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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.

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.

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anti-His_6 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 (BaiedUrfe, 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 (Tet^5) 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/Tet^5 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 5000*g* 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/Tet^5 medium and allowed to grow overnight at 37 °C shaker. The overnight culture was diluted (1:100) in fresh 4 × 1 L TB/Tet^5 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 section. 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 5000*g* 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-His_6 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 27,000*g* 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 27,000*g* for 30 min at 4 °C. The pellet was resuspended in lysis buffer and washed thrice at 27,000*g* 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 27,000*g* 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_2PO_4, 10 mM Tris–Cl, pH 8.0
Buffer C	8 M urea, 100 mM NaH_2PO_4, 10 mM Tris–Cl, pH 6.3
Buffer D	8 M urea, 100 mM NaH_2PO_4, 10 mM Tris–Cl, pH 5.9
Buffer E	8 M urea, 100 mM NaH_2PO_4, 10 mM Tris–Cl, pH 4.5
TA buffer	50 mM Tris, pH 8.0, 0.4 M L-arginine