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論文概要
(Summary of the Thesis/Dissertation)

Doctoral Program in Life Science Innovation
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1. Title of the Thesis/Dissertation :
Identification and Characterization of Zn^{2+} Dependent DNAzymes

2. Summary (1,000 - 1,200 words in English)
Nucleic acid enzymes refer to a single-strand RNA, named ribozyme, and a single-stranded DNA, named DNAzyme, which exhibit a catalytic activity utilizing cofactors such as metal ions. Although ribozymes have been isolated from living beings in the early 1980s, DNAzymes have never been identified in nature. Since around 1990, some ribozymes and also DNAzymes were obtained by using molecular evolution approach with a repetitive *in vitro* selection method [SELEX (Systematic Evolution of Ligands by Exponential Enrichment) method]. Remarkably, in 1997, Joyce et al. have identified RNA-cleaving DNAzymes named 8-17 and 10-23 systematically obtained by the *in vitro* selection. Dozens of DNAzymes that have different sequences and enzymatic properties have been identified until now, and the DNAzymes are often utilized for applied research and technology for analytical tools. In 2013, Breaker et al. firstly reported an efficient DNA-cleaving DNAzyme named I-R3 which has ability to selectively and rapidly hydrolyzed DNA using a unique *in vitro* selection system with a 50-nt randomized DNA pool designed in a circle. The I-R3DNAzyme is compact in size (10 bases in the catalytic core region and 7 bases in the core substrate region) and efficiently cleaves DNA substrates in the presence of 2 mM Zn^{2+} at pH 7.0 (k_{bos}= ~1 min^{-1}).

First, I attempted to develop an in vitro selection method for obtaining nucleic acid enzymes by using the previously reported DNAzyme (I-R3) which has a 10-nt catalytic core sequence and cleaves DNA in the presence of Zn^{2+} ion. I designed an N10-DNA library having a 10-nt randomized sequence in the catalytic core region and a substrate DNA having 7-nt in a core substrate and selected sequences that possess cleavage activity by the designed *in vitro* selection method. After determining a large number of sequences from the selected DNA library by Deep sequence, I analyzed the enriched motives. As the result, I confirmed that the sequence motif of the I-R3 was enriched, demonstrating that the designed screening system can be used as the selection method for nucleic acid enzymes.

Next, I attempted to obtain novel RNA-cleaving DNAzymes different from I-R3 DNAzyme sequence using a substrate in which a part of the DNA substrate sequence was replaced with RNA. I carried out the *in vitro* selection experiment using the N10-DNA library and the reaction condition including 1 mM $ZnCl_2$ at 37°C that was used in the previous experiment. As expected, I was able to obtain an enriched motif sequence that could cleave RNA. The obtained motif sequence has a 10-nt catalytic core sequence targeting the 7-nt core substrate region. Then I tried to minimize the identified DNAzyme based on motif analysis and secondary structure prediction. The original DNAzyme was shortened stepwise by confirming the cleavage activity by an *in vitro* cleavage experiment. The final minimized DNAzyme, named minGAA, consisted of the smallest unit which is 2-nt (5′-AC-3′) of the catalytic core targeting a 3-nt (5′-rArArG-3′) of the core substrate. The optimum cleavage condition of minGAA was at pH 7.5 in the presence of Zn^{2+} at 37°C, which is the same condition used in the screening.

Despite the minimum structure, minGAA showed a cleavage rate (k_{bos}= 0.20 ± 0.01 min^{-1}) comparable to that of the reported DNAzymes and a high Zn^{2+} ion selectivity. To examine sequence specificity of the catalytic core sequence, 16 DNAzyme sequences (minGAA and 15 mutants, 5′-NN-3′) were synthesized. I compared the cleavage activity of the minGAA with that of 15 mutants that have different 2-nt catalytic core sequences. Furthermore, I performed an *in vitro* selection experiment using the N3-DNA library to investigate the optimal catalytic core sequence. As the results of both experiments, I confirm that the minGAA (5′-AC-3′) was the optimal sequence, and the motif of the catalytic core sequence was found to be 5′-AB-3′ or 5′-GR-3′. Next, to examine the sequence specificity in the core substrate, I designed three point-substituted substrates and compared the cleavage activity for the original substrate with that of them. In addition, I underwent an in vitro selection experiment using a 3-nt randomized substrate sequence to be cleaved by minGAA and investigated the sequence specificity in the core substrate of minGAA. As the result, I was able to obtain correlated results between two independent experiments and the sequence specificity in the core substrate of minGAA was predicted to be 5′-HAG-3′. Currently, I and collaborators have been undergoing on the elucidation of the cleavage mechanism of minGAA by using of NMR analysis and X-ray crystal structure analysis.

Succeeding, I attempted to obtain DNAzyme efficiently cleaving between pyrimidine bases (5′-rPy↓rPy-3′) using our screening system, because few RNA-cleaving DNAzyme efficiently hydrolyzing between pyrimidine bases have not reported yet. I designed a screening system with an N16-DNA library targeting the substrate having 5′-rCrCrA-3′ in the core substrate, which is different from the reported experimental procedures. A selection experiment was conducted with the expectation of obtaining DNAzymes with the enough cleavage activity between pyrimidine bases. After three cycles of the selection, the selected DNA library was analyzed by Deep sequencing and motif software. As a result, I found two enriched motif sequences and selected two DNAzyme sequences, named ZincDz1 and ZincDz2 as a representative sequence from each motif (motif 1 and motif 2) and characterized the catalytic properties of each DNAzyme. As pH 7.5, ZincDz1 and ZincDz2 exhibited the maximum substrate cleavage activity with 0.3 mM ~ 1 mM and 1 mM Zn^{2+}, respectively. And in the presence of 1 mM Zn^{2+}, ZincDz1 and ZincDz2 showed the maximum substrate cleavage activity at pH 7.0 ~ pH 7.5, and pH 7.5, respectively. On monovalent and divalent metal ion selectivity, both DNAzymes showed high zinc ion selectivity. I performed *in vitro* cleavage experiments and subsequent a gel electrophoresis using a marker to determine the cleavage site in the core substrate by ZincDz1 and ZincDz2. I also confirmed the detailed cleavage sites and the chemical structure at the ends of the substrates by LC-Mass analysis. As a result, I revealed that ZincDz1 and ZincDz2 cleave the substrate at 5′-rCrCrA↓-3′ and 5′-rC↓rCrA-3′ in the core substrate, respectively. I focused on ZincDz2 which could cleave between pyrimidine bases and analyzed the sequence specificity in the core substrate in more details. As the result of the cleavage analysis for all combinations of cleaved bases, it was found that ZincDz2 efficiently cleaves between any bases with substrate sequence specificity of "5′-rN↓rNrrPu-3′" in the core substrate. Furthermore, I identified a highly active mutant, ZincDz2-v2 designed based on the point mutation analysis in the catalytic core. The ZincDz2-v2 exhibited more efficiently cleave activities between all bases than ZincDz2. I would like to investigate the catalytic properties of ZincDz2-v2 in more detail, analyze the structure, and clarify the mechanism of the sequence tolerant cleavage of ZincDz2-v2 in future work.

Although applications in vitro utilizing these DNAzyme may be limited because of the zinc selectivity, it is proposed that the characteristic properties of the DNAzymes such as the minimum structure and efficiently cleaving between any bases might be useful in industrial uses. In future, I would like to obtain useful novel nucleic acid enzymes that exhibit efficiently working in biological condition or cleaving a DNA strand for applications like therapeutics and diagnostics by conducting more detailed cleavage and structural analysis.