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# A novel catalytic function of synthetic IgG-binding domain (Z domain) from staphylococcal protein A: Light emission with coelenterazine

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## Abstract

The synthetic IgG-binding domain (Z domain) of staphylococcal protein A has a catalytic function to oxidize coelenterazine to emit light like a coelenterazine-utilizing luciferase. The Z domain derivatives (ZZ-gCys, Z-gCys and Z-domain) were purified and the luminescence properties were characterized by comparing with coelenterazine-utilizing luciferases, including *Renilla* luciferase, *Gaussia* luciferase and the catalytic domain of *Oplophorus* luciferase. Three Z domain derivatives showed luminescence activity with coelenterazine and the order of the initial maximum intensity of luminescence was ZZ-gCys (100%) > Z-gCys (36.8%) > Z-domain (1.1%) > bovine serum albumin (0.9%) > staphylococcal protein A (0.1%) and the background value of coelenterazine (0.1%) in our conditions. The luminescence properties of ZZ-gCys showed the similarity to that of *Gaussia* luciferase, including the luminescence pattern, the emission spectrum, the stimulation by halogen ions and non-ionic detergents, and the substrate specificity for coelenterazine analogues. In contrast, the luminescence properties of Z-gCys were close to the catalytic domain of *Oplophorus* luciferase. The catalytic region of the Z domain for the luminescence reaction might be different from the IgG-binding region of the Z domain.

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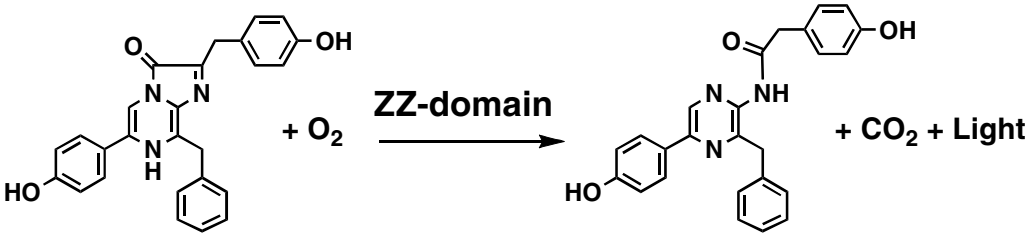
**Abbreviations:** ZZ-gCys, ZZ domain with a cysteine residue at the carboxyl terminus; Z-gCys, Z domain with a cysteine residue at the carboxyl terminus; Z-domain, a monomer of Z domain; MCS, multiple cloning site; $I_{max}$, initial maximum intensity of luminescence: rlu, relative light units; PBS, 10 mM phosphate buffer (pH 7.4) containing 137 mM NaCl and 2.7 mM KCl.

## Introduction

Protein A of *Staphylococcus aureus* has a high specific affinity to immunoglobulin G (IgG) and binds the Fc region of IgG from most mammalian species (1). Protein A has been used in the IgG purification and the immunological assay (1). The gene fragment coding for a functional analogue of IgG-binding domain B of protein A, designated Z domain (58 amino acid residues), was chemically synthesized and expressed in *Escherichia coli* cells (2). The ZZ domain consisting of two repeat sequences of the Z domain with 116 amino acid residues shows the binding ability to IgG (2) and was used as an affinity tag for protein purification (3).

Recently, we used the ZZ domain as a soluble partner in the cold inducible expression system of *E. coli* cells (4, 5) and succeeded in expressing the heterogeneous proteins efficiently as a soluble form in the cytoplasm of *E. coli* cells by using a novel expression vector of pCold-ZZ-X (4). To study the formation of the fluorescent chromophore possessing an imidazolone structure in green fluorescent protein (GFP) (6-9), we expressed the fusion protein of the ZZ domain to GFP (ZZ-GFP) at 15 $^{o}$C in *E. coli* cells and 139 mg of the purified ZZ-GFP was obtained from 2 L of cultured cells (unpublished). In the course of characterizing the biochemical properties of ZZ-GFP, we found that ZZ-GFP has a catalytic function like a luciferase with coelenterazine (a luciferin). Previously, we purified recombinant GFP from *E. coli* cells (6,7) and examined the luminescence activity of GFP with coelenterazine under various assay conditions. However, GFP did not show any luminescence activity with coelenterazine (unpublished). From these results, we presumed that the ZZ domain of ZZ-GFP could catalyze the oxidation of coelenterazine to emit light in the following reaction scheme.




**Coelenterazine** **Coelenteramide**

Coelenterazine, an imidazopyrazinone (3,7-dihydropyrazinon-3-one) compound, is widely distributed in marine organisms (10,11) and is known as a luciferin for many luciferases including *Renilla* (12), *Oplophorus* (13), *Periphylla* (14), *Gaussia* (15), *Metridia* (16) and

*Conchoecia* (17) (referred to as "coelenterazine-type luciferase" in this paper). Coelenterazine also serves as a light–emitting substrate for the $Ca^{2+}$-binding photoproteins such as aequorin (18, 19).

On the other hand, we have prepared recombinant aequorin and *Gaussia* luciferase having a reactive cysteine residue to conjugate with a maleimide-activated ligand and used them for bioluminescent immunoassays (20, 21). In the similar way, we prepared the ZZ domain possessing a reactive cysteine residue at the carboxyl terminus, designated ZZ-gCys, and used ZZ-gCys as an affinity ligand to purify IgG (unpublished). Under these circumstances, we examined the luminescence activity of ZZ-gCys with coelenterazine and found that the ZZ domain catalyzes the oxidation of coelenterazine to emit light like a coelenterazine-type luciferase (12-17).

In this report, we purified the Z domain derivatives, ZZ-gCys, Z-gCys and Z-domain and characterized their luminescence properties by comparing with those of coelenterazine-type luciferases. As a result, we concluded that the catalytic properties of ZZ-gCys and Z-gCys are similar to that of *Gaussia* luciferase (22) and *Oplophorus* luciferase (23), respectively, and a small protein/polypeptide can catalyze the oxidation of coelenterazine.

## MATERIALS AND METHODS

***Construction of the expression vectors for ZZ domain (ZZ-gCys) and Z domains (Z-gCys and Z-domain) in E. coli cells***. The strategy for constructing the expression vectors was summarized in Fig. 1. The cold inducible expression vectors of pCold-ZZ-X (4) and pCold-ZZ (4) were used for the ZZ domain (ZZ-gCys, the ZZ domain possessing a flexible linker and a cysteine residue at the carboxyl terminus) and the Z domains (Z-gCys and Z-domain), respectively. The expression vector of pCold-ZZ-X (4) has the sequences of a six-histidine tag for the nickel chelate affinity purification, the ZZ domain (116 amino acid residues), and the multiple cloning site (MCS). The protein expression was under the control of the *cspA* promoter and the *lac* repressor from a pCold II vector (24). To express ZZ-gCys, the oligonucleotide for a flexible linker sequence with a cysteine residue (GGGGSGGGGSGGGGC: gCys) was chemically synthesized and inserted into MCS of pCold-ZZ-X as follows: the synthetic oligonucleotides of gCys Linker-F (5' AG CTT GGT GGT GGT GGT TCT GGT GGT GGT GGT TCT GGT GGT GGT GGT TGC TAA GCT AGC CTG CA 3', new *Nhe*I site underlined) and gCys Linker-R (5' G GCT AGC TTA GCA ACC ACC ACC ACC AGA ACC ACC ACC ACC AGA ACC ACC ACC ACC A 3') for "gCys Linker" were synthesized (Fig. 1) and inserted into the *Hin*dIII/*Pst*I site of pCold-ZZ-X to give pCold-ZZ-gCys (Fig. 2-A). The expressed ZZ-gCys had the sequences of histidine-tag, ZZ

>Figure 1<

domain, followed by the flexible linker with a new cysteine residue at the carboxyl terminus. For expressing Z-gCys and Z-domain in *E. coli* cells, the vectors of pCold-Z-Cys and pCold-Z were prepared by self-ligation after digesting with *Bgl*II (Fig. 1).

***Expression and purification of ZZ domain (ZZ-gCys) and Z domains (Z-gCys and Z-domain) from E. coli cells.*** The bacterial strain BL21 (Novagen, Madison, WI) was used as a host. The procedures for the protein expression were essentially the same as previously described (4,5). Briefly, the seed culture of BL21 possessing an expression vector was grown in 10 mL of Luria-Bertani broth containing ampicillin (100 μg/mL) at 37 °C for 18 h, and was transferred into 400 mL of LB broth containing 20 μL of antifoam (Disfoam CE457: NOF Co., Tokyo, Japan) in a 3 L of Sakaguchi flask, incubated for 2.5 h at 37 °C and then cooled on an ice-water bath for 1 h. After adding isopropyl-β-thiogalactopyranoside (Wako Pure Chemicals, Osaka, Japan) to the culture medium at a final concentration of 0.2 mM, the bacterial cells were incubated at 15 °C for 17 h. The cells harvested by centrifugation at 4,700*g* for 5 min were suspended in 200 mL of 50 mM Tris-HCl (pH 7.6) and were disrupted by sonication using a Brabson model 250 sonifire (Danbury, CT) for 9 min (3 min x 3) in an ice-water bath. After centrifugation at 12,000*g* for 20 min at 4 °C, the supernatant obtained was applied on an IgG-Sepharose 6 Fast Flow column (ϕ 2.5 x 6.0 cm and ϕ1.5 x 6.5 cm for 2 L and 0.8 L of cultured cells, respectively: GE Healthcare, Uppsala, Sweden) equilibrated with 20 mM Tris-HCl (pH 7.4) containing 150 mM NaCl and 0.5% (w/v) Tween 20 (TBST) at room temperature. After washing the column with 300 mL of TBST and 100 mL of ammonium acetate (pH 5.0), the adsorbed proteins were eluted by stepwise with 10 mL of 0.5 M acetic acid (pH 3.4). The eluted fraction was analyzed by SDS-PAGE and the protein fractions were dialyzed against 4 L of 100 mM sodium phosphate buffer (pH 7.3) at 4 °C for 72 h. For long-term storage, the protein dialyzed was stored at -80 °C.

***Determination of luminescence activity*.** Recombinant protein A (rPA50, Lot. RN092399) was obtained from RepliGen Co. (Waltham, MA). Bovine serum albumin (Fraction V: A3059-100G, Lot. 116K0757) and human IgG from human serum (Lot. 44204601) were obtained from Sigma Co. (St. Louis, MO) and Oriental Yeast Co. Ltd. (Tokyo, Japan), respectively. Recombinant *Renilla* luciferase (25) and *Gaussia* luciferase (22) was purified as previously described. Coelenterazine was obtained form JNC Corp. (Tokyo, Japan) and the C-2 modified coelenterazine analogues were prepared as previously described (26). (*S*)-Cypridina luciferin was obtained from Prolume Ltd. (Pinetop, AZ). The reaction mixture (100 μL) contained a luciferin (coelenterazine, its analogue, or Cypridina luciferin;1 μg

dissolved in 1 μL of ethanol) in 30 mM Tris-HCl (pH 7.6)–10 mM EDTA and the luminescence reaction was initiated by the addition of 1-15 μL of a protein solution into the reaction mixture. The luminescence intensity (n = 2) was measured using an Atto (Tokyo, Japan) AB2200 luminometer (Ver.2.07, rev.4.21) equipped with a Hamamatsu R4220P photomultiplier in 0.1 s intervals for 60 s without an attenuation filter. As recombinant aequorin shows 4.8 x $10^{15}$ photons/mg protein (27), we used recombinant aequorin as a light standard for the luminometer. The initial maximum intensity of luminescence ($I_{max}$) of 1 pg of the purified recombinant aequorin (27) showed 1.3 x $10^3$ relative light unit (rlu)/0.1 s and one rlu was estimated to be 3.7 x $10^3$ photons/0.1 s in our assay conditions. To examine the effects of NaCl concentrations and halogen ions on luminescence activity, 50 mM sodium phosphate buffer (pH 7.5) was used instead of 100 mM Tris-HCl (pH 7.6). For determining the optimal pH, the following buffers were used: 100 mM sodium acetate buffer (pH 5.0), 100 mM sodium phosphate buffer (pH 6.0, 6.5, 7.0 and 7.5) and 100 mM Tris-HCl buffer (pH 7.0, 7.5, 8.0 and 8.5).

***Protein analysis.*** Protein concentration was determined by the dye-binding method of Bradford (28) using a commercially available kit (Bio-Rad, Richmond, CA) and bovine serum albumin as a standard (Pierce; Rockford, IL). The concentration of purified ZZ-gCys, Z-gCys and Z-domain was determined by the absorbance at 280 nm in 0.1% solution. The calculated absorbance values of 0.166, 0.131 and 0.1500, which were obtained using a program ProtParam (http://web.expasy.org/protparam/), were used for ZZ-gCys, Z-gCys and Z-domain. SDS-PAGE analysis was carried out under reducing or non-reducing conditions using a 16% separation gel (TEFCO, Tokyo, Japan) (29) and the gels were stained with a colloidal CBB staining kit (TEFCO).

***Measurement of luminescence emission spectrum.*** The luminescence emission spectra of ZZ-gCys, Z-gCys and Z-domain with coelenterazine were measured on a Jasco FP-6500 fluorescence spectrophotometer (emission band width, 20 nm; response, 0.5 sec; sensitivity, medium; scan speed, 1000 nm/min) at 22~25 $^{o}$C with the excitation light source turned off. The reaction mixture (500 μL) contained 0.5 mg of the purified proteins in 30 mM Tris-HCl (pH 7.6)-10 mM EDTA and the luminescence reaction was initiated by the addition of 5 μg of coelenterazine dissolved in 5 μL of ethanol. The corrected luminescence emission spectrum was obtained according to the manufacture's protocol.

***Identification of oxidation products of coelenterazine by HPLC analysis.*** The reaction mixture (0.5 mL) contained 0.5 mg of ZZ-gCys and 10 μg of coelenterazine (dissolved in 10 μL of ethanol) in 30 mM Tris-HCl (pH 7.6)-10 mM EDTA. After the luminescence reaction for 1 min at 25 °C, 100 μL of the reaction mixture was added to 0.5 mL of ether and stored at -80 °C over 10 min. The ether layer recovered by centrifugation at 10,000 rpm for 3 min was dry down *in vacuo*. The resultant precipitate was dissolved in 20 μL of ethanol and 5 μL was analyzed by HPLC. The analytical conditions were follows: Agilent (CA, USA) 1100 series HPLC system. column: Wakosil 5C4 (5 mm, 4.6 mm × 250 mm, Wako Pure Chemicals); eluting solvent: $CH_3CN$ containing 0.1% trifluoroacetic acid; gradient elution: 40% $CH_3CN$ for 10 min, 40-50% $CH_3CN$ for 20 min, 50-80% $CH_3CN$ for 10 min and 80% $CH_3CN$ for 10 min; flow rate: 0.5 mL/min; detector: 225 nm, 330 nm and 450 nm. The retention times of coelenterazine, coelenteramide, and coelenteramine were 14.3 min, 21.0 min, 13.6 min, respectively. The amounts of coelenterazine, coelenteramide, and coelenteramine were determined by using the authentic solutions of synthetic compounds (30).

## RESULTS AND DISCUSSION

***Expression and purification of ZZ-gCys, Z-gCys and Z-domain.*** The ZZ-gCys, Z-gCys and Z-domain were expressed in *E. coli* cells using the cold inducible expression vectors of pCold-ZZ-gCys, pCold-Z-gCys and pCold-Z, respectively, (Fig. 1), and were purified from the soluble fractions of *E. coli* cells in a single step using an IgG Sepharose column. As the amounts of the purified Z-gCys and Z-domain were not determined accurately by the dye-binding method of Bradford (28), the amount of protein was estimated by using the calculated absorbance value at 280 nm in 0.1% solution as described in *Materials and Methods*. The yield of the purified ZZ-gCys was 96.4 mg from 2 L of cultured cells, which was in good agreement with that by the dye-binding method. From 0.8 L of cultured cells, the yields of Z-gCys and Z-domain estimated were 29.4 mg and 21.1 mg, respectively (Table 1). On SDS-PAGE analysis under reducing conditions, the single bands of ZZ-gCys (19 kDa), Z-gCys (11 kDa) and Z-domain (10.5 kDa) were observed and the purities were estimated to be over 95% (Fig. 3A). Under non-reducing conditions, however, the dimer of Z-gCys with a disulfide bond was detected at the molecular size of 23.5 kDa, showing over 50% density on a gel (Fig. 3B, lane 2). On the other hand, the percentage of the ZZ-gCys dimer was lower than that of Z-gCys. The density of ZZ-gCys dimer (32.5 kDa) on a gel was ~10% of the purified ZZ-gCys. The dimer formation of ZZ-gCys increased slowly at 4 °C over 1 month (Fig. 3B, lane 1). From this reason, we used the protein fractions containing a dimer for assays.

***Luminescence activity of ZZ-gCys, Z-gCys and Z-domain.*** The luminescence activities of ZZ-gCys, Z-gCys and Z-domain with coelenterazine as a substrate were examined by comparison with recombinant staphylococcal protein A, bovine serum albumin (BSA) and recombinant *Renilla* luciferase. As shown in Table 2, ZZ-gCys and Z-gCys showed luminescence activity with coelenterazine and Z-domain had measurable luminescence activity. In contrast, protein A did not show the luminescence activity and the initial maximum intensity of luminescence ($I_{max}$) was same to that of the background level from coelenterazine (Table 2). BSA had very weak luminescence activity with coelenterazine as previously reported (31,32), but was only 0.9% of $I_{max}$ to ZZ-gCys. The $I_{max}$ value of ZZ-gCys was 2.7 times higher than that of Z-gCys. The integrated luminescence activity of ZZ-gCys for 60 s was 5.6 times lower than that of Z-gCys, due to the different luminescence pattern from Z-gCys (Fig. 4). The order of $I_{max}$ was ZZ-gCys (100%) > Z-gCys (36.8%) > Z-domain (1.1%) > bovine serum albumin (0.9%) > staphylococcal protein A (0.1%) and the background value of coelenterazine (0.1%). The dimer of Z-gCys gave the higher luminescence activity than that of the monomer of Z-domain. The luminescence intensities of ZZ-gCys and Z-gCys were dependent on the amounts of proteins (Table 2). Further, the luminescence activity of ZZ-gCys was lost by heat treatment at 95 $^{o}$C for 10 min (Table 3). Also Z-gCys and Z-domain lost luminescence activity by heat treatment (data not shown). These results indicated that the Z domain has a catalytic function to emit light and the light-emitting process with coelenterazine is an enzymatic reaction like a coelenterazine-type luciferase. However, the $I_{max}$ value of ZZ-gCys was over 60,000 times lower than that of recombinant *Renilla* luciferase (25) (Table 2). On the other hand, the $I_{max}$ value of *Renilla* luciferase was ~10 times lower than that of *Gaussia* luciferase and was ~100 times higher than that of the catalytic 19 kDa domain of *Oplophorus* luciferase as previously reported [33].

***Oxidation process of coelenterazine by ZZ-gCys.*** To investigate the oxidation process of coelenterazine by ZZ-gCys, the luminescence products of coelenterazine were analyzed by reversed-phase HPLC. The reaction mixture containing 0.5 mg of ZZ-gCys and 10 μg of coelenterazine was incubated only for 1 min in 30 mM Tris-HCl (pH 7.6)-10 mM EDTA, avoiding the auto-oxidation of coelenterazine. The luminescence products of coelenterazine were identified as coelenteramide and coelenteramine, and the reaction mixture contained unreacted coelenterazine (88%), coelenteramide (5%) and coelenteramine (7%). The catalytic ability of luminescence by ZZ-gCys was low, but it was interesting that the value of 7% for coelenteramine was unexpectedly high. As coelenteramine was not hydrolyzed from coelenteramide in our reaction conditions, coelenteramine might be produced directly from

coelenterazine after the oxygenation of coelenterazine [33]. Presumably, the low luminescence activity of the Z and ZZ domains might be explained by low oxidation efficiency of coelenterazine and less stabilization of the light-emitting species of coelenteramide.

***Luminescence patterns and luminescence emission spectra of ZZ-gCys, Z-gCys and Z-domain with coelenterazine.*** The luminescence pattern of a luciferin-luciferase reaction gives an insight into the kinetics property of a luciferase, as previously described in the case of the firefly luciferase-luciferin reaction (34). The coelenterazine-type luciferases from *Renilla* (25), *Oplophorus* (23) and *Conchoecia* (17) show the steady-state kinetics with a glow luminescence pattern. In contrast, *Gaussia* and *Periphylla* luciferases show a rapid decay pattern with flash-light emission in the presence of excess coelenterazine (14, 15, 35). As shown in Fig. 4A, light emission by ZZ-gCys rose quickly followed by a rapid decay, and then continuous weak-glow luminescence was observed. This luminescence pattern was similar to that of *Gaussia* luciferase and *Periphylla* luciferase. The rapid decay of luminescence in these proteins might be explained by the product inhibition of coelenteramide, the oxidation product of coelenterazine. On the other hand, Z-gCys and Z-domain showed the continuous glow luminescence, similar to *Renilla*, *Oplophorus* and *Conchoecia* luciferases (Fig. 4A).

The luminescence emission spectra of ZZ-gCys, Z-gCys and Z-domain with coelenterazine were measured under the same conditions at pH 7.6, and the corrected emission peaks and a full width at half maximum of spectra (FWHM) were determined (Fig. 5). The emission peaks of ZZ-gCys, Z-gCys and Z-domain were at 490 nm (FWHM = 80 nm), 460 nm (FWHM = 77 nm) and 466 nm (FWHM= 83 nm), respectively. The luminescence emission spectrum of ZZ-gCys was different from that of Z-gCys and Z-domain. Interestingly, the emission peak and FWHM of ZZ-gCys was close to that of *Gaussia* luciferase ($\lambda$max = 488 nm, FWHM = 81 nm) (22). In contrast, the luminescence emission spectra of Z-gCys and Z-domain were close to that of the catalytic 19 kDa domain of *Oplophorus* luciferase ($\lambda$max = 460 nm, FWHM = 72 nm) (23). The different luminescence emission spectra of them were caused by the emitting species of coelenteramide, the oxidation product of coelenterazine. To understand the luminescence mechanism of coelenterazine and Cypridina luciferin by a luciferase, the studies on the chemiluminescence of imidazopyrazinone derivatives have been performed in organic solvents (10, 36, 37). The light-emitting species of coelenteramide have been proposed by the fluorescence spectral analyses. In our findings, the different light-emitting spectra between the Z domain and the ZZ domain might be explained by the light-emitting species of coelenteramide; amide anion of coelenteramide ($\lambda_{max}$ 450 - 470 nm) for Z domain and phenolate anion ($\lambda_{max}$ 480 nm) for ZZ domain. However, it is difficult to

explain how difference spectra were produced by an identical domain in the Z domain and the ZZ domain.

***Effects of IgG binding to Z domain on luminescence activity.*** It is known that the Z domain has an ability to bind IgG (1, 2) and the Z domain derivatives were purified by an IgG Sepharose column. To investigate whether the binding of IgG to the Z domain inhibits luminescence activity of ZZ-gCys, Z-gCys and Z-domain, the effect of IgG on luminescence activity was examined (Table 4). If the catalytic region of the Z domain with coelenterazine might be identical to the IgG-binding region, the luminescence activity should be decreased significantly. However, the addition of IgG to ZZ-gCys at a 1:1 molar ratio gave about 1.5 time stimulation of luminescence intensity (Fig. 4C), and the luminescence activity of Z-gCys was not stimulated or inhibited. These results suggested that the catalytic region of the Z domain with coelenterazine was different from the IgG-binding region of the Z domain. Previously, the solution structures of the B and Z domains of staphylococcal protein A have been determined by NMR spectroscopy (38-41). These domains are composed of an antiparallel three α-helices (helix 1, helix 2 and helix 3) and the Fc portion of IgG binds the helix 1 and helix 2 in the B and Z domains. The luminescence stimulation of ZZ-gCys by IgG could be explained by binding IgG to the helix 1 and helix 2 of Z domain and the protein structure of ZZ-gCys could be stabilized. Presumably, a part of the helix 3 of the Z domain was related to the luminescence reaction. On the other hand, the stimulation of luminescence intensity was also observed in the presence of BSA, and might be explained by the protein stabilization (Table 4).

***Optimal pH for the luminescence reaction by ZZ-gCys and Z-gCys with coelenterazine.*** The luminescence activity ($I_{max}$) of ZZ-gCys and Z-gCys was determined under various pH conditions. The optimal pH for ZZ-gCys was found to be around 7.5 in 100 mM Tris-HCl buffer, and over 80% activity was observed between pH 7.0 and 8.0. Thus, the pH profile of ZZ-gCys was close to that of *Gaussia* luciferase (22). On the other hand, Z-gCys showed the optimal pH at 8.0 in sodium phosphate buffer and Tris-HCl buffer, and over 80% activity was observed between pH 7.0 and 9.0 (Supplementary Fig. S1) .

***Stimulation of luminescence activity of ZZ-gCys by halogen ions and non-ionic detergent.*** Previously, we reported that the luminescence intensity of recombinant *Gaussia* luciferase was stimulated by non-ionic detergents, sodium chloride, and halogen ions (22). To compare the luminescence property of ZZ-gCys with *Gaussia* luciferase, the effects of Tween 20, sodium chloride and halogen ions on the luminescence activity of ZZ-gCys were examined.

The $I_{max}$ value of ZZ-gCys was 4-folds stimulated by 0.01% Tween 20 (Fig. 4B and Fig. 6A). The effective concentration of 0.01% Tween 20 on ZZ-gCys was similar to that of *Gaussia* luciferase. Further, 100 mM NaCl stimulated the luminescence intensity of ZZ-gCys significantly (Fig. 6B, Table 5). Further, the halogen ions such as $Cl^-$, $Br^-$, and $I^-$ played as a stimulator on the luminescence activity of ZZ-gCys (Table 6). The stimulation of halogen ions was the same to *Gaussia* luciferase (22) and the mechanism of stimulation was not clear. In contrast, the luminescence intensities of Z-gCys and Z-domain were not stimulated by Tween 20 and halogen ions. These results strongly suggested that the catalytic process of luminescence by ZZ-gCys might be similar to that by *Gaussia* luciferase.

***Substrate specificities of ZZ-gCys, Z-gCys and Z-domain for coelenterazine analogues and Cypridina luciferin.*** The substrate specificities of ZZ-gCys, Z-gCys and Z-domain were characterized using the C-2 modified coelenterazine analogues (26) and were compared with the coelenterazine-type luciferases (33). As a result, ZZ-gCys showed narrow substrate specificity, indicating highly specific for coelenterazine (Table 7). This narrow substrate specificity of ZZ-gCys was similar to that of *Gaussia* luciferase, as previously reported (22, 33). On the other hand, Z-gCys and Z-domain had broad specificity for coelenterazine analogues and this was similar to *Oplophorus* luciferase (23,25, 33). Among coelenterazine analogues, *ameh*-coelenterazine is only used for the catalytic19 kDa protein of *Oplophorus* luciferase (33), but not for *Renilla* luciferase, *Gaussia* luciferase and aequorin (33). Other imidazopyrazinone-type luciferin, (*S*)-Cypridina luciferin, was not utilized for the luminescence reactions by ZZ-gCys, Z-gCys and Z-domain, as same as by *Gaussia* luciferase and *Oplophorus* luciferase.

***Similarity of the luminescence reaction of the Z domain to coelenterazine-type luciferase.*** It is known that coelenterazine-type luciferases from *Renilla, Oplophorus* and *Gaussia* do not have any similarities in their primary structures. The luminescence mechanism of coelenterazine catalyzed by coelenterazine-type luciferases is essentially the same (10). The differences with luminescence efficiency and emission spectrum might be explained by the environment of the light-emitter species in a protein (enzyme) (36, 37). As we described above, the Z domain and the ZZ domain clearly showed luminescence activity with coelenterazine. The primary structure of the Z domain with 58 amino acid residues has no significant homology with that of coelenterazine-type luciferases. However, the luminescence properties of the ZZ domain and the Z domain were close to *Gaussia* luciferase and *Oplophorus* luciferase, respectively. The secreted *Gaussia* luciferase consists of 168 amino acid residues, having two

repeat domain with 71 amino acid residues (22), and each domain in *Gaussia* luciferase shows the weak luminescence activity of luciferase. The catalytic domain of *Oplophorus* luciferase is 169 amino acid residues and composes of two structural units (23). These results suggested that a small protein/polypeptide could have a catalytic ability to oxidize coelenterazine and the folded structure of a luciferase is of considerable important to a catalytic function of luminescence.

***Conclusion.*** We found that the synthetic IgG-binding domains (ZZ domain and Z domain) derived from staphylococcal protein A have a catalytic function to oxidize coelenterazine to emit light like a coelenterazine-utilizing luciferase. The ZZ domain showed the similar luminescence properties to *Gaussia* luciferase, including the luminescence pattern, the emission spectrum, the stimulation by halogen ions and non-ionic detergents, and the substrate specificity for coelenterazine analogues. On the other hand, the luminescence properties of the Z domain were close to the catalytic domain of *Oplophorus* luciferase. The catalytic region for the luminescence reaction might be different from the IgG-binding region of the Z domain.

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## Supplementary Material:

Figure S1 can be found online on DOI: xxxxx

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**Table 1. Purification of ZZ-gCys, Z-gCys and Z-domain from *E. coli* cells using IgG Sepharose column.**

| Protein | Purification steps | Total volume (mL) | Total protein (mg) |
|---|---|---|---|
| ZZ-gCys | Soluble fraction (12,000*g* sup) from 2 L of cultured cells | 200 | 880 |
| | Dialysis fraction after IgG Sepharose column (ϕ2.5 x 6 cm) | 22 | 94.6 [a] |
| Z-gCys | Soluble fraction (12,000*g* sup) from 0.8 L of cultured cells | 80 | 384 |
| | Dialysis fraction after IgG Sepharose column (ϕ1.5 x 6.5 cm) | 19 | 29.4 [a] |
| Z-domain | Soluble fraction (12,000*g* sup) from 0.8 L of cultured cells | 80 | N.D.[b] |
| | Dialysis fraction after IgG Sepharose column (ϕ1.5 x 6.5 cm) | 10 | 21.1 [a] |

[a] Protein concentration is determined by the absorbance at 280 nm, as described in *Materials and Methods.*

[b] N.D. Not determined.

**Table 2. Luminescence activities of ZZ-gCys, Z-gCys and Z-domain using coelenterazine as a substrate.**

| Protein | Protein conc. (μg/100 μL) | Luminescence intensity (rlu) [b] | | | |
|---|---|---|---|---|---|
| | | $I_{max}$ | | Integration for 60 s | |
| None | 0 | 15 | (0.1) [a] | 3,638 | (0.8) [a] |
| ZZ-gCys | 1 | 2,847 | | 66,199 | |
| | 5 | 16,266 | (100) [a] | 456,992 | (100) [a] |
| | 15 | 59,865 | | 2,632,829 | |
| Z-gCys | 1 | 990 | | 251,305 | |
| | 5 | 5,991 | (36.8) [a] | 2,565,133 | (561) [a] |
| | 15 | 21,488 | | 8,536,031 | |
| Z-domain | 1 | 41 | | 4,721 | |

| | | | | | |
|---|---|---|---|---|---|
| | 5 | 172 | (1.1) [a] | 26,888 | (5.9) [a] |
| | 15 | 1,137 | | 314,559 | |
| Protein A | 5 | 14 | (0.1) [a] | 3,024 | (0.7) [a] |
| | 100 | 28 | | 8,350 | |
| Bovine serum albumin (BSA) | 5 | 144 | (0.9) [a] | 64,261 | (14.1) [a] |
| | 100 | 2,033 | | 1,015,256 | |
| *Renilla* luciferase | 0.005 | 1,075,980 | | 225,961,817 [c] | |

[a] Relative luminescence intensity to ZZ-gCys (100% as 5 μg protein used).

[b] The reaction mixture (100 μL) contains 1 μg of coelenterazine (1 μg/μL dissolved in ethanol) in 30 mM Tris-HCl (pH 7.6)-10 mM EDTA. The luminescence reaction is started by the addition of the protein solution into the reaction mixture and luminescence intensity was recorded by a luminometer for 60 s in 0.1 s intervals.

[c] Integration for 30 s in 0.1 s intervals.

**Table 3. Effect of heat treatment on the luminescence activity of ZZ-gCys.**

| Protein (5 μg/100 μL) | Heat treatment [a] | Luminescence intensity [b] ($I_{max}$, rlu) (%) |
|---|---|---|
| None | - | 20 (-) |
| ZZ-gCys | - | 13,572 (100) |
| | + | 366 (0.26) |
| Protein A | - | 14 (0) |
| | + | 14 (0) |
| BSA | - | 144 (0.09) |
| | + | 71 (0.04) |

[a] Protein solutions are heated at 95 $^{o}$C for 10 min and used for assays.

[b] Assay conditions are the same as described in Table 2.

**Table 4. Effects of IgG and BSA on the luminescence activities of ZZ-gCys, Z-gCys and Z-domain.**

| Conditions [a] | Protein molar ratio to IgG or BSA | Luminescence intensity ($I_{max}$, rlu) (%) |
|---|---|---|
| IgG | 0:1 | 38 |
| BSA | 0:1 | 711 |
| ZZ-gCys | 1:0 | 9,808 (100) |
| ZZ-gCys + IgG | 1:1 | 16,107 (164) |
| ZZ-gCys + BSA | 1:1 | 22,705 (231) |
| Z-gCys | 1:0 | 1,070 (100) |
| Z-gCys + IgG | 1:1 | 1,062 (100) |
| Z-gCys + BSA | 1:1 | 2,221 (208) |
| Z-domain | 1:0 | 52 (100) |
| Z-domain + IgG | 1:1 | 138 (265) |
| Z-domain + BSA | 1:1 | 840 (162) |

[a] The reaction mixture (100 μL) contains IgG or BSA with Z-domain, Z-gCys or ZZ-gCys (5 μg) in PBS and incubate at room temperature for 20 min, and then the luminescence reaction was started by the addition of 1 μg of coelenterazine (1 μg/μL dissolved in ethanol) into the reaction mixture. The luminescence intensity was recorded by a luminometer.

**Table 5. Effect of sodium chloride on the luminescence activities of ZZ-gCys, Z-gCys and Z-domain.**

| NaCl (mM) | Luminescence intensity ($I_{max}$, rlu) [a] | | |
|---|---|---|---|
| | ZZ-gCys | Z-gCys | Z-domain |
| 0 | 159 | 9,949 | 199 |
| 100 | 28,620 | 9,495 | 339 |

[a] The reaction mixture (100 μL) with or without 100 mM NaCl contains 1 μg of coelenterazine in 50 mM sodiium phosphate buffer (pH 7.5) and the luminescence reaction is started by the addition of ZZ-gCys, Z-gCys and Z-domian (10 μg protein).

**Table 6. Effect of halogen ions on the luminescence activity of ZZ-gCys.**

| Assay conditions (Salt conc. 50 mM) | Relative luminescence intensity ($I_{max}$, %) [a] |
|---|---|
| 100 mM Tris-HCl (pH 7.6) | 100.0 |
| 50 mM Sodium phosphate (pH 7.5) | 0.6 |
| + $(NH_4)_2SO_4$ | 11.2 |
| + $MgSO_4$ | 9.8 |
| + $MgCl_2$ | 86.4 |
| + KF | 0.7 |
| + NaF | 10.7 |
| + KCl | 126.6 |
| + NaCl | 124.9 |
| + KBr | 127.7 |
| + NaBr | 128.6 |
| + KI | 74.6 |
| + NaI | 74.9 |

[a] The reaction mixture (100 µL) contains 50 mM halogen ions and 1 µg of coelenterazine in 50 mM sodium phosphate buffer (pH 7.5) and the luminescence reaction is started by the addition of ZZ-gCys (10 µg protein).

**Table 7. Substrate specificities of ZZ-gCys, Z-gCys and Z-domain for coelenterazine analogues.**

| Coelenterazine analogue [a] | Relative luminescence intensity ($I_{max}$, %) [b] | | | |
|---|---|---|---|---|
| | Z | Z-gCys | ZZ-gCys | *Gaussia* luciferase |
| Coelenterazine | 100 | 100 | 100 | 100 |
| *bis-* | 64.3 | 68.0 | 0.1 | 0.03 |
| *hcp-* | 45.1 | 54.2 | 0.1 | 0.04 |
| *n-* | 49.9 | 43.2 | 0.7 | 0.8 |
| *h-* | 116 | 136 | 5.3 | 5.9 |
| *3iso-* | 37.8 | 52.4 | 7.0 | 7.8 |
| *3meo-* | 75.2 | 99.9 | 1.4 | 2.2 |
| *cf3-* | 35.1 | 41.9 | 0.6 | 0.6 |
| *i-* | 46.8 | 41.3 | 0.7 | 1.0 |
| *et-* | 40.4 | 40.7 | 0.6 | 0.7 |
| *meo-* | 54.0 | 70.1 | 7.8 | 6.8 |

| | | | | |
|---|---|---|---|---|
| *me-* | 62.2 | 65.2 | 3.1 | 3.5 |
| *3me-* | 62.0 | 70.5 | 1.4 | 1.7 |
| *ameh-* | 9.6 | 8.4 | 0.1 | 0.1 |

[a] Abbreviations of coelenterazine analogues are used from ref. 25 and 26.

[b] Assay conditions are the same as described in Table 2.

## Figure legends

**Fig. 1. A schematic representation of strategy for constructing the expression vectors for ZZ-gCys, Z-gCys and Z-domain.**

Z, IgG binding domain of the Z domain (58 amino acid residues); His-tag, six histidine-tagged sequence for Ni-chelate affinity chromatography; MCS, a multiple cloning site; gCys Linker, a flexible linker sequence with a cysteine residue (-SH) at the carboxyl terminus (GGGGSGGGGSGGGGC).

**Fig. 2. Expression vector for ZZ-gCys in *E. coli* cells.**

A. Plasmid map of pCold-ZZ-gCys expression vector. *cps*A, the promoter of cold shock protein A; TEE, translational enhancing element; His-Tag, six histidine-tagged sequence; ZZ domain, IgG binding domain (116 amino acid residues); gCys Linker, a flexible linker sequence with a cysteine residue.

B. Amino acid sequence of the N- and C-terminal regions of ZZ-gCys.

**Fig. 3. SDS-PAGE analyses of purified ZZ-gCys, Z-gCys and Z-domain.** SDS-PAGE analyses were performed under reducing conditions (A) and non-reducing conditions (B). Lane 1, ZZ-gCys (5 μg); lane 2, Z-gCys (5 μg), lane 3, Z-domain (5 μg). The numbers on the left margin represent the molecular weight marker proteins (TEFCO): β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (69,0 kDa), glutamic dehydrogenase (55.0 kDa), lactic dehydrogenase (36.5 kDa), carbonic anhydrase (29.0 kDa), trypsin inhibitor (20.1 kDa), lysozyme (14.3 kDa), and aprotinin (6.5 kDa).

**Fig. 4. Luminescence reaction of ZZ-gCys, Z-gCys and Z-domain with coelenterazine.**

A. Luminescence patterns of ZZ-gCys, Z-gCys and Z-domain (5 μg). Assay conditions are the same as in Table 2.

B. Luminescence patterns of ZZ-gCys (a:5 μg and b: 15 μg), BSA (5 μg), and ZZ-gCys (5 μg)

with 0.01% Tween 20. Assay conditions are the same as in Table 2.

C. Luminescence pattern of ZZ-gCys (5 μg) at the presence of a 1:1 molar ratio to IgG (21 μg) or BSA (19 μg). Assay conditions are the same as in Table 4.

**Fig. 5. Luminescence emission spectra of ZZ-gCys, Z-gCys and Z-domain with coelenterazine.**

The measurement conditions are described in *Materials and Methods*.

**Fig. 6. Stimulation of the luminescence activity of ZZ-gCys by Tween 20 (A) and NaCl (B).**

The assay conditions are described in *Materials and Methods*.

**Fig. S1.** Effect of pH on the luminescence activity of Z-domain (A), Z-gCys (B) and ZZ-gCys (C). The reaction mixture (100 μL) contains 5 μg of protein and 1 μg of coelenterazine in various pH solutions. Closed circle, 100 mM Tris-HCl buffer; Open circle, 100 mM sodium phosphate buffer; Closed triangle, 100 mM sodium

phosphate buffer.

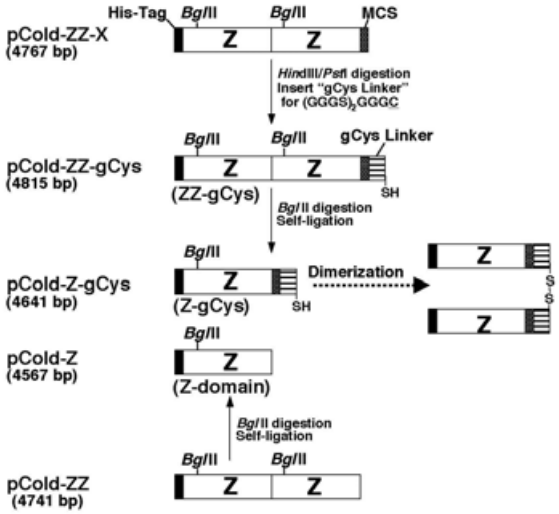


Fig. 1 Inouye & Sahara-Miura

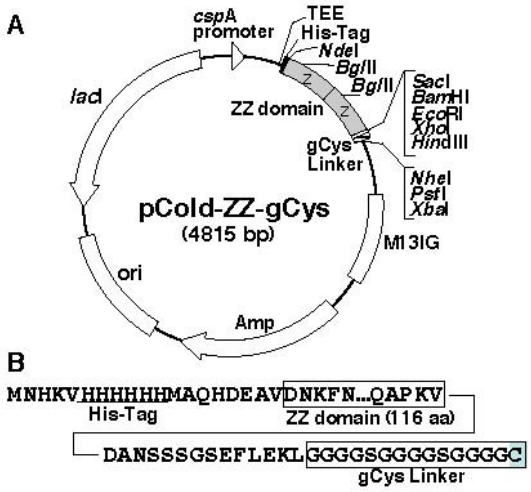


Fig. 2 Inouye & Sahara-Miura

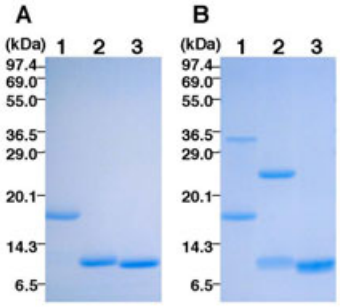


Fig. 3 Inouye & Sahara-Miura

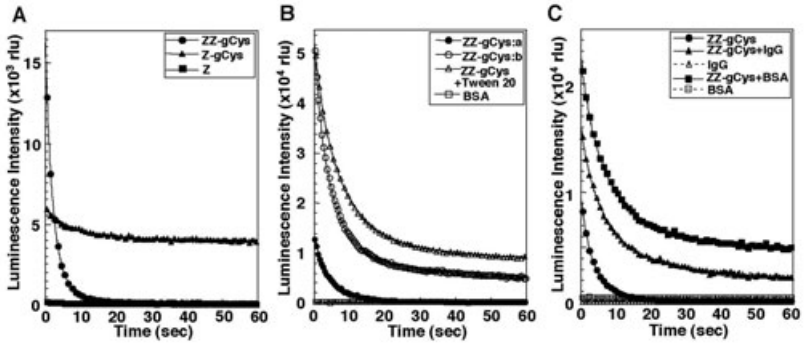


Fig. 4 Inouye & Sahara-Miura

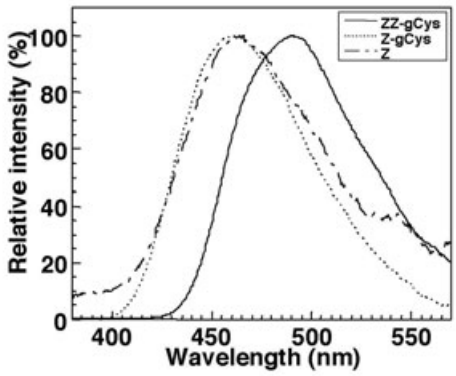


Fig. 5 Inouye & Sahara-Miura

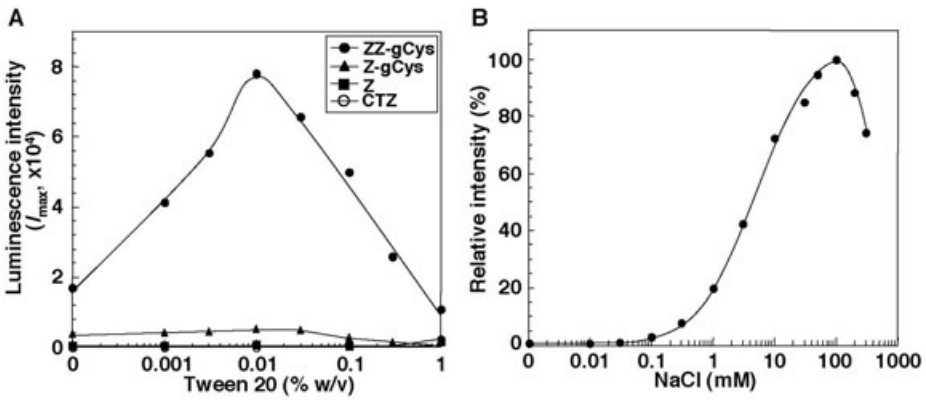


Fig. 6 Inouye & Sahara-Miura