Supplementary Information

Application of Photoactive Yellow Protein as a Photoresponsive Module for Controlling Hemolytic Activity of Staphylococcal α-Hemolysin

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1. Materials and Methods

Construction of an expression vector for apo-PYP-fused Staphylococcal \( \alpha \)-Hemolysin (apo-PYP-Hla) and its cysteine mutant D108C/K154C (N-PYP-Hla-SS). A DNA fragment encoding staphylococcal \( \alpha \)-toxin without the signal sequence was amplified using KOD-Plus DNA polymerase (TOYOBO, Osaka, Japan), with \( S. \) aureus strain Mu50 (ATCC 700699) genomic DNA as the template and the following primers:

- \( \alpha \)-Hemolysin-S (5′-CATGCCATGCGAGATTCTGATATTAATATTAAACCGG-3′),
- \( \alpha \)-Hemolysin-AS (5′-CCGCTCGAGATTTGTCATTCTCTTTTTTTCCCAATCG-3′),

(recognition sites for the restriction enzyme are underlined). The PCR products were inserted into the NcoI and XhoI sites of the pET28-b vector (Merck, Whitehouse Station, NJ). A His\(_6\) tag was fused at the C terminus for purification by nickel-chelate affinity chromatography. A EcoRI site was introduced upstream of staphylococcal \( \alpha \)-toxin gene with KOD-Plus-Mutagenesis Kit (TOYOBO, Osaka, Japan) (pET28-b-Hla vector). Then, a DNA fragment encoding photoactive yellow protein was amplified using KOD-Plus DNA polymerase with the following primers:

- EcoRI_Hla_Back (5′-GAATTCAGCAGATTCTGATATAATTAAACCGG-3′),
- NcoI_Hla_Forward (5′-CCATGCGAGATTTGTCATTCTCTTTTTTTCCCAATCG-3′),

(recognition sites for restriction enzyme are underlined). The amplified fragment was cloned into the pET28-b-Hla vector, and then the stop codon and the EcoRI site were removed using a KOD-Plus-Mutagenesis Kit (TOYOBO). The expression vector for the cysteine mutant apo-PYP-Hla D108C/K154C was constructed by using KOD-Plus-Mutagenesis Kit (TOYOBO). The DNA oligonucleotide primers used for mutation of Asn108 and Lys154 to Cys were as follows: Hla_D108C_Back (5′-TGCAACAAAGAGATATGAGTACTTTAACTTATGG-3′),

Hla_D108C_Forward (5′-AATCGAATTTCTTGAGTAATCACGATAT-3′),

Hla_K154C_Back (5′-TGCAAAATTTTAGAGCCCCAATCG-3′),

Hla_K154C_Forward (5′-GAAATCAGGTTGACATATT CAGTG -3′). The constructs were verified by DNA sequencing service (Sigma-Aldrich, Japan).

Expression and purification of apo-PYP-Hla and its cysteine mutant D108C/K154C
(N-PYP-Hla-SS). *Escherichia coli* BL21(DE3) transformed with the expression vector was grown at 28 °C in 2× YT medium containing 16 g/L Tryptone, 10 g/L yeast extract, 5 g/L NaCl, and 30 mg/L kanamycin. After growing the culture up to an optical density of 0.8 at 600 nm, IPTG (a final concentration: 500 mM) was added to the culture to induce expression of the desired protein. Then the culture was incubated overnight and centrifuged at 7000 rpm for 15 min at 4 °C. After removal of the supernatant, the resulting pellet was resuspended in 50 mL Tris-HCl buffer (50 mM, pH 8.0, containing 200 mM NaCl). The resulting cell suspension was sonicated for 20 min on ice and then centrifuged at 40,000 ×g for 30 min at 4 °C. The supernatant was loaded on 1 mL of resin (Ni Sepharose 6 Fast Flow, GE Healthcare), which was then washed with 20 mL of washing buffer (5 mM Imidazole, 200 mM NaCl, and 50 mM Tris-HCl, pH 8.0). The target protein was eluted with 10 mL of elution buffer (250 mM imidazole, 200 mM NaCl, and 50 mM Tris-HCl, pH 8.0) at 4 °C. Fractions containing the desired protein were collected, dialyzed against Tris-HCl buffer (50 mM, pH 8.0 containing 200 mM NaCl) and then further purified on a size-exclusion chromatography (HiLoad™ 26/60 Superdex 200, GE Healthcare). The purity and homogeneity of the protein were evaluated by means of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The concentration of proteins was evaluated by Pierce® BCA™ Protein Assay Kit (Thermo Fisher Scientific Inc., USA).

**Synthesis of p-coumaric anhydride** — To a dry dichloromethane (DCM; 20 mL) solution of *p*-coumaric acid (328 mg, 2.0 mmol) was added 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; 192 mg, 1.0 mmol) at 0 °C, and then the resulting mixture was stirred for 5 h at 20 °C. Then, to the reaction mixture was added tetrahydrofuran (THF; 20 mL) and the resulting mixture was stirred overnight. The reaction mixture was washed with water (2 × 10 mL) and brine (10 mL) to allow isolation of *p*-coumaric anhydride in 47% yield (289 mg, 0.93 mmol).

^1^H NMR (400 MHz, CDCl3 containing 0.03% TMS, 22°C): δ 7.79 (d, J = 15.6 Hz, 2H), 7.48 (d, J = 8.8 Hz, 4H), 6.87 (d, J = 8.8 Hz, 4H), 6.38 (d, J = 15.6 Hz, 2H) ppm; MALDI-TOF MS (CHCA, positive mode): *m/z*: calculated for C_{18}H_{14}O_{5}: 310.32; found: 333.59 [M+Na]^+, 349.44 [M+K]^+. 
Reconstitution of the PYP domain and purification of N-PYP-Hla — To a Tris-HCl buffer (50 mM, pH 8.0, containing 200 mM NaCl) solution of apo-PYP-Hla (3 mg/mL), was added a DMF solution of p-coumaric anhydride (10-fold molar excess), and the resulting mixture was stirred overnight at 4 °C. The reaction mixture was filtered to remove insoluble substances, and then subjected to size-exclusion chromatography (HiLoad™ 26/60 Superdex 200, GE Healthcare) to allow isolation of the reconstituted protein N-PYP-Hla.

Hemolytic activity assay — Preserved blood collected from sheep (Nippon Biotest Laboratories inc., Tokyo, Japan) was centrifuged at 1,500 ×g for 5 min and washed several times with PBS. The erythrocytes were then resuspended in PBS (pH 7.4) so that the resulting mixture has a turbidity of 0.6 at 700 nm (≈ 0.06% erythrocytes, v/v) with a volume of 2.95 mL. Then the resulting suspension was incubated at 25 °C for 1 h. To the erythrocyte suspension thus prepared, was added a Tris-HCl buffer (50 mM, pH 8.0, containing 200 mM NaCl) solution of the protein (50 μL), and the activity was evaluated by measuring the decrease in turbidity at 700 nm in the dark and under irradiation with visible light (λ = 450 ± 10 nm; ASAHI SPECTRA LAX-102), respectively.

Transmission electron microscopy — An aliquot of a solution comprising N-PYP-Hla (0.1 mg/mL) in Tris-HCl (50 mM, pH 8.0, containing 200 mM NaCl and 6.25 mM sodium deoxycholate) was mounted on a Cu300 grid with a film of carbon-coated parlodion, and then the grid was washed with the same buffer as that for the sample. The excess solution was removed with filter paper. Then, the grid was negatively stained with 2% uranyl acetate for 10 min. The grid was examined under a Transmission Electron Microscope JEM1010 (JEOL, Japan) at an accelerating voltage of 80 kV. The electron micrographs were recorded on Fuji FG electron image film (11.8 × 8.2 cm, Fujifilm, Japan)².
2. Hemolytic Activity of Wild-type Hla

![Graph](image)

**Fig. S1.** Time course curves of hemolysis of sheep red blood cells by wild-type Hla in PBS (pH 7.4) at 25 °C without (blue) and under irradiation with visible light ($\lambda = 450$ nm ± 10 nm) (green). The suspension of erythrocytes in PBS (pH 7.4; OD$_{700}$ = 0.6; 2.95 mL), incubated at 25 °C for 1 h, was treated with a Tris-HCl buffer (50 mM, pH 8.0, containing 200 mM NaCl) solution of wild-type Hla (0.1 µM). OD$_{700}$ was monitored every 15 s. Data are means ± standard deviations (every 150 s for clarity) of 3 independent experiments.
3. Hemolytic Activity of N-PYP-Hla before and after Visible-Light Irradiation

Fig. S2. Time course curves of hemolysis of sheep red blood cells by N-PYP-Hla in PBS (pH 7.4) before and after irradiation with visible light (\( \lambda = 450 \text{ nm} \pm 10 \text{ nm} \)) for 3 h at 25 °C. The suspension of erythrocytes in PBS (pH 7.4; OD\(_{700} = 0.6; 2.95 \text{ mL} \)) incubated at 25 °C for 1 h, was treated with a Tris-HCl buffer (50 mM, pH 8.0, containing 200 mM NaCl) solution of no-irradiated and irradiated N-PYP-Hla (0.18 \( \mu \text{M} \)). OD\(_{700} \) was monitored in the dark every 300 s.
4. Change in Absorbance of N-PYP-Hla at 445 nm Corresponding to the Chromophore in Response to Irradiation

![Graph showing absorbance change](image)

**Fig. S3** Reversible change of absorbance at 445 nm of N-PYP-Hla (16 μM) (corresponding to the absorption of p-coumaric thioester in trans-configuration) in Tris-HCl buffer (pH 8.0) containing 200 mM NaCl and 6.25 mM sodium deoxycholate. The absorbances of N-PYP-Hla were monitored under irradiation with visible lights, where those at the ground state were measured by using neutral density (ND) filter between the light source and the sample (filled circles) and those under irradiation were measured without the filter (filled squares). The UV cut-off filter (λ < 310 nm) was used for reducing the photodamage to the sample.
5. Hemolytic Activity of N-PYP-Hla-SS

**Fig. S4.** Time course curves of hemolysis of sheep red blood cells by N-PYP-Hla-SS in PBS (pH 7.4) at 25 °C. The suspension of erythrocytes in PBS (pH 7.4; OD$_{700}$ = 0.6; 2.95 mL), incubated at 25 °C for 1 h, was treated with a Tris-HCl buffer (50 mM, pH 8.0, containing 200 mM NaCl) solution of N-PYP-Hla-SS (0.20 µM). After further incubation for 1 h in the dark at 25 °C, 10 mM dithiothreitol (DTT) was added to the resulting mixture, whereby OD$_{700}$ was monitored in the dark (blue) or under irradiation with visible light ($\lambda$ = 450 nm ± 10 nm) (green). OD$_{700}$ in the absence of DTT was also monitored in the dark (orange).
6. References
