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C6-Deoxy coelenterazine analogues as an efficient substrate for glow luminescence reaction of nanoKAZ: The mutated catalytic 19 kDa component of *Oplophorus* luciferase

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ABSTRACT

The codon-optimized gene for the mutated 19 kDa protein (nanoKAZ), which is the catalytic component of *Oplophorus* luciferase, was expressed in *Escherichia coli* cells and the recombinant protein was highly purified. The secretory expression of nanoKAZ from CHO-K1 cells was performed by fusing the secretory signal peptide sequence of *Gaussia* luciferase to the amino-terminus of nanoKAZ. The substrate specificity for the purified nanoKAZ and the nanoKAZ secreted into the cultured medium was determined, indicating that *bis*-coelenterazine (*bis*-CTZ) and newly synthesized *6h-f*-coelenterazine (*6h-f*-CTZ) are an efficient substrate for the glow luminescence reaction of nanoKAZ.

1. Introduction

The luminescence of the deep-sea shrimps *Oplophorus gracilirostris* is produced by a luciferin–luciferase reaction [1,2]. The secreted luciferase from *O. gracilirostris* catalyzes the oxidation of coelenterazine (a luciferin) to emit blue light ($\lambda_{max} \sim 460$ nm) [1–3] (Fig. 1A). The purified *Oplophorus* luciferase was estimated to be 106 kDa protein and was found to be composed of the 19 and 35 kDa proteins by Western blot analysis using anti-*Oplophorus* luciferase antibody [3]. This result suggested that the native luciferase would be a complex of two units of each protein. The cDNAs cloning for the 19 and 35 kDa proteins revealed that they consist of 196 amino acid residues and 359 amino acid residues with their putative signal peptide sequences for secretion, respectively. The primary structures of the 19 and 35 kDa proteins have no significant homology to the coelenterazine-utilizing luciferases from *Renilla* [4] and *Gaussia* [5,6], and the Ca^{2+}-binding photoproteins such as aequorin [7]. The independent expression of the 19 and 35 kDa proteins in *Escherichia coli* cells and COS-7 cells indicated that the 19 kDa protein of *Oplophorus* luciferase (assigned "19kOLase") is the catalytic component for the luminescence reaction [3]. However, the 19kOLase was mainly expressed as inclusion bodies in *E. coli* cells and the 19kOLase with a putative signal sequence for secretion did not express into the cultured medium from various mammalian cells (unpublished result). Later, the 19kOLase was purified from inclusion bodies of *E. coli* cells and the enzymatic properties were characterized including the substrate specificity for coelenterazine analogues [8]. The 19kOLase showed broad substrate specificity [8], similar to native *Oplophorus* luciferase [9]. Especially, *bis*-coelenterazine [10], a unique analogue of coelenterazine, was found to be a suitable substrate for *Oplophorus* luciferase [8,10], but not for other coelenterazine-utilizing luciferases [11] and the photoproteins [11]. Recently, the mutated 19kOLase with 16 amino acid substitutions, named nanoLuc, was reported and the luminescence activity of nanoLuc was relatively higher than that of the native 19kOLase [12]. Further, nanoLuc with the signal peptide sequence for secretion derived from interleukin 6 was shown to secrete into the culture medium of HEK293 and CHO cells [12].

In this paper, we prepared a new codon-optimized gene for the mutated 19kOLase (assigned "*nanoKAZ*" in this paper), which has an identical amino acid sequence to nanoLuc. The fused protein of nanoKAZ with IgG-binding domain (ZZ domain) was expressed

Abbreviations: 19kOLase, the catalytic 19 kDa protein of *Oplophorus* luciferase; nanoKAZ, the mutated 19kOLase; CTZ, coelenterazine; GLase, *Gaussia* luciferase; FWHM, full width at half maximum; I_{max}, maximum intensity of luminescence; rlu, relative light units.

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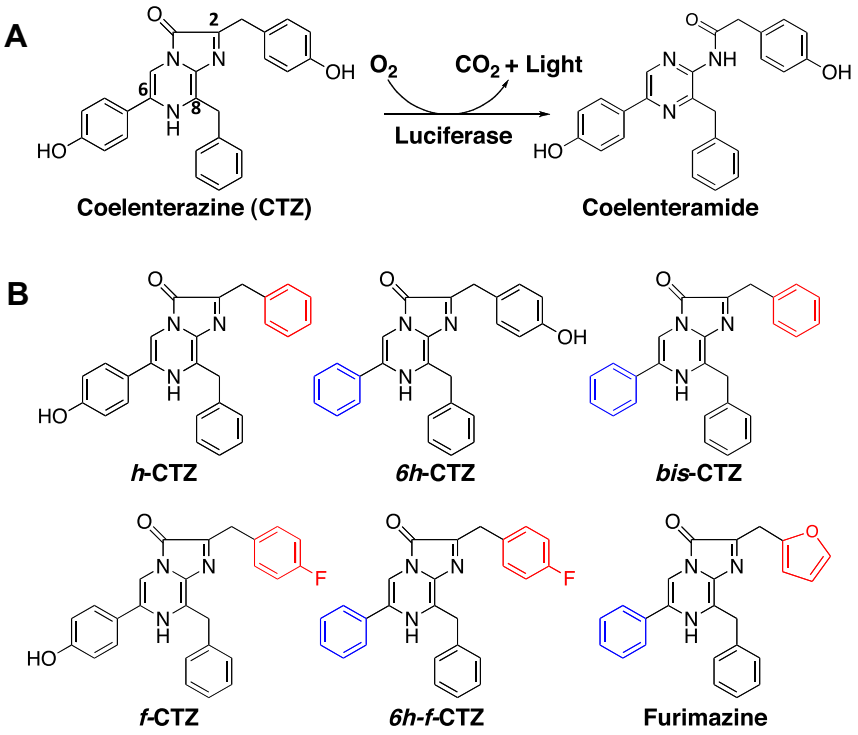

Fig. 1. Luminescence reaction of coelenterazine with molecular oxygen catalyzed by luciferase and the chemical structures of coelenterazine analogues. (A) Oxidation of coelenterazine with molecular oxygen by a coelenterazine-utilizing luciferase. (B) C2- and/or C6-modified coelenterazine analogues used in this study.

in the cytoplasm of *E. coli* cells as a soluble form and purified. Further, the secretory expression of nanoKAZ in CHO-K1 cells was performed using nanoKAZ fused with the signal peptide sequence of *Gaussia* luciferase [6]. The substrate specificity of the purified nanoKAZ from *E. coli* cells and the secreted nanoKAZ in CHO-K1 cells was investigated for coelenterazine analogues, demonstrating that *6h-f*-coelenterazine and *bis*-coelenterazine (Fig. 1) are more suitable substrate for the glow luminescent reaction than the previously reported furimazine.

2. Materials and methods

2.1. Materials

The sources of chemicals were as follows: isopropyl β-thiogalactopyranoside (IPTG), ethylenediaminetetraacetic acid disodium salt (EDTA-2Na), (±)-dithiothreitol (DTT), imidazole, Ham's F-12 medium, the solution of penicillin G and streptomycin (Wako Pure Chemicals, Osaka, Japan), fetal calf serum (Biowest, France); chelate Sepharose Fast Flow, PreScission protease (GE-Healthcare Bio-Science, Piscataway, NJ); FuGENE (Promega, Madison, WI); coelenterazine (CTZ), *h*-coelenterazine (*h*-CTZ), *bis*-coelenterazine (*bis*-CTZ) (JNC Co., Tokyo, Japan); *f*-coelenterazine (*f*-CTZ) (Prolume, Pinetop, AZ); *hcp*-coelenterazine (*hcp*-CTZ) [13], furimazine [12] and the C2-modified coelenterazine analogues [14], were synthesized as previously reported; *6h*-Coelenterazine (*6h*-CTZ) and *6h-f*-coelenterazine (*6h-f*-CTZ) were newly synthesized by the coupling reaction of deoxy-coelenteramine and α-ketoacetal derivatives (Supplementary data).

2.2. Expression and purification of nanoKAZ from bacterial cells

The codon-optimized gene for nanoKAZ (GeneBank Accession No. AB823628) was chemically synthesized and used in this study. The amino acid sequence of nanoKAZ was identical to that of nanoLuc [12], but the identities of the nucleotide sequence of *nanoKAZ* to that of the gene for the native 19kOLase and *nanoLuc* were 72% and 82%, respectively. To express nanoKAZ in *E. coli* cells, we used the cold induced expression vector, pCold-ZZ-P-X [15], which consists of a histidine tag sequence for a nickel chelate affinity chromatography, the cleavage sequence of human rhinovirus 3C protease (PreScission protease) between ZZ domain and a target protein, followed by multiple cloning sites under the controlled of the cold shock protein A (*csp*A) promoter and the *lac* operator. The *Eco*RI/*Xba*I fragment of *nanoKAZ* was inserted into the *Eco*RI/*Xba*I site of a pCold-ZZ-P-X vector to give pCold-ZZ-P-nanoKAZ (Fig. 2A).

The seed culture of *E. coli* strain BL21 (Novagen, Madison, WI) possessing pCold-ZZ-P-nanoKAZ vector was grown in 10 mL of Luria–Bertani broth containing ampicillin (50 μg/mL) at 37 °C for 18 h. This seed culture was transferred into 400 mL of LB broth in a 3 L flask, incubated at 37 °C for 3 h and then cooled on an ice-water bath for 1 h. After adding of IPTG to the culture medium at the final concentration of 0.2 mM, the bacterial cells were incubated at 15 °C for 20 h. The cells were harvested from 800 mL culture medium by using a Hitachi model CR20GIII high-speed refrigerated centrifuge (Tokyo, Japan) at 2750*g* for 10 min, and the pellet was suspended in 80 mL of 50 mM Tris–HCl (pH 7.6), and disrupted by sonication using a Branson model 250 sonifier (Danbury, CT) 3 times for 3 min on ice. The soluble fraction (70 mL) of cell extracts obtained by centrifugation at 15,800*g* for 10 min was applied on a nickel chelate column (column size; 2.5 cm × 6 cm), equilibrated with 50 mM Tris–HCl (pH 7.6). After washing the column with 250 mL of 50 mM Tris–HCl (pH 7.6), His-ZZ-P-nanoKAZ was eluted with 0.1 M imidazole in 50 mM Tris–HCl (pH 7.6). The yield of His-ZZ-P-nanoKAZ was 37.2 mg from 800 mL of cultured cells with over 95% purity on SDS–PAGE analysis (Fig. 2C, lane 3).

To obtain nanoKAZ from His-ZZ-P-nanoKAZ, 2.7 mg of the purified His-ZZ-P-nanoKAZ was digested with 3 μg of PreScission protease in 50 mM Tris–HCl (pH 7.6) containing 150 mM NaCl, 1 mM

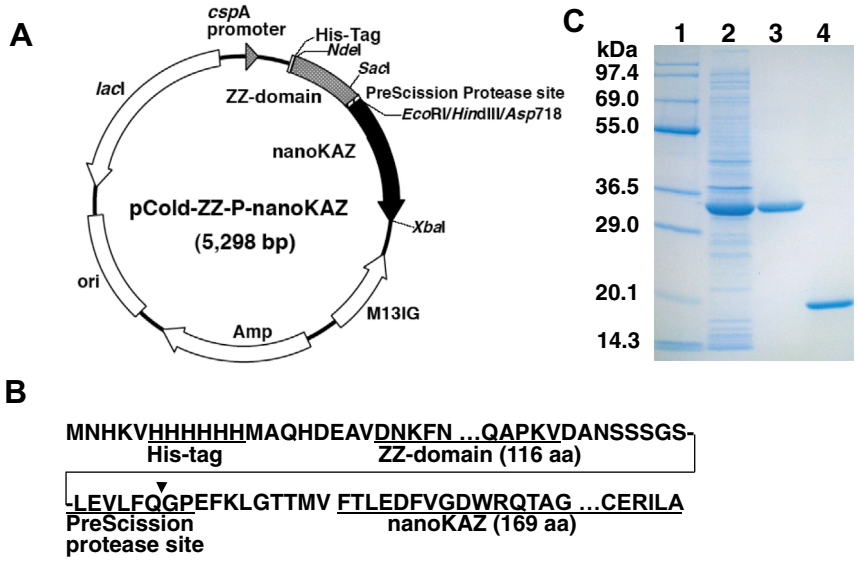

Fig. 2. Expression and purification of nanoKAZ from *E. coli* cells using a pCold-ZZ-P vector. (A) Plasmid map of pCold-ZZ-P-nanoKAZ to express the fusion protein of nanoKAZ to ZZ domain in *E. coli* cells. (B) Schematic representation of amino acid sequence of His-ZZ-P-nanoKAZ expressed in *E. coli* cells. (C) SDS–PAGE analysis of proteins at various stages of nanoKAZ purification. Lane 1, molecular weight markers (TEFCO): 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); lane 2, the soluble fraction at 15,800 g from crude extracts (29 μL of cultured cells, 10.7 μg protein); lane 3, the purified His-ZZ-P-nanoKAZ eluted from a nickel-chelate column (1.7 μg protein); lane 4, the flow-through fraction of the purified nanoKAZ from a nickel-chelate column (1.9 μg protein), after digesting of His-ZZ-P-nanoKAZ with PreScission protease.

EDTA and 1 mM DTT for 18 h at 4 °C. The reaction mixture was directly applied on a nickel column (column size; 0.8 cm × 4 cm) to separate nanoKAZ from the cleaved His-ZZ domain and undigested His-ZZ-P-nanoKAZ. The flow-through fraction (1 mL) containing nanoKAZ (0.7 mg) was obtained and the purity was determined by SDS–PAGE analysis (Fig. 2B, lane 4).

2.3. Secretory expression of nanoKAZ from CHO-K1 cells by fusing the signal peptide sequence of Gaussia luciferase

To express nanoKAZ into the culture medium, we first constructed a pcDNA3-GLsp vector that was a derivative of pcDNA3 having the signal peptide sequence of *Gaussia* luciferase for secretion. Briefly, the signal peptide sequence of *Gaussia* luciferase was obtained from pcDNA3-GLuc (Prolume) by PCR procedures with a primer set of GLsp-1R/EcoRI (5′ ggc GAA TTC GGT GGG CTT GGC CTC GGC CAC 3′ *Eco*RI site underlined) and T7 primer (5′ TAA TAC GAC TCA CTA TAG GG 3′). The resultant fragment was digested with *Eco*RI and *Hin*dIII and was inserted into the *Eco*RI/*Hin*dIII site of pcDNA3 (Invitrogen, Carlsbad, CA) to give pcDNA3-GLsp. The *Eco*RI/*Xba*I fragment of nanoKAZ was ligated to the *Eco*RI/*Xba*I site of pcDNA3-GLsp to produce pcDNA3-GLsp-nanoKAZ (Fig. 3A and B). A CHO cell line was cultured in Ham's F-12 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 units of penicillin G/mL and 0.1 mg of streptomycin/mL at 37 °C in a humidified atmosphere of 5% CO_2. The cells (2.2×10^5 cells) in a 6 cm plate were transfected by the transfection reagent of FuGENE (7.5 μL) with 2.5 μg of pcDNA3-GLsp-nanoKAZ plasmid. After incubating for 48 h, the conditioned medium was collected and stored at −20 °C before use. For serum-free medium, Ham's F-12 medium was changed with MCDB201 (Sigma) after transfection for 24 h and incubated for 47 h.

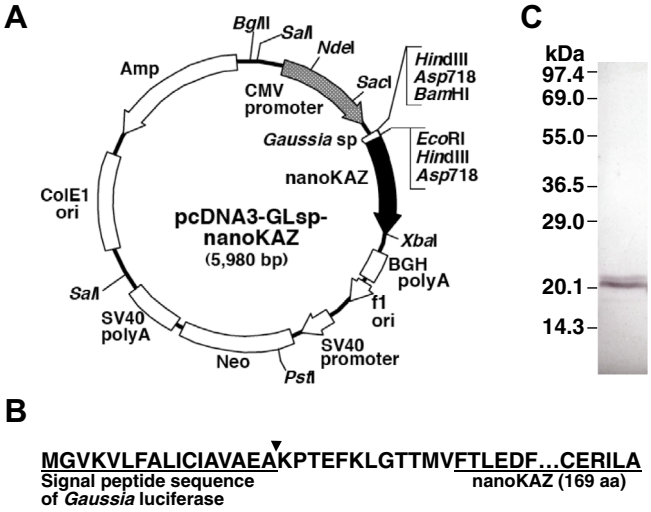

Fig. 3. Secretory expression of nanoKAZ into the culture medium from CHO-K1 cells by fusing the signal peptide sequence of *Gaussia* luciferase. (A) Plasmid map of pDNA3-GLsp-nanoKAZ to express nanoKAZ in the culture medium from CHO-K1 cells. (B) Amino acid sequence of the amino terminal region for expressing nanoKAZ using pDNA3-GLsp-nanoKAZ. Underlines indicate the signal peptide sequence of *Gaussia* luciferase and nanoKAZ, respectively. The vertical arrow indicates the cleavage site of the signal peptide sequence of *Gaussia* luciferase. (C) Western-blot analysis of nanoKAZ secreted into the culture medium using anti-*Oplophorus* luciferase antibody. The secreted nanoKAZ into the culture medium was obtained by incubating with the serum-free medium of MCDB201 medium (Sigma) for 47 h. Twenty microliters of the cultured medium was run on the gel. The numbers on the left margin represent the apparent kDa of the molecular weight markers, which were determined by using the unstained protein markers (TEFCO) with the pre-stained molecular weight markers (Bio-Rad) on the membrane.

2.4. Assay for luminescence activity

The luminescence activity of luciferase was determined using an Atto (Tokyo, Japan) AB2200 luminometer (Ver.2.07, rev4.21) equipped with a Hamamatsu R4220P photomultiplier and a 0.23% neutral density filter. The reaction mixture (100 μL) contained coelenterazine or its analogue (1 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 the luciferase solution into the reaction mixture and the luminescence

Table 1
Purification of nanoKAZ-P expressed as the fusion protein of ZZ-domain in the bacterial cells using the cold induction system.

Purification step	Total volume (mL)	Total protein (mg) (%)	Total activity ($\times 10^8$ rlu) (%)	Specific activity ($\times 10^8$ rlu/mg)
Crude extracts (15,800g sup)	70	150.5 (100)	420 (100)	2.8
1st. Ni-chelate column (Adsorbed fraction)	20	37.2 (25)	196 (46)	5.3
Digested fraction with PreScission protease	1.5	2.7 (100)	13.8 (100)	5.1
2nd. Ni-chelate column (Flow-through fraction)	1.0	0.7 (26)	8.4 (61)	12.0

intensity was recorded in 0.1 s intervals for 60 s. The maximum intensity of luminescence (I_{max}) was shown as a relative light unit (rlu), and 1 rlu was estimated to be 1.8×10^6 photons from the I_{max} value of the purified recombinant aequorin [16].

2.5. Bioluminescence spectral analysis

The bioluminescence spectrum was measured with a fluorescence spectrophotometer FP-6500 (Jasco, Tokyo, Japan) at 26 °C with the excitation light source turned off (emission bandwidth, 20 nm; sensitivity, medium; response, 0.5 s; scan speed, 2000 nm/min). All spectra were corrected according to the manufacture's protocol. The reaction mixture (1 mL) contained 5 μg of coelenterazine or its analogue in the buffer (30 mM Tris–HCl (pH 7.6)–10 mM EDTA) and the luminescence reaction in the quartz cuvette was started by adding 5 μL of the purified nanoKAZ (1.6 μg protein).

2.6. Protein analysis

SDS–PAGE analysis was carried out under reducing conditions using a 12% separation gel (TEFCO, Tokyo, Japan). Western blot analysis was performed using anti-*Oplophorus* luciferase serum (×1000 dilution) as previously described [3]. Protein concentration was determined by using a commercially available kit (Bio-Rad, Richmond, CA) and bovine serum albumin as a standard (Pierce, Rockford, IL).

3. Results and discussion

3.1. Expression and purification of nanoKAZ from E. coli cells

To express the mutated 19kOLase of *Oplophorus* luciferase in *E. coli* cells and CHO-K1 cells, we synthesized a codon-optimized gene for the mutated 19kOLase and named it "*nanoKAZ*". The gene product of *nanoKAZ* has the identical amino acid sequence to that of nanoKAZ [12] with 72% identity of the nucleotide sequence to *nanoKAZ*. Previously, we succeeded in expressing the foreign proteins efficiently as a soluble form in *E. coli* cells by fusing an IgG binding domain of protein A (ZZ domain) as a soluble partner in the cold-induced expression system [15]. An expression vector, pCold-ZZ-P-X, consisting of a histidine tag sequence for the nickel chelate affinity purification, an IgG binding domain of protein A (ZZ domain), and the cleavage site of PreScission protease followed by the multiple cloning sites, was applied to express nanoKAZ in *E. coli* cells. The bacterial cells having pCold-ZZ-P-nanoKAZ (Fig. 2A and B) was expressed and 37.2 mg of His-ZZ-P-nanoKAZ with over 95% purity was obtained from 800 mL of the cultured cells (Fig. 2C, lane 3 and Table 1). The purified His-ZZ-P-nanoKAZ was digested with PreScission protease to remove the ZZ domain and the purified nanoKAZ was obtained from a flow-through fraction of a nickel chelate column (Fig. 2C, lane 4).

3.2. Comparison of luminescence properties of the purified nanoKAZ with other coelenterazine-utilizing luciferase

The specific activity of nanoKAZ was determined by using native coelenterazine as a substrate and was compared with those of other coelenterazine-utilizing luciferases including *Renilla* luciferase, *Gaussia* luciferase and 19kOLase as previously reported [17]. As summarized in Table 2, the specific activity of nanoKAZ was about 10-fold lower than that of *Gaussia* luciferase, and was about 100-fold higher than that of 19kOLase. The emission maximum of nanoKAZ at 457 nm was close to that of 19kOLase at 460 nm, suggesting that the excited species of coelenteramide in the protein molecule might be similar to these luciferases. The effect of concentrations of nanoKAZ on the luminescence activity was determined in our assay conditions. When an excess amount of coelenterazine (1 μg, 2.4 nmol) was present in the reaction mixture, nanoKAZ (0.03 μg, 1.5 pmol) showed a glow luminescence pattern. In contrast, high concentration of nanoKAZ (1 μg, 50 pmol) showed a fast decay pattern of luminescence, due to lack of sufficient amounts of coelenterazine (Supplementary Fig. 1S).

3.3. Secretory expression of nanoKAZ from mammalian cells using the signal peptide sequence of Gaussia luciferase

To express nanoKAZ into the culture medium from CHO-K1 cells, we constructed a pcDNA3-GLsp-nanoKAZ vector (Fig. 3A) having the signal peptide sequence for secretion of *Gaussia* luciferase at the amino terminal region of nanoKAZ (Fig. 3B). After CHO-K1 cells were transfected with pcDNA3-GLsp-nanoKAZ, the luciferase activity was observed in the cultured medium and the secreted nanoKAZ was identified by Western blot analysis using anti-*Oplophorus* luciferase antibody (Fig. 3C). A major band with a faint band was detected around 20 kDa and the molecular sizes on the membrane were in good agreement with the estimated molecular weight of 20.2 and 21.9 kDa for secreted nanoKAZ and nanoKAZ with the uncleaved signal peptide sequence, respectively. Under the culture conditions with the serum-free MCDB201 medium, the cells were partially lysed and the uncleaved nanoKAZ was observed.

Table 2
Comparison of luminescence properties among the purified recombinant coelenterazine-utilizing luciferases using coelenterazine as a substrate.

Recombinant protein	Average mass	Expression system (Vector/host bacterial cell)	Specific activity (I_{max}, $\times 10^8$ rlu/mg)	Emission maximum (FWHM[a]) (nm)	Reference
nanoKAZ	20,026	Obtained from His-ZZ-P-nanoKAZ	1.2×10^9 (1.0)	457 (73)	This work
His-ZZ-P-nanoKAZ	37,092	pCold-ZZ-P-nanoKAZ (EX)/BL21	5.3×10^8 (0.45)	457 (73)	This work
19kOLase (wild KAZ)	20,254	pCold-KAZ/BL21	1.0×10^7 (0.01)	460 (72)	[17]
Renilla luciferase	37,697	pCold-RL/BL21	8.8×10^8 (0.73)	485 (93)	[17]
Gaussia luciferase	19,976	pCold-hGL/BL21	1.1×10^{10} (9.3)	488 (81)	[17]

[a] Full width at half maximum.

3.4. Substrate specificity of nanoKAZ for C2-modified coelenterazine analogues

Coelenterazine has an imidazopyrazinone (3,7-dihydroimidazopyrazin-3-one) core structure with two *p*-hydroxyphenyl groups at the C2- and C6-position and a benzyl group at the C8-position (Fig. 1). Previously, we reported that *Oplophorus* luciferase and 19kOLase show a broad substrate specificity in catalyzing the oxidation of various coelenterazine analogues including *bis*-coelenterazine (*bis*-CTZ), a C2,C6-dideoxy analogue, which emitted the light efficiently [8–10]. Recently, we newly synthesized eight C2-modified coelenterazine analogues (Supplementary Fig. 2S) [14] and examined the substrate specificities of *Renilla* luciferase, *Gaussia* luciferase and 19kOLase by using them [17]. In this study, we firstly examined the substrate specificity for the purified nanoKAZ using these coelenterazine analogues. The order of the initial luminescence intensity (I_{max}) for the purified nanoKAZ (relative I_{max} to CTZ) was *f*-CTZ (18.4) > *h*-CTZ (17.7) > *3meo*-CTZ (11.6) > *3me*-CTZ (11.5) > *meo*-CTZ (8.9) > *me*-CTZ (5.2) = *cf3*-CTZ (5.2) > *n*-CTZ (4.9) > *et*-CTZ (4.4) > *i*-CTZ (3.3) > *3iso*-CTZ (1.5) > CTZ (1.0) > *αmeh*-CTZ (0.1) (Table 3 and Supplementary Table 1S). A similar order was obtained from the luminescence reaction for the secreted nanoKAZ in the culture medium (Table 3 and Supplementary Table 1S), showing that *f*-CTZ and *h*-CTZ are a good substrate for both the purified and the secreted nanoKAZ from the cell. The reaction kinetics of the purified nanoKAZ and the secreted nanoKAZ with the efficient C2-analogues such as *f*-CTZ, *h*-CTZ, *3meo*-CTZ, *3me*-CTZ and *meo*-CTZ showed the fast-decay pattern of luminescence (Fig. 4 and Supplementary Fig. 2S). These results indicated that a substituent on phenyl group at the C2-position of coelenterazine does not largely affect the fast decay pattern of luminescence for nanoKAZ.

3.5. Substrate specificity of nanoKAZ for C6-deoxy coelenterazine analogues

Recently, a C6-deoxy coelenterazine analogue with the furan group at the C2-position, named furimazine (Fig. 1), was synthesized and shown as a good substrate for nanoLuc producing a glow-type luminescence [12]. Based on the structure of furimazine, we predicted that a substrate lacking the hydroxy group at the C6-phenyl moiety of coelenterazine might give the glow luminescence pattern of nanoKAZ and synthesized *6h-f*-coelenterazine (*6h-f*-CTZ) and *6h*-coelenterazine (*6h*-CTZ) to confirm this idea (Fig. 1 and Supplementary data). The luminescence properties of nanoKAZ with *bis*-CTZ, *h*-CTZ, *6h*-CTZ, *f*-CTZ, *6h-f*-CTZ and furimazine were characterized and were summarized in Table 3. The relative intensity of I_{max} for the purified nanoKAZ was in the order of: *f*-CTZ (18.4) > *h*-CTZ (17.7) > *bis*-CTZ (11.2) > *6h-f*-CTZ (10.1) > furimazine (5.5) > CTZ (1.0) > *6h*-CTZ (0.6). A similar result was obtained from the nanoKAZ secreted into the culture medium (Table 3). However, the different luminescence patterns were observed between the purified nanoKAZ and the cultured medium of secreted nanoKAZ (Fig. 4A and B). This difference might be explained by the protein instability of the purified nanoKAZ in an aqueous solution and/or the partial product inhibition of nanoKAZ by oxyluciferin (coelenteramide analogues). In contrast, the nanoKAZ secreted in

Table 3
Substrate specificity of the purified nanoKAZ for C2- and/or C6-modified coelenterazine analogues.

Substrate	Relative intensity I_{max} (Int.[a])		Emission maximum of purified nanoKAZ (FWHM[b]) (nm)
	Purified nanoKAZ from *E. coli* cells	Secreted nanoKAZ from CHO-K1 cells	
CTZ	1.0[c] (1.0)[d]	1.0[e] (1.0)[f]	457 (73)
bis-CTZ	11.2 (7.7)	10.3 (7.7)	453 (73)
h-CTZ	17.7 (10.6)	17.0 (10.1)	455 (71)
6h-CTZ	0.6 (0.5)	0.8 (0.7)	454 (75)
f-CTZ	18.4 (9.2)	19.5 (11.1)	453 (71)
6h-f-CTZ	10.1 (6.0)	10.1 (10.1)	449 (71)
Furimazine	5.5 (4.4)	5.5 (5.7)	453 (73)

[a] Integrating for 60 s in 0.1 s intervals.
[b] Full width at half maximum.
[c] 3.1×10^4 rlu/0.1 s.
[d] 1.4×10^7 rlu/60 s.
[e] 1.2×10^4 rlu/0.1 s.
[f] 6.5×10^6 rlu/60 s.

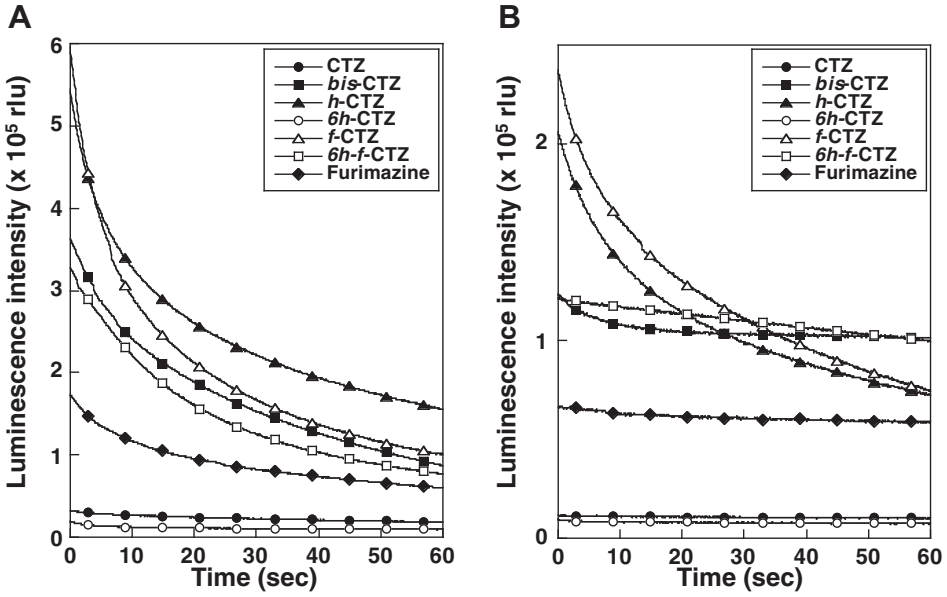

Fig. 4. Luminescence patterns of the purified nanoKAZ from *E. coli* cells and the secreted nanoKAZ from CHO-K1 cells with the C2- and/or C6-modified coelenterazine analogues. (A) Luminescence patterns of the purified nanoKAZ from *E. coli* cells. The purified nanoKAZ (0.003 μg) was used for assay in 100 μL of 30 mM Tris–HCl (pH 7.6)–10 mM EDTA containing 1 μg of coelenterazine analogue. (B) Luminescence patterns of the secreted nanoKAZ from CHO-K1 cells. The culture medium (5 μL) containing nanoKAZ was used at the same assay conditions.

the culture medium was stabilized by the medium containing 10% fetal bovine serum and could show the glow pattern of luminescence with *bis*- and *6h-f*-CTZ. As we predicted, the C6-deoxy analogues, *bis*-CTZ, *6h*-CTZ and *6h-f*-CTZ showed the glow luminescence patterns with nanoKAZ, and *bis*-CTZ and *6h-f*-CTZ showed about 2-fold higher luminescence intensity than furimazine (Fig. 4). Furthermore, the chemical stabilities of CTZ, *bis*-CTZ, *6h-f*-CTZ and furimazine were examined by incubation with 30 mM Tris–HCl (pH 7.6) at 37 °C for 22 h. The results of HPLC analysis indicated that over 95% of CTZ and furimazine were decomposed under these conditions, whereas 38% and 49% of *bis*-CTZ and *6h-f*-CTZ, respectively, remained intact (unpublished result). Taken together, *bis*-CTZ and *6h-f*-CTZ would be a suitable substrate for a reporter assay using nanoKAZ or nanoLuc secreted from mammalian cells.

Acknowledgments

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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.2013.06.026.

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