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Super induction of dengue virus NS1 protein in *E. coli*

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ABSTRACT

The non-structural protein 1 (NS1) of dengue virus is a useful target for diagnostics of dengue infection since the protein is abundantly circulating in blood during the acute phase of the disease. Prior work has established that secreted NS1 levels in plasma correlates with viremia levels and hence can also be used to diagnose patients at the risk for developing dengue hemorrhagic fever. Thus detection of non-structural dengue antigens may be of benefit for an early rapid diagnosis of dengue infection due to its long half life in the blood. Here we describe a simple and efficient method for the expression of NS1 in *Escherichia coli*, which could potentially be used to develop monoclonal and bispecific antibodies for point of care diagnostics. *E. coli* codon optimized synthetic full-length NS1 gene of dengue serotype 1 (DEN-1) was successfully cloned and expressed in very high-level as inclusion bodies. The NS1 protein was successfully affinity purified and refolded as a recombinant NS1 (rNS1) protein in *E. coli* and yield was 230–250 mg/L of bacterial culture. The rNS1 protein was used to immunize mice for hybridoma development. The polyclonal antiserum from animals immunized with this rNS1 protein was found to specifically recognize the rNS1, thus demonstrating the immunogenic nature of the protein. The rNS1 protein purified from *E. coli* could be useful for developing a sensitive serum diagnostic assay to monitor dengue outbreaks.

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Introduction

Dengue fever is an important mosquito-borne viral disease of humans. This has been a recurrent phenomenon throughout the tropics in the past decade. During 2002, more than 30 Latin American countries reported over a million dengue fever (DF)¹ cases with large number of dengue hemorrhagic fever (DHF). Annually, there are an estimated 100 million dengue virus infections worldwide [1]. Increasingly cases of the more severe and potentially lethal DHF and dengue shock syndrome (DSS) are reported with children bearing much of the disease burden. Dengue virus is endemic in at least 100 countries worldwide and causes more human cases than any other mosquito-borne virus. The mortality rate of DHF in most countries is 5%, primarily among children and young adults. In several Asian countries, this virus is the leading cause of hospitalization and death in children. Hence, there is an urgent need for diagnostic, prophylactic and therapeutic reagents to manage DHF.

The dengue virus non-structural NS1 protein is a 46–50 kDa glycoprotein expressed in infected mammalian cells. All non-structural

proteins are intracellular proteins with the exception of dengue NS1 protein, which exists as secreted as well as a membrane-associated protein. Both forms are demonstrated to be immunogenic [2–4]. It was also reported that NS1 is one of 7 NS proteins produced during viral replication. It possesses not only group specific but also type specific determinants and has been recognized as an important antigen in dengue infection [2,4,5]. A high circulating level of NS1 was demonstrated in the acute phase of dengue by antigen capture ELISAs [2,6]. The precise function of dengue NS1 protein remains unclear. However, antigen detection of non-structural dengue antigens may be of benefit for an early stage rapid diagnosis of infection due to its long half life in the blood.

The usefulness of this study was to clone and express of DEN-1 full-length NS1 gene in *Escherichia coli*, for future development of monoclonal antibodies exploiting hybridoma and quadroma technology for rapid point of care applications. In this study, we report the successful cloning and very high-level expression of the NS1 protein and purification from *E. coli* as inclusion bodies and subsequent refolding.

Materials and methods

Chemicals

Restriction endonucleases and modifying enzymes were purchased from New England Biolabs (Mississauga, Canada). The

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¹ Abbreviations used: DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome; TB, 1.2% tryptone, 2.4% yeast extract, 0.4% (v/v) glycerol and 25 mM Hepes pH 7.2; Ni-NTA, nickel-nitrilotriacetic acid; MAb, monoclonal antibody; EDTA, ethylene diamine tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; h, hour; HRPO, horseradish peroxidase; ECL, enhanced chemiluminescence; BSA, bovine serum albumin.

anti-His₆ MAb was purchased from Novagen Inc. (Madison, USA). Prestained low range protein molecular weight markers, 40% acrylamide: bisacrylamide, glycine and protein assay reagents were purchased from Bio-Rad (Mississauga, Canada). ECL nitrocellulose membrane, X-ray film and Western blotting reagent were purchased from Amersham Pharmacia Biotech (Baie d'Urfe, Quebec, Canada). Glutathione (reduced and oxidized), sodium deoxycholate, L-arginine, GAM-HRPO, urea and other general molecular biology grade reagents were purchased from Sigma (Oakville, Canada). Ni-NTA agarose, plasmid DNA isolation and gel extraction kits were obtained from Qiagen (Mississauga, Canada).

Construction of plasmid (pDS21NS1)

The NS1 full-length nucleotide sequence of dengue (DEN-1) was codon optimized for *E. coli* expression and chemically synthesized by GENEART Inc., Germany. The codon optimized NS1 gene containing plasmid obtained from GENEART Inc. and the expression vector pBM802 [7] were digested with NdeI and EcoRI, gel purified and ligated. The ligation mixtures were transformed in *E. coli* top 10 cells and bacterial colonies were analyzed by plasmid DNA isolation and restriction digestion fragment mapping [8].

Recombinant clones analysis

Single bacterial colonies were cultured in 2 ml TB medium [8] containing 5 µg/ml of tetracycline (Tet⁵) and were incubated overnight at 37 °C with shaking (250 rpm). The overnight culture was diluted to 1/100th volume in 10 ml fresh TB/Tet⁵ medium and grown at 37 °C. The bacterial culture was induced when the optical density (OD_{600nm}) reached approximately 0.5–0.6 with arabinose [0.2% (w/v)] overnight (~16 h) at 37 °C, where as in control sample arabinose was not added. The bacterial culture of test and control samples were harvested by centrifugation at 5000g for 10 min at 4 °C and the total cell lysate was prepared [8]. Total cell protein (TCP) was analyzed by SDS-PAGE using 10% polyacrylamide gel [9] with a Mini Protean III apparatus (Bio-Rad). The protein gel was stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 in 10% acetic acid and 45% methanol and destained with 10% acetic acid and 30% methanol.

Expression optimization (inducer, temperature and time)

The expression of the NS1 protein was optimized for three different temperatures, time durations and inducer (arabinose) concentrations. Bacterial growth condition was similar to that described above. For arabinose dose optimization, the bacterial culture was induced with different concentrations of arabinose [2%, 0.2%, 0.02%, 0.002% and 0.0002% (w/v)] and allowed to grow overnight (~16 h) at 30 °C. For temperature optimization, the bacterial culture was induced with arabinose [0.2% (w/v)] and allowed to grow overnight (~16 h) at three different temperatures (37, 30 and 24 °C). For time optimization, the bacterial culture was induced with arabinose [0.2% (w/v)] and allowed to grow for 0 h, 2 h, 4 h, 6 h and overnight (~16 h) at 30 °C. Total cell proteins from each optimization experiment were analyzed by SDS-PAGE and Western blot to select the ideal condition for optimum protein expression.

Medium scale expression and purification of rNS1 protein

A single bacterial colony was inoculated in 10 ml TB/Tet⁵ medium and allowed to grow overnight at 37 °C shaker. The overnight culture was diluted (1:100) in fresh 4 × 1 L TB/Tet⁵ medium and grown at 37 °C until an OD_{600nm} of 0.5–0.6 was reached. Expression was done by optimized conditions as described in the previous sec-

tion. Induction was initiated by adding 0.2% (w/v) arabinose and bacterial culture was incubated for 16 h with vigorous shaking at 30 °C. Bacterial culture was harvested by centrifugation at 5000g for 20 min at 4 °C and total cell protein (TCP) from induced and uninduced culture was analyzed by SDS-PAGE and Western blot probed with anti-His₆ MAb.

Purification of inclusion bodies (IB)

The purification of inclusion bodies was done according to previously published method [10]. Briefly, 19.6 g of bacterial wet pellet from 4 L bacterial culture was suspended in 196 ml PBS (10 ml PBS per g of pellet) and completely lysed by passing through a French Press (20,000 psi). The total cell lysate was clarified by centrifugation at 27,000g for 30 min at 4 °C and supernatant was collected as total soluble protein. The pellet was resuspended in lysis buffer (Table 1) and then 2% sodium deoxycholate was added. The mixture was incubated at room temperature for 30 min with gentle shaking and centrifuged at 27,000g for 30 min at 4 °C. The pellet was resuspended in lysis buffer and washed thrice at 27,000g for 20 min at 4 °C to completely remove sodium deoxycholate.

IB solubilization and immobilized metal affinity chromatography (IMAC) purification

Inclusion bodies were solubilized in denaturing buffer B (Table 1) for 1 h at room temperature with gentle shaking. Solubilized denatured rNS1 proteins from insoluble materials were separated by centrifugation at 27,000g for 30 min at 4 °C. Final yield of solubilized denatured protein was determined by protein assay using BSA as the standard protein [11]. A Ni-NTA column was prepared by loading the Ni-NTA agarose on a plastic column (Bio-Rad) and equilibrated with 10 bed volumes of buffer B. Twenty milligrams of solubilized denatured rNS1 protein was loaded on the column and the column was washed with 5–10 bed volumes with buffer C. After complete wash, bound protein was eluted with buffer D and buffer E. All the eluted fractions were analyzed by SDS-PAGE prior to refolding.

Refolding

Protein assay was done to quantitate the amount of protein eluted from the Ni-NTA column and it was estimated ~0.4–0.6 mg/ml with a total amount of ~12–14 mg. Refolding was done in three different concentrations to evaluate the best refolding condition. The eluted protein was adjusted to 100, 75 and 50 µg/ml with refolding TA buffer and refolding was done by dialysis in TA buffer in the presence of 1.0 mM GSH (glutathione, reduced), 0.1 mM GSSG (glutathione, oxidized) for 3 days with two changes at 4 °C. Final dialysis was done in PBS pH 7.4 at 4 °C.

Western blot analysis

TCP, inclusion bodies, IMAC eluted fractions or refolded rNS1 protein were electrophoresed on SDS-PAGE using 10% polyacryl-

Table 1
Buffer used for the inclusion bodies purification and refolding.

Buffer	Composition
Lysis buffer	50 mM Tris, pH 8.0, 200 mM NaCl, 1 mM EDTA
Buffer B	8 M urea, 100 mM NaH ₂ PO ₄ , 10 mM Tris-Cl, pH 8.0
Buffer C	8 M urea, 100 mM NaH ₂ PO ₄ , 10 mM Tris-Cl, pH 6.3
Buffer D	8 M urea, 100 mM NaH ₂ PO ₄ , 10 mM Tris-Cl, pH 5.9
Buffer E	8 M urea, 100 mM NaH ₂ PO ₄ , 10 mM Tris-Cl, pH 4.5
TA buffer	50 mM Tris, pH 8.0, 0.4 M L-arginine

amide gel and then electroblotted onto Hybond ECL nitrocellulose membranes [12]. The nitrocellulose membrane was blocked with 5% skim milk in PBST (0.1% Tween 20 in 1× PBS, pH 7.3) for overnight at 4 °C. The membrane was washed four times with PBST and incubated with anti-His₆ MAb for 1 h. After washing, the membrane was incubated with HRPO labeled goat anti-mouse IgG (GAM-HRPO) for 1 h. Finally, the membrane was washed with PBS and enhanced chemiluminescence based detection was performed to visualize the binding.

In gel digestion

Protein identification was done at the Institute for Biomolecular Design, University of Alberta, Edmonton, Alberta, Canada as described earlier [13].

Results

NS1 gene cloning and small scale expression

The full-length codon optimized NS1 gene was cloned in the correct reading frame with the His₆ tag at the C-terminal and designated as pDS21NS1 for high-level expression of proteins as inclusion bodies in *E. coli* [7]. The correct size recombinant clones were selected for protein expression. The plasmid containing full-length NS1 gene was isolated for expression. Expression results showed that all the NS1 clones selected were expressing the target protein of approximately 46 kDa at different levels when analyzed by SDS-PAGE where as in control sample (without arabinose) there was no expression of the target protein (Fig. 1). The expression of rNS1 protein was confirmed by Western blot probed with anti-His₆ MAb (data not shown). The best NS1 clone was chosen for the expression optimization and further studies.

Expression optimization (inducer, temperature, and time)

The NS1 protein was successfully expressed as inclusion bodies in *E. coli*. The optimal conditions for rNS1 protein expression were 0.2% (w/v) arabinose concentrations (Fig. 2A), 30 °C temperature (Fig. 2B) and 16 h induction time (Fig. 2C).

Medium scale expression of NS1 protein

The bacterial expression vector pBM802 was designed for high-level expression of recombinant protein in *E. coli* as inclusion bodies. The medium scale expression of the rNS1 was performed and there was high-level expression in *E. coli* when analyzed by

SDS-PAGE (Fig. 3). Inclusion bodies were prepared from the bacterial pellet by a French Press. Following complete bacterial cell lysis, the insoluble inclusion bodies were separated from total soluble protein by centrifugation. The pellet was washed with sodium deoxycholate and subsequently washed with lysis buffer to remove any sodium deoxycholate. The final yield of denatured soluble inclusion bodies was estimated to be approximately 230–250 mg/L of initial bacterial culture. The purity of the inclusion bodies along with different washes was analyzed by SDS-PAGE (data not shown).

NS1 protein purification and refolding

The rNS1 protein was predominantly expressed in *E. coli* as inclusion bodies. The rNS1 protein was isolated from inclusion bodies from bacterial shake flask culture and purified by IMAC chromatography under denaturing conditions (Fig. 4A). The purity of the eluted rNS1 protein was analyzed by SDS-PAGE prior to refolding. Renaturing conditions, protein concentrations in the refolding buffer as well as suitable buffer compositions are important to simulate correct folding, formation of the proper disulfide bond and proper association of different domains. It has been reported that the presence of arginine in refolding buffer played an important role in solubilization, inhibiting the aggregation of refolding intermediates and thus increases the yield of the refolded protein [14]. It has also been clearly demonstrated in the literature that addition of GSH/GSSG into the refolding buffer facilitates disulfide bond formation and thus enhances renaturation of the protein [15]. The refolding step was done for 3 days by dialysis and any aggregate formed during refolding was removed by centrifugation. The supernatant was collected as refolded rNS1 for further use. Thus the *in vitro* refolding proved to be successful in recovering soluble protein expressed in *E. coli* as inclusion bodies. The purification steps and yield of rNS1 protein from 1 L of *E. coli* culture are summarized in Table 2. The purity of the refolded rNS1 protein was evident from the SDS-PAGE (Fig. 4B) and Western blot (Fig. 4C) with a single band of approximate molecular weight of 46 kDa. Its purity was estimated to be greater than 90%.

In gel digestion

Protein identification from the generated LC/MS data was done by searching the NCBI (National Center for Biotechnology Information) non-redundant database [Database: NCBI nr 20071130 (5678482 sequences; 1961803296 residues)] using Mascot Daemon search methodology (<http://www.matrixscience.com>). Mascot search results showed significant hits for the DEN-1 polyprotein which includes NS1 [gi|20135604 polyprotein (dengue virus type 1)].

Discussion

Rapid diagnostics of any infectious disease can lead to early therapeutic intervention of probable cases as well as suspect cases. In many viral diseases, virus shedding is greatest during the early symptomatic phase, i.e. around and immediately following the onset of symptoms. It has been reported that the NS1 antigen was found circulating from the first day after the onset of symptoms up to day 9. The NS1 levels ranged from 0.04 to 2 µg/ml in acute phase serum samples (from day 0 to 7), and the level for a convalescent phase serum (day 8 and later) was 0.04 µg/ml. In secondary phase infection, NS1 levels ranged from 0.01 to 2 µg/ml and were not detectable in convalescent phase sera. An antigen capture ELISA has been developed for detection of serum NS1 early in primary and secondary dengue infection [6]. According to these studies the

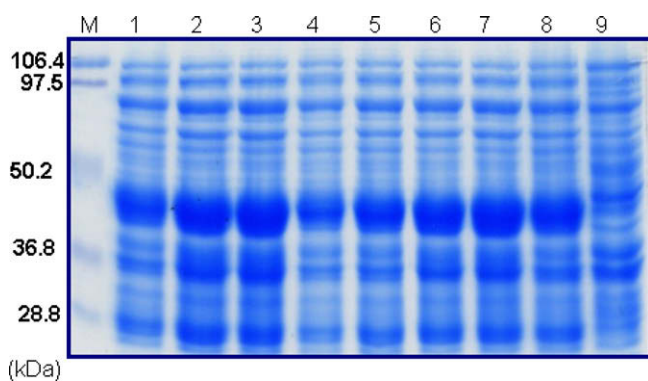


Fig. 1. SDS-PAGE analysis of different NS1 clones expression in *E. coli*. Lane M: standard protein molecular weight markers, lanes 1–8: clone# 1, 3, 4, 5, 7, 9, 11, and 12, respectively, lane 9: control.

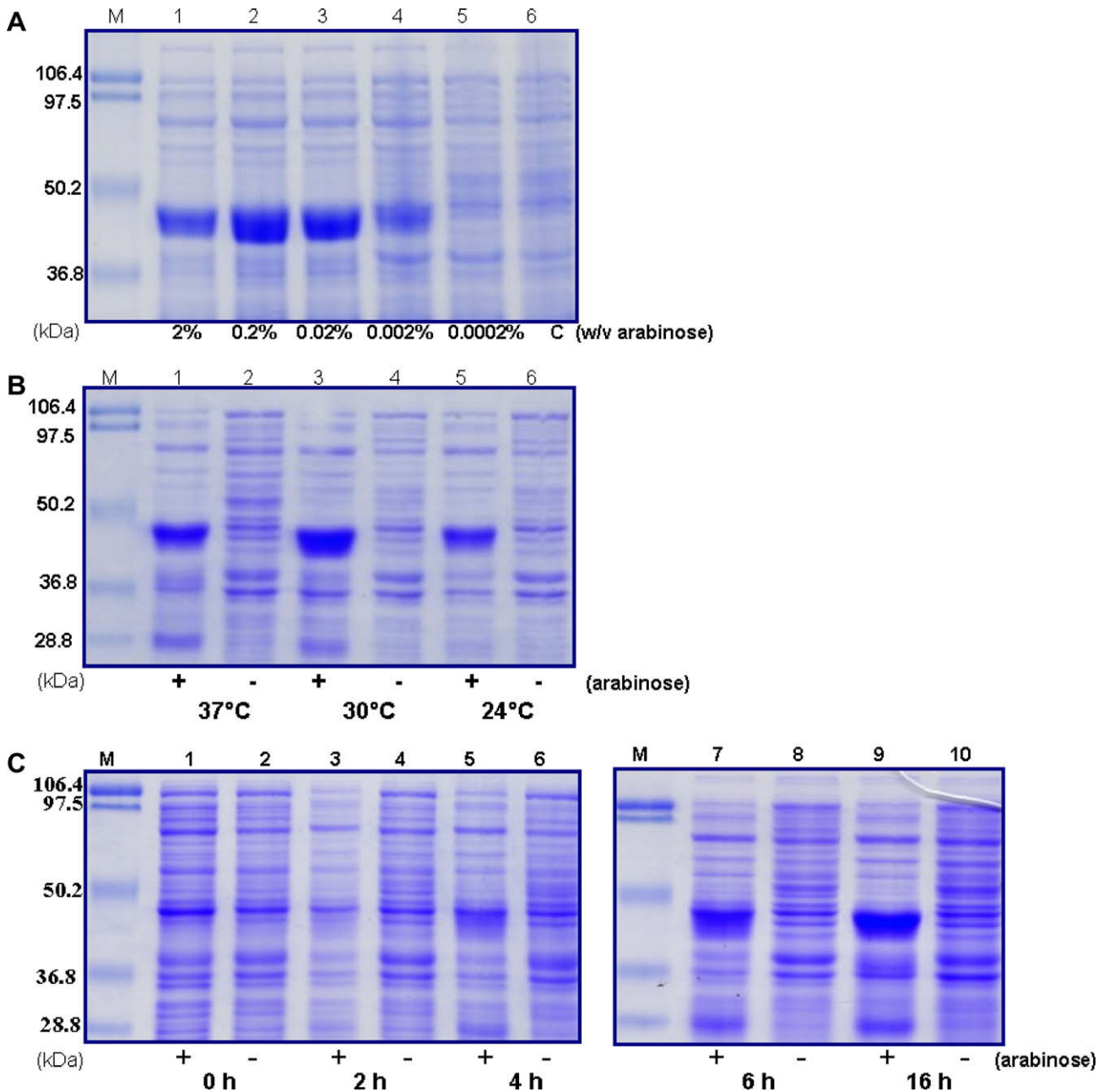


Fig. 2. Expression optimization. (A) SDS-PAGE analysis of arabinose concentration dependent rNS1 protein expression. Bacterial cultures were induced with different concentrations of arabinose and grown at 30 °C for overnight. Lane M: standard protein molecular weight markers, lanes 1–5: bacterial cultures were induced with 2%, 0.2%, 0.02%, 0.002%, 0.0002% arabinose (w/v), respectively, lane 6: control. (B) SDS-PAGE analysis of temperature dependent rNS1 protein expression. Bacterial cultures were induced with 0.2% arabinose (w/v) and grown at different temperatures (37, 30 and 24 °C) for overnight. Lane M: standard protein molecular weight markers, lanes 1 and 2: 37 °C, lanes 3 and 4: 30 °C, lanes 5 and 6: 24 °C. “+” indicates arabinose was added, “-” indicates arabinose was not added (control). (C) SDS-PAGE analysis of time dependent rNS1 protein expression. Bacterial cultures were induced with 0.2% arabinose (w/v) and grown at 30 °C for different time period. Lane M: standard protein molecular weight markers, lanes 1 and 2: 0 h, lanes 3 and 4: 2 h, lanes 5 and 6: 4 h, lanes 7 and 8: 6 h, lanes 9 and 10: overnight (16 h). “+” indicates arabinose was added, “-” indicates arabinose was not added (control).

presence of NS1 in human sera can be confirmed between days 0 and 9 [16,17] and with a peak at days 6–10 [18].

Dengue NS1 antigen testing is suggested as a helpful tool for the early diagnosis of dengue infection after the onset of fever [19,20]. Commercially available dengue NS1 antigen capture ELISA has been evaluated for the detection of NS1 from patient’s samples in different stages [17,20–22]. It is therefore an important antigen for rapid viral diagnosis.

It has been reported in the literature that different expression systems have been exploited for NS1 expression and with a typical

yield of 10–30 mg/L in bacteria [23], 25 mg/L of multi-epitope dengue protein in bacteria [24] and 70 mg/L in yeast [25]. Successful expression of NS1 protein has also been reported in both baculovirus and mammalian expression systems [26–29].

Here, we have also exploited the bacterial expression system for the production of rNS1 protein. Bacterial expression is perhaps the most commonly employed expression system for the production of non-glycosylated recombinant proteins. The organism is relatively simple to manipulate and the small scale analysis of many different parameters can be optimized in a short period of time. This

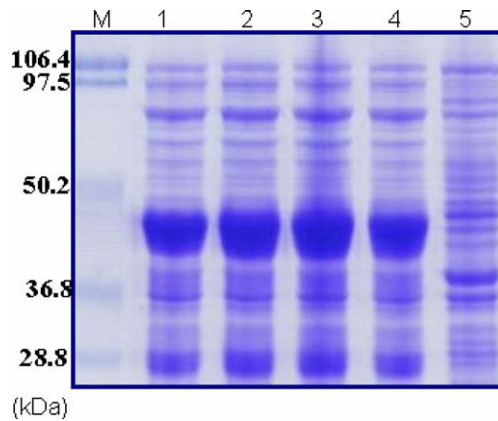


Fig. 3. SDS–PAGE analysis of medium scale production of rNS1 protein. Lane M: standard protein molecular weight markers, lanes 1–4: bacterial culture flask 1–4 (f1–f4), respectively, lane 5: control.

allows the rapid identification and optimization of several growth and induction conditions for medium scale production. Many eukaryotic genes cannot be expressed efficiently in the *E. coli* host due to the difference in codon preference as well as toxicity of foreign protein and mRNA instability for the expression of protein encoded by the gene. It is also a very well known fact that heterologously expressed eukaryotic proteins are not post-translationally modified when it is expressed in *E. coli*. It is also difficult to express as soluble protein or facilitate the secretion of expressed protein into the culture medium. In addition, proteins expressed in large amounts tend to precipitate, forming inclusion bodies [10,30,31] and present an advantage with respect to higher yield and especially the purification of expressed protein.

It has been demonstrated in the literature that genes can be codon optimized to the host translational system with the significantly higher expression level than native genes [7]. Based on this knowledge, we have obtained the codon optimized NS1 gene from GENEART Inc. for expression in *E. coli*. In the present study, we have cloned and purified the dengue NS1 protein in *E. coli* for the development of monoclonal antibody and subsequently bispecific antibodies for early diagnostic applications. NS1 gene was cloned under the control of the pBAD promoter for 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 [11] to be approximately 230–

Table 2

Purification of dengue rNS1 protein from *E. coli* in 1 L culture.

Purification steps	Total protein (mg)	rNS1 (mg)	Recovery (%)
Inclusion bodies	250	232	92.8
IMAC elutions ^a	20	12	60
Refolding	12	12	100

^a In each batch of IMAC purification, 20 mg of protein was used.

250 mg/L of initial bacterial culture which is 10- to 25-fold higher compared to previous reports using the native NS1 gene sequence. We have expressed several viral antigens in *E. coli* exploiting codon optimized genes and the NS1 expression is the most robust yield we have achieved (Table 3). The bacterial cell lysis by French Press and washing steps with detergent were used successfully to purify inclusion bodies from soluble protein. SDS–PAGE analysis clearly demonstrated that lysis by French Press and several washings with lysis buffer increased the purity of the inclusion bodies since the bulk of the *E. coli* soluble proteins were separated. The purity of inclusion bodies was also judged by SDS–PAGE and Western blot (data not shown). The purification method exploits the immobilized metal affinity chromatography (IMAC) under denaturing conditions. The effectively adsorbed His-tagged protein could be purified to homogeneity [10]. IMAC purification under denaturing conditions yielded significant amount of pure rNS1 protein with a single band as judged by SDS–PAGE and with a typical yield of 60–80% of starting denatured inclusion bodies (Table 2). Most recombinant proteins are expressed in *E. coli* as inclusion bodies and different refolding methods have been reported to renature proteins from inclusion bodies [10,32]. The IMAC eluted rNS1 protein was refolded in TA buffer in the presence of a redox pair (GSH/GSSG) with three different protein concentrations (Table 4). There are 12 cysteines conserved among flaviviruses that form disulfide bond (S–S) which suggesting their role in structure and function of the protein. The crystal structure of NS1 protein is yet not known but Wallis et al. has predicted 6 disulfide bonds present

Table 3

Expression yields of various codon optimized genes.

Protein	Yield (mg/L)	Reference
SARS-CoV NP	70	[7]
Dengue NS1	230–250	This report
H5N1 HA1	15–20	Unpublished

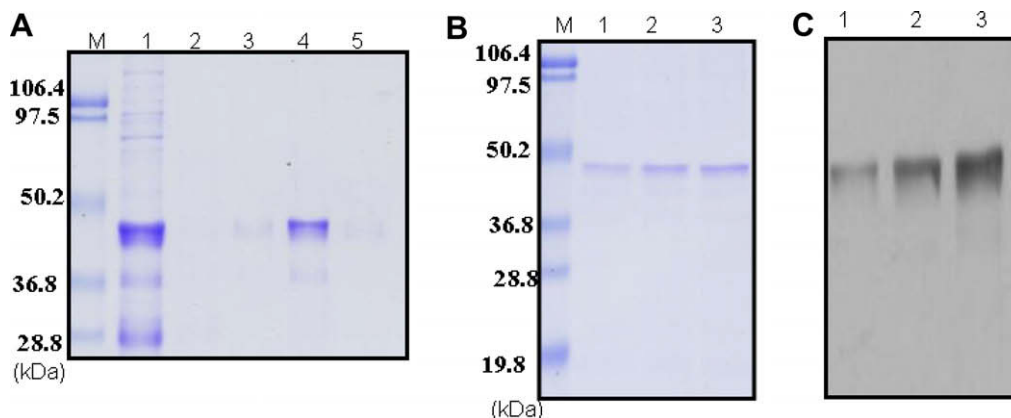


Fig. 4. IMAC purification and refolding. (A) SDS–PAGE analysis of IMAC purified rNS1 protein. Lane M: standard protein molecular weight markers, lane 1: unbound protein, lanes 2 and 3: washes, lanes 4 and 5: elutions. (B) SDS–PAGE analysis of refolded rNS1 protein. Lane M: standard protein molecular weight markers, lanes 1–3: recovered rNS1 protein refolded at 50, 75, 100 µg/ml, respectively. (C) Western blot analysis of refolded rNS1 protein probed with anti-His₆ MAb. Lanes 1–3: recovered rNS1 protein refolded at 50, 75, 100 µg/ml, respectively.

Table 4
Different refolding conditions and yield.

Protein	GSH:GSSG (mM)	Refolding TA buffer	Protein conc. in refolding buffer (µg/ml)	Yield (%)
rNS1	1:0.1	50 mM Tris-Cl, pH 8.0, 0.4 M L-arginine	100	No aggregation (100)
rNS1	1:0.1	50 mM Tris-Cl, pH 8.0, 0.4 M L-arginine	75	No aggregation (100)
rNS1	1:0.1	50 mM Tris-Cl, pH 8.0, 0.4 M L-arginine	50	No aggregation (100)

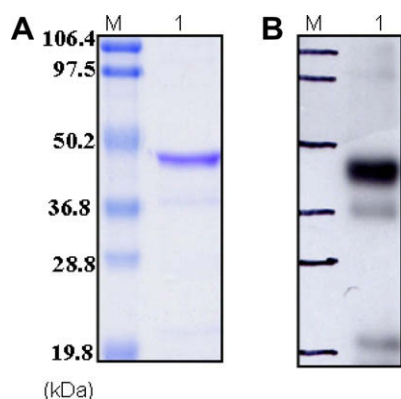


Fig. 5. SDS-PAGE analysis of refolded rNS1 protein. (A) Lane M: standard protein molecular weight markers, lane 1: refolded rNS1 protein. (B) Western blot analysis of refolded rNS1 protein probed with mouse anti-NS1 polyclonal antibodies. Lane 1: refolded rNS1 protein.

in the dengue NS1 protein [33]. Therefore the addition of GSH/GSSG into the refolding buffer which maintained the oxidizing environment enhances S–S bond formation and hence increases the solubility of the recombinant protein [15,34]. The protein concentration plays a crucial role in refolding conditions and it was observed that when the concentration was above 100 µg/ml, aggregation was evident. The purity of the refolded protein was judged by SDS-PAGE (Fig. 4B) and Western blot (Fig. 4C) data indicated that anti-His₆ MAb reacted with a single band of ~46 kDa, suggesting that we had successfully purified rNS1. The identity of purified rNS1 protein was confirmed by *in vitro* gel digestion, mass spectrometry, and NCBI non-redundant database searching. The refolded rNS1 antigen is being used to immunize mice to develop monoclonal antibodies. The polyclonal antibodies from mouse serum were strongly reacting with recombinant antigen (Fig. 5A) in Western blot (Fig. 5B). The rNS1 protein will be useful to select a pair of monoclonal antibodies with non-overlapping different specificities for antigen capture ELISA and point of care rapid assays [35]. In addition, the rNS1 antigen based ELISA could be the basis of developing a sensitive serum diagnostic to monitor dengue outbreaks by detecting the early human anti-dengue antibodies.

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