

# Identification and characterization of the RNA helicase activity of Japanese encephalitis virus NS3 protein

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**Abstract** The NS3 protein of Japanese encephalitis virus (JEV) contains motifs typical of RNA helicase/NTPase but no RNA helicase activity has been reported for this protein. To identify and characterize the RNA helicase activity of JEV NS3, a truncated form of the protein with a His-tag was expressed in *Escherichia coli* and purified. The purified JEV NS3 protein showed an RNA helicase activity, which was dependent on divalent cations and ATP. An Asp-285-to-Ala substitution in motif II of the JEV NS3 protein abolished the ATPase and RNA helicase activities. These results indicate that the C-terminal 457 residues are sufficient to exhibit the RNA helicase activity of JEV NS3.

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**Key words:** Japanese encephalitis virus; RNA helicase; NS3 protein; ATPase

## 1. Introduction

Japanese encephalitis virus (JEV) is an arthropod-borne flavivirus and is a causative agent of central nervous diseases such as meningitis and severe encephalitis [1]. JEV belongs to the genus flavivirus of the family Flaviviridae. JEV has a positive, single-strand RNA genome of about 11 kb in length and the genome RNA is translated into a single polypeptide precursor which is subsequently processed to produce three structural (C, M and E) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) proteins.

The flavivirus NS3 protein is a multifunctional protein, which possesses several enzymatic activities. The serine protease activity was identified within about one fourth of the N-terminal domain of the NS3 protein of flaviviruses [2–4]. The remaining C-terminal region of the NS3 protein contains typical sequence motifs presumably responsible for NTP binding, NTP hydrolysis and RNA helicase. Among flaviviruses, an RNA-stimulated nucleoside triphosphatase (NTPase) activity was identified in the NS3 proteins of yellow fever virus, West Nile virus, dengue virus and JEV [4–9]. To date, however, the RNA helicase activity of flavivirus was demonstrated only in the NS3 protein of dengue virus type 2 [4]. The RNA-stimulated NTPase and RNA helicase activities were also identified in the NS3 proteins of viruses which are genetically related to

flavivirus such as hepatitis C virus (HCV), hepatitis G virus (HGV) and bovine viral diarrhoea virus, a pestivirus (BVDV) [10–19]. The roles of such enzymatic activities of the flavivirus NS3 protein in viral replication are poorly understood.

In this study, we demonstrated that an N-terminally truncated form of the JEV NS3 protein expressed in *Escherichia coli* has an RNA helicase activity. The optimal enzymatic activity of the JEV NS3 helicase has been studied. Moreover, the site-directed mutagenesis at Asp-285 in motif II has revealed that this residue is essential not only for RNA-stimulated ATPase activity but also for the RNA helicase activity.

## 2. Materials and methods

### 2.1. Construction of NS3 expression plasmids

A cDNA fragment was amplified using a sequence derived from the Japanese JEV strain JaO0566. PCR amplification was carried out using the primers 5'-TCG AAT TCC AGC GCC ATC GTG CAG-3' and 5'-ACG TCG ACT CTC TTT CCT GCT GC-3'. The 1.4 kb PCR-amplified DNA fragment was digested with *EcoRI* and *SalI*, and cloned into the corresponding restriction site of pET-21b (Novagen, Madison, WI, USA). The expression plasmid encodes amino acids 163–619 of the JEV NS3 protein with a His-tag at the C-terminus. As a positive control for the enzymatic activities of the JEV NS3 protein, we constructed a truncated form of the recombinant HCV NS3 protein as reported previously [20].

### 2.2. Expression and purification of NS3 proteins

The expression plasmids containing JEV and HCV NS3 genes were independently transformed into *E. coli* BL21(DE3)pLysS cells (Stratagene, La Jolla, CA, USA). After the IPTG induction at 37°C for 3 h, the cells were collected by centrifugation. The cells were resuspended in buffer B (10 mM Tris-HCl buffer (pH 8.5), 100 mM NaCl, 0.25% Tween 20) and disrupted by sonication for 5 min on ice. The soluble fraction of the clarified cell lysate was mixed with TALON metal affinity resin (Clontech, Palo Alto, CA, USA). After gentle mixing for 1 h at 4°C, the resin was collected by a brief centrifugation and washed with buffer B. Resin-bound protein was eluted with 2 volumes of buffer B containing 400 mM imidazole. Eluted protein fractions were dialyzed against dialysis buffer (10 mM Tris-HCl (pH 8.5), 100 mM NaCl, 10% Glycerol) at 4°C. Purified JEV and HCV NS3 proteins were designated NS3JEV-wild and NS3HCV-wild, respectively.

### 2.3. Site-directed mutagenesis

Site-directed mutation was introduced by QuikChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instruction. The nucleotide sequence of the entire coding region was determined and the desired mutation was confirmed. The mutant plasmid was transformed into *E. coli* BL21(DE3)pLysS cells and the resultant mutant protein was designated NS3JEV-AEAH.

### 2.4. Preparation of the substrate for RNA helicase assay

The <sup>32</sup>P-labeled RNA fragment was synthesized by the Riboprobe

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System-T7 (Promega, Madison, WI, USA) using a pSP72 DNA fragment linearized with *Bam*HI as a template DNA. Plasmid pGEM-3Zf(+) (Promega) was linearized with *Eae*I and was used to prepare a non-radiolabeled RNA fragment. The RNA transcripts were resuspended together in 100  $\mu$ l of annealing buffer (10 mM Tris-HCl (pH 8.5), 100 mM NaCl, 8 fmol of each transcript), and were hybridized by boiling for 5 min and then incubated at room temperature overnight.

### 2.5. RNA helicase assay

The RNA helicase assay was carried out in 20  $\mu$ l of helicase buffer containing 25 mM MES (pH 6.0), 2 mM DTT, 2 mM MgCl<sub>2</sub>, 5 mM ATP, 1.25 units of RNase inhibitor (Promega), 0.32 fmol of the RNA substrate and the purified NS3 protein. The reaction mixture was incubated at 37°C for 30 min, and 5  $\mu$ l of loading buffer (100 mM Tris-HCl (pH 7.4), 5 mM EDTA, 0.5% SDS, 50% glycerol, 0.1% xylene cyanol, 0.1% bromophenol blue) was added to terminate the reaction. A 10  $\mu$ l loading mixture was analyzed by 10% native polyacrylamide gel electrophoresis. The autoradiographic pattern was obtained with the BAS 2000 Image Analyzing System (Fuji Photo Film, Tokyo, Japan) and the labeled RNA was quantified using MacBAS software (Fuji Photo Film).

### 2.6. Colorimetric ATPase assay

The amount of free phosphate moiety released from ATP was measured as described previously [21]. Briefly, 50  $\mu$ l/well of reaction mixture containing 10 mM MOPS buffer (pH 6.5), 2 mM ATP, 1 mM MgCl<sub>2</sub>, and purified NS3 protein (0.8 pmol) was incubated in a 96 well microtiter plate at room temperature for 30 min. The reaction was stopped by adding 100  $\mu$ l/well of dye solution (water:0.081% malachite green:5.7% ammonium molybdate in 6 N HCl:2.3% polyvinyl alcohol=2:2:1:1, v/v). After the addition of 25  $\mu$ l/well of 30% sodium citrate, the absorbance at 620 nm with a reference wavelength at 492 nm was measured.

## 3. Results

### 3.1. Expression and purification of the truncated JEV NS3 protein

To identify and characterize the helicase activity of JEV NS3, we prepared an expression plasmid spanning 457 amino acids of the C-terminus of JEV NS3. Similarly, the corresponding region of the HCV NS3 gene was subcloned into the pET-21b vector, and the recombinant HCV NS3 protein

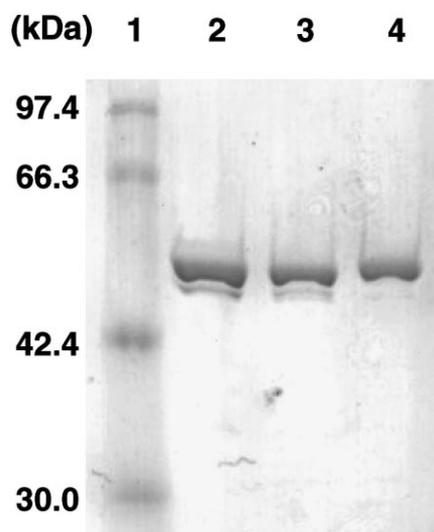


Fig. 1. SDS-PAGE analysis of purified JEV and HCV NS3 proteins. Each purified protein (2  $\mu$ g/lane) was subjected to SDS-PAGE, and the gel was stained with Coomassie blue. Lane 1, molecular weight marker; Lane 2, NS3JEV-wild; Lane 3, NS3JEV-AEAH mutant; Lane 4, NS3HCV-wild.

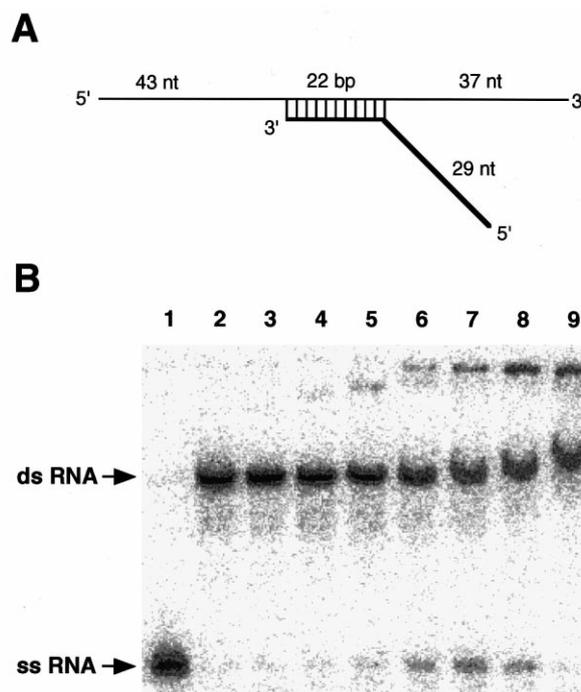


Fig. 2. RNA helicase assay of the wild-type JEV NS3. A: Partially double-stranded RNA substrate for the helicase assay. B: RNA helicase assays were performed as described in Section 2. Lane 1, boiled RNA substrate; Lane 2, untreated RNA substrate (negative control); Lanes 3–9, an RNA helicase assay was performed with increasing concentrations (0, 0.25, 0.5, 1, 2, 4 and 8 pmol) of purified JEV NS3 protein.

was also expressed in *E. coli* as a positive control for the helicase activity. The SDS-PAGE analysis indicated that the purified JEV and HCV NS3 proteins were 54 kDa (Fig. 1, lanes 2 and 4). The purified NS3JEV-wild protein had an ATPase activity which was significantly stimulated by poly(U) (data not shown) as reported previously [8].

### 3.2. RNA helicase activity of the JEV NS3 protein

For RNA helicase assay, we used an RNA substrate containing a partially double-stranded region (22 bp) and both 5' and 3' single-stranded overhanging sequences (Fig. 2A). An unwinding activity of the RNA substrate was demonstrated in 1–4 pmol purified NS3JEV-wild protein (Fig. 2B, lanes 6–8). As shown in Fig. 2B, decreased RNA unwinding activities were observed in the reactions with 4–8 pmol of the JEV NS3 protein. The slower migration rate of labeled dsRNA in lanes 7 and 8 suggests that any protein-RNA complex still remained after the addition of SDS sample buffer to the reactions with higher concentrations of the protein. A similar potent affinity for dsRNA was recently reported for a mutant HCV NS3 helicase [22]. In the absence of ATP (lane 6) or MgCl<sub>2</sub> (lane 7), no RNA helicase activity was demonstrated in the JEV NS3 protein (Fig. 3). In this assay, an identical amount (2 pmol) of the NS3HCV-wild protein (Fig. 3, lane 3), which served as a positive control, showed more RNA helicase activity than the JEV NS3 protein (lane 5).

### 3.3. Characterization of the RNA helicase of the JEV NS3 protein

To characterize the RNA helicase activity of the purified JEV NS3 protein, an RNA unwinding activity was examined

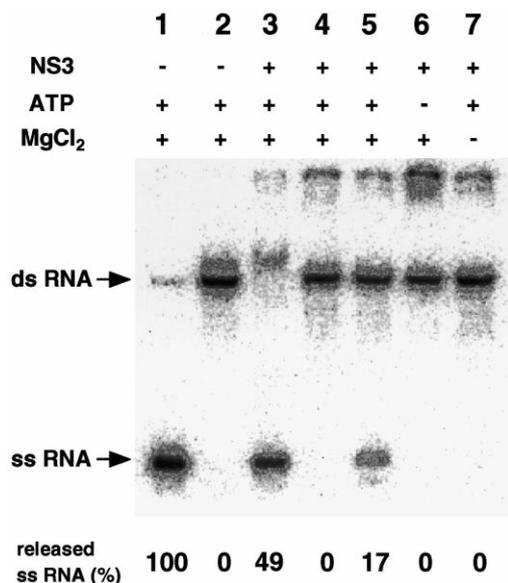


Fig. 3. RNA helicase assay of the wild-type and mutant NS3 proteins. RNA helicase assays were performed as described in Section 2 with 4 pmol of each purified protein. Lane 1, boiled RNA substrate; Lane 2, untreated RNA substrate; Lane 3, NS3HCV-wild; Lane 4, NS3JEV-AEAH mutant; Lane 5, NS3JEV-wild; Lane 6, NS3JEV-wild without ATP; Lane 7, NS3JEV-wild without MgCl<sub>2</sub>. The amount of labeled RNA released from the boiled substrate was set as 100%.

in various assay conditions. As shown in Fig. 4A, the RNA helicase activity was dependent on the divalent cations, and the optimal concentrations of Mg<sup>2+</sup> and Mn<sup>2+</sup> were 2 mM and 3 mM, respectively. On the other hand, increasing concentrations of potassium ion inhibited the RNA helicase activity. A similar phenomenon was reported for HCV NS3 and BVDV p80 helicases [12,14,17,18]. Increasing concentrations of Ca<sup>2+</sup> also decreased the RNA helicase activity of JEV NS3 (Fig. 4C). The optimal pH of JEV NS3 helicase was 6.0 (Fig. 4D) which was slightly lower than that of HCV and BVDV helicases, 7.0 and 6.5, respectively [12,14,17,18]. The RNA helicase activity of JEV NS3 required ATP and the optimal ATP concentration was 5 mM in the presence of 2 mM MgCl<sub>2</sub> (Fig. 4E). Higher concentrations of ATP (up to 8 mM) had an inhibitory effect as reported for the adeno-

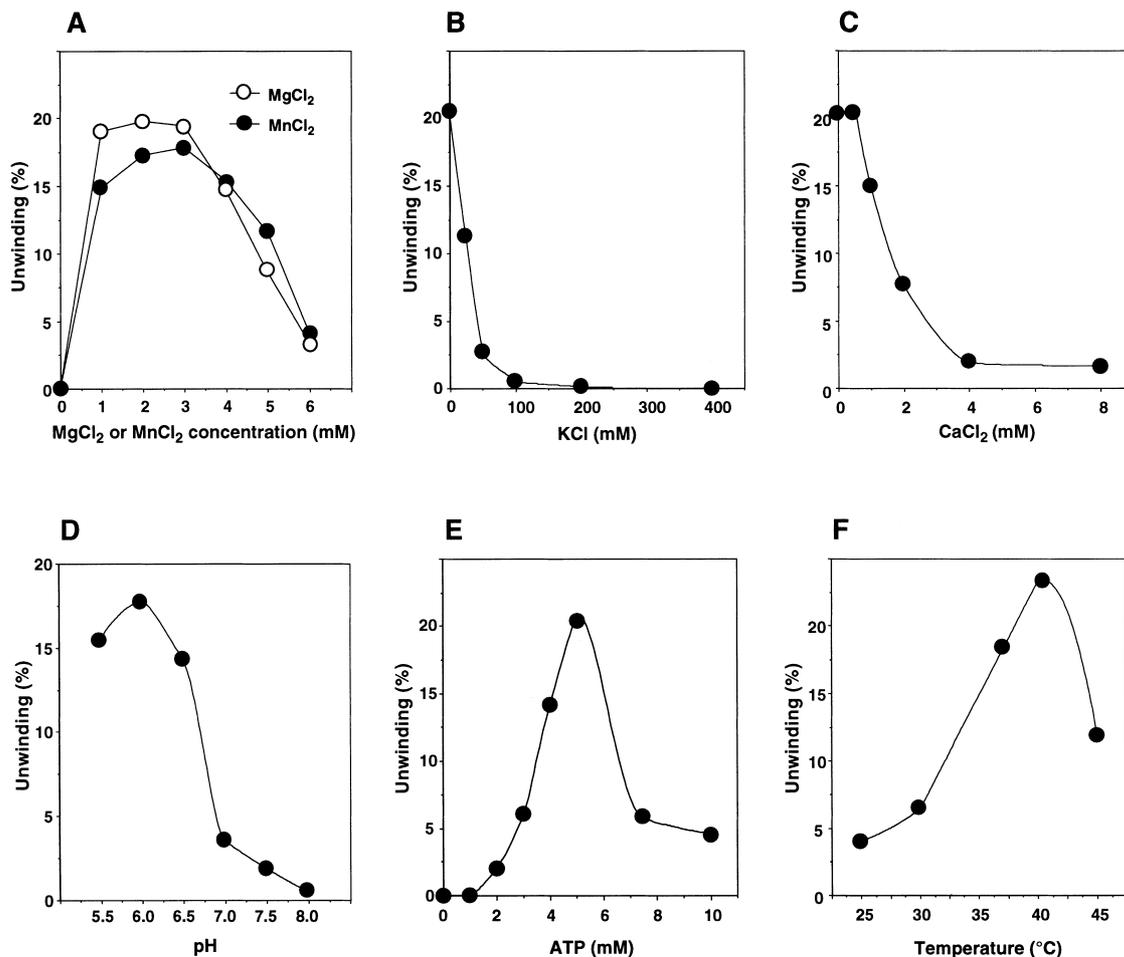


Fig. 4. Characterization of JEV NS3 helicase. The RNA helicase activity of wild-type JEV NS3 was assayed in the standard helicase reaction mixture with 2 pmol of the NS3JEV-wild protein. A: Optimization of the concentration of MgCl<sub>2</sub> or MnCl<sub>2</sub> in the presence of 3 mM ATP. B: Effect of KCl concentration in the presence of 2 mM MgCl<sub>2</sub>. C: Effect of CaCl<sub>2</sub> concentration in the presence of 2 mM MgCl<sub>2</sub>. D: Optimization of pH. E: Effect of ATP concentration. F: Optimization of the reaction temperature.

associated virus Rep68 helicase [23]. The helicase activity of JEV NS3 could be detected at 37–45°C, and the optimal temperature for the reaction was 40°C (Fig. 4F). In conclusion, the truncated form of the JEV NS3 protein showed similar enzymatic characteristics to those previously described for HCV NS3 and BVDV p80 helicases.

#### 3.4. Mutagenesis at Asp-285 in motif II of JEV NS3

To confirm the intrinsic RNA helicase activity of a truncated form of the JEV NS3 protein, we have introduced an alanine-substitution at Asp-285 in motif II which is presumably one of the most important sequence motifs for the NTP hydrolysis. The purified NS3JEV-AEAH mutant protein (Fig. 1, lane 3) exhibited no detectable ATPase activity (data not shown). The NS3JEV-AEAH mutant protein showed no RNA helicase activity (Fig. 3, lane 4), indicating that the Asp-to-Ala substitution completely abolished both enzymatic activities. The result also confirms that the apparent RNA helicase activity is due to the NS3JEV-wild protein rather than contaminated proteins derived from the bacterial cell lysates.

#### 4. Discussion

Although RNA-stimulated NTPase and RNA binding activities of JEV NS3 has been identified using recombinant NS3 proteins expressed in *E. coli*, an RNA helicase activity has not been detected [7,8]. In the present study, we showed that the truncated form of the JEV NS3 protein possessed an RNA helicase activity as well as the poly(U)-stimulated ATPase activity.

Recently, the NS3 protein of dengue virus type 2, one of the arthropod-borne flaviviruses, was shown to possess RNA helicase activity, together with serine protease and RNA-stimulating NTPase activities by using the recombinant proteins expressed in *E. coli* [4]. However, biochemical characterization of the RNA helicase has not been described. Moreover, the truncated NS3 protein of dengue virus has been shown to exhibit significantly weaker RNA helicase activity than that reported previously for HCV NS3 helicase and for BVDV p80 helicase [12,14,20,24]. Up to 27 pmol of the purified NS3 protein of dengue virus was required before it exhibited weak but detectable RNA helicase activity [4]. In this study, however, 1–4 pmol of a truncated form of the JEV NS3 protein had obvious RNA helicase activity (Fig. 2). The difference of apparent RNA helicase activities between the NS3 proteins of dengue virus and JEV may be due to the way each protein was purified. To purify and characterize the recombinant NS3 proteins of dengue virus the proteins from insoluble fractions, expressed in *E. coli*, were purified in the presence of 6 M guanidine, and renatured to active form after their purification [4]. In this study, we have purified the truncated forms of wild and mutant JEV NS3 proteins directly from soluble fractions expressed in *E. coli* BL21(DE3)pLysS. By using such a simplified purification system, a more detailed characterization of the RNA helicase activity of JEV NS3 has become possible. Another possible explanation for higher RNA helicase activity of JEV NS3 is that the reaction condition was not optimal for the dengue NS3 helicase. The optimization for the helicase activity of dengue NS3 has not been described [4].

The flavivirus NS3 protein contains GKT/S (motif I) and

DEXH (motif II) motifs which are commonly found in the DEXH protein subfamily of the DEAD-box protein family [25–27]. From the crystal structure of HCV NS3, it appears that motif II interacts with motif I which forms a phosphate-binding loop [28–30]. The side chains of the residues in motif II may be involved in binding  $\beta$ -phosphate of ATP and  $Mg^{2+}$  which presumably make a complex with  $\beta$  and  $\gamma$  phosphates of the ATP molecule. Mutational and crystallographic analyses of HCV NS3 also suggest that motif II is involved in the coupling of the ATPase and RNA helicase activities via conformational changes of the NTP-binding pocket [18,24,31].

However, for flavivirus NS3 proteins including JEV NS3, the crystal structure has not been solved and no mutational analysis has been so far reported. The mutagenesis at Asp-285 in this study implies that the residue is essential for both ATPase and RNA helicase activities. For other DEXH helicases, a corresponding Asp-to-Ala substitution in motif II was shown to cause severe defects in RNA unwinding and ATPase activities [18,32,33]. Further mutational analysis of JEV NS3 will address the contribution of each domain to the ATP hydrolysis, RNA binding and RNA helicase activities. The expression and purification system for the JEV NS3 protein described in this study will provide useful material for such biochemical studies.

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