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Note

Ferric iron reductase activity of LuxG from *Photobacterium leiognathi*

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Photobacterium leiognathi LuxG 단백질의 철(III) 이온 환원 효소 활성도

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ABSTRACT: In order to identify the biochemical characteristics of LuxG, the *lux*G gene from bioluminescence bacteria of *Photobacterium leiognathi* ATCC 25521 was isolated by PCR-Amplification and inserted into pQE30 vector containing the T5 promoter and 6X His-tag system. The resulting recombinant plasmid was transformed into *Escherichia coli* to over-express the *lux*G gene and purify the gene product. The purified LuxG protein demonstrated ferric iron reductase activity and the kinetic parameters of K_m and V_{max} for FMN as well as the NADPH substrates of ferric iron reductase were determined, respectively.

Key words: bioluminescence, ferric iron reductase, flavin, LuxG, *Photobacterium*

The light-emitting reaction in bacteria involves oxidation of reduced flavin mononucleotide ($FMNH_2$) and long-chain fatty aldehydes by luciferase, resulting in emission of blue-green light (Meighen, 1988, 1991) (Fig. 1).

$$FMNH_2 + RCOH + O_2 \rightarrow FMN + H_2O + RCOOH + light$$
(Bacterial luciferase)

The long-chain fatty aldehyde substrate is provided by the fatty acid reductase complex (LuxCDE). The other substrate, $FMNH_2$, is produced by flavin reductase, which exists in mostly bioluminescent bacteria (Meighen, 1991, 1994).

$$FMN + NAD(P)H + H^+ \rightarrow FMNH_2 + NAD(P)^+$$
(Flavin reductase)

It was reported that LuxGs from marine bioluminescence bacteria have an amino acid homology with NAD(P)H:flavin oxidoreductase coded by *fre* gene in *Escherichia coli* (Andrew *et al*., 1992). It was also shown that the NAD(P)H: flavin oxidoreductase of *E. coli* catalyzes the reduction of free flavins; FMN, FAD, or riboflavin, which in turn, transfer their electrons to physiological ferric complexes: ferrisiderophores, ferric citrate and ferritins (Spyrou *et al*., 1991).

Iron is essential for the growth of almost all living organisms.

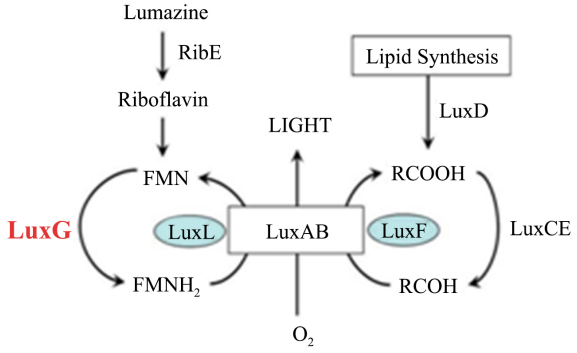

Fig. 1. Overall light emitting reaction in bioluminescence bacteria of *Photobacterium* species. The enzymes and proteins are RibA, GTP cyclohydrolase II; RibB, DHBP synthase; *Rib*H, lumazine synthase; RibE, riboflavin synthase; LuxAB, heterodimer of luciferase; LuxCDE, fatty acid reductase complex; LuxF, non-fluorescent flavoprotein; LuxG, flavin reductase; LuxL, lumazine protein (Lee *et al*., 1994).

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Although iron is an abundant element, its bioavailability in aerobic environments is limited due to the insolubility of ferric iron (Coves and Fontecave, 1993). It is especially interesting to note that iron regulates luminescence in *Photobacterium* species from marine bioluminescent bacteria, whereas iron does not have any influence on luminescence in *Xenorhabdus luminescens* (Meighen, 1994). Regulation of luminescence by iron is controversial in that its physiological effects on luminescence vary depending on species (Haygood and Nealson, 1985). Iron has also been proposed to be critical to the function of luciferase as an electron carrier (Coves and Fontecave, 1993; Meighen, 1994).

A *lux*G gene knockout mutant of *P. leiognathi* TH1 exhibited a much dimmer luminescent phenotype compared to the native strain (Nijvipakul *et al*., 2008), indicating that it supplies flavin substrate used by luciferase in luminous reactions. The LuxGs from *Vibrio* species are highly similar in amino acid sequence to *fre*-like flavin reductase, however, the LuxGs constitute a group of proteins distinctly different from that for *fre* enzyme (Zenno and Saigo, 1994). Therefore, it is not appropriate to define the function of LuxG as just a flavin reductase. In this study, the LuxG from *P. leiognathi* ATCC 25521 was purified by using the 6X-His tag system to establish the function of LuxG as a ferric iron reductase. These results can be important clues to an investigation on the integration of regulation of bioluminescence by flavin dependence and iron regulation.

To amplify the *lux*G gene by PCR (Polymerase Chain Reaction), the plasmid PlXba pT7-3 (Lee *et al*., 1991) containing the *lux* genes from *P. leiognathi* ATCC 25521 was used as template and the primers of forward (5′-CATCACGGATCC ATCATGATTTTTAATTG-3′) and reverse (5′-GCTAATT AAGCTTTTAATTACGTATAG-3′) were used. The restriction sites of *Bam*H1 and *Hin*dIII were underlined on the primers. The pQE30 plasmid was used for cloning vector, and *E. coli* XL-1 blue was used as a cloning strain to obtain recombinant plasmid. After PCR and through the gel extraction, DNA and pQE30 vector were cleaved with *Bam*H1 and *Hin*dIII restriction enzymes. After the restriction, ligation and transformation were done with *E. coli* XL-1 blue competent cell. The pQE plasmid belong to the pDS family of plasmids containing an optimized regulatory promoter/ operator element, consisting of the *E. coli* phage T5 promoter and two *lac* operator sequences (Stüber *et al*., 1990).

The constructed recombinant plasmid pQE30 with the *lux*G gene from *P. leiognathi* ATCC 25521, was transformed with the protein expression strain of *E. coli* M15 and grown under conditions described in the protocol supplied by Qiagen. Frozen cells (mass 5 g) obtained from the culture were thawed for 30 minutes on ice and then suspended in 30 ml of lysis buffer (75 mM Tris-Cl; pH 8.0, 500 mM NaCl, 10% glycerol). Protease inhibitor phenylmethanesulphonyl fluoride (PMSF) was added at a concentration of 1 mM and incubated on ice for 30 min. The resultant suspension mixture was sonicated on ice five times for 5 min, followed by centrifugation of the lysate at 10,000 × *g* for 1 h at 4°C. The pellet was dissolved by lysis buffer 2 (75 mM Tris-Cl; pH 8.0, 500 mM NaCl, 10% glycerol, 8 M urea) and incubated for 1 day by using a magnetic stirrer. After incubation, centrifugation of the lysate was carried out at 10,000 × *g* for 1 h at 4°C. The supernatant was filtered to obtain a clear lysate. To remove urea, added to solubilize LuxG during cell extraction, sequential dialysis was performed for 3 h against dialysis buffer containing step-wise decreasing concentrations of urea (4 M, 2 M, and 1 M). For the final step, dialysis was carried out in buffer without urea for 1 day at 4°C. Each dialyzed protein was condensed by centrifugal filtration (cutoff MW 10 kDa) from Millipore, and the concentration was measured using a Quanti-iT^{TM} protein assay kit from Invitrogen.

The pQE30 vector has an N-terminus 6X His-tagged and is a simple system for protein purification via Ni column absorption and elution with imidazole. For protein purification, Biologic-LP from Bio-Rad was used while Ni-Super-flow from Qiagen was used as the column. Equilibrium buffer was flowed into the nickel-charged column, after which the lysate was loaded into the Ni-Superflow at a flow rate of 1 ml/min. The flow-through fractions were collected and later analyzed by SDS-PAGE. The column was washed with washing buffer (75 mM Tris-Cl; pH 8.0, 500 mM NaCl, 10% glycerol, 50 mM imidazole). In both cases, fractions (50 ml each) were collected and analyzed by SDS-PAGE. The purified protein was eluted with elution buffer (75 mM Tris-Cl; pH 8.0, 500 mM NaCl, 10% glycerol, 250 mM imidazole). Collected elute fractions were each analyzed by 15% SDS- PAGE to confirm the molecular weight of the protein and levels of purity (Fig. 2). Fractions containing the purified protein of interest were

pooled together and concentrated.

To confirm whether or not purified LuxG protein has ferric iron reductase activity, enzymatic experiments of ferric iron reductase activity was carried out based on the absorption spectrum of the ferrous complex of ferrozine showing a single peak with maximum absorbance at 562 nm. The molar absorptivity of ferrozine was 27.9 $mM^{-1}cm^{-1}$ at 562 nm (Stookey, 1970; Fontecave *et al*., 1994). The enzymatic assay was performed in reaction buffer [75 mM Tris-Cl; pH 8.0, 10% (v/v) glycerol, 1 mM DTT] adding 100 μM ferric citrate, 2 mM ferrozine, 20 μM FMN, and 200 μM NADPH, respectively. All enzyme activities showed average values from the separated measurements. As shown in Fig. 3A, the concentration of reduced ferric iron increased as reaction time passed. The rate of reaction was expressed as specific activity based on the concentration of ferric iron reduction with the same concentration of protein.

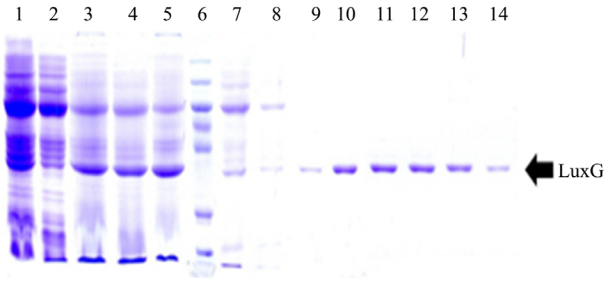

Fig. 2. SDS-PAGE analysis of purified recombinant LuxG. Purified wild-type LuxG was analyzed by Coomassie blue-stained 12% acryl-amide gel. **1**; Cell extract, **2**; Supernatant before denaturation, **3**; Lysate after denaturation, **4**; Supernatant after denaturation, **5**; Pellet after denaturation, **6**; Protein size marker (100 kDa, 70 kDa, 50 kDa, 40 kDa, 30 kDa, 20 kDa, 15 kDa), **7**; Flow-through, **8**; Washing (50 mM imidazole buffer), **9**; Washing (90 mM imidazole buffer), **10**; Elution 1, **11**; Elution 2, **12**; Elution 3, **13**; Elution 4, **14**; Elution 5.

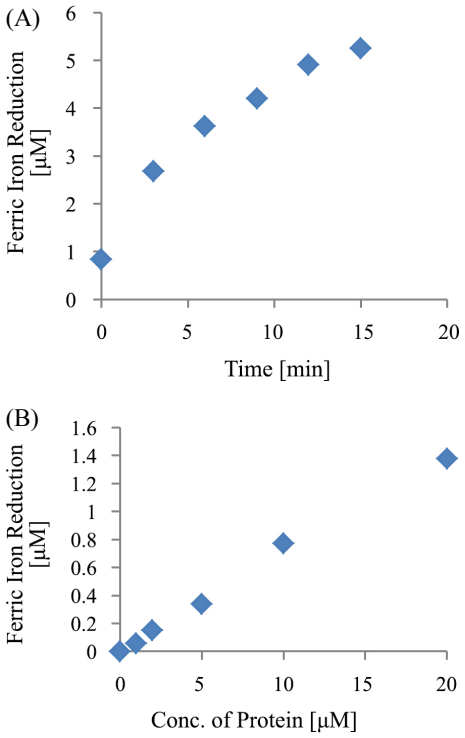

Fig. 3. Ferric iron reductase activities of 2.5 μM LuxG with increasing time (A) and different protein concentrations (B). Assay reactions were performed in reaction buffer (75 mM Tris-Cl; pH 8.0, 10% (v/v) glycerol, 1 mM DTT), 100 μM ferric citrate, 2 mM ferrozine, 200 μM NADPH, and 20 μM FMN.

The degree of ferric iron reduction was tested at a higher concentration of LuxG. As the concentration of LuxG increased, activity of ferric iron reduction increased, confirming that the LuxG protein has ferric iron reductase activity (Fig. 3B). In addition, the activity was stimulated with increasing ferric citrate concentration as a source of ferric iron (data not shown). To investigate enzyme characteristics of the LuxG as a ferric iron reductase, the kinetic parameters of K_m and V_{max} values for the substrates FMN and NADPH were determined by increasing the substrates, respectively. As shown in Fig. 4, the ferric iron reductase activity shows rectangular hyperbolic curve with increasing of FMN substrate to 50 μM under saturating concentrations of NADPH. The values of K_m and V_{max} for FMN were 3 μM and 13 nmol mg-protein^{-1} sec^{-1}, respectively, based on the dependence of the enzyme activity on the concentration of FMN substrate (Fig. 5). Similarly, the ferric iron reductase activity was observed on hyperbolic curve with various concent-

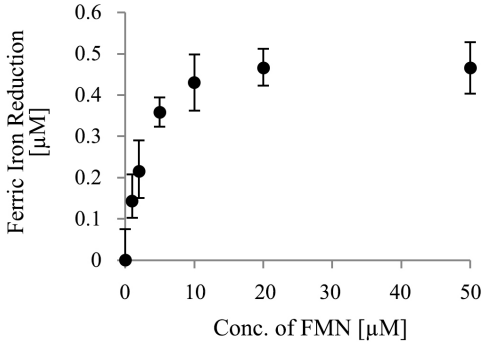

Fig. 4. Effect of FMN concentration on ferric iron reductase activity. Assay reactions were performed with a protein concentration of 5 μM in reaction buffer (75 mM Tris-Cl; pH 8.0, 10% (v/v) glycerol, 1 mM DTT), 100 μM ferric citrate, 2 mM ferrozine, 200 μM NADPH for 5 min. FMN concentration differs from 1 to 50 μM. Values are the means ± SD of three independent experiments.

rations of NADPH (1 to 300 μM) under saturating of FMN substrate (Fig. 6). The value of K_m was 32 μM while the V_{max} for NADPH was 24 nmol mg-protein^{-1} sec^{-1}, respectively, calculated from the data of enzyme activity with the increasing of NADPH substrate in Lineweaver-Burk plot (Fig. 7).

The results in this study showing that the LuxG protein has ferric iron reductase activity, in addition to the suggested flavin reductase activity, were important with regard to the regulation of bioluminescence. The kinetic parameters of ferric iron reductase are compatible to the values of flavin reductase. The K_m for FMN and NADH for FMN reductase from the LuxG in *P. leiognathi* TH1 were calculated 2.7 μM and 15.1 μM, respectively (Nijvipakul *et al*., 2008). Numerous studies have been directed towards the question of whether or not the pathway catalyzed by the flavin reductase corresponds to the *in vivo* pathway of transfer of electrons to the luminescent reaction. Fe^{3+} is formed when microbes use Fe^{2+} as the electron donor in oxygen respiration or as an electron source for photosynthesis (Fontecave *et al*., 1994; Schroder *et al*., 2003). Fe^{2+} is highly unstable in aerobic neutral aqueous solutions and spontaneously reacts with oxygen to give the stable Fe^{3+} form, however, due to the insolubility of ferric hydroxide, iron is often growth-limiting (Nielands, 1981).

Flavin reductase has been found in various bacteria, including *Agrobacterium tumefaciens*, *Azotobacter vinelandii*, *Bacillus megaterium*, *B. subtilis*, and *E. coli* (Fontecave *et al*., 1994). Assimilatory ferric iron reductase is an essential component of the iron assimilatory pathway, which generates more soluble ferrous iron, and is incorporated into cellular proteins (Nieland, 1981; Schroder *et al*., 2003). Luminescence in *P. leiognathi* and *P. phosphoreum* is subject to control by iron restriction (Meighen, 1994). In *P. phosphoreum*, an iron regulated outer membrane protein may be part of a high affinity iron uptake system (Lűmmen and Winkler, 1986).

The riboflavin synthesis genes are closely linked with the *lux* operon in *Photobacterium* species (Lee and Meighen, 1992; Lee *et al*., 1994). In addition, the *lux*G gene is located on the *lux* operon in marine bioluminescent bacteria whereas terrestrial bioluminescence bacteria *X. luminescens* does not have the gene. As biosynthesis of riboflavin is repressed by iron, it raises the possibility that iron affects luminescence indirectly by controlling the synthesis of FMN (riboflavin 5'-phosphate) (Meighen, 1994). Therefore, the data presented in this study that detection of ferric iron reductase activity from the LuxG in *P. leiognathi* can be particularly relevant in luminescent *Photobacterium* species, which are related to iron regulation.

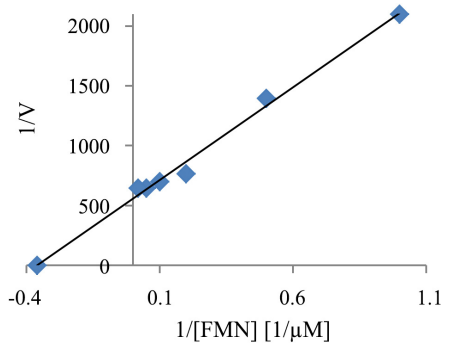

Fig. 5. Lineweaver-Burk plot for FMN dependent ferric iron reductase assay. The reaction rate (V) was calculated from the increase in absorption at 562 nm for 5 min with the same concentrations of LuxG and is expressed as specific activity based on the concentration of ferric iron reduction per second.

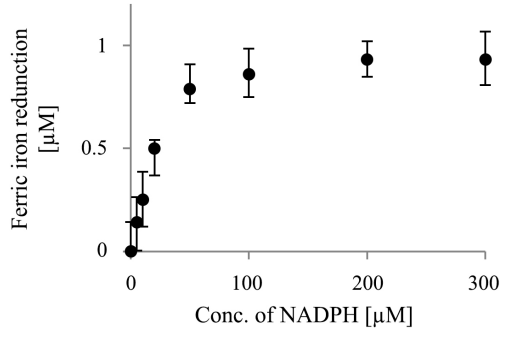

Fig. 6. Dependence of NADPH for ferric iron reductase activity. Concentrations of protein and FMN were 5 μM and 20 μM, respectively, and NADPH concentration varied from 0 to 300 μM. Other experimental conditions were the same as those described in the legends of Figs. 3 and 4.

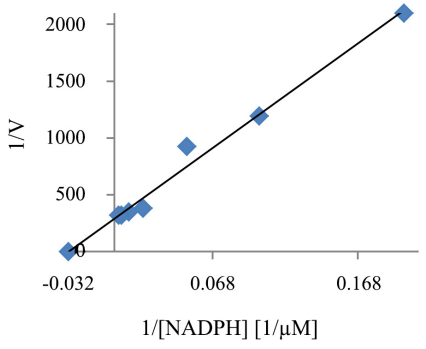

Fig. 7. Lineweaver-Burk plot for NADPH-dependent ferric iron reductase assay. Other experimental conditions were the same as those described in the legends of Figs. 3 and 4.

적 요

본 연구에서는 발광 세균에 존재하는 LuxG 단백질의 효소학적 성질을 알아내기 위하여 *Photobacterium leiognathi* ATCC 25521의 *lux*G 유전자를 중합효소연쇄반응으로 증폭시켜 T5 프로모터와 6X His-tag 시스템을 지닌 pQE 벡터에 삽입시킨 재조합플라스미드를 제조하여 대장균에 형질전환 후 과발현시켜 단백질을 분리, 정제 하였다. 정제된 단백질의 효소학적 실험 결과, 이 단백질은 FMN과 NADPH 기질에 대한 ferric iron reductase의 기능을 갖고 있음을 확인하였으며 이들 기질에 대한 효소 활성도 상수 K_m 및 V_{max} 값을 결정하였다.

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