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| 단 보 |

Construction, Expression, and Purification of N-Terminal Variants of Lumazine Protein from *Photobacterium leiognathi*

Kyoung-Suk Kang, So-Young Kim, Ji-Sun Choi, Young-Doo Kim, Robert Pokoo, Ki-Seok Nam, and Chan Yong Lee*

Department of Biochemistry, Chungnam National University, Daejeon 305-764, Republic of Korea

발광세균 *Photobacterium leiognathi*의 돌연변이 아미노-말단 루마진 단백질들의 제조, 발현 및 정제

강경숙 · 김소영 · 최지선 · 김영두 · 로버트 포쿠 · 남기석 · 이찬용*

충남대학교 생화학과

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Lumazine protein is a fluorescent protein isolated from the bioluminescent bacteria of *Photobacterium* species. To generate minimal size of lumazine protein with possessing fluorescent characteristic, the gene coding for the wild type N-terminal domain of lumazine protein (N-LumP 118) containing amino acids up to 118 from *Photobacterium leiognathi* was produced. In addition, the genes coding for the variant proteins of N-LumP 118, replaced with one tryptophan amino acid (N-LumP 118 V41W, S48W, T50W, D64W, and A66W), were also constructed by Polymerase Chain Reaction and Site Directed Mutagenesis. These proteins were expressed in *Escherichia coli* by transformation with recombinant plasmids and purified by 6X-His tagging system. Spectroscopic studies have show that the purified proteins are capable of binding to the fluorescent ligand 6,7-dimethyl-8-ribityllumazine, resulted in showing of fluorescent characteristic with the minimal size of protein. From these studies, the mutant proteins containing single tryptophan amino acid residue, possessing its own intrinsic flouophore character at the different position, will be able to the use as a probe for further studies to deduce their three dimensional structure and the binding modes.

***Keywords*:** *Photobacterium*, bioluminescence, lumazine protein, site directed mutagenesis

Bioluminescence refers to the emission of light without external source in specific environments (Herring, 1987; Campbell, 1989). The luminescent bacteria emits the blue-green light during the reduced riboflavin phosphate ($FMNH_2$) and long chain fatty-aldehyde are oxidized as the reaction scheme below (Hastings and Nealson, 1977; Meighen, 1988, 1991).

$$\mathbf{FMNH_2 + RCHO + O_2 \rightarrow FMN + H_2O + RCOOH + light}$$

It was revealed that several genes involved in the riboflavin (vitamin B_2) biosynthesis were present at the downstream of *lux* operon in *Photobacterium* species (Fig. 1A) (Lee *et al*., 1994; Meighen, 1994). The riboflavin biosynthesis in bioluminescent bacteria is essential as it is the precursor of flavin substrate for the light emitting reaction. In addition, the fact that the gene (*rib*E) codes for the riboflavin synthase is interested in the aspect of molecular genetics as it shows 30% amino acid homology with lumazine protein (O'Kane *et al.*, 1991; Lee *et al.*, 2007) and the *lum*P gene coding the lumazine protein is transcribed in opposite direction with *lux*C gene (Lee and Meighen, 2000) (Fig. 1B).

Lumazine protein consisting of 185 amino acids is a fluorescent protein and was found in *Photobacterium phosphoreum* at the second half of 1970 (Gast and Lee, 1978; Small *et al*., 1980). The strong fluorescent protein forms non-covalent bond interacted with 6,7-dimethyl-8-ribityllumazine (O'Kane *et al*., 1985; O'Kane and Lee, 1985) (It was hereafter referred to as lumazine) known to be a precursor for direct biosynthesis of

*For correspondence. E-mail: cylee@cnu.ac.kr; Tel.: +82-42-821-5482; Fax: +82-42-822-7548

vitamin B_2 (Koka and Lee, 1979; Fischer and Bacher, 2005).

This protein is considered to be generated through riboflavin gene duplication and is under the superfamily of riboflavin synthase (O'Kane *et al.*, 1991). The lumazine protein has a monomeric structure showing the intramolecular sequence similarity between N-terminal and C-terminal half domain (Fig. 2A). It was reported that the protein binds to one molecule of lumazine in N-terminal half (Illarionov *et al.*, 2007; Chatwell *et al.*, 2008). In this study, to generate a minimal size of fluorescent lumazine protein and to provide a basis for the future binding study, the wild type and the mutant genes for the half of the N-terminal region of lumazine protein from *Photobacterium leiognathi*, were designed by PCR (Polymerase Chain Reaction) and site directed mutagenesis and expressed to be purified.

Studies have shown that asparagine 101 and isoleucine 102, located beyond N-terminal region, are involved in binding of the lumazine ligand in additions to the amino acids such as serine 48, threonine 50, and alanine 66 at the binding sites in N-terminal half of lumazine protein (Illarionov *et al.*, 2007; Chatwell *et al.*, 2008) (Figs. 2A and 2B). Therefore, the gene coding for the N-terminal lumazine protein (N-LumP 118) extending to the region of these two amino acids of asparagine 101 and isoleucine 102 was synthesized using pPhl36 (Illarionov *et al.*, 1994) as a template in PCR. In order to amplify the gene coding for the 118th position of amino acids in lumazine protein, designed forward primer (5′-CTTTAACA AGGATCCGTAATGTTTAG-3′) and reverse primer (5′-GGG ATATCAATCACTGCAGATTAACCTGCGTC-3′) from Solgent Co. were used. *Bam*HI and *Pst*I restriction sites on these primers were indicated by underlined. PCR conditions were as followed: pre-denaturation 95℃ 5 min, denaturation 95℃ 30 sec, annealing 60℃ 30 sec, extension 72℃ 1 min 20 sec, final extension 72℃ 7 min denaturation, annealing, extension. This program set-up was run 24 cycles repeatedly. Generation of mutants such as N-LumP 118 V41W, S48W, T50W, D64W, and A66W were produced from the recombinant plasmid containing the gene for the wild type lumazine protein by point mutagenesis in the PCR reaction (Fig. 3). The primers used in mutation were manufactured by Solgent and Finnzymes cooperations as listed in Table 1.

Confirmation of mutants was detected by restriction digestions using *Sna*BI and *Eco*RV restriction enzyme whose restriction sites were located on the primers (Table 1). Initial analysis of the agarose gel electrophoresis gave a clue that the mutation had occurred by new generated additional restriction sites. These mutant genes were cut with *Bam*HI and *Pst*I restriction enzymes and ligated with pQE30 vector that had also been cut with the above restriction enzymes (Fig. 3). The DNA was sent to Solgent Co. in order to confirm the sequence of the mutants of interest by automatic base-sequence analyzer.

The recombinant plasmids, containing pQE30 vector inserted with the gene coding for N-LumP 118 of wild and mutant types, were inserted into *E. coli* XL-1 blue by transformation using the heat shock method and the resultant mixture was on Luria Bertani (LB) plates containing 100 μg/ml of ampicillin. Single colony out of the numerous colonies obtained was also further cultured in LB (tryptone 10 g, yeast-

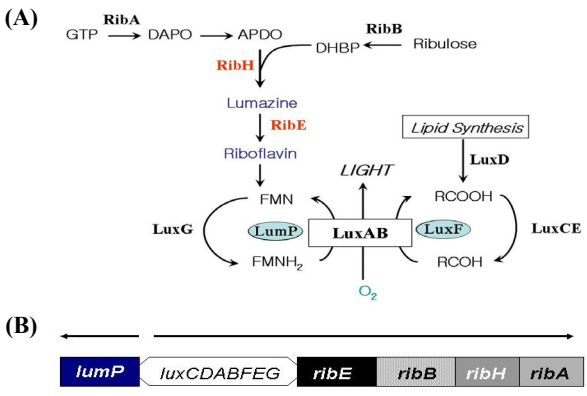

Fig. 1. (A) The genes involved in bacterial bioluminescence reaction. The functions of the gene products are as follows: *lux*ABCDE genes coding for *lux* proteins (luciferase and fatty acid reductase complex) involved in bioluminescence reaction, *lum*P for lumazine protein, *rib*A for GTP cyclohydrolase II, *rib*B for dihydroxy-butanone 4-phosphate synthase, *rib*H for lumazine synthase, and *rib*E for riboflavin synthase, and *luxF* for non-fluorescent flavoprotein. (B) Gene organization of *lux* operon region of *P. leiognathi.* Arrows indicate the direction of transcription.

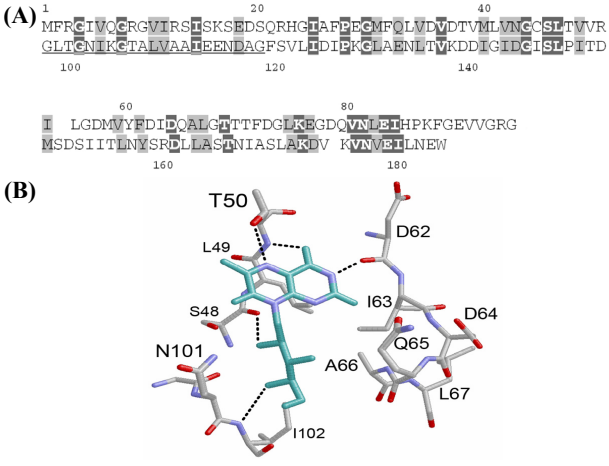

Fig. 2. (A) Internal amino acid sequences between N-terminal half and C-terminal half of lumazine protein from *P. leiognathi.* Identical amino acids are marked in box and similar amino acids shown in grey letters. The amino acids from the number of 97 to 118 containing N101 and I102 are underlined. (B) Model of binding site topology of lumazine protein of 20 kDa with 6,7-dimethyl-8-ribityllumazine obtained from author's previous paper (Illarionov *et al.*, 2007).

Table 1. Oligonucleotides used as primer for site directed mutagesesis of the gene for N-terminal domain of lumazine protein from *P. leiognathi*. Restriction sites of *Eco*RV (GATATC) and *Sna*BI (TACGTA) are underlined.

V41W
5′-AGTATCTACATCAACAAGTTGAAACATACCCTC-3′
S48W
5′-ATGGCCTAAAATACGTACAACTGTCAACCAACAACCATTTAC-3′
T50W
5′-CGCCTAAAATACGTACAACCCACAATGAACAACC-3′
D64W
5′- GATATCAAAGTACACCATATCGCCTAAAATACG-3
A66W
5′ GTTGTCGTACCCAACCATTGATCGATATCAAAGTA-3′

extract 5 g, NaCl 10 g/L) media containing similar antibiotics as above.

The recombinant plasmid DNA was then transformed into M15 competent cell for protein expression. After heat-shock, they were streaked and incubated on LB plates with kanamycin (25 μg/ml) and ampicillin (100 μg/ml). The absorbance at a wavelength of 600 nm of the cultured media was measured during the culturing of the cell containing the recombinant plasmid. IPTG inducer of known concentration was added at the OD value of 0.6. The expression levels of the protein were analyzed by loading the samples on SDS-PAGE electrophoresis. The composition of the media for protein expression are as follows: LB and TB (Terrific Broth) (tryptone 12 g, yeast-extract 24 g, 4 ml glycerol, 2.31 g KH_2PO_4, 12.54 g K_2HPO_4/L). These media were used interchangeably to identify the best media for protein expression.

A high yeast-extract containing media TB provided the better expression than LB for protein expression with stable pH by using sodium phosphate in the experiment (Fig. 4). Probably, it is due to the fact that TB media is suitable condition for growing in *E. coli* M15 host cell. The grown cell media was centrifuged for a known period of time at a specific gravity to separate the supernatant from the pellets (precipitate). The precipitate was dissolved in lysis buffer solution containing 50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole and this was followed with sonication.

After cell sonication, it was observed that proteins expressed by N-LumP 118 wt and S48W genes were found on the supernatant in step of cell lysis. In contrast, N-LumP 118 V41W, D64W, and A66W were found on pellet in step of cell lysis (data not shown). Hence, in order to obtain the protein from the precipitate, it was dissolved in a buffer solution containing 100 mM NaH_2PO_4, 10 mM Tris-Cl, and 8 M urea at pH 8.0 according to the manual (Palmer and Wingfield, 1995). After all the pellets had evenly dissolved, the resultant solution was mixed with Ni-NTA resin from Qiagen in the column. The

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                M   R   G   S   H   H   H      7
AAATTAACT ATG AGA GGA TCG CAT CAC CAT    60

 H   H   H   G   S   V   M   F   R   G    17
CAC CAT CAC GGA TCC GTA ATG TTT AGA GGT  90
            BamHI
 I   V   Q   G   R   G   V   I   R   S    27
ATT GTT CAA GGT CGT GGT GTT ATT CGA TCC 120

 I   S   K   S   E   D   S   Q   R   H    37
ATT TCA AAA AGT GAA GAT AGT CAG CGA CAT 150

 G   I   A   F   P   E   G   M   F   Q    47
GGG ATT GCA TTC CCT GAG GGT ATG TTT CAA 180

 L   V   D   V   D   T   V   M   L   Y    57
CTT GTT GAT GTA GAT ACT GTA ATG TTA GTA  210

 N   G   C   S   L   T   V   V   R   I    67
AAT GGT TGT TCA TTG ACA GTT GTC CGT ATT  240

 L   G   D   M   V   Y   F   D   I   D    77
TTA GGC GAT ATG GTG TAC TTT GAT ATC GAC  270

 Q   A   L   G   T   T   T   F   D   G    87
CAA GCA TTG GGT ACG ACA ACA TTT GAT GGA  300

 L   K   E   G   D   Q   V   N   L   E    97
TTA AAA GAA GGC GAT CAG GTA AAC CTA GAG  330

 I   H   P   K   F   G   E   V   V   G   107
ATC CAC CCT AAA TTT GGT GAA GTG GTT GGC  360

 R   G   G   L   T   G   N   I   K   G   117
CGT GGT GGA TTA ACA GGA AAT ATT AAA GGC 390

 T   A   L   V   A   A   I   E   E   N   127
ACT GCG CTG GTC GCA GCA ATA GAA GAG AAT 420

   D   A   G   *                        130
  GAC GCA GGT TAA TCTGCAGCCAAGCTTAAT  450
                    PstI
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Fig. 3. Nucleotide sequences and their corresponding translated amino acid sequences of N-LumP 118 wt. The nucleotide sequence and its translated amino acid sequences (MRGSHHHHHHGSV) of 6X-His tagging system are included. The amino acid sequences replaced with tryptophan were marked in underlined-bold.

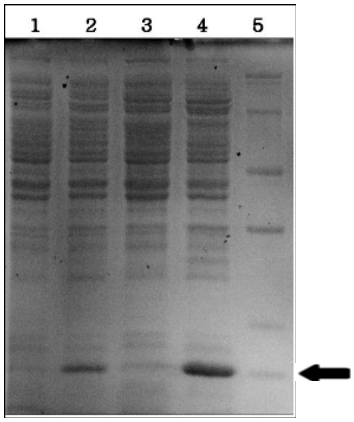

Fig. 4. SDS-PAGE analysis of N-terminal lumazine protein (N-LumP 118) obtained from different growing conditions. Lanes: 1, N-LumP 118 D64W no IPTG in LB media; 2, N-LumP 118 D64W with IPTG induction at 30℃ overnight in LB; 3, N-LumP 118 D64W no IPTG in TB media; 4, N-LumP 118 D64W with IPTG induction at 30℃ overnight in TB media; 5, Protein size marker (14, 20, 24, 29, 36, 45 kDa, respectively).

purified protein was eluted with imidazole of concentration 20 mM and 50 mM, respectively. In each step of protein elution, the eluent was analyzed for the presence of protein by SDS-PAGE analysis.

From the procedure above, the proteins were separated and purified by using buffer solutions at different pH conditions. Undesirable proteins from the sonicated sample were washed off using a buffer of pH 6.3 whiles elution of protein of interest was done at pH 5.9 and pH 4.5. It was observed that some of the protein of interest was present in each of the above mentioned steps. After confirming the separated and purified proteins on SDS-PAGE (Fig. 5), they were emptied into a dialysis bag containing 50 mM NaH_2PO_4 5 L buffer and dialysed at 4℃ overnight. The proteins obtained after the dialysis step, were further concentrated to 1 ml and stored at 4℃.

The quantification of proteins was tested by using Qubit™ from Invitrogen. To determine the protein concentration precisely, the absorption and literatures (Illarionov *et al.*, 1994; Lee *et al.*, 2007) value of molar extinction coefficient (ε_{280} =5,800 $M^{-1}cm^{-1}$) were also counted. To remove the lumazine ligand bound to the proteins during purifications, the solutions containing 100 mM phosphate, pH 7.0, and the N-LumP proteins were dialyzed against 100 mM phosphate pH 7.0, containing 4 mM dithiothreitol and 6 M urea. For refolding, the apoprotein was dialyzed overnight against 100 mM phosphate, pH 7.0 containing 0.3 mM dithiothreitol.

As the analysis of spectroscopic properties of wild type and mutant of N-LumP, the possibility of binding of N-terminal lumazine protein (N-LumP 118) to lumazine ligand was investigated by measuring the absorbance from the protein and ligand. The protein and its ligand lumazine were mixed together at a concentration ratio of 1:5, after which the resultant mixture was evenly shaken and dialyzed in a dialysis bag at 4℃ for 8 h. The dialysis bag contained 500 ml of 50 mM NaH_2PO_4. The absorbance spectra of the proteins were analyzed at a wavelength of range spanning 250 nm to 650 nm using Agilent 8453 Spectrophotometer.

As shown in Fig. 6, the following solutions 27 μM N-LumP 118 wt and N-LumP S48W, T50W, and A66W incubated with lumazine recorded a maximum peak around at 410 nm. These results indicate that wild type and mutants N-terminal lumazine proteins bind to the lumazine ligand. Comparing to the absorbance peak of free lumazine ligand, the λ_{max} (maximum absorption) of lumazine (407 nm) generally shifted over a longer wavelength upon binding of ligands to N-LumP (409-415 nm). Similarly, the accurate ratio of binding was determined based on the value of maximum absorption and literature value of molar extinction coefficient of lumazine ligand (ε_{408}=10,700 $M^{-1}cm^{-1}$). The value of 28 μM of lumazine ligand was obtained from the calculation, suggesting that the molar ratio of binding of N-LumP protein to lumazine was 1:1.

Fluorescence intensities are also measured using FP-750 series of Fluorescence Spectrometer (JASCO). The emission spectra of the fluorescent ligand bound proteins at 407 nm of fixed excitation were shown to maximum fluorescence intensity at 490 nm region (data not shown). The following results of relative fluorescence intensities were recorded: free lumazine 1.0; N-LumP 118 wt, 1.10; N-LumP 118 V41W,

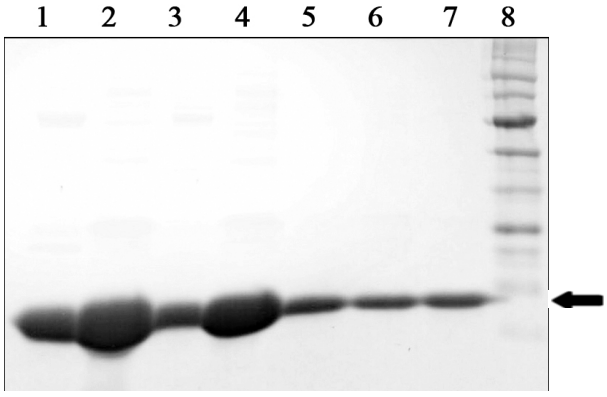

Fig. 5. SDS-PAGE analysis of wt and mutants N-terminal lumazine protein (N-LumP 118) after purification. Lanes: 1, N-LumP 118 wt; 2, N-LumP 118 V41W; 3, N-LumP 118 S48W; 4, N-LumP 118 T50W; 5, N-LumP 118 D64W[1]; 6, N-LumP 118 D64W[2]; 7, N-LumP 118 A66W; 8, Protein size marker (15, 20, 30, 40, 50, 70, and 100 kDa, respectively). The following results were recorded: N-LumP 118 wt protein, 334 μM; V41W, 704 μM; S48W, 222 μM; T50W, 567 μM; D64W [1], 121 μM; D64W [2], 81 μM; A66W 100 μM, respectively.

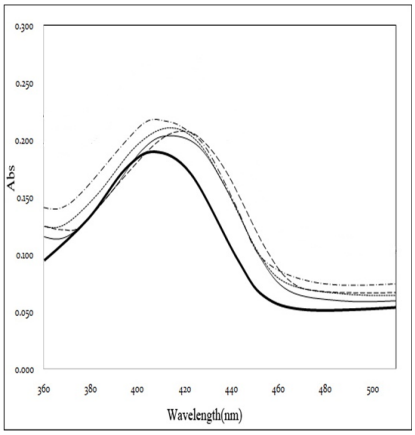

Fig. 6. Absorbance spectra of 6,7-dimethyl-8-ribityllumazine, free or in complex with variants of N-terminal domain of lumazine protein. Thick solid line, free lumazine (30 μM); Thin solid line, N-LumP 118 wt; Dotted line, N-LumP 118 V41W; Dashed line, N-LumP 118 S48W; Dash-dotted line, N-LumP 118 T50W. All protein concentrations were adjusted to 28 μM.

0.75; N-LumP 118 S48W, 0.90; N-LumP 118 T50W, 0.90; N-LumP 118 A66W, 0.75. Considering to the previous result that the fluorescence intensity was quenched to 10% by binding N-terminal domain of riboflavin synthase (Lee *et al.*, 2007), it can be suggested that all mutants N-LumP still possess the capability of fluorescent character. The observations, slight decreasing fluorescence intensities of N-LumP tryptophan mutants comparing to wild type, are in agreement with the fact that the artificial introduced aromatic acid residue (tryptophan) are close to protein-bound fluorophore. From these results, fluorescence quenching could be due to resonance transfer between the lumazine ligand and aromatic side chain.

The mutant proteins contain only one tryptophan amino acid residue. Therefore, it was also tested emission spectra arose from the excitation of the fluorescent tryptophan amino acids by the fixed at 295 nm (Fig. 7). Each tryptophan containing mutant proteins (N-LumP 118 V41W, S48W, and T50W) shows different pattern due to the specific environment around each tryptophan amino acid residue. The fluorescence emission peak of each purified protein were also changed quite differently by adding 1 M urea, indicated that the environmental conditions leading to urea denaturation are peculiar among these proteins (Fig. 7). From these results, it can be raised the possibility that these proteins can be used as a probe for the detection of conformational change by its fluorescent character.

In summary, generating minimal size of lumazine protein with possessing fluorescent characteristic, wild type N-terminal half of lumazine protein as well as variant proteins mutated at putative binding sites of amino acids with tryptophan were constructed by PCR and site directed mutagenesis. The proteins were purified by 6X-His tagging affinity system to test the binding properties of the lumazine ligand as well as the fluorescent characteristics. These proteins will be helpful for further studies to deduce the three dimensional structure such as distance and orientations between protein and lumazine ligand as well as the binding modes of N-terminal domain of lumazine protein by using fluorescence character of tryptophan amino acid and of fluorescent ligand.

적 요

루마진 단백질은 발광 세균인 *Photobacterium* 종에서 추출된 형광성 단백질이다. 형광성을 지닌 최소 크기의 *Photobacterium leiognathi* 야생형 아미노-말단 도메인 루마진 단백질(N-terminal domain of lumazine protein 118 wt)과 여러 영역에 tryptophan을 생성시킨 돌연변이 단백질들(N-LumP 118 V41W, S48W, T50W, D64W, A66W)을 코드하는 유전자들을 위치 지정 돌연변이(Site Directed Mutagenesis)와 중합효소 연쇄 반응(Polymerase Chain

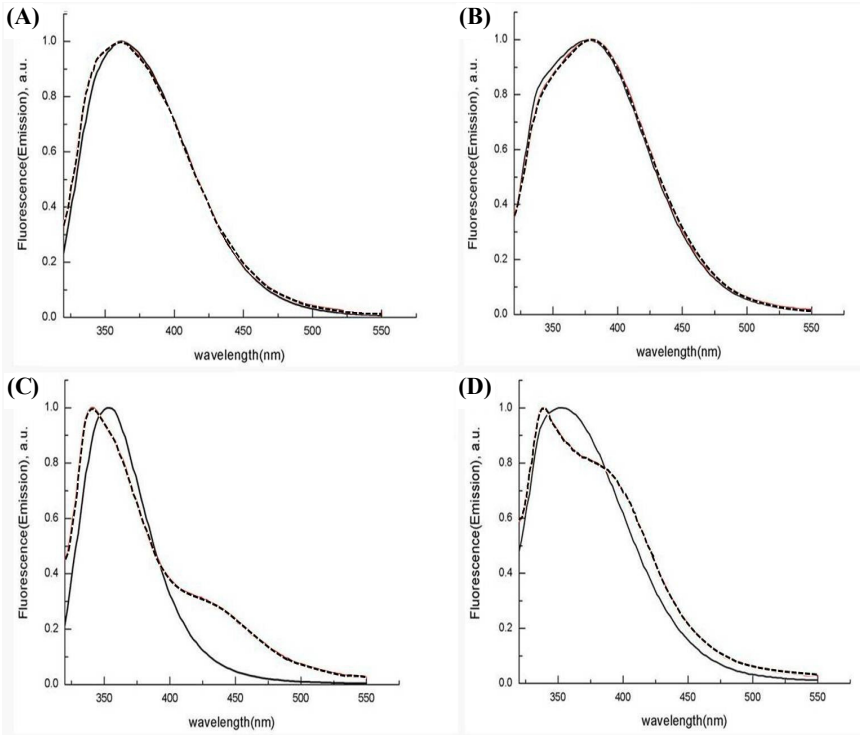

Fig. 7. Fluorescence emission spectra of wt and mutants N-LumP 118 (solid line) and N-LumP in the presence of 1 M urea (dashed line). Excitation at 295 nm. (A) N-LumP 118 wt, (B) N-LumP 118 S48W, (C) N-LumP 118 T50W, (D) N-LumP 118 A66W. The concentration of each protein is 5 μM, respectively.

Reaction)을 통해 제조하였다. 위의 유전자들이 포함된 재조합 플라스미드를 대장균에 형질 전환시켜 과발현시키는 최적의 조건을 찾았으며, 발현된 야생형 및 돌연변이 아미노-말단 영역 루마진 단백질을 6X-His tag system을 이용하여 정제 하였다. 흡광 및 형광 분광광도계를 이용한 실험 결과 이들 단백질들은 리간드인 6,7-dimethyl-8-ribityllumazine과 결합하여 형광성을 보유함을 보였다. 따라서 이들은 형광성을 지니게 되는 최소 크기의 루마진 단백질일 뿐만 아니라 형광성을 지닌 아미노산인 tryptophan이 여러 위치에 유일하게 존재함으로써 배향성 및 거리 등의 단백질의 구조 및 결합에 관한 심도 있는 연구에 탐침자로써 유용하게 활용 될 수 있을 것이다.

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