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Electronic Supplementary Information

Amino group binding peptide aptamer with double disulphide-bridged loops selected by *in vitro* selection using cDNA display

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Materials and methods

Library construction

The full construction of the DNA library coding cysteine-rich peptides is shown in Fig. S1 (a). The amino acid composition of the library was adjusted by nucleotide mixture. Random amino acids comprised the codon triplets XYZ, where X, Y, and Z indicate nucleotide mixture, and two patterns of mixing ratios were used as follows: X: A 44%, T 0%, G 38%, C 22%; Y: A 16%, T 30%, G 20%, C 34%; Z: A 45%, T 15%, G 30%, C 10% or X: A 50%, T 0%, G 50%, C 0%; Y: A 16%, T 33%, G 20%, C 31%; Z: A 35%, T 18%, G 32%, C 15%. The two DNA libraries were prepared by joining the three DNA fragments by overlap extension polymerase chain reaction. These two DNA libraries were used as an initial library in the mixture. The total theoretical percent appearance of amino acids in the randomized region is shown in figure S1 (b).

cDNA display preparation from library DNAs

The library DNAs were transcribed by T7 RNA polymerase in a RiboMAX Large Scale RNA Production Systems instrument (Promega, Madison, WI, USA). Reactions were terminated by adding DNase and products were purified with an RNA purification column (FavorPrep After Tri-Reagent RNA Clean-Up Kit, Favorgen, Ping-Tung, Taiwan). The 3′-terminal regions of the mRNA molecules were hybridized to the complementary strands of the short biotin segment puromycin (SBP)-linker DNA under annealing conditions (heating to 90 °C for 1 min followed by incubation at 70 °C for 1 min, then cooling to 25 °C) in ligation buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT) and 1 mM ATP). Then, T4 RNA ligase (0.4–2.0 U/pmol mRNA, Takara bio, Japan) and T4 polynucleotide kinase (0.25 U/pmol mRNA, Takara bio, Otsu, Japan) was added and the mixture incubated at 25 °C for 1 h. 2× Binding buffer (20 mM Tris-HCl pH 7.5, 2 M NaCl, 2 mM EDTA, 0.1% Tween20) was added to the ligation reaction mixture and the mixture incubated with streptavidin-coated magnetic beads (SA beads, Dynabeads MyOne Streptavidin C1, Invitrogen, Carlsbad, CA, USA) in a ratio of 3.75 μL of SA-beads to 1 pmol of mRNA-linker conjugates at 20

°C for 15 min. After washing with 1× binding buffer once, the SA beads were incubated at 30 °C for 25 min in a cell-free translation mixture with a rabbit reticulocyte lysate (Retic Lysate IVT Kit, Ambion, Austin, TX, USA) in a ratio of 10 μL of SA beads to 20 μL of the cell-free translation mixture. To facilitate puromycin incorporation to the C-terminus of the peptide, KCl and $MgCl_2$ were added to the mixture (each final concentration of 800 and 80 mM) and incubated at 37 °C for 60 min. Then, to release ribosomes binding to the mRNA, pure 0.5 M EDTA solution was added (final concentration of 83 mM) and the mixture incubated at r.t. for 10 min. The SA beads were washed three times with 1× binding buffer and one time with a buffer for reverse transcription reaction that contains a reverse transcription enzyme (ReverTraAce, Toyobo, Osaka, Japan). A reverse transcription reaction mixture (1 mM dNTP, 2.5 U/μL ReverTra Ace in the buffer) was mixed with the SA beads at a ratio of 15 μL of SA beads to 10 μL of the mixture, and the total mixture incubated at 42 °C for 30 min. Then the SA beads were incubated in a his-tag wash buffer (20 mM sodium phosphate pH 7.4, 0.5 M NaCl, 5 mM imidazole, 0.05% Tween20) at 4 °C overnight, his-tag wash buffer with 5 U/μL RNase T1 (Ambion) was mixed with the SA beads at a ratio of 30 μL of SA beads to 10 μL of the his-tag wash buffer containing RNase T1, and incubated at 37 °C for 15 min. Supernatant containing cDNA display molecules was collected. To purify the cDNA display molecules, the solution containing the cDNA display molecules was incubated with Ni-NTA magnetic beads (His Mag Sepharose Ni, GE Healthcare, Pittsburgh, PA, USA) at 10 °C for over 2 h. The Ni-NTA magnetic beads were washed twice with his-tag wash buffer and the binding cDNA display molecules were eluted by incubation at r.t. for 10 min in his-tag elution buffer (20 mM sodium phosphate pH 7.4, 0.5 M NaCl, 250 mM imidazole, 0.05% Tween20). The above elution step was performed twice under the same conditions. Before the affinity selection of disulphide-rich peptides from the cDNA display library, the his-tag elution buffer was changed to a selection buffer (50 mM Tris-HCl, 1 M NaCl, 1 mM EDTA, 0.1% Tween20) with 1 μM L-cystine using Micro Bio-Spin Columns with Bio-Gel P-6 in Tris Buffer (BioRad, Hercules, CA, USA).

Affinity selection

The above cDNA display library was screened to identify peptides selectively binding to amino groups using Dynabeads M-270 Amine (Invitrogen). In the initial round, a cDNA display library was prepared from 150 pmol of library mRNA in 70 μL of selection buffer. This mixture was incubated at r.t. for 90 min using a tube rotator (AS ONE, Osaka, Japan) with 50 μL of Dynabeads M-270 Amine which was prewashed using selection buffer with 100 nM L-cystine. The beads were washed four times using selection buffer with 100 nM L-cystine. To elute amino group binding cDNA display molecules, the beads were incubated at r.t. for 1 h in 400 μL of selection buffer containing 0.1 M DTT. Then this mixture was incubated r.t. for 1 h using a micro tube mixer (TOMY SEIKO, Tokyo, Japan) in 400 μL of selection buffer with 1% SDS. The cDNA display molecules in each eluate were ethanol precipitated using Quick-precip Plus (Edge BioSystems, Gaithersburg, MD, USA). To prepare library DNAs for the next round of *in vitro* selection, T7 promoter reconstructed library DNAs were prepared from the above precipitated cDNA display molecules by PCR. A total of four rounds of *in vitro* selection were performed according to the above protocol with minor changes as follows: the cDNA display library was prepared from 30 pmol mRNA in the second round and 20 pmol in third and fourth rounds; the target beads volume was successive decreased from 50 μL to 30 μL, and cDNA molecules which bound to the materials of the beads were removed by prescreening using Dynabeads M-270 Carboxylic Acid (Invitrogen). After the fourth round of selection was performed, the sequence composition of the library DNAs was confirmed by direct sequencing. Then, the library DNAs were cloned using pGEM-T Easy Vector Systems (Promega) and NEB 5-alpha Competent E. coli (High efficiency) (New England Biolabs, Ipswich, MA, USA), and a total of 90 clone DNAs were sequenced by Operon Biotechnologies (Tokyo, Japan).

Peptides

The peptides analysed in this study are listed in Table 1. A FAM-G_3S linker was introduced to the N-terminal of all synthesized peptides for ease of detection. Three disulphide isomers of CP1 (2SS): CP1 (2SS)-α, β, and γ; and two kinds of fragmented peptides, the N-terminal or C-terminal loop

regions of CP1 (2SS)-α: CP1 (1SS)-NTR and CP1 (1SS)-CTR were chemically synthesized by Toray Research Center (Tokyo, Japan). Two kinds of serine variants of CP1 (2SS)-α: CP1 (1SS)-A and B; and an alanine variant of CP1 (2SS)-α: CP1 (Cys→Ala) were chemically synthesized by SCRUM Corporation (Tokyo, Japan). All peptides were certified at more than 90% purity.

Pull-down assay using amino group modified magnetic beads

10 μL of Dynabeads M-270 Amine and of Dynabeads M-270 Carboxylic acid were washed with 100 μL of the selection buffer. Each peptide solution (30 μL, 2 μM) was incubated with each magnetic bead at 25 °C for 2 h using a thermo block rotator (SNP-24B, Nissinrika, Tokyp, Japan). The beads were washed two times with 100 μL of the selection buffer and the remaining peptides were eluted by incubation at 85 °C for 3 min in 20 μL of SDS sample buffer (125 mM Tris-HCl pH 6.8, 8 M urea, 4% (w/v) SDS, 6% (w/v) sucrose, optimal amount of xylene cyanol). The eluates were subjected to tricine-SDS-PAGE and visualized with a fluorescence image analyser (Pharos FX, BioRad).

Binding assay against a glass slide

Peptide solutions (20 μL) in the selection buffer (final concentration of 20 μM) were spotted on a glass slide (MAS coated glass slide, Matsunami Glass, Osaka, Japan) and incubated at r.t. for 2 h. The remaining solutions were removed and each spotted region was washed four times with 20 μL of the selection buffer by pipetting. The glass slide was placed in the selection buffer for 1 h. Then the glass slide was removed from the buffer and peptides remaining on the glass slide were visualized with a fluorescence image analyser.

Binding assay against agarose beads

100 μL portions of amino group modified agarose-resin beads (Calboxylink Coupling Gel, 50% slurry, Thermo Fisher Scientific, Waltham, MA, USA) was poured into a 1.5 mL microcentrifuge tube and washed three times with 900 μL of selection buffer by centrifugation of the beads and

removing the supernatant. After removing the supernatant by pipetting, each peptide solution were added to the tube (final concentration was approximately 65 μM, final volume was approximately 80 μL), then mixed and incubated at 25 °C for 2 h. After washing the agarose beads twice with 300 μL of selection buffer, the 2× SDS sample buffer was added to elute the binding peptides. The SDS sample buffer was separated from the agarose beads with an empty column (MicroSpin Empty Columns, GE Healthcare). A sample of the eluate (3%) was subjected to tricine-SDS-PAGE and visualized by the fluorescence image analyser.

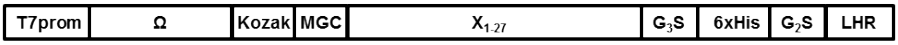
Binding assay against agarose beads with several kinds of modifications

50 μL portions of non-modified agarose resin beads (Pierce Control Agarose Resin, Thermo Fisher Scientific, 50% slurry), amino group modified agarose-resin beads (Calboxylink Coupling Gel, 50% slurry, Thermo Fisher Scientific), and carboxyl group modified agarose-resin beads (ECH Sepharose 4B, 50% slurry, GE Healthcare) were each poured into 1.5 mL microcentrifuge tubes and washed three times with 400 μL of the selection buffer by centrifugation of the beads and removal of the supernatants. After removing the supernatants by pipette, 50 μL of a CP1 (2SS)-α solution (20 μM) was added to each tube, then mixed and incubated at 25 °C for 2 h. After washing each kind of agarose bead four times with 300 μL of the selection buffer, 25 μL of the 2× SDS sample buffer was added to elute the binding peptides. A 10 μL portion of each supernatant was subjected to tricine-SDS-PAGE and visualized using the fluorescence image analyser.

Circular dichroism (CD) analysis

CD spectra were recorded at 25 °C in 0.1 mm cell from 250 to 195 nm at 50 nm/min on a circular dichroism spectrometer (J-720, JASCO, Tokyo, Japan). Peptides were dissolved (0.2 mg/ml) in 20 mM sodium phosphate buffer, pH 7.4. Spectra were measured four times and averaged for each sample, and an equally averaged buffer baseline was subtracted.

(a)



(b)

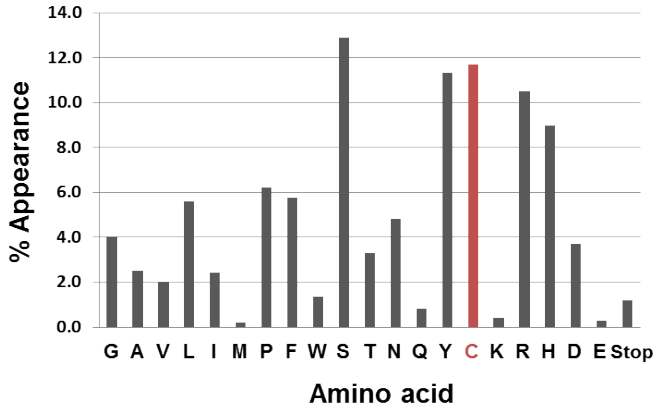


Figure S1. The library DNA construct and amino acid composition of the random region. (a) Construct of library DNA coding cysteine-rich peptides. Symbols and abbreviations are defined as follows: T7prom, T7 promoter; Ω, translation enhancer of tobacco mosaic virus; Kozak, Kozak sequence for translation initiation; MGC, N-terminal three constant amino acids; $X_{1\text{-}27}$, twenty seven randomized amino acids; G_3S and G_2S, glycine-serine linker; 6×His, hexahistidine tag; and LHR, hybridization region for puromycin-linker DNA. (b) Theoretical percent appearance of amino acids in the random region.

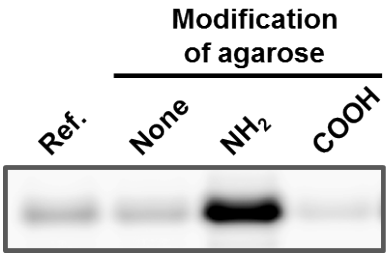


Figure S2. Pull-down assay using agarose resin beads modified with each functional group. CP1 (2SS)-α was incubated with non-modified agarose-resin beads, amino group (NH_2) or carboxyl group (COOH) modified agarose-resin beads respectively at 25 °C for 2 h in the selection buffer (20 mM Tris-HCl pH 7.4, 1 M NaCl, 1 mM EDTA, 0.1% Tween20). After washing the each agarose-resin beads, the remaining CP1 was eluted with SDS sample buffer. The eluates were subjected to tricine-SDS-PAGE and visualized with the fluorescence image analyser. CP1 (2SS)-α (1 pmol) was loaded as a reference (Lane: Ref.).

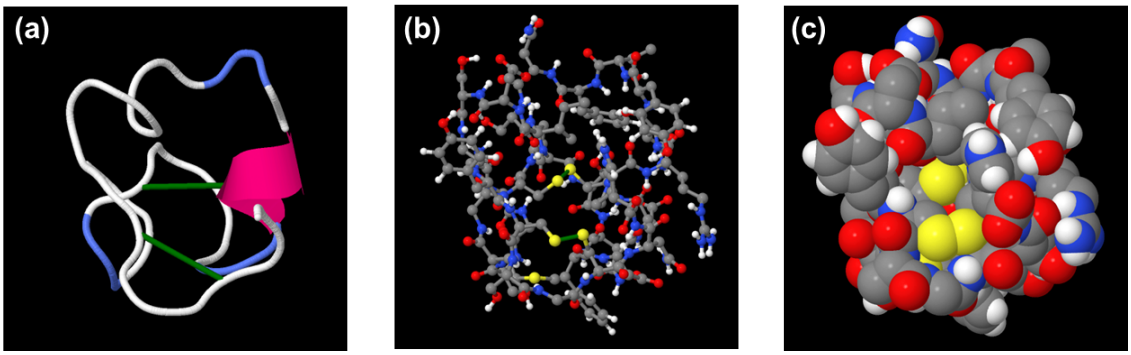


Figure S3. A possible conformation of the CP1 (2SS)-α obtained by PEP-FOLD.[1] Ribbon model (a), ball and stick model (b), and space-filling model (c) are depicted using Jmol software. Secondary structures in (a) are coloured as follows: α-helix, magenta; β-turn, light blue. Carbon, nitrogen, oxygen, and sulphur atoms in (b) and (c) are shown in grey, blue, red, and yellow, respectively. Disulphide bonds are shown in green.

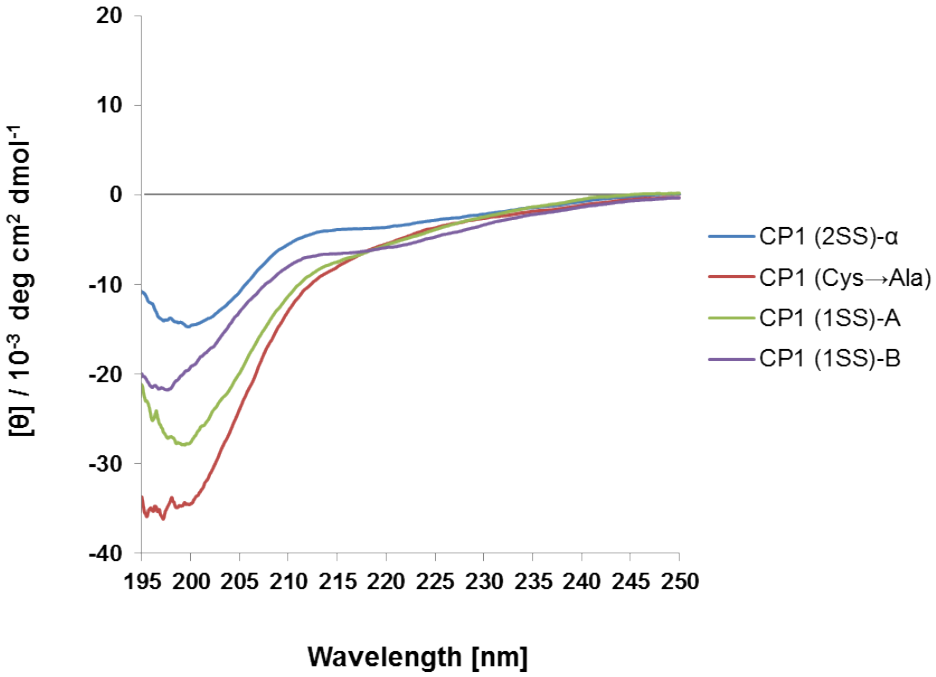


Figure S4. Circular dichroism analyses of CP1 derivative peptides. Each peptide concentration was 0.2 mg/mL in 20 mM sodium phosphate buffer, pH 7.4. The temperature was set at 25 °C.

References

[1] (*a*) J. Maupetit, P. Derreumaux and P. Tufféry, *Nucleic Acids Res.*, 2009, **37**, W498-503; (*b*) J. Maupetit, P. Derreumaux and P. Tuffery, *J. Comput. Chem.*, 2010, **31**, 726-738; (*c*) P. Thévenet, Y. Shen, J. Maupetit, F. Guyon, P. Derreumaux and P. Tufféry, *Nucleic Acids Res.*, 2012, **40**, W288-293.