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Luminescence enhancement of the catalytic 19 kDa protein (KAZ) of *Oplophorus* luciferase by three amino acid substitutions

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ABSTRACT

To characterize the luminescence properties of nanoKAZ, a 16 amino acid substituted mutant of the catalytic 19 kDa protein (KAZ) of *Oplophorus* luciferase, the effects of each mutated amino acid were investigated by site-specific mutagenesis. All 16 single substituted KAZ mutants were expressed in *Escherichia coli* cells and their secretory expressions in CHO-K1 cells were also examined using the signal peptide sequence of *Gaussia* luciferase. Luminescence activity of KAZ was significantly enhanced by single amino acid substitutions at V44I, A54I, or Y138I. Further, the triple mutant KAZ-V44I/A54I/Y138I, named eKAZ, was prepared and these substitutions synergistically enhanced luminescence activity, showing 66-fold higher activity than wild-KAZ and also 7-fold higher activity than nanoKAZ using coelenterazine as a substrate. Substrate specificity of eKAZ for C2- and/or C6-modified coelenterazine analogues was different from that of nanoKAZ, indicating that three amino acid substitutions may be responsible for the substrate recognition of coelenterazine to increase luminescence activity. In contrast, these substitutions did not stimulate protein secretion from CHO-K1 cells, suggesting that the folded-protein structure of KAZ might be different from that of nanoKAZ.

1. Introduction

The secreted luciferase of the deep-sea shrimp *Oplophorus gracilirostris* catalyzes the oxidation of coelenterazine to emit blue light (λ_{max} = ~460 nm), according to the following reaction scheme [1–3]:

$$\text{Coelenterazine} + O_2 \xrightarrow{\textit{Oplophorus}\ \text{luciferase}} \text{Coelenteramide} + CO_2 + h\nu$$

Native *Oplophorus* luciferase consists of the 19 and 35 kDa proteins with a molecular weight of 106 kDa [1,2]. The cDNA cloning of *Oplophorus* luciferase revealed that the 19 and 35 kDa proteins consist of 196 and 359 amino acid residues including the putative signal peptide sequences for secretion, respectively [2]. The gene expressions of these proteins in *Escherichia coli* and mammalian cells clearly showed that the 19 kDa protein of *Oplophorus* luciferase (designated as KAZ) is the catalytic component for the luminescence reaction [2]. The primary structure of KAZ comprises two domains (I and II) with repeated sequences (Fig. 1). When KAZ was expressed in *E. coli* cells, the recombinant protein was mainly expressed as inclusion bodies [3]. We have recently succeeded in expressing KAZ as a soluble form in *E. coli* cells by fusing KAZ to a synthetic IgG-binding domain (ZZ domain) of protein A derived from staphylococcal protein A, using a cold-inducible expression system [4,5]. On the other hand, KAZ with a putative signal peptide sequence for secretion was not secreted into the culture medium from mammalian cells and was retained in the cytoplasm [2]. Interestingly, *Oplophorus* luciferase and KAZ showed broad substrate specificities for various coelenterazine analogues, and the substrate specificity was distinct from other coelenterazine-type luciferases including *Renilla* and *Gaussia* luciferases [6–9], and the Ca^{2+}-binding photoprotein aequorin [8].

Recently, Hall et al. reported that a KAZ mutant prepared by 16 amino acid substitutions, named "nanoLuc," showed higher luminescence intensity than wild-KAZ [10], and proposed that *nanoLuc* is a potential candidate for a reporter assay. More recently, we have synthesized the codon-optimized gene for nanoLuc and named it "*nanoKAZ*" (GenBank accession No. AB823628), which has 72% identity to *nanoLuc* [11]. The fused protein of nanoKAZ

Abbreviations: KAZ, the catalytic 19 kDa protein of *Oplophorus* luciferase; nanoKAZ, a KAZ mutant substituted with 16 amino acid residues; eKAZ, a triple mutant of KAZ with V44I, A54I and Y138I; CTZ, coelenterazine; GLase, *Gaussia* luciferase; GLsp, the signal peptide sequence of *Gaussia* luciferase for secretion; I_{max}, maximum intensity of luminescence; rlu, relative light units.

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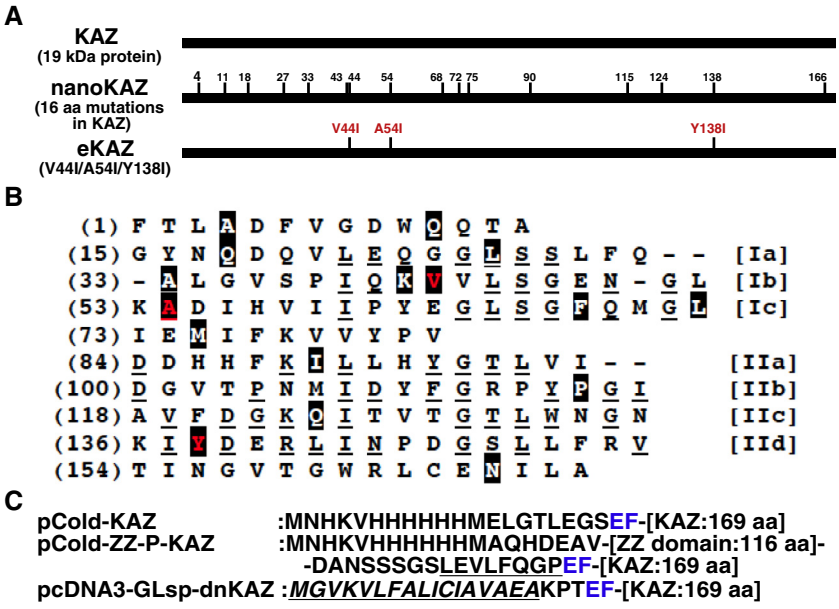

Fig. 1. Expression of the catalytic 19 kDa protein (KAZ) of *Oplophorus* luciferase and its mutants in *E. coli* and CHO-K1 cells. (A) Schematic representation of KAZ, nanoKAZ, and KAZ-V44I/A54I/Y138I mutant (eKAZ). (B) Amino acid sequence of 19 kDa protein (KAZ) of *Oplophorus* luciferase with two repeat domains [I] and [II]. Letters in black boxes indicate the mutated positions of KAZ in this study. Identical amino acids and amino acid groups having similar physical and chemical properties are underlined. The groups are defined as follows: A, S, T, P, and G; N, D, E, and Q; H, R and K; M, L, I, and V; F, Y, and W. Gaps (-) were inserted to increase sequence similarity. The numbers in parentheses on the left indicate the amino acid residues. (C) Expression vectors of pCold-KAZ, pCold-ZZ-P-KAZ, and pcDNA3-GLsp-dnKAZ with the amino acid sequence at the amino-terminal regions. The underline in pCold-ZZ-P-X vector indicates the recognition site of PreScission protease. The italic sequence underlined in pcDNA-GLsp vector indicates the signal peptide sequence of *Gaussia* luciferase for secretion.

to ZZ domain was expressed as a soluble form in *E. coli* cells using a pCold-ZZ-P-X vector [5] and nanoKAZ was highly purified after removing the ZZ domain [11]. Furthermore, nanoKAZ possessing the signal peptide sequence of *Gaussia* luciferase for secretion expressed efficiently into the culture medium of Chinese hamster ovary-K1 (CHO-K1) cells [11], and the expression level of nanoKAZ into the medium was about 1.5-fold higher than that of nanoLuc (unpublished). Using the purified nanoKAZ from *E. coli* cells and the secreted nanoKAZ from CHO-K1 cells, we have characterized the luminescence properties including the luminescence pattern and substrate specificity, and found that *bis*-coelenterazine (*bis*-CTZ) and *6h-f*-coelenterazine (*6h-f*-CTZ) are more suitable substrates than coelenterazine for the glow luminescence assay with nanoKAZ and nanoLuc in mammalian cells [11].

In this manuscript, we described the effect of each of 16 amino acids mutated in nanoKAZ on luminescence activity and substrate specificity of KAZ. We found that the luminescence activity of KAZ was significantly enhanced by the mutations at V44I, A54I, or Y138I. Moreover, the triple mutant of KAZ with V44I, A54I, and Y138I, named "eKAZ," showed 7-fold higher activity than nanoKAZ using coelenterazine, but these substitutions did not stimulate protein secretion from mammalian cells.

2. Materials and methods

2.1. Materials

Coelenterazine (CTZ), *h*-coelenterazine (*h*-CTZ), *bis*-coelenterazine (*bis*-CTZ) (JNC Co., Tokyo, Japan); *f*-coelenterazine (*f*-CTZ) (Prolume, Pinetop, AZ); *6h*-coelenterazine (*6h*-CTZ), *6h-f*-coelenterazine (*6h-f*-CTZ), and furimazine were synthesized as previously reported [11].

2.2. Site-specific mutagenesis

To substitute the amino acid residues in the 19 kDa protein (KAZ) of *Oplophorus* luciferase, site-specific mutagenesis was performed by the overlap extension method with two-step polymerase chain reactions (PCR) using the synthetic oligonucleotide primers (Supplementary Table S1) [12]. PCR was carried out using Takara EX Taq DNA polymerase (Takara-Bio, Kyoto, Japan) with pCold-KAZ [3] or pCold-ZZ-KAZ [4] as a template under the following conditions: 25 cycles at 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min. In brief, two fragments obtained by the first PCR step were separated by agarose electrophoresis on a mini-gel and were recovered from the gel by the freeze–thaw method. A portion of aliquot (2 μL) was used for the second PCR step and the fragment of mutated KAZ cDNA was obtained. The nucleotide sequences were confirmed by DNA sequence analysis.

2.3. Expression of KAZ mutants in E. coli cells using pCold II and pCold-ZZ-P-X vectors

To express KAZ mutants in *E. coli* cells, we used the cold-inducible expression vectors, pCold II (Takara-Bio) [13] and pCold-ZZ-P-X [5]. A pCold-ZZ-P-X vector has a histidine tag sequence for nickel-chelate affinity chromatography, the cleavage sequence of human rhinovirus 3C protease (PreScission protease) between the ZZ domain and a target protein, followed by the multiple cloning sites under the control of the cold shock protein A (*csp*A) promoter and the *lac* operator [5]. The *Eco*RI/*Xba*I fragment of mutated KAZ cDNA was inserted into the *Eco*RI/*Xba*I site of pCold II and pCold-ZZ-P-X to yield the expression vectors of pCold-KAZ mutants and pCold-ZZ-P-KAZ mutants, respectively. The *E. coli* strain BL21 (Novagen, Madison, WI) possessing the expression vector was grown in 5 mL of Luria–Bertani (LB) broth containing ampicillin (50 μg/mL) at 37 °C for 18 h. Then, 0.1 mL of the seed culture was transferred into LB broth (10 mL), incubated at 37 °C for 3 h, and cooled on an ice-water bath for 1 h. After addition of IPTG to the culture medium at the final concentration of 1 mM, the bacterial cells were incubated at 15 °C for 20 h. The bacterial cells were collected by centrifugation from 1 mL of culture medium and the pellet was suspended in 0.5 mL of 30 mM

Tris–HCl (pH 7.6)–10 mM EDTA. The crude extracts were prepared by sonication using a Branson model 250 sonifier (Danbury, CT) for 3 s on ice. The soluble fraction was separated by centrifugation at 12,000*g* for 2 min and used for protein analysis. The purification of ZZ-P-KAZ-V44I/A54I/Y138I (ZZ-P-eKAZ) was performed by nickel-chelate column chromatography as previously described [11].

2.4. Secretory expression of KAZ mutants in CHO-K1 cells using the signal peptide sequence of Gaussia luciferase

To express KAZ mutants in CHO-K1 cells, we used a pcDNA3-GLsp vector that is a derivative of pcDNA3 having the signal peptide sequence of *Gaussia* luciferase for secretion, as previously described [11]. The *Eco*RI/*Xba*I fragment of mutated KAZ cDNA obtained by PCR procedures was ligated to the *Eco*RI/*Xba*I site of pcDNA3-GLsp to produce pcDNA3-GLsp-KAZ mutant. A CHO-K1 cell line was cultured in Ham's F-12 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (Biowest, France), 100 units of penicillin G/mL, and 0.1 mg of streptomycin/mL (Wako Pure Chemicals, Osaka, Japan) at 37 °C in a humidified atmosphere of 5% CO_2. Cells (1×10^5 cells) in a 6-well plate (Nunc) were transfected by the transfection reagent of FuGENE (3 μL) (Promega, Madison, WI) with 1 μg of pcDNA3-GLsp-KAZ mutant plasmid and 0.1 μg of pGL4.13 [Luc2/sv40] (Promega) as an internal control. After incubation for 24 h, the conditioned medium and cell extracts were used for determination of luminescence activity. To prepare cell extracts, the cultured cells were washed with 3 mL of D-PBS (–KCl) (Wako Pure Chemicals), suspended in 1 mL of D-PBS (–KCl), and collected with a scraper. Cells were disrupted by sonication and used as cell extracts.

2.5. Assay for luminescence activity

The luminescence activity of luciferase was determined using an Atto (Tokyo, Japan) AB2200 luminometer (Ver.2.07, rev4.21) in the presence or absence of a 0.23% neutral density filter, determined by using a laser at 544 nm. The reaction mixture (100 μL) contained coelenterazine or its analogue (1 or 0.5 μg dissolved in 1 μL of ethanol) in 30 mM Tris–HCl (pH 7.6)–10 mM EDTA, and the luminescence reaction was started by the addition of 1–5 μL of luciferase solution into the reaction mixture and the luminescence intensity was recorded in 0.1 s intervals for 60 s. The maximum intensity of luminescence (I_{max}) is shown as relative light units (rlu), and 1 rlu was estimated to be 1.8×10^6 photons/0.1 s from the I_{max} value of the purified recombinant aequorin [14].

2.6. Protein analysis

SDS–PAGE analysis was carried out under reducing conditions using a 12% separation gel (TEFCO, Tokyo, Japan). Electrophoresis was run at 25 mA for 70 min, and the gel was stained with a colloidal CBB staining kit (TEFCO). Protein concentration was determined with a commercially available kit (Bio-Rad, Richmond, CA) and bovine serum albumin as a standard (Pierce, Rockford, IL).

3. Results and discussion

3.1. Soluble expression and characterization of a single substituted KAZ mutant in E. coli cells

Previously, we expressed recombinant 19 kDa protein (wild-KAZ) in *E. coli* cells using a pTrcHis B vector (Invitrogen) under the control of the trc promoter at 37 °C [2] and a pCold II vector under the control of the cold shock protein promoter at 15 °C [3]. In both expression systems, wild-KAZ was expressed mainly as inclusion bodies and was purified with a nickel-chelate column after solubilization of inclusion bodies with 2 M urea [3]. To identify the key amino acid residues for stimulating the luminescence activity of nanoKAZ, we introduced the site-specific mutations at 16 independent amino acid positions of wild-KAZ, which are observed in nanoKAZ (=nanoLuc; Fig. 1, Table 1). The mutated fragment was inserted into a pCold II vector for expression in *E. coli* cells. As we expected, all single-mutated KAZ proteins were expressed as inclusion bodies and the protein expression level in *E. coli* cells varied (Fig. 2B). In contrast, nanoKAZ was expressed only in soluble form (Fig. 2A, lane 18). The luminescence activities in soluble and insoluble fractions were determined using coelenterazine as a substrate (Table 1). The enhancement of luminescence activity was observed in the soluble fractions of the mutated A54I and Y138I of wild-KAZ, but the expressed proteins in the soluble fraction were not detected on a SDS–PAGE gel (Fig. 2A). Thus, it was difficult to identify the critical amino acid residues for enhancing the luminescence activity of wild-KAZ under insoluble expression conditions. From these reasons, we introduced a pCold-ZZ-P-X vector to express the mutants as a soluble form [4,5,11] and all single-substituted KAZ mutants were fused to a ZZ domain (ZZ-P-KAZ mutants) and were expressed in soluble form, similar to the case of wild-KAZ [4]. The expression of ZZ-P-KAZ mutants in crude extracts of *E. coli* cells was confirmed by SDS–PAGE analysis under reducing conditions, indicating that ZZ-P-KAZ mutants were expressed as the same level as ZZ-P-nanoKAZ in *E. coli* cells (Fig. 3A).

The soluble expressed ZZ-P-KAZ in crude extracts was detected partially as a dimer on a SDS–PAGE gel under non-reducing conditions, and the luminescence activity was lost by dimerization (unpublished). On the other hand, the luminescence activity of ZZ-P-KAZ in crude extracts increased about 2-fold after incubation on ice over 2 h. Presumably, ZZ-P-KAZ in crude extracts might be refolded and showed high luminescence activity. From these results, the luminescence assay for ZZ-P-KAZ was carried out using crude extracts in the presence of 1 mM DTT, followed by incubation on ice over 3 h. As summarized in Table 2, significant increases in luminescence activity over wild-KAZ were observed in mutants

Table 1
Expression of the single amino acid substituted 19 kDa protein (KAZ mutant) in *E. coli* cells using pCold II vector.

Entry No.[a]	KAZ mutant (pCold II)	Relative luminescence activity (I_{max})[b]	
		Sup	Ppt
1	KAZ (wild-type)	0.1	0.01
2	-A4E	0.1	0.01
3	-Q11R	0.1	0.02
4	-Q18L	0.1	0.01
5	-L27V	0.01	0.001
6	-A33N	0.1	0.04
7	-K43R	0.1	0.01
8	-V44I	0.4	0.03
9	-A54I	8.3	0.74
10	-F68D	0.3	0.01
11	-L72Q	0.4	0.03
12	-M75K	0.3	0.02
13	-I90V	0.1	0.02
14	-P115E	0.9	0.06
15	-Q124K	0.4	0.04
16	-Y138I	1.8	0.08
17	-N166R	0.3	0.05
18	nanoKAZ	100[c]	0.05

[a] Entry numbers correspond to the column numbers on the SDS–PAGE gel in Fig. 2.
[b] Cell extracts were separated by centrifugation and the supernatant and precipitate were used for assay with coelenterazine as a substrate in the presence of a 0.23% neutral density filter.
[c] 6.1×10^5 rlu/0.1 s/10 μL of cultured cells.

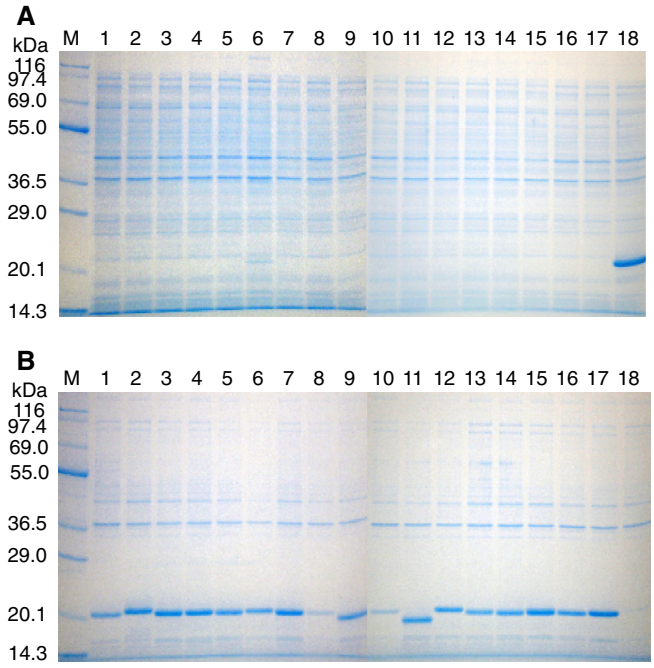

Fig. 2. SDS–PAGE analysis of single amino acid substituted KAZ mutants expressed in *E. coli* cells using pCold II vector. (A) Soluble fractions of crude extracts at 12,000*g* supernatant. (B) Insoluble fractions of crude extracts at 12,000*g* precipitate. Column numbers at the top of the gel correspond to the entry numbers in Table 1. Column M, molecular weight markers (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), and lysozyme (14.3 kDa).

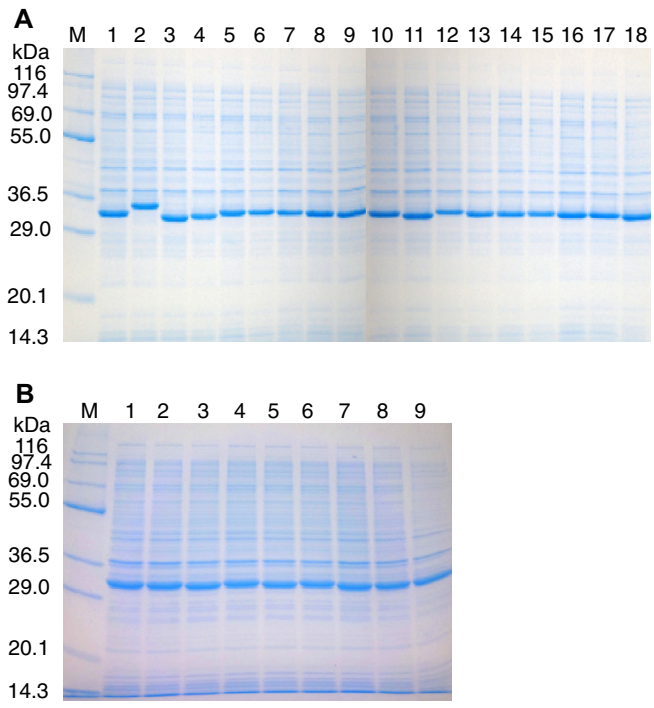

Fig. 3. SDS–PAGE analysis of KAZ mutants expressed in *E. coli* cells using pCold-ZZ-P-X vector. (A) Crude extracts of the single amino acid substituted KAZ mutants expressed in *E. coli* cells. Column numbers at the top of the gel correspond to the entry numbers in Table 2. (B) Crude extracts of the substituted KAZ mutants at V44I, A54I, and/or Y138I, expressed in *E. coli* cells. Column numbers at the top of the gel correspond to the entry numbers in Table 4.

of V44I (6.6-fold), A54I (8.6-fold), A115E (4.6-fold), and Y138I (5.9-fold). Recently, Hall et al. reported that the secretory expression of nanoLuc into the culture medium from mammalian cells is 2.5×10^6-fold higher than that of wild-KAZ using furimazine as a substrate [10]. However, our results indicated that the catalytic ability of nanoKAZ (=nanoLuc) was only some 10-fold higher than that of wild-KAZ using coelenterazine as a substrate.

3.2. Secretory expression and luminescence activity of single amino acid substituted KAZ mutants in CHO-K1 cells

To confirm the enhancement of luminescence activity of wild-KAZ by a single amino acid substitution, which was observed in *E. coli* cells, the secretory expression of a single substituted KAZ in CHO-K1 cells was performed using the signal peptide sequence of *Gaussia* luciferase for secretion, as previously described [11], and the luminescence activities in culture medium and cell extracts were determined using coelenterazine as a substrate (Table 3). None of the 16 single substituted KAZ mutants showed efficient secretion into culture medium from CHO-K1 cells, similar to the case of wild-KAZ [2]. In cell extracts, the mutations at V44I, A54I, and Y138I in wild-KAZ enhanced the luminescence activity by 8.2-, 29.9-, and 12.9-fold, respectively. From the results of KAZ mutants expressed in *E. coli* and CHO-K1 cells, we concluded that the substitutions at V44I, A54I, and Y138I in wild-KAZ enhanced luminescence activity, suggesting that these amino acid residues among the 16 substitutions of nanoKAZ (=nanoLuc) may play critical roles in luminescence enhancement.

3.3. Expression of triple amino acid substituted KAZ (eKAZ) in E. coli and CHO-K1 cells and luminescence properties using coelenterazine and its analogues

To investigate synergistic effects on the enhancement of luminescence activity by multiple mutations in wild-KAZ, we introduced the double and triple mutations at the positions of V44I, A54I, and/or Y138I in wild-KAZ. The ZZ-fusion proteins of wild-KAZ with multiple mutations were expressed in soluble form with

Table 2
Soluble expression of the single amino acid substituted 19 kDa protein (KAZ mutant) in *E. coli* cells using pCold-ZZ-P-X vector.

Entry No.[a]	KAZ mutant (pCold-ZZ-P-X)	Relative luminescence activity (I_{max})[b]
1	KAZ (wild-type)	1.0[c]
2	-A4E	0.5
3	-Q11R	1.8
4	-Q18L	0.3
5	-L27V	0.1
6	-A33N	1.0
7	-K43R	0.6
8	-V44I	6.6
9	-A54I	8.9
10	-F68D	2.7
11	-L72Q	2.5
12	-M75K	1.3
13	-I90V	1.8
14	-P115E	4.6
15	-Q124K	3.7
16	-Y138I	5.9
17	-N166R	3.6
18	nanoKAZ	9.4

[a] Entry numbers correspond to the column numbers on the SDS–PAGE gel in Fig. 3A.
[b] Cell extracts were incubated in the presence of 1 mM DTT for 5 h and luminescence activity was determined using coelenterazine as a substrate in the presence of a 0.23% neutral density filter.
[c] 3.2×10^4 rlu/0.1 s/2 μL of cultured cells.

Table 3
Secretory expression of the single amino acid substituted 19 kDa protein (KAZ mutant) in CHO-K1 cells using pcDNA3-GLsp vector.

Entry No.	KAZ mutant[a] (pcDNA3-GLsp)	Relative luminescence activity (I_{max})[b]	
		Culture medium	Cell extracts
1	KAZ (wild-type)	>0.01	1.0[c]
2	-A4E	>0.01	1.0
3	-Q11R	>0.01	1.6
4	-Q18L	>0.01	0.3
5	-L27V	>0.01	0.3
6	-A33N	>0.01	1.0
7	-K43R	>0.01	1.6
8	-V44I	>0.01	8.2
9	-A54I	>0.01	29.9
10	-F68D	>0.01	3.4
11	-L72Q	>0.01	4.9
12	-M75K	>0.01	1.9
13	-I90V	>0.01	4.1
14	-P115E	>0.01	3.9
15	-Q124K	>0.01	3.2
16	-Y138I	>0.01	12.9
17	-N166R	>0.01	2.6
18	nanoKAZ	1261	81.9

[a] KAZ mutant with the secretory signal peptide sequence of *Gaussia* luciferase was expressed transiently in CHO-K1 cells using a pcDNA3-GLsp vector.
[b] After culture for 44 h, cell extracts and cultured medium were used for assay with coelenterazine as a substrate in the absence of a 0.23% neutral density filter.
[c] 7.0×10^3 rlu/0.1 s/500 cells.

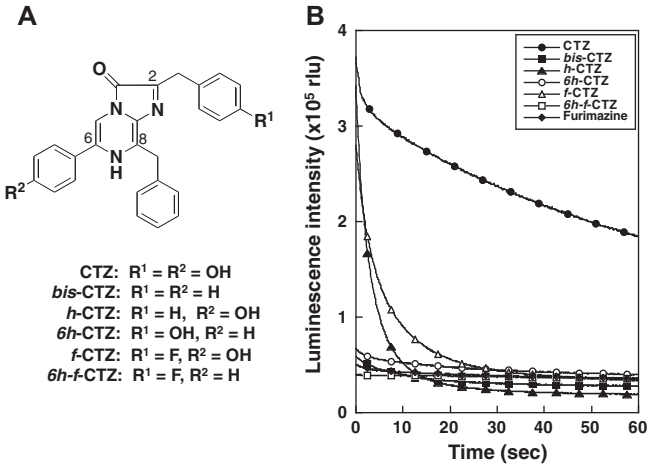

Fig. 4. Luminescence patterns of ZZ-P-KAZ-V44I/A54I/Y138I (ZZ-P-eKAZ) using coelenterazine and its C2- and/or C6-modified analogues. (A) Structures of coelenterazine and its analogues used in this study. (B) Luminescence patterns of purified ZZ-P-eKAZ using coelenterazine and its analogues. The purified ZZ-P-eKAZ (0.04 μg) was used for assay in 100 μL of 30 mM Tris–HCl (pH 7.6)–10 mM EDTA containing 1 μg of coelenterazine or its analogue.

the similar amounts of recombinant proteins in *E. coli* cells and crude extracts were used for luminescence assays (Fig. 3B). Unexpectedly, when coelenterazine was used as a substrate, the relative intensity of I_{max} for ZZ-P-KAZ-V44I/A54I/Y138 (ZZ-P-eKAZ) was 66.7-fold higher than that for wild-KAZ. In contrast, nanoKAZ showed only 9.1-fold higher activity than wild-KAZ (Table 4).

The substrate specificities were compared among the mutants of V44I, A54I, and/or Y138I with wild-KAZ using C2- and/or C6-modified coelenterazine analogues (Fig. 4A, Table 4). As a result, a similar synergistic effect on luminescence activity was observed for all coelenterazine analogues used. The preferred substrates for ZZ-P-eKAZ were coelenterazine (CTZ: 66.7-fold), *h*-CTZ (83.1-fold), and *f*-CTZ (43.8-fold), which all possess a *p*-hydroxyl group at the C6-phenyl moiety of coelenterazine. In contrast, ZZ-P-nanoKAZ showed high luminescence activity with *h*-CTZ (161.2-fold), *bis*-CTZ (112.0-fold), *f*-CTZ (146.3-fold), *6h-f*-CTZ (111.2-fold) and furimazine (65.1-fold), which commonly possess a hydrophobic benzyl group without a hydroxyl group at the C2-position of coelenterazine (Table 4). These specificities were in good agreement with those observed for the purified nanoKAZ [11]. Thus, although the recognition moieties of coelenterazine may be different between eKAZ and nanoKAZ, appropriate interactions of eKAZ or nanoKAZ with the functional groups at C2- and/or C6-positions in coelenterazine seem to be important to achieve high luminescence activity.

To confirm the substrate specificity, ZZ-P-eKAZ was purified by a nickel-chelate column, as previously described [11]. The substrate specificity of purified ZZ-P-eKAZ was similar to that of crude extracts from *E. coli* cells (Supplementary Table S2). This indicates that the ZZ domain fused to KAZ does not significantly affect the substrate specificity. Furthermore, the luminescence emission spectra of the purified proteins of ZZ-P-KAZ, ZZ-P-eKAZ, and ZZ-P-nanoKAZ were determined using coelenterazine as a substrate (Supplementary Fig. S1). All emission spectra were almost identical to that of nanoKAZ as previously reported [11] with the emission peak at 458 ± 1 nm, suggesting that the light-emitting species in all protein molecules might be the coelenteramide in a similar state. On the other hand, these substitutions did not stimulate protein secretion from CHO-K1 cells (Table 5).

In conclusion, the single amino acid substitutions of wild-KAZ at V44I, A54I, and Y138I resulted in luminescence enhancement and the triple mutant of wild-KAZ at V44I, A54I, and Y138I (eKAZ) showed higher luminescence activity than wild-KAZ and nanoKAZ, when coelenterazine was used as a substrate (Tables 4 and 5). The results of this study will help us to understand the structure–function relationship of *Oplophorus* luciferase with coelenterazine after determining the protein structure of KAZ or nanoKAZ.

Table 4
Substrate specificities of KAZ mutants of V44I, A54I and/or Y138I for coelenterazine analogues.

Entry No.[a]	KAZ mutant (pCold-ZZ-P-X)	Relative luminescence activity, I_{max} (Int.)[b]						
		CTZ	*h*-CTZ	*6h*-CTZ	*bis*-CTZ	*f*-CTZ	*6h-f*-CTZ	Furimazine
1	KAZ (wild-type)	1.0[c] (1.0)[d]	1.1 (0.71)	0.10 (0.15)	0.60 (0.94)	0.91 (0.75)	0.56 (0.67)	0.37 (0.53)
2	-V44I	7.2 (5.9)	3.7 (2.2)	0.40 (0.57)	1.1 (1.4)	2.1 (2.0)	0.55 (0.69)	0.63 (0.87)
3	-A54I	8.4 (9.0)	11.8 (6.9)	1.6 (2.1)	3.0 (3.4)	8.2 (4.9)	1.6 (2.0)	2.0 (2.1)
4	-Y138I	7.5 (5.9)	3.9 (3.0)	0.21 (0.31)	1.3 (1.5)	2.4 (2.4)	0.62 (0.78)	0.60 (0.78)
5	-V44I/A54I	15.3 (12.7)	17.9 (8.7)	4.5 (5.6)	5.7 (5.9)	11.3 (5.2)	3.2 (4.0)	3.4 (4.3)
6	-V44I/Y138I	17.1 (9.9)	19.1 (9.7)	4.3 (5.3)	7.9 (8.3)	13.3 (7.4)	3.6 (4.2)	5.1 (5.4)
7	-A54I/Y138I	23.1 (19.1)	24.2 (12.9)	5.8 (6.1)	9.5 (9.2)	19.7 (9.2)	5.2 (5.7)	5.9 (5.8)
8	-V44I/A54I/Y138I (eKAZ)	66.7 (66.7)	83.1 (33.2)	12.9 (11.1)	13.3 (11.9)	43.8 (9.5)	9.5 (9.6)	10.6 (9.2)
9	nanoKAZ	9.1 (7.9)	161.2 (109.1)	6.8 (4.8)	112.0 (90.2)	146.3 (75.5)	111.2 (68.1)	65.1 (66.8)

[a] Entry number corresponds to the column number on the SDS–PAGE gel in Fig. 3B.
[b] The protein was expressed in *E. coli* cells using a pCold-ZZ-P-X vector. Cell extracts were incubated in the presence of 1 mM DTT for 4 h and used for assay in the presence of a 0.23% neutral density filter.
[c] 4.0×10^4 rlu/0.1 s/2 μL of cultured cells.
[d] 1.4×10^7 rlu/60 s.

Table 5
Secretory expression of the 19 kDa protein (KAZ mutant) substituted at V44I, A54I and Y138I in CHO-K1 cells.

KAZ mutant[a] (pcDNA3-GLsp)	Relative luminescence activity (I_{max})[b]	
	Cell extracts	Culture medium
KAZ (wild-type)	0.3	>0.01
-V44I/A54I	13.9	0.07
-V44I/Y138I	11.2	>0.01
-A54I/Y138I	16.0	0.03
-V44I/A54I/Y138I (eKAZ)	16.2	1.7
nanoKAZ	12.6	100[c]

[a] The protein with the secretory signal peptide sequence of *Gaussia* luciferase was expressed transiently in CHO-K1 cells using a pcDNA3-GLsp vector.
[b] After culture for 49 h, cell extracts and cultured medium were prepared and used for assay with coelenterazine as a substrate in the absence of a 0.23% neutral density filter.
[c] 4.2×10^6 rlu/0.1 s/500 cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.133.

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