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Methods for detecting ATP hydrolysis and nucleic acid unwinding of Japanese encephalitis virus NS3 helicase

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A B S T R A C T

Japanese encephalitis virus (JEV) is a mosquito-borne zoonotic pathogen that is prevalent in south-east Asia. Because there is no specific antiviral agent, JEV still causes a high rate of neurologic sequelae and mortality in humans. The helicase encoded by the NS3 gene of JEV has emerged recently as a novel antiviral target for treatment. In this study, a soluble recombinant JEV helicase protein was expressed and purified. Methods for detecting the ATP hydrolysis and nucleic acid unwinding activity were developed by luminescence and fluorescence resonance energy transfer (FRET). The concentrations of enzyme, substrate, capture strand, ATP, and divalent ions were optimised in the ATPase and helicase reactions. The feasibility of using these two methods for high-throughput screening of NS3 helicase inhibitors is discussed.

1. Introduction

Japanese encephalitis virus (JEV), which is prevalent in south-east Asia, is a mosquito-transmitted, zoonotic flavivirus that cause 50,000 human cases and 10,000 deaths per year (Barrett, 2008). The fatality rate of JE ranges from 10 to 50%, and approximately 50% of cases develop permanent neuropsychiatric sequelae, especially in infants and children (Mackenzie et al., 2004). Inactivated and live-attenuated JEV vaccines have been developed for many years (Hoke et al., 1988; Xin et al., 1988), and vaccination is considered to be the most effective control measure against JEV (Tsai, 2000). However, vaccination is not effective for all of the clinical isolates (Ku et al., 1994). Antiviral agents are needed to reduce the death rate and neurological sequelae resulting from JEV infections (Gould et al., 2008).

JEV has a single-strand positive-sense RNA genome containing 10,976 nucleotides encoding three structural and seven nonstructural proteins (Sumiyoshi et al., 1987). The nonstructural protein 3 (NS3) is a bifunctional protein with both protease and helicase activity (Luo et al., 2008a). The C-terminal two-thirds of JEV NS3 has catalytic domains for helicase, nucleoside 5′-triphosphatase (NTPase), and 5′-terminal RNA triphosphatase activities (Wang et al., 2009). The main function of NS3 helicase is to unwind double-stranded genomic RNA during the process of virus replication (Luo et al., 2008b). The unwinding function was found to be essential for the replication of DENV based on genetic knockout studies (Matusan et al., 2001). Although flavivirus helicase and human DDX3 share identical residues, a tremendous divergence in sequence is found outside of the conserved motifs (Kwong et al., 2005), which makes it possible to design specific antiviral inhibitors targeting the viral helicase.

The flaviviral helicase should be an optimal target for drug development, mainly because the structure and function have been well characterised (Sampath and Padmanabhan, 2009). Several NS3 helicase inhibitors, such as paclitaxel and trifluoperazine (Borowski et al., 1999), tetrachlorobenzotriazole (TCBT) and tetrabromobenzotriazole (TBBT) (Borowski et al., 2003), ivermectin (Mastrangelo et al., 2012), and ST-610 (Byrd et al., 2013), have been identified. However, flaviviral helicase-specific inhibitors are very limited with regard to drug development.

In this study, methods for detecting the ATP hydrolysis and nucleic acid unwinding activity of JEV helicase were established. The reaction conditions were optimised to develop a high-throughput screening (HTS) assay.

2. Materials and methods

2.1. Expression and purification of the JEV NS3 helicase

The JEV helicase/NTPase domain (amino acid residues 180–619 of NS3) was cloned into the expression vector pET-30a to

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produce pET30-helicase. The plasmid pET30-helicase was transformed into *E. coli* BL21 (DE3) pLysS competent cells (Promega, Madison, WI, USA) and induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma–Aldrich, St. Louis, MO, USA). The NS3 helicase in the supernatant was purified by Ni^{2+} affinity column chromatography and gel filtration chromatography.

2.2. SDS–PAGE and Western blotting

The purified JEV NS3 helicase protein was electrophoresed by 12% SDS–PAGE. Western blotting was performed with a His-tagged Mab (Proteintech, Chicago, IL, USA) or NS3-specific Mab (constructed in our laboratory) as the primary antibody, followed by the addition of HRP-conjugated goat anti-mouse IgG (Boster, Wuhan, China) as the secondary antibody. The protein bands were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA).

2.3. ATPase activity assay

The Kinase-Glo Plus Luminescent Kinase Assay kit (Promega) was used to detect the ATPase activity of JEV NS3 helicase. The kit provides a homogeneous non-radioactive method for determining the activity of kinase by quantifying the amount of ATP remaining in solution. To establish a method for assessing the ATPase activity, NS3 helicase in reaction buffer (20 mM Tris–HCl pH 8.0, 50 mM NaCl, and 10 mM $MgCl_2$) was added to a 96-well black plate (Jet Bio-Filtration, Guangzhou, China). Then, 100 μM ATP was added to each well, and made a total volume of 100 μL with deionised water. The plate was submitted to horizontal shaking for 60 s to ensure thorough mixing and incubated in a 37 °C incubator. At the end of reaction, an equal volume of Kinase-Glo reagent was added to the NS3 helicase reaction mixture. After mixing and incubation at room temperature for 10 min, the luminescence of each well was measured by a 1450 MicroBeta TriLux (Perkin Elmer, Waltham, MA, USA).

2.4. Unwinding activity assay

The nucleic acid unwinding assay was established using a fluorescence resonance energy transfer (FRET) assay as described previously (Boguszewska-Chachulska et al., 2004). Two complementary DNA molecules were synthesised as substrates, one was a 3′ BHQ-2-labelled 22-mer DNA (5′-GGTTCTGAGGGTGGCGGTACTA-3′), and the other was a 5′ Cy5-labelled 36-mer DNA (5′-TAGTACCGCCACCCTCAGAACCTTTTTTTTTTTTTT-3′). A capture strand (5′-TAGTACCGCCACCCTCAGAACC-3′) was employed to prevent re-annealing of the two separated substrate DNAs. The substrate DNAs for the FRET assay were prepared by mixing at a 1.2:1 (BHQ-2:Cy5) molar ratio. The mixture was heated briefly to 95 °C and then cooled slowly in a 37 °C incubator. The efficiency of annealing was evaluated by detecting the fluorescence units at 670 nm with excitation at 635 nm in a TECAN Infinite F200 (TECAN, Seestrasse, Switzerland). In the unwinding activity assay, the purified helicase in reaction buffer (20 mM Tris–HCl pH 8.0 and 50 mM NaCl) was added to a 96-well black plate, and then the divalent ions, the annealed substrates, the capture strand, and ATP were added to each well to a final volume of 100 μL. Then, the plate was shaken for 60 s on an orbital shaker and incubated in a 37 °C incubator. Finally, the fluorescence value of each well was detected. The influence of the concentration of helicase, substrate, ATP, capture strand, and divalent ions on the unwinding activity was evaluated by adjusting each condition.

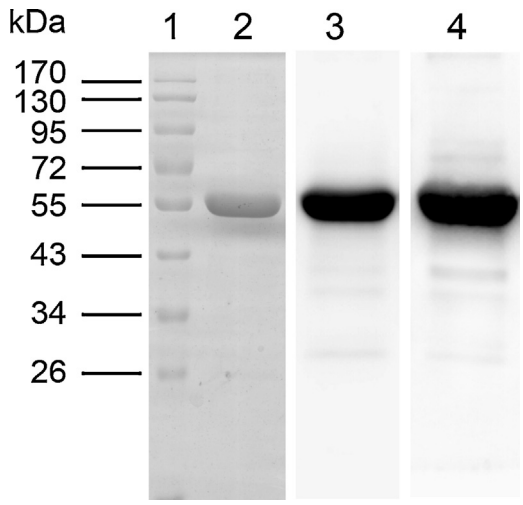

Fig. 1. SDS–PAGE and Western blot analysis of the purified NS3 helicase protein. The expressed and purified NS3 helicase protein was analysed by electrophoresis on a 12% SDS–PAGE gel and then stained by coomassie brilliant blue. The eluted protein migrated on SDS–PAGE as a single major band of approximately 56.3 kDa (lane 2). The protein on the gel was electrotransferred to a nitrocellulose membrane and detected by an NS3-specific Mab (lane 3) and a His-tagged Mab (lane 4).

3. Results

3.1. Expression, purification, and identification of JEV NS3 helicase protein

The recombinant NS3 helicase was expressed in BL21 (DE3) pLysS competent cells and then purified by Ni^{2+} affinity column chromatography followed by gel filtration chromatography. The protein was identified by SDS–PAGE and Western blotting. The SDS–PAGE analysis showed that the protein was more than 95% pure with an expected molecular mass of 56.3 kDa (Fig. 1, lane 2). A protein band of identical molecular weight was detected by Western blotting using a His-tagged Mab or NS3-specific Mab (Fig. 1, lanes 3 and 4).

3.2. ATP hydrolysis activity of NS3 helicase

3.2.1. Time course of the ATP hydrolysis reaction

To elucidate the time course of the ATPase reaction, 100 μM ATP was added to 3 μM helicase protein in the presence of 10 mM $MgCl_2$. The amount of remaining ATP was detected at different times from 0 to 180 min. The results showed that most of the ATP (98.9%) was hydrolysed by helicase during the first 10 min of the reaction. There was no obvious reduction in the luminescence from 10 to 180 min of reaction (Fig. 2A).

3.2.2. ATP hydrolysis reaction in different concentrations of NS3 helicase

To find a suitable concentration for the ATPase reaction, different concentrations (0–4.8 μM) of NS3 helicase were added to the ATP hydrolysis reaction buffer containing 10 mM $MgCl_2$ and 100 μM ATP. The luminescence units of the control group (no helicase) were the highest and considered as 0% ATP hydrolysed. When the concentration of helicase was 0.3 or 0.6 μM, 84.5% and 41.2% of the ATP was hydrolysed in 30 min. Almost all of the ATP (99.9%) was hydrolysed when the concentration of helicase exceeded 2.4 μM (Fig. 3).