Supplementary Information

Creation of A Caspese-3 Sensing System Using A Combination of Split-GFP and Split-Intein

Seiji Sakamoto,* Mika Terauchi, Anna Hugo, Tanner Kim, Yasuyuki Araki and Takehiko Wada*
Institute of Multidisciplinary Research for Advanced Materials, Tohoku University, Katahira 2-1-1, Aoba-ku, Sendai 980-8577, Japan
e-mail: sakamoto@tagen.tohoku.ac.jp; hiko@tagen.tohoku.ac.jp

1. Experimental Section

General Materials. All chemicals and solvents were of reagent or HPLC grade. All materials were obtained from Sigma-Aldrich unless otherwise noted. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. KOD Plus Neo2 DNA polymerase and dNTPs were obtained from Toyobo (Tokyo, Japan). Isopropyl-β-D(-)-thiogalactopyranoside (IPTG), dL-dithiothreitol (DTT), tris(2-carboxyethyl)phosphine (TCEP), and PURESYSTE classic II were purchased from Wako Pure Chemical Industries (Osaka, Japan). A caspase-3 inhibitor (Ac-DNLD-CHO) was obtained from Peptide Institute, Inc. (Osaka, Japan). Recombinant active caspase-3, and -9 were purchased from Medical & Biological Laboratories (Nagoya, Japan). One unit of the recombinant caspase-3 or -9 is defined as the amount of enzyme that cleaves 1 nmol of each caspase substrate [DEVD-pNA or LEHD-pNA (pNA: pnitroanaline)] per hour at 37 °C in 50 mM HEPES buffer (pH 7.2) containing 100 mM NaCl, 10 mM DTT, 10 mM EDTA, 0.1% CHAPS and 5% glycerol. MALDI-TOF MS was measured on a Bruker autoflex III mass spectrometer by using 3,5-dimethoxy-4-hydroxycinnamic acid as a matrix.

Plasmid Construction and Expression of GFPN. An E. coli strain XL1-BLUE was used as the bacterial host for the construction of all plasmids. A DNA sequence encoding a N-terminal fragment (1–214) of GFP OPT (GFPN) was prepared by PCR amplification from full-length GFP OPT as a
template using primers, 5’-GC ATA TCG GAT CCG AGT AAA GGA GAA GAA CTT TTC-3’ and 5’-T CGA ATT CTC GAG TCA CTT TTC GTT GGG ATC TTT C -3’. The PCR product was ligated into pET22b(+) vector via the BamHI/XhoI restriction sites. The NdeI/BamHI site of the obtained plasmid was replaced with the DNA fragment ATG CAC CAT CAT CAC CAT GGC TCT TCG coding a 6× His tag (MHHHHHHHGSS). Sequence of the construct was verified by dye-terminator sequencing.

The GFPN protein was expressed in BL21(DE3) at 25 °C overnight under the control of T7 promoter with 0.5 mM IPTG. The proteins were purified with Ni-NTA (Qiagen) and Sephadex G-25 (GE Healthcare) columns. The purity and MW were confirmed by SDS PAGE analysis (Figure S2).

The DNA and amino acid sequences of GFPN are shown in Figure S3.

Plasmids Construction of DnaB Split-Inteins for Preparing Cyclic GFP C-Terminal Fragments, cM4(DEVD) and cM4(DEVG). The plasmid construct encoding DnaB split-intein was prepared by PCR using a pTWIN vector (New England BioLabs) as a template with appropriate primers. The PCR product encoding an intein C-terminal fragment (residues 106–154) was cloned into a modified pET22b vector (pET22b-6H-MCS-6H, Figure S4), which contains NsiI and EagI restriction sites in its MCS, via the NdeI/NsiI restriction sites. Subsequently, the PCR product coding an intein N-terminal fragment (1–105) was ligated via the EagI/XhoI sites to render pET22b-sDnaB. The DNA insert for cpM4(DEVD) was created with Klenow fragment DNA polymerase I using synthetic oligo DNAs, 5’-CAC AAC TCG GCC GCT GGT ATC ACC GGT GAT GAG GTG GAC GGT CGC GAT CAC ATG -3’ and 5’-GCC AGA GAT GCA TAC ATA TTC GTG CAG AAC CAT GTG ATC GCG ACC GTC CAC CTC -3’. The reaction product was ligated into the pET22b-sDnaB plasmid via EagI/NsiI sites to render a pET22b-sDnaB-cpM4(DEVD) plasmid. The DNA sequence encoding DEVD in the pET22b-sDnaB-cpM4(DEVD) was replaced with one encoding DEVG by overlap-extension PCR using primers 5’-GAT GAG GTG GgC GGT CGC GAT CAC ATG -3’ and 5’-GCG ACC GcC CAC CTC ATC ACC GGT GAT -3’ to confer pET22b-sDnaB-cpM4(DEVG). The intein-disabled version of construct, pET22b-sDnaB-cpM4(DEVG)-T70A/H73A, was created by overlap extension PCR using primers 5’-GCA GCA GCA AAT GCT AGA TTT TTA ACT ATT
GAT GG -3' and 5'- TCT AGC ATT TGC TGC TGC CTT GAT AGT TCT ACC TA -3'. Sequences of constructs were verified by dye-terminator sequencing. The DNA and amino acid sequences of split-intein constructs are shown in Figure S5-S7.

**SDS-PAGE Analysis of Split-Intein Fusions Expressed in *E. coli*.** All split-intein fusions were expressed in BL21(DE3) at 25 °C overnight under the control of T7 promoter with 0.5 mM IPTG. The cells were pelleted by centrifugation, and frozen at -20 °C overnight. Cells were resuspended in a bind buffer [20 mM Tris HCl (pH 7.9), 0.5 M NaCl, 5 mM imidazole, 0.5 mM TCEP] on ice bath and lysed using standard sonication protocols. The soluble fractions were loaded onto Ni-NTA agarose beads (Qiagen) pre-equilibrated with a bind buffer. After washing the column with 10 volumes of bind buffer and 6 volumes of wash buffer [20 mM Tris HCl (pH 7.9), 0.5 M NaCl, 30 mM imidazole, 0.5 mM TCEP], the proteins were eluted with 6 volumes of elution buffer [20 mM Tris HCl (pH 7.9), 0.5 M NaCl, 1 M imidazole, 0.5 mM TCEP]. Each eluted fraction was analyzed by SDS-PAGE to check the processing status of split-intein constructs.

**Preparation and Isolation of Cyclic GFP C-terminal Fragments Using PURESYSTEM Classic II.** In vitro transcription and translation of the intein construct was carried out using a pET22b-sDnaB-cpM4(DEVD), pET22b-sDnaB-cpM4(DEVD) or pET22b-sDnaB-cpM4(DEVD)-T70A/H73A plasmid (500 ng) in PURESYSTEM classic II (BioComber) in a total volume of 50 µL at 37 °C for 1 hr. The reaction mixture was diluted with 50 µL of water and incubated with 10 µL of a Ni-NTA agarose slurry (QIAGEN) at 4 °C for 3 hr to remove the His-tagged proteinous factors. The mixture was applied on an ultrafiltration membrane unit (Vivacon 500 100K, Sartorius stedim) and centrifuged at 4 °C and 1500 g for 1 hr for removal of ribosome. The flow-through was lyophilized and the residue was dissolved in 5% acetonitrile/water containing 0.1% trifluoroacetic acid. This was further cleaned using MonoTip C18 (GL Science) and analyzed by MALDI-TOF MS. cM4(DEVD), m/z 2354.39 [(M+H)^+] (Calcd. = 2354.15); cM4(DEVG), m/z 2296.56 [(M+H)^+] (Calcd. = 2296.51).
Caspase Sensing Using A Split-Intein Mediated Cyclic Peptide, cM4(DEVD). In vitro transcription and translation of cM4(DEVD) was carried out using a pET22b-sDnaB-cpM4(DEVD) plasmid (500 ng) in PURESYSTEM classic II in a total volume of 50 µL at 37°C for 1 hr. The 10 µL of reaction solution was mixed with a GFP N-terminal fragment (final protein concentration was 1 µM) in 50 mM HEPES buffer (pH 7.2) containing 100 mM NaCl, 10 mM DTT, 10 mM EDTA, 0.1% CHAPS and 5% glycerol with and without a caspase-3 inhibitor, Ac-DNLD-CHO. The proteolysis reaction was started by the addition of caspase-3 (1 u) in a total volume of 200 µL. The samples were subsequently incubated at 25 °C. Fluorescence spectra were measured on a JASCO FP-6500 fluorescence spectrophotometer at 25 °C. The concentration of cM4(DEVD) was estimated by comparing the maximum fluorescence intensity of the reconstituted split-GFP with that of an original split-GFP sample of known concentration.
**II. Supporting Figures**

**Figure S1.** (A) Fluorescence spectra of the GFPN/cM4(DEVD) mixture after 6 h incubation at 25 °C with various concentrations of caspase-3. (B) A plot of fluorescence intensity change ($\Delta F$) at 508 nm of the GFPN/cM4(DEVD) mixture as a function of caspase-3 concentration. The reconstitution reaction and the fluorescence measurement were performed as in Figure 3. $\Delta F = F - F_0$, where $F$ and $F_0$ denote fluorescent intensities after and before incubation, respectively. Arbitrary fluorescence units (A.U.).

**Figure S2.** SDS-PAGE of the purified proteins. 15% Polyacrylamide gel was used for the analysis. A gel was stained with Coomassie Brilliant Blue.
**Figure S3.** The DNA and amino acid sequence of GFPN on pET22b-6H-GFPN. Mutations (S30R, Y39I, F64L, S65T, F99S, N105K, E111V, I128T, Y145F, M153T, V163A, K166T, I171V, S205T, and A206V) are shown in **Red.** The linker and 6× His Tag are shown in **green and blue,** respectively.
pET22b-6H-MCS-6H

Figure S4. The DNA and amino acid sequence of a modified pET22b(+) vector, pET22b-6H-MCS-6H.
Figure S5. The DNA and amino acid sequence of sDnaB-cpM4(DEVD) on pET22b-sDnaB-cpM4(DEVD). DnaB C-intein (106-154), cpM4, caspase-3 substrate (DEVD), and DnaB N-intein (1-105) sequences are shown in cyan, red, pink, and blue, respectively.
Figure S6. The DNA and amino acid sequence of sDnaB-cpM4(DEVG) on pET22b- sDnaB-cpM4(DEVG). DnaB C-intein (106-154), cpM4, DEVG, and DnaB N-intein (1-105) sequences are shown in cyan, red, pink, and blue, respectively.
**Figure S7.** The DNA and amino acid sequence of sDnaB-cpM4(DEVD)-T70A/H73A on pET22b-sDnaB-cpM4(DEVD)-T70A/H72A. **DnaB C-intein** (106-154), **cpM4**, **caspase-3 substrate** (DEVD), and **DnaB N-intein** (1-105) sequences are shown in **cyan**, **red**, **pink**, and **blue**, respectively.