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Identification of two catalytic domains in a luciferase secreted by the copepod *Gaussia princeps*

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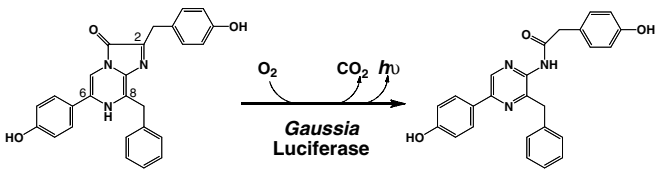
Received 14 October 2007
Available online 5 November 2007

Abstract

Gaussia luciferase secreted by the copepod *Gaussia princeps* catalyzes the oxidation of coelenterazine to produce blue light. The primary structure of *Gaussia* luciferase deduced from the cDNA sequence shows two repeat sequences of 71 amino acid residues, suggesting the luciferase consists of two structural domains. Two domains in *Gaussia* luciferase were expressed independently in *Escherichia coli* cells, purified and characterized. We found that both domains have luminescence activity with coelenterazine, and the catalytic properties including luminescence spectrum, optimal pH, substrate specificity and luminescence stimulation by halogen ions (Cl^-, Br^- and I^-) are identical to intact *Gaussia* luciferase. Thus, *Gaussia* luciferase has two catalytic domains for the luminescence reaction.

Keywords: Protein refolding; Coelenterazine; Bioluminescence; Halogen ions; Secretion

The deep-sea copepod *Gaussia princeps* (T. Scott), of the family *Metridinidae,* produces bright extracellular luminescence from a luciferase–luciferin reaction [1–4]. The secreted luciferase of *G. princeps* catalyzes the oxidation of coelenterazine (luciferin; an imidazopyrazinone compound) to emit blue light [5,6] in the following reaction:



Abbreviations: hSGL, *Gaussia* luciferase with the signal peptide sequence; hGL, *Gaussia* luciferase without the signal peptide sequence (1–168 amino acids); hGL-27/97, the catalytic domain of *Gaussia* luciferase at the position of 27–97; hGL-98/168, the catalytic domain of *Gaussia* luciferase at the position of 98–168; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; I_{max}, maximum intensity; rlu, relative light units; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis.

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doi:10.1016/j.bbrc.2007.10.152

In 1999, the cDNA for *Gaussia* luciferase from *G. princeps* was isolated by expression cloning in *Escherichia coli* cells [7], and *Gaussia* luciferase consists of 185 amino acid residues including a signal peptide sequence for secretion [7–9]. Recently, the cDNA clones of *Metridia* luciferase from the luminous marine copepod *Metridia longa* in the family *Metridinidae* were isolated and the primary structure of *Metridia* luciferase shows high similarity to that of *Gaussia* luciferase [10]. *Gaussia* luciferase has no significant sequence homologies with other luciferases including coelenterazine-type luciferases from *Renilla reniformis* [11] and *Oplophorus gracilorostris* [12]. The small *Gaussia* luciferase does not require any cofactors and is a useful reporter protein/gene [13–17].

Long repeat sequences were found in luciferases from the ostracod *Cypridina* (presently *Vargula*) *hilgendorfii* [18–20] and the dinoflagellate *Gonyaulax polyedra* (presently *Lingulodinium polyedrum*) [21,22]. *Cypridina* luciferase consists of 555 amino acid residues having two repeat sequences (217 and 206 amino acid residues), which are homologous to the von Willebrand factor type D domain. The amino terminal domain showed luminescence activity [20], suggesting that the carboxy terminal domain might

have luminescence activity. *Gonyaulax* luciferase has three tandem homologous domains with 377 amino acid residues in 1241 amino acid residues and these domains show the catalytic activity of luminescence reaction [22]. In *Gaussia* luciferase, the primary structure deduced from the cDNA sequence shows two short repeat sequences of 71 amino acid residues containing 4 conserved cysteine residues, suggesting the *Gaussia* luciferase gene may have arisen by duplication. The duplicated amino acid sequences in *Gaussia* luciferase might be the structural domain. In the present report, we examined whether the small repeat domains of *Gaussia* luciferase have catalytic function to oxidize coelenterazine to emit light, and characterized the catalytic properties of two domains including luminescence spectrum, optimal pH, substrate specificity and luminescence stimulation by halogen ions.

Materials and methods

Materials. The sources of chemicals were as follows: urea, imidazole, $NiSO_4 \cdot 6H_2O$, ethylenediaminetetraacetic acid disodium salt (EDTA·2Na), isopropyl-β-thiogalactopyranoside (IPTG), dithiothreitol (DTT) and *n*-coelenterazine (*n*-CTZ) (Wako Pure Chemicals, Osaka, Japan); chelate Sepharose Fast Flow (Amersham Biosciences, Piscataway, NJ); coelenterazine (CTZ), *h*-coelenterazine (*h*-CTZ) and *Bis*-coelenterazine (*Bis* -CTZ) (Chisso Co., Yokohama, Japan); *hcp*-coelenterazine (*hcp*-CTZ), *f*-coelenterazine (*f*-CTZ) and *cp*-coelenterazine (*cp*-CTZ) (Promega Co. Madison, WI); *e*-coelenterazine (*e*-CTZ) and methoxycoelenterazine (*MeO*-CTZ) was kindly provided by Dr. K. Teranishi (Mie University, Japan). All other chemicals were of the highest commercial grade available.

Protein analysis. SDS–PAGE analysis was carried out under reducing conditions using 12% or 16% separation gels (TEFCO), as described by Laemmli [23]. Protein concentration was determined by the dye-binding method of Bradford [24] using a commercially available kit (Bio-Rad, Richmond, CA) and bovine serum albumin as a standard (Pierce; Rockford, IL).

DNA sequence analysis. The nucleotide sequence was determined with an Applied Biosystems model 310 DNA sequencer using a BigDye terminator v1.1 cycle sequencing kit.

Construction of the expression vectors for Gaussia luciferase and catalytic domains in E. coli cells. To express *Gaussia* luciferase (hSGL and hGL) and two repeat domains (hGL-27/97 and hGL-98/168, as in Fig. 1C), the expression vector pColdII (Takara-Bio, Kyoto, Japan) containing the promoter of cold shock protein A (*csp*A) and the *lac* operator was used [25]. Briefly, the coding regions of hSGL, hGL, hGL-27/97 and hGL-98/168 (Fig. 1B) were obtained from pcDNA3-hGL containing humanized *Gaussia* luciferase (Prolume Ltd.; Pinetop, AZ) as a template by PCR amplification (25 cycles; 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C) using Ex-Taq polymerase (Takara-Bio). Primer sets are as follows: hSGL, GL4-N/SacI (5′-gcc-GAG-CTC-GGA-GTC-AAA-GTT-CTG-TTT-GCC-3′; SacI site underlined) and GL2-C/EcoRI (5′-gcc-GAA-TTC-TTA-GTC-ACC-ACC-GGC-CCC-CTT-3′; EcoRI site underlined); hGL, GL5-N/SacI (5′-gcc-GAG-CTC-AAG-CCC-ACC-GAG-AAC-AAC-GAA-3′; SacI site underlined) and GL2-C/EcoRI (5′-gcc-GAA-TTC-TTA-GTC-ACC-ACC-GGC-CCC-CTT-3′; EcoRI site underlined); hGL-27/97, GL13-N-27/EcoRI (5′-ggc-GAA-TTC-CGC-GGG-AAG-TTG-CCC-GGC-AAG-3′; EcoRI site underlined) and GL8-C-71/XbaI (5′-cgg-TCT-AGA-TTA-GTC-GAC-GAT-CGC-CTC-GCC-3′; XbaI site underlined); hGL-98/168, GL15-N-97/EcoRI (5′-ggc-GAA-TTC-ATT-CCT-GAG-ATT-CCC-GGG-TTC-3′; EcoRI site underlined) and GL7-C/XbaI (5′-gcc-TCT-AGA-TTA-GTC-ACC-ACC-GGC-CCC-CTT-3′; XbaI site underlined). The PCR fragments digested with the restriction enzymes were inserted into pColdII vector to give pCold-hSGL, pCold-hGL, pCold-hGL-27/97 and pCold-hGL-98/168 (Fig. 1B). The expressed protein had a histidine-tagged sequence at the amino terminus for nickel-chelate affinity chromatography (Fig. 1C). The host *E. coli* strain used was BL21 (Novagen, Madison, WI).

Expression and purification of Gaussia luciferase and catalytic domains from E. coli cells. The seed culture of the bacterial strain possessing expression vector was grown in 10 ml of Luria–Bertani broth containing

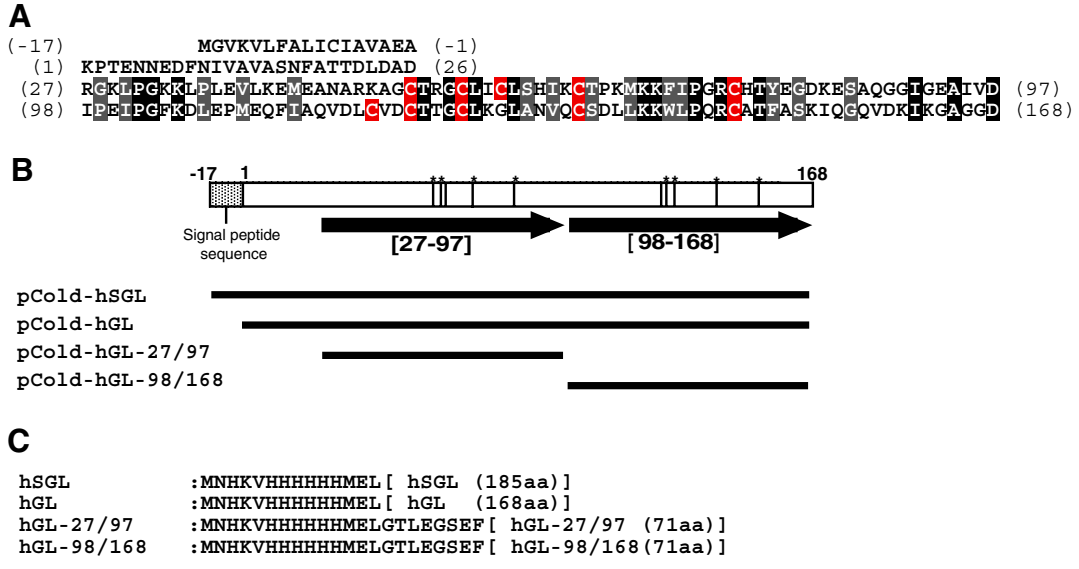

Fig. 1. Structure of *Gaussia* luciferase and its expression in *E. coli* cells. (A) Alignment of the amino acid sequences in *Gaussia* luciferase. Letters in the black box and the shaded box indicate identical amino acids and amino acid groups having similar physical and chemical properties, respectively. 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. The numbers in parenthesis indicate amino acid positions. (B) Schematic representation of the *Gaussia* luciferase gene and its expression in *E. coli* cells. The two arrows with positions indicated in square brackets show the repeat domains of *Gaussia* luciferase. Vertical bars in the open box are cysteine residues and asterisks (∗) indicate conserved cysteine residues in the repeat sequences. Horizontal lines show the regions expressed using the pColdII vector. (C) Expression of histidine-tagged *Gaussia* luciferase and its repeat domains in *E. coli* cells. The amino terminal sequences in *Gaussia* luciferase and their repeat domains expressed in *E. coli* cells are given.

ampicillin (50 μg/ml) at 37 °C for 18 h. The seed culture was transferred into 400 ml of LB broth in a 3 l flask, incubated for 4.5 h and then cooled on an ice-water bath for 1 h. After adding of IPTG at the final concentration of 0.2 mM to the culture medium, the bacterial cells were incubated at 15 °C for 17 h. The cells harvested by centrifugation at 5000*g* for 5 min were dissolved in 80 ml of 20 mM Tris–HCl (pH 7.6) and disrupted by sonication using a Branson model 250 sonifier (Danbury, CT) for 6 min (2 min × 3) in ice-water bath. After centrifugation at 12,000*g* for 20 min, the expressed proteins from the supernatant (soluble fraction) and the precipitate (insoluble fraction) were purified as follows:

(i) *Purification from soluble fractions.* The soluble fraction (80 ml) was applied on a nickel-chelate column (2.5 × 6.5 cm), equilibrated with 50 mM Tris–HCl (pH 7.6). After washing the column with 500 ml of 50 mM Tris–HCl (pH 7.6), the adsorbed proteins were stepwise eluted with 50 mM Tris–HCl (pH 7.6) containing imidazole. *Gaussia* luciferase (hGL) and two domains (hGL-27/97 and hGL-98/168) were eluted by 0.1 M imidazole and 0.3 M imidazole, respectively.

(ii) *Purification from insoluble fractions.* The precipitate was dissolved in 50 ml of 20 mM Tris–HCl (pH 7.6)-6 M urea, and the urea-soluble fraction obtained by centrifugation at 12,000*g* for 10 min was applied on a nickel-chelate column (2.5 × 6.5 cm), equilibrated with 20 mM Tris–HCl (pH 7.6)-6 M urea. The adsorbed proteins were stepwise eluted with in 50 mM Tris–HCl (pH 7.6)-6 M urea containing imidazole. *Gaussia* luciferase (hGL) and two domains (hGL-27/97 and hGL-98/168) from insoluble fractions were eluted by 0.1 M imidazole and 0.3 M imidazole, respectively. After combining active fractions, the purity of protein was determined by SDS–PAGE analysis.

Determination of luminescence activity. (i) The total reaction mixture (100 μl) contained coelenterazine (0.5 μg, dissolved in 1 μl of ethanol) in 30 mM Tris–HCl (pH 7.6)-10 mM EDTA. The reaction was started by addition of protein solution (1 μl) and the initial maximal light intensity was measured using an Atto (Tokyo, Japan) AB2200 luminometer equipped with a R4220P photomultiplier (Hamamatsu K.K., Japan). The maximum intensity (I_{max}) of 1 ng of the purified recombinant aequorin showed 8.8×10^5 rlu.

(ii) To determine the effects of ions on luminescence activity, 100 mM sodium phosphate buffer (pH 7.5) containing various ions was used.

Measurements of bioluminescence spectra. Bioluminescence emission spectra were measured on a Jasco FP-6500 fluorescence spectrophotometer (emission band width, 20 nm; response, 0.5 s; sensitivity, medium; scan speed, 2000 nm/min) at 22–25 °C with the excitation light source turned off, as previously described [26].

Mass spectrometry. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was performed on an AutoFLEX (Bruker Daltonics) in the positive reflector mode using sinapic acid (Invitrogen) as a matrix, as previously described [27].

Results and discussion

Prediction of two catalytic domains in Gaussia luciferase

In the primary structure of *Gaussia* luciferase, the repeat sequences with 71 amino acid residues were found at positions 27–97 (assigned hGL-27/97) and 98–168 (assigned hGL-98/168), respectively (Fig. 1A). It is of interest that 4 of 5 cysteine residues were conserved in the repeat sequences (Fig. 1B, marked asterisks), suggesting that the repeat sequences correspond to two structural domains. By treatment of *Gaussia* luciferase with reducing reagents such as 1 mM dithiothreitol and 0.01% 2-mercaptoethanol, the luminescence activity of *Gaussia* luciferase was lost completely. The chemical modification of the thiol group by 1 mM *N*-ethylmaleimide and 1 mM iodoacetamide gave no significant loss of luminescence activity (data not shown). These results suggest that cysteine residues might be in disulfide bond(s), essential for the functional structure of *Gaussia* luciferase, but not for the luminescence reaction.

Expression and purification of two domains of Gaussia luciferase in E. coli cells

To investigate the catalytic function of *Gaussia* luciferase, the entire region and the repeat regions of *Gaussia* luciferase were expressed in *E. coli* cells using the cold-induction system [25] with the expression vectors pCold-hSGL, pCold-hGL, pCold-hGL-27/97 and pCold-hGL-98/168 (Fig. 1C). After induction by adding of IPTG and cold-shock treatment at 15 °C (Fig. 2, lanes 4 and 5), the bacterial cells with pCold-hSGL possessing the signal peptide sequence for secretion in mammalian cells were lysed. Presumably, the signal peptide sequence of *Gaussia* luciferase may affect the bacterial membranes and induced cell lysis. On the other hand, the crude extracts of the bacterial cells with pCold-hGL showed luminescence activity and a 25 kDa band was observed on SDS–PAGE (data not shown). After centrifugation of crude extracts at 12,000*g* for 5 min, the 25 kDa protein was found in the precipitate and the supernatant. Thus, *Gaussia* luciferase was mainly expressed as inclusion bodies in *E. coli* cells (Figs. 2 and 3). Using pCold-hGL-27/97 and pCold-hGL-98/168 as expression vectors, the expressed domains, hGL-27/97 and hGL-98/168, formed mainly as inclusion bodies and both crude extracts showed weak luminescence activity with coelenterazine.

The recombinant proteins expressed in *E. coli* cells had a six-histidine tag sequence at the amino terminus for purification by nickel-chelate affinity chromatography. The soluble fractions of hGL, hGL-27/97 and hGL-98/168 were applied on a nickel-chelate column and purified. From insoluble fractions, the precipitate was dissolved in 6 M urea and purified on a nickel-chelate column, equilibrated with 6 M urea. In SDS–PAGE analysis under reducing conditions, the mobility of hGL-98/168 from soluble and insoluble fractions was faster than hGL-27/97 (Fig. 2B, lanes 4 and 8). To confirm the molecular weight of proteins from the soluble fractions, the purified proteins were subjected to MALDI-TOF-MS analyses. The observed masses *m/z* for hGL, hGL-27/97 and hGL-98/168 were 20019.1, 10742.8 and 10354.5, respectively, in good agreement with the calculated average masses of 19977.1, 10696.5 and 10309.9, respectively. Thus, hGL-98/168 on the gel was not a truncated product and the behavior of hGL-98/168 on gels might be caused by the structure of protein.

Two domains in Gaussia luciferase have luminescence activity

The specific activities of recombinant proteins purified from soluble and insoluble fractions from bacterial cells are summarized in Table 1. hGL expressed as a soluble form showed significant luminescence activity, suggesting

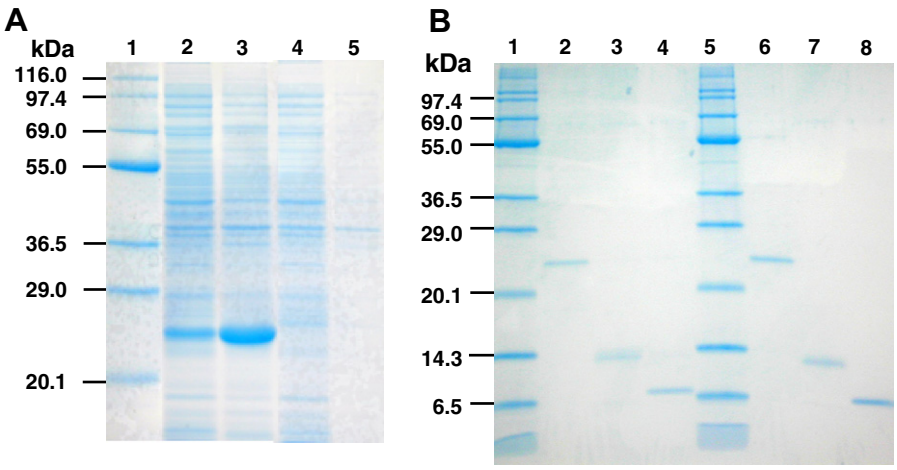

Fig. 2. SDS–PAGE analysis of recombinant *Gaussia* luciferase. (A) Expression of *Gaussia* luciferase with and without its own signal peptide sequence in *E. coli* cells. Lane 1, molecular weight markers (TEFCO, Japan): β -galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (69.0 kDa), glutamic dehydrogenase (55.0 kDa), lactic dehydrogenase (36.5 kDa), carbonic anhydrase (29.0 kDa) and trypsin inhibitor (20.1 kDa). Lane 2, 12,000*g* supernatant of crude extracts from pCold-hGL/BL21 (100 μl cultured cells). Lane 3, 12,000*g* precipitate of crude extracts from pCold-hGL/BL21 (100 μl cultured cells). Lane 4, 12,000*g* supernatant form pCold-hSGL/BL21 (100 μl cultured cells). Lane 5, 12,000*g* precipitate from pCold-hSGL/BL21 (100 μl cultured cells). (B) Purified *Gaussia* luciferase and the repeat domains from soluble and insoluble fractions in *E. coli* cells. Lanes 1 and 5, molecular weight markers (TEFCO, Japan). Purified proteins from soluble fractions: lane 2 (0.5 μg protein), lane 3 (1.0 μg protein) and lane 4 (0.5 μg protein) are from pCold-hGL, pCold-hGL-27/29, pCold-hGL-98/168, respectively. Purified proteins from insoluble fractions: lane 6 (0.5 μg protein), lane 7 (0.75 μg protein) and lane 8 (0.5 μg protein) are from pCold-hGL, pCold-hGL-27/29, pCold-hGL-98/168, respectively.

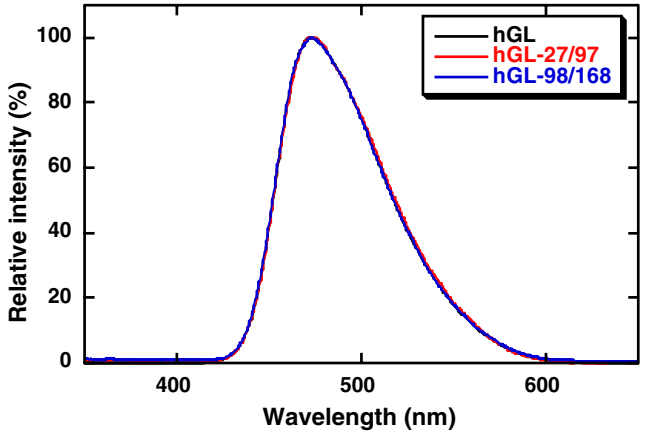

Fig. 3. Bioluminescence spectra of hGL, hGL-27/97 and hGL-98/168. The amount of protein in 1 ml of 30 mM Tris–HCl (pH 7.6)-10 mM EDTA was as follows: hGL, 140 μg; hGL-27/97, 80 μg; hGL-98/168, 210 μg.

that active hGL might be refolded during expression in *E. coli* cells. Furthermore, two domains of hGL-27/97 and hGL-98/168 purified from a soluble fraction, showed luminescence activity of 2.29% and 1.15%, compared to hGL. Thus, two domains of hGL-27/97 and hGL-98/168 have the catalytic function to emit light independently, even if the disulfide bond(s) in hGL, hGL-27/97 and hGL-98/168 did not form correctly.

Characterization of the catalytic domains of Gaussia luciferase

As shown in Fig. 3, the light emission spectra of hGL, hGL-27/97 and hGL-98/168 using coelenterazine were superimposed and the emission peaks were at 473 nm with a full width at half maximum of 66 nm. This result suggests that the light-emitting species of coelenteramide (oxyluciferin) produced by the oxidation of coelenterazine with hGL, hGL-27/97 and hGL-98/168 is identical.

The optimal pH of hGL, hGL-27/97 and hGL-98/168 with coelenterazine was determined and they were found to be around 7.5 (Fig. 4A). This result suggests that the catalytic residues in the oxidation reaction might be similar in the two catalytic domains of *Gaussia* luciferase.

Table 1
Purification of *Gaussia* luciferase (hGL) and two repeat domains (hGL-27/97 and hGL-98/168) from soluble and insoluble fractions in *E. coli* cells

Proteins	Purified fraction	Purified proteins (mg)	Total activity ($I_{max} \times 10^9$ rlu)	Specific activity ($\times 10^9$ rlu/mg)	Relative specific activity (%)
hGL[a]	Soluble	15.0	768.5	51.23	100.00
	Insoluble	217.7	3095.7	14.22	27.76
hGL-27/97 [b]	Soluble	2.0	2.3	1.17	2.29
	Insoluble	15.4	48.1	3.12	6.09
hGL-98/168 [b]	Soluble	9.2	5.4	0.59	1.15
	Insoluble	36.8	0.6	0.02	0.03

[a] Purified from 2 l of cultured cells.
[b] Purified from 0.8 l of cultured cells.

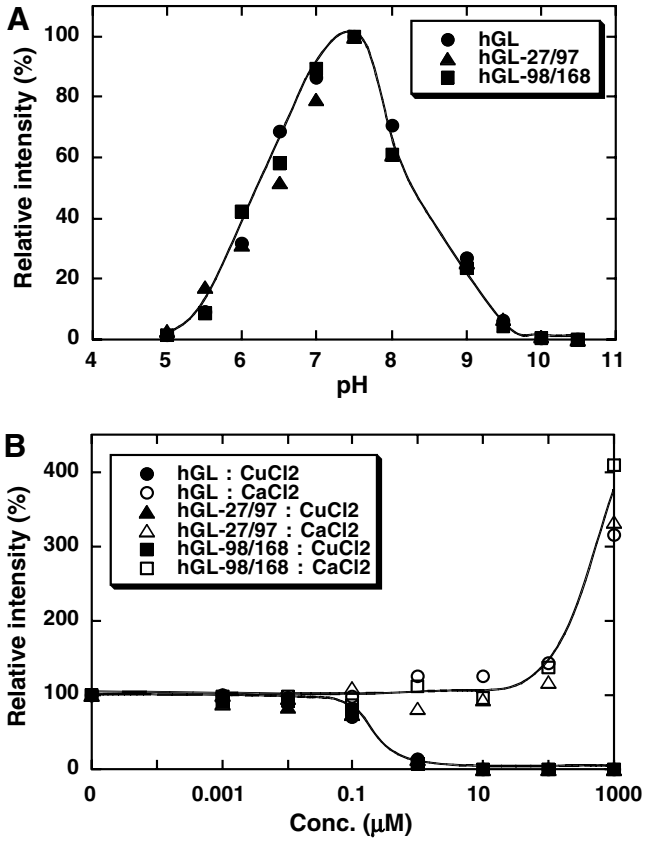

Fig. 4. Enzymatic properties of hGL, hGL-27/97 and hGL-98/168. (A) Optimum pH of hGL, hGL-27/97 and hGL-98/168. Symbols are as follows: hGL (●), hGL-27/97 (▲) and hGL-98/168 (■). The buffers were: 0.1 M sodium acetate buffer (pH 5.5–6.0), 0.1 M sodium phosphate buffer (pH 6.5–8.0), 0.1 M Glycine–NaOH buffer (pH 8.5–10.5). (B) Effects of $CuCl_2$ and $CaCl_2$ on luminescence activity of hGL, hGL-27/97 and hGL-98/168. Symbols are as follows: hGL + $CuCl_2$ (●), hGL + $CaCl_2$ (○), hGL-27/97 + $CuCl_2$ (▲), hGL-27/97 + $CaCl_2$ (Δ), hGL-98/168 + $CuCl_2$ (■) and hGL-98/168 + CaCl2 (□).

The effect of ions on luminescence intensity of hGL was investigated by incubation with 100 mM sodium phosphate buffer (pH 7.5) containing 1 mM salts, and their relative luminescence intensities were; None, 1.00; NaCl, 3.90; $MgCl_2$, 5.67; MgSO4, 0.98; KCl, 3.95; $CaCl_2$, 5.37; $FeCl_2$, 4.30; $NiCl_2$, 1.52; $CuCl_2$, 0.01; $CuSO_4$ 0.003; $ZnCl_2$, 3.62; $ZnSO_4$, 0.51; RbCl, 3.73; $SrCl_2$, 5.51, $CdCl_2$, 3.47; $(NH_4)_2SO_4$, 1.03; NH_4Cl, 4.11. Thus, the heavy metal ion Cu^{2+} showed strong inhibition of luminescence activity and the monovalent ion Cl^- stimulated luminescence activity significantly. The divalent ion SO_4^{2-} had little effect on luminescence activity. To confirm inhibition and stimulation on luminescence activity, dose response curves for $CuCl_2$ and $CaCl_2$ were determined using hGL, hGL-27/97 and hGL-98/168. As shown in Fig. 4B, hGL, hGL-27/97 and hGL-98/168 were over 95% inhibited by 1 μM $CuCl_2$, whereas $CaCl_2$ stimulated luminescence activity over a concentration of 0.1 mM. Further, the effects of other halogens including F^-, Br^- and I^- on luminescence activity were examined and summarized in Table 2. The

Table 2
Effects of halogen ions on *Gaussia* luciferase (hGL) and two catalytic domains (hGL-27/97 and hGL-98/168)

Salt conc. (5 mM)	Relative intensity		
	hGL[a]	hGL-27/97[a]	hGL-98/168[a]
None	1.00	1.00	1.00
$(NH_4)_2SO_4$	0.95	0.77	0.85
$MgSO_4$	0.94	0.94	1.00
$MgCl_2$	5.77	5.52	10.68
KF	0.90	0.90	0.62
NaF	0.99	0.74	0.69
KCl	4.81	5.50	6.61
NaCl	4.76	4.84	6.71
KBr	7.59	8.42	16.76
NaBr	8.18	9.12	13.82
KI	5.54	5.32	7.75
NaI	6.02	5.27	6.91

[a] Purified proteins from soluble fractions were used and the amounts of hGL, hGL-27/97 and hGL-98/168 were 0.32 μg, 0.08 μg and 0.04 μg, respectively.

halogens Br^- and I^- stimulated luminescence activity of hGL, hGL-27/97 and hGL-98/168 and the stimulation efficiency was $Br^- > I^- > Cl^-$. However, F^- from NaF and KF did not stimulate luminescence activity. The mechanism of stimulation by the halogen ions Br^-, I^- and Cl^- was not clear.

Substrate specificities for hGL, hGL-27/97 and hGL-98/168 were determined using coelenterazine analogs (Table 3). *Gaussia* luciferase showed narrow substrate specificity, indicating highly specific for coelenterazine. Similar substrate specificities were observed in the cases of hGL-27/97 and hGL-98/168. This substrate specificity of *Gaussia* luciferase is different from other coelenterazine-type luciferases such as *Renilla* luciferase [28], *Oplophorus* luciferase [28] and *Periphylla* luciferase [29]. From these results, we conclude that *Gaussia* luciferase has two catalytic domains for the luminescence reaction and the catalytic properties of the domains, hGL-27/97 and hGL-98/168, were almost identical to *Gaussia* luciferase.

Table 3
Substrate specificities of *Gaussia* luciferase (hGL) and two catalytic domains (hGL-27/97 and hGL-98/168)

Substrate	Relative luminescence activity (%)		
	hGL[a]	hGL-27/97[a]	hGL-98/168[a]
CTZ	100.00	100.00	100.00
h-CTZ	1.27	2.70	0.25
hcp-CTZ	0.02	0.01	0.02
cp-CTZ	0.03	0.07	0.03
f-CTZ	0.08	1.63	0.04
fcp-CTZ	0.02	0.00	0.02
n-CTZ	0.16	0.14	0.03
Bis-CTZ	0.02	0.02	0.07
MeO-CTZ	0.42	0.75	1.28
e-CTZ	0.02	0.08	0.32

[a] Purified proteins from soluble fractions were used and the amounts of hGL, hGL-27/97 and hGL-98/168 were 0.02 μg, 0.08 μg and 0.005 μg, respectively.

Acknowledgments

The authors thank Dr. Y. Oba for mass spectrometry. This study was supported in part by the Program for Promotion of Fundamental Studies in Health Science of the National Institute of Biomedical Innovation (NIBIO) of Japan.

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