Supporting information

Isomeric replacement of a single aspartic acid induces a marked change in protein function: the example of ribonuclease A

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1. Preparation of the intein fusion RNase A cDNA.

A complementary DNA (cDNA) of RNase A was purchased from Open Biosystems, Inc. RNase A cDNA was inserted into plasmid pCMV-SPORT6 (pRNase), which was then cloned into DH10B TonA *E. coli*. The RNase A cDNA sequence corresponding to amino acids 1–109 was amplified by PCR. During PCR, recognition sequences for *Nde*I and *Sap*I were added to, respectively, the 5'- and 3'-ends of the RNase A cDNA. In addition, a His-tag sequence and a recognition sequence for factor Xa were added between the *Nde*I recognition sequence and the RNase A cDNA to increase the purification efficiency and to remove the initiating methionine and His-tag from the expressed protein, respectively. An overview of the method is shown in Fig. 3. The PCR primers used in each reaction are shown in Table 1.

Table S1. Primers used in this study

Primer		Sequence
1st PCR	Forward	GAAGGTCGTAAGGAAACTGCAGCAGCCAAG
	Reverse	CTCTTCCAGCCACAATGATGTGTTTATTCG
2nd PCR	Forward	TCACCACATCGAAGGTCGTAAGGAAACTGC
	Reverse	GGTGGTTGCTCTTCCAGCCACAATGATG
3rd PCR	Forward	CATCATCACCATCACCACATCGAAG
	Reverse	GGTGGTTGCTCTTCCAGCC
4th PCR	Forward	GGTGGTCATATGCATCATCACCA
	Reverse	GGTGGTTGCTCTTCCAGCC
Mutation	Forward	GATGATGAAGAGTCGGAACCTGACC
	Reverse	GGTCAGGTTCCGACTCTTCATCATC
Colony PCR	Forward[#]	TAATACGACTCACTATAGGG
	Reverse[*]	GATTGCCATGCCGGTCAAGG
Sequencing[#]		TAATACGACTCACTATAGGG

Primers to be annealed to [#]T7 promoter and [*]intein sequence

2. Introduction of a mutation into the *Sap*I site in RNase A cDNA.

Site-directed mutagenesis was performed to introduce a mutation into the *Sap*I site in the RNase A cDNA clone. For mutagenesis, the following PCR reagents were mixed and water was added to a total volume of 50 µl: 3 µl of pRNase (4 µg/ml), 1 µl of forward and reverse primers (10 µM each), 5 µl of 10×PCR buffer for KOD-Plus-Neo, 5 µl of dNTPs (deoxyribonucleotides, 2 mM), 3 µl of $MgSO_4$ (25 mM), and 1 µl of KOD-Plus-Neo (1.0 U/µl; TOYOBO, Japan). The PCR was carried out at 94°C for 2 minutes, followed by 30 cycles of 98°C for 10 seconds, 50.5°C for 30 seconds, and 68°C for 3 minutes. Next, 50 µl of PCR product was incubated with 1 µl of *Dpn*I (Takara Bio, Japan) at 37°C for 2 hours. The resulting product was transformed into *E. coli* HST08 Premium Competent Cells (Takara Bio, Japan) and seeded on Luria-Bertani (LB) agar plates containing 100 µg/ml of ampicillin. A single colony was picked and cultured in LB medium containing 100 µg/ml of ampicillin. The pRNase with a silent mutation in the *Sap*I site was extracted using PureLink Quick Plasmid Miniprep Kit (Invitrogen, Carlsbad, CA, USA) and sequenced by a 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad CA, USA).

3. Cloning of intein fusion RNase A (1–109) cDNA.

Primers were dissolved in Tris-EDTA (TE) at a stock concentration of 10 µM. The pRNase plasmid was diluted in TE at a stock concentration of 355 ng/µl. For the first PCR, the following reagents were mixed and water was added to a total volume of 50 µl: 1 µl of plasmid DNA (355 ng/µl), 1.25 µl of each primer (10 µM), 25 µl of 2×Gflex PCR Buffer (Mg^{2+}, dNTP plus), and 1 µl of Tks Gflex DNA polymerase (1.25 U/µl, Takara Bio, Japan). The first PCR was performed using a PTC-200 cycler (MJ Research) at 94°C for 2 minutes, followed by 25 cycles of 98°C for 10 seconds, and 68°C for 15 seconds. Next, 1 µl of the first PCR product was mixed with the same PCR reagents except for the primers, and the second PCR was carried out at 94°C for 2 min, followed by 30 cycles of 98°C for 10 seconds, and 68°C for 15 seconds. The third and fourth PCRs were performed in the same way as the second, except that different primers was used (see Table 1). Each PCR product was checked by electrophoresis using a 1% agarose gel (Nippon gene) dissolved in Tris-Acetate-EDTA buffer. The fourth PCR product was purified by using UltraPure Phenol:Chloroform:Isoamyl Alcohol (25:24:1) (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions.

The purified fourth PCR product and pTXB1 plasmid (New England BioLabs, Massachusetts, USA) were digested by *Sap*I and *Nde*I restriction enzymes at 37°C for 16 hours. The digested products were purified by gel electrophoresis and extracted by using a PureLink Quick Gl Extraction Kit (Invitrogen, Carlsbad, CA, U.S.A). The digested pTXB1 plasmid (25 fmol) and the digested PCR product (125 fmol) were mixed, TE was added to a total volume of 5 µl and then 5 µl of a DNA Ligation Kit was added (Takara Bio, Japan). The resulting construct was transformed into NEB turbo *E. coli* (New England BioLabs, Ipswich, MA, USA) and seeded on an LB agar plate containing 100 µg/ml of ampicillin. After overnight incubation at 37°C, single colonies were picked and the

presence of the insert was checked by colony direct PCR. The following reagents were mixed and water was added to a total volume of 10 µl per tube: 1×Buffer for Blend Taq, 200 µM dNTPs, 0.3 mM each primer, 1.25 U of Blend Taq (TOYOBO, Japan). The PCR was performed at 94°C for 4 min, followed by 30 cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 2 min. The plasmid containing the cDNA of intein fusion RNase A (pRNase-int) was sequenced by a 3130xl Genetic Analyzer.

A

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GGCAGAAACTGCCTTCTCTCTCTCAGACATCAAACTAGAGACCCAGGTTTCTCCAGGGGAGTGCGGTCATCATGGCTCTGAAGTCCCTGGTCCTGTTGTCGCTGTTGGTCCTGG
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B

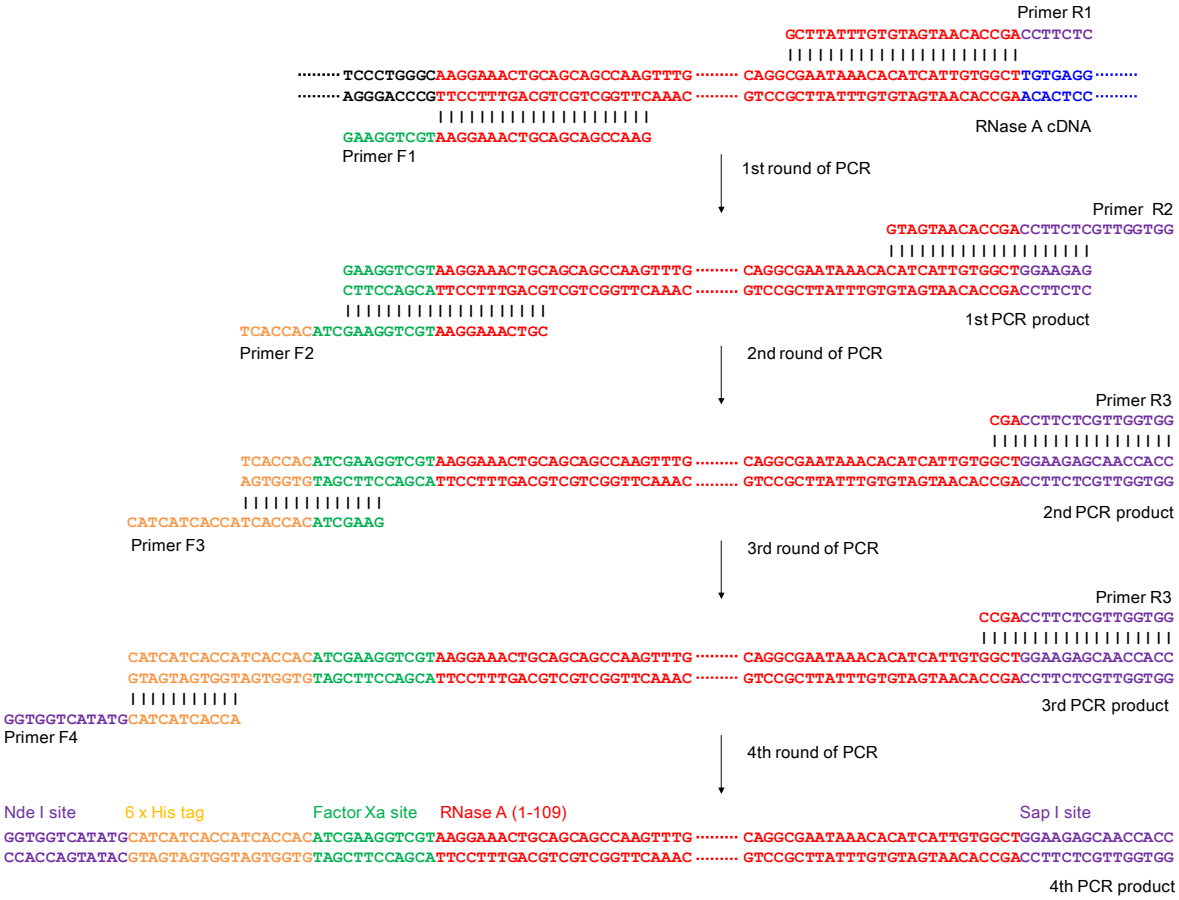

Figure S1. cDNA sequence of RNase A and overview of the stepwise addition of cDNA sequence. (A) DNA sequence of RNase A inserted in the pCMV-SPORT6 plasmid. Red and blue letters indicate the sequence of RNase A(1–109) and RNase A(110–124), respectively. (B) Overview of the stepwise addition of cDNA sequence. Green, orange, and purple letters indicate the factor Xa recognition site, His tag, and adaptor sequence, respectively.

4. Expression of intein-fused RNase A (1–109) for protein ligation.

pRNase-int was transformed into T7 Express lysY/Iq Competent *E. coli* (New England BioLabs, Ipswich, MA, USA) and then seeded on an LB agar plate containing 100 μg/ml of ampicillin. Single colonies were picked and the presence of the plasmid was checked by using colony direct PCR as described above. A colony containing the target plasmid was transferred to 2 ml of LB medium containing 100 μg/mL of ampicillin and incubated at 37°C overnight. The overnight culture was then transferred to 1 L of LB medium and incubated at 37°C with shaking until an OD_{600} of approximately 0.5 was reached. Expression was induced by adding 0.6 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the cells were incubated for 9 hours at 25°C with shaking at 180 rpm. The cells were harvested by centrifugation at 6,000×g for 15 minutes at 4 °C, and then resuspended in 5 ml of lysis buffer (20 mM Tris-HCl pH7.4, 500 mM NaCl, 8 M Urea). The expressed protein was extracted by using an ultrasonic generator (UD-201, TOMY) on ice, and the supernatant was collected by centrifugation at 12,000×g for 30 minutes at 4°C. The supernatant was dialyzed against a dilution buffer (20 mM Tris-HCl pH7.4, 500 mM NaCl, 2 M Urea).

5. Purification of intein-fused RNase A (1–109).

Intein-fused RNase A (1–109) was purified by affinity chromatography using a HisPrep FF16/10 column (GE Healthcare Life Sciences) with a linear gradient of 0-250 mM imidazole in the presence of 20 mM Tris-HCl pH7.4, 500 mM NaCl, and 2 M Urea at a flow rate of 1 ml/min using an AKTA prime instrument (GE Healthcare Life Sciences). Elution of intein-fused RNase A was monitored at 280 nm and the protein was collected manually (Figure S2).

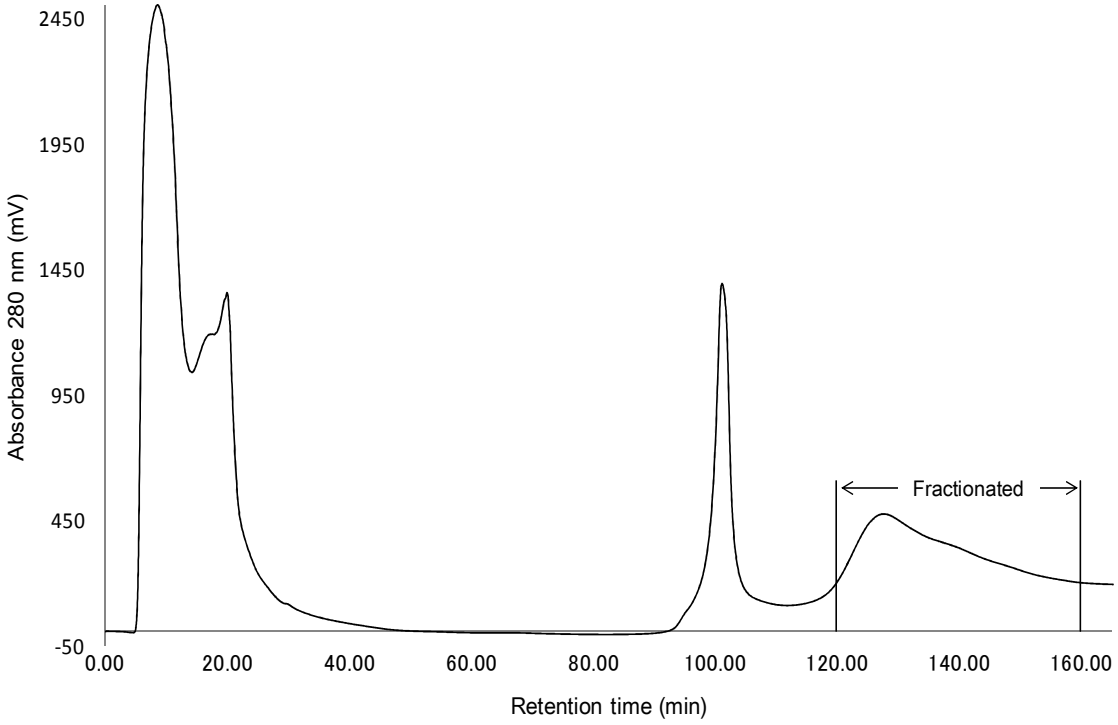

Figure S2. Elution profile of intein fusion RNase A (1-109) using FF16/10 column with a linear gradient of 0-250 mM imidazole in the presence of 20 mM Tris-HCl pH7.4, 500 mM NaCl, and 2 M urea at a flow rate of 1 ml/min. Protein eluted between 120 and 160 min was collected.

6. **EPL of intein-fused RNase A (1–109) and RNase A peptide (110–124).**

To cleave intein from the intein-fused RNase A(1–109) protein, 50 mM sodium 2-mercaptoethanesulfonate (MESNA) was added to purified RNase A(1–109) solution. Cleavage efficiency was checked by SDS-PAGE using a 15% gel. Approximately 80% of intein was cleaved from RNase A (1–109) by overnight incubation at 4°C. After cleavage of intein, expressed protein ligation was carried out by adding 10 mg of synthesized RNase A peptide (110–124) to the RNase A (1–109) solution. EPL was performed at 4°C for 10 hours. Ligation efficiency was checked by SDS-PAGE using a 15% gel. The yield of RNase A (1–124) proteins containing the four different Asp isomers was approximately 50%. Ligation product was purified by affinity chromatography using a HisPrep FF16/10 column as described for the purification of intein-fused RNase A (1–109).

7. **Cleavage of the 6×His tag from RNase A.**

To remove urea from the ligation solution, RNase A was dialyzed against factor Xa catalysis buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM $CaCl_2$) using a dialysis membrane (Spectra/Por 7, 1,000 MWCO, Spectrum Labs). After dialysis, 50 µg of factor Xa (Merck Millipore, Darmstadt, Germany) was added to the RNase A solution, which was incubated at 25°C for 16 hours to cleave the 6×His tag from the RNase A fusion protein. As a final step, complete RNase A was purified by ion exchange chromatography using a CM-650M column (Tosoh Bioscience) with a linear gradient of 0–1 M NaCl in the presence of 20 mM Tris-HCl pH 7.0 at a flow rate of 2 ml/min and detection at 280 nm using an AKTA prime instrument.