Enhanced Prokaryotic Expression of Dengue Virus Envelope Protein

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ABSTRACT – Purpose. To highlight the expression and purification of the recombinant dengue virus type-1 antigen exploiting the codon optimized full length envelope for increased yield in *E. coli*. **Methods.** A 6x His tag was inserted at the C terminus to facilitate purification. The purified protein was recognized in Western blot by Monoclonal antibody specific for the tag. The *in vitro* refolded recombinant protein was used to immunize mice for the development of hybridomas and also analyzed for its biological functionality with heparan sulfate binding assay. **Results**. The polyclonal anti-sera from the immunized mice were found to recognize the envelope protein thereby establishing the immunogenicity of the protein. **Conclusion**. The purified envelope protein could potentially be used towards dengue diagnostics and vaccine development efforts.

INTRODUCTION

Dengue virus is the most important arthropod-borne human pathogen. The incidence of dengue fever epidemics has increased significantly over the last few decades, and it is estimated that up to 100 million cases occur annually. In addition, a severe form of the disease, dengue hemorrhagic fever (DHF), has emerged in the same period causing 500,000 cases worldwide each year (1, 50). There are four different but antigenically closely related serotypes of dengue virus (DEN-1, 2, 3 and 4), and it is believed that DHF may result from secondary infection by different virus serotypes, in a process known as antibody mediated disease enhancement (2). This feature has made vaccine development efforts against the dengue virus a difficult issue. Nevertheless, researchers around the world are exploring different approaches towards dengue vaccine development based on recombinant viral proteins expressed in different systems, inactivated viruses, conventional live attenuated viruses, antigen encoding plasmids and viral vectors encoding antigen genes (3).

The envelope (Env) protein of the dengue virus is the most studied antigen. Several studies confirm that the Env protein can be an effective vaccine candidate. Multiple strategies including subunit Env vaccines, DNA vaccines and attenuated viruses are being undertaken to develop a suitable vaccine for controlling the viral infection (4-7).

The dengue virus Env protein is around 50-55 kDa in size, with the N terminal corresponding to the ectodomain that is exposed on the virion surface. The C terminal constitutes the trans-membrane hydrophobic domain and helps anchor the molecule on the lipid bilayer. The envelope is a multifunctional protein with important roles in host cell surface receptor binding (8). The Env protein is also very important from the viewpoint of humoral immunity against the virus (9). Hence, efforts are made to express the Env protein in heterologous systems in order to develop it as a vaccine candidate.

The goal of this study was efficient cloning and expression of DEN-1 full length Env gene in *E. coli* that could be used to develop monoclonal antibodies (MAbs) by hybridoma technology or as a viable antigen for diagnostic and vaccine purposes. In this study, we report the successful cloning and high-level expression of the Env gene, purification from *E. coli* as inclusion bodies and its subsequent refolding. After purification with an affinity column and subsequent refolding, the protein was tested for its capability to induce robust humoral immune response in mice and virus blocking capability. The functionality of the refolded protein was further determined with heparan sulfate binding assay. Dengue virus infectivity has been reported to be dependent on the Env protein binding to heparan sulfate in the target cell (10).

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The recombinant antigen has been evaluated as a diagnostic reagent. This article illustrates enhanced expression and functionality of the recombinant protein.

MATERIALS AND METHODS

Vector and Chemicals

Restriction enzymes were procured from New England Biolabs (Mississauga, Canada). The anti-His_6 was purchased from Novagen Inc (Madison, USA). 40% acrylamide: bisacrylamide, prestained low range protein molecular weight markers and protein assay reagents were obtained from Bio-Rad (Mississuaga, Canada). Hybond ECL nitrocellulose membrane, X-ray film and the ECL Western blotting reagents were obtained from Amersham Pharmacia Biotech (Baied Urfe, Quebec, Canada). Glutathione (GSH) and Glutathione disulphide (GSSG) were purchased from Boehringer Mannheim. Baby Hamster Kidney (BHK 21) cells, C6/36 mosquito cells were obtained from American Type Culture Collection (Virginia, USA), Dengue-1 virus (Hawaii strain) was maintained in the C6/36 cells. Briefly, monolayers of C6/36 were incubated with virus at a multiplicity of infection of 0.01 and incubated at 26°C in 5% CO_2 for 5 days, Heparan sulfate, sodium deoxycholate, arginine, Goat anti Mouse-HRPO (GAM-HRPO), urea and other general reagents were obtained from Sigma (Oakville, Canada). Ni-NTA agarose, plasmid DNA isolation kit and gel extraction kit were obtained from Qiagen (Mississauga, Canada).

Construction of Plasmid (pDS20Env)

Codon optimized Env nucleotide sequence for *E. coli* expression was chemically synthesized from GENEART, Germany. The codon optimized Env gene comprising of the plasmid and the expression vector pBM802 were NdeI and EcoRI digested followed by gel purification and ligation. The ligation mixtures were subsequently transformed in *E. coli* Rosetta (ATCC 87064, *E. coli* DE3) by a heat shock method for recombinant protein expression. Screening of the resultant transformants was done by plasmid DNA isolation and restriction digestion (11).

Analysis of recombinant clones

Individual bacterial colonies were grown in 2 mL Terrific Broth (TB) medium (1.2% tryptone, 2.4% yeast extract, 0.4% (v/v) glycerol and 25 mM Hepes pH 7.2) comprising tetracycline and chloramphenicol, 5 μ gmL^{-1} and 34 μ gmL^{-1}, respectively, followed by overnight incubation at 37°C with shaking at 250 rpm. The culture was diluted 100 fold the next day in fresh TB medium comprising 5 μ gmL^{-1} tetracycline and 34 μ gmL^{-1} chloramphenicol and grown at 37°C with brisk shaking at 250 rpm. The bacterial culture was induced with 0.2% (w/v) of arabinose on reaching optical density (OD_{600nm}) of approximately 0.5-0.6 and was thereafter allowed to grow overnight (~16 h) at 37°C with shaking at 250 rpm. The bacterial culture was harvested by centrifugation at 5,000 x g for 10 min at 4°C and the total cell protein lysate was prepared (12). The total cell protein was analyzed by SDS-PAGE using 10% polyacrylamide gel, according to Laemmli's method (13) and stained.

Expression optimization (Temperature, Time and Inducer)

The Env protein expression was optimized for three different sets of temperatures (37°C, 30°C and 24°C), varied time durations and inducer (arabinose) concentrations. Bacterial growth conditions were identical as defined earlier. The bacterial culture was induced with 0.2% (w/v) of arabinose at optical density ($OD_{600\ nm}$) of 0.5-0.6 and allowed to grow overnight (~16 h) at three different temperatures. For time optimization, the bacterial culture was induced with 0.2% (w/v) of arabinose after an absorbance of 0.5 was reached. The cultures were further allowed to grow for 0 h, 2 h, 4 h, 6 h and overnight (~16 h) at 37°C. The bacterial cultures were induced with different concentrations of arabinose (2%, 0.2%, 0.02%, 0.002% and 0.0002%) and allowed to grow over night (~16 h) at 37°C. The total cell proteins obtained from each experiment were analyzed by SDS- PAGE and Western blot.

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Medium scale expression and purification of the viral antigen

A fresh single colony of *E. coli* transformants containing pDS20Env was inoculated in 10 mL TB medium containing 5 μ gmL^{-1} of tetracycline and 34 μ gmL^{-1} chloramphenicol and allowed to grow overnight at 37°C in an incubator shaker at 250 rpm. The overnight culture was diluted 100 times in 1 L TB medium containing 5 μ gmL^{-1} of tetracycline and 34 μ gmL^{-1} chloramphenicol and grown at 37°C with shaking at 250 RPM until an

$OD_{600\ nm}$ of 0.5–0.6 was reached. Induction was done by arabinose addition to a final concentration of 0.2% (w/v) and bacterial culture was incubated for 16 h with vigorous shaking (250 rpm) at 37°C. Pellets were collected by centrifugation at 5,000 X g for 20 min at 4°C. Total cell proteins from both uninduced and induced culture were analyzed by SDS–PAGE and Western blot.

Processing of inclusion bodies

The bacterial pellet (3 g) was resuspended in 30 mL PBS and lysed by French Press (20,000 psi). The cell lysate was clarified by centrifugation at 27,000 X *g* for 30 min at 4°C and the supernatant was discarded. The pellet was again resuspended in 50 mL lysis buffer (50 mM Tris, pH 8.0, 200 mM NaCl and 1 mM EDTA) and adjusted to 2% sodium deoxycholate, incubated at room temperature for 30 min and centrifuged at 27,000 X g for 30 min at 4°C. The pellet was resuspended in lysis buffer and washed three times at 27,000 X g for 20 min at 4°C.

Protein purification (IMAC) under denaturing conditions

Inclusion bodies from the above step were solubilized in denaturing buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 8.0) for 1 h at room temperature with occasional shaking. Solubilized proteins were separated from insoluble material by centrifugation at 27,000 X g for 30 min at 4°C and the final yield of solubilized protein was determined by Bradford assay. An IMAC based separation system was prepared by loading 9 mL of Ni–NTA agarose on a column. The column was equilibrated with 5 bed volumes of denaturing buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 8). Denatured soluble protein was loaded on the column and the column was washed initially with 5 bed volumes of washing buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 6.3). Bound protein was eluted with elution buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 4.5). The various eluted fractions were analyzed by SDS–PAGE to measure the purity.

Refolding

Protein assay was done by Bradford method to quantify the amount of eluted protein from the IMAC column. The eluted protein was diluted to 75 μ gmL^{-1} and 60 μ gmL^{-1} with Tris Arginine (TA) dialysis buffer (50 mM Tris, pH 8.0, 0.4 M L-arginine) to identify the appropriate refolding condition. Refolding was done by dialysis in TA buffer in the presence of 1.0 mM GSH and 0.1 mM GSSG, and changed three times over three days at 4°C. Final dialysis was done in PBS at 4°C. Any aggregation was removed by centrifugation and the supernatant was collected as a soluble refolded protein.

Western blot analysis

Total cell proteins, inclusion bodies, IMAC eluted fractions or refolded Env proteins were separated on SDS–PAGE using 10% polyacrylamide gel and transferred onto hybond ECL nitrocellulose membranes [14] with a transblot apparatus (Bio-Rad) according to the manufacturer's instructions. The membrane was blocked with 5% skim milk in PBST (0.1% Tween 20 in PBS, pH 7.3) for 1 h. The membrane was washed four times with PBST and incubated for 1 h with anti-His_6 MAb. After washing four times with PBST, the membrane was incubated with GAM-HRPO for 1 h. Finally, the membrane was washed with PBST four times and ECL-based detection was performed according to manufacturer's instructions.

In gel digestion

Protein identification by LC-MS was performed at the Institute of Biomolecular design, University of Alberta, Edmonton, Alberta, Canada.

Heparan Sulfate binding assay

Refolded protein was analysed for recognition of heparan sulfate by ELISA. Briefly, 96–well Maxisorp plates were coated with 0.5 μ gmL^{-1} heparan sulfate and blocked with 3% bovine serum albumin (BSA). Heparan sulfate coated wells were incubated with refolded protein for 1 h at 37°C. Serial dilutions of refolded Env protein were analysed. Then, wells were washed four times with PBST and incubated with 100 μl of diluted (1:1,000 in 5% BSA) anti-His_6 MAb for 1 h at room temperature. The wells were washed again and incubated with anti-mouse IgG HRP (at 1:1,000 dilutions in 5% BSA) for 1 h at room temperature. The color reaction was developed with TMB. The optical density was measured at 650 nm. Two negative controls (all assay components minus the recombinant protein and the other minus heparan sulfate) and a blank (containing only the detection reagents) were also included in the assay. The assay was repeated 3 times.

Immunization of mice

Groups of five balb/c mice were intra-peritoneally injected with 25 µg of purified Env protein in PBS emulsified with an adjuvant (protocol number 074/09/09, approved by Health Science Animal Protocol Committee, University of Alberta). The protein was administered to the mice on days 0, 14 and 28 using Freund's complete adjuvant for the first administration and Freund's incomplete adjuvant for the second administration. The final injection was with 10 µg of protein in PBS. Mice were bled one week after the last administration and serum samples were then collected for further analysis.

Preparation of Dengue virus type 1 stock

The culture supernatant, obtained from dengue 1 virus (Hawaii strain)-infected C6/36 culture after removal of cellular junk by centrifugation, was used as the source of virus. The stock was titrated on BHK cells grown in culture plates. The virus dilution at which 50% of the infected wells resulted in Cytopathic Effect was done by an established method (39) and viral titers were formulated as the reciprocal of the dilution in terms of Tissue Culture Infective Doses ($TCID_{50}$) (31).

Virus binding blocking assay

The ability of the Env protein to block dengue virus type 1 binding to cells in tissue culture was performed (31). BHK cells were immobilized in 96-well plates (2×10^3 cells/well). At about 60% confluency, they were pre-incubated for 30 min at 37 °C with 200 µl of 1× PBS containing either the test (Env) or control proteins. We used BSA as a control. Protein concentration in each case was from 0 to 20 µg per well. Following pre-incubating with the proteins 50 µL dilute Dengue type 1 virus (equivalent to 250 $TCID_{50}$) was added to each well and incubated for 30 min at 37 °C. After this, the protein/virus mixture was aspirated out the cells were washed three times with 1× PBS and fresh medium (200 µL/well) was added followed by incubation for 3 days in a humidified 10% CO_2 incubator at 37 °C. After 3 days the wells were scored for the presence or absence of cytopathic effects (CPE) by microscopic examination.

Envelope Protein as a diagnostic reagent

We spiked different concentrations of anti-dengue monoclonal antibody 8A5 in rabbit serum and neat serum was used as blank. We also used serum spiked with SARS antibodies as negative control. We blinded the tubes and labeled the samples 1-7 randomly. The purified full length Env protein was diluted to 10 μgmL^{-1} in 0.1M bicarbonate buffer (pH 9.6) and 96–well Maxisorp plates were coated overnight at 4°C. The coated plates were washed with PBS and blocked with 2% BSA for 2 h at 37°C. The plates were washed again with PBS. 100 µL of the samples were added to the wells and incubated for 2 h at 37°C. The plates were washed again with PBS and anti-mouse IgG conjugated to Horseradish peroxidase was added (1:10000 dilution) for 30 min at 37°C. The plates were washed again with PBS and TMB substrate added and absorbance was read at 650 nm. The assay was done in triplicates.

Evaluation of assay with clinical samples

Clinical samples from dengue infected patients being difficult to procure, we simulated the assay with 21 human serum samples collected from TB patients at diagnosis prior to initiating drug therapy who were part of a cohort of newly diagnosed TB patients from the Tuberculosis Trials Consortium Study Group 22 between 1995 and 1998 (48, 49). Anti dengue mAb was spiked in the samples in different concentrations. Non-spiked samples were used as control. 96–well plates were coated overnight at 4°C with full length Env protein diluted to 10 μgmL^{-1} in 0.1M bicarbonate buffer (pH 9.6). The plates were then washed with PBS and blocked with 2% BSA for 2 h at 37°C. The plates were washed again with PBS. 100 µL of the samples were added to the wells and incubated for 2 h at 37°C. The plates were washed again with PBS and anti-mouse IgG conjugated to Horseradish peroxidase was added (1:10000 dilution) for 30 min at 37°C. The plates were washed again with PBS and TMB substrate added and absorbance was read at 650 nm. The assay was done in triplicates.

RESULTS

Env gene cloning and expression

The full length Env gene was successfully cloned with the C-terminal His_6 tag and denoted as pDS20Env for higher expression of proteins in *E. coli* in the form of inclusion bodies (15). Recombinant clones pertaining to the right size were selected for protein expression. The full length Env gene comprising plasmid was isolated for expression. Expression results of various clones

showed that all the Env full length clones selected expressed the protein of interest at approximately 54 kDa at varying levels as determined by SDS-PAGE analysis (data not shown). This was confirmed by Western blot probed with anti-His_6 MAb (Figure 1). In the control sample, no expression of Env protein was observed. The best Env clone was selected for expression optimization and further studies.

Expression optimization (Temperature, Time and Inducer)

The Env protein was successfully expressed as a recombinant protein in *E.coli*. The optimal conditions for Env protein expression were 0.2% (w/v) arabinose concentration (Figure 2a and 2b), 37°C temperature (Figure 3a and 3b) and 16 h induction time (Figure 4).

Medium scale expression of Env protein

The recombinant Env was expressed and the bulk of the antigen was in the inclusion bodies (Figure 5). Inclusion bodies were prepared from bacterial pellet by French press. After completion of cell lysis, the insoluble inclusion bodies were separated from the soluble bacterial protein by centrifugation and thereafter the pellets were washed with sodium deoxycholate. Any remaining sodium deoxycholate were removed from the inclusion bodies by subsequent washes with lysis buffer. The purity of the inclusion bodies along with the different washes was analyzed by SDS-PAGE (data not shown). The final yield of purified soluble inclusion bodies was estimated by Bradford protein assay to be approximately 15-20 μ gmL^{-1} of bacterial culture.

Purification and Refolding

The insoluble protein was isolated from inclusion bodies with a final purified protein yield in the range of 15-20 mgL^{-1} of bacterial shake flask culture and purified by immobilized metal–affinity chromatography (IMAC) under denaturing conditions. The purification method involved IMAC for separating recombinant proteins from major bacterial contaminants. Employing this method, pure proteins were eluted out from the IMAC affinity column as determinated in the methods section. The eluted protein showed a single band of approximate molecular weight of 54 kDa, with purity greater than 90% (Figure 6). Folding of proteins and disulphide bond formation and association of various domains require renaturing conditions and suitable buffers. The presence of arginine in the refolding buffer helped in solubilization, inhibiting aggregation of refolding intermediates and thereby increasing the yield (16). Refolding was done over three days by dialysis. No aggregation was observed after refolding. The supernatant was collected as refolded Env protein for future use. Hence, the *in vitro* refolding was successful in recovering the soluble protein expressed in *E.coli* in form of inclusion bodies.

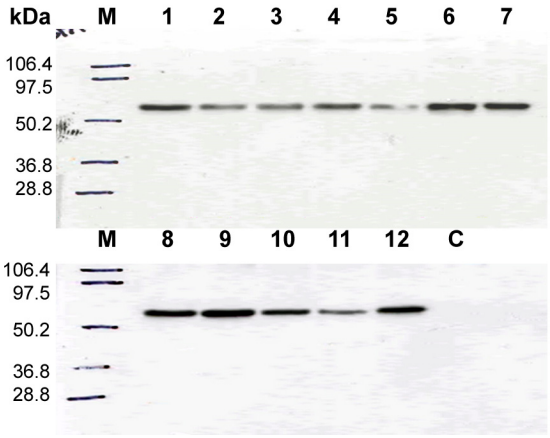

Figure 1. Envelope protein expression. Lane M: Marker, Lanes 1-12: Clones # 1-12 and Lane C is Control.

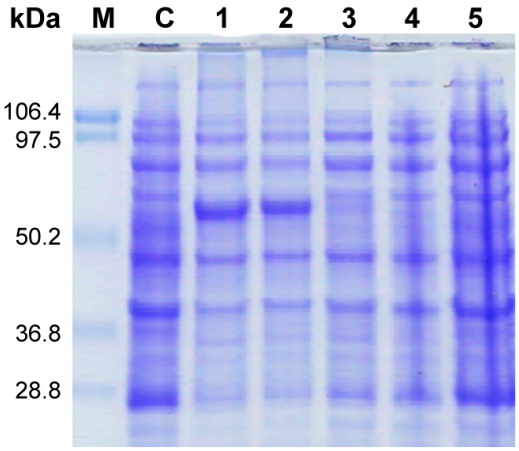

Figure 2a. Arabinose dose optimization of envelope protein expression **a** SDS-PAGE. Lane M: prestained marker, Lane C: Control, Lanes 1-5: 2%, 0.2%, 0.02%, 0.002%, 0.0002%.

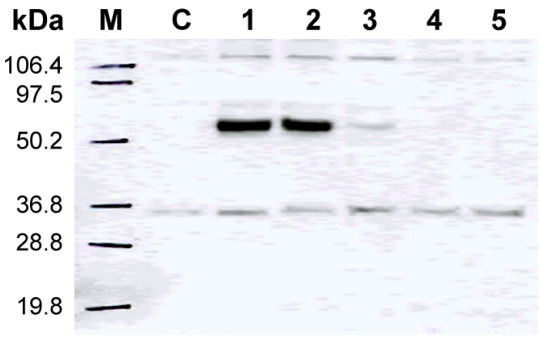

Figure 2b. Western blot. Lane M: prestained marker, Lane C: Control, Lanes 1-5: 2%, 0.2%, 0.02%, 0.002%, 0.0002%.

In Gel Digestion

Non-redundant National Center for Biotechnology Information (NCBI) database was searched for protein identification from the LC/MS data according to established protocol (11). Significant hits were obtained from the search for the DEN- 1 polyprotein which included Env (gi)130423 polyprotein (Dengue virus type 1).

Immunogenicity of the purified Env protein

Mice were immunized intra peritoneally (60 µg at two week intervals) Sera from immunized mice were analyzed by ELISA (Figure 7) and Western blot (Figure 8). The results indicate development of robust humoral immunity in mice. Immunized mice sera could also bind to the purified and refolded dengue Env protein on Western blot.

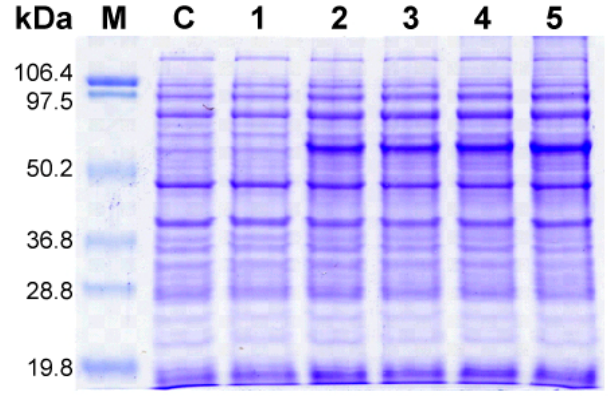

Figure 3a. Time induction of envelope protein expression **a** SDS-PAGE. Lane M: prestained marker, Lane C: Control, Lanes 1-5: 0 h, 2 h, 4 h, 6 h, Overnight.

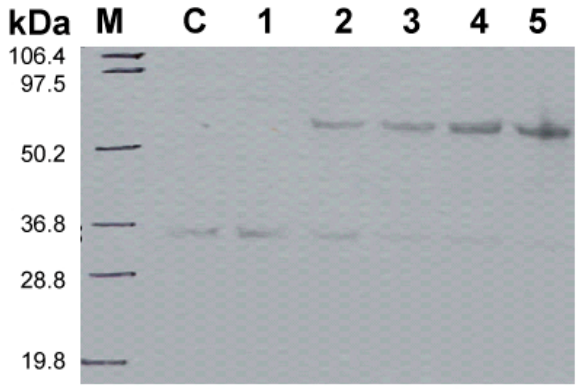

Figure 3b. Western blot. Lane M: prestained marker, Lane C: Control, Lanes 1-5: 0 h, 2 h, 4 h, 6 h, Overnight.

Refolded Env protein neutralizes Dengue virus – 1 infection

After refolding, the recombinant protein's biological functionality was assessed by virus blocking assay. Pre-incubated BHK cells with various concentrations of refolded Env protein were infected with Dengue type 1 virus. Total blocking was observed at concentrations 16 µg and higher (Figure 10). This substantiates a successful refolding of the recombinant protein and its potential as a vaccine candidate for its ability to neutralize virus infection.

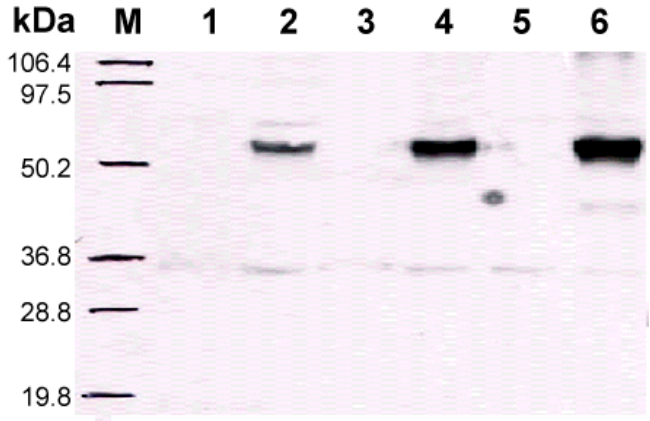

Figure 4. Temperature optimization of envelope protein expression by Western blot. Lane M: prestained marker Lane 1: 24°C Control, Lane 2: 24°C Test, Lane 3: 30°C Control, Lane 4: 30°C Test, Lane 5: 37°C Control, Lane 6: 37°C Test.

Binding Assay

The recombinant protein was found to be biologically functional based on the heparan sulfate

binding assay (Figure 9). Denatured protein was also analyzed in a parallel assay (data not shown) wherein the binding was not as significant as previously reported by pattnaik and co-workers (17).

Diagnostic evaluation of anti-dengue antibodies using recombinant antigen

The recombinant env protein was used in indirect ELISA for detection anti dengue antibodies in the blinded spiked samples. We tested 5 samples with varying dengue antibody concentrations along with blank and negative control. The assay could positively detect the 5 samples that had spiked dengue antibodies and the remaining two samples yielded negative result. The specificity of the ELISA was found to be 100% (Table 2).

Simulation of diagnostic immunoassay with clinical samples

The Env antigen was also evaluated in an indirect immunoassay to detect anti dengue Envelope antibodies spiked in clinical samples. The assay was able to successfully detect antibodies in the nanogram range. Control sample that were not spiked with dengue antibodies gave negative result.

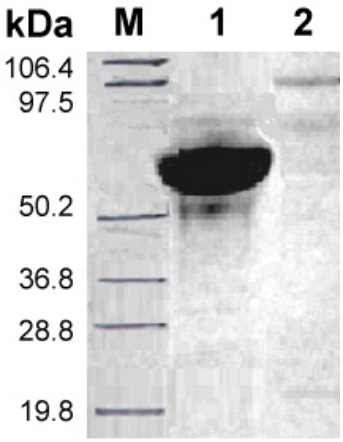

Figure 5. Accumulation of envelope protein in inclusion bodies (Lane 1) and soluble fractions (Lane 2) by Western blot. M: prestained marker.

DISCUSSION

The Env protein of Dengue viruses is widely recognized as a major subunit vaccine component. The Env protein is a multifunctional protein with proven roles in host cell surface receptor binding (18). It is also one of the important targets to induce protective and prolonged immune response [19].

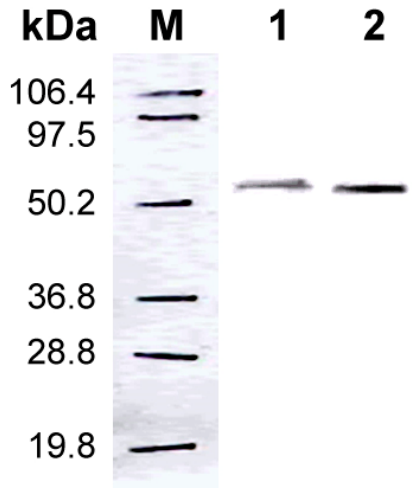

Figure 6. Refolded envelope protein by Western blot. Lane M: prestained marker, Lane 1: refolded envelope protein (75 µ gmL^{-1}), Lane 2: envelope protein (60 µ gmL^{-1}).

The dengue envelope comprises of three structurally distinct domains: I, II and III. Domain III is known to have a role in host cell receptor binding for viral attachment and also in inducing protective antibodies against dengue virus infection in mice (17, 19). This dengue Envelope protein is therefore considered an important antigen for vaccine development and use as a reagent for diagnostic purposes (24, 25, 42, 43). Earlier studies have used different expression systems involving yeast, insect cells and *E coli* for Env expression (20-22). In most of the previous expression systems and purification methods the recombinant proteins were associated with fusion tags such as glutathione S-transferase (GST) and maltose binding proteins for expressing the protein in soluble form with subsequent affinity purification (23). Most studies show that yields of the Dengue Env protein to be low associated with significantly high cost of production. Low expression levels and difficulty in purification has been a major hindrance in the development of Env based subunit vaccines (24). The baculovirus based expression of the antigen has been reported to form aggregates (25). The Env glycoprotein is also very important for vaccine and therapeutic aspects due to the presence of neutralizing epitopes (26-27).

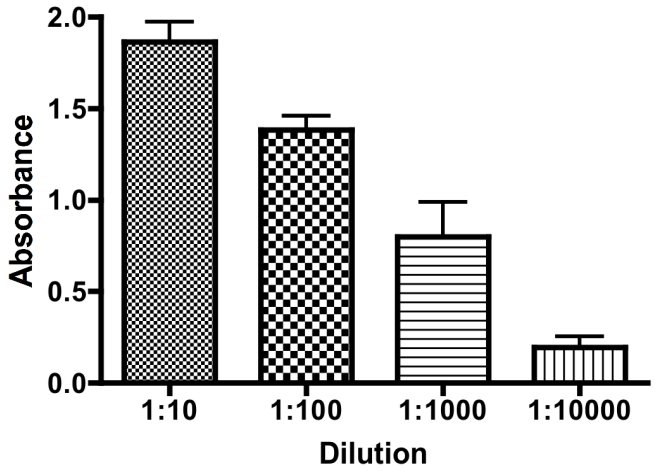

Figure 7. Indirect ELISA of mice sera immunized by Dengue envelope protein at absorbance of 650 nm. Analysis of humoral immune response to dengue envelope protein.

In the present study we have used the *E coli* system for the production of Env protein, which is one of the most frequently used for recombinant protein expression. An advantage of the *E.* coli system is its convenient expression capability and low cost. It is known that *E. coli* expressed eukaryotic protein folding is difficult to obtain as a soluble protein.

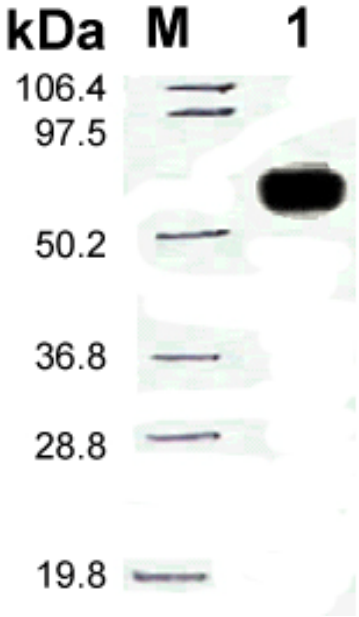

Figure 8. Western blot analysis of refolded envelope protein probed with mouse anti-Env polyclonal antibodies. Lane M: prestained marker, Lane 1: refolded envelope protein.

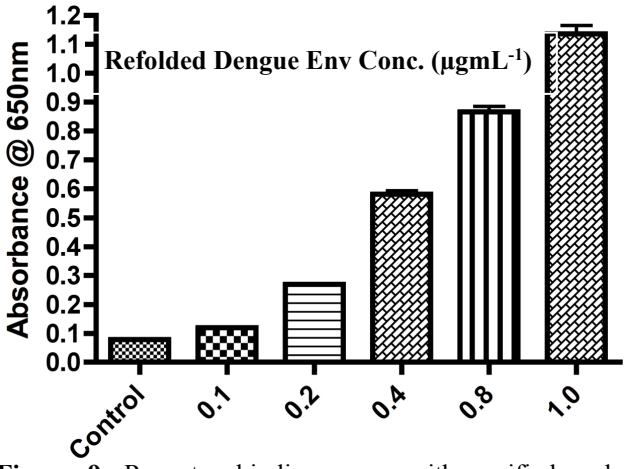

Figure 9. Receptor binding assay with purified and refolded Dengue envelope protein. ELISA plate wells were coated with 0.5 μgmL^{-1} cell free soluble heparin sulfate, except the control wells. Different concentrations of refolded protein were used. Refolded protein bound to heparin sulfate in a concentration dependent manner.

Large-scale expression of proteins is conducible to precipitation thereby forming inclusion bodies [28-29]. Protein purification from inclusion bodies gives a better yield and is relatively simple to purify as previously demonstrated in our laboratory. Earlier studies show that codon optimized genes boost expression level than the native gene (28).

In this study, we have obtained codon optimized Env gene chemically for expression in *E. coli*. Codon optimization substantially enhances

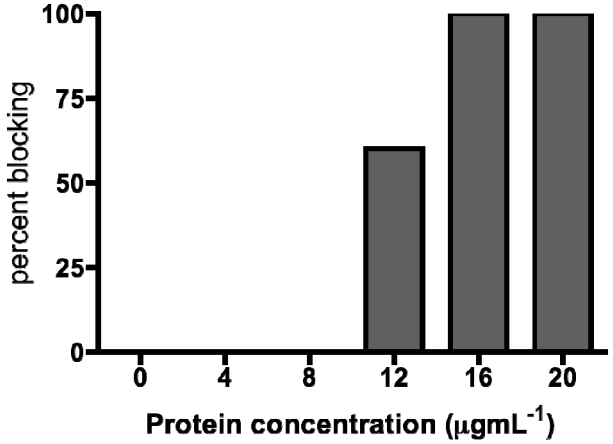

Figure 10. Biological function assessment of refolded Env protein. BHK cells were pre-incubated with various concentrations of Env protein and BSA (control) and then infected with dengue virus type 1 (250 $TCID_{50}$ per well). Afetr 3 days, the wells were examined under a microscope for cytopathic effects. Three wells were examined for each concentration.

gene expression that leads to higer level of protein expression. Codon optimization was carried out using optimizer software in Geneart. They follow a deterministic sliding window algorithm for multiparameter sequence optimization. The target sequences are determined with a quality attribute taking codon usage, GC content, mRNA structure and species-specific sequence motifs into consideration. The first codon of the best candidates' variation window is made absolute and the window is shifted by one codon position towards the 3' end (30). We have cloned and purified the Dengue Env in *E. coli* for the development of MAbs for primarily translational applications. The Env protein could also be used for dengue detection and therapeutic and vaccine applications. The 6x His tag was inserted at the C-terminal end of the recombinant protein to augment easy purification. This tag has negligible immunogenicity and hence need not be removed from the recombinant protein (31). We also analyzed a range of conditions to validate the optimal temperature, time and inducer concentration for highest protein expression (11).

The Env gene was cloned in presence of a promoter for high-level expression of recombinant protein as inclusion bodies. The pBM802 vector was used for cloning under the control of the pBAD promoter that is nothing but arabinose promoter hence arabinose was used as inducer (32). This promotes high level expression of recombinant protein as inclusion bodies in the bacterial cytoplasm. The final yield of purified inclusion bodies was estimated by Bradford protein assay (33) and estimated to be approximately 15-20 mgL^{-1} (Table 1) of initial bacterial culture which is 5-10 fold higher compared to previous studies using the native E gene sequence (19,22,31). The purity of inclusion bodies was determined by SDS-PAGE and Western blot. French Press lysis of cells and subsequent washing steps with detergent were adopted to purify the inclusion bodies from soluble proteins. SDS-PAGE analysis (data not shown) demonstrated that lysis by French Press and further washing with PBS substantially enhanced the purity of the inclusion bodies as a large amount of the *E. coli* soluble proteins could be separated. The purification method comprising of IMAC under denaturing conditions adsorbed the His-tagged protein (23). IMAC purification under denaturing conditions produced better amounts of pure Env protein with one band as determined by SDS-PAGE analysis. Bradford assay estimated a final concentration of around 80% of the initial amount. Recombinant proteins are expressed in *E. coli* as inclusion bodies and various refolding methods have been described to renature the proteins from inclusion bodies (23).

The IMAC purified Env protein was refolded using TA buffer consisting of a redox pair (GSH/GSSG) with two different protein concentrations (75 μ gmL^{-1} and 60 μ gmL^{-1}). No aggregation was visible with both the concentrations during the process of refolding. SDS-PAGE and Western blot analyses were used to determine the purity of the refolded protein. Anti-His_6 MAb reacted with a single band of ~54 kDa, suggesting a successful purification of recombinant Env. The identity of purified protein was further confirmed by *in vitro* gel digestion, mass spectrometry, and NCBI non-redundant database search which proved that the purified protein band to be the dengue polyprotein of our interest. The protein was also probed with dengue Env MAbs thus confirming proper refolding. The Env protein was also successful in inducing a robust humoral immune response in balb/c mice as evidence from the high antibody titers and western blot analysis. The refolded Env protein was used as an antigen to immunize mice for development of MAbs. The polyclonal antibodies from mice sera were strongly binding with the recombinant protein in Western blot (Figure 8). The conformation of the Env protein plays an important role during viral infection (34). We did not analyze the crystal structure of the recombinant Env protein but putting it in perspective to findings by various research groups we can predict our refolded protein to be having a postfusion conformation. It has been reported that exposure to detergent and acidic pH would lead to trimeric postfusion conformation (35). As our procedure involves similar parametric conditions we can anticipate the structural conformation. In addition anti dengue MAbs 8A5 and 12A1 were sourced from our collaborators in the United States to probe our refolded Env protein in Western blot as well as standard ELISA and we found desired level of interaction (data not shown). Subsequent analysis of the functional integrity of the refolded protein was done by a dengue virus-blocking assay. The assay demonstrated the ability of the recombinant Env protein to completely neutralize dengue virus infectivity. Cells in the presence of the recombinant protein were protected

from Dengue type 1 virus challenge (Figure 10). It can be concluded that this could be the result of competition between the recombinant protein and the dengue type 1 virus for the host cell surface receptors. This phenomenon is consistent with the earlier findings of Jaiswal and associates (31).

The functionality of the recombinant protein was further analyzed with heparan sulfate based assay. The recombinant protein may also be used as a diagnostic antigen. Our blinded optimized diagnostic assay resulted in 100% specificity. The specificity and sensitivity of the assay was further established in the analysis with the clinical samples.

Table 1. Comparative analysis of production of full length Env protein

Dengue Envelope Type	Affinity Tag	Host	Yield	Reference
Dengue 1 Envelope	His_6	*E. coli*	15-20 mg L^{-1}	This article
Dengue 1 Envelope	GST	*E. coli*	2 mg L^{-1}	[22]
Dengue 1 Envelope	GST	*Pichia*	0.1 mg L^{-1}	[22]
Dengue 2 Envelope	HBsAg	*Pichia*	0.5 mg L^{-1}	[38]
Dengue 2 Envelope	MBP	Sf9	1 mg10^{-9} cells	[21]

Table 2. Range of OD_{650} values in blinded diagnostic assay

Range of OD value	Blinded Sample No	Original Sample
0.736 - 0.910	1	8 ngmL^{-1} anti den MAb
0.588 - 0.692	2	6 ngmL^{-1} anti den MAb
0.000 - 0.096	3	Neat Serum
0.211 - 0.289	4	2 ngmL^{-1} anti den MAb
0.951 - 1.078	5	10 ngmL^{-1} anti den MAb
0.000 - 0.127	6	10 ngmL^{-1} SARS MAb
0.473 - 0.535	7	4 ngmL^{-1} anti den MAb

We obtained detection limit as low as 0.4 ng/mL (data not shown). Further optimization would offer a rapid alternative to whole virus antigen based diagnostic assays. This expression system can be exploited for other recombinant proteins as well which would be biologically functional and also in developing a dengue vaccine owing to high yield protein production at a very reasonable cost. The recombinant Env protein will be used to develop several MAbs with different specificities from immunized mice. MAbs against dengue Env is known to protect mice against dengue infection (36).

In summary the Den-1 full length Envelope protein was efficiently expressed in the *E.*coli system. We hope to develop several MAbs (37, 38) in future to develop an oligoclonal antibody cocktail based therapeutic vaccine against all the dengue virus serotypes. The full length Env protein could also be used for dengue diagnostic studies (39-45). Development of drugs inhibiting the Env mediated dengue infection can be exploited with a functional recombinant protein. In addition to this there are many targeting approaches and the development of nanocarrier vaccine have been reviewed (46, 47). This would be highly inexpensive procedure for the development of antigens for large-scale use in terms of therapeutics and diagnostics.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conceived and designed the experiments: MRS, AG, DD. performed the experiments: AG, DD and RBM. Analyzed the data: AG, DD, MRS and HHS. Contributed reagents/materials/analysis tools: MRS, HHS. Wrote the paper: AG. All authors read and approved the final manuscript.

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