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Development of robust *in vitro* RNA-dependent RNA polymerase assay as a possible platform for antiviral drug testing against dengue

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ABSTRACT

NS5 is the largest and most conserved protein among the four dengue virus (DENV) serotypes. It has been the target of interest for antiviral drug development due to its major role in replication. NS5 consists of two domains, the N-terminal methyltransferase domain and C-terminal catalytic RNA-dependent RNA polymerase (RdRp) domain. It is an unstable protein and is prone to inactivation upon prolonged incubation at room temperature, thus affecting the inhibitor screening assays. In the current study, we expressed and purified DENV RdRp alone in *Esherichia coli* (*E. coli*) cells. The N-terminally His-tagged construct of DENV RdRp was transformed into *E. coli* expression strain BL-21 (DE3) pLysS cells. Protein expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.4 mM. The induced cultures were then grown for 20 h at 18 °C and cells were harvested by centrifugation at 6000 x g for 15 min at 4 °C. The recombinant protein was purified using HisTrap affinity column (Ni-NTA) and then the sample was subjected to size exclusion chromatography, which successfully removed the degradation product obtained during the previous purification step. The *in vitro* polymerase activity of RdRp was successfully demonstrated using homopolymeric polycytidylic acid (poly(rC)) RNA template. This study describes the high level production of enzymatically active DENV RdRp protein which can be used to develop assays for testing large number of compounds in a high-throughput manner. RdRp has the *de novo* initiation activity and the *in vitro* polymerase assays for the protein provide a platform for highly robust and efficient antiviral compound screening systems.

1. Introduction

Dengue is currently considered as one of the most important mosquito-borne diseases worldwide. According to recent estimates, the global incidence of dengue infections has increased from 50 to 100 million to almost 400 million cases per year [1]. Despite the enormous efforts towards the development of dengue vaccine, there is still no licensed vaccine available. This is primarily due to the complex pathology of the disease and immune mediated responses against the four distinct serotypes [2]. Presently, many promising vaccine candidates are in pre-clinical and clinical development stages including live attenuated, inactivated, recombinant subunit, DNA, and viral vectored vaccines [3]. The development of therapies for DENV requires both an understanding of the viral life cycle and rational strategies for identifying antiviral inhibitors. Both viral and host proteins essential for viral replication cycle are potential targets for antiviral drug development [4].

Dengue related infections are caused by the dengue viruses, which belongs to the family *Flaviviridae* and include small enveloped viruses with a diameter of about 50 nm [5]. Dengue virus contains an 11 kb positive-sense, single-stranded RNA genome. The genome consists of a single open reading frame which encodes three structural proteins (capsid C, pre-membrane/membrane (prM/M), and envelope (E) protein), and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [6]. The structural proteins form the viral particle and the non-structural proteins participate in the replication of the RNA genome, virion assembly and invasion of innate immune response [7]. NS5, with a molecular weight of around 104 kDa is the largest of dengue virus proteins containing 900 amino acids. NS5 is the most conserved protein of the dengue proteome as it shares a minimum of 67% amino acid sequence across all four dengue serotypes [8,9]. NS5 is essential for RNA replication and performs enzymatic activities required for capping and synthesis of RNA genome of virus. It consists of two domains with distinct functions, the N-terminal methyl transferase (MTase) and the C-terminal RNA-dependent RNA

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polymerase (RdRp) catalytic domain [9,10]. The tertiary structure of RdRp consists of palm, thumb and finger subdomains. The catalytic site contains conserved aspartic residues and coordinated magnesium cations and nucleotide incorporation occurs through two metal ion catalytic mechanism [9].

The MTase activity of NS5 is responsible for both guanine N-7 and ribose 2-O methylations [11]. The replication of flaviviral RNA occurs through the formation of replicative intermediates synthesized by the RdRp, an essential component of a replication complex [12]. Recombinant NS5 full-length protein along with C-terminal domain alone have been shown to possess RdRp activity *in-vitro*, which was also evaluated in a study with NS5 protein of DENV-1 expressed in *E. coli* as a glutathione-S-transferase (GST) fusion. The *in vitro* demonstration of polymerase activity in recombinant NS5 protein has made it possible to study and identify the host and virus factors involved in the dengue virus genome replication [13,14]. Flavivirus RdRp initiates the RNA synthesis *via de novo* initiation mechanism in which first nucleotide serves as a primer to provide the 3′-hydroxyl group which differs from a primer (an oligonucleotide, a protein linked to nucleotides, or intra-molecular self-priming) dependent mechanism used by other viruses, such as poliovirus and SARS-CoV [15,16].

Owing to the central role of polymerase enzymes in viral life cycles, RdRps have been the target of considerable interest for antiviral drug development [17]. RdRp is an unstable protein and is prone to inactivation upon prolonged incubation at room temperature (RT) thereby affecting the inhibitor screening assays. It has been observed that addition of linker residues to the N-terminal end of the RdRp domain stabilizes the protein and enhances its *de novo* polymerase initiation activity and thermostability [18]. Mutation studies of the linker residues have indicated their importance for viral replication, consequently, accelerating the development of antivirals against dengue. The 74 kDa RdRp domain of DENV-2 has been expressed in *E. coli* and purified with a high quantity of the protein. It was observed that the protein was active with considerably similar enzymatic characteristics as its full-length counterpart [13]. Previous studies with NS5 RdRp of DENV-3 have described high quantity production of the protein with >95% purity, yielding 3 mg protein from 1 L of culture [9]. In the current study, expression and purification of the RdRp domain of DENV-2 NS5 protein from *E. coli* was carried out and the polymerase activity of the recombinant protein was successfully demonstrated using an *in vitro* transcribed RNA. Based on its multifunctional role in viral replication, NS5 has been identified as an interesting drug target for the development of therapies against dengue. The aim of this investigation was to demonstrate the *in vitro* polymerase activity of the purified recombinant RdRp (NS5) protein. This assay will provide a platform for screening and development of antiviral compounds specifically targeting the polymerase protein.

2. Materials and methods

2.1. Construct for DENV-2 RdRp (NS5) protein

The genomic construct of RdRp domain of NS5 protein for expression in *E. coli* was kindly provided by Dr. Richard J Kuhn (Department of Biological Sciences, Purdue University). The region encoding the catalytic RdRp domain (amino acid residues, 273–900) was amplified using the genomic clone of DENV-2 strain 16681 (Genbank Accession No. U87411) by PCR. The amplicon was then cloned into the expression vector pET30a to allow expression of recombinant protein carrying an N-terminal TEV protease cleavable His6 affinity tag in *E. coli* cells. The pET30a expression vector used in this construct had been modified for ligation-independent cloning (LIC) by Dr. Etti Harms, high-throughput facility (HTP) [19].

2.2. Expression of DENV-2 RdRp in E. coli

The N-terminally His-tagged RdRp domain of DENV-2 NS5 were transformed into *E. coli* expression strain BL-21 (DE3)pLysS cells. The transformed cells were first grown in small scale culture of 5 mL to test for the expression and solubility of DENV-2 NS5. Large scale culture was then grown in 1 L LB medium containing 50 μg/mL of Kanamycin (Kan) and 35 μg/mL of Chloramphenicol (Cam) overnight at 37 °C, till an OD_{600} of 0.7–0.8 was reached. Protein expression was induced using 0.4 mM IPTG. The induced cultures were then grown for 20 h at 18 °C and cells were harvested by centrifugation at 6000*g* for 15 min at 4 °C. Samples were analyzed for expression on 10% SDS-PAGE gel (Bio-Rad Laboratories) and visualized by coomassie staining according to Laemmli method [20].

2.3. Purification of DENV-2 RdRp

The harvested pellets were resuspended in 50 mL of lysis buffer containing Buffer A (20 mM sodium phosphate pH 7.0, 0.5 M NaCl, 50 mM L-Arginine, 50 mM L-Glutamic acid, 5 mM Imidazole) and 1 mM PMSF, 5 mM β-Mercaptoethanol (ME), 0.01 mg/mL each of DNaseI and RNaseA. The Samples were lysed by sonication on ice at amplitude of 60% using sonicator machine (Hielscher Ultrasonics GmbH, USA). The lysates were centrifuged at 25,000*g* for 45 min at 4 °C. The supernatants were clarified by filtration through 0.45 μm filter unit and the clarified supernatants were loaded onto a Ni-NTA agarose beads (Qiagen) column pre-equilibrated with Buffer A, and incubated for 1 h at 4 °C. Column was washed with 40 mL wash1 buffer (Buffer A + 30 mM imidazole + 1 M NaCl) and then 20 mL of wash 2 buffer (Buffer A + 50 mM imidazole + 1 M NaCl). The protein was eluted with 20 mL of 100 mM and 500 mM imidazole in buffer A. Finally, the column was washed with 1 M imidazole to remove any bound protein and then with excess of deionized water.

Samples were collected at various stages during purification, analyzed on a 10% SDS-PAGE gel and visualized by coomassie staining. The fractions containing the purified protein were pooled after buffer exchange (50 mM Tris buffer pH 8.0, 500 mM NaCl, 5 mM βME, 5% glycerol) and were concentrated using a 50 kDa centrifugal concentrator. The concentrated protein was applied onto a Superdex 200 10/300 HL size-exclusion column (GE Healthcare, USA), pre-equilibrated with the exchange buffer. Fractions containing the purified recombinant RdRp protein were pooled and the protein was concentrated using a 50-kDa vivaspin concentrator (GE Healthcare, USA). The final concentration of protein was assessed by measuring absorbance at 280 nm using nanodrop (Thermo Scientific, USA).

2.4. In vitro RdRp polymerase assay

The purified protein (1 μM) diluted in RdRp buffer containing 50 mM HEPES, 10 mM KCl, 5 mM $MnCl_2$, 5 mM $MgCl_2$ and 10 mM DTT, was then tested for *in vitro* RdRp activity assessed by liquid scintillation counter (Beckman LS6500, USA). Following optimization, the polymerase assays were carried out with RdRp protein using poly(rC) RNA as template (Amersham Biosciences, USA) and monitored by filter-binding and scintillation counting as previously described [13]. The final RdRp assays were performed in RdRp buffer containing 1 μM NS5, 0.1 mg/mL poly(rC) RNA, 20 U RNase OUT, 0.2 μg Actinomycin D in a volume of 50 μL. Reactions were initiated by addition of 5 μM GTP, 0.25 μi [^{3}H]-GTP pre-incubated at 30 °C. The reactions were incubated at 30 °C for 1 h, terminated by addition of 10 mM EDTA and spotted onto DE-81 filter discs (Whatman). The filter discs were air dried, washed thrice with 0.3 M ammonium formate (pH 8.0) and once with ethanol and air dried