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Unraveling activity of crucial domain HABD protein in dengue virus

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ABSTRACT

Dengue virus (DENV) causes dengue, which is a very common mosquito-borne viral disease. The global incidence of dengue has increased dramatically in recent decades. About half of the world's population is now at risk. This virus is widespread throughout the tropics, which are influenced by rainfall, temperature, and humidity; however, severe dengue has a higher risk of death when not managed timely. To describe Dengue virus helicase ATP binding domain (HABD) protein in biochemically characterized. Sequences analysis, structure modeling, secondary structure prediction, ATPase assay, unwinding assay, RNA binding assay. HABD has RNA-dependent ATPase and helicase activity which are crucial proteins that participate in the unwinding of double-stranded DNA or RNA by utilizing ATP. RNA binding proteins and DEAD-box RNA helicases have been revealed to contribute to viral replication. Moreover, DEAD-box RNA helicases have been demonstrated to be involved in several features of cellular metabolism of RNA, for example, transcription, splicing, biogenesis, ribosomal processing of RNA, etc. In the present study, we have mainly focused on the Dengue virus's helicase ATP binding domain (HABD) and observed that HABD contains RNA-dependent ATPase and unwinding activity at different concentrations and time points.

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Introduction

Dengue is a viral disease (mosquito-borne) dengue fever caused by a dengue virus (DENV), a single positive-stranded RNA virus belonging to the genus Flavivirus and family Flaviviridae. The Flavivirus genus also includes other viruses, for example, JEV (Japanese encephalitis virus), YFV (Yellow Fever Virus), and WNV (West Nile Virus) (1). DENV genome codes a primary polyprotein which gets cleaved into three crucial structural proteins (C, capsid; pr/M, membrane; E, envelope) and seven nonstructural (NS) proteins such as nonstructural (NS1), nonstructural (NS2A), nonstructural (NS2B), nonstructural (NS3), nonstructural (NS4A), nonstructural (NS4B), and nonstructural (NS5) by the host, and viral proteases. The NS proteins further assemble to form a replication complex at the endoplasmic reticulum (ER), where viral RNA synthesis and protein translation occur (2). The recently formed viral RNA is then encapsulated with the assistance of structural proteins and passes through the Golgi to the cell surface to be released outside of the cells (2).

DENV has been spread around 100 countries that are located in tropical and subtropical areas. This virus infects more than 60 million people annually. However, there is no effective treatment available to cure this disease (3-7). Till now, there are four different kinds of virus serotypes that are very close to each other and can cause dengue. However, it is believed that recovery from infection provides enduring immunity alongside that serotype. Approximately every year, 50-100 million dengue fever cases are reported due to transmission of viral contamination by Aedes mosquitos. The nearness of five other saved themes places NS3 in superfamily 2 of RNA helicases/NTPases, as per the order of helicases into three noteworthy superfamilies (8, 9).

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Practically, the helicase and NTPase exercises of the nonstructural (NS3) protein have been portrayed for a few individuals from the Flaviviridae, which also includes hepatitis C infection (10, 11)**,** Dengue infection (12), West Nile infection (13), yellow fever infection (14), and *Japanese encephalitis* infection (15). DENV NS3 protein is a multifunctional protein where its N-terminal encodes a protease that mediates viral polyprotein processing; however, its C-terminal consists of DExD/H box helicases. In contrast, decreased activity of protease or helicase in DENV NS3 impaired virus replication, implying the importance of NS3 in the virus life cycle (16). Helicases belong to the class of chemicals which are essential to every single living life form. Several RNA binding proteins, including DEAD-box RNA helicase (DDX6), NF90, and ER13, have been identified by RNA affinity chromatography or thiouridine cross-linking spectrometry technologies, which participate in the DENV life cycle (17). They are engine proteins that move directionally along the nucleic acid phosphodiester bond, separating two annealed nucleic acid strands by utilization of energy ATP (18). Helicases have been arranged in 7 superfamilies' (SF1-SF7). The majority of the proteins tie ATP, and, in this way, each one of them conveys the traditional Walker A (phosphate-restricting circle or P-circle) and Walker B (Mg^{2+}-restricting aspartic amino acid) themes. In both superfamilies (SF1 and SF2), motifs I&II are the conserved Walker A and B sequences characteristic of ATPases (10). These two superfamilies develop an expansive number of DNA & RNA helicases from archaea, eubacteria, eukaryotes, and infections that appear to be dynamic as monomers or dimers. RNA and DNA helicases are viewed as proteins that catalyze the impartiality of double-stranded nucleic acids (RNA/DNA) in a vitality subordinate approach (19). The different structures of SF1& SF2 helicases present a typical center with two alpha-beta RecA-like spaces. The essential homology with the RecA recombination protein covers the five adjacent parallel beta-strands and the pair alpha-helices. ATP attaches to the amino-proximal alpha-beta area, where Walker A (the theme I) and Walker B (theme II) are found (10, 19). The N-terminal space additionally contains theme III (S-A-T), which was proposed to assist in connecting ATPase and helicase exercises. The carboxy-terminal alpha-beta area is fundamentally the same as the proximal one even though it is run-down of an ATP-restricting site, recommending that it might have initially emerged through quality duplication of the first. These studies provide a direction for future research to understand the basic biology of RNA in the virus (dengue) (7, 10). RNA helicases play a fundamental role in most of the procedures that are intricate in RNA digestion, for example, ribosome biogenesis, pre-mRNA grafting, and interpretation inception. They likewise assume a vital job in detecting viral RNAs (20). RNA helicases are associated with the mediation of antiviral-resistant reactions since they can distinguish remote RNAs invertebrates. About 80% of all infections are RNA infections, and they possess RNA helicases (21-25).

In the present examination, we report each biochemical representation of the HABD (Helicase ATP Binding Domain) in the Dengue virus. HABD domain contains the brand nucleic acid ATPase, as well as unwinding activities. The findings detailed here will impel our insight into the nucleic acid metabolic procedures in the Dengue virus.

Materials and Methods

We purchased T4 DNA ligase, DNA polymerase and restriction from NEB, MA, and the USA for the cloning. All the molecular grade reagents, including agarose, antibiotics, and enzymes like proteinase K, lysozyme and other chemical reagents were acquired from Sigma, USA.

Sequence analysis of HABD Protein

HABD sequences of the dengue viruses were rescued from the genome database NCBI and BLASTp. The formats (FASTA) of the rescued sequences were used for additional investigation. Multiple sequence alignment was done among HABD (201 amino acid, 22kDa protein). The rescued sequences were studied by *in silico* methods, and numerous domains were physically consigned and arranged via integrated software, InterProScan (26). Antigenic region and secondary structure are predicted using the emboss plugin of generous prime, superfamily, and domains are predicted using InterProScan plugin of generous prime (27-29). We also identified no of charged amino acid and atomic

composition of target protein (HABD) by using of available tools Protparam (https://web.expasy.org/cgi-bin/protparam/protparam).

Molecular modeling

The entire sequence (amino acid) of Helicase ATP Binding Domain (HABD) has been submitted to the Swiss model homology-modeling server (http://swissmodel.expasy.org/)(30, 31). Thus, the structural modeling of HABD was submitted with the help of crystal structure PDB number 5yvw.1 as the template.

Secondary structure prediction

The secondary structure of the HABD (201 amino acids) was made with the help of the server http://bioinf.cs.ucl.ac.uk/psipred/. It is very extensive that α- helix, strands, and coils are attainable in the helicase domain (32, 33).

HABD recombinant protein

The recombinant His-Tag protein HABD was purchased from ABCAM. This protein was used for the SDS -PAGE analysis and also helped in further studies.

Hydrolysis of ATP

The hydrolysis of ATP, which was catalyzed by HABD, was mixed with buffer by determining the formation of ^{32}P from [γ32P] ATP. The reaction was performed for 2 hours at 37ºC in the occurrence of all purified proteins and 100nM of M13 mp19 ssDNA. The reaction mixture of [γ32P] ATP (specific activity 222 TBq.mmol-1) and cold ATP (1 mM) was stopped by keeping in the ice. One of each reaction mixture was spotted onto a polyethyleneimine-cellulose thin-layer strip, and chromatography was used for performing in 0.5 M LiCl, and 1M formic acid for 20 minutes at room temperature. The strip was dried at room temperature and was also exposed to hyper film to identify the hot spots of ATP and Pi. Alpha Imager-EP/Image-J software (http://rsbweb.nih.gov/ij/) was used for quantitation. All the experiments were done at least two times, and quantification data were used for calculating the standard deviation (SD) with the help of Microsoft Excel 2010, and the mean value was used for preparing the graph (34, 35).

Unwinding assay

The helicase substrate was prepared using the method described earlier (36). The RNA helicase substrate was made with the help of the RNA oligonucleotides synthesized. Using bacteriophage polynucleotide kinase (T4), five units purchased from NEB, England, and 13-mer oligonucleotide with concentration 25ng were labeled at the 5 prime ends. This labeled oligonucleotide was annealed with oligonucleotides (39-mer) by the standard procedure. The substrate (duplex RNA) was purified using the method detailed previously. (35-37).

Data analysis

The densitometry calculations of band intensities in gel images were done with the help of ImageJ 1.48 software (downloaded from http://imagej.nih.gov/ij/download.html) followed by further analysis in Excel. GraphPad Prism was used for plotting graphs for reaction rates versus time.

Results and discussion

Sequence analysis and domain Organization

With the help of bioinformatics available tools (Inter Pro Scan SMART, Panther, and Pfam), we identified conserved domains like NS1, NS2A, NS2B, NS3, NS4A, NS4B (non-structural proteins) (Figure 1A and 1B) where NS3 protein contains helicase ATP binding domain. The sequence (amino acid) of the helicase ATP binding domain is 201 amino acids. The molecular weight of the target protein is 22438.69 and the theoretical PI is 6. We have analyzed the sequence (amino acid) of the conserved domain (Helicase); we found it appropriate to the DEAH family (Figure 1C). The result clearly shows the domain DEAH family highlighted in a red color sequence. Our study on secondary structure depicts that it contains 10 blocks of the alpha helix, contains 83 residues and 13blocks contain 43 residues which make a beta-sheet. There are 46 amino acids involved in making a coil. However, 28amino acid makes the turn in the protein secondary structure. In this structure, 87 residues participate in forming 8 antigenic regions. Flavi_DEAD (Pfam) is having a length of 148 amino acids, while the signal cleavage is having 13 amino acids (Figure 1D). In parallel, we also identified the no of a positively charged amino acid (Asp + Glu: 28)

and negatively charged amino acid (Arg + Lys): 25. Illustrate the detail with the bar diagram in figure 1E. On other hand the, we anticipate the atomic composition of the HABD target protein. The bar diagram clearly showed the C, H, N, O and S with their numbers (Figure 1F).

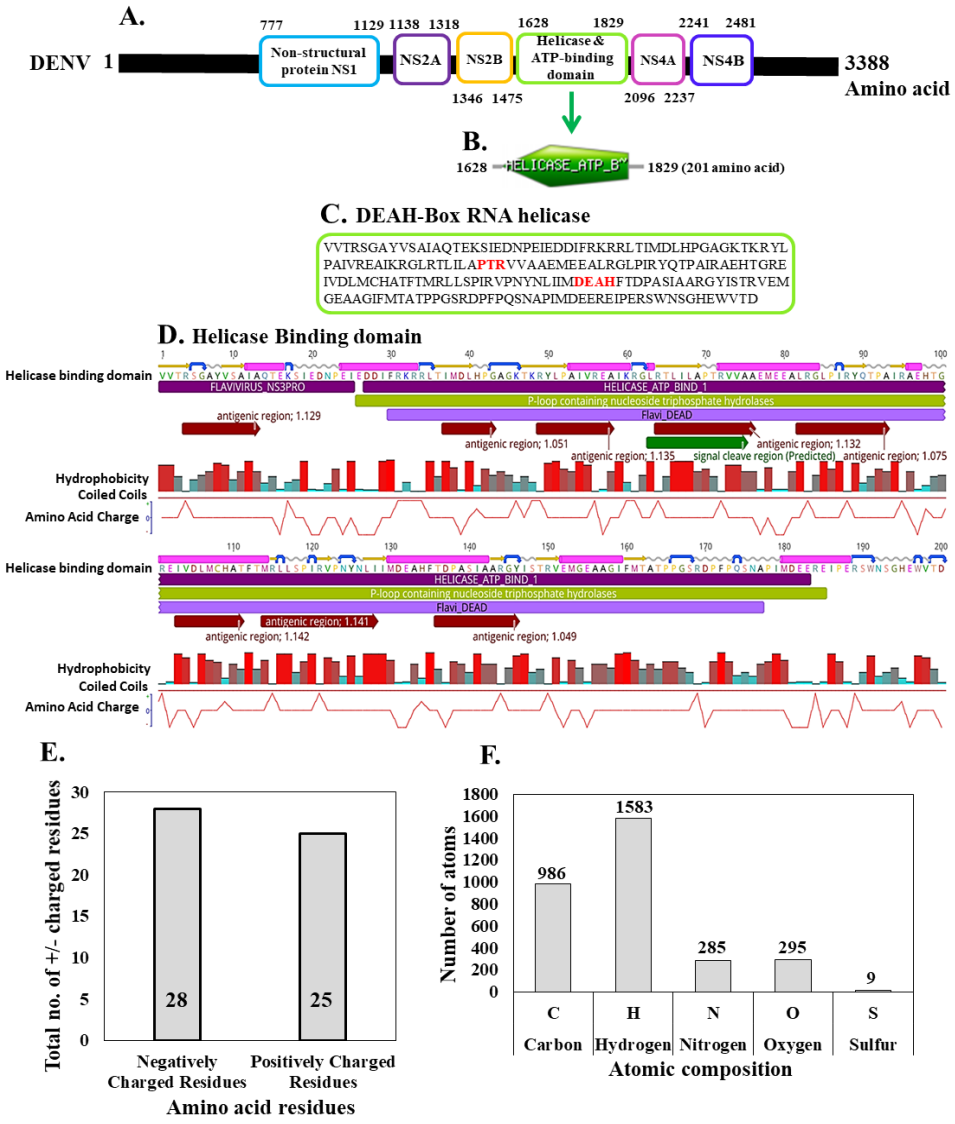

Figure 1. Details of the Helicase domain. (A) Amino acid sequence arrangement of the crucial area of Dengue Virus (DEV, 3388 amino acid). (B) Helicase domain (201 amino acid) identification of Inter pro (https://www.ebi.ac.uk/interpro/search/sequence-search) program. (C) DEAD-box RNA helicases interpretation of the references to the color box (Red). (D) Helicase Binding domain of Dengue Virus predicted secondary structure of the protein; Alpha helix in pink color, beta-strand in red arrow, the coil in zig zag gray color and turn is plotted in blue color turned arrow, predicted antigenic region and its score in red color, signal cleavage domain in green color, Hydrophobicity of the whole sequence predicted in the bar graph, the line graph predicts amino acid charge in red color. Predicted superfamily in corn color, Pfam is predicted and plotted in the blue color rod. (E) A total number of charged amino acid residues. (F) The bar diagram represents the atomic composition of HABD protein.

Structure modeling and secondary structure prediction

The sequence of amino acids consented to the Swiss model homology- modeling server (http://swissmodel.expasy.org/) (30, 38), for structural modeling. Therefore, the structural modeling of HABD was tendered with the help of crystal structure PDB number 5yvw.1 as a template. Figure 2A (i) represents the structure of the helicase ATP Binding domain (HABD), whereas Figure 2A (ii) represents the template (5yvw.1). The HABD and the template (i & ii) of the modeled structure were superimposed in Figure 2A (iii). We have also done the secondary structure prediction analysis, as shown earlier (21).

Significantly, a-helix, b- sheets, b- turn, and the coils are available in the helicase domain (Figure 2B). This indicates that the predicted secondary structure of the helicase ATP binding domain exhibits the presence of Strand (6), Helix (11), and Coil (17) (Figure 2B).

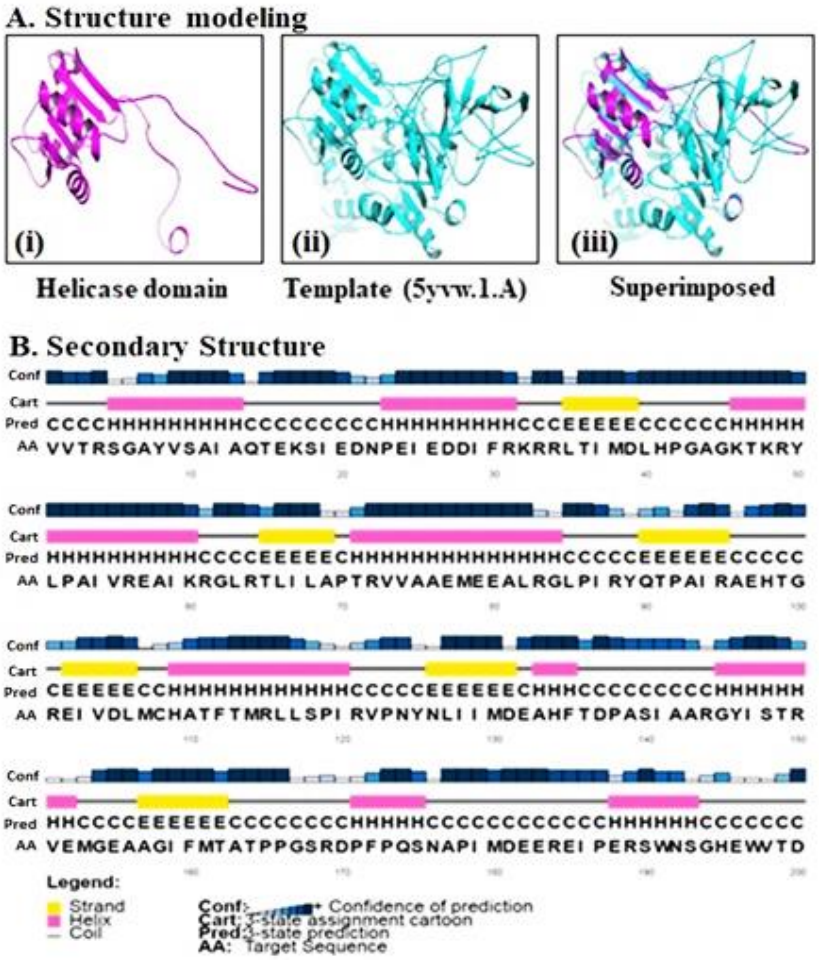

Figure 2. Structure modeling and secondary structure prediction. For obtaining the structure, the sequence of amino acids of the helicase domain was predicted to the Swiss model server (A) (i) Helicase domain (ii) Template (iii) superimposed image. (B) By using PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/), the secondary structure was predicted, and the secondary structure was obtained.

Hydrolysis of ATP by using purified protein

We purchased the new batch of recombinant protein (His-tagged) with 22 kDa from Abcam (Cambridge, MA), and the purity of the purchased protein was tested by SDS-PAGE analysis, as shown in Figure 3A. This purified recombinant protein was used for all the enzymatic assays. The concentration-dependence of RNA-dependent ATPase movement was tested using 10 to 200 nM of HABD protein, and the released percent of Pi (radioactive inorganic phosphate) from [γ32P] ATP was estimated. The outcomes of purified protein demonstrate that HABD contains concentration (10, 20, 30, 40, 50, 80, 100, and 200 nM) dependent ATPase activity (Figure 3B). For time-dependent ATPase activity, the ATPase response utilizing 150 nM of purified HABD protein at different time intervals (10, 20, 30, 40, 50, 60, and 90 minutes) was performed. The percent Pi from [γ-32P] ATP revealed linearity up to 60 minutes, and the enzymatic activity did not increase more for prolonged incubation up to 90 minutes (Figure 3C). Collectively, these results indicate that ATP hydrolysis is HABD concentration-dependent as well as time-dependent up to 60 min.

ATP hydrolysis is required to provide chemical energy for effective RNA duplex unwinding (39). Previously, Zika virus helicase showed intrinsic NTPase activity, which provides the energy for unwinding viral RNA replication (40). Practically all the helicases have specific nucleotide necessity, and

nucleotide hydrolysis is firmly coupled to ATPase enzymatic activity (41). To validate the unwinding activity of HABD (Helicase ATP binding domain) protein (200 nM), various nucleotides, deoxynucleotides (dNTPs), and triphosphates (NTPs) were used and performed for ATPase assay. The results appear ATP hydrolysis with ATP and dATP, and a considerable amount was observed in the presence of UTP (Figure 3D, lanes 2, 3, and 4, individually). In contrast, a negligible amount of hydrolysis of ATP was observed in the presence of dTTP, CTP, dGTP, and GTP as shown in Figure 3D (lanes 2, 5, 6 and 7). Taken together, our observation indicates that ATP and dATP potentially catalyze ATP hydrolysis, which might be further utilized in viral replication.

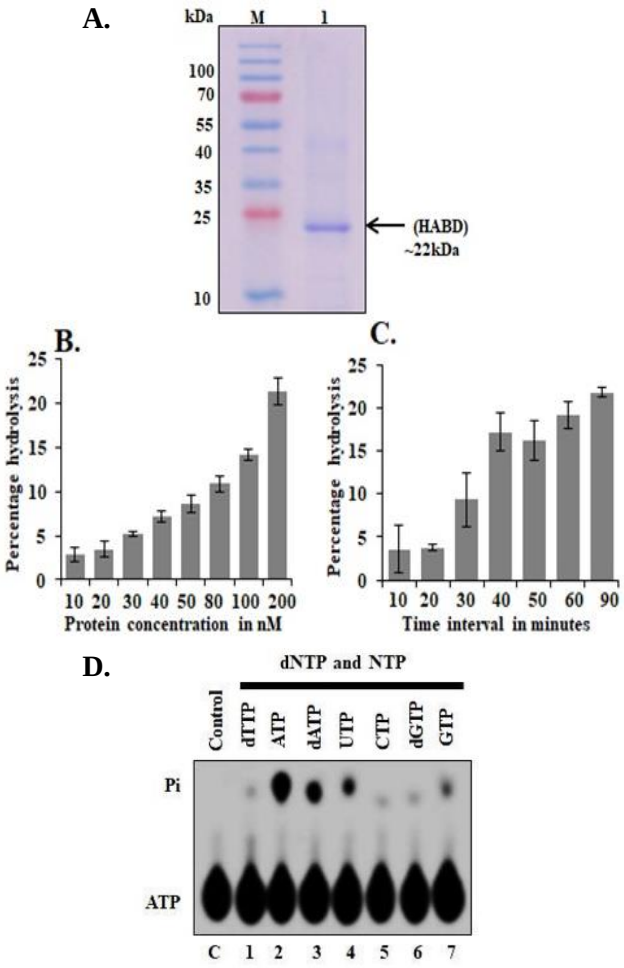

Figure 3. ATPase assay with purified protein. (A) Coomassie blue-stained gel of purified protein. Lane M is a protein marker; lanes 1 contain purified protein (100nM). (B) ATPase activity of the protein in the presence of RNA. The reactions mixture with increasing concentrations (lanes are 10, 20, 30, 40, 50, 80, 100 and 200 nM) of purified protein. The bar diagram shows the quantitative enzyme activity data as percent ATP hydrolysis. (C) Time dependence (lanes are 10, 20, 30, 40, 50, 60, and 90 min, respectively) of ATPase activity of the purified protein. The bar graph clearly shows the quantitative enzyme activity data as percent ATP hydrolysis. (D) ATP-dependent assay. ATPase activity in the presence of different nucleotides was used in Lane 1-7. C is the control reaction without HABD protein.

Enzymatic activity by using purified protein

Helicases have been shown to unwind RNA-RNA duplexes which function by utilizing energy from ATP hydrolysis, as shown in Figure 5; however, HABD role in this process is elusive. Here, in diverse groupings of HABD protein are developed, the substrate (duplex RNA-RNA) RNA helicase association was examined (41). The results demonstrate that with an increasing concentration of HABD protein (10– 100 nM), RNA unwinding activity was increased (Figure 4A). The bar diagram shows unwinding activity in a concentration-dependent manner (Figure 5A). The enzymatic activity (RNA helicase) was observed at around 80%. Similarly, the time-dependent RNA activity was also achieved, and the effects demonstrate that in presence of HABD (100 nM), RNA unwinding activity was increased at a time-dependent manner and the most significant movement was accomplished in 60 minutes (Figure 4B) as depicted by bar diagram.

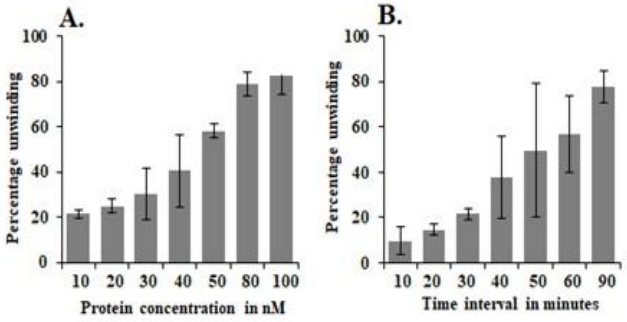

Figure 4. Unwinding activity of the purified protein (HABD). (A) Concentration dependence of RNA helicase activity of HABD. A different concentration (10–100 nM) of purified HABD was used for the assay. The activity is shown as percentage unwinding. The bar graph shows the quantitative enzyme activity data as percent unwinding. (B) Time dependence (10–90 minutes) of RNA helicase activity of purified HABD. The bar graph clearly shows the quantitative enzyme activity data as percent unwinding.

Dengue virus NS3, a multifunctional protein, contains protease, helicase, ATPase and RTPase (RNA 5’ triphosphatase) activities (6, 7, 14). A C-

terminal domain of NS3 includes three subdomains that retain RNA helicase/RTPase activities (6, 7, 14). RNA helicase activity of NS3 (NS3H) is going to the superfamily 2 of RNA helicases/NTPases containing DEx(D/H) motifs. There are conserved sequence motifs present in subdomains (I&II) in another superfamily. However, subdomain III of viral NS3 protein forms the covering of the ssRNA-binding groove. Here, we observed the presence of a-helix, b-sheets, b- turn, and coils in the helicase domain which is a conserved domain of NS3 (Figure 2B), indicating that the secondary structure of the helicase ATP binding domain displays Strand (6), Helix (11), and Coil (17) (Figure 2B).

Studies have demonstrated that NS3 helicase activity is responsible for the duplex RNA unwinding by using the energy generated from ATP hydrolysis (33). Additionally, the functional activity of NS3 helicase and NTPase has been verified in diverse Flaviviridae families (6, 7, 14), including the virus (Hepatitis C) NS3 protein (8, 9). Several RNA and DNA viruses such as HIV, HCV, CMV, adenovirus, HEV, influenza A virus, and swine fever virus have been studied to be exploited RNA helicase for unwinding DNA and RNA and its numerous biological functions including cellular (DNA&RNA) metabolic processes, RNA processing and transport (1, 42). Here, we investigated that helicase contains ATP binding domain (HABD) and demonstrated that with increasing concentration of HABD, the RNA unwinding enzymatic activity was increased. Subsequently, at a particular concentration of HABD, increased RNA unwinding was observed in a time-dependent manner which implies that RNA unwinding activity is HABD concentration and time-dependent (Figure 4).

Helicases are indispensable for the replication of several RNA viruses and to drive a duplex of RNA and DNA unwinding. Helicases commonly hydrolyze NTP, normally ATP, to provide energy (40, 43). However, our study uncovered that among other dNTPs or NTPs, ATP and dATP potentially catalyze ATP hydrolysis (Figure 3D) where this ATP hydrolysis is HABD concentration and time-dependent (Figure 3) that further enhanced helicase activity which in turn utilized for viral replication and other metabolic functions which requires more in-depth study.

Conclusions

Dengue is a viral disease (mosquito-borne) dengue fever because of a dengue virus (DENV), a single positive-stranded RNA virus that belongs to the genus Flavivirus and its own circle of relatives Flaviviridae. This virus is great at some stage in the tropics, which can be stimulated through rainfall, temperature, and humidity; however, extreme dengue has a better chance of dying while now no longer controlled in a timely. In this study, the biochemical research discovered the critical steps to reap new insights into DENV enzyme features and regulation. Overall, this investigation demonstrates the enzymatic helicase (RNA) activity of the Helicase ATP binding domain (HABD) protein, which belongs to the DEAH family. In addition, HABD protein illustrates enzymatic activity (ATPase) withinside the presence of RNA. Among other dNTP and NTP, ATP and dATP showed maximum energy, which assist in unwinding the RNA duplex. Also, this HABD protein can be beneficial for mitochondrial RNA splicing, translation, and genome maintenance.

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Interest conflict

The authors declare that they have no conflict of interest.

Author Contribution

AP design the concept and did the experimental work including manuscript writing and the final draft. BEG helps to write the first draft. MAE contributed to enzymatic assays. HA helped to design the manuscript. TMM contributed to the analysis, and editing manuscript. WHA helped to edit the manuscript. ASA wrote the first draft.

Abbreviations

DENV (Dengue virus), HABD (Helicase ATP binding domain), NS (Nonstructural), dNTPs (Deoxynucleotides) and SD (Standard deviation).

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