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Selection of an RNA Molecule That Specifically Inhibits the Protease Activity of Subtilisin[1]

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RNA ligands (RNA aptamers) to a protease subtilisin were selected from pools of random RNA by SELEX (systematic evolution of ligands by exponential enrichment) and by use of a subtilisin-immobilized Sepharose column. After eight rounds of selection, RNA aptamers were isolated by cloning to a plasmid vector. We characterized one of the selected RNA molecules. This RNA aptamer displayed specific inhibition toward the subtilisin activity, even when the assay for subtilisin was performed using the chromogenic small peptide as substrate, and almost no inhibitory activity toward trypsin and chymotrypsin, although these enzymes are serine proteases similar to subtilisin. These findings indicate that this RNA can differentially recognize the surfaces of similar proteases. Kinetic analysis of the RNA aptamer revealed that the inhibition constant (K_i) toward subtilisin was 2.5 μM.

Key words: aptamer, *in vitro* selection, protease inhibitor, SELEX, subtilisin.

The term "RNA aptamer" is used to describe an RNA molecule obtained by *in vitro* selection that binds to a target ligand (*1*). Several rounds of selective amplification from a large "combinatorial library" of RNA molecules bearing random sequences yields RNA aptamers that possess the greatest affinity with the target molecule (*1*). This selection procedure is also called SELEX (systematic evolution of ligands by exponential enrichment) (*2*). When an enzyme is used as the target molecule, the selected RNA aptamers are expected to be new types of inhibitors or modifiers of its specificity. In recent years, many aptamers for different biomolecules have been reported (for review, Refs. *3* and *4*). However, this technique has yet to be applied to industrially useful enzymes as target ligands. To explore the possibility of industrial application of RNA aptamers, we decided to attempt to obtain RNA inhibitors for such industrially useful enzymes as amylases and proteases. These are usually extracellular microbial enzymes with relatively high activities, but it is not known whether RNA aptamers act as inhibitors for such highly active enzymes. Here we used a protease, subtilisin, as a target ligand. Subtilisin is a bacterial alkaline serine protease produced by *Bacillus* species and is widely used industrially (*5, 6*). An RNA inhibitor of subtilisin may be useful as a stabilizer for subtilisin solution or as a molecular switch for proteolytic reaction process, because the RNA can be easily removed from the reaction mixture by use of ribonuclease, alkaline conditions, or antisense nucleic acids. Although a protein inhibitor of subtilisin is known (*7*), it may be difficult to remove from enzyme solution without damaging the enzyme. Thus, RNA aptamers for subtilisin may provide a new tool for industrial application; and at the same time they may facilitate basic investigation into a new type of molecular interactions. Subtilisin and its protein protease inhibitor constitute a well-known model system to study protein-protein interaction and protein engineering, and much information about this enzyme is now available (*7*). Therefore, if RNA aptamers to subtilisin are obtained, they may be useful in a system to elucidate artificial RNA-protein interactions. As a first step for this, we have tried to obtain RNA aptamers to subtilisin.

Here we report that our selection method is useful to create RNA aptamers to subtilisin and that one selected RNA aptamer specifically inhibits subtilisin activity. This is the first reported RNA inhibitor of bacterial extracellular protease.

MATERIALS AND METHODS

Materials—Subtilisin BPN′ was purchased from Nagase Sangyo. Trypsin and chymotrypsin were from Wako Junyaku. The substrates for proteases were from Sigma. CNBr-activated Sepharose 4B was purchased from Pharmacia. T7 RNA polymerase and RNasin (human placenta ribonuclease inhibitor) were from Toyobo. RNase-free DNase (RQ-DNase) was purchased from Promega. The yeast RNA was from Boehringer-Mannheim. All other enzymes and chemicals were purchased from commercial

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sources. Subtilisin-immobilized Sepharose 4B was prepared by coupling of CNBr-activated Sepharose 4B with subtilisin as instructed by the supplier.

The Synthetic DNAs—The template DNA used for the synthesis of the initial random RNA population was constructed with oligonucleotides R-SETP-5 (5′-TG*TAATACGACTCACTATA*GGGCGAATTCGAGCTCGG-3′), R-SET100 (5′-AAGCTTGCATGCCTGCAG(N_{47})CCGAGCTCGAATTCGCCC*TATAGTGAGTCGTATTA*-3′) (N=G, A, T, or C), and R-SETP-3 (5′-CTCAAGCTTGCATGCCTGCAG-3′). R-SET100 contains 47 nucleotides of randomized sequence (N_{47}) flanked by fixed sequences and a sequence for a promoter of T7 RNA polymerase (shown in italics) at the 3′-terminus. R-SETP-3 contains the 5′-terminal fixed sequence present in R-SET100 with an extra sequence at the 5′-terminus. R-SETP-5 contains the sequence complementary to the 3′-terminal 35 nucleotides of R-SET100. These DNAs have *Hin*dIII and *Eco*RI sites (underlined) to facilitate subcloning after selection.

Preparation of RNA Pools—RNAs were synthesized by *in vitro* transcription using T7 RNA polymerase. The first RNA pool was prepared from the reaction mixture containing 0.14 μg of R-SET100 (approximately 10^{12} DNA molecules) as template, 40 mM Tris-HCl (pH 7.6), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 40 units of RNasin, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.5 mM UTP, 0.037 μg of R-SETP-5, and 140 units of T7 RNA polymerase in a total volume of 50 μl. The mixture was incubated for 4 h at 37°C, then 1 μl (1 unit) of RNase-free DNase was added, and the incubation was continued for 10 min at 37°C. RNA was extracted with phenol, precipitated with ethanol, and collected by centrifugation. Approximately 5 μg (0.18 nmol=10^{14} molecules) of RNA was obtained, and 0.5 μg (10^{13} molecules) of this RNA was used as the first RNA pool for SELEX. The RNA pools of the second and subsequent generations were prepared by the same transcription reaction, except that 0.1–0.5 μg of PCR amplified DNA from the RNA of the previous generation was used instead of synthetic DNAs.

In Vitro Selection—The selection of RNA was performed using a subtilisin-immobilized Sepharose 4B column (4 mm in diameter×7 mm in height). The column was equilibrated with binding buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl). RNAs (0.5 μg) were dissolved in 60 μl of the binding buffer and heated at 95°C for 2 min, chilled in ice, then incubated for 20 min at room temperature. This RNA solution was poured into the column and incubated with the resin for 5 min at room temperature. The column was then washed with more than 30 column volumes of binding buffer, and the bound RNAs were eluted with 0.5 mg/ml subtilisin in binding buffer. The eluted RNAs were reverse transcribed using primer R-SETP-3 and First-Strand cDNA synthesis Kit (Pharmacia). The cDNA molecules were then amplified by PCR, purified by phenolization, and transcribed *in vitro* using T7 RNA polymerase to synthesize the RNA pool for the next generation as described above. After the eighth selection cycle, the PCR-generated 100-bp DNA was isolated from agarose gels and digested with *Eco*RI and *Hin*dIII. The fragments were ligated to a pGEM-3Z (Promega). *Escherichia coli* JM 109 cells were transformed and plasmids from individual bacterial clones were sequenced. RNAs were also prepared from these plasmids. For this, run-off transcription was performed using a *Hin*dIII-digested plasmid as template as described (*8*).

Assay for Proteases—Subtilisin was assayed using a synthetic peptide, 3-carboxypropionyl-(succinyl)-Ala-Ala-Pro-Phe-*p*-nitroanilide, as substrate. Subtilisin was dissolved in 50 mM sodium phosphate/1 mM EDTA buffer, pH 6.0, to give concentrations of 0.3–15 μM (subtilisin solution). The synthetic peptide was dissolved in 50 mM HEPES-NaOH/$CaCl_2$, pH 7.5, to give a concentration of 0.1 mM (substrate solution). The subtilisin reaction was started by the addition of 30 μl of subtilisin solution to 280 μl of substrate solution. The mixture was incubated for 5 min at 40°C and the absorbance of 410 nm of the mixture was measured. When RNAs were included in the reaction mixture, they were added to the substrate solution to give final concentrations of 0–2.5 μM. Trypsin was assayed using a synthetic peptide, α-*N*-benzoyl-DL-Arg-*p*-nitroanilide, as substrate. The trypsin reaction was started by the addition of 20 μl of 25 μg/ml trypsin solution into 390 μl of the substrate and RNA solution containing 1 mM substrate, 0–2.5 μM RNA, 20 mM Tris-HCl, pH 8.0, and 1 mM $CaCl_2$. The reaction mixture was incubated for 30 min at 37°C. The reaction was terminated by the addition of 20 μl of 1 N HCl, and the absorbance at 410 nm of the mixture was measured. Chymotrypsin was assayed using a synthetic peptide, *N*-succinyl-Phe-*p*-nitroanilide, as substrate. The chymotrypsin reaction was started by the addition of 20 μl of 5 μg/μl trypsin solution into 390 μl of the substrate and RNA solution containing 1 mM substrate, 0–2.5 μM RNA, and 20 mM Tris-HCl, pH 7.8. The reaction mixture was incubated for 30 min at 37°C. The absorbance at 410 nm of the mixture was measured.

Kinetic Analysis—To analyze the mode of inhibition of the RNA aptamer, kinetic analyses were performed. The reaction mixture contained 1.5 μM RNA, 350 nM subtilisin, and 0.087, 0.13, 0.17, or 0.22 mM substrate. Other components in the reaction mixture and the assay methods were the same as described above.

RNA Secondary Structure—The secondary structure of RNA was computer-generated using the software GENETYX-MAC 8.0.

RESULTS

We chose an enzymatically well-defined enzyme, subtilisin BPN′, as a target ligand for *in vitro* selection. Although a basic protein, this enzyme is not known to bind nucleic acids as part of its natural function. First, a Sepharose 4B column containing immobilized subtilisin was used to obtain subtilisin-binding aptamer as described in "MATERIALS AND METHODS." The first population of RNAs contained a randomized sequence of 47 bases in the middle of the molecule that was flanked by defined sequences at both ends. This RNA preparation was prepared by *in vitro* transcription and used as the first population for SELEX. After eight rounds of *in vitro* selection, selected RNAs were reverse transcribed, and resulting cDNAs were PCR-amplified and subcloned into pGEM-3Z. Of 20 clones sequenced, only 4 clones were found to have appropriate inserts (79-bp from *Eco*RI to *Hin*dIII sites including recognition sequences for these restriction enzymes). Cloning of selected RNAs from the eighth generation was very inefficient, although the cloning from the third genera-

Sequence		20	40	60	80
1	GGGCGAAUUCGAGCUCGG	GCCACUCGCUCAACACGGUAAGUAGAGACCUAGUGGUACAUAAAGGA			CUGCAGGCAUGCAAGCU
2	GGGCGAAUUCGAGCUCGG	GCCACUCGCUCAACACGGUAAGUAGAGACCUCGUGGUACAUAAAGGA			CUGCAGGCAUGCAAGCU
3	GGGCGAAUUCGAGCUCGG	CCAACAGAACACAAUUCACAGUACCAGUCACACCAACUAACACACCC			CUGCAGGCAUGCAAGCU

Fig. 1. **Selected RNA sequences.** After eight rounds of *in vitro* selection and subsequent subcloning of amplified cDNAs, cloned DNAs were sequenced. Three sequences (1-3) were found. Predicted RNA sequences are shown. Vertical lines indicate the junctions between constant regions used for primer binding (1-18 and 66-82) and selected sequences (19-65). Different bases in sequences 1 and 2 (position 50) are shown by bold letters.

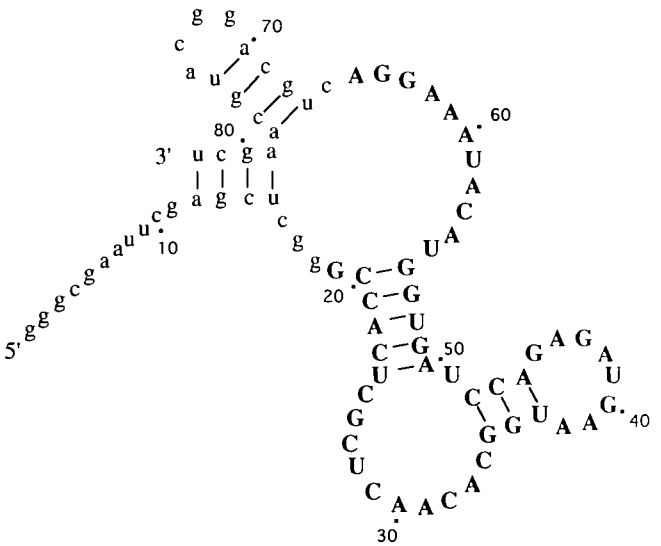

Fig. 2. **Computer-generated secondary structure of RNA-1.** Lower case letters show primer binding sequences.

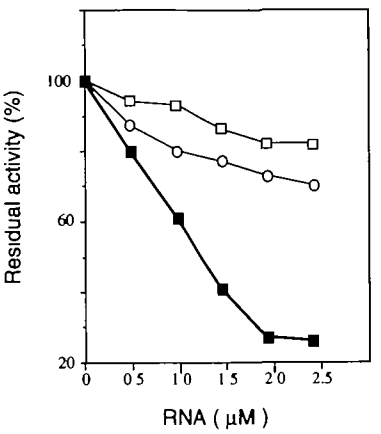

Fig. 3. **Inhibitory effects of RNAs on subtilisin activity.** Subtilisin was assayed using a chromogenic small peptide substrate as described under "MATERIALS AND METHODS." Filled squares, selected RNA-1; open squares, non-selected RNAs (the first generation); open circles, yeast RNAs.

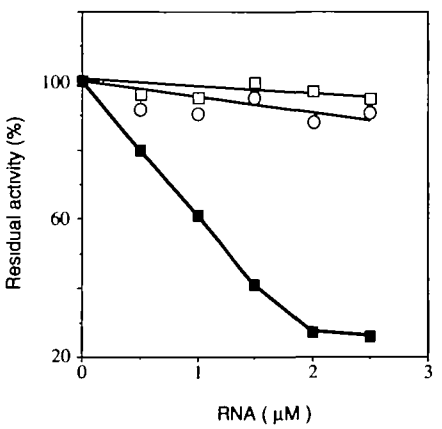

Fig. 4. **Effect of RNA-1 on proteolytic activities of various serine proteases.** RNA-1 (0-2.5 μM) was added to the reaction mixture for subtilisin, trypsin or chymotrypsin and assayed as described under "MATERIALS AND METHODS." Filled squares, subtilisin; open squares, chymotrypsin; open circles, trypsin.

tion, which was done as a control experiment, proceeded smoothly. We do not yet know the reason for this inefficient cloning of the eighth generation. Two of the 4 clones had sequence 1 shown in Fig. 1. Another clone had sequence 2, which differs from sequence 1 only in a single nucleotide substitution from A to C at position 50 (Fig. 1). The final clone had a completely different sequence (sequence 3 in Fig. 1). Here we examined in more detail the RNA of sequence 1, hereafter called RNA-1. RNA-1 was prepared by *in vitro* transcription of the corresponding plasmid linearized by *Hin*dIII using T7 RNA polymerase. A computer-generated secondary structure for RNA-1 is shown in Fig. 2, although we do not know whether this secondary structure is critical for the binding. The minimal free energy was calculated to be -22.16 kcal/mol.

Direct binding of RNA-1 to subtilisin was confirmed using the subtilisin-immobilized Sepharose 4B column. The purified RNA-1 (0.37 nmol) was dissolved in 60 μl of the binding buffer and heated at 95°C for 2 min, chilled in ice, then incubated for 20 min at room temperature. This RNA solution was poured into the subtilisin-immobilized column (4 mm in diameter × 7 mm in height). Under these conditions, more than 93% of RNA-1 was retained by the column. The RNA-1 bound to the column could be completely eluted with 1.8 ml of 36 μM subtilisin solution (data not shown). This is direct evidence that RNA-1 has subtilisin-binding ability.

The inhibitory effect of RNA-1 on the subtilisin activity

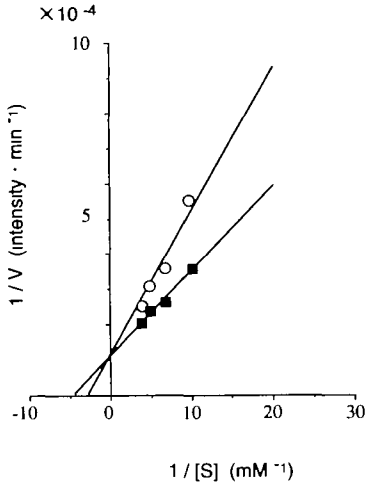

Fig. 5. **Lineweaver-Burk plot of subtilisin reactions with RNA-1 inhibitor.** Assays were done as described under "MATERIALS AND METHODS." Filled squares, minus RNA-1; open circles, plus RNA-1.

was tested using synthetic chromogenic substrate (Fig. 3). Subtilisin activity was clearly inhibited by RNA-1 in a concentration-dependent manner. Figure 3 also shows that the non-selected RNAs and yeast RNAs also slightly inhibited subtilisin activity. The non-selected RNAs are RNAs of the first generation prepared by *in vitro* transcription of the synthetic DNAs, R-SET100 and R-SETP-5. Since subtilisin is a basic protein, these RNAs may bind non-specifically to it, thereby causing slight inhibition of its activity. However, the inhibition by RNA-1 could be clearly distinguished from that by the other RNAs (Fig. 3). Subtilisin activity was 75% inhibited by 20 μg of RNA-1 under the conditions employed.

Is RNA-1 a specific inhibitor of subtilisin or a general inhibitor of serine proteases? The inhibitory effects of RNA-1 on two bovine pancreatic serine proteases, trypsin and chymotrypsin, were tested. All reactions were performed using chromogenic small peptides as substrate as described in "MATERIALS AND METHODS." While the residual activity of subtilisin decreased with the increasing concentration of RNA-1, only slight inhibition of trypsin and chymotrypsin activities was observed (Fig. 4). The inhibitory action of RNA-1 was thus subtilisin-specific.

To analyze subtilisin inhibition by RNA-1, subtilisin reaction using the chromogenic substrate was measured in the presence or absence of RNA-1. A Lineweaver-Burk plot of the results revealed the inhibition of subtilisin activity by RNA-1 to be competitive (Fig. 5). The apparent inhibition constant of RNA-1 for subtilisin was calculated to be 2.5 μM.

DISCUSSION

The present study demonstrates that an RNA can specifically bind subtilisin and inhibit its proteolytic activity. This is the first example of an RNA aptamer to an extracellular bacterial enzyme. This SELEX began with an RNA population transcribed from 10^{12} different DNA template molecules. This number may generally be too small to select RNA aptamers. Burgstaller and Famulok attempted to select RNA aptamers that bind to β-nicotinamide mononucleotide (NMN) and found no NMN-binding species, even though they started the SELEX with a complexity of 10^{15} different molecules (*9*). The initial complexity we used here might be the smallest complexity in the SELEX methods so far reported.

DNA and RNA aptamers for human serine proteases have been reported (*10-13*). The development of a DNA aptamer for human thrombin was the first demonstration that nucleic acids have sufficient structural plasticity to bind to a wide variety of proteins with non-nucleic acid-binding nature (*10*). Here we again demonstrated the plasticity of RNA in the subtilisin-binding RNA, since subtilisin was not known to bind nucleic acids. However, many aptamers to human proteases so far reported do not inhibit proteolytic activities when a small peptide substrate is used for enzyme assay (*11, 12*). These aptamers have been reported to bind to different sites from the protease catalytic sites. To confer an inhibitory effect, an aptamer to human neutrophil elastase was designed to contain an inhibitor group, valyl phosphonate (*13*). Recently, Gal *et al.* reported an RNA aptamer consisting of only four common nucleotides (A, G, U, and C) that binds to human activated protein C and inhibits its protease function (*14*). Human activated protein C is also a trypsin-like serine protease that plays an important role with thrombin as a regulator in the blood clotting cascade. Since this RNA aptamer non-competitively inhibited the cleavage of a small peptide by activated protein C, it was concluded that the binding site of this aptamer is different from the substrate-binding site (*14*).

Our aptamer, RNA-1, clearly inhibited the proteolytic reaction of subtilisin, when the small peptide was used as substrate. Since trypsin and chymotrypsin were not inhibited by RNA-1 (Fig. 4), RNA-1 can discriminate subtilisin from other serine proteases. The primary as well as the tertiary structures of subtilisin BPN′ are different from those of trypsin and chymotrypsin. From this result, although we do not yet know the exact site on subtilisin for RNA-1 binding, it is clear that the binding target of RNA-1 is not the catalytic triad (the buried active site consisting of residues Ser---His---Asp), a catalytic site common to serine proteases (*5*). Kinetic analysis revealed that this inhibition was competitive (Fig. 5). Therefore, in contrast to the aptamers to human serine proteases described above, our aptamer, RNA-1, is thought to bind to or near the substrate-binding site of the enzyme. Subtilisin recognizes substrates mainly by its substrate-binding cleft and the S1-S4 subsites that are arranged along the cleft (*15*). The target of RNA-1 may include these subsites. Identification of the target site will be analyzed and discussed in another paper.

Gal *et al.* reported that their aptamer for activated protein C did not bind to thrombin and other proteins, and thus their aptamer was specific for the activated protein C (*14*). Also, as described above, RNA-1 is a specific inhibitor of subtilisin. These specificities were obtained by chance without taking special methods during the selection process. We can expect to obtain a more specific binder when the selection procedure is well designed from the standpoint of the molecular discrimination (*16*).

The apparent inhibition constant of RNA-1 for subtilisin was shown to be 2.5 μM. This constant is comparable to the

binding constants for RNA aptamers to small molecules so far reported (*17-19*), but almost ten-fold larger than that of the DNA aptamer to human thrombin (*10*). *In vitro* selection or evolution to obtain RNA aptamers for subtilisin with higher affinity is in progress.

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