Heliyon 10 (2024) e35449

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Enzymatic characterization and dominant sites of foot-and-mouth disease virus 2C protein

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ARTICLE INFO

Keywords:
FMDV
2C protein
Nuclease
Helicase
RNA chaperone

ABSTRACT

Foot-and-mouth disease virus (FMDV) 2C protein is a conserved non-structural protein and crucial for replication of the virus. In this study, FMDV 2C protein was prepared and the enzymatic activities were investigated in detail. The protein could digest ssDNA or ssRNA into a small fragment at about 10 nt, indicating that the protein has nuclease activity. But it did not show digestion to blunt-end dsDNA or dsRNA. The nuclease activity of 2C protein could be inhibited in 2 mM Zn^{2+} or Ca^{2+} while enhanced by Mg^{2+} or Mn^{2+}. FMDV 2C protein exhibited unwinding activity to all the three kinds of dsDNA and dsRNA (5′ protruded, 3′ protruded, and blunt-end). The unwinding velocity to 5′ protruded dsRNA was higher than to the blunt-end dsRNA. 2C protein only showed unwinding activity in high concentration of Mg^{2+}, but no unwinding activity in physiological concentrations of Mg^{2+} and Ca^{2+}, as well as in cell lysate. The 2C protein could catalyze two structured ssRNA to form double strand, thus it was proved to have RNA chaperone activity. The Mg^{2+} and ATP in different concentrations did not show promotion to the RNA chaperone activity. Finally, six mutant proteins (K116A, D160A, D170A, N207A, R226A, and F316A) were constructed and the enzymatic activities were analyzed. All the six mutations reduced the ATPase activity, D170A and F361A could inactivate the nuclease activity, while the N207A and F316A could inactivate the helicase activity. Our study provides a comprehensive understanding of the enzymatic activities of FMDV 2C protein.

1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious disease of even-toed ungulates caused by foot-and-mouth disease virus (FMDV). FMDV belongs to the *Aphthovirus* genus in the family *Picornaviridae*. The family *Picornaviridae* is one of the largest virus families, containing five subfamilies, namely *Caphthovirinae*, *Kodimesavirinae*, *Ensavirinae*, *Paavivirinae*, and *Heptrevirinae*. Many important human and animal viruses are included in this family, such as poliovirus (PV), hepatitis A virus (HAV), Coxsackievirus B3 (CVB3), Enterovirus 71 (EV71), and FMDV. FMDV can induce both acute and chronic infection, as well as asymptomatic yet persistent infection [1,2]. There are seven serotypes in FMDV, namely A, O, C, Asian 1, South African Territorial 1 (SAT 1), SAT 2, and SAT 3 [3]. It is challenging for commercial vaccinations to offer effective defense against so many viral serotypes. FMDV genome is a

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https://doi.org/10.1016/j.heliyon.2024.e35449
Received 4 April 2024; Received in revised form 21 July 2024; Accepted 29 July 2024
Available online 30 July 2024

positive-sense, single-stranded RNA with approximately 8500 nucleotides. The genome has one open reading frame (ORF) and two non-coding regions, as well as a sequence of poly-A. The ORF is translated into a polyprotein. The polyprotein was cleaved into four fragments (Lpro, P1, P2, and P3) and then further processed into a series of mature virus proteins [2,4,5]. P2 and P3 fragments contain all the non-structural proteins (NSPs) and these NSPs are related to virus replication and pathogenicity [6–8].

FMDV 2C protein is located in the P2 fragment and generated by 3C protease cleavage [9,10]. 2C protein has 318 amino acids and the sequence shows relative low homology (~60 %) to the other picornaviruses [2,3,11]. The protein possesses an amphipathic helix at its N-terminal for membrane-bound and this region is crucial for membrane vesicle and replication complex (RC) formation [12]. The main body of 2C was an ATPase domain containing Walker A and B, motif C and R-finger [3]. The C-terminal of 2C protein is a Zinc-finger equivalent region (ZFER) and pocket binding loop (PBL). The PBL domain is important for the enterovirus replication complex to bind to the lipid droplets to form a replication compartments [13]. A synthesized 14-mer peptide derived from PBL can inhibit FMDV 2C ATPase activity and disrupt FMDV 2C-induced lipid drops clustering, and thus have potential antiviral activity [3].

Picornavirus 2C is a multifunctional protein with multiple enzymatic activities. The NTPase activity of 2C protein has been widely described [12,14]. According to sequence analysis, the 2C protein has the conserved motif of SF3 helicase. EV71 2C protein is reported to have RNA unwinding activity in a bidirectional manner by its middle domain [15]. At the same time, the protein also has RNA chaperone activity by its C-terminus [15,16]. Recently, a study reported ribonuclease activity in several picornavirus 2C proteins, including EV71, PV, and FMDV [11]. The RNA helicase and chaperone activities of FMDV 2C protein has not been investigated yet.

In the current study, FMDV 2C protein was prepared by prokaryotic expression and Ni^{2+} affinity chromatography. The characters of enzymatic activities including nuclease, ATPase, helicase, and RNA chaperoning, were investigated. The optimal reaction conditions for nuclease and helicase were explored. Some new enzyme characteristics were found in FMDV 2C protein.

2. Materials and methods

2.1. Expression and purification of recombinant proteins

The FMDV 2C (97–318 aa) and its mutation genes were cloned into prokaryotic expression plasmid pET-28a. The plasmid was transferred into *E. coli* BL21 (DE3) competent cells for expression. The expressed protein was purified by Ni^{2+} affinity chromatography. The purified protein was identified by SDS-PAGE and Western blot. Finally, the protein buffer was replaced with buffer containing 50 mM HEPES (pH 7.5) and 50 mM NaCl by ultrafiltration.

2.2. ATPase activity assay

The ATPase activity assay was carried out using Kinase-Glo Plus Luminescent Kinase Assay Kit (Promega, USA) as described previously [17–19]. In short, the Kinase-Glo substrate was resolved in Kinase-Glo buffer, thoroughly mixed, and kept at −20 °C. 20 μM protein and 100 μM ATP was added in reaction buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$). The reaction was held in 37 °C for 30 min. The Kinase-Glo reagent was added to the mixture and the chemiluminescence was measured using multimode plate reader (PerkinElmer, USA). The chemiluminescence value of non-protein control group was recorded as Lc and the value of experimental groups was recorded as Le. The relative ATPase activity was calculated as (1-Le/Lc) %.

2.3. Preparation of oligonucleotide substrates

The single-stranded DNA and RNA with or without Hexachloro-fluorescein (HEX) tag were synthesized. The sequences of these oligonucleotides were provided in Table 1. The oligonucleotides were diluted into 10 μM. The dsDNA or dsRNA was prepared by mixing the two complementary strands, heating to 95 °C for 3 min, and then slowly down to room temperature. The 5′ protruded dsDNA or dsRNA was generated by DNA1F/RNA1F and DNA1R/RNA1R; 3′ protruded dsDNA or dsRNA was generated by DNA1F/RNA1F and DNA2R/RNA2R; and blunt-end dsDNA or dsRNA was generated by DNA1F/RNA1F and DNA3R/RNA3R.

In the fluorescence resonance energy transfer (FRET) assay for helicase activity, a BHQ-1 labeled reverse strand was applied to

Table 1
The test's oligonucleotide substrates.

Oligonucleotide	Sequence (5′–3′)	Label
RNA1F	GCUAGUCACUGUUCGAGCACCA	5′HEX
RNA1R	UAGAUAGCCAUAGAUAGCAUUGGUGCUCGAACAGUGACUAGC	
RNA2R	UGGUGCUCGAACAGUGACUAGCUAGAUAGCCAUAGAUAGCAC	
RNA3R	UGGUGCUCGAACAGUGACUAGC	
DNA1F	GCTAGTCACTGTTCGAGCACCA	5′HEX
DNA1R	TAGATAGCCATAGATAGCATTGGTGCTCGAACAGTGACTAGC	
DNA2R	TGGTGCTCGAACAGTGACTAGCTAGATAGCCATAGATAGCAC	
DNA3R	TGGTGCTCGAACAGTGACTAGC	
DNA4R	TAGATAGCCATAGATAGCATTGGTGCTCGAACAGTGACTAGC	3′BHQ-1
DNA5R	TGGTGCTCGAACAGTGACTAGC	3′BHQ-1
ssRNA+	GCUAGUCACUGUUCGAGCACCAAUGCUAUCUAUGGCUAUCUA	5′HEX

generate the double-stranded DNA (Table 1). The 5′ protruded dsDNA was generated by DNA1F and DNA4R and the blunt-end dsDNA was generated by DNA1F and DNA5R.

2.4. Nuclease assay

The nuclease assay was conducted as earlier described [11]. The reaction mixture consisted 0.2 μM nucleic acid and 20 μM 2C protein. The reaction buffer for nuclease assay was 50 mM HEPES (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, and 20 U RNase inhibitor. The total volume of reaction was 10 μL. The reaction was carried out at 37 °C for 90 min and stopped by adding 1 μL of 10 × stop buffer (1.2 mg/mL proteinase K, 1.0 % sodium dodecyl sulfate). Then the product was mixed with 10 × RNA loading buffer and submitted to 15 % native-PAGE electrophoresis. Gel images were produced by FLA-5100 (Fujifilm, JPN) or Typhoon 5 imaging system (GE, USA).

2.5. Helicase activity assay

Helicase activity assay was performed in accordance with the previous reports [15,20]. The 2C protein in 20 μM was mixed with 0.2 μM HEX-labeled double-stranded substrate. The reaction buffer for helicase activity assay was 50 mM HEPES (pH 7.5), 50 mM NaCl, 20 mM $MgCl_2$, 100 μM ATP, 1 mM dithiothreitol, and 20 U RNase inhibitor. The total volume of reaction was 10 μL. The reaction was carried out at 37 °C for 60 min and stopped by adding 1 μL of 10 × stop buffer (1.2 mg/mL proteinase K, 1.0 % sodium dodecyl sulfate). Then the product was mixed with 1.1 μL of 10 × RNA loading buffer and submitted to 15 % native-PAGE electrophoresis. Gel images were produced by FLA-5100 or Typhoon 5 imaging system.

2.6. RNA annealing assay

The RNA annealing experiment was performed according to the previous study [15]. Two complementary single-stranded DNAs or RNAs were synthesized. The oligonucleotide was designed to forming a stem-loop structure by itself (Fig. 5A). One of the oligonucleotides was labeled with HEX. Each of the oligonucleotide was heated to 95 °C for 3 min, and then slowly down to room temperature, allowing to form stem-loop structure. The protein in 0.8 μM was mixed with the two single-stranded and structured nucleic acid in 0.2 μM. The reaction buffer for helicase activity assay was 50 mM HEPES (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, 20 U RNase inhibitor. The total volume of reaction was 10 μL. The reaction was carried out at 37 °C for 10 min. Then 10 × stop buffer (1.2 mg/mL proteinase K, 1.0 % sodium dodecyl sulfate) was added to the mixture and analyzed by 15 % native-PAGE electrophoresis. Gel images were produced by FLA-5100 or Typhoon 5 imaging system.

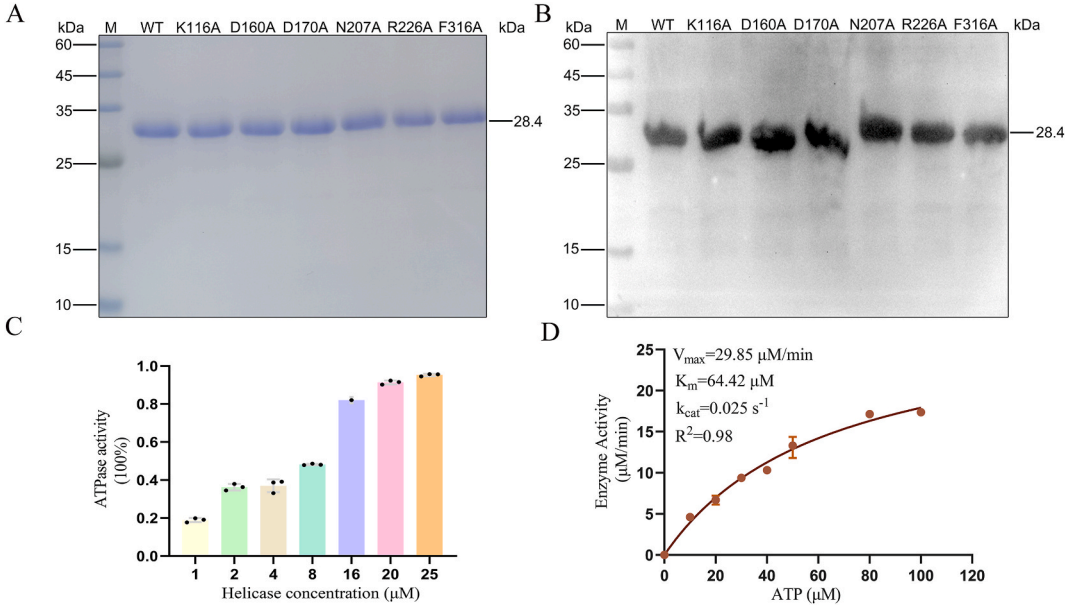


Fig. 1. Expression, purification, and ATPase activity of FMDV 2C protein. (A–B) Identification of the purified protein by SDS-PAGE (A) and Western blot (B) (See Supplementary Fig. 1A and B for uncropped blot). (C) The ATP hydrolysis in different concentrations of 2C protein. (D) The velocity of ATP hydrolysis was determined under different substrate concentrations and the reaction curve was drawn.

2.7. Unwinding assay by FRET

A FRET assay was established to quantitatively assess the helicase activity of 2C protein. Two complementary DNA strands were synthesized and labeled with HEX and BHQ-1, respectively (Table 1). DNA1F and DNA5R produced blunt-end dsDNA, and DNA1F and DNA4R made 5′ protruded dsDNA. The two strands of DNA were mixed in ratio of 1:1.2 (HEX: BHQ-1) (molar ratio), heated to 95 °C for 3 min, and then slowly down to room temperature. The annealing efficiency was evaluated by detecting the fluorescence value of annealed DNA. The 2C protein in 20 mM was mixed with 0.2 μM FRET substrate and incubated at 37 °C in a white 96-well plate. The reaction plate was placed in a VICTOR Nivo Multimode Plate Reader (PerkinElmer, USA). The fluorescence value (580 nm emission and 530 nm excitation) was read at every minute within 90 min.

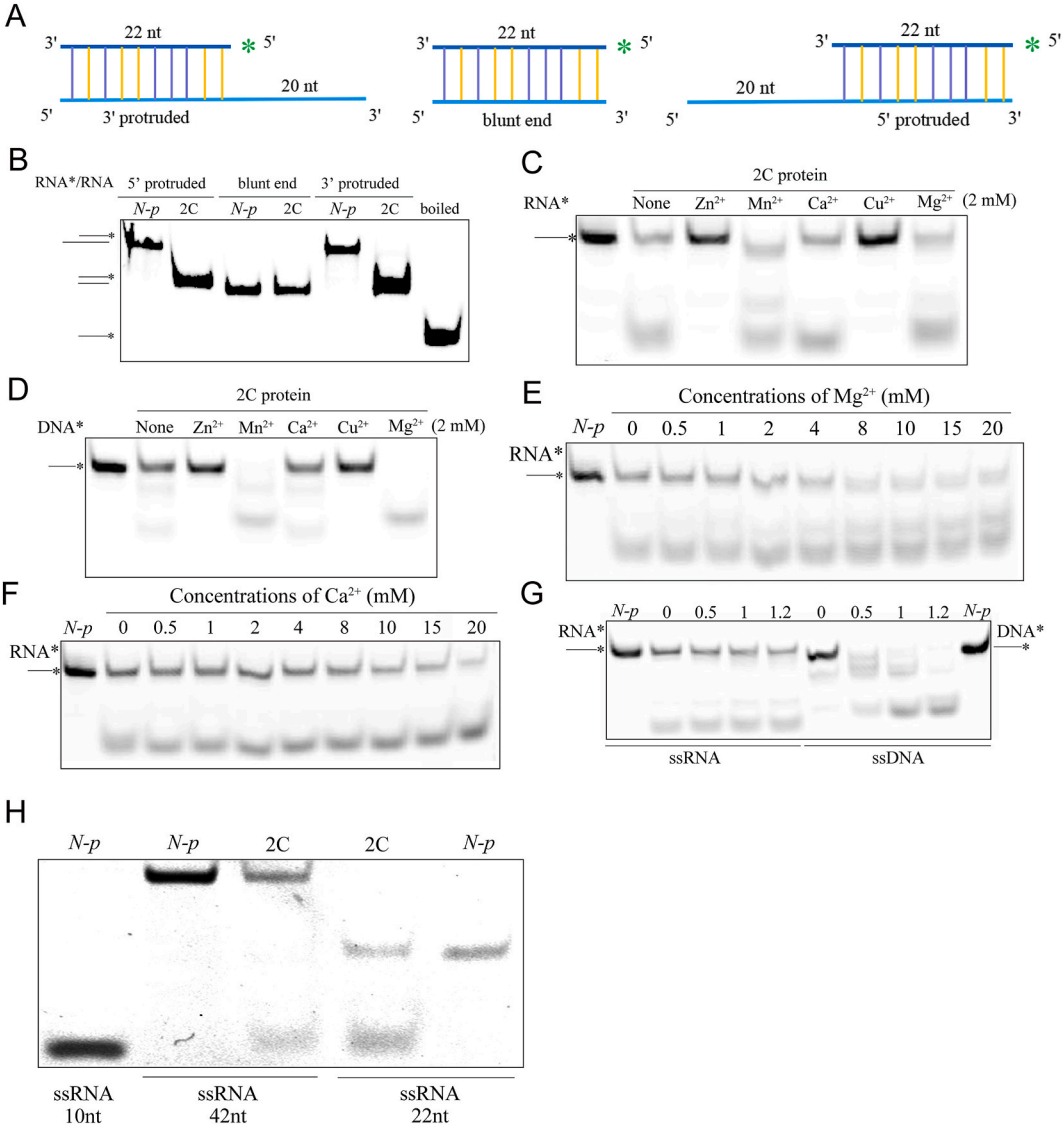


Fig. 2. The nuclease activity of FMDV 2C protein. (A) Schematic diagram of different kinds of substrate dsRNA. Left, 3′ protruded dsRNA; middle, blunt-end dsRNA; right, 5′ protruded dsRNA. Asterisk represents the HEX label. (B) Ribonuclease activity acts only on single-stranded. Only single strands of 3′ protruded and 5′ protruded dsRNA can be digested, resulting in fragments of the same length as the blunt-ended dsRNA. (C–D) The effect of different metal ions on 2C digestion activity to RNA (C) or DNA (D). (E) The effect of different concentrations of Mg^{2+} on ribonuclease activity of 2C protein. (F) The effect of different concentrations of Ca^{2+} on ribonuclease activity of 2C protein. (G) The reaction product of nuclease on ssDNA and ssRNA. (H) Two RNA fragments (42 nt and 22 nt) were digested by the FMDV 2C protein. Both reactions produced a small RNA fragment in the same length near the 10 nt ssRNA marker. *N-p* stands for non-protein control (See Supplementary Figs. 2A–G for uncropped).

3. Results

3.1. Protein expression, purification, and activity detection

The recombinant 2C protein and its mutant proteins (K116A, D160A, D170A, N207, R226A, and F316A) were expressed by prokaryotic expression system and purified by Ni^{2+} affinity chromatography. The purified proteins were identified by SDS-PAGE and Western blot. SDS-PAGE results showed that the molecular weight of these proteins was consistent with expectation and all the proteins showed a high purity (Fig. 1A). All the proteins had reaction with His-tagged antibody in Western blot (Fig. 1B) (Supplementary Fig. 1A and B for uncropped). The ATPase activity of wild type protein was detected by a kinase assay kit which could quantify the ATP amount in solution. The chemiluminescence values decreased with the increase of 2C protein (Fig. 1C), indicating that the protein possessed ATPase activity. Then, the enzyme kinetic of ATPase was investigated by adding different concentrations of ATP in the reaction mixture. The Michaelis-Menten curve was drawn in Fig. 1D and the V_{max} and K_m were calculated to be 29.85 μM/min and 64.42 μM, respectively.

3.2. Nuclease activity of FMDV 2C protein

Previous study revealed that 2C protein of picornavirus could digest U-rich ssRNA and thus had ribonuclease activity [11]. In the current study, more properties of ribonuclease activity were investigated. To identify the digest direction, different forms of double-stranded RNAs (3′ protruded, blunt-end, and 5′ protruded) were prepared as shown in Fig. 2A. These RNAs were added in Mg^{2+} free solutions with or without 2C protein. The 2C protein would not have helicase activity in the Mg^{2+} free buffer. The results showed that the length of both 3′ protruded RNA and 5′ protruded RNA shortened after 2C digestion, and the length of digested RNA was similar with the blunt-end dsRNA (Fig. 2B). While the length of blunt-end RNA was not changed compared with the 2C absent group (Fig. 2B). These results indicated that the FMDV 2C protein could digest ssRNA by both 5′ to 3′ and 3′ to 5′ direction, while it could not digest the dsRNA (Fig. 2B, Supplementary Fig. 2A for uncropped). To elucidate the effect of metal ions to ribonuclease activity, different divalent metal ions (Cu^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, and Mg^{2+}) in 2 mM were added in the digestion reaction. Compared with the no metal ion control, the Zn^{2+} and Cu^{2+} showed complete inhibition to the ribonuclease activity (Fig. 2C). The ribonuclease activity did not change visible in Mn^{2+}, Ca^{2+} and Mg^{2+} (Fig. 2C) (Supplementary Fig. 2B for uncropped).

The digestion activity of FMDV 2C protein to ssDNA was also investigated in this study. In divalent metal ion free buffer, the 2C protein exhibited weak degradation to ssDNA (Fig. 2D), indicating that 2C protein possessed DNase activity. In the buffer with 2 mM Mn^{2+} or Mg^{2+}, the activity could be enhanced to digest all ssDNA, while the presence of Zn^{2+} and Cu^{2+} could inhibit the activity (Fig. 2D). According to these results, FMDV 2C protein can degrade both RNA and DNA, and thus it is not only a ribonuclease, but a nuclease (Fig. 2D, Supplementary Fig. 2C for uncropped). The nuclease activities to ssDNA and ssRNA were then detected in different concentrations of Mg^{2+} and Ca^{2+}. It was shown that the activity would be slightly inhibited when the concentration of Mg^{2+} exceeded

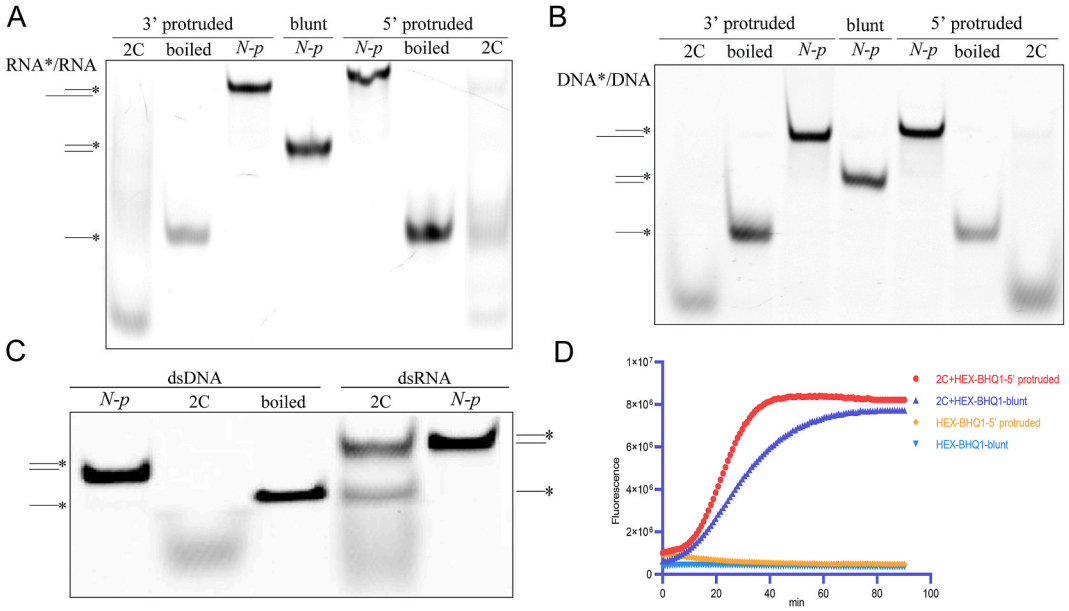


Fig. 3. Helicase activity of FMDV 2C protein. (A) Unwinding assay of 2C protein to 5′ protruded and 3′ protruded dsRNA. (B) Unwinding assay of 2C protein to 5′ protruded and 3′ protruded dsDNA. (C) The efficiency of unwinding activity to dsRNA and dsDNA. The boiled RNA or DNA was set as control. (D) FRET assay of unwinding activity. One dot represents the fluorescence value at a certain time point. Asterisk represents the HEX label. *N-p* represents non-protein control (See Supplementary Figs. 3A–C for uncropped).

4 mM (Fig. 2E) (Supplementary Fig. 2D for uncropped). High concentration of Ca^{2+} (20 mM) did not show any effect to the activity (Fig. 2F) (Supplementary Fig. 2E for uncropped).

In the nuclease assay, a small RNA or DNA fragment was always observed after reaction (Fig. 2C-F). Then different length of RNA (42 nt and 22 nt) was applied in the digestion assay. A same length of small fragment was generated based on the two different substrates (Fig. 2G) (Supplementary Fig. 2F for uncropped). To identified the length of this product, a synthesized ssRNA (10 nt) was loaded into the gel as a marker and the digested product was found to have similar length with this ssRNA (Fig. 2H) (Supplementary Fig. 2G for uncropped). These results showed that the 2C protein of FMDV could digest ssRNA into an approximate 10 nt fragment.

3.3. Helicase activity of FMDV 2C protein

The FMDV 2C protein was predicted to have helicase SF3 motif as previous studies [3,12,21]. But there had no report on the helicase activity of FMDV 2C protein yet. In our study, different forms of dsRNA (5′ protruded, 3′ protruded, and blunt-end) were added to FMDV 2C protein. The FMDV 2C protein showed unwinding activity to both 5′ and 3′ protruded dsRNA (Fig. 3A). The ssRNA generated by the 2C helicase activity could be further digested into a shorted RNA fragment by the ribonuclease activity of FMDV 2C

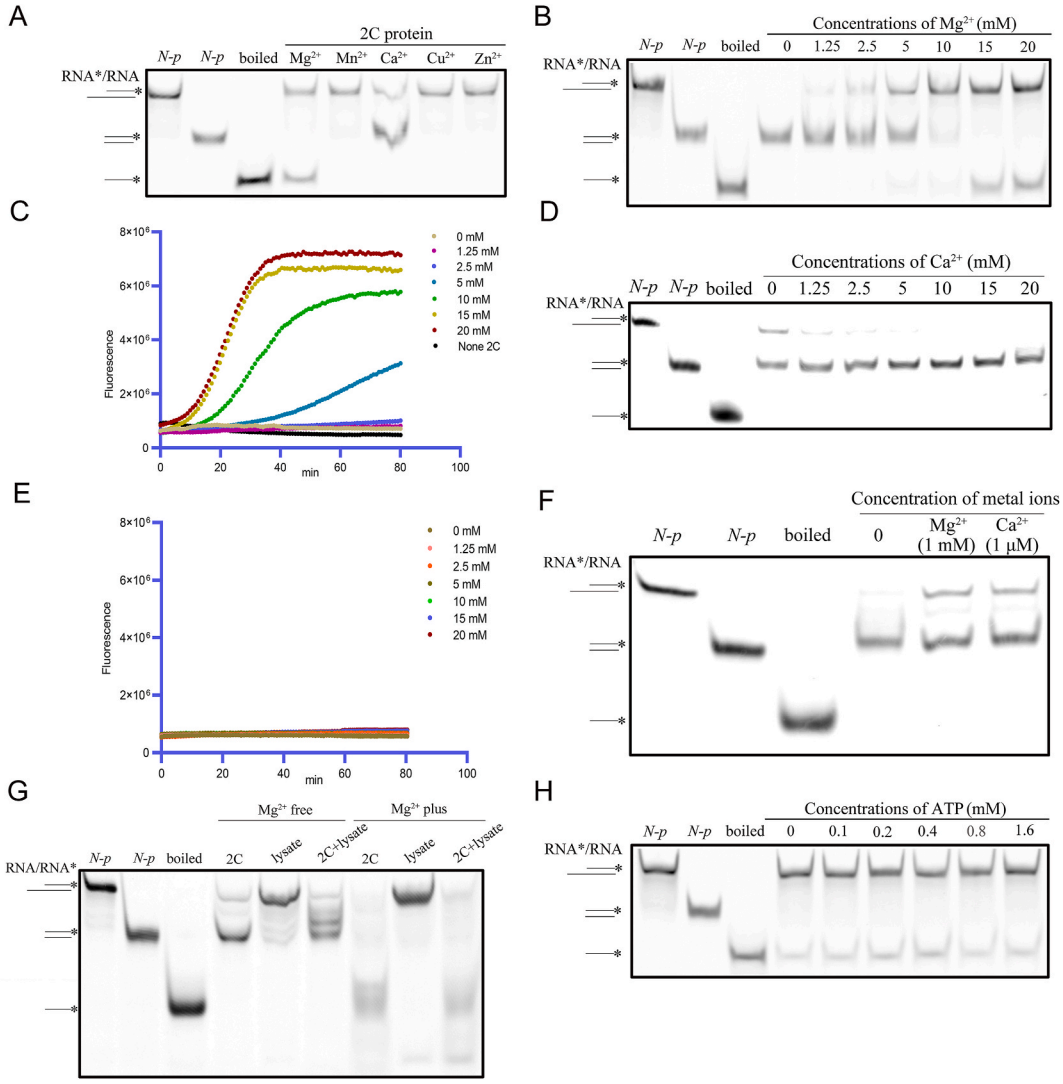


Fig. 4. The influence factors for helicase activity of FMDV 2C protein. (A) The unwinding activity of 2C protein in different divalent metal ions in 20 mM. (B and C) The unwinding activity in different Mg^{2+} concentration detecting by electrophoresis (B) and FRET assay (C). (D and E) The unwinding activity in different concentrations of Ca^{2+} detecting by electrophoresis (D) and FRET assay (E). (F) The unwinding activity of 2C protein in physiological concentration of Mg^{2+} and Ca^{2+}. (G) The unwinding activity of 2C in cell lysate. The activities of 2C protein in cell lysate (Mg^{2+} free) or cell lysate adding with 20 mM Mg^{2+} (Mg^{2+} plus) was detected. (H) The unwinding activity of 2C protein in different concentration of ATP detecting by electrophoresis (See Supplementary Figs. 4A–F for uncropped).

protein (Fig. 3A) (Supplementary Fig. 3A for uncropped). Similar results were acquired in the DNA unwinding assays (Fig. 3B) (Supplementary Fig. 3B for uncropped). In addition, the 2C protein was found to have unwinding ability to blunt-end dsDNA and dsRNA (Fig. 3C). In the same reaction conditions, FMDV 2C protein could unwind and degrade all the dsDNA into a small fragment (Fig. 3C). While these reactions to dsRNA was not completed, the three forms RNA (double strand, single strand, and small fragment) was all found in the reaction product (Fig. 3C) (Supplementary Fig. 3C for uncropped). These results indicated that the unwinding efficiency to blunt-end dsDNA was higher than the dsRNA in the same condition. Collectively, FMDV 2C protein could unwind all kinds of dsDNA and dsRNA.

A FRET assay was established to quantitative detect the activity of 2C helicase. Two strands of ssDNA were labeled with HEX and BHQ-1, respectively (Table 1). The annealed dsDNA (5′ protruded and blunt-end) was mixed with 2C protein for reaction. The unwinding activity was measured by detecting the fluorescence value at different reaction time. As shown in Fig. 3D, the fluorescence values persistently increased in a certain time, while the wells without 2C protein could not detect the change of fluorescence value. These results suggested that 2C protein could unwind the 5′ protruded and blunt-end dsDNA. In 5′ protruded DNA, it took 40 min to the maximum fluorescence value, while it took 60 min for the blunt-end DNA (Fig. 3D). This result indicated that the 2C protein had higher unwinding efficiency to protruded dsDNA than blunt-end dsDNA.

3.4. *Factors affecting the helicase activity*

The helicase activity of FMDV 2C protein in different conditions was studied. Different divalent metal ions in 20 mM were added to the reaction mixture, respectively. The 2C protein in Mn^{2+}, Ca^{2+}, Cu^{2+}, or Zn^{2+} did not show unwinding activity (Fig. 4A). The protein only exhibited unwinding activity in 20 mM Mg^{2+} (Fig. 4A) (Supplementary Fig. 4A for uncropped). Then different concentrations of Mg^{2+} were added into the reaction mixture, the unwinding activity began to be detectable when the Mg^{2+} concentration reached to 5 mM and enhanced with the increase of Mg^{2+} to 20 mM (Fig. 4B) (Supplementary Fig. 4B for uncropped). The velocity was investigated in different concentrations of Mg^{2+} by FRET assay. The unwinding velocity showed a dose-dependent effect when the concentration of Mg^{2+} under 15 mM (Fig. 4C). The velocity was almost identical in 15 and 20 mM Mg^{2+} (Fig. 4C). The unwinding activity was also detected in different concentrations of Ca^{2+}. There was no unwound single strand detected in 0–20 mM Ca^{2+} buffer (Fig. 4D). Only the protruded ssRNA was digested by nuclease activity of 2C protein to generate a blunt-end dsRNA (Fig. 4D) (Supplementary Fig. 4C for uncropped). FRET assay also showed that the fluorescence value did not increase in presence of different concentrations of Ca^{2+} (Fig. 4E). Then 1 mM Mg^{2+} and 1 μM Ca^{2+} was added in reaction mixture to mimic physiological concentration, the 2C protein did not show RNA unwinding activity but possessing nuclease activity (Fig. 4F) (Supplementary Fig. 4D for uncropped).

To tested the enzymatic activities of 2C protein in the environment of cytoplasm, BHK-21 cells were collected, resuspended in HEPES (pH 7.5) buffer, and ultrasonic lysed on ice. The supernatant was mixed with FMDV 2C protein. The nuclease activity could be detected in a certain decrease in the cell lysate (Fig. 4G). But there had no ssRNA present in the mixture, indicating that no unwinding activity in the cell lysate (Fig. 4G). As a control, when 20 mM Mg^{2+} was added into the cell lysate, clearly unwinding and nuclease activity could be detected (Fig. 4G) (Supplementary Fig. 4E for uncropped). To clarify the role of ATP in the unwinding reaction, different concentrations of ATP was added into the reaction mixture. The unwinding activity did not show visible change in different groups, even if in the ATP absent group (Fig. 4H) (Supplementary Fig. 4F for uncropped). These results suggested that Mg^{2+} in a certain concentration was essential for the helicase activity, and the Mg^{2+} concentration required for the unwinding activity of 2C protein was

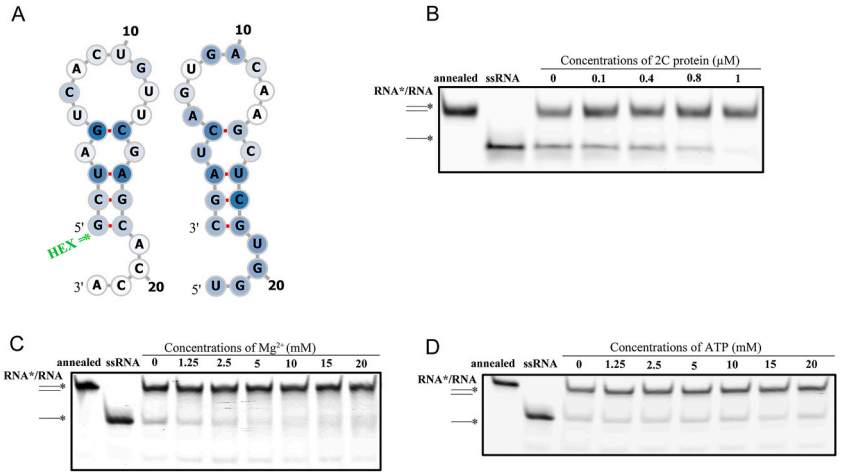


Fig. 5. The chaperone activity of FMDV 2C protein. (A) The sequence and structure for substrates of chaperone activity assay. Two complementary ssRNA strands were synthesized. Each of the ssRNA could form a stem-loop structure by itself. One of the RNA strands was labeled with HEX. (B) The chaperone activity assay of 2C protein. Different concentrations of 2C protein were added into the RNA substrates. The structured ssRNAs could anneal to form double strand RNA and the amount of double strand increased with the 2C concentration. (C) The influence of Mg^{2+} on chaperone activity of 1 μM 2C protein. (D) The influence of ATP on chaperone activity of 1 μM 2C protein (See Supplementary Figs. 5A–C for uncropped).

higher than that in the cytoplasm.

3.5. ATP-independent RNA chaperone activity of FMDV 2C protein

The RNA chaperone activity of FMDV 2C protein was investigated in our study. A 22-nt ssRNA and its complementary strand were heated and cooled down to form stem-loop structure, respectively (Fig. 5A). Then the two RNAs were mixed with 2C protein in different concentrations for annealing assay. There had no Mg^{2+} in the annealing buffer and thus no helicase activity would present in this condition. In the non-protein control, some single strand RNA was found to annealed to form double strand (Fig. 5B). Compared with the control group, the RNA with 2C protein showed higher ability to form double strand and the hybridizing efficiency increased with protein concentration (Fig. 5B) (Supplementary Fig. 5A for uncropped). To explore the influence factors for the chaperone activity, different concentration of Mg^{2+} and ATP were added in the reaction buffer. Mg^{2+} within a certain concentration range (1.25–10 mM) could promote the chaperone activity (Fig. 5C), while the ATP did not show any effect on this reaction (Fig. 5D). An RNA fragment smaller than the ssRNA was also observed in the annealing assays (Fig. 5C and D), suggesting that the nuclease activity was also presented in these reactions (Fig. 5C and D, Supplementary Fig. 5B and C for uncropped).

3.6. Dominant sites for the different enzymatic activities in FMDV 2C protein

The amino acids responsible for different enzyme activities in 2C protein was investigated. Combined the previous studies on picornavirus 2C protein [22] and the conservative site analysis (Fig. 6A), six conserved amino acids in 2C protein were selected for mutation analysis (K116, D160, D170, N207, R226, and F316). The mutant genes were generated by site-directed mutagenesis, expressed in *E. coli*, purified by Ni^{2+} affinity chromatography, and identified by SDS-PAGE and Western blot (Fig. 1A). The ATPase activity of all the mutant proteins were significantly reduced compared with the wild type protein ($P < 0.0001$) (Fig. 6B), suggesting that all these sites were related with the ATPase activity. For the nuclease activity, the degradation efficiency to ssRNA was clearly reduced in D170A and F316A mutant protein (Fig. 6C). While the mutation at N207 and R226 could enhance the activity of nuclease, because more ssRNAs could be degraded compared with the wild type protein (Fig. 6C) (Supplementary Fig. 6A for uncropped). As to the helicase activity, the mutations of N207A and F316A could eliminate the unwinding activity of 2C protein (Fig. 6D). Because the N207A could enhance the nuclease activity, a dsRNA equivalent to the length of blunt-end was present in the digested product. F316A could inactivate the nuclease, so the input protruded dsRNA presented in the digested product (Fig. 6D) (Supplementary Fig. 6B for uncropped). In the chaperone activity assay, the mutations of D170A, N207A, R226A, and F316A showed enhancement effects to this activity (Fig. 6E) (Supplementary Fig. 6C for uncropped).

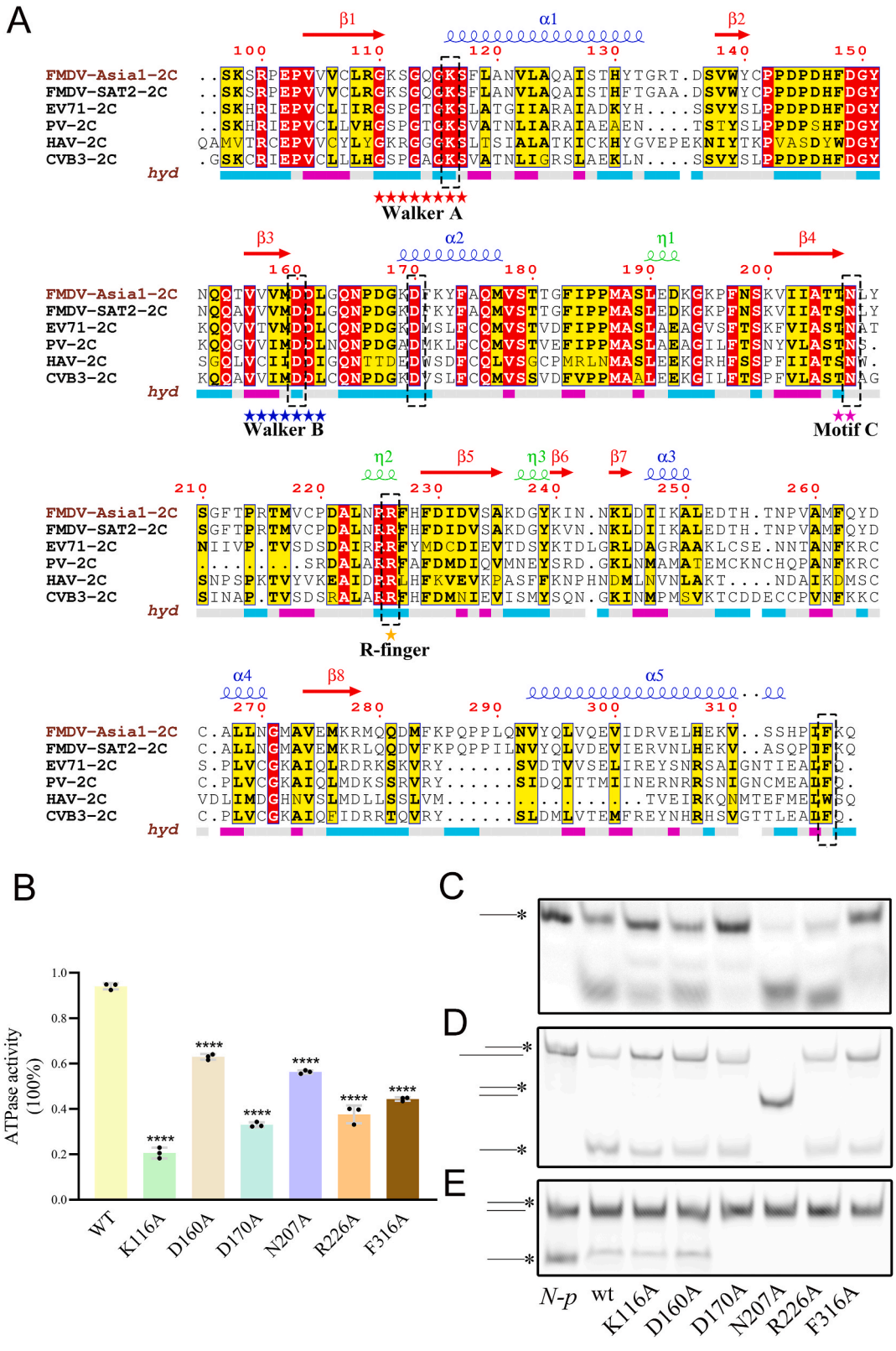
4. Discussion

Helicase is a kind of enzyme that hydrolyzes NTP to unwind dsDNA and dsRNA. The helicase participates in many processes of nucleic acids metabolism of, including replication, repair, recombination, mRNA transcription, RNA processing, and translation [23,24]. Six superfamilies (SF1-SF6) may be distinguished among helicases based on their differences in sequence, structure, and function [25,26]. There have conserved motifs in helicase responsible for binding and hydrolysis of NTP, binding of metal divalent ions, and binding of nucleic acids, respectively. Several viruses have been shown to encode helicases, including NS3 helicase of flavivirus, NSP13 helicase of coronavirus, NPH-I/II helicase of poxviridae, Rep helicase of circovirus, and the 2C helicase in picornavirus [17,18,27–30]. Since viral helicase is crucial for virus replication, it has been regarded as an ideal target for the development of antiviral drugs [31,32]. FMDV 2C protein was regarded as a helicase by the sequence analysis, but the unwinding activity has not reported yet.

It has been proved that 2C protein of picornavirus was a multifunctional protein and played multiple roles in the replication of virus. It can interact with the virus genome or other proteins, directly participating in the formation of virus replication complex [33–36]. The 2C protein also has various enzyme activities and may participate in some biochemical reactions of viral RNA replication [11,12,15,16]. At the same time, 2C protein can also interact with many cellular proteins and participates in virus replication, cellular metabolism, and inflammatory responses [9,37,38]. Due to the important functions of 2C protein, it is recognized as an important drug target in picornavirus [3]. Several antiviral agents have been identified to inhibit enterovirus by targeting the 2C protein [39–41].

Compared with the other viral helicases, such as coronavirus nsp13 and flavivirus NS3, FMDV 2C protein shows many differences. The traditional viral helicases possess nucleic acid binding, ATPase, and unwinding activities according to previous reports [18,19,29,42,43]. While the 2C protein of FMDV shows more biological functions than the other viral helicases. It has nuclease activity and can digest ssDNA or ssRNA into a small fragment (Fig. 2). The protein is also proved to have RNA chaperone activity (Fig. 5), and can help structured single-stranded RNA to form double strand. In addition, the helicase activity of 2C protein is also quite different from that of other viruses. The 2C protein can unwind double strand not only in 5′ to 3′ direction, but also in 3′ to 5′ direction (Fig. 3). 2C protein can unwind both protruded double strand and blunt-end double strand (Fig. 3). While most of viral helicases only unwind double strand RNA in one direction, and cannot unwind the blunt-end double strand [30]. Although FMDV 2C protein has more biological activity than other helicases, the role of these activities in virus replication, pathogenesis, and immunity is still unknown.

Previous study has reported the ribonuclease activity of hepatitis A virus (*Picornaviridae* family) 2C protein. The protein could digest a 35 nt ssRNA into a small fragment [11]. Our current study has enriched the understanding of 2C protein enzyme activity. We found that FMDV 2C protein could digest not only ssRNA but also ssDNA (Fig. 2). So, the 2C protein of FMDV should be a nuclease. Our studies also optimized the reaction condition for 2C nuclease. The results indicated that metal ions made obvious different impacts on this activity (Fig. 2). It is shown that Ca^{2+} could inhibit the nuclease while Mg^{2+} could promote this activity. In cytoplasm, there has



(caption on next page)

Fig. 6. The dominant amino acids for ATPase, nuclease, helicase, and RNA chaperoning activity. (A) The amino acid alignment of Picornavirus 2C protein. The mutant sites were labeled with dash line box. (B) The ATPase activity of mutant proteins. **** represents extremely significant difference with wide type protein ($P < 0.0001$). (C) The nuclease activity of mutant proteins. The input ssRNA was digested into a smaller fragment. The amount of this fragment could reflect the activity of nuclease. (D) The helicase activity of mutant proteins. The activity was assessed by comparing the amount of double strand RNA. (E) The RNA chaperone activity of 2C protein and its mutant. The two single strand RNAs was added into the proteins. The RNA chaperone activity was evaluated by the double strand RNA (See Supplementary Figs. 6A–C for uncropped).

very low concentration of free Ca^{2+} (0.01–0.1 μM) but presents a certain concentration of free Mg^{2+} (0.5–1.2 mM). These ionic environment in the cytoplasm is favorable for the activity of nuclease according our results. In addition, we first reported that the single-stranded nucleic acid could be digested into a short form at about 10 nt by the FMDV 2C protein (Fig. 2). The role of these small RNA fragments in virus replication and cellular metabolism is an area that deserves in-depth study.

In unwinding activity assay, FMDV 2C protein showed strict dependency on Mg^{2+}. High unwinding activity could be detected only when the concentration of Mg^{2+} exceeded 10 mM (Fig. 4B&C). This result is different from the previously reported helicase activity of EV71 2C protein. The EV71 2C protein can exhibit partial helicase activity in the absence of Mg^{2+}, but shows high helicase activity when Mg^{2+} is present at 1–5 mM [15]. In the normal condition, the concentration of Mg^{2+} in the cytoplasm is hard to reach 10 mM. We also applied a cell lysate as helicase reaction buffer, and the unwinding activity was not detected in this condition (Fig. 4G). According to these results, FMDV 2C protein might possess nuclease activity and RNA molecular chaperone activity in cytosol environment, but cannot exhibit helicase activity. For EV71, the helicase activity of 2C protein was proved to be crucial for virus replication. The helicase-defective mutations in 2C protein could inactivate the replication of EV71 [15]. It has not been reported whether the helicase activity of the 2C protein was necessary for FMDV replication and how the 2C protein exhibited helicase activity in cells. Some vesicular structures in cells may provide the microenvironment for helicase activity. Previous study described that FMDV 2C protein could interact with cellular vimentin protein and form a cage-like structure, and this might modulate the host cell microenvironment to allow for the unwinding reaction of 2C protein [44].

Collectively, our study reveals that the FMDV 2C protein has nuclease activity and it can digest the ssDNA or ssRNA and generate a final product at about 10 nt. The results also reveal that FMDV 2C protein possess bidirectional unwinding activity to both dsDNA or dsRNA. In addition, FMDV 2C protein is proved to have RNA chaperone activities. The optimized reaction conditions and the dominant sites for enzymatic activities was also explored in this study. These results are beneficial for further research on the 2C protein function in cells, inhibitor screening, and mechanism of virus replication.

Data availability statement

All data generated in this study has been included in this article.

CRediT authorship contribution statement

Saisai Zhou: Writing – original draft, Software, Methodology, Investigation, Formal analysis, Conceptualization. **Nankun Liu:** Investigation, Formal analysis. **Yang Tian:** Software, Investigation. **Hong Pan:** Investigation, Formal analysis. **Yang Han:** Validation. **Zhen Li:** Validation. **Jinhua Zhang:** Investigation. **Shuaiyin Guan:** Visualization. **Huanchun Chen:** Resources, Project administration. **Yunfeng Song:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This study was supported by the National Key Research and Development Program of China (2021YFD1800303) and the National Natural Science Foundation of China (31972658).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e35449.

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