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Note

Site Directed Mutagenesis of LuxG as a Flavin Reductase from Bioluminescent Bacteria of *Photobacterium leiognathi*

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Riboflavin is a water-soluble vitamin that is involved in respiration physiologically, in a form of coenzyme FMN (riboflavin 5'-phosphate) and FAD (flavin adenine dinucleotide), contributing to various oxidation-reduction reactions.[1] It serves the important roles of delivering and accepting of hydrogen atoms, protons, or hydride ions through several organic metabolites, such as amino acids, fatty acids, and pyrimidine.[2] The core part of the isoalloxazine ring in flavin acts as a donor and acceptor for two electrons.[3]

Genes that encode for proteins involved in the light of bioluminescent bacteria are grouped by location and referred as *lux* operons. The operon consists of *lux*AB, encoding α, β subunits of luciferase, and *lux*CDE, encoding acyl-CoA reductase, acyl-transferase, acyl-protein synthetase subunits of the fatty acid reductase complex.[4,5] This *lux* gene system (*lux*CDABE) is found in common all the operons from bioluminescent bacteria. The *lux*CDABE can be modified not only by regulatory genes (*lux*R and I) but also genes that are involved in flavin binding and metabolism (*lux*F, *lux*L, *lux*Y, *lux*G, and *lux*H).[6,7] The *Photobacterium* species has been a particularly interesting subject since it is brightest species and has at least more than three-fold the activity of luciferase than that of the *Vibrio* species.[8,9]

Riboflavin genes (*rib*E, B, H, and A), which exist downstream of *lux*G in the *Photobacterium* species, will have a relationship with the condition of the highest luminous intensity in bioluminescent bacteria[10] (*Fig*. 1). As reduced riboflavin 5'-phosphate ($FMNH_2$) is the substrate for bacterial luciferase, it is significant that riboflavin synthesis provides the flavin substrate in the bacterial bioluminescence reaction.[7,9]

From the reaction shown below, the luminous reaction in bioluminescent bacteria produce a blue-green light as $FMNH_2$ and a long chain of fatty aldehyde is oxidized by luciferase.[4,5]

$$FMNH_2 + RCHO + O_2 \rightarrow FMN + H_2O + RCOOH + light$$
(bacterial luciferase)

The long chain fatty aldehyde substrate is provided by the fatty acid reductase complex (LuxCDE). The other substrate, $FMNH_2$, is reproduced by flavin reductase, which exists primarily in bioluminescent bacteria. The regeneration of $FMNH_2$ catalyzed by flavin reductase is significant for continuous emission of light (*Fig*. 1).

$$FMN + NADP(H) + H^+ \rightarrow FMNH_2 + NADP^+$$
(flavin reductase)

Flavin reductase has been found in various bacteria, including *Agrobacterium tumefaciens*, *Azotobacter vinelandii*, *Bacillus megaterium*, *Bacillus subtilis*, and *Escherichia coli*.[8] The *lux*G is present on *lux* operons in marine bioluminescent bacteria; however, the terrestrial bioluminescent bacteria *Xenorhabdus luminescens* does not have that gene. As LuxG of *Photobacterium leiognathi* has an amino acids homology with NAD(P)H:flavin oxidoreductase in *E. coli*,[12] and the LuxG from *P. leiognathi* exhibits fla-

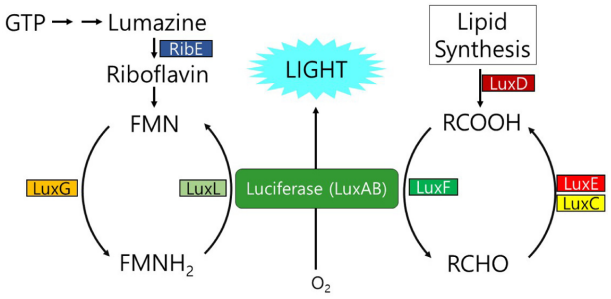

***Figure* 1.** Overall light-emitting reaction in bioluminescent bacteria of the *Photobacterium* species. The enzymes are RibE, riboflavin synthase; LuxAB, heterodimer of luciferase; LuxCDE, fatty acid reductase complex; LuxF, non-fluorescent flavoprotein; LuxG, flavin reductase; and LuxL, lumazine protein.

```
                                                                  ###50
MIFNCKVKK VEAS DSHIYKVFIKPDKCFDFKAGQYVIVYLNGKNLPFSIANCP
--L---II- I--S ECN-F---------LN-------LA--D--K---------
--VDGR-S-I-L--IKNN------TVNSPIK-I---F-M-TI---KC--------
-LCSIEK    I-PL TSF-FR-LL---QP-E-R----IN-S-SFGS------S--

                                                      100
TCNELLELHVGGSVKESAIEAISHFINAFIYQKEF TIDAPHGDAWLRDESQSPL
-----I-----S----T-VKS----LD--VNSS-I Q------N----ED-N---
-K-HEI---I-S-N-DCSLDI-EY-VD-LVEEVAI EL-----N----S--NN--
SNGAF----I---DISKKNTLVMEELTNSWGCGNMVEVSEAR-K-------VK--

    #####                                    150
LLIAGGTGLSYINSILSCCISKQLSQPIYLYWGVNNCNLLYADQQLKTLAAQYRN
-----------------N-VNRN-PRS--V------ID-----T---A-SSDFN-
-----------------TN-LNRNIP-D-------K-SS---E-EE-LE-SLNNK-
--V-----M--TL---KNSLAQGFN----V---AKDMEN--VHDE-VDI-LENK-

                                           200 ###
INYIPVVENLNTDWQGKIGNVIDAVIEDFSDLSDFDIYVCGPFGMSRTAKDILIS
VK-V--L--FDNN-Y--K------I----G---E--------Q--T-SVREK-T-
LH----I-DKSEE-I--K-T-L---M---T--AH---------M-AKT-KEK---
VS-V--T-ISTCPQYA-Q-K-LEC-MS--RN--EF---L---YK-VEV-R-WFCD

            ###
QKKANIGKMYSDAFSYT*
L---DTD--FA---AYM*
E---KSEQ-FA---A-V*
KRG-EPEQLYA---A-L*
```

***Figure* 2.** Amino acid sequence comparison of various LuxG proteins. The LuxG sequences are listed in order of percent homology to *P. leiognathi* LuxG from the top to the bottom: 1. *P. leiognathi* ATCC 25521, 2. *P. phosphoreum* NCMB844 66%; 3. *V. fischeri* ATCC 7744 55%, 4. *V. harveyi* B392 39%. The *P. leiognathi* LuxG amino acids are numbered, and residues identical to the *P. leiognathi* sequences are replaced by bars (-). # indicate the putative flavin and adenine nucleotide binding sites based on the previous publication.[12,14]

vin reductase activity,[13] it is suggested that LuxG regenerates flavin substrates used by luciferase in luminous reactions.

For the purpose of studying of the enzymatic characteristic of LuxG as a flavin reductase, the LuxG proteins from *P. leiognathi* were purified using the 6X-His tag system. In addition, to check the active and binding sites of flavin reductase, site-directed mutagenesis of the amino acids that are among the most highly conserved regions of LuxG (shown in *Fig.* 2) was performed to analyze the activities of these proteins. After ligation into pQE30 using the same procedure described above, the nucleotide sequence of the constructed recombinant plasmids was confirmed by automated Sanger's dideoxy sequencing methods in Bioneer Co. The whole nucleotide sequence of *lux*G and its translated amino sequence is shown in *Fig.* 3.

The nucleotide sequences of the 6X His tag system in the pQE30 vector are underlined on top. The numbers of nucleotide and amino acid sequences are denoted from the original start codon of the *lux*G sequence. The nucleotide sequences of the codon of amino acids for site-directed mutagenesis are also underlined.

After cell sonication, it was observed that proteins expressed by wild-type LuxG were found on the pellet in the step of cell lysis (lane 5 in *Fig.* 4). Hence, to obtain the protein from the precipitate (lanes 7 and 8 in *Fig*. 4), it was dissolved in a buffer solution (pH 8) containing 100 mM of NaH_2PO_4, 10 mM of Tris-Cl, and 8 M of urea.[15] To determine the quantity of the expression, the cells grown with or without induction were analyzed by SDS-PAGE. A high-expression protein with molecular weight of 25.5 kDa that corresponds to the calculated value was found in the pellet after sonication (lane 4 in *Fig.* 4).

After purification, the refolding process to remove the urea and imidazole by dialysis under the appropriate buffer conditions described above was formed. Through the Ni-super flow column, the protein was eluted with the imidazole buffer. The collected eluate fractions were analyzed on 15% SDS-gel to confirm the molecular weight of the protein and their level of purity.

Separated wild- and mutant-type LuxG proteins were confirmed by SDS-PAGE (data not shown). The concentrations of the purified proteins were as follows: LuxG wild-type 1.27 mM; LuxG F47Y, 1.55 mM; LuxG G112A, 0.73 mM; LuxG G113A, 2.06 mM; LuxG T114S, 1.40 mM;

```
M  R  G  S  H  H  H  H  H  H  G  S  I  M  I  F  N  C  K  V          7
ATG AGA GGA TCG CAT CAC CAT CAC CAT CAC GGA TCC ATC ATG ATT TTT AAT TGC AAG GTT   21
K  K  V  E  A  S  D  S  H  I  Y  K  V  F  I  K  P  D  K  C         27
AAA AAA GTC GAA GCA TCT GAC AGC CAT ATT TAC AAA GTG TTT ATT AAG CCT GAC AAA TGC   81
F  D  F  K  A  G  Q  Y  V  I  V  Y  L  N  G  K  N  L  P  F         47
TTT GAT TTT AAA GCG GGT CAA TAT GTA ATT GTG TAT CTC AAT GGA AAA AAT TTG CCG TTT  141
S  I  A  N  C  P  T  C  N  E  L  L  E  L  H  V  G  G  S  V         67
TCT ATT GCT AAC TGC CCA ACT TGT AAT GAG CTC CTT GAA TTA CAT GTA GGA GGT TCG GTA  201
K  E  S  A  I  E  A  I  S  H  F  I  N  A  F  I  Y  Q  K  E         87
AAA GAA TCC GCC ATT GAA GCT ATT TCG CAC TTT ATT AAT GCA TTT ATT TAT CAA AAA GAA  261
F  T  I  D  A  P  H  G  D  A  W  L  R  D  E  S  Q  S  P  L        107
TTT ACA ATC GAT GCA CCA CAC GGT GAT GCA TGG CTG AGA GAT GAA AGC CAA TCA CCT TTA  321
L  L  I  A  G  G  T  G  L  S  Y  I  N  S  I  L  S  C  C  I        127
CTA CTT ATA GCA GGA GGG ACA GGT TTA TCA TAT ATC AAT AGC ATT TTA AGT TGT TGT ATT  381
S  K  Q  L  S  Q  P  I  Y  L  Y  W  G  V  N  N  C  N  L  L        147
AGT AAA CAG TTA TCT CAG CCT ATC TAT CTT TAT TGG GGA GTA AAT AAC TGT AAT TTA CTC  441
Y  A  D  Q  Q  L  K  T  L  A  A  Q  Y  R  N  I  N  Y  I  P        167
TAT GCT GAT CAA CAA CTA AAA ACA CTC GCC GCA CAA TAC AGA AAT ATA AAT TAT ATT CCT  501
V  V  E  N  L  N  T  D  W  Q  G  K  I  G  N  V  I  D  A  V        187
GTG GTA GAG AAT TTA AAT ACT GAC TGG CAG GGA AAA ATT GGT AAT GTT ATT GAC GCG GTT  561
I  E  D  F  S  D  L  S  D  F  D  I  Y  V  C  G  P  F  G  M        207
ATT GAA GAT TTT TCA GAT TTA TCT GAC TTT GAT ATC TAT GTC TGC GGG CCA TTT GGT ATG  621
S  R  T  A  K  D  I  L  I  S  Q  K  K  A  N  I  G  K  M  Y        227
AGC CGG ACT GCG AAA GAT ATT CTG ATC TCA CAG AAA AAG GCG AAT ATA GGA AAA ATG TAT  681
S  D  A  F  S  Y  T  *     234
TCT GAT GCA TTT AGC TAT ACG TAA   705
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***Figure* 3.** The nucleotide sequences and corresponding translated amino acid sequence of wild-type LuxG.

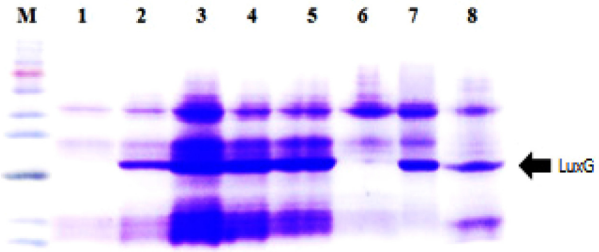

***Figure* 4.** SDS-PAGE analysis of the wild-type LuxG protein during cell extraction. M; Protein marker (11 kDa, 17 kDa, 25 kDa, 35 kDa, 48 kDa, 63 kDa), 1; Before induction, 2; After induction, 3; After lysis, 4; After sonication, 5; Pellet, 6; Supernatant, 7; Pellet after denaturation, 8; Supernatant after denaturation. The wild-type LuxG protein during cell extraction was analyzed using Coomassie blue-stained 15% acryl-amide gel.

LuxG 115A, 1.49 mM; LuxG C202S, 1.14 mM; LuxG203A, 2.32 mM; LuxG F231Y, 1.22 mM, respectively.

To measure flavin reductase of LuxG protein, an experiment was performed to detect the enzyme activity using by increasing the reaction time in the reaction mixture containing 200 μM of NADPH and 20 μM of FMN with 2.5 μM of LuxG protein. As a result, the concentration of enzymatic product of $NAD(P)^+$ was increased as time passed. An initial experiment was done to confirm whether purified wild-type LuxG proteins have the function of flavin reductase. Enzyme reactions with the wild- and mutant-type LuxG were performed at 25 °C in a buffer containing 75 mM of Tris-Cl, a pH of 8.0, 10% glycerol, and 1 mM of DTT.

The enzymatic reaction activities were determined by measuring the differences in absorbance of NAD(P)H and $NAD(P)^+$ at 340 nm, as NAD(P)H strongly absorbs the light of this wavelength, but $NAD(P)^+$ does not absorb the light at this wavelength. The decrease of NADPH was calculated using the molar extinction coefficient 6.22 $mM^{-1}cm^{-1}$. To obtain more detailed understanding of this, the difference in the degree of activity based on the concentration of LuxG was also tested. The flavin reductase activity was shown to be stimulated linearly by adding LuxG protein, also indicating the protein has a function of flavin reductase as predicted (data not shown).

Based on the finding that LuxG is a flavin reductase, the kinetic parameters of Km and Vmax values for two substrates, FMN and NADPH, were measured, respectively. As shown in *Fig.* 5, the enzyme activity was shown on a hyperbolic curve as the FMN concentration increased in the saturated condition of NADPH at 200 μM.The values for Km and Vmax for FMN are 6 μM and 0.53 μmol $mg^{-1}sec^{-1}$, respectively. Similarly, the values of Km and Vmax for NADPH are 25 μM and 2.56 μmol $mg^{-1}sec^{-1}$, respectively, measured by the increase in the NADPH substrate at the saturated 20 μM FMN (*Fig.* 6). All the enzyme activities were the average value from the three times measurements.

In an amino acid sequence comparison of LuxG with flavin $NADP^+$ reductase, two stringently conserved areas are apparent[11] (*Fig.* 2). The first is the AGGTG motif beginning at amino acid 111. These residues are believed to be involved in adenine nucleotide binding.

The other conserved area beginning at position 198 is believed to constitute part of the flavin binding site in

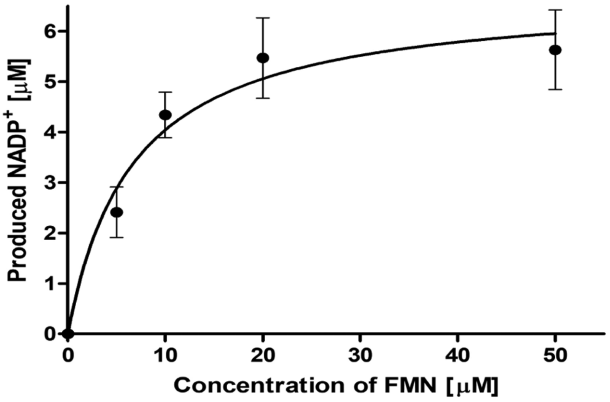

***Figure* 5.** Dependence of the flavin reductase activity on FMN. Assay reactions were performed in a reaction buffer (75 mM of Tris-Cl (a pH of 8.0), 10% (v/v) glycerol, 1 mM of DTT), and 200 μM of NADPH. The protein concentration was 2.5 μM. The rate of the reaction was calculated based on the absorption reduction at 340 nm for 3 min and is expressed as specific activity by the concentration of $NADP^+$ produced per second.

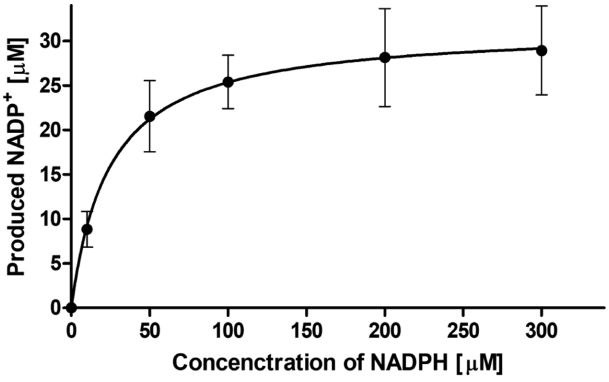

***Figure* 6.** Dependence of the flavin reductase activity on NADPH. Assay reactions were performed in a reaction buffer and 20 μM of FMN. The protein concentration was 2.5 μM. The NADPH concentration was increased from 0 to 300 μM. The other experimental conditions were the same as in *Fig.* 5.

these proteins. These amino acids are indicated by arrows in the putative adenine nucleotide binding (A111 to G115) and in the flavin binding sites (P46 to S48, C202 and G203, and D229 to F231), and these areas are the most highly conserved regions between the LuxG proteins, as shown in *Fig.* 2.

To check the active and binding sites of flavin reductase, the mutant LuxGs that changed those amino acids (F47, G112, G113, T114, G115, C202, G203, and F231) by the PCR and the site-directed mutagenesis were generated. The difference in the flavin reductase activities of wild- and mutant-type LuxG proteins was compared. Each reaction was performed in a reaction buffer (75 mM of Tris-Cl, 10% glycerol, and 1 mM of DTT at a pH of 8.0)

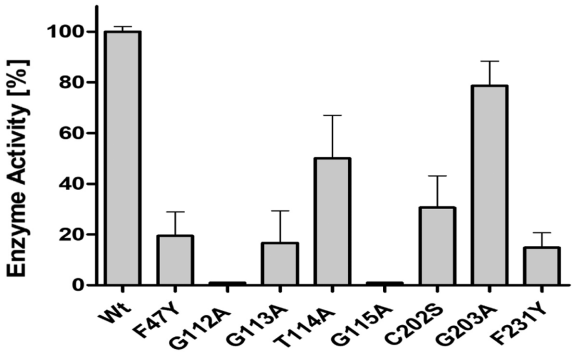

***Figure* 7.** Comparison of the flavin reductase activity of wild-type and mutant LuxGs. Assay reactions were performed in 75 mM of Tris-Cl (a pH of 8.0), 10% (v/v) glycerol with 200 μM of NADPH and 20 μM of FMN added as substrates. The rate of the reaction was calculated based on the absorption reduction at 340 nm and is expressed as specific activity described in the text.

and contained 200 μM of NADPH and 20 μM of FMN as substrates. The enzyme activities were measured using the same procedure as the previous experiment by adding 2.5 μM of wild- and mutant-type flavin reductase.

The relative enzyme activities of the mutant type of proteins of LuxG compared to those of wild-type LuxG are as follows: The enzyme activity of wild-type flavin reductase (LuxG): LuxG F47Y 20%, LuxG G112A 1%, LuxG G113A 17%, LuxG T114S 50%, LuxG G115A 1%, LuxG C202S 31%, LuxG G203A 79%, and LuxG F231Y 15%, respectively (*Fig.* 7). The activities are plotted as a percentage of the wild-type LuxG value. In particular, the activities of LuxG G112A and LuxG G115A substantially reduced to the background level of the reaction mixture without protein. From these results, 112nd and 115th glycine residues, the nicotine adenine nucleotide-binding sites, have the greatest impact on the enzyme activity.

It can be assumed that the adenine nucleotide-binding sites, particularly glycine 112 and glycine 115, have the most important amino acids to maintain the structural integrity of flavin reductase for the binding of NADPH substrate. These data are correlated quite well with the crystal structure of the flavin reductase from *V. fischeri*, supporting the amino acids' involvement in adenine nucleotide-binding sites to the protein.[14]

In conclusion, this study demonstrated the enzymatic characteristics of LuxG protein from bioluminescence bacteria of *Photobacterium leiognathi*. The *lux*G genes were amplified by PCR and inserted into pQE30 vector which have 6X His-Tag system. By making recombinant plasmid, it was transformed into *E. coli* to purify the over-expressed *lux*G genes product. To identify the active site and enzymatic

***Table* 1.** Primers for site-directed mutagenesis. Codons specifying modified amino acid residues are shown in bold. Novel restriction sites are underlined

Primer	novel site	Nucleotide sequences
Forward	*Bam*H1	5'-CATCACGGATCCATCA TGATTTTTAATTG-3'
Reverse	*Hind*III	5'-GCTAATTAAGCTTTTAATTA CGTATAG-3'
F47Y	*Hinf*I	5'-TTTCAGTTAGCAATA GAAtACGGCAgATTcTTTCC-3'
G112A	*Hpy*188III	5'-TAAACCTGTCCCTgCTGCT ATcAGgAGTAAAGG-3'
G113A	*Hpy*188III	5'-ATAAACCTGTCgCTCCTGCT ATcAGgAGTAAAGG-3'
T114S	*Hpy*188III	5'-TGATAAACCgcTCCCTCCT GCTATcAGgAGTAAAGG-3'
G115A	*Hpy*188III	5'-TGATAAagCTGTCCCTCCTG CTATcAGgAGTAAAGG-3'
C202S	*Hpy*188III	5'-GATATcTATGTCaGCG GGCCATTTGGTAT-3'
G203A	*Eco*RV	5'-GATATcTATGTCTGCGcGC CATTTGGTAT-3'
F231Y	*Eco*RV	5'-AGCTAATTAAGCTTTTAATTAC GTATAGCTAtATGCATCAGA-3'

characteristics of LuxG, several mutant types of *lux*G have been constructed by site directed mutagenesis for clarifying the relationship between protein structure and function. The putative amino acids involved in substrate binding sites were substituted to other amino acid to check how protein function can be changed from wild type LuxG. The flavin reductase activities of mutated LuxG have been decreased comparing to the wild type LuxG. Especially, the activities of LuxG G112A and LuxG G115A have substantially decreased to background level, indicating these amino acids are crucial for the binding of substrates.

EXPERIMENTAL

The pQE30 plasmid from Qiagen was used as the cloning vector, and *E. coli* XL-1 blue was used as a cloning strain to obtain recombinant plasmid DNA. *Bam*H1 and *Hind*III were used as the restriction enzymes purchased from New England Biolabs. A polymerase chain reaction (PCR) premix from Genet-Bio, and a PCR primer from Genotech were used. To amplify the *lux*G genes, primers obtained from Genotech were used. DNA was amplified using PCR with PlXba pT7-3 plasmid[16] as a template (*Table* 1). The PCR were composed of five steps: pre-denaturation 95 ºC for 5 min, denaturation 95 ºC for 20 sec, annealing 56 ºC for 10 sec, extension 72 ºC for 40 sec, and post-extension 72 ºC for 5 min. Denaturation, annealing, and extension were performed in 30 cycles. After the PCR and the gel extraction, the amplified DNA and the pQE30 vector were cleaved with *Bam*H1 and *Hind*III restriction enzymes for the ligation. The transformations were done with *E. coli* XL-1 blue competent cells. The oligonucleotides synthesized from Genotech were used as primer for performing site-directed mutagenesis (*Table* 1). PCR was performed twice for the generation of mutants using flanking and mutant primers for forward and reverse.

For the first step of PCR using the forward primer (reverse primer for C202S and G203A) and the mutant primer, the DNA was amplified in 30 cycles. The product of PCR was purified with gel extraction. For the second step, the product from the first step was used for the primer, and for the counterpart primer, the reverse primer (forward primer for C202S and G203A) was used for 30 cycles of amplification. The constructed recombinant plasmid pQE30 with wild- and mutant-type *lux*G gene was transformed into *E. coli* M15 and grown in the conditions described below. Bacteria were cultured in a Luria-Bertani (LB) medium containing ampicillin (100 μg/ml) and kanamycin (25 μg/ml) at 37 ºC in shaking volumetric flasks until the optical density of 0.6 at a wavelength of 600 nm. Isopropyl-β-D- thiogalactopyranoside (IPTG) was added to a concentration of 1 mM. This was followed with incubation at 25 ºC for 6 hours. The other procedures of protein purification were the same as described in the previous paper.[17,18]

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