Organic experiment Unit.4

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1 Purpose and background

Hairpin rebozyme is one of the RNA enzymes that cleaves substrate RNA. To demonstrate the activity, first, we prepared template DNAs for hairpin ribozyme and substrate RNA. Then, hairpin ribozyme and substrate RNA were synthesized in vitro by transcription of template DNA. The cleavage activity was demonstrated using the RNAs. Reaction rate and the requirement for Mg^{2+} were also examined in this experiment.

2 Synthesis of DNA templates for hairpin ribozyme and substrate RNA

2.1 Experimental

1. Two reaction solutions were prepared in thermal-cycler tubes (table.1), and mixed by pipetting gently.
2. The solutions were heated in the thermal-cycler: 95°C, 60 sec, then (95°C, 40 sec, 51°C, 40 sec, 72°C, 40 sec), 3 cycle.
3. 3 μL of each reaction solution and 3 μL of 2x DNA loading buffer were mixed and loaded in the separate wells of a 3% agarose gel.
4. Electrophoresis was performed at 100 V for 10 min.
5. After electrophoresis, we observed the DNA products using a UV transilluminator.
6. The rest of the reaction solutions were transferred into new 600 μL tubes, and 120 μL of PCI solution was added.
7. The solutions were centrifuged at 15000×g for 5 min at 25°C, after being mixed by pipetting and tapping.
8. The upper aqueous phase was transferred into new 600 μL tubes, and 120 μL of chloroform/isoamyl alcohol(24:1) solution was added.
9. The solutions were centrifuged at 15000×g for 5 min at 25°C, after being mixed by pipetting and tapping.
10. The upper aqueous phase was transferred into new 600 μL tubes, and 12 μL of NaCl solution (3 M) and 300 μL of ethanol were added.
11. The solutions were centrifuged at 15000×g for 10 min at 25°C.
12. The supernatant was removed immediately, and 500 μL of 70% ethanol was added.
13. The solutions were centrifuged at 15000×g for 2 min at 25°C.
14. The supernatant was removed immediately, and the DNA pellet was dried.
15. DNA pellet was dissolved into 30 μL of ultrapure water after it completelly dried.

表 1: Reaction solutions for template DNA synthesis. ”H” and ”S” indicate ”Hairpin ribozyme RNA” and ”Substrate RNA”, respectively.

Reagent	concentration	H / μL	S / μL	final conc.
Ultrapure water	-	54	54	-
Taq reaction buffer	10x	12	12	1x
$MgCl_2$ aq.	25 mM	12	12	2.5 mM
dNTP solution	5 mM each	6	6	0.25 mM
forward DNA primer for H	10 μM	12	0	1 μM
reverse DNA primer for H	10 μM	12	0	1 μM
forward DNA primer for S	10 μM	0	12	1 μM
reverse DNA primer for S	10 μM	0	12	1 μM
Taq DNA polymerase	-	12	12	-
total		120	120	

2.2 Results and Discussion

Template DNAs were synthesized and purified in this experiment. After the dsDNAs were synthesized by Taq polymerase, proteins (the Taq polymerase) were removed by washing with PCI and a chloroform/isoamyl alcohol (24:1) solution. Proteins denature upon exposure to phenol, becoming insoluble in both the aqueous and phenolic phases. Only DNA is soluble in the water layer, and the phenol remains in the water layer is removed by extracting with chloroform/isoamyl alcohol(24:1) solution[*1]. Then, DNAs were salted out using concentrated NaCl and ethanol. DNA has a positive charge, so DNA can be salted out by adding concentrated salt such as NaCl. The white pellet was precipitated after ethanol was added. It should be pure DNA.

3 Transcription of hairpin ribozyme and substrate RNA

3.1 Experimental

1. Two reaction solutions were prepared in thermal-cycler tubes (table.2), and mixed by pipetting gently.
2. The reaction solutions were incubated at 37°C overnight.
3. 12 μL of EDTA solution (500 mM) was added into the reaction solution and well mixed to remove the turbidity.
4. 15 μL of NaCl solution (3 M) and 150 μL of 2-propanol were added into the reaction solution and mixed by pipetting.

[*1] Question 1

5. The solutions were centrifuged at 15000×g for 5 min at 25°C.
6. The supernatant was removed immediately, and the DNA pellet was dried.
7. The pellet was dissolved in 5 μL of ultrapure water.
8. 5 μL of 2x RNA loading buffer was added and mixed.
9. The solution for the acrylamide gel was prepared in a 15-mL tube (table.3).
10. 75 μL of 10% APS and 7.5 μL of TEMED were added into the gel solution and mixed.
11. The gel solution was poured into the gel plate immediately.
12. 10-well comb was put into the gel plate and left until it completely solidified.
13. The reaction solution was loaded into the acrylamide gel.
14. Urea in the well was removed by pipetting.
15. Electrophoresis was performed at 250 V for 35 min.
16. We observed the gel plate by UV after electrophoresis and excised the RNA region.
17. The piece of the gel was transferred into a new 600-μL tube, and ground into a fine paste.
18. 500 μL of NaCl (500 mM) / EDTA (1 mM) was added into the reaction solution and mixed, then it was shaken for 30 min using a rotisserie.
19. The gel/solution mixture was transferred to the upper unit of a filter tube and centrifuged at 8000×g for 2 min at 4°C.
20. The solution in the bottom unit was transferred to a new 1.5-mL tube, 1 mL of ethanol was added, and mixed by pipetting.
21. The solutions were centrifuged at 15000×g for 2 min at 4°C.
22. The supernatant was removed immediately, and 1 mL of 70% ethanol was added.
23. The solutions were centrifuged at 15000×g for 2 min at 4°C.
24. The supernatant was removed immediately, and the DNA pellet was dried.
25. The pellet was dissolved in 10 μL of ultrapure wate after it completelly dried.
26. The UV absorption of the two samples was measured.
27. The two solutions were diluted by ultrapure water into 10 μM (Hairpin) and 50 μL (Substrate) solutions.

3.2 Resalts and Discussion

After the transcription reaction was performed overnight, the reaction solution was turbid. As the turbidity was removed by adding EDTA solution, the turbidity was thought to be caused by Mg^{2+} ion. This suggests that the turbidity was an aggregation of RNA and proteins.[*2]

For the electrophoresis gels of RNA, we used urea. Urea is needed because it weakens the hydrogen bond within RNA and keeps the RNA straight chain formation. Urea weakens the hydrogen bond within RNA because it is highly polarized and can form hydrogen bonds with RNA. RNA favors complex secondary structure, but in a complex structure, it is difficult to separate them by length in electrophoresis[*3].

[*2] Question 2
[*3] Question 3

表 2: Reaction solutions for RNA synthesis. ”H” and ”S” indicate ”Hairpin ribozyme RNA” and ”Substrate RNA”, respectively.

Reagent	concentration	H / μL	S / μL	final conc.
Ultrapure water	-	31.5	31.5	-
T7 buffer	-	19.5	19.5	-
DTT	100 mM	15	15	-
$MgCl_2$ aq.	250 mM	18	18	30 mM
NTP solution	25 mM each	30	30	5 mM
DNA template for H	-	30	0	-
DNA template for S	-	0	30	-
Taq DNA polymerase	-	6	6	-
total		150	150	

表 3: Reaction solutions for RNA synthesis. ”H” and ”S” indicate ”Hairpin ribozyme RNA” and ”Substrate RNA”, respectively.

Reagent	concentration	volume	final conc.
acrylamide/bisacrylamide (19:1)	40%	3 mL	15%
TBE buffer	5x	1.6 mL	1x
Urea	-	3.36 g	-
Ultrapure water	-	up to 8 mL	-
total	-	8 mL	-

In the preparation of PAGE gel, APS and TEMED work as radical initiators. First, the O−O bond in APS cleaves (shown in fig.3), and TEMED becomes a stable radical. The TEMED radical attacks the acrylamide and works as a radical initiator[*4].

The result of the UV absorption measurement was shown in fig.2 and table.4. The concentration of each sample was calculated by assuming that the absorbance at 260 nm (A260) is proportional to the concentration of the RNA sample, and A260 = 1 is equivalent to 40 ng/μL. Also, the absorbance at 280 nm was recorded as A280, and A260/A280 was calculated as an index of the purity of the RNA samples. According to [1], pure RNA typically has an A260/A280 of 2.0, and a small change (0.2-0.3) occurs by pH change. The A260/A280 values of our samples indicated that they are pure RNA samples.

[*4] Question 4

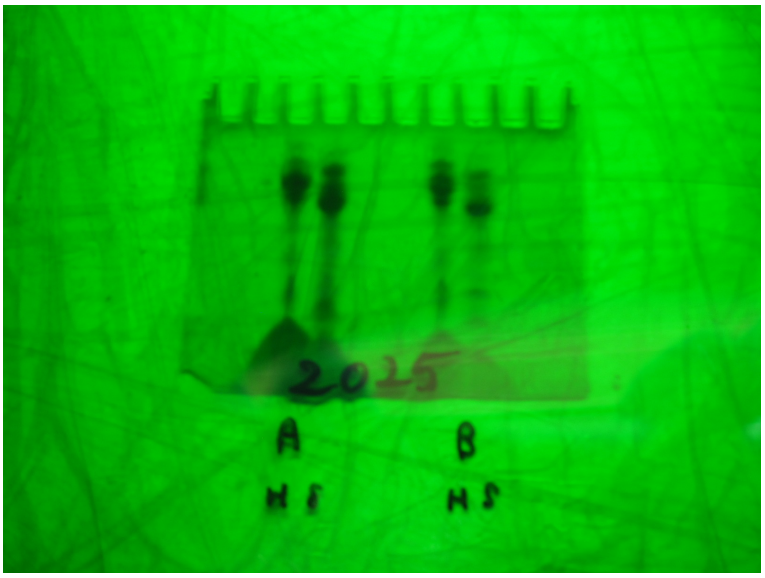
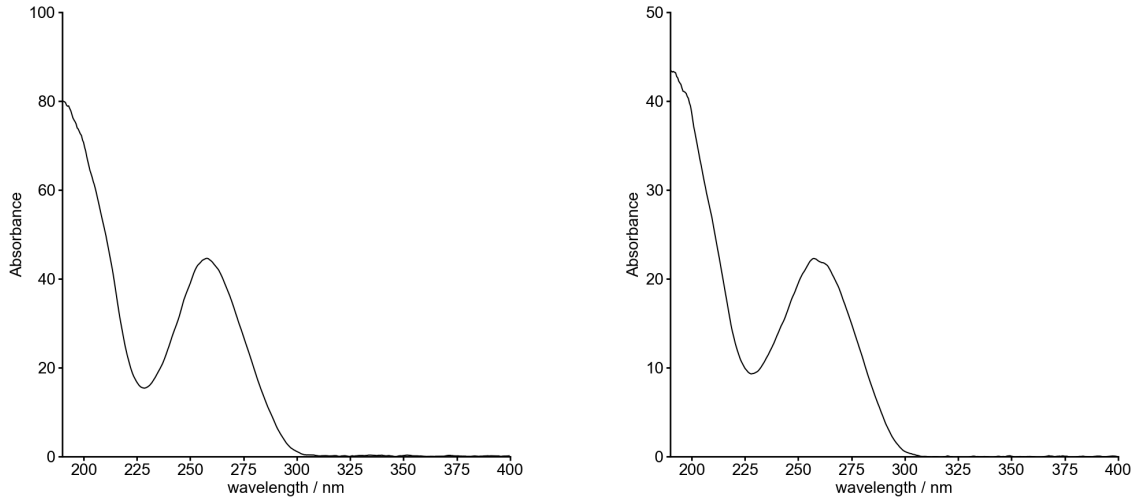


図 1: Agalose gel after the electrophoresis in sec 3.2. The right side (B), our sample Hairpin / Substrate were shown.

表 4: UV absorbance values of refined RNA samples and calculated concentration of each sample

	A260	A280	A260/A280	Mw / kDa	conc. / μM
Hairpin	43.947	19.632	2.24	17.24	102
Substrate	21.925	11.259	1.95	11.85	74.0



Hairpin RNA Substrate RNA

図 2: UV-Vis spectrum of hairpin RNA and substrate RNA

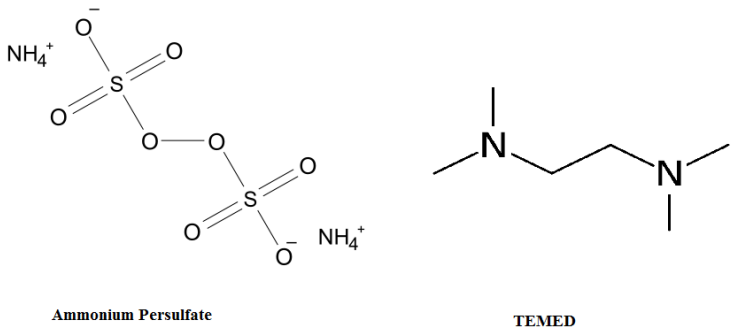


図 3: The structures of APS and TEMED

4 Cleavage of substrate RNA by hairpin robozyme

4.1 Experimental

1. Reaction solutions (A-F) in a 600-mL tube (table.5).
2. The reaction solutions were incubated at 95°C for 2 min.
3. $MgCl_2$ solution (50 mM) or ultrapure water was added to the reaction solutions.
4. The reaction solutions were incubated at 37°C for several minutes (written in table.5).
5. 10 μL of RNA 2x loading buffer was added after the incubation.
6. An acrylamide gel was prepared in the same manner as 4-3.
7. 10 μL of each sample was loaded into the well of the gel plate.
8. Electrophoresis was performed at 250 V for 40 min.
9. The gel was strained by incubating in ethidium bromide solution (0.5 μg/mL) for 10 min.
10. We observed the gel plate under UV.

表 5: Reaction solutions for cleavage of substrate RNA

Reagent	concentration	A / μL	B / μL	C / μL	D / μL	E / μL	F / μL	final conc.
Ultrapure water	-	2	2	2	2	2	2	-
Tris-HCl (pH 7.5)	100 mM	4	4	4	4	4	4	40 mM
hairpin ribozyme	10 μM	2	0	2	2	2	2	2 μM
substrate RNA	50 μM	0	2	2	2	2	2	10 μM
$MgCl_2$ aq.	50 mM	2	2	2	2	2	0	10 mM
ultrapure water	-	0	0	0	0	0	2	-
total	-	10	10	10	10	10	10	-
incubated time	-	30 min	30 min	0 min	5 min	30 min	30 min	-

4.2 Resalts and Discussion

By mistake, we put the reaction tube in the wrong place in the heat block, and the first 5 minutes of incubation were insufficient. So, solution D is not incubated sufficiently. We prepared another solution D' and incubated it for 5 minutes. Other samples were incubated for 30 minutes after we noticed the problem and moved them to the right heat block.

The result of electrophoresis after incubation is shown in fig.4. 4 major bands were observed. Band 1 and 2 is attributed to the Hairpin RNA and substrate RNA, respectively. And bands 3 and 4 show the existence of shorter 2 RNAs than the substrate RNA, which are attributed to the cleaved RNAs. In addition to that, when bands 3 and 4 appear, band 2 is thinner, suggesting that bands 3 and 4 are the cleaved RNA of the substrate RNA. In this experiment, samples D, D', and E showed the cleaved products. This result shows that the cleavage reaction of substrate RNA occurs only in the presence of the Hairpin RNA and Mg^{2+}.

In addition to that, only incubation for 5 min (even the heating is insufficient), the cleavage reaction occurs. On the other hand, even after a 30-minute reaction, in sample E, band 2 remains, suggesting the cleavage reaction is not finished.

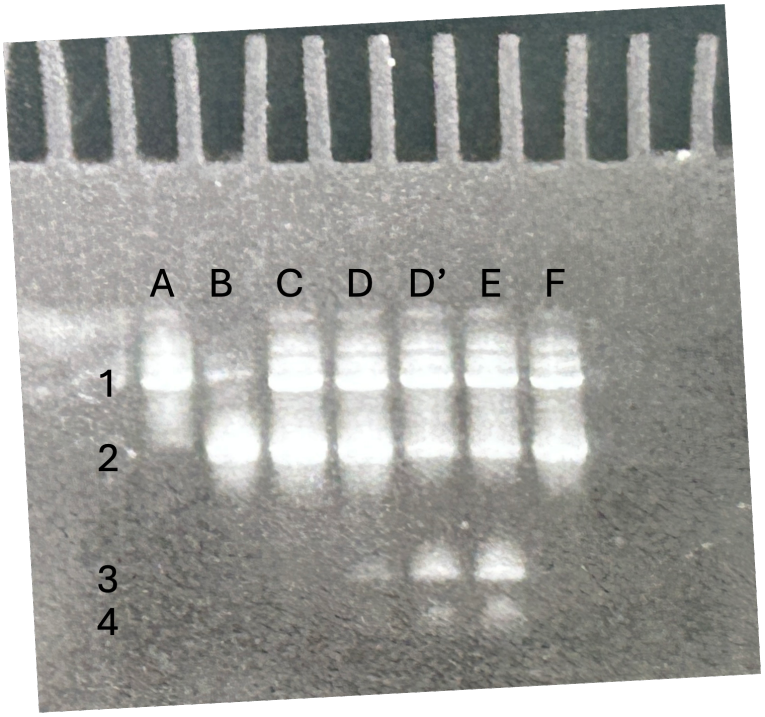


図 4: PAGE gel picture after incubation

The reaction mechanism proposed for the cleavage reaction is shown in fig.5 [3]. According to [3], Mg^{2+} itself does not involve the hydrolysis, only stabilizes the transition formation, and this is unique to hairpin rebozyme. And the cleavage reaction can be considered an acid/base reaction. Ribozyme

is considered to work as an acid and base in hydrolysis. Generating the favorable confirmation for the reaction by hybrization is also considered to be important for the activation of the reaction[*5].

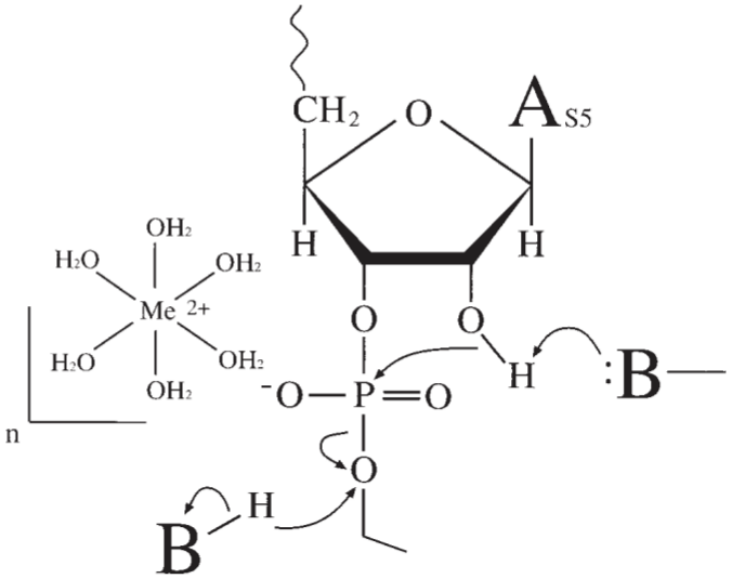


図 5: proposed reaction mechanism of cleaved reaction.

Reference

[1] Thermo Fisher Scientific - NanoDrop products, https://dna.uga.edu/wp-content/uploads/sites/51/2019/02/Note-on-the-260_280-and-260_230-Ratios.pdf

[2] What are the uses of APS and TEMED in SDS PAGE protocol? - InfoBiochem https://www.infobiochem.com/uses-of-aps-and-temed-in-sds-page-protocol/

[3] Shippy, Richard and Lockner, Randy and Farnsworth, Margaret and Hampel, Arnold, The hairpin ribozyme, Molecular Biotechnology, 1999(12). 1. 117-129.

[*5] Question 5