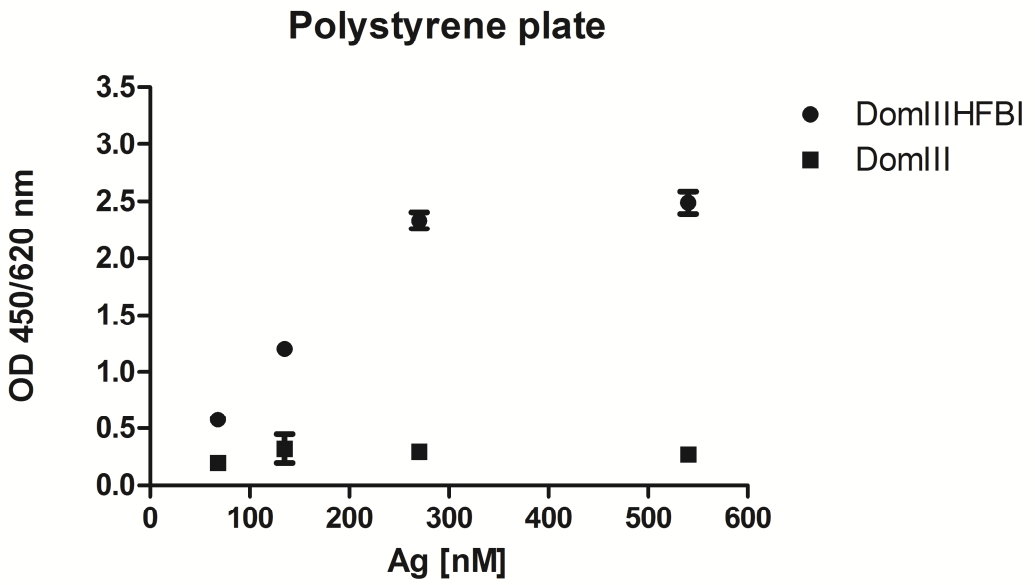

Polyhedrin promoter
GP67
DomIII
HFBI
Polyhedrin promoter
GP67
GFP
HFBI

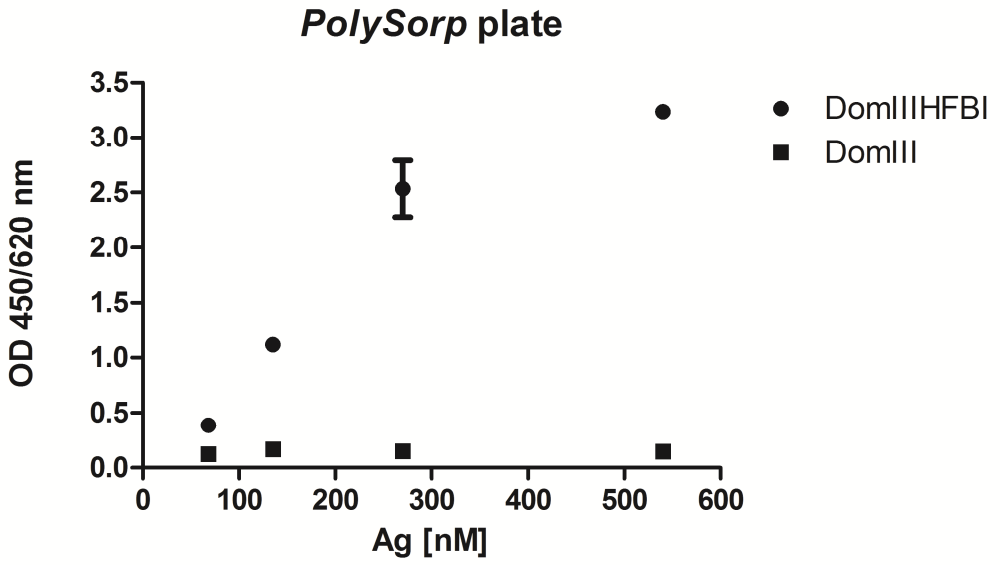









Polystyrene plate
OD 450/620 nm
Ag [nM]
DomIIIHFBI
DomIII
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
0
100
200
300
400
500
600


PolySorp plate
OD 450/620 nm
Ag [nM]
DomIIIHFBI
DomIII
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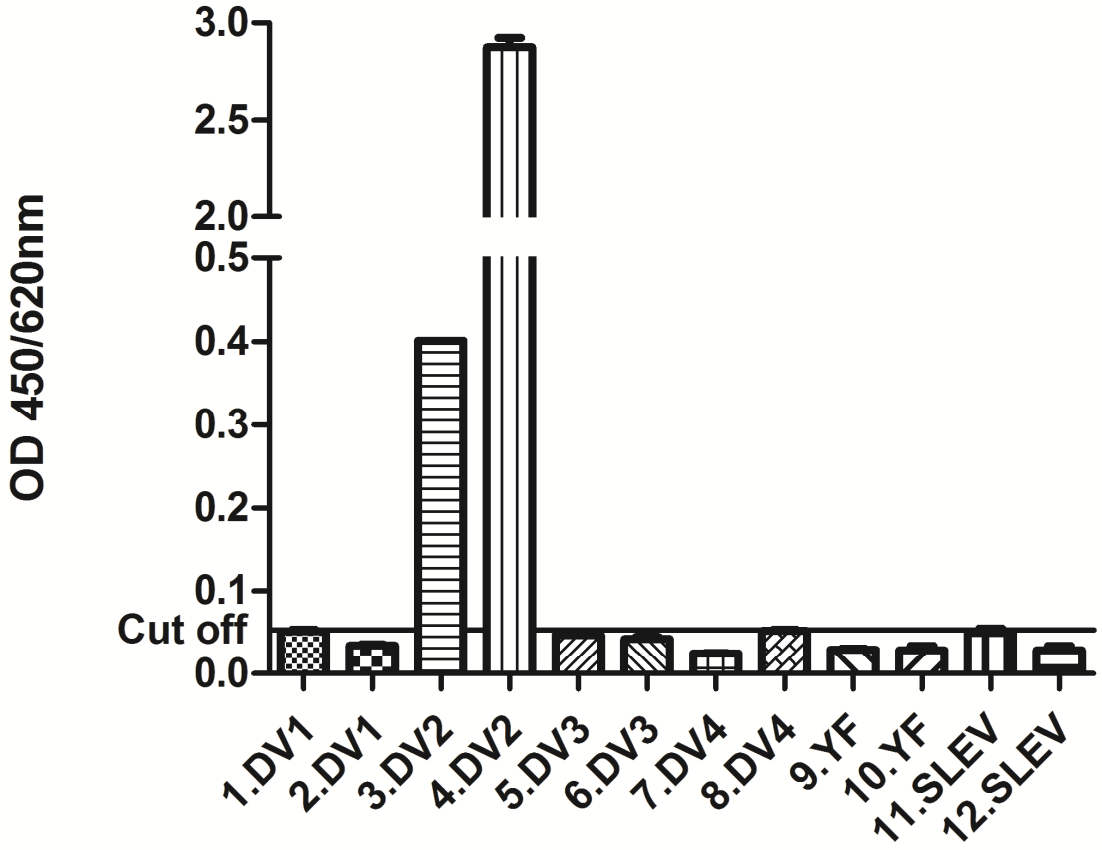
0.5
1.0
1.5
2.0
2.5
3.0
3.5
0
100
200
300
400
500
600


OD 450/620nm
3.0
2.5
2.0
0.5
0.4
0.3
0.2
0.1
Cut off
0.0
1.DV1
2.DV1
3.DV2
4.DV2
5.DV3
6.DV3
7.DV4
8.DV4
9.YF
10.YF
11.SLEV
12.SLEV

Highlights

- The domain III of DENV-2 fused to HFBI was expressed in *Rachiplusia nu* larvae.
- Surfactant-based ATPS allowed to purify DomIIIHFBI directly from larval extracts.
- Hydrophobin I enabled domain III immobilization to hydrophobic surface plates.
- The immobilized antigen was recognized by anti-DENV-2 IgG from serum samples.