Preparation of Antibodies against Soluble Recombinant Dengue E Proteins Fused with Glutathione’s Transferase

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**Abstract**

The envelope glycoprotein (E) of dengue (DENV) viruses mediates viral attachment and entry through membrane fusion. In this study, three fragments (domain 3a, domain 12 and domain 123a) of DENV envelope protein were inserted into plasmid pGEX-6p-1 to express glutathione’s transferase (GST) fusion proteins in *Escherichia coli* (*E. coli*) strain BL21. High yields of soluble recombinant proteins were obtained as follows: 20 mg/L of GST-E3a protein, 5 mg/L of GST-E12 protein, and 3 mg/L of GST-E123a protein. Although they all failed to protect vero cells from DENV infection in virus-binding blocking assay, three proteins could be recognized by mouse serum against DENV in western blot analysis. Then, polyclonal antibodies against either GST-E12 or GST-E3a were raised in New Zealand rabbits, and used in immunofluorescence staining. The infected vero cells showed typical cytoplasmatic fluorescence near the nucleus where the virus replication took place. The study indicated that soluble GST fusion E proteins might lack some biological activities of natural dengue E protein. But polyclonal antibodies against GST-E3a and GST-E12 might be helpful tools for further study of DENV pathogenesis in vitro.

**Keywords:** Dengue virus, envelope protein, glutathione’s transferase (GST).

**Introduction**

Dengue virus type 2 (DENV-2), a member of the Flavivirus, is an infectious pathogen with wide-reaching effects in tropical and subtropical areas across the globe. The DENV has a single-stranded, positive-sense RNA molecule of about 11 kb in size. The genome encodes three structural proteins (capsid, precursor of membrane, and envelope) and seven non-structural proteins that are essential for intracellular replication. The envelope of mature virions contains a metastable, icosahedral arrangement of dimer of the E protein, which is responsible for both receptor binding and fusion activity. Among the three structural domains of E protein, Domain 1 and 2 are linearly discontinuous: Domain 1 includes amino acids (aa) 1–52, aa 132–193 and aa 280–296, which is divided by Domain 2 (approximately aa 52–132 and aa 193–280). They are involved in stabilizing the E-glycoprotein dimer and contain the internal fusion peptide. Domain 3 includes two parts, so-called 3a (aa 296–394) and 3b (aa 395–495, transmembrane). It is an immunoglobulin (Ig)-like domain suggested to be the putative receptor-binding site.
To verify the “E protein-host cell receptor” interaction in vitro, intense efforts have been devoted in heterologous expressions of E protein, especially in E. coli. Recombinant E proteins with small tags were most commonly purified, such as S-E protein,[5] His-E protein,[6] and His-E protein domain 3.[7,8] Biological studies showed that they could either effectively protect cells against DENV infection, or bind to the 40 and 45 KD proteins in C6/36 cells, suggesting these proteins retained the abilities to bind to host cellular receptors. 26 KD of Glutathione’s transferase (GST), a larger tag, had also been fuse-expressed with E protein, but its blocking activity has not been accessed.[9]

In this study, three fragments of the E protein were fuse-expressed with GST tag. High levels of soluble recombinant protein were produced in E. coli strain BL21. Purified recombinant proteins showed some antigenecity in western blotting, but failed to protect vero cells from DENV infection, indicating that soluble GST fusion E proteins might lack some biological activities. However, polyclonal antibodies against these proteins successfully detected E proteins in infected vero cells, indicating their further applications to study the role of E protein in DENV pathogenesis.

Materials and methods

Cell lines and virus

DENV-2 (DENV-2, strain TR1751) isolated from a dengue patient was kindly provided by Dr Oya A (National Institute of Infectious Disease, Japan). The virus was propagated in Aedes albopictus mosquito cells (C6/36) and then stocked at –80 °C. C6/36 cells were grown at 28 °C in Dulbecco’s modified Eagle’s minimum essential medium (DMEM, Gibco) supplemented with 10% of fetal bovine serum (FBS). Vero cells were grown in Eagle’s minimum essential medium (MEM) supplemented with 5% of FBS.

Construction of DENV-2 E protein deletion mutants

As shown in Figure 1, three fragments of DENV-2 E protein were corresponding to the DENV-2 E protein domain 1 + domain 2 + domain 3a (E123a, 394a.a.), domain 1 + domain 2 (E12, 290a.a.), and domain 3a (E3a, 104a.a.) respectively.[3] Clones encoding these E deletion mutants were constructed by PCR amplification. All forward primers contained an EcoRI site, and all reverse primers carried an XbaI site. The respective forward and reverse primers were as follows: for E123a amplification, 5’ CGGAATTC ATGCCCTGTATAGGAATAGC 3’ and 5’ CCGCTCGAGTCATTTTCTGTCACAATTGGGTT 3’; for E12 amplification, the same forward primer as that for E123a and 5’ CCGCTCGAGTCAGTCCATTCTCAGCCTGACT 3’; for E3a amplification, 5’ CGGAATTC AACTACAGCT TAAAGGGAT 3’ and the same reverse primer as that for E123a. PCR amplifications were carried out by using the pMD19T-E plasmid (constructed and stored in our laboratory) as a template and 25 cycles with steps of 1 minute (min) at 94 °C, 1 min at 50 °C, and 2 min at 72 °C. PCR products were isolated and purified by agarose gel electrophoresis and were digested with EcoRI and XbaI. The resulting fragments were cloned into pGEX-6P-1 (Amersham) vector that had been digested with EcoRI and XbaI. All constructs were sequenced to confirm that the correct bases were present. All primers were synthesized by TAKARA, Japan.

Optimizing expression of soluble GST fusion proteins

Several colonies of E. coli strain BL21 transformed with recombinant plasmids were picked and transferred into separate tubes containing 2 ml of 2xYT medium. When cultures grow to OD600 of 0.6–0.8, isopropyl
thiogalactoside (IPTG) was added to the final concentrations of 1 μM, 5 μM, 10 μM, and 20 μM respectively. Cultures were continued to be incubated at 25 °C for additional 2 hours (h) and cells from 1.5 ml of culture were harvested by centrifuging for 30 seconds at 12 000 rpm. The pellets were resuspended in 300 μl of 0.1 mol/L (M) phosphate buffer saline (PBS) and sonicated on ice. After centrifuging for 5 min at 12 000 rpm, each supernatant was transferred into a new tube. Then, 20 μl of pre-washed glutathione sepharose 4B (Amersham) were added to the supernatant and incubated for 10 min at room temperature. Bound fusion protein was eluted off the beads with 15 μl elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0) and analyzed by SDS-PAGE.

Large-scale expression and purification of GST-fusion proteins

E. coli strain BL21 transformed with recombinant plasmids was grown in 2xYT medium at 37 °C overnight. The overnight culture was diluted 1:20 into fresh 2xYT medium, and IPTG was immediately added to a final concentration of 5 μM to induce gene expression. After continued incubation for an additional 24 h at 25 °C, the cells were harvested and centrifuged for 10 min at 10 000 rpm. The pellets were completely resuspended by adding 50 μl of PBS per ml of culture and sonicated on ice in short bursts. Then Triton X-100 was added to a final concentration of 1%, mixed gently to aid in the solublization of fusion protein. After centrifuge at 12 000 rpm for 10 min, supernatants were transferred into a new container.

Batch purification of recombinant proteins using glutathione sepharose 4B (Amersham) was performed following the manufacturer’s instruction. Briefly, the beads were washed with PBS and adjusted to 50% slurry before use. Then beads were incubated with the supernatant at 4 °C, with gentle agitation overnight. After a thorough wash, bound fusion protein was eluted off the beads with elution...
buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0). The concentrations of the eluted proteins were determined by spectrophotometer (U-0080D, Hitachi) and analysed by SDS-PAGE.

**Western blotting**

Samples were subjected to a 12% SDS-polyacrylamide gel and electrophoretically transferred onto nitrocellulose membranes (Dingguo, China). After incubation with 5% non-fat milk in Tris Tween Buffered Saline (TTBS) (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) for 60 min, the membranes were incubated with rabbit anti-DENV-2 E polyclonal antibody (produced by our laboratory) overnight at 4 °C. The membranes were then washed three times in TTBS buffer and incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG, Beijing Zhongshan Golden Bridge, China) in 1:1000 dilutions at room temperature for 1 h. Blots were washed three times again with TTBS and developed with DAB (3,3’-Diaminobenzidine, Sigma).

**Virus-binding block assay**

Vero cells were seeded in 24-well plates and incubated at 37 °C for 16 h. After the cells reached 100% confluence, they were pre-incubated with 200 µl of DMEM containing different concentrations (0, 5, 15, 25, 50 and 100 µg/well respectively) of three recombinant proteins of soluble GST protein at 4 °C for 2 h. Then, cells were subsequently infected with 50 µl of DENV-2 (about 150~200 plaque formation unit, PFU) at 4 °C for 2 h. Finally, the protein/virus mixture was aspirated and overlay medium (MEM with 2% methylcellulose and 2% FBS) was added to the well and incubated at 37 °C with 5% CO₂ for 7 days. The cells were scored for the presence of cytopathic effects by staining with crystal violet solution. Three independent infections were performed and three wells were tested for each protein concentration in each experiment.

**Immunofluorescence staining**

Polyclonal antibodies against GST-E12 and GST-E3a were raised in New Zealand rabbits and used in immunofluorescence staining of DENV-infected vero cells. Briefly, at 48 h post-infection, the cells were fixed with 4% paraformaldehyde at room temperature for 20 min. Then cells were incubated with monoclonal antibody cross-reacting with DENV-2 E protein and polyclonal antibodies against either GST-E3a or GST-E12, at 4 °C overnight. After being washed with PBS three times, the cells were further incubated with appropriate secondary antibody, i.e. FITC-conjugated rabbit anti-mouse IgG (Sigma), TRITC-conjugated goat anti-rabbit IgG (Sigma), at room temperature for 1 h. The cells were observed by fluorescence microscope (BX51, Olympus).

**Results**

**Recombinant plasmids were correctly constructed and induction dose of IPTG for soluble recombinant proteins were optimized**

Three fragments of 1182 bp, 870 bp, 312 bp in size had been generated and ligated into GST fusion protein expression vector pGEX-6p-1 as described above. To verify the insertion of the E coding sequence in the expression vector, the recombinant plasmids were digested with both EcoRI and XbaI restriction enzymes and released the corresponding fragments (data not shown). The sequences
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of three fragments revealed 100% homology with that of DENV-2 (strain TR1751) E protein.

Then, the pGEX-6p-E123a or pGEX-6p-E12 or pGEX-6p-E3a construct was transformed into E. coli strain BL21 (DE3), and the induction dose of IPTG for soluble expression of GST-fusion proteins was optimized. After being induced with different concentrations of IPTG, the total cell lysates and soluble proteins from these transformants were analyzed by SDS-PAGE with Coomassie blue staining. As the sizes of GST-E123a (about 62 KD) and GST-E12 (about 59 KD) were very close, their expression levels under each IPTG inductions were similar (data not shown). Here, we only reported the results of induction experiments on GST-E3a, the smallest, and GST-E123a, the largest.

Induced with four tested concentrations of IPTG, the total expression of both GST-E3a and GST-E123a scaled up dramatically as the concentrations went higher (Figure 2A). Triple or more times of recombinant proteins were produced under 10 μM (lane 3, 9) and 20 μM (lane 4, 10) of IPTG induction, comparing to 5 μM (lane 2, 8) and 1 μM (lane 1, 7).

However, the high-level expression of foreign fusion proteins in E. coli often results in the formation of an insoluble product, rather than the soluble one. As shown in Figure 2B, major bands of soluble GST-E3a, approximately 29 KD, were revealed under all IPTG induction doses (lane 1, 2, 3, 4). Although the soluble expression level of GST-E3a increased significantly when induction dose changed from 1 μM (lane 1) to 5 μM (lane 2), but no significance was seen between 5 μM and 10 μM (lane 3). As to GST-E123a, the large size tended to lower its soluble expression level. There was only a thin band of 62 KD observed under 1 μM of IPTG induction (lane 7). The band became thinner under 5 μM of IPTG induction (lane 8), and disappeared when the IPTG induction concentration reached 10 μM and 20 μM (lane 9, 10). Considering both the protein size and the “soluble protein/total protein” ratio, expression of all three GST fusion proteins were induced with 5 μM of IPTG in subsequent large-scale productions.

Soluble recombinant proteins prepared in large scale were tested for their antigenicities

E. coli strain BL21 carrying pGEX-6p-E123a or pGEX-6p-E12 or pGEX-6p-E3a was grown in the presence of 5 μM of IPTG, at 25 °C for 24 h. The concentrations of soluble recombinant proteins were determined by spectro-photometer and analysed by SDS-PAGE. The yields of recombinant proteins from 1L medium were as follows: GST-E123a, 3 mg/L; GST-E12, 5 mg/L; GST-E3a, 20 mg/L.

To demonstrate the antigenic properties of the recombinant proteins, western blot assays using mouse antibody against DENV-2 was performed (Figure 3). The bands under 43 KD (lane 1, 2) could be unspecific reactions between mouse serum and bacteria proteins, which falsely bound to glutathione sepharose beads by over-sonication. However, the bands of 62 KD (lane 1, arrow), 59 KD (lane 2, arrow) and 29 KD (lane 3, arrow) were still very clear, representing GST-E123a, GST-E12 and GST-E3a respectively.

Additionally, two New Zealand rabbits were immuned with GST-E12 and GST-E3a respectively. Polyclonal serum against each recombinant protein were produced and applied to immunofluorence staining (Figure 4), with the monoclonal antibody against DENV E as positive control. The infected cells showed typical cytoplasmatic fluorescence near the nucleus where virus replication took place, while the non-infected were dark. The results indicated that the antigenicities of GST fusion proteins did retain to some extent.
Figure 2: SDS-PAGE analysis of protein expressions after induction with different concentrations of IPTG

[After being induced with 1 μM (lane 1, 7), 5 μM (lane 2, 8), 10 μM (lane 3, 5, 9) and 20 μM (lane 4, 10) of IPTG, protein expressions were analyzed by SDS-PAGE]

(A) Whole cell lysates of E. coli strain BL21 transformed with plasmids pGEX-6p-E3a (lane 1 to 4), pGEX-6p-1 (lane 5) and pGEX-6p-E123a (lane 7 to 10) were directly boiled with Lammlie buffer. In both gels (A and B), protein molecular weight markers were run in lane 6. Their sizes (in KD) are indicated on the left. The arrow on the right indicated the position of the GST-E123a protein band (about 62 KD). The lower arrow on the left indicated the position of the GST-E3a protein band (about 29 KD)

(B) Soluble GST-E3a (lane 1 to 4), original GST (lane 5) and GST-E123a (lane 7 to 10) proteins were eluted from the glutathione sepharose beads. The upper arrow on the left indicated the host protein DnaK that was usually co-purified with GST fusions (about 70 KD)
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Recombinant proteins failed to block DENV binding the vero cells

Since three recombinant proteins were soluble expressed, no denaturing or refolding procedure was needed in purification. To measure their biological function, purified recombinant proteins were serial diluted and directly applied to virus-binding assay. As shown in Figure 5, pre-incubation of GST, GST-E3a, GST-E12 or GST-E123a all failed to decrease the plaque number significantly. Despite the serial-diluted protein concentrations, the PFUs were all around 130, even at the dose of 100 μg/well. The results indicated that three GST-E fusion proteins could not effectively compete with DENV-2 virions in the infection of host cells. Thus, the receptor-binding function of the purified recombinant E proteins was not retained. Our data were not consistent with three recent studies, which reported that both His tag-E protein domain III and S tag-E protein could antagonize the binding of DENV virus to BHK cells.[6,7,8]

Figure 3: Western blotting for recombinant E proteins expression

Soluble proteins purified from E. coli cells transformed with pGEX-6p-E123a (lane 1), pGEX-6p-E12 (lane 2), and pGEX-6p-E3a (lane 3) were detected with mouse anti DENV serum

Figure 4: Immunofluorescence staining for DENV E protein

[Vero cells infected with DENV-2 were incubated with monoclonal antibody cross-reacting with DENV-2 E protein (A), polyclonal antibody against GST-E3a (B) or polyclonal antibody against GST-E12 (C) respectively. And the cells were further incubated with either FITC-conjugated rabbit anti-mouse IgG (A) or TRITC-conjugated goat anti-rabbit IgG (B, C)]
Discussion

The *E. coli*-based fusion protein system has the highest expression potential as it can afford inexpensive scale up. In recent years, more than 20 kinds of tags have been developed for recombinant proteins,\(^{[10]}\) from 4 amino acid of polycysteine to 116 KD of *lacZ*. A proper system for the interest protein may share the following features: one-step purification, easy removal of the tags, and, the most important, a minimal effect on its original biological activity.

To verify the interaction of DENV E protein and host cell receptor *in vitro*, several strategies had been developed to produce recombinant E proteins in *E. coli*. One approach was to use a small peptide tag (as poly-his and S) to achieve high-level expression of fusion proteins. Most of the proteins were in the form of inclusion body, and further denature and recover procedures were needed. Another approach was to use larger protein tag (as GST) to increase the expression of fusion proteins in soluble form, which could be affinity-purified in one step. However, this advantage has been largely compromised with low expression levels. Data showed that recombinant his-E domain 3 protein had been produced at the level of 30 mg/L,\(^{[8]}\) while a soluble 81 KD of GST-E protein only 2 mg/L.\(^{[9]}\)

As previous studies showed, the fuse expressions of S tag or GST tag with the full length of E protein were very inefficient. In this study, we chose to express non-transmembrane fragments of the E protein fused with GST in *E. coli* system. Induction dose of IPTG had been optimized to increase the expression of soluble recombinant proteins. At the IPTG dose of 5 μM, more than 90% of recombinant E proteins were expressed in soluble form. After being induced for 24 h at 25 °C, three soluble proteins of 29 KD, 59KD and 62 KD were yielded at the level of 20 mg/L, 5 mg/L and 3 mg/L respectively. The results suggested that induction at lower IPTG dose for a longer time could maximize the soluble expression of GST fusion proteins.

The epitopes of the recombinant E protein were commonly detected with polyclonal or monoclonal antibodies to determine the structural integrity. In our study, all of three purified proteins could be detected by mouse serum against DENV in western blot. Additionally, polyclonal antibodies against either GST-E12 or GST-E3a could detect the E proteins in DENV-infected cells, indicating that the antigenicities of GST fusion proteins did retain to some extent. However, competitive virus-binding assay was a more direct method to test the biological function of purified proteins. Regardless of the serial concentrations, all of these proteins failed to inhibit virus plague formation in vero cells, including the GST-E domain 3a protein that was presumably competitive with wild-type virions for host cell receptor binding. This result suggested that purified GST fusion proteins did not retain the natural three-dimensional structure, at least partially in the region functioning for cell surface binding and entry. The failures might...
result from the dimerization of soluble GST fusion proteins and improper processing of proteins (such as glycosylation and disulfide bridging). Compared with GST fusion proteins, both of his-E domain 3 and S-E protein, no matter soluble or insoluble, could successfully block the virus binding to the host cells. These results suggested that the smaller tag was less likely to interfere with the structure of fused proteins. showed some antigenecities, they could not compete with DENV viral particles for infection on vero cells. The results indicated that soluble GST fusion E proteins might lack some biological activities, and should not be applied in vitro instead of natural DENV E protein. However, polyclonal antibodies against E12 and E3a might be helpful tools for further study of DENV pathogenesis.

### Conclusion

Here, we reported the optimization of soluble GST fusion proteins expression. Although all of three purified recombinant GST-E proteins showed some antigenecities, they could not compete with DENV viral particles for infection on vero cells. The results indicated that soluble GST fusion E proteins might lack some biological activities, and should not be applied in vitro instead of natural DENV E protein. However, polyclonal antibodies against E12 and E3a might be helpful tools for further study of DENV pathogenesis.

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### References


