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Bacterially expressed and refolded envelope protein (domain III) of dengue virus type-4 binds heparan sulfate

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Abstract

An arboviral infection like dengue fever/dengue hemorrhagic fever (DHF) with high morbidity and mortality rate are extensively prevalent in several parts of the world. Global efforts have been directed towards development of vaccine for prevention of dengue. However, lack of thorough understanding about biology and pathogenesis of dengue virus restricts us from development of an effective vaccine. Here we report molecular interaction of domain III of envelope protein of dengue virus type-4 with heparan sulfate. A codon optimized synthetic gene encoding domain III of dengue virus type-4 envelope protein was expressed in *Escherichia coli* and purified under denaturing conditions, refolded and purified to homogeneity. Refolded Den4-DIII was characterized using biochemical and biophysical methods and shown to be pure and homogeneous. The purified protein was recognized in Western analyses by monoclonal antibody specific for the 6× His tag as well as the H241 monoclonal antibody. The *in vitro* refolded recombinant protein preparation was biologically functional and found to bind cell free heparan sulfate. This is the first report providing molecular evidence on binding of dengue-4 envelope protein to heparan sulfate. We developed a homology model of dengue-4 envelope protein (domain III) and mapped the possible amino acid residues critical for binding to heparan sulfate. Domain III envelope protein of dengue virus is a lead vaccine candidate. Our findings further the understanding on biology of dengue virus and will help in development of bioassay for the proposed vaccine candidate.

Keywords: Dengue virus; Envelope protein; Domain III; Expression; Purification; Refolding; Heparan sulfate

1. Introduction

The last decade witnessed emergence of several viral diseases with pronounced virulence thereby causing severe epidemics. Majority of such diseases belong to the group of vector-borne infections due to various factors such as massive urbanization and change in ecosystem [1]. Vector borne viral diseases constitute most of the re-emerging diseases. Among the arboviral infections, dengue fever with high morbidity and mortality are extensively prevalent in several parts of tropical countries [2]. Dengue virus infection and its more severe manifestations, namely, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) have emerged as significant global public health threats, especially as there is neither an effective antiviral therapy for its treatment nor a vaccine for its prevention [3]. Dengue virus is transmitted to humans by infected mosquitoes, mainly *Stegomyia aegypti* and *S. albopicta*. The global prevalence of dengue viruses and their mosquito vector has grown dramatically in the recent years [4]. This has been attributed to several factors such as lack of effective vector control measures, uncontrolled urbanization coupled with concurrent population growth and increased air travel. The disease is now endemic in more than 100 tropical and sub-tropical countries [5]. WHO estimates that approximately 50 million cases of dengue infections may occur every year and about 2.5 billion people are estimated to be at risk [6].

As the prospects of reversing the recent trend of increased epidemic activity and geographic expansion of dengue are not very promising, disease prevention through the development of effective vaccines assumes greater significance. Vaccine development

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for dengue and DHF is a very challenging task because of the fact that four antigenically distinct virus serotypes (Den-1, -2, -3 and -4) may cause disease, and protection against any one or two of the dengue virus serotypes can increase the risk of more serious disease through the antibody dependant enhancement (ADE) phenomenon [7,8]. Nevertheless, several laboratories worldwide are exploring multiple approaches towards developing dengue vaccines on the basis of conventional live attenuated viruses [9–11], inactivated viruses [12–15] infectious clone derived attenuated viruses [16–18], DNA vaccine [19,20], vectored vaccine [21–23], and recombinant DEN viral proteins expressed in heterologous systems [24–31]. Each and every approach of dengue vaccine development has its own advantages and disadvantages.

Recently intense efforts are being directed at heterologous expression of the envelope protein in order to develop it as a possible sub-unit vaccine candidate. Most of the studies reporting expression of envelope protein in either bacteria or yeast systems have resulted in low yields, apparently due to low expression levels [24,26,29]. Several studies have used dengue 2 envelope protein for heterologous expression [25,27,32,33]. Few also report heterologous expression of homologous domain of other flavi viruses [34–37]. However, most of the studies have not explored *in vitro* refolding of the protein, or if attempted refolding, failed to report biophysical and chemical characterization of the refolded protein [33]. Efforts to use denatured/non-refolded envelope protein as vaccine though elicited good antibody response but failed to offer protection [38]. Neutralizing epitopes on envelope protein are known to be conformation dependant [39], hence an ideal subunit vaccine using envelope protein need to be correctly folded.

Envelope protein of dengue virus is known to play critical role in receptor binding [40]. It binds to cell surface carbohydrate of the target cells, resulting in receptor mediated endocytosis and internalization of virus [41]. Envelope (E) protein of dengue virus type 2 (Den2) is known to bind cell surface heparan sulfate [42,43]. Antibodies binding to domain III of dengue virus E protein have been shown to block adsorption of virus to target cells [44,45]. The domain III of the envelope protein is particularly important from the viewpoint of vaccine development as it contains multiple serotype-specific conformation-dependent, neutralizing epitopes and the host cell receptor recognition site. Studies on structure of envelope protein have also indicated involvement of critical amino acids in receptor–ligand interaction [43,46]. Several studies address interaction of dengue virus to cell bound surface receptor (heparan sulfate), however, none have shown interaction of envelope protein to cell free receptor. Crystal structure of E protein of dengue virus type 4 (Den4) is not available, neither any study is known to report on its ability to bind heparan sulfate.

We have explored the feasibility of expressing a small, biologically critical domain of the dengue virus type 4 envelope protein in *Escherichia coli*. In this paper, we show that expressing domain III alone rather than the full-length E molecule in *E. coli*, results in high levels of recombinant protein synthesis. This paper describes the expression, purification, and evaluation of the biological function of the D4EIII protein, the functional receptor binding domain of Den4. The objective of the study is to obtain high quality, well characterized refolded D4EIII for use in vaccine development studies. We also report development of a simple ELISA based biological functional assay for the refolded D4EIII protein.

2. Experimental

2.1. Instruments

The chromatographic system used throughout this study was the fast protein liquid chromatography (FPLC) system; model AKTA-Explorer from Amersham Biosciences AB (Uppsala, Sweden). Centrifuge, model Sorvall Evolution RC was from Kendro Laboratory Products (Newtown, CT, USA). The ultrasonic homogenizer Vibracell VCX 750 model was from Sonics (Newtown, CT, USA). Protein sequencer, model 491 and Automated DNA sequencer, model ABI 310 was from Applied Biosystems (Foster City, CA, USA). The electrophoresis apparatus Mini-Protean III, electroporator Gene Pulser Xcell and Semidry trans blot apparatus (Transblot SD) were from Bio-Rad (Hercules, CA, USA). Image analysis Gel Doc 2000 and Quantity one software version 4.4.0 were from Bio-Rad Laboratories Inc. [Segrate (Milan), Italy]. LC–MS system model LCQ advantage, LC surveyor pump, Finnigan Surveyor PDA detector, MSD-ion trap and spectrophotometer model Biomate3 were from Thermo Electron Corporation (Madison, WI, USA). ELISA microplate reader was from Bio-Tek Instruments Inc. (Winooski, Vermont, USA).

2.2. Materials and chemicals

E. coli host strain BLR(DE3) and the plasmid pET30a+ were from Novagen (Madison, WI, USA). The synthetic gene encoding domain III of envelope protein of dengue virus type 4 was custom synthesized from Biotech Desk, Hyderabad, India. PCR kit (PCR core systems) was from Promega (Madison, WI, USA). Rapid ligation kit and restriction enzymes were from Fermentas International Inc. (Ontario, Canada). Streamline Chelating, the XK 16/20, Superdex 75 10/300 GL and LMW Gel filtration calibration kit were from Amersham Biosciences, Sweden. Anti-His mAb (catalog #34660) was from Qiagen (Valencia, CA, USA). DEN-4 virus-specific H241 monoclonal antibody (mAb) was from Chemicon International (Temecula, CA, USA). Dialysis bags, anti-mouse IgG HRP conjugate, dehydrated Luria Bertani broth for preparation of bacterial growth media, L-Arginine, Urea, Guanidine hydrochloride (GuHCl), all other salts, additives and chemicals were from Sigma Chemical (St. Louis, MO, USA). C-8 RP-HPLC column was from SGE Inc. (Austin, TX, USA). BCA protein assay kit, Ellman's reagent and Cysteine standard were from Pierce (Rockford, IL, USA). UNOsphere S cation exchange media, Immuno-Blot PVDF membranes were from Bio-Rad Laboratories Inc. (Hercules, CA, USA). ELISA plates (Nunc–Immuno Plate MaxiSorp Surface) were from Nunc Brand products (Roskilde, Denmark). Amicon Ultra centrifugal filter devices were from Millipore (Bedford, MA,

USA). HPLC grade acetonitrile chromasolve was from Fluka (Buchs/Switzerland).

2.3. Preparation of the cellular extract containing D4EIII

2.3.1. Construction of pET-D4EIII plasmid

A DNA fragment (0.33 Kb) encoding the carboxy terminal portion (aa300–400) of dengue virus type-4 (Den4) E protein (which encompasses domain III, aa300–395) fused to hexahistidine at the C-terminal end (D4EIII) were amplified by polymerase chain reaction (PCR) using primers 5′-acg agt cat atg tgc tca gga aag ttc tca a-3′ and 5′–acg agt gtc gac tca atg gtg atg gtg atg gtg ctt gcc aat gga act ccc t-3′ as forward and reverse primer, respectively and the plasmid containing the synthetic gene encoding amino acids of D4-61NIID strain of Den4 as template. PCR product was digested with *Nde*I and *Sal*I and the resultant *Nde*I-*Sal*I fragment was cloned downstream of T7 promoter of *E. coli* expression vector pET30a+ to yield plasmid pET-D4EIII. The N-terminal methionine of the recombinant D4EIII expressed by this construct is provided by the expression vector. The insert as well as the junction between the insert and vector sequences were sequenced in both directions. *E. coli* BLR(DE3) cells were transformed with plasmid pET-D4EIII by electroporation. The resultant transformants were selected on kanamycin + tetracycline plates and subjected to preliminary polymerase chain reaction (PCR)-screening using insert-specific primers. The positive clones were used for expression of D4EIII.

2.3.2. Expression screening

Several of the PCR-positive clones were inoculated into 3 ml test-tube cultures and allowed to grow at 37 °C in a shaker at 200 rpm. Cultures in logarithmic phase (at OD of 0.6–0.7 at 600 nm) were induced for 4 h with 1 mM isopropylthiogalactoside (IPTG). After induction, equivalent numbers of cells from the various cultures (normalized on the basis of OD_{600} values) were lysed in sample buffer and analyzed by SDS–PAGE [47]. Un-induced control cultures were analyzed in parallel. One clone that expressed maximal levels of the predicted recombinant protein was chosen for further experiments.

2.3.3. Localization of recombinant D4EIII protein in induced cells

For initial experiments (designed to determine the solubility of the recombinant protein), 10 ml cultures were set up in wide mouth test tubes and induced with 1 mM IPTG at 0.6 OD_{600} for 4 h. Aliquots of the induced culture were lysed separately under native and denaturing conditions as follows. To prepare a native lysate, induced cells (1.0 OD_{600}) were re-suspended in 200 μl native lysis buffer (50 mM potassium phosphate pH 8.0, 250 mM NaCl) and sonicated for 2 min. The lysate was centrifuged at 13,000 × *g* for 15 min and the resulting supernatant (S) was transferred into a fresh tube. The pellet (P) was solubilized by boiling in 200 μl SDS–PAGE sample buffer. To prepare a lysate under denaturing conditions, another equivalent aliquot of induced cell culture was processed in parallel, using denaturing lysis buffer (50 mM potassium phosphate pH 8.0, 250 mM NaCl, 8 M GuHCl). Fractions S and P were obtained from the denaturing lysate as done earlier. Equivalent aliquots of all four resultant fractions were analyzed by SDS–PAGE.

2.3.4. Preparation of inclusion bodies from shake-flask culture

Mother culture was set up by inoculating 10 ml LB medium containing 50 μg kanamycin ml^{-1} and 12.5 μg tetracycline ml^{-1} with 25 μl glycerol stock of BLR(DE3) cells transformed with the pET-D4EIII expression vector. The culture was grown overnight in a shaker-incubator at 37 °C, at 200 rpm. One litre LB (containing 50 μg kanamycin ml^{-1} and 12.5 μg tetracycline ml^{-1}) in a 5 l Haffkine flask was inoculated with mother culture @1% and placed in the shaker-incubator at 37 °C at 200 rpm. When the OD_{600} of the culture reached 0.6–0.7 (a small aliquot of the un-induced culture was set aside for subsequent SDS–PAGE analysis), it was induced by the addition of IPTG to a final concentration of 1 mM. Induction was allowed to proceed for 4 h before harvesting the cells. Aliquots of the induced and un-induced cell cultures were analyzed by SDS–PAGE prior to the preparation of inclusion bodies (IBs).

The induced culture was centrifuged in a Sorvall SLC-6000 rotor at 7900 × *g* for 15 min at 4 °C. The cell pellet (∼3 g wet weight) was washed once with 250 ml cell wash buffer (10 mM Tris–HCl pH 8.0, 10 mM EDTA, 100 mM NaCl) and re-suspended thoroughly in 100 ml chilled cell lysis buffer (10 mM Tris pH 7.5, 5 mM Benzamidine–HCl, 5 mM EDTA, 100 mM NaCl, 5 mM DTT, 100 μg ml^{-1} Lysozyme) containing 1 mM freshly prepared phenylmethylsulfonyl fluoride (PMSF), and lysed by sonication using five one min pulses with 2 min in ice between each pulse. The lysate was centrifuged in a Sorvall SLC-1500 at 26,000 × *g* for 30 min at 4 °C and supernatant removed (a small aliquot of the supernatant was set aside for subsequent SDS–PAGE analysis). The cell pellet containing the inclusion bodies (IBs) was washed with IB wash buffer (50 mM Phosphate buffer pH 6.0, 5 mM EDTA, 200 mM NaCl, 0.5 M urea, 1% Triton*X*-100) followed by another wash with buffer containing 50 mM phosphate buffer pH 6.0, 1 mM EDTA and 1 M NaCl and centrifuged as before. The supernatant was carefully removed and the IB pellet was stored frozen at −80 °C until further use.

2.3.5. Solubilization of IBs

The IB pellet was thawed and re-suspended in about 20 ml of solubilization buffer (10 mM Tris–HCl, pH 8.0, 100 mM NaH_2PO_4, 100 mM NaCl, 8 M GuHCl), stirred on a magnetic stirrer for 6–8 h at room temperature (RT), and clarified by centrifugation at 25,800 × *g* for 45 min at 4 °C using Sorvall SLC-1500 rotor. The supernatant containing the solubilized IBs was collected and its protein concentration was determined with reference to standard bovine serum albumin (BSA) in the BCA assay [48]. A blank reaction was performed in parallel to correct for the effect of GuHCl in the colorimetric assay. We also estimated the total protein by taking absorbance at 280 nM in spectrophotometer and calculating the protein concentration using absorbance coefficient of 0.802 (0.802 OD_{280} = 1 mg ml^{-1}).

2.4. Chromatographic procedure-1

2.4.1. Preparation of the immobilized metal affinity chromatography (IMAC)

The Streamline chelating resin was packed in an XK16/20 column. Slurry was prepared with 10 mM Tris, 100 mM NaCl, pH 8.0, in a ratio of 75% settled gel to 25% buffer. The column was filled through the outlet with a few centimeters of buffer and was closed. The gel slurry was poured into the column in one continuous motion. The remainder of the column was filled with buffer and the top piece mounted and connected to a pump. The bottom outlet of the column was opened and the pump was set at 1 ml min^{-1}. The packing flow rate was maintained for three bed volumes after a constant bed height was reached. The final volume of the gel was 4.0 ml. The column was washed with three column volumes of distilled water. The column was charged with three column volumes of 50 mM $NiCl_2$ solution. The column continued to be washed with three column volume of distilled water and equilibrated with equlibration buffer (10 mM Tris, 100 mM NaCl, 100 mM NaH_2PO_4, 6 M Guanidine HCl, pH 8.0).

2.4.2. Immobilized metal affinity chromatography procedure

Solubilized IBs were loaded to the pre-equilibrated streamline chelating column at a flow rate of 1 ml min^{-1}. The column was washed with equilibration buffer at pH 6.3, at a flow rate of 1 ml min^{-1} until UV baseline was reached. The bound protein was eluted using a pH gradient starting at pH 6.3 and ending at pH 4.0. The elution buffer has similar composition as equilibration buffer, but pH was different. The emergence of protein in the fractions was monitored by spectrophotometer by taking OD at 280 nm. Hundred microlitres of each fraction was ethanol precipitated and analyzed by SDS–PAGE. The relevant fractions were pooled, and concentrated using centrifugal ultra filtration devices and adjusted to 4 mg ml^{-1} with equilibration buffer.

2.5. Refolding of affinity purified D4EIII

Purified, denatured D4EIII was refolded by 100 fold dilution in freshly prepared refolding buffer containing 50 mM phosphate buffer, pH 5.8, 1 M urea, 1 mM EDTA, 0.5 M arginine, 2 mM cystine base and 0.6 mM cystamine dihydrochloride so that the final concentration of D4EIII was 40 μg ml^{-1}. Routinely, 5 ml affinity purified D4EIII (20 mg protein) was added rapidly to 1 L refolding buffer that was kept stirring during the addition. One subsequent additions of 5 ml (20 mg protein) was made after 1 h of first addition, so as to reach a final concentration of 40 μg ml^{-1} D4EIII in refolding buffer (a total of 40 mg purified D4EIII). The refolding vessel was flushed with N_2 gas and the refolding reaction was allowed to incubate for a period of 24 h at 10 °C, without stirring. The refolded preparation was dialyzed against 50 mM phosphate buffer (pH 5.8), containing 1 M urea (freshly prepared) for 48 h, with buffer changes every 12 h to remove arginine and co-solvent. The dialyzed sample (1400 ml) was clarified by filtration through a 0.22 μ membrane. Following removal of arginine and clarification, as the sample was very dilute (0.035 mg protein ml^{-1}), it was subjected to concentration and batch purification by ion-exchange chromatography.

2.6. Ion-exchange chromatography procedure

The UNOsphere S cation exchange media (UnoS) was flow packed in an XK16/20 column at a constant flow rate of 1 ml min^{-1}. The packing flow rate was maintained for three bed volumes after a constant bed height was reached. The final volume of the gel was 3.6 ml. The column was washed with three column volumes of distilled water equilibrated with equlibration buffer (50 mM phosphate buffer pH 5.8, 1 M urea). Refolded, dialyzed and clarified D4EIII was loaded to the pre-equilibrated UnoS column. The column was washed with wash buffer (50 mM phosphate buffer pH 5.8, 50 mM NaCl), at a flow rate of 1 ml min^{-1} until UV baseline was reached. The bound protein was eluted using a NaCl gradient starting at 50 mM and ending at 1 M. The elution of protein in the fractions was monitored by spectrophotometer by taking OD at 280 nm and each fraction was analyzed by SDS–PAGE. The fractions showing refolded D4EIII were pooled, and concentrated using centrifugal concentrator and adjusted to 1 mg ml^{-1}.

2.7. Analytical procedures

2.7.1. Electrophoresis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) [47] using a Mini-protean III apparatus and Tris–glycine–SDS buffer were used to monitor the purification during chromatographic procedures. Electrophoresis was performed for 90 min at 100 V using 16%T and 3%C polyacrylamide gels. Detection was performed with Coomassie brilliant blue R250.

2.7.2. Reverse phase chromatography and mass spectral analysis

Reverse phase C8 column (Wakosil II 5C8RS, 5 μm, 100 mm × 4.6 mm) was initially equilibrated with 95% eluent A (0.05% trifluoroacetic acid (TFA) in water) and 5% eluent B (0.05% TFA in 90% acetonitrile, 10% water). Ten microlitres of refolded D4EIII was loaded on to column. The bound protein was eluted using a linear gradient from 95% eluent A + 5% eluent B reaching 5% eluent A + 95% eluent B in 40 min. The eluted fraction from HPLC was used as sample feed for mass spectra analysis. Mass analysis was carried out by electro-spray ionization mass spectrometry. Helium was used as collision gas and nitrogen as sheeth gas. The following parameters were set in the system. Spray voltage: 4.5 KV, capillary temperature: 280 °C, capillary voltage: 3 V, tube lens (offset): 25 V, Ionization energy: 10–30%, mass analyzer: ion trap, scan mode: positive. The molecular mass was estimated after deconvolution of the spectra using the built in software.

2.7.3. N-terminal sequencing of refolded, recombinant D4EIII

Automated Edman degradation was carried out using an Applied Biosystem 491 protein sequencer following standard method [49]. Around 100 pmol of purified refolded D4EIII was used for N-terminal sequencing reaction.

2.7.4. Detection of free thiol

Analysis of free thiols in D4EIII was carried out using 5,5′-dithiobis(2-nitrobenzoic acid) by Ellman's method [50]. The sensitivity of the assay was determined using known amounts of cysteine. The assay could clearly detect free thiols up to a concentration of 30 μM. Presence of free thiols in refolded D4EIII was determined in the presence of 6 M GuHCl to promote side chain availability.

2.7.5. Mobility of refolded D4EIII before and after reduction with DTT

Refolded D4EIII (10 μg) was denatured by addition of denaturing buffer (10 mM Tris–HCl, pH 8.0, 100 mM NaH_2PO_4, 100 mM NaCl, 8 M GuHCl) and reduced with 100 mM dithiothreitol (DTT). Reduced D4EIII was alkylated with 5% iodoacetamide for 30 min at 37 °C. Reduced and alkylated D4EIII was precipitated using ethanol, dissolved in SDS–PAGE buffer containing β-mercaptoethanol and separated by SDS–PAGE as described before. Refolded D4EIII was also precipitated with ethanol and dissolved in non-reducing SDS–PAGE buffer lacking β-mercaptoethanol or other reducing agents. Both correctly folded D4EIII and reduced D4EIII were separated by SDS–PAGE and detected by Coomassie staining.

2.7.6. Gel permeation chromatography

The Superdex 75 10/300 GL column was equilibrated with 50 mM phosphate buffer pH 5.8 containing 250 mM NaCl at a constant flow rate of 1 ml min^{-1}. Retention time of different standard proteins in the column at a flow rate of 1 ml min^{-1} was standardized using LMW gel filtration calibration kit. Refolded D4EIII was loaded (100 μl) to the pre-equilibrated and pre-calibrated Superdex 75 column. Elution of D4EIII from the column was monitored at 280 nm and elution time was compared with the standard proteins.

2.7.7. Western blotting

Purified and refolded D4EIII protein was run on 16% SDS–PAGE (sample buffer lacking any reducing agent), and transferred electrophoretically to PVDF membrane. The membrane was blocked overnight with 5% BSA. After overnight blocking, the membrane was incubated with primary antibody (either H241 mAb at 1:500 dilution or anti-His mAb at 1:1000 dilution) for 60 min at RT. The membrane was washed three times with PBS containing 0.05% Tween-20 (PBST) and then incubated in anti-mouse IgG-HRP conjugate solution at a dilution of 1:2000 for 60 min at RT. The membrane was washed as above and the protein bands were visualized by incubating in chromogenic substrate (3,3′-diaminobenzidine tetrahydrochloride) and hydrogen peroxide for 5–6 min at RT. The membrane thus developed was washed with double distilled water, dried and observed for appearance of brown color bands.

2.7.8. Recognition by patient sera

Sera collected from dengue patients were used for recognition of refolded recombinant D4EIII by dot-ELISA. Briefly, Nitrocellulose strips (NC strips) were coated with 400 ng refolded D4EIII protein. Coated strips were blocked with 5% bovine serum albumin in phosphate-buffered saline, overnight at 4 °C. Coated strips were kept standing in wells of microtitre plates wells filled with 200 μl of patient sera diluted to 1: 1000 in phosphate buffered saline containing 0.05% Tween-20 and 0.001% phenol red and incubated for 60 min at 37 °C. The NC strip was washed five times, 3 min each with PBST and then incubated in anti-human IgG-HRP conjugate solution at a dilution of 1:5000 for 60 min at RT. Healthy human sera were used for recognition of D4EIII by dot-ELISA in the similar way, as controls. The strips were washed as above and visualized by following method explained in section 2.7.7. NC strips thus developed were dried and observed for appearance of brown color dots.

2.7.9. Functional bioassay

Refolded recombinant D4EIII was tested for recognition of heparan sulfate by ELISA. Briefly, Immuno Plate MaxiSorp wells were coated with 0.5 μg ml^{-1} heparan sulfate and blocked as described above. Heparan sulfate coated wells were incubated with refolded D4EIII diluted in blocking buffer for 60 min at 37 °C. Serial dilutions (1:2 fold) of D4EIII starting with 2 μg ml^{-1} were tested. Then, wells were washed four times with PBST and incubated with 100 μl of diluted (1:1000 in 5% BSA) penta-His mAb for 1 h at RT. The wells were washed as before and incubated with anti-mouse IgG conjugated to horseradish peroxidase (at 1:1000 dilution in 5% BSA) for 1 h at RT. The color reaction was developed with *o*-phenylenediamine dihydrochloride and hydrogen peroxide as the chromogenic substrate. The reaction was stopped by addition of 2 M sulfuric acid and OD was recorded at 490 nm in a microplate ELISA reader. 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 analyzed in parallel.

2.8. Homology modeling

The protein sequence of envelope protein (domain III) was obtained from the GenBank database (accession no. AB111090). A structural model of carboxy terminal portion (aa300–400) of the envelope protein sequence of Den4 was obtained by means of three-dimensional position-sensitive scoring matrix (3D-PSSM) fold recognition server V 2.6.0. The obtained model is viewed and refined using InsightII® molecular modeling package (version: 95, MSI and version 2000, Accelrys) and the Homology® module was used to build and optimize the model. Amino acid residues predicted to bind heparan sulfate [43] were mapped on to the homology model structure.

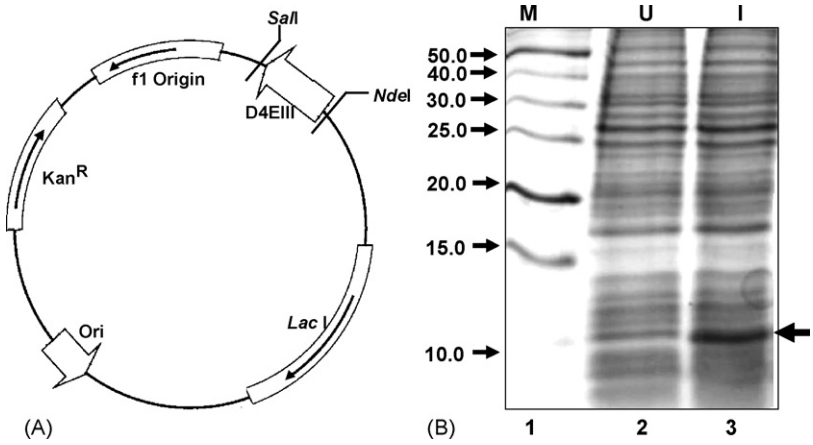

Fig. 1. [A] Map of plasmid pET-D4EIII. In this plasmid, the cDNA encoding dengue virus type-4 envelope domain III, D4EIII, is cloned in-frame with the initiator codon provided by pET30a+. Restriction sites used for cloning are indicated. Abbreviations are as follows: Lac I, Lac repressor gene; Kan^R, Kanamycin marker; Ori, replication origin sequences. The arrows indicate direction of transcription. [B] SDS–PAGE analysis of D4EIII protein expression. The panel depicts Coomassie stained polypeptide profiles of *E. coli* lysates prepared before (lane 2) and after (lane 3) IPTG induction. Protein molecular weight markers were run in lane 1. Their sizes (in kDa) are shown at the left of the panel. The arrow on the right indicates the position of the D4EIII protein. Abbreviations are as follows: M, markers; U, un-induced; and I, induced.

3. Results and discussion

3.1. Expression of the dengue virus type-4 envelope (domain III) protein in E. coli

The bacterial vector designed to express domain III of the E protein of DEN-4 virus is shown in Fig. 1A. This vector was generated by fusing domain III-encoding sequences in-frame with the translation initiator codon and the 6× His tag of pET30a+, under the control of IPTG-inducible phage T7 promoter. This construct is predicted to encode a 106aa recombinant protein with a molecular weight of 11.68 kDa. This construct was transformed into BLR(DE3) host cells, which provide phage T7 RNA polymerase for expression of heterologous genes. Small-scale cultures of the positive clones (selected on the basis of PCR screening) were subjected to IPTG induction to identify clones capable of expressing the predicted 11.68 kDa recombinant protein. A typical induction experiment comparing the polypeptide profiles of un-induced (lane 2) and IPTG-induced (lane 3) *E. coli* cultures is shown in Fig. 1B. It is evident that IPTG induction results in the expression of a unique 11.6 kDa protein. Of the BLR(DE3) clones identified, one was selected which expressed the recombinant protein maximally; plasmid DNA was isolated from it and verified to be correct by restriction analysis and sequencing (data not shown). This clone was used in further experiments. We optimized expression conditions by testing several different IPTG concentrations (0.2–1.5 mM) and induction duration (1–4 h). We found 1 mM IPTG with 4 h induction time is most effective for protein yield (data not shown). Therefore, in all subsequent experiments induction was carried out with 1 mM IPTG for 4 h.

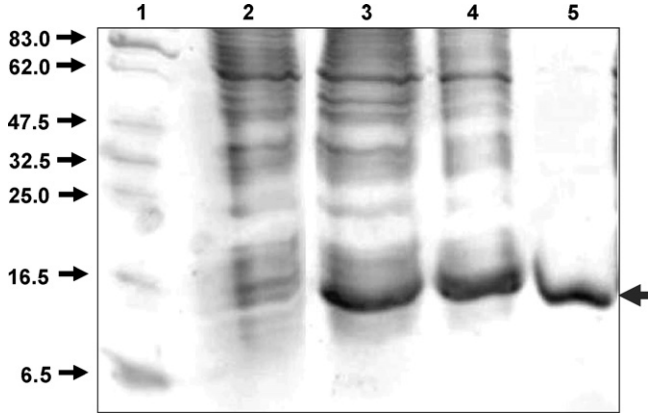

Fig. 2. Localization of D4EIII protein expression. An aliquot of induced culture was lysed under native conditions and the resultant soluble (lane 2) and pellet (lane 3) fractions were subjected to SDS–PAGE followed by Coomassie staining. In parallel, another aliquot was lysed under denaturing conditions, and the resulting soluble (lane 4) fractions were analyzed similarly. Lane 5 represented affinity (ICAM) purified D4EIII. Protein molecular weight markers were run in lane 1. Their sizes (in kDa) are indicated on the left. The arrow on the right indicates the position of the rD2EIII protein band.

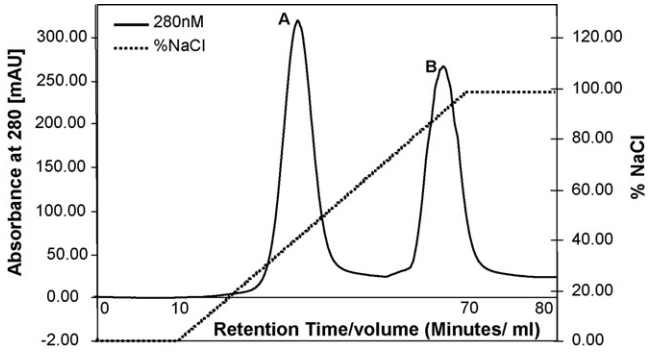

Fig. 3. Ion exchange chromatogram (elution profile) of refolded D4EIII. Column: UNOsphere S cation exchange media flow packed in an XK16/20 column. Sample: refolded, dialyzed and clarified D4EIII; buffer A: 50 mM phosphate buffer pH 5.8, 50 mM NaCl; buffer B: 50 mM phosphate buffer pH 5.8, 1 M NaCl; and a linear gradient of NaCl from 50 mM to 1 M, detection at 280 nm; flow rate: 1 ml min^{-1} (buffer A–B). Peak A represent monomeric form of D4EIII and peak B represent aggregated fractions.

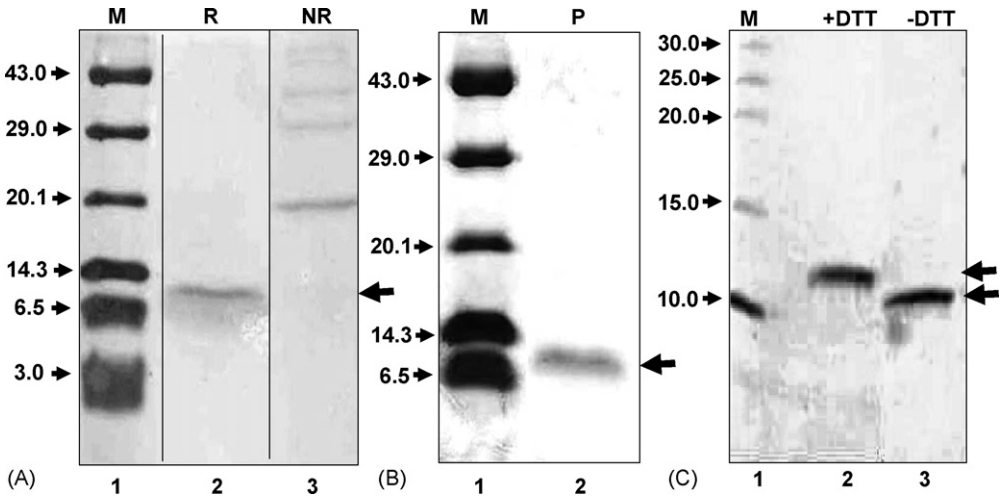

Fig. 4. [A] Analysis of fraction eluted from Ion-exchange chromatography (UnoS). Refolded D4EIII eluted from column in two different peaks. Eluted fractions representing the 2nd peak (B) was analyzed on SDS–PAGE followed by Coomassie staining to visualize protein bands. Before loading to gel one aliquot of protein was treated at reducing condition, i.e. sample buffer containing β-mercaptoethanol (lane 2), and other aliquot at non-reducing condition, i.e. sample buffer lacking β-mercaptoethanol (lane 3). [B] Analysis of purified D4EIII on SDS–PAGE. Refolded D4EIII, eluted from ion exchange chromatography, represented by 1st peak (A), was analyzed on SDS–PAGE followed by Coomassie staining. Purified protein is seen in lane 2. [C] Mobility of refolded and purified D4EIII before and after reduction. Refolded D4EIII has slower mobility by SDS–PAGE after reduction with dithiothreitol (+DTT) and alkylation with iodoacetamide (lane 2) indicating presence of disulfide linkages. Protein molecular weight markers were run in lane 1 (M) of each panel. Their sizes (in kDa) are indicated on the left. The arrow on the right indicates the position of the D4EIII protein band.

To devise an appropriate purification strategy, we proceeded to examine the relative distribution of the expressed recombinant protein in the soluble and insoluble fractions. For this purpose, we checked presence of protein in supernatant as well as pellet after sonication and centrifugation. The data are shown in Fig. 2. When the cells were lysed under native conditions, virtually all the expressed recombinant protein was associated with the pellet fraction, demonstrating that the D4EIII protein is insoluble (Fig. 2, lane 3). The native lysate supernatant, representing the soluble fraction, had no discernible recombinant protein (Fig. 2, lane 2). Analysis of the GuHCl lysate confirmed the presence of the major proportion of the 11.6 kDa protein band in the GuHCl supernatant (Fig. 2, lane 4). As the localization experiment showed that the D4EIII protein is predominantly found in the insoluble fraction, we attempted to purify the D4EIII protein under denaturing conditions. IBs were harvested and purified from the induced and lysed cell mass and solubilized in 8 M GuHCl containing buffers and directly purified by IMAC under denaturing conditions. The protein could be eluted out with the pH gradient and gel analysis showed it to be ~95% pure (Fig. 2, lane 5). The D4EIII protein yield was 42 mg l^{-1} of shake flask culture.

3.2. Refolding and purification of D4EIII

The affinity purified D4EIII were rapidly diluted (1:100) in refolding buffer. After refolding, the sample was dialyzed extensively to eliminate arginine and co-solvents. This step is

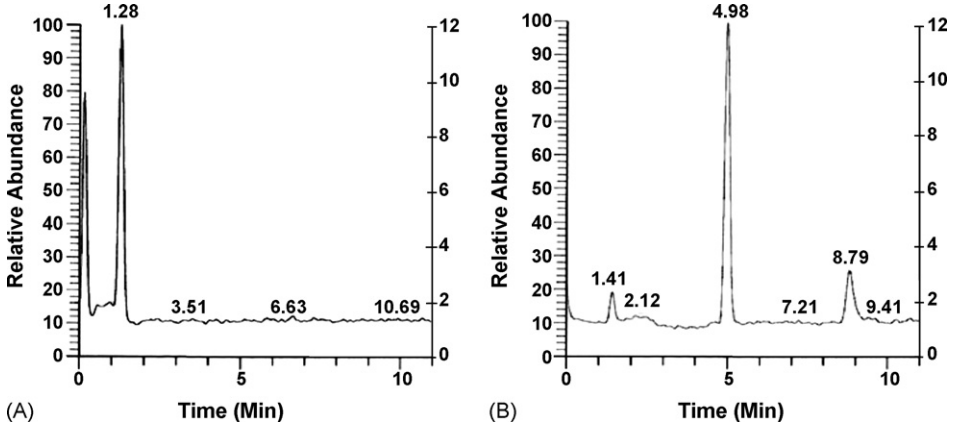

Fig. 5. Reverse phase chromatography profile of refolded and purified D4EIII. Column: reverse phase C8 column (Wakosil II 5C8RS, 5 μm, 100 mm × 4.6 mm). Sample: 10 μl of refolded D4EIII; eluent A: 0.05% trifluoroacetic acid (TFA) in water; eluent B: 0.05% TFA in 90% acetonitrile, 10% water; and a linear gradient of eluent B from 5% to 95%, detection at 280 nm; flow rate: 1 ml min^{-1} (buffer A–B). [A] Elution profile of refolded D4EIII. [B] Elution profile of reduced and denatured D4EIII. Refolded D4EIII elutes as a single, symmetric peak suggesting it contains a single, homogeneous population of conformers. Reduction of refolded D4EIII resulted in a shift in elution time by 3.7 min.

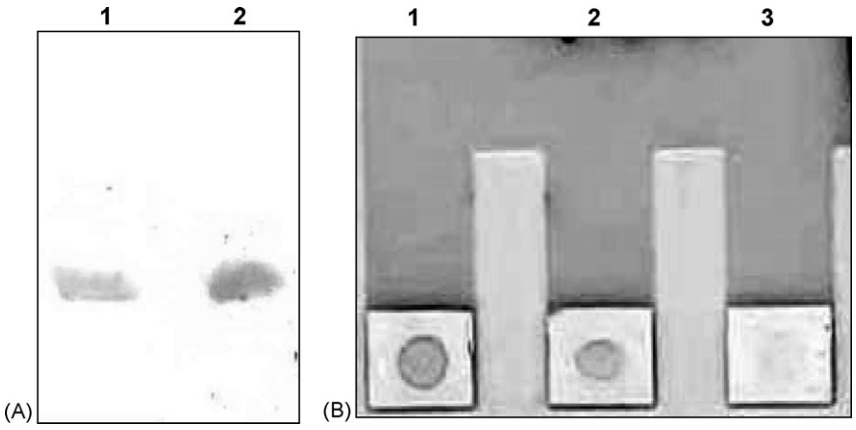

Fig. 6. Immunoblot analysis of refolded D4EIII by antibodies. [A] Aliquots of the refolded and purified D4EIII protein were electrophoresed on a denaturing polyacrylamide gel, electro-transferred onto a PVDF membrane, and subjected to Western analysis using anti-His mAb (lanes 1), H241 mAb (lanes 2). [B] Purified D4EIII was coated on to nitrocellulose strips and blotted using polyclonal anti-dengue virus antiserum collected from dengue patients. Strip 1 was coated with dengue virus, strip 2 with refolded D4EIII and developed with dengue patient sera. Healthy human sera did not recognize refolded D4EIII (strip 3).

necessary as the presence of arginine can interfere with the subsequent ion-exchange chromatography. The dialyzed and clarified refolded D4EIII was subjected to batch purification and concentration by cation exchange chromatography. The chromatographic profile is presented in Fig. 3. D4EIII eluted in two different peaks. Upon SDS–PAGE analysis it was found that the 1st peak (A) represents monomeric fractions and the second peak (B) represents aggregated fractions (Fig. 4A). When SDS–PAGE analysis of aggregated fractions was carried out under reducing conditions, and a single band of 11.6 kDa was observed (Fig. 4A). This indicated that the aggregated fraction likely to represent a disulfide-linked species. Aggregation due to cross disulfide linking is a common phenomenon during refolding. Monomer and aggregated D4EIII in a proportion of 60:40 was recovered after ion-exchange chromatography. However, refolding conditions could further be optimized to increase the monomer yield and reduce aggregation during refolding. Monomeric form of D4EIII was concentrated to result a single major protein band of the expected size (11.68 kDa) that was purified to homogeneity as can be seen from Fig. 4B.

3.3. Biochemical, biophysical and functional characterization of refolded and purified D4EIII

Refolded D4EIII was separated by SDS–PAGE and the purified protein migrates with an apparent molecular mass of ~12 kDa on denaturing SDS–PAGE gels (Fig. 4B). Densitometric scanning of the Coomassie blue stained SDS–PAGE gels indicate that the purity of refolded protein is greater than 98%. Refolded and purified D4EIII was characterized using a variety of biochemical and biophysical methods. N-terminal sequencing of recombinant D4EIII yields the expected sequence, namely MCSGKFSIDKEMAET. No other sequence is detected. Refolded D4EIII migrates slower on SDS–PAGE

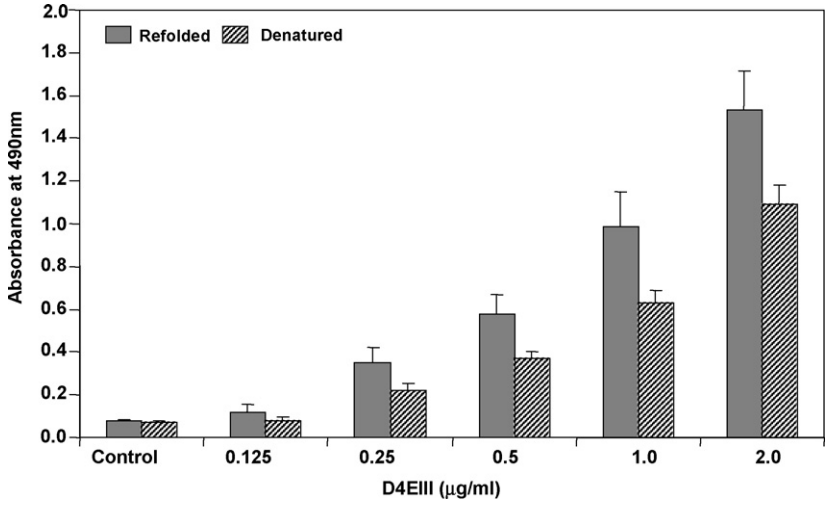

Fig. 7. Receptor binding assay with refolded and purified D4EIII. ELISA plate wells were coated with 0.5 µg ml^{-1} cell free soluble heparan sulfate, except the control wells. Different concentrations of refolded and denatured (separately) D4EIII were used to bind to immobilized heparan sufate and detected by using penta His mAb as primary antibody and anti-mouse IgG HRP conjugate as secondary antibody. The error bars indicate mean standard error of five independent experiments. Refolded D4EIII bound to heparan sulfate in a concentration dependant manner. Reduced and denatured D4EIII bound to heparan sulfate, but less than the refolded D4EIII.

gels after reduction with DTT indicating presence of disulphide bonds (Fig. 4C). Free thiol content was estimated by method of Ellman [50] to further examine the oxidation state of refolded D4EIII. Free thiols can be clearly detected up to thiol concentrations of 30 μM in this assay. No free thiols are detected in refolded D4EIII at a protein concentration of 500 μM. Given that D4EIII contains two cysteines, around 95% of cysteines are thus disulphide linked. The homogeneity of refolded D4EIII was analyzed by reverse phase chromatography, which can separate different conformers of the same protein based on differences in surface hydrophobicity. Refolded D4EIII elutes in a single, symmetric peak by reverse phase chromatography on a C-8 column suggesting that purified D4EIII is homogenous (Fig. 5A). Reduction of refolded D4EIII with DTT results an increase in elution time by 3.7 min confirming presence of disulphide links in the refolded protein (Fig. 5B). RP-HPLC results suggest presence of a dominating structure. However, different structures could be in an equilibrium, which would not allow separation by RP-HPLC, specifically when the structural changes are fast.

The retention time of purified D4EIII on a superdex 75 column is consistent with an apparent molecular mass of 11.6 kDa indicating that purified D4EIII does not contain aggregates or multimers. Mass analysis by LC–MS also confirmed the exact molecular weight of the refolded D4EIII (data not shown). The refolded purified D4EIII was subjected to Western blotting with anti-His mAb and H241 mAb (Fig. 6A, lanes 1 and 2, respectively). H241 mAb is a dengue virus type-4 envelope protein (domain III) specific antibody [51]. Recognition of our recombinant protein by H241 mAb confirms its epitope organization. Additionally, polyclonal sera from dengue virus infected humans also recognized the D4EIII protein (Fig. 6B, lane 2). Serum drawn from control (healthy human) failed to pick up the recombinant protein in this assay (Fig. 6B, lane 3). Having physically characterized the D4EIII protein we assessed its functional status. To this end, we utilized an indirect bioassay to assess the ability of the purified recombinant protein to bind to heparan sulfate, the proposed host cell surface receptor for dengue virus attachment [43]. This assay showed that the D4EIII protein could effectively bind to heparan sulfate in a concentration dependant manner (Fig. 7). We observed that binding to heparan sulfate is partially dependant on protein conformation as denatured Den4-DIII also exhibited binding to heparan sulfate, but ∼30% less in comparison to refolded protein. This variation may not be due to protein concentration or different purities, as same amount protein of defined purity is used to compare heparan sulfate binding of refolded and denatured D4EIII. This deviation may be due to certain structure of expressed protein or denatured D4EIII may partially contain structural specificity for heparan sulfate. The physico–chemical methods used to characterize the protein after *in vitro* refolding though indicate presence of a dominating structure, does not unequivocally confirm that the binding of D4EIII to heparan sulfate is due to a single structure attained by *in vitro* refolding. As the structure for dengue type-4 envelope protein is not known, it is difficult to predict that our refolded protein adapted correct structure. Dengue virus is known to bind cell surface heparan sulfate, leading to receptor mediated endocytosis and internalization of virus to the target cells. All previous studies provided evidence on dengue virus (Den2) to cell interaction [52–55]. In this study, we demonstrated binding of the viral protein (receptor binding domain of Den4) to soluble cell free receptor (heparan sulfate). This is the first report of its kind.

We performed homology modeling of the amino acid sequences of envelope protein (domain III) of dengue virus type 4 (D4EIII) using 3D-PSSM. The modeled structure is presented in Fig. 8A. Five different amino acids in dengue 2 domain III (aa300–aa400) have been predicted to play role in heparan sulfate binding [43]. On the basis of the earlier reports we predicted four amino acids, i.e. K305, K310, R393, K394 in D4EIII, may

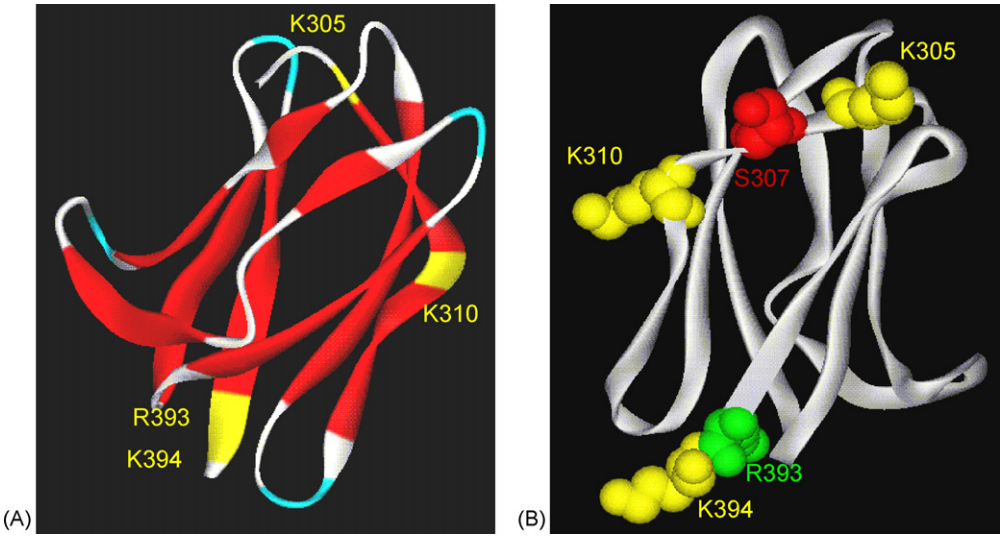

Fig. 8. [A] Homology model structure of domain III of Den4 E protein. Homology modeling of the amino acid sequences of envelope protein (domain III) of dengue virus type-4 (D4EIII), based on crystal structure of dengue2 E protein was generated using 3D-PSSM. [B] Putative heparan sulfate binding amino acids in modeled domain III of dengue virus type 4. Conserved lysine residues with in the putative receptor binding domain shown in yellow (space fill). Arginine residue at position 393, a conservative replacement from K393 of den2 is shown in green (space fill). Serine residue at position 307, a non-conservative radical amino acid change from K307 of den2 is shown in red (space fill).

play critical role in receptor binding (Fig. 8B). As most the amino acids that are predicted to play role in binding to heparan sulfate are positively charged and exposed, may be responsible for observed binding of denatured D4EIII. However, K307 in dengue 2 that is predicted as GAG binding amino acid has been changed to S307 in dengue 4. Being a non-conservative radical change, amino acid at position 307 on dengue 4 (D4EIII) may not play any role is receptor binding.

4. Conclusion

In conclusion, we have expressed and purified biologically functional recombinant domain III of the dengue virus type-4 envelope protein. A similar strategy could lead to the production of recombinant domains III of the envelope proteins of the remaining three serotypes. Since the expression system uses *E. coli* as the heterologous host, the process is amenable to inexpensive scale-up. Further we demonstrated that recombinant domain III of dengue virus type-4 binds to soluble cell free heparan sulfate. Domain III envelope protein of dengue virus is a lead vaccine candidate. Our findings further the understanding on biology of dengue virus and will help in development of bioassay for the proposed vaccine candidate. This could be valuable for the development of sub-unit vaccine for prevention of dengue infections.

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