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Real-time monitoring of RNA helicase activity using fluorescence resonance energy transfer *in vitro*

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ABSTRACT

We have developed a continuous fluorescence assay based on fluorescence resonance energy transfer (FRET) for the monitoring of RNA helicase activity *in vitro*. The assay is tested using the hepatitis C virus (HCV) NS3 helicase as a model. We prepared a double-stranded RNA (dsRNA) substrate with a 5′ fluorophore-labeled strand hybridized to a 3′ quencher-labeled strand. When the dsRNA is unwound by helicase, the fluorescence of the fluorophore is emitted following the separation of the strands. Unlike in conventional gel-based assays, this new assay eliminates the complex and time-consuming steps, and can be used to simply measure the real-time kinetics in a single helicase reaction. Our results demonstrate that Alexa Fluor 488 and BHQ1 are an effective fluorophore–quencher pair, and this assay is suitable for the quantitative measurement of the RNA helicase activity of HCV NS3. Moreover, we found that several extracts of marine organisms exhibited different inhibitory effects on the RNA and DNA helicase activities of HCV NS3. We propose that this assay will be useful for monitoring the detailed kinetics of RNA unwinding mechanisms and screening RNA helicase inhibitors at high throughput.

Introduction

RNA helicases are ubiquitous enzymes that utilize the energy derived from nucleoside triphosphate (NTP) hydrolysis to unwind double-stranded RNA (dsRNA), and in some cases, disrupt RNA–protein complexes [1,2]. RNA helicases are found in viruses, bacteria, archaea, and eukaryotes. The functions of RNA helicases are required in a variety of key cellular processes involving RNA, such as transcription, RNA splicing, ribosome biogenesis, RNA export, RNA editing, and RNA turnover [3,4]. The dysregulations of RNA helicase expression are observed in various types of cancer [5]. Moreover, RNA helicases have recently emerged as novel targets for the treatment of viral infections such as human immunodeficiency virus (HIV) and hepatitis C virus (HCV) because the helicase activities are essential for viral replication [6,7]. Therefore, the development of an assay that can be used for both detailed kinetic unwinding mechanisms and high-throughput screening for RNA helicase inhibitors is strongly desired.

The conventional assays for the monitoring of RNA helicase activity use a radiolabeled dsRNA as a substrate [8–12]. In these assays, after incubating an RNA helicase with the radiolabeled substrate and terminating the reaction, the unwound RNA strands are separated by gel electrophoresis and visualized by autoradiography. The gel-based assays are laborious and time-consuming, and only yield an end-point for each reaction performed. In the monitoring of DNA helicase activity, continuous fluorescence assays have been developed [13–16]. Typically, these assays are based on the observation of fluorescence resonance energy transfer (FRET) [17,18]. In these assays, a dsDNA substrate is used with a 5′ fluorophore-labeled strand hybridized to a 3′ quencher-labeled strand. The fluorescence of the fluorophore is quenched by FRET and is recovered following the separation of the dsDNA substrate because of the DNA helicase. The fluorescence assays do not require complex and time-consuming steps, and can be used to simply measure the real-time kinetics in a single helicase reaction. However, to our knowledge, no fluorescence assays have been applied to the monitoring of RNA helicase activity using a dsRNA substrate.

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Here, we have developed a continuous fluorescence assay based on FRET for the monitoring of RNA helicase activity, and demonstrated its feasibility using HCV NS3 helicase as a model. HCV is a single-stranded (+) RNA virus belonging to the family *Flaviviridae* [19]. The HCV NS3 helicase belongs to the DExH/D protein family and can unwind dsRNA, dsDNA, and RNA–DNA heteroduplexes in a 3′–5′ direction on a 3′ overhang region, using any NTPs or dNTPs as the energy sources [10,20,21]. We prepared a dsRNA substrate with a 5′ fluorophore-labeled strand hybridized to a 3′ quencher-labeled strand and optimized the fluorophore–quencher pair for the assay using three different fluorophores. Moreover, we applied this assay to the screening for HCV NS3 helicase inhibitors from extracts of marine organisms, and found that several extracts of marine organisms exhibited different inhibitory effects on RNA and DNA helicase activities.

Materials and methods

Purification of recombinant HCV NS3 helicase. The recombinant HCV NS3 helicase was expressed and purified following the protocol established in our previous report [16]. The expression plasmid (pT7/His-NS3) containing an N-terminal His-tagged full-length HCV NS3 gene derived from the HCV 1b genotype was transformed in *Escherichia coli* BL21 (DE3) Codon Plus RIL. Protein concentrations were determined by absorbance at 280 nm using an ND-1000 UV/Vis spectrometer (NanoDrop Technologies).

Oligonucleotides. Alexa Fluor 488 (maximum excitation/emission = 495/519 nm)-, 6-FAM (max ex/em = 494/516 nm)-, and BHQ1-labeled RNA strands were purchased from Japan Bio Services Co., Ltd. BODIPY FL (max ex/em = 505/513 nm)-labeled RNA and DNA strands were purchased from J-Bio 21 Corporation. Unlabeled DNA strands were purchased from Hokkaido System Science Co., Ltd.

Preparation of extracts from marine organisms. Specimens of marine invertebrates, mainly sponges, were collected by one of the authors (JT) either in Kendari, South-East Sulawesi, Indonesia in 2000 or in Yaeyama area, Okinawa, Japan in 2003. The Indonesian materials were obtained under collaboration with Dr. Rachmaniar Rachmat, LIPI-Oceanology, Indonesia and with Professor Motomasa Kobayashi, Osaka University and were kept in ethanol after collection. The Okinawan materials were kept frozen until extraction. The specimens were extracted thrice either with ethanol or acetone, and the EtOAc soluble portions were obtained after concentration and partition. The representative extracts with codes 1–13 were from Indonesia, while those with codes14–20 were from Okinawa.

Fluorescence quenching efficiency. A dsRNA was prepared with the three fluorescent strands (5′-UAGUACCGCCACCCUCAGAAC-CUUUUUUUUUUUUUU-3′ with Alexa Fluor 488, BODIPY FL, or 6-FAM coupled to the 5′-end) and the quencher strand (5′-GGUUCUGAGGGUGGCCCUACUA-3′ with BHQ1 coupled to the 3′-end) at a 1:2 M ratio. dsRNAs were formed in 25 mM MOPS–NaOH (pH 6.5) by heating at 100 °C for 5 min, incubated at 65 °C for 30 min, and then incubated at 25 °C overnight. Then, the fluorescence intensities were measured using a LightCycler 1.5 (Roche). The reaction mixture contained 200 nM dsRNA, 25 mM MOPS–NaOH (pH 6.5), 3 mM $MgCl_2$, 2 mM DL-dithiothreitol (DTT), 4 U RNasin (Promega), and 5 mM ATP in 20 μL of reaction volume. The fluorescence intensity of each aliquot was measured at 37 and 95 °C. The fluorescence intensity at 37 °C was divided by that at 95 °C to normalize well-to-well variations in fluorescence measurement.

Fluorescence helicase assay using dsRNA substrates. A typical fluorescence helicase assay using dsRNA substrates was performed in 25 mM MOPS–NaOH (pH 6.5), 3 mM $MgCl_2$, 2 mM DTT, 4 U RNasin (Promega), 50 nM dsRNA substrate, 5 mM ATP, and 100 nM DNA capture strand (5′-TAGTACCGCCACCCTCAGAACC-3′) in 20 μL of reaction volume. The unwinding reaction was started by adding HCV NS3 helicase (40–200 nM) and was carried out at 37 °C for 30–65 min using a LightCycler 1.5 (Roche). The fluorescence intensity was recorded every 5 s from 0 to 5 min, and then every 30 s from 5 to 65 min. The kinetics data of the fluorescence signal were fitted to Eq. (1):

$$F(t) = C + A[1 - \exp(-k_{obs}t)], \quad (1)$$

where $F(t)$ is the fluorescence intensity at time t; C is the initial fluorescence intensity; A is the amplitude; and k_{obs} is the observed rate constant. Then, the helicase activity was calculated as the initial reaction velocity by multiplying A with k_{obs} using Eq. (2):

$$F(0) = Ak_{obs}. \quad (2)$$

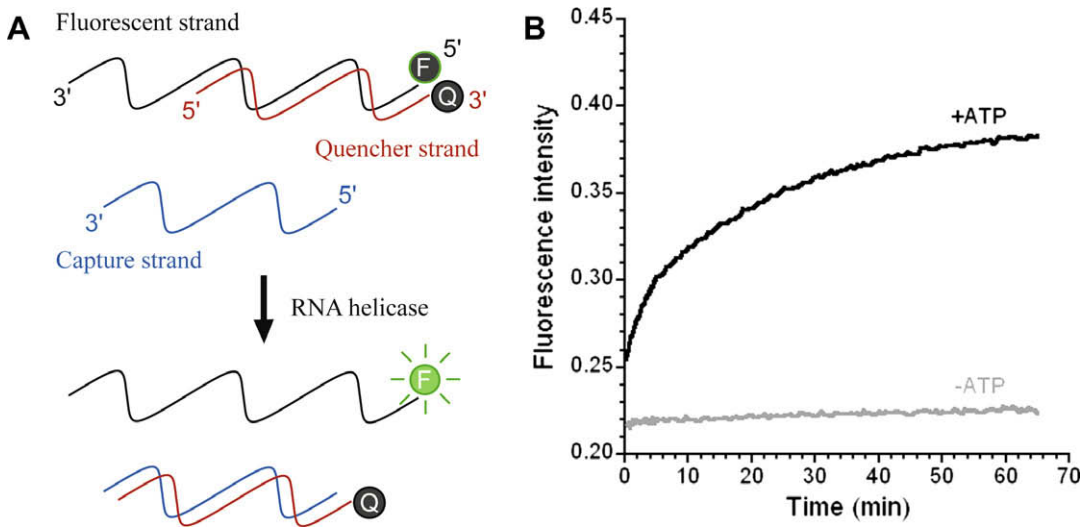

Fig. 1. (A) Schematic representation of fluorescence helicase assay based on fluorescence resonance energy transfer (FRET). The fluorophore (F) is covalently attached to the 5′-end in the fluorescent strand and quenched by the quencher (Q) that is covalently attached to the 3′-end in the quencher strand via FRET. When the double-stranded RNA (dsRNA) substrate is unwound by the helicase, the fluorophore (F) emits bright light upon its release from the quencher (Q). The capture strand, which is complementary to the quencher strand, prevents the reannealing of the unwound duplex. (B) ATP dependence of NS3 helicase activity evaluated by fluorescence helicase assay. The NS3 helicase (120 nM) was incubated with the dsRNA substrate (50 nM) in the presence (black line) or absence (gray line) of 5 mM ATP.

Table 1
Fluorescence quenching efficiencies of different fluorophores by BHQ1 in dsRNA substrates.

Fluorophore	F_0	F	Fluorescence quenching efficiency (%)	S/B
Alexa Fluor 488	1.38 ± 0.01	0.0732 ± 0.0002	94.7 ± 0.0	18.9 ± 0.1
BODIPY FL	1.38 ± 0.00	0.0759 ± 0.0006	94.5 ± 0.1	18.1 ± 0.2
6-FAM	1.52 ± 0.05	0.108 ± 0.001	92.9 ± 0.3	14.0 ± 0.6

Fluorescence quenching efficiency was calculated using the equation $(F_0 - F)/F_0 \times 100$. F, fluorescence intensity (arbitrary unit); F_0, fluorescence intensity in the absence of quencher strand; S/B, signal-to-background ratio. Results represent the average ± SD (standard deviation) of triplicate samples.

Fluorescence helicase assay using dsDNA substrates. A typical fluorescence helicase assay using dsDNA substrates was performed as described in Ref. [16] with slight modification. The dsDNA substrate was prepared with the fluorescent strand (5′-CTACTACCCCCACCCTCACAACCTTTTTTTTTTTTTT-3′ with BODIPY FL coupled to the 5′-end) and the complementary strand (5′-GGTTGTGAGGGTGGGGGTAGTAGGG-3′) at a 1:3 M ratio. dsDNA was formed in 20 mM Tris–HCl (pH 7.5) by brief heating at 90 °C, followed by slow cooling to room temperature. The fluorescence helicase assay was performed in 30 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 0.075% Triton X-100, 10 nM dsDNA substrate, 1 mM ATP, and 125 nM capture strand (5′-CTACTACCCCCACCCTCACAACC-3′) in 20 μL of reaction volume. The unwinding reaction was started by adding HCV NS3 helicase (240 nM) and was carried out at 37 °C for 25 min using a LightCycler 480 (Roche). The fluorescence intensity was recorded every 5 s. The helicase activity was calculated as the initial reaction velocity from the linear part of the progress curve using the linear regression method. A linear equation $Y = A + BX$ was fitted to the experimental data, where Y is the helicase activity expressed in fluorescence units, X is the reaction time, and B is the slope or the initial velocity.

Results and discussion

Optimization of the fluorophore–quencher pair

A scheme of the proposed assay is shown in Fig. 1A. The dsRNA substrate with a 3′ single-strand overhang was prepared by

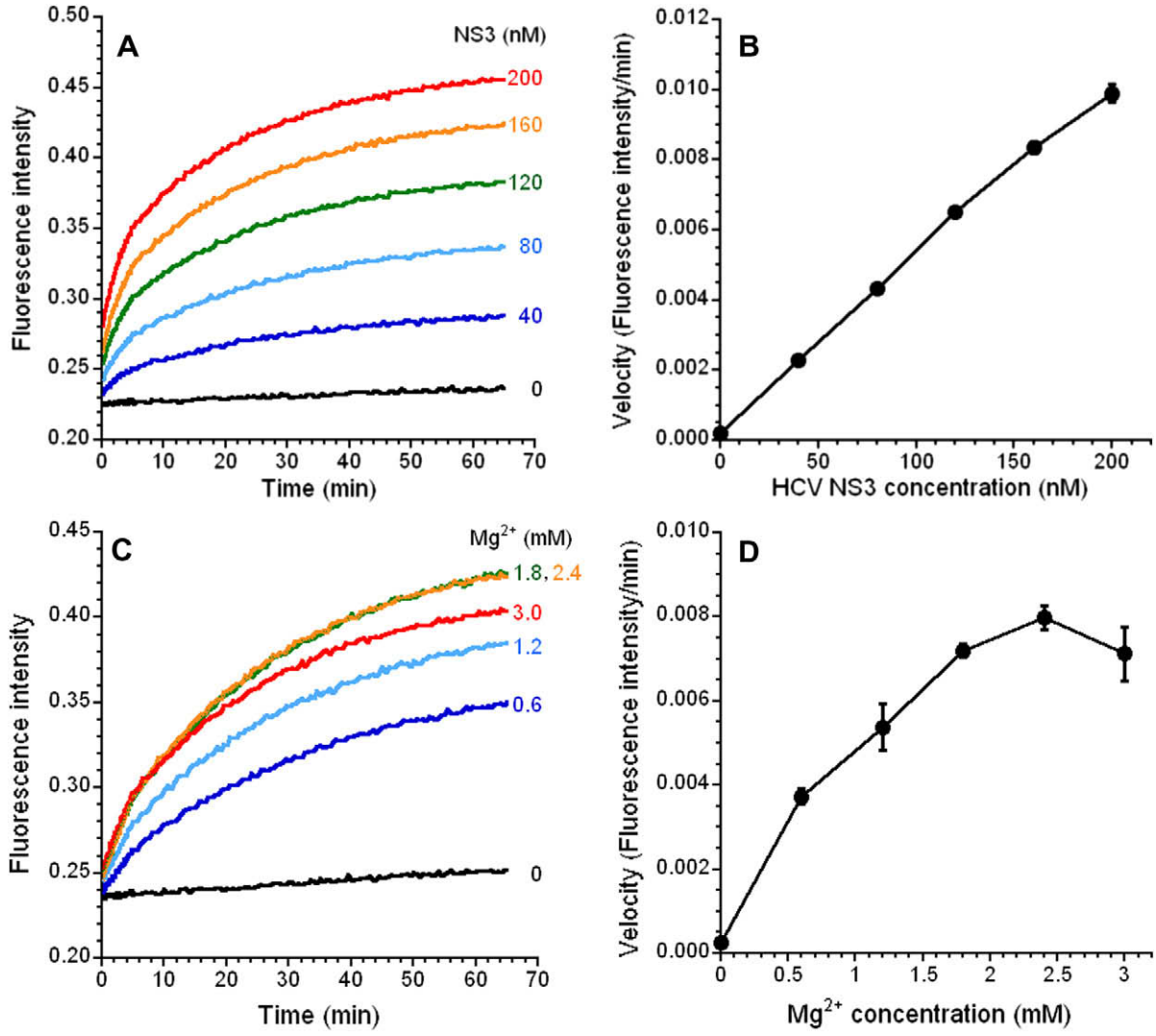

Fig. 2. Effect of NS3 helicase concentration (A,B) and Mg^{2+} (C,D) on kinetics and initial velocity of fluorescence emission evaluated by fluorescence helicase assay. (A) Kinetic analysis. RNA helicase reaction mixtures contained 5 mM ATP and the indicated concentrations of NS3 helicase. These graphs show representative traces for all the mixtures in duplicate determinations. (B) Initial velocities of fluorescence emission. The data are presented as the mean initial velocity ± standard deviation (SD). (C) Kinetic analysis. RNA helicase reaction mixtures contained 5 mM ATP, 120 nM NS3 helicase, and the indicated concentrations of Mg^{2+}. These graphs show representative traces for all the mixtures in triplicate determinations. (D) Initial velocities of fluorescence emission. The data are presented as the mean initial velocity ± SD.

annealing two oligonucleotides, a 5′ fluorophore-labeled 36-nucleotide donor and a 3′ quencher-labeled 22-nucleotide quencher. The fluorescence of the fluorophore is quenched by FRET or contact-mediating quenching between the fluorophore and the quencher, and is recovered following the separation of the dsRNA substrate from the RNA helicase. Thus, a high signal-to-background ratio (S/B), namely, a high fluorescence quenching efficiency, is critical for the sensitive monitoring of helicase activity. To optimize the fluorophore–quencher pair of the dsRNA substrate, we measured the fluorescence quenching efficiencies of three different fluorophores, Alexa Fluor 488, BODIPY FL, and 6-FAM, and one nonfluorescent quencher, BHQ1. BHQ1 has maximal absorption at 534 nm, and it is considered as a 'true dark quencher' with a much higher S/B than other quenchers such as DABCYL and TAMRA [18]. Thus, we chose BHQ1 as the quencher. As shown in Table 1, the fluorescence quenching efficiencies of Alexa Fluor 488 and BODIPY FL were relatively similar, whereas that of 6-FAM was significantly lower. Similar tendencies in S/B values were found. The highest fluorescence quenching efficiency and S/B were observed when Alexa Fluor 488 was used; therefore, we chose Alexa Fluor 488 as the fluorophore for further experiments.

Fluorescence helicase assay

The fluorescence helicase assay was then used for measuring the RNA helicase activity of the recombinant HCV NS3 protein. When the 120 nM HCV NS3 helicase was added, fluorescence was emitted in the presence of 5 mM ATP, whereas no increase in fluorescence intensity occurred in the absence of ATP (Fig. 1B). These results indicate that the observed fluorescence emission arose from the unwinding of dsRNA by the NS3 helicase because ATP hydrolysis is required for dsRNA unwinding by NS3 helicase [10,20,21]. The results also indicate that no contaminating proteins that can disrupt the assay, such as nucleases, were detected in the NS3 helicase. In addition to the DNA capture strand, we also tested the RNA capture strand for the assay. The observed fluorescence emissions with the RNA capture strand were slightly higher than those with the DNA capture strand (data not shown); however, a DNA strand is more cost-effective and easier to handle than an RNA strand. We considered that the DNA capture strand is suitable for high-throughput screening.

Next, we evaluated the effect of NS3 helicase concentration on the performance of the fluorescence helicase assay. Serial dilutions of the NS3 helicase, ranging from 40 to 200 nM, were assayed with 5 mM ATP, and the initial velocity of fluorescence emission in each test was measured. Fig. 2A shows that both the fluorescence intensity and initial velocity of the fluorescence emission increased with NS3 helicase concentration. In the absence of the NS3 helicase, no increase in the fluorescence intensity was observed. An almost linear dependence of the initial velocity of the fluorescence emission on NS3 helicase concentration was observed up to 200 nM (Fig. 2B). These results further supported that the assay can be used to quantitatively measure RNA unwinding activity caused by NS3 helicase.

Then, the effect of Mg^{2+} concentration on the performance of the fluorescence helicase assay was studied. Various concentrations of Mg^{2+}, ranging from 0.6 to 3.0 mM, were examined with 5 mM ATP and 120 nM NS3 helicase, and the initial velocity of the fluorescence emission in each test was measured. As shown in Fig. 2C and D, the initial velocity of the fluorescence emission reached a maximum at 2.4 mM Mg^{2+} and slightly decreased at 3 mM Mg^{2+}. In the absence of Mg^{2+}, no increase in the fluorescence intensity was observed. These results are consistent with those of previous studies using conventional gel-based assay [20,22].

Inhibition of NS3 helicase activity

To test the feasibility of the new assay to screen potential NS3 helicase inhibitors, we incubated the 120 nM NS3 helicase with various concentrations of ATP-γ-S, which is known as a helicase inhibitor [16,23], ranging from 2.5 to 12.5 mM. As shown in Fig. 3, the increase in the concentrations of ATP-γ-S significantly decreased the helicase activity. At about 5.4 mM ATP-γ-S, approximately 50% of the original helicase activity was inhibited. This result is expected because ATP-γ-S is a competitive inhibitor of NS3 ATPase and 5 mM ATP was used in this experiment.

Then, we screened for possible inhibitory substances from extracts of marine organisms using the new assay, employing the dsRNA substrates. The fluorescence helicase assay using dsDNA substrates [16] was also applied to the same samples, and the results were compared. The results of an initial screening of 20 selected representative samples are shown in Fig. 4. Both the helicase activities with the dsRNA and dsDNA substrates were

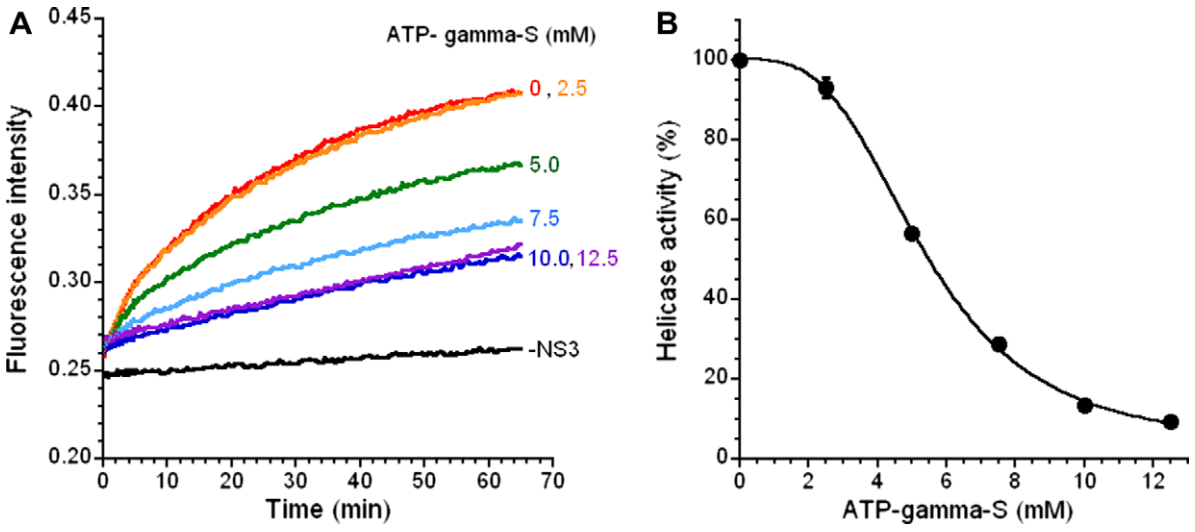

Fig. 3. Inhibition of NS3 helicase activity by ATP-γ-S. (A) Kinetic analysis. RNA helicase reaction mixtures contained 5 mM ATP, 120 nM NS3 helicase, and the indicated concentrations of ATP-γ-S. These graphs show representative traces for all the mixtures in triplicate determinations. (B) Helicase activity was calculated from control reactions without ATP-γ-S. The data are presented as the mean helicase activity ± SD.

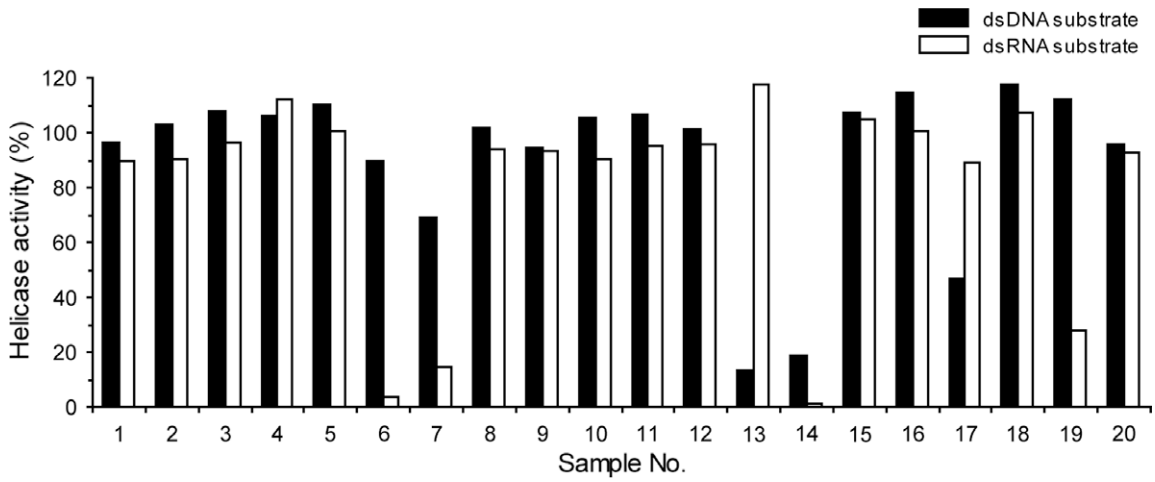

Fig. 4. Screening for inhibition of NS3 helicase activity by extracts of marine organisms. With the dsRNA substrates, the reaction was performed in 20 μL in individual wells containing 120 nM NS3 helicase and 2 μL of the extracts. With the dsDNA substrates, the reaction was performed in 20 μL in individual wells containing 240 nM NS3 helicase and 4 μL of the extracts. Fluorescence emission rate (helicase activity) was calculated relative to the control value determined without inhibitors.

inhibited in 1 out of the 20 samples (sample no. 14 in Fig. 4). Unexpectedly, the helicase activities with the dsRNA substrates were more inhibited than those with the dsDNA substrates in 3 out of the 20 samples (nos. 6, 7, and 19 in Fig. 4). By contrast, the helicase activities with the dsDNA substrates were more inhibited than those with the dsRNA substrates in 2 out of the 20 samples (nos. 13 and 17 in Fig. 4). These results indicate that several extracts of marine organisms exhibited different inhibitory effects on the RNA and DNA helicase activities of HCV NS3. Recently, to obtain candidate anti-HCV drugs, dsDNA substrates have been used for the high-throughput screening of HCV NS3 helicase inhibitors because the HCV NS3 helicase can unwind not only dsRNA but also dsDNA [16,24,25]. Strictly speaking, these previous strategies can be used to measure directly the DNA helicase activity, but not the RNA helicase activity. Thus, potential inhibitory substances for RNA helicase activity, but not for DNA helicase activity, may be missed out in such screenings.

Conclusion

Here, we describe a new fluorescence assay for the measurement of RNA helicase activity using dsRNA substrates *in vitro*. Alexa Fluor 488 and BHQ1 were selected as the fluorophore–quencher pair. The new assay developed in this study enables the real-time and high-throughput measurement of RNA helicase activity and does not require time-consuming procedures as in the case of conventional gel-based assays. Our results demonstrate that this new assay is suitable for the quantitative measurement of the RNA helicase activity of HCV NS3. We believe that the present assay is valuable for monitoring the detailed unwinding kinetics of RNA helicases and screening RNA helicase inhibitors at high throughput.

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