

## Supplementary Information

### Materials and Methods

#### Materials

pET-32b(+) was purchased from Novagen (Darmstadt, Germany). pMAL-c4E was purchased from New England Biolabs (Ipswich, MA, USA). BL21 star (DE3) pLysS and EKMax were purchased from Invitrogen (Carlsbad, CA, USA). A synthetic gene of phosphite dehydrogenase was purchase from GenScript (Piscataway, NJ, USA). 4-Amidinophenylmethanesulfonyl fluoride hydrochloride (APMSF) was purchased from Wako Pure Chemical Industries (Oosaka, Japan). Mineral oil was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fmoc-NH-SAL resin, Fmoc-protected amino acids and hydroxybenzotriazole dihydrate (HOBt•2H<sub>2</sub>O) were purchased from Watanabe Chemical Industries (Hiroshima, Japan). N-methylpyrrolidone (NMP), N,N'-diisopropylcarbodiimide (DIC), trifluoroacetic acid (TFA) and triisopropylsilane (TIS) were purchased from Tokyo Kasei (Tokyo, Japan).

#### Construction of fusion proteins

A modified pET-32b(+), with is modified to have a second NcoI site after a *SacI* site, was digested with *Bam*HI and *SacI*. The digested pET-32b(+) was ligated with the dsDNA encoding Wzip1 peptide

sequence, which was prepared from the mixture of two oligo DNAs, wzip1-f and wzip1-r, that were boiled for 3 min and subsequently allowed to cool to room temperature. The original *NcoI* site of pET-32b(+) was removed from the obtained plasmid by PCR using two primers, enterow-f and enterow-r. The resulting plasmid, pTWzip1, was verified by DNA sequencing. The MBP gene amplified from pMAL-c4E by PCR using two primers, MPB-f and MBP-r, was cloned into pTWzip1 using the *NcoI* and *NotI* sites. The resulting plasmid, pTWzip1M, which was the expression vector for T1M, was verified by DNA sequencing. A T2M expression vector, pTWzip2M, was obtained by inserting the dsDNA, which was prepared from two oligo DNAs, wzip2-f and wzip2-r, between the *BamHI* and *NcoI* sites of pTWzip1M. A TCM expression vector, pTCM, was obtained using two oligo DNAs, control-f and control-r, as above.

The plasmids, pTWzip1M, pTWzip2M and pTCM, were digested with *AgeI*, removing the sequence encoding MBP and self-ligated. The resulting plasmids, pTW1, pTW2 and pTC, which encoded Trx-1, Trx-2 and Trx-C, respectively, were verified by DNA sequencing.

An MBP-1 expression vector was obtained by removing the sequence encoding GSKKWTWLPAT from pTWzipM1 using PCR and two primers, MBP1-f and MBP1-r. The resulting plasmid, pM1, was verified by DNA sequencing. MBP-2 and MBP-C expression vectors, pM2 and pMC,

were also obtained from pTWzipM2 and pTCM using primers, MBP2-f and MBP2-r and MBPC-f and MBPC-r, respectively.

A gene of His<sub>6</sub> tag attached phosphite dehydrogenase (PTDH) was amplified from an expression vector, which had been obtained by cloning a gene of PTDH into pET-15b, using primers, T7\_p and PTDH\_r. The amplified gene was inserted into pTW2 and pTC using *Xba*I and *Bam*HI by exchanging with a gene of Trx. The resulting plasmids, pPTDH2 and pPTDHC, which encoded PTDH-GSKKWTWTWLPATGG and PTDH-GSKKAAALPATGG, respectively, were verified by DNA sequencing.

A gene of CyPet, which had been prepared by mutating ECFP, was cloned between *Sac*I and *Not*I sites of pTWzip1. The sequence encoding GSKKWTWLPAT was removed from the resulting plasmid using PCR and two primers, MBP1-f and MBP1-r. The resulting plasmid, pTW1CyPet, which encoded GGWTWQESS-CyPet, was verified by DNA sequencing. GGAAQESS-Cypet and GGWTWTW-CyPet expression vectors were obtained from pTW1CyPet by PCR using primers CyPetC\_f and CyPetC\_r, and CyPet2\_f and CyPet2\_r, respectively. Oligo DNAs to the construct plasmids are summarized in Supporting Table 1.

### **Expression and purification of proteins**

*Escherichia coli* BL21 star (DE3) pLysS was transformed with an expression vector. A single colony of cells was inoculated into 5 ml of LB medium containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol and incubated at 37 °C overnight. The culture was added to 1 L of TB medium containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol and the cells were grown at 37 °C. When the OD<sub>600</sub> reached ~0.7, 1 mmol of IPTG was added and the culture was continued at 30 °C overnight. The cells were harvested by centrifugation.

T1M, T2M or TCM expressing cells were resuspended in buffer A (20 mM sodium phosphate, pH 7.4, containing 150 mM NaCl, 40 mM imidazole and 0.04% Triton X-100) and were lysed by ultrasonication. The soluble fraction of the lysate was loaded onto a HisTrap FF crude column which was pre-equilibrated with buffer A. The column was washed with buffer A and the protein was eluted with buffer B (20 mM sodium phosphate, pH 7.4, containing 150 mM NaCl, 500 mM imidazole and 0.04% Triton X-100). The fractions containing the target protein were combined and loaded onto a MBP Trap HP column, which was pre-equilibrated with buffer C (20 mM Tris-HCl, pH 7.4, containing 200 mM NaCl and 1 mM EDTA). After washing the column with buffer C, the protein was eluted with buffer D (20 mM Tris-HCl, pH 7.4, containing 200 mM NaCl, 1 mM EDTA and 10 mM maltose). The fractions containing the target protein were combined and concentrated with an Amicon Ultra-15 centrifugal unit

(30,000 NMWL). The concentrated protein was subjected to size exclusion chromatography on a Superdex 75 column equilibrated with buffer E (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl). The purified protein was concentrated with the centrifugal unit and the concentration was determined using The Better Bradford Assay Kit (Thermo Scientific).

Trx-1, Trx-2 or Trx-C was partially purified with a HisTrap FF crude column as described above. The partially purified protein was dialyzed against buffer F (50 mM Tris-HCl, pH 8.0) and loaded onto a HiTrap DEAE FF column, which was pre-equilibrated with buffer G (10 mM Tris-HCl, pH 7.2). After washing the column with buffer G, the protein was eluted with a linear gradient of NaCl (0–500 mM). The fraction containing the target protein was combined and concentrated with a centrifugal unit. The concentrated protein was subjected to size exclusion chromatography on a Superdex 75 column equilibrated with buffer E. The purified protein was concentrated with a centrifugal unit and the concentration was determined using The Better Bradford Assay Kit (Thermo Scientific). PTDH-GSKKWTWTWLPATGG and PTDH-GSKKAAALPATGG were purified as above, except that anion exchange chromatography was performed using a HiTrap Q FF column with 20 mM Tris-HCl buffer, pH 7.6.

MBP-1, MBP-2 or MBP-C expressing cells were resuspended in buffer C and were lysed by

ultrasonication. The soluble fraction of the lysate was loaded onto a MBP Trap HP column, which was pre-equilibrated with buffer C. After washing the column with buffer C, the protein was eluted with buffer D. The eluted protein was incubated with EKMax at 20 °C overnight. The digested protein with EKMax was loaded onto a HisTrap FF crude column which was pre-equilibrated with buffer A. The unbound fractions were combined and incubated with 0.1% APMSF at 4 °C for 30 min to inactivate EKMax. The protein concentrated using a centrifugal unit was subjected to size exclusion chromatography on a Superdex 75 column equilibrated with buffer E. The purified protein was concentrated with a centrifugal unit and the concentration was determined using The Better Bradford Assay Kit (Thermo Scientific). GGAAQESS-Cypet and GGWTWTW-CyPet were purified as above, except that affinity chromatography was performed using a HisTrap FF crude column with buffer A and B.

SrtA was expressed and purified as described previously.<sup>12</sup> Briefly, SrtA was expressed in BL21 Star (DE3) using the plasmid, pET30b-SrtA. SrtA was purified using a HisTrap FF crude column and a Superdex 75 column. The concentration of purified SrtA was determined using The Better Bradford Assay Kit (Thermo Scientific).

### Peptide synthesis and purification

Peptides were manually synthesized using a standard Fmoc-based solid-phase peptide synthesis protocol. Fmoc deprotection was carried out with 20% piperidine in DMF, and coupling reactions were carried out with a mixture of Fmoc-amino acids, diisopropylcarbodiimide (DIC) and HOBt in DMF. Following chain assembly, global deprotection and cleavage from the resin was carried out with TFA containing 2.5% triisopropylsilane (TIS) and 2.5% H<sub>2</sub>O. The crude peptide products were precipitated by H<sub>2</sub>O and purified by reversed-phase HPLC using a semi-preparative YMC-Pack ODS-A C18 column with a linear gradient of 0.1% aqueous TFA and CH<sub>3</sub>CN containing 0.1% TFA. The peptide was identified by MALDI-TOF MS (Matrix; CHCA): obsd 1957.99 (Ctl). obsd 2292.65 (Wzip1). obsd 2866.35 (Wzip2). obsd 2046.72 (HP5W4).

### Circular Dichroism Measurements

Circular dichroism (CD) stock solutions were prepared by dissolving approximate amounts of peptide in 20 mM potassium phosphate buffer (pH 8.0). The concentration of the stock solution was measured by the UV absorption of tryptophan ( $\epsilon_{280} = 5580 \text{ M}^{-1} \text{ cm}^{-1}$ ). CD samples were diluted appropriately to obtain 30  $\mu\text{M}$  solutions of the peptide in buffer. Mineral oil was put on samples. Spectra were recorded

using a Jasco J-820 spectropolarimeter with a 0.10 cm path length cell. Typical spectral accumulation parameters were a scan rate of 100 nm/min and a 0.1 nm step resolution over the range of 200–250 nm with 16 scans averaged for each spectrum. For melting experiments, CD stock solutions were prepared by dissolving peptides in 20 mM potassium phosphate buffer (pH 8.0) and the temperature was increased from 0 to 100 °C with 10 °C increments. Each step was equilibrated at the target temperature for 5 min before acquiring data. CD data are presented in residue molar ellipticity units ( $\text{deg cm}^2 \text{ residue dmol}^{-1}$ ).

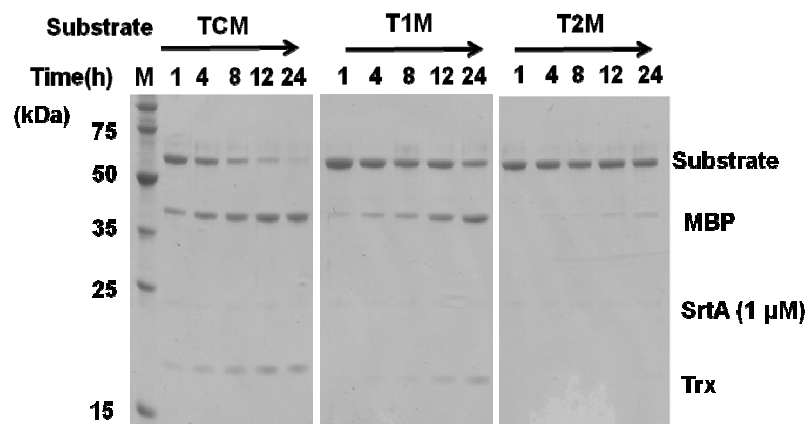
#### **SrtA-mediated protein cleavage and ligation**

Fusion proteins (60  $\mu\text{M}$ ) composed of Trx and MBP were digested by SrtA (1 or 30  $\mu\text{M}$ ) using triglycine (1 mM) as a nucleophile in buffer H (50 mM Tris-HCl, pH 8.0, containing 150 mM KCl and 10 mM  $\text{CaCl}_2$ ). The fusion proteins were also incubated with SrtA in the absence of nucleophiles to evaluate the hydrolysis reaction. A typical ligation reaction mixture contained SrtA (1 or 5  $\mu\text{M}$ ), LPATGG-tagged Trx (60  $\mu\text{M}$ ) and GG-tagged MBP (60  $\mu\text{M}$ ) in buffer H. All reactions were conducted at 25 °C. Reaction products were separated by SDS-PAGE and quantified using ImageJ (Biophotonics Int., 11, 36–42).

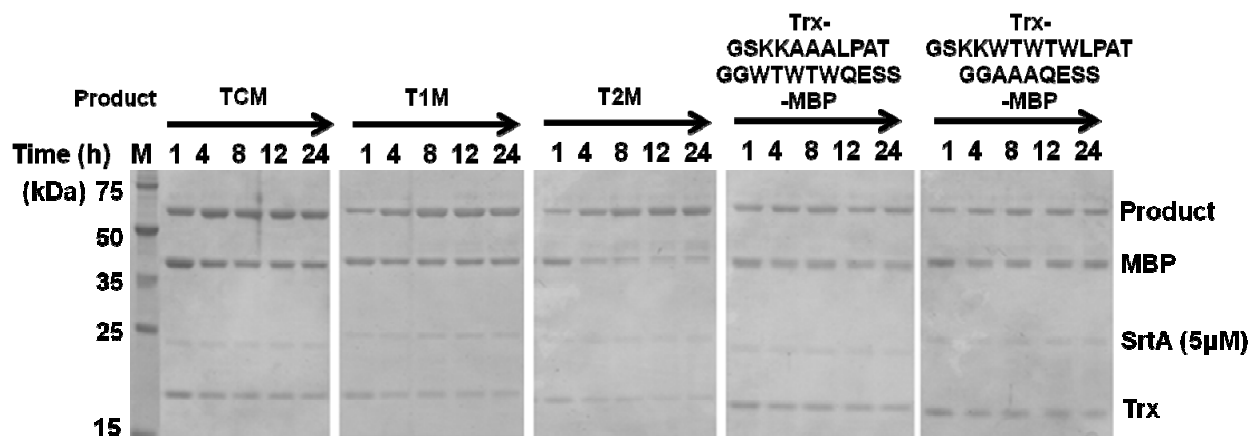


## Figures

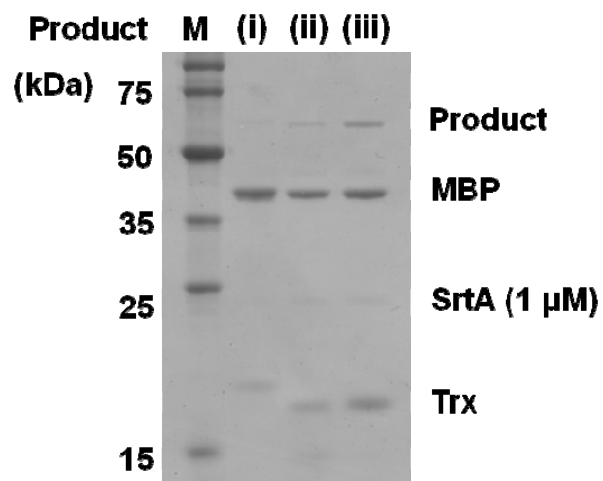
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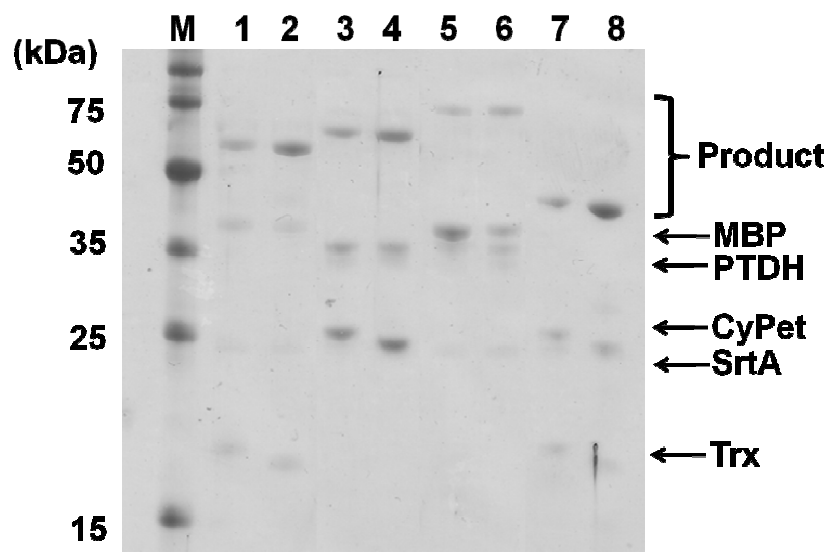
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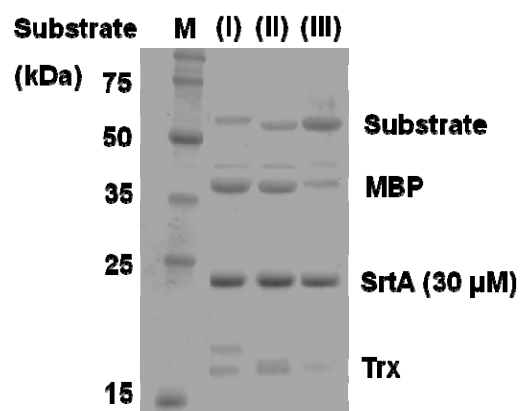
c)



d)



**Figure S1. SDS-PAGE analysis of SrtA-mediated protein cleavage and ligation.** (a) The SrtA-mediated cleavage reaction in the presence of 1mM triglycine. Fusion proteins were incubated with 1  $\mu$ M SrtA and 1 mM triglycine at 25 °C. (b) The SrtA-mediated ligation. Each substrate was incubated with 5  $\mu$ M SrtA at 25 °C. (c) The SrtA-mediated ligation in the presence of a competitive inhibitor. Each substrate was incubated with 1  $\mu$ M SrtA and 250  $\mu$ M diglycine at 25 °C. Products: i) TCM, ii) T1M and iii) T2M. (d) The SrtA-mediated ligation of various combination of proteins. Lane 1, Trx-C and MBP-C; lane 2, Trx-2 and MBP-2; lane 3, PTDH-GSKKAAALPATGG and GGAAAQESS-CyPet; lane 4, PTDH- GSKKWTWTWLPATGG and GGWTWTWQESS-CyPet; lane 5, PTDH-GSKKAAALPATGG and MBP-C; lane 6, PTDH-GSKKWTWTWLPATGG and MBP-2; lane 7, Trx-C and GGAAAQESS-CyPet lane 8, Trx-2 and GGWTWTWQESS-CyPet.



**Figure S2. SDS-PAGE analysis of the hydrolysis reaction at equilibrium.** Fusion proteins

were incubated with 30  $\mu$ M SrtA at 25  $^{\circ}$ C. Substrates: i) TCM, ii) T1M and iii) T2M.

## Tables

**Table S1. Summary of oligo DNAs used for vector construction.**

Name	Sequence
wzip1-f	GATCCAAGAAATGGACCTGGCTGCCGGCGACCGGTGGCTGGACCTGGCAGGA GAGCT
wzip1-r	CTCCTGCCAGGTCCAGCCACCGGTCGCCGGCAGCCAGGTCCATTCTTG
enteroW-f	CGACAAGGGATCCAAGAAATGGACC
enteroW-r	TTGGATCCCTTGTCGTCGTCGTCGGT
MBP-f	GCTCCATGGGAGGAGGAGGCTCCATGAAATCGAAGAAGG
MBP-r	AATTGCGGCCGTTCAACCGGTTCAAGTCTGCGCGCGTCTTTCAGG
wzip2-f	GATCCAAGAAATGGACCTGGACCTGGCTGCCGGCGACCGGTGGCTGGACCTG GACCTGGCAGGAGAGCTC
wzip2-r	CATGGAGCTCTCCTGCCAGGTCCAGGTCCAGCCACCGGTCGCGGGCAGCCAG GTCCAGGTCCATTCTTG
Control-f	GATCCAAGAAAGCCGCCGCCCTGCCGGCGACCGGTGGCGCCGCCGCCAGGA GAGCTC

Control-r	CATGGAGCTCTCCTGGGCGGCGGCCACCGGTCGCCGGCAGGGCGGCGGCT TTCTTG
MBP1-f	CGACAAGGGTGGCTGGACCTGGCAG
MBP1-r	CAGCCACCCTTGTCGTCGTCGTCGGT
MBP2-f	CGACAAGGGTGGCTGGACCTGGACC
MBP2-r	CAGCCACCCTTGTCGTCGTCGTCGGT
MBPC-f	CGACAAGGGTGGCGCCGCCGCCAG
MBPC-r	GCGCCACCCTTGTCGTCGTCGTCGGT
T7_p	TAATACGACTCACTATAGGG
PTDH_r	AGTTTCTTGGATCCGCACGCCGCCGGTTCCGC
CyPetC_f	GGCGCCGCCGCCAGGAGAGCTCCATGGTG
CyPetC_r	CTGGGCGGCGGCCACCCTTGTCGTTCGTC
CyPet2_f	TGGCTGGACCTGGACCTGGCAGGAGAGC
CyPet2_r	GTCCAGGTCCAGCCACCCTTGTCGTCGGTC

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