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Note

Fluorescent Properties of the Tryptophan Containing Variants of Leucine-responsive Regulatory Proteins

Eui Ho Lee, Robert Pokoo, Eugeney Oh, Sunjoo Lim, and Chan Yong Lee*
Department of Biochemistry, Chungnam National University, Daejeon 34134, Korea.
**E-mail: cylee@cnu.ac.kr*
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Leucine responsive regulatory protein (Lrp) is a global regulatory protein and its activity is not only restricted to transcriptional control, but can also affect other DNA transactions, such as site-specific recombination.[1,2] Also, Lrp protein can either activate or repress gene expressions, and depending on the type of gene that is regulated by Lrp, L-leucine can play a stimulatory, inhibitory or neutral role.[3,4,5] L-leucine amino acid has been identified as the most abundant and readily available building block of proteins, and so it serves as a good reporter to study the activity and presence of proteins in enteric bacteria.[6,7]

Lrp from *Escherichia coli* has been identified to consist of 164 amino acids,[8] and it crystallizes as an octamer, but assembles as a tetramer of dimers[9,10,11] (*Fig.* 1). The Lrp monomer is shown to consist of three regions, namely: (a) the N-terminal HTH domain, the C-terminal domain, and the flexible linker peptide.[3,4] The N-terminal domain, also referred to as the helix-turn-helix domain, is the DNA binding unit of the Lrp protein and consists of three α-helices. The C-terminal domain of Lrp consists of a fold similar to that of a regulation of amino acid metabolism (RAM) domain; it is involved in the allosteric regulation of amino acid metabolism in prokaryotes.[12,13] The RAM domain of Lrp contains the binding site for the effector leucine and is also referred to as the leucine response domain.

With the exception of cofactors such as FMN, FAD, NAD and porphyrins, which exhibit fluorescence, proteins contain only three amino acids that exhibit fluorescence properties, namely tryptophan, tyrosine and phenylalanine.[14] This property has been attributed to the presence of a benzene ring in their primary structures. Tryptophan amino acid is an important intrinsic fluorescent probe that can be used to estimate the nature of the micro-environment of the tryptophan within the protein.[14] Therefore, the objective of this research study is to express and purify wild-type and tryptophan-generated variants of leucine-responsive regulatory proteins (Lrp) and to elucidate more information about its binding by spectroscopic characteristics with tryptophan

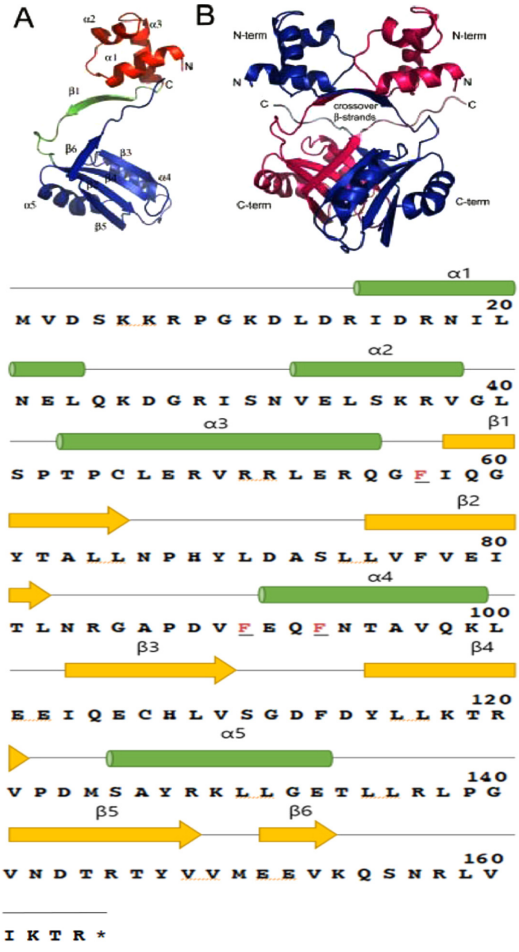

***Figure* 1.** (upper) A representation of the *Escherichia coli* Lrp monomer (A) and Lrp dimer (B).[11,15] (bottom) Amino acid sequence of wild-type Lrp protein and predicted secondary structure from *E. coli*.[9,13] Targeted amino acid sequences for site-directed mutagenesis replaced with tryptophan are underlined.

fluorescence spectroscopy and by molecular modelling of the protein.

The Lrp wild-type and variant genes expressed very well at suitable conditions of growth in BL-21 strain (DH-5α). Ampicillin antibiotic was used because the cloning vector of pQE30 has an ampicillin resistance region in its structure. Hence, it ensures the selective growth of bacteria containing the *lrp* gene. It was observed that IPTG induction of 0.5 mM at an optical density of 0.7 showed a high expression of the protein. Concentrations of IPTG higher than 0.5 resulted in a large fraction of the expressed proteins showing up in the pellet (data not shown). A high protein expression of molecular weight 18 kDa that corresponds to the calculated value of Lrp was identified on 12% SDS-PAGE. In addition, it was confirmed that in the absence of IPTG, there was low expression of Lrp protein by all genes, as shown in *Fig*. 2.

The recombinant proteins obtained from the supernatant fraction were higher than those in the pellet after centrifugation was performed a few minutes after sonication. A large culture of 2 L of Terrific Broth (TB) media was inoculated with an overnight culture at 37 °C. Lysis of the cells and purification of the proteins from the culture yielded an appreciably high concentration of protein. The quantification of proteins was tested by using QubitTM from Invitrogen. To determine the protein concentration precisely, the absorbance and literature[16] values of molar extinction coefficient 5,400 $M^{-1} \cdot cm^{-1}$ was also counted. The wavelength scanning of tryptophan fluorescence is widely used as a tool to monitor changes in proteins and also to make inferences regarding their local three-dimensional structures. Hence, the fluorescence of a protein molecule can be used as a diagnostic tool to identify the conformational state of protein under investigation. Tryptophan amino acids are generally excited at a wavelength of 280 nm. Most of the emissions released are due to the excitation of tryptophan residues, with a few emissions due to tyrosine and phenylalanine. Fluorescence intensities are measured using an LS-45 series fluorescence spectrometer (Perkin Elmer).

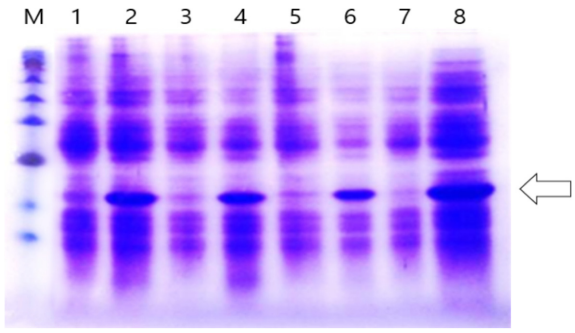

***Figure* 2.** 12% SDS-PAGE analysis of both wild-type and mutant Lrp proteins expressed by IPTG induction. Lanes M, Protein marker (15, 20, 30, 40, 50, 70, and 100 kDa, respectively); 1, No IPTG induction and incubation (*lrp* Wt); 2, IPTG induction and incubation (*lrp* Wt); 3, No IPTG induction and incubation (*lrp* F57W); 4, IPTG induction and incubation (*lrp* F57W); 5, No IPTG induction and incubation (*lrp* F90W); 6, IPTG induction and incubation (*lrp* F90W); 7, No IPTG induction and incubation (*lrp* F93W); 8, IPTG induction and incubation (*lrp* F93W).

To investigate the effective spectroscopic analysis of Lrp, the site-directed mutagenesis of amino acids including phenylalanine 57, phenylalanine 90, and phenylalanine 93 was performed (*Fig*. 1B). Phenylalanine 57 is located at the short turn between α3 and β1 and phenylalanine 90 at the tight turn between β2 and α4, whereas phenylalanine 93 resides inside of α4. This method included the replacement of the above phenylalanine amino acid with tryptophan and resulted in the synthesis of tryptophan-mutant forms of Lrp. Since Lrp wild-type does not possess tryptophan amino acid, the fluorescence properties of newly generated endogenous tryptophan residues at different positions can serve as local intrinsic probes for investigating the dynamic nature of the protein. As shown in *Fig*. 3, the variants Lrp changing of phenylalanine (F) to tryptophan (W) amino acid residue give higher fluorescence intensity than that of Lrp Wt. Lrp F90W shows the highest fluorescence out of the tryptophan containing Lrp (scan 1 in *Fig*. 3). In addition, the highest emission peak of Lrp F90W was observed to have shorter wavelength than that of Lrp F57W, Lrp F93W, and Lrp Wt. Fluorescence analysis showed that the maximum peak of fluorescence from Lrp F90W was shifted shortest to 362 nm. Lrp F57W, Lrp F93W, and Lrp Wt showed maximum fluorescence emission at 375 nm, 378 nm, and 390 nm, respectively (scans 2, 3, and 4 in *Fig*. 3). Three amino acids in protein, tryptophan, tyrosine and phenylalanine, exhibit fluorescence properties attributed to the presence of a benzene ring in their primary structures. The tryptophan emission spectrum is dominant over the weaker fluorescence of tyrosine and phenylalanine.[14] Although phenylalanine and tyrosine are present in the wild-type protein, the fluorescence spectra of wild-type Lrp was low in this condition (scan 4 in *Fig*. 3).

For obtaining precise information about the three-dimensional spatial arrangement of tryptophan amino acid, the quenching of tryptophan's fluorescence by acrylamide was investigated. A final concentration of 10μM of Lrp Wt and mutants were prepared and 0.2, 0.4, 0.6, 0.8, and 1.0 M of acrylamide were added to enhance the change of tryptophan's fluorescence. In order to accurately compare the extent of quenching, Stern-Volmer plot was plotted. The graph shows

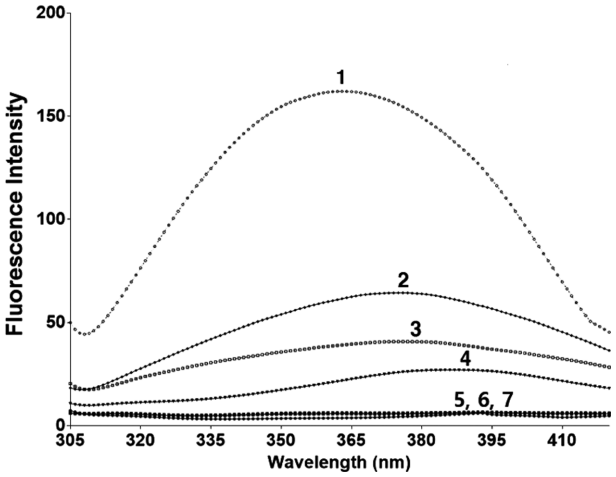

***Figure* 3.** Fluorescence emission spectra of Lrp Wt (scan 4), Lrp F57W (scan 2), Lrp F90W (scan 1), Lrp F93W (scan 3), DNA (scan 5), leucine (scan 6), and DNA plus leucine (scan 7). Excited at 280 nm. The protein concentrations of Lrp and DNA were 2 μM, respectively, and L-leucine was 2 mM. The complementary double-stranded DNA was formed by thermal annealing of two synthetic oligomers (5'-AAGGAGAATATTATGCTATGG-3' and 5'-CCATAGCATAATATTCTCCTT-3') in TE buffer. The DNA were designed from the specific Lrp-binding DNA sequences from *ilv*IH operon.[17,18]

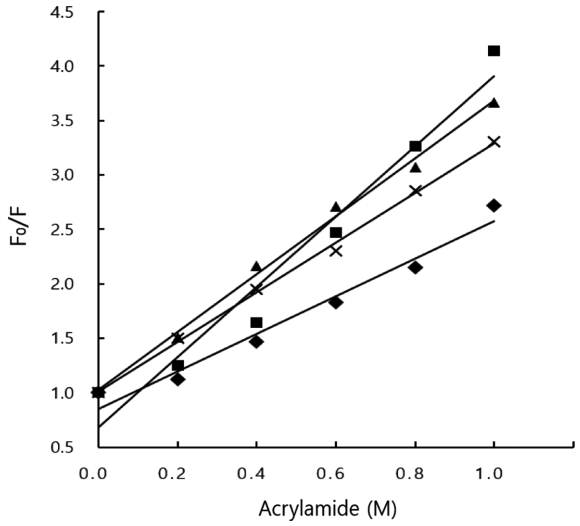

***Figure* 4.** Stern-Volmer plot of fluorescence quenching studies with Lrp Wt and mutants by acrylamide. Lrp Wt (◆), Lrp F57W (■), Lrp F90W (▲), and Lrp F93W (×). Fluorescence quenching of Lrp Wt and mutants by adding acrylamide were performed by excitation at 280 nm with 10 μM protein, respectively.

the concentration of acrylamide added on the X-axis, fluorescence intensity before the addition of acrylamide of different concentrations, divided by the fluorescence intensity after the addition of different concentrations of acrylamide on the Y-axis (*Fig*. 4). From this plot, it was observed that Lrp F57W recorded the highest degree of quenching of its fluorescence intensity. This implies that acrylamide had a huge effect on Lrp F57W, because its structure has an exposed surface. It was also observed that tryptophan fluorescence of Lrp F93W protein showed less effects of quenching of by acrylamide (*Fig*. 4). By using Stern-Volmer equation, the K_{sv} Stern-Volmer constants of Wt and mutant Lrp were deduced, respectively.

$$F_o/F=1+K_{sv}[Q]$$

F_o : fluorescence intensity without quencher
F: fluorescence intensity at the concentration quencher
[Q] : concentration of quencher
K_{sv} : Stern-Volmer quenching constant.

The Stern-Volmer constants for acrylamide quenched Lrps are as follows. Lrp Wt, 1.7 M^{-1}; Lrp F57W, 4.0 M^{-1}; Lrp F90W, 2.8 M^{-1}; Lrp F93W, 2.2 M^{-1}. By comparing the values obtained, Lrp F57W recorded about 2 times acrylamide quenching than Lrp F93W. The variation of the slope *Ksv* of the plots reflects the different overall accessibility of the intrinsic tryptophan residue to solvent. As shown in *Fig*. 1, the Lrp monomer is shown to consist of three regions: a N-terminal HTH domain, a flexible linker peptide, and a C-terminal domain.[4] These activities of Lrp protein are sometimes influenced by L-leucine, which binds to it at the C-terminal domain.[1] The RAM domain of Lrp contains the binding site for the effector leucine and is also referred to as the leucine response domain.[15] The amino acids of phenylalanine 57, phenylalanine 90, and phenylalanine 93, which were mutated to tryptophan, were located at different positions and surrounded by their own secondary and tertiary Lrp structures. Since Lrp wild-type does not possess tryptophan amino acid, the fluorescence properties of newly generated endogenous tryptophan residues at different positions can serve as local intrinsic probes for investigating the dynamic nature of the protein.

It was observed that Lrp Wt recorded very low fluorescence spectra even when it was bound to 21-mer DNA or L-leucine. The reason attributed to this characteristic feature is that the Lrp Wt amino acid sequence does not contain an amino acid that exhibits high fluorescence properties such as tryptophan. Even though phenylalanine and tyrosine are present in the amino acid sequence of the wild-type protein, they exhibit low fluorescence properties. The fluorescence properties of the endogenous tryptophan residue of Lrp can serve as local intrinsic probes for the investigation of the dynamic nature of the protein by binding of leucine. We can find a big difference in fluorescence inten-

sity between Wt and mutants Lrp. The fluorescence intensity of Wt Lrp was increased by adding DNA leucine, whereas the intensity of tryptophan containing Lrp mutants decreased in these conditions. Hence, the result observed in the above results, for L-leucine amino acid has been identified as the most abundant and readily available building block of proteins, so it functions as a good reporter to study the activity and presence of proteins in enteric bacteria.[6,19]

The fluorescence spectrum of Lrp F57W, Lrp F90W, and Lrp F93W was also measured in the presence of DNA and leucine that are bound together. As a control, Lrp itself, DNA, and leucine themselves were measured separately to test fluorescence intensity. The fluorescence spectra of Lrp Wt and Lrp mutants unbound or bound to the complementary double-stranded DNA was plotted. Lrp F90W recorded the same highest maximum peak of fluorescence intensity as the fluorescence spectra produced by the complex of Lrp F90W+DNA. In the same vein, the fluorescence spectra emitted by Lrp F90W+DNA+leucine complex was low compared to the above two fluorescence spectra recorded (*Fig*. 5). In the same respect, the fluorescence spectra emitted by Lrp F57W and Lrp F93W with DNA in the presence of L-leucine was low compared to the fluorescence spectra of Lrp itself. From these results, it was inferred that the binding of DNA to Lrp F90W resulted in a change of conformation of the protein, especially at its binding region and the surface groups. It was stated that the N-terminal domain, also referred to as the helix-turn-helix domain, is the DNA binding unit of the Lrp protein consisting of three

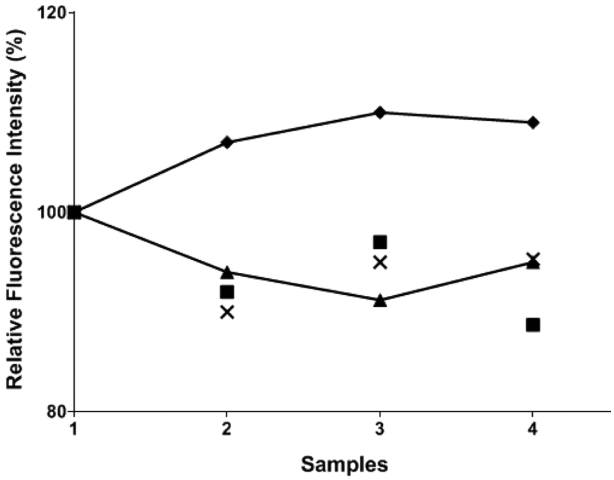

***Figure* 5.** Relative fluorescence intensity of Lrp Wt (◆) as well as Lrp F57W (■), Lrp F90W (▲), and Lrp F93W (x) by adding leucine and DNA. Relative fluorescence intensity was calculated by the normalization of fluorescence intensity of Lrp itself. The leucine and the DNA do not show any fluorescence. Samples 1, Lrp itself; 2, Lrp plus leucine; 3, Lrp with DNA; 4, Lrp plus leucine with DNA.

α-helices, with a conserved tight turn between the second and third helices.[8]

For tryptophan residues, the intensity and spectra of their fluorescence emission are dependent on the polarity and microenvironment. To show a correlation of these experimental results with the orientation and distance of the chromophores, the 3-D modeling of the proteins are constructed (*Fig*. 6). Tryptophan containing variant proteins showed slightly different emission values for the maxi-

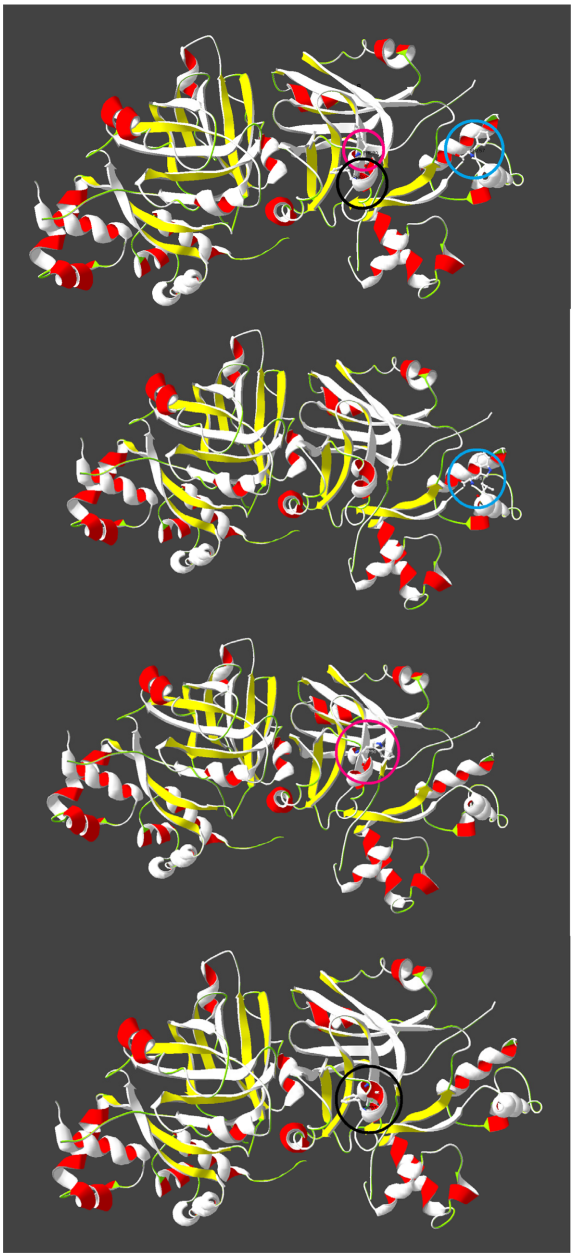

***Figure* 6.** 3D modelling of wild type and tryptophan containing Lrp variants. The mutated phenylalanine amino acids are marked on the circle. From top to bottom, Lrp Wt, Lrp F57W, Lrp F90W, and Lrp F93W are presented.

mum peak of fluorescence intensity. The extent of conformational change affecting the accessibility of individual tryptophan residues was tested with acrylamide through the quenching experiment. Acrylamide diminishes the fluorescence of tryptophan through the collision with tryptophan or complex formation. Based on these experiments, it can be explained that the 3-D modelling and fluorescence data are quite correlated. In conclusion, amongst the tryptophan mutated proteins, Lrp F57W especially, was most sensitive to acrylamide. In the case of Lrp F90W protein, the maximum peak was shifted to the shortest wavelength according to the degree of accessibility of indole group of tryptophan to the excitation of light and the emission fluorescence of its tryptophan appeared to have the shortest wavelength.

EXPERIMENTAL

E. coli XL-1 Blue competent cell and BL21 expressing strains were purchased from RBC Bioscience Co. The primers used for site-directed mutagenesis and polymerase chain reaction were synthesized and provided by Bioneer Co. T4 DNA ligase, restriction enzymes and their corresponding buffers were also purchased from New England Biolabs. Plasmid miniprep kits, PCR purification kits and gel extraction kits were obtained from Solgent Company. Bronsted Lowry protein concentration determination reagents and protein markers were purchased from Bio-Rad Co. The construction of wild-type (pCV294) gene coding for the 6X His-tagged with Lrp was described in our previous paper.[18,19] DNA containing the gene coding for the Lrp of pCV294 was used as the template for site-directed mutagenesis to obtain the following mutants: *lrp* F57W, *lrp* F90W, and *lrp* F93W, by using their corresponding primers (*Table* 1). For site-directed mutagenesis, the Mega PCR methodology was used. In the first PCR method, the forward primer CATCACGGATCCGTAGATAGGAAGAA (*Bam*HI sites are underlined) and the respective flanking mutant primers in *Table* 1 were added to the template DNA and Taq polymerase. PCR was run for about 30 cycles under the following conditions: Pre-denaturation at 95 °C for 5 minutes, denaturation at 95 °C for 1 minute, annealing at 58 °C for 1 minute, extension at 72 °C for 2 minutes and then final extension at 72 °C for 7 minutes. The amplified DNA was run on agarose gel and the sites of interest were cut, purified and eluted using the Gel Extraction Kit from Solgent Co. The eluted DNA was then used as a forward primer and in addition to GCTAATTAAGCTTCCGTGTTAGCGCGTC (*Hind*III sites are underlined), the reverse primer for the second stage of PCR, an additional 30 cycles of PCR were performed to obtain the desired mutant plasmid under the same PCR conditions used for the first PCR analysis. The amplified mutant DNA was once again analyzed on 1% agarose gel to identify the desired mutant DNA at the 495 base pair mark and digestion by appropriate restriction enzyme located in the mutant primer. The purified DNA plasmid of each mutant form was digested with restriction enzymes *Bam*H1 and *Hind*III, respectively, and the resultant products were also analyzed on 1% agarose gel by electrophoresis. The digested plasmid DNA of both wild-type and mutants were cut out and purified. The same steps and conditions were repeated in digesting the cloning vector (pQE30) using the above-named restriction enzymes. The mutant forms of DNA containing the Lrp gene were ligated with pQE30 cloning vector digested with the same restriction enzymes. The ligated product was then transformed into XL-1 Blue competent cells and plated on ampicillin LB-Agar plates. Colonies from each plate were selected, inoculated in ampicillin Luria-Bertani (LB) media and cultured at 37 °C for 15 hours. The growth media obtained was spun and the plasmid DNA was extracted using plasmid miniprep kits. The recombinant wild-type plasmid and mutants were confirmed to be present by performing restriction enzyme digestion. The plasmids confirmed to have inserts present were then sent to Bioneer Co. (Daejeon, Korea) for sequencing to confirm. Single colonies from recombinant *E. coli* strains that were transformed with mutants and wild-type gene of Lrp, were cultured in 2 L of terrific broth (TB) media (tryptone 12 g, yeast extract 24 g, 4 ml glucose, 2.31 g KH_2PO_4 and 12.54 g K_2HPO_4) containing ampicillin (100 μg/ml) at 37 °C in shaking volumetric flasks until an optical density of 0.7 was recorded at a wavelength of 600 nm. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a concentration of 0.5 mM. This was followed by incubation at 25 °C for 10 hours.

***Table* 1.** Primers for site-directed mutagenesis in this study

Mutation site on wild-type gene	Restri-ction enzyme	Mutation primers (restriction site is marked in bold) 5'→3'
lrp F57W	*Bgl*II	GTAT**AGATCT**GAATCCACC CTTGTCTTTCC
lrp F90W	*Kpn*I	GGTATTGAAT**GGTACC**CA CACATCCGGTGCGC
lrp F93W	*EcoR*I	GTACAGCG**GAATTC**CATTG TTCAAACACATCC

The Modeling of Wild Type and Variant LRPs

Leucine-responsive regulatory protein modeling was performed by using DeepView/Swiss-PdbViewer v.4.1.0. The

LRP mutants were based on crystal structure of *E. coli* Leucin-regulatory protein from PDB (protein data bank acc. Code 2GQQ).

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