Development of an Inducible Secretory Expression Vector and Host System for High Yield Production of Recombinant Protein

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Research

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Abstract

Background

Escherichia coli has been the most widely used recombinant protein expression system due to the availability of various protein expression vectors and ease of genetic manipulation. However, recombinant proteins expressed in *E. coli* are often contaminated with lipopolysaccharide (LPS) highly toxic to humans and must be removed from FDA-approved biologics, a process which requires extensive and expensive procedures. Gram-positive bacteria possess a single layer of cytoplasmic membrane free of LPS which make it ideal for producing recombinant protein. However, a lack of inducible protein expression systems limits a large-scale protein production in Gram-positive bacteria.

Results

The HptARS is a three-component regulatory system in *Staphylococcus aureus* which senses extracellular glucose-6-phosphate and activates the *uhpT* gene promoter to facilitate uptake of extracellular G6P. To construct an inducible and secretory protein expression vector system, the promoter of the *uhpT* gene and the N-terminal signal peptide sequence of the *hlb* gene was fused in-frame with a C-terminal 6x-histidine sequence. For constitutive expression, we generated *S. aureus* expression host strain lacking the *uhpT* gene which could not uptake extracellular G6P, resulting in constitutive activation of HptARS system. With this newly established expression vector system and host strain, we demonstrated large-scale production of biologically active and highly pure staphylococcal leukotoxin E.

Conclusion

Extracellular expression of recombinant protein in LPS-free bacteria has a tremendous advantage in industrial production of FDA-approved biologics. Our newly established inducible and secretory expression vector system and *S. aureus* host strain will be useful to produce recombinant proteins for vaccine applications and other industrial purposes.

Introduction

A large-scale production of recombinant protein is important for biopharmaceutical companies and other industries. *Escherichia coli* has been the most widely used recombinant protein expression agent due to the availability of various protein expression vectors and ease of genetic manipulation. In *E. coli*, recombinant proteins are synthesized in the cytoplasm and excreted by the SecB-dependent type II secretion system, in which pre-pro proteins are carried to the inner membrane, transferred across to the periplasm where they are folded and excreted by non-specific periplasmic leakage [1, 2]. However, many recombinant proteins are trapped in the periplasm [3], which requires enzymatic or mechanical disruption for protein purification. During these processes, many recombinant proteins are mechanically or enzymatically damaged causing contamination with lipopolysaccharides (LPS) which are toxic to humans and very difficult to completely remove [4].

In contrast to Gram-negative bacteria, Gram-positive bacteria possess a single layer of cytoplasmic membrane free of LPS [5]. Thus, transposition of a target protein across the cytoplasmic membrane results in direct secretion into the culture media [6]. Secretion of proteins in Gram-positive bacteria is mostly mediated by the Sec-dependent pathway in which the N-terminal signal peptide of target protein is recognized by the signal recognition particle (SRP) that transfers the target protein to the cytoplasmic membrane. And then, the target protein is translocated across the cytoplasmic membrane by the Sec translocase and cleaved by the signal peptidase (SPase) at the alanine-X-alanine motif in the signal peptide sequence, resulting in release of target protein to the culture media [7].

Secretion of recombinant proteins into the culture media provides immense benefits for downstream processes and reduces production costs. It can prevent accumulation of recombinant protein-containing inclusion bodies as well as simplifying protein purification. Several Gram positive bacteria including *Bacillus*, *Lactococcus*, and *Streptomyces* have been used in industry for the production of a variety of recombinant proteins [8]. However, a high production yield was still difficult to achieve due to the lack of inducible protein expression system.

Recently, we characterized the hexose phosphate transport (Hpt) system in *Staphylococcus aureus* [9]. The Hpt system is composed of the UhpT, hexose phosphate transporter, and a three-component regulatory system (HptARS). We demonstrated that the HptA senses extracellular hexose phosphates such as glucose-6-phosphate (G6P) which activates the two-component regulatory system, the HptS (a histidine kinase) and HptR (a transcriptional factor). Extracellular G6P is recognized by HptA which induces a cascade of phosphorylation events of HptS, followed by HptR. The promoter region of *uhpT* gene contains a defined -35 (TATTA) and –10 (TATAT) promoter element and ribosomal binding site (GAGGTG) and a binding site (GTTCAGTATTTTGGATAATTTAATAATTTT) for the phosphorylated HptR [10]. The binding of phosphorylated HptA activate the *uhpT* promoter to express UhpT more than 1,000-fold without activation [9, 10]. These findings led us to develop an inducible secretory expression vector system in *S. aureus* with potential for broad application.

Materials And Methods

Bacterial strains, plasmids, and oligonucleotides

Chromosomal DNA from *S. aureus* strain RN4220 was used as template to amplify the *uhpT* gene promoter, N-terminal signal peptide sequence of the hlb gene, the LukE gene. The *E. coli-S. aureus* shuttle vector, pOS1, was obtained from Dr. Taeok Bae (Indiana University). The pMAD temperature sensitive homologous recombination plasmid and LuxABCDE luminescent reporter plasmid were purchased from Addgene. *E. coli* DH5α strain was used for cloning and plasmid preparation.

All *S. aureus* strains were grown in 1 % (w/v) Casamino acids and Yeast extract (CY) broth supplemented with chloramphenicol (25 μg/ml), if necessary. All *E. coli* strains were grown in Luria-Bertani (LB) broth supplemented with ampicillin (100 μg/ml), if necessary. All oligonucleotides were synthesized by IDT DNA and listed in Table 1.

Construction of inducible and secretory plasmid

The pOS1 plasmid was cleaved with PstI/BmtI restriction enzymes and oligonucleotides containing a new multi-cloning site and the 6 histidine residues (MCS_HisF/MCS_HisR) were directly ligated using Gibson assembly [11]. A DNA fragment containing the *uhpT* gene promoter and the HptA binding site was amplified by PCR using primers (UhpTpF_EcoRI/UhpTpR). A DNA fragment containing the signal peptide sequence of Hlb was amplified by PCR using primers (UhpTp_HlbF/HlbsigR_BamHI). The two PCR products were joined together by a 21-bp overlapping segment between the UhpTp_HlbF and UhpTpR primers using SOWing PCR method. The joined PCR product was digested and cloned into the EcoRI/BamHI sites in the plasmid above, resulting in a pKS62.

Construction of *S. aureus* expression host strain lacking UhpT

The *uhpT* gene was deleted from *S. aureus* strain RN4220 by an allelic replacement using homologous recombination. Briefly, DNA fragments upstream and downstream of the *uhpT* gene were amplified by PCR using primers (UhpTupF_SalI/ UhpTupR_MluI and UhpTDnF_EcoRI/UhpTDnR_SmaI, respectively) and cloned into a pMAD-CM, a temperature sensitive shuttle vector system. The resulting plasmid was electroporated into *E. coli* DH5a and then into *S. aureus* strain RN4220. *S. aureus* strain RN4220 harboring the constructed plasmid was cultured at 43°C (non-permissive temperature for the replication of pMAD-CM to promote the first homologous recombination, followed by culturing at 37°C to promote the second recombination, resulting in deletion of the *uhpT* gene by allelic replacement.

To measure the induction of target gene expression by G6P under the control of the *uhpT* promoter, a DNA fragment containing the *uhpT* gene promoter region was amplified by PCR using primers (UhpTpF_EcoRI/Lux_UhpTpR_BamHI) and cloned into a corresponding site in a promoterless bioluminescent plasmid (pLuxABCDE) [12]. The resulting plasmid was electroporated to *S. aureus* strain RN4220 or RN4220 lacking the *uhpT* gene (D*uhpT*). Strains harboring a constructed plasmid were cultured in CY broth supplemented 2% G6P. The bioluminescent signal was measured using Cytation 5 (BioTek Instrument).

Expression and purification of staphylococcal leukotoxin E

Staphylococcal leucocidin E (LukE) gene was amplified by PCR using primers (LukEF_BamHI/LukER_XhoI) and cloned into the corresponding site in the pKS62. The resulting plasmid was electroporated into *E. coli* DH5a, followed by the *S. aureus* RN4220 D*uhpT* strain. *S. aureus* RN4220 D*uhpT* strain was cultured in CY broth without or with supplementation of G6P (2% w/v) at 37 ^{o}C for 18 hours with shaking at 200 rpm. Culture supernatants were collected by centrifugation at 12,000 rpm. Proteins in the culture supernatants were concentrated by TCA (10%, w/v) precipitation and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For protein purification, culture supernatants were sterilized by filtration (0.45μM, Millipore) and directly applied to a Ni-NTA (nickel-nitrilotriacetic acid) column using His-Bind Purification Kit (Novagen) as suggested by the manufacturer.

Cytotoxicity assay

A cytotoxicity assay was performed to verify the biological activity of recombinant LukE expressed in our system. Briefly, bovine leukocytes were purified from whole bovine blood by lysing red blood cells with Tris-NH_4Cl buffer. Purified bovine leukocytes were adjusted to 1×10^6/ml in serum free RPMI media. Cells were co-incubated with purified LukE (1μg/ml), LukD (1μg/ml), or both LukD and LukE for 30 min and then propidium iodine solution (1μM) was added to the culture. The fluorescent intensity as an indication of membrane damage was measured using Cytation 5 (BioTek Instrument).

Results

Design and construction of an inducible and secretory expression vector system

To induce extracellular expression of the target gene by G6P and purification by affinity chromatography, we designed a protein expression vector in which expression of the target gene is induced by G6P under control of the staphylococcal *uhpT* promoter, followed by an N-terminal secretory signal peptide and C-terminal 6x histidine residues (Figure 1). For these purposes, the oligonucleotide containing a multi-cloning site and the 6x histidine sequence was directly cloned into the *E. coli-S. aureus* shuttle vector pOS1 plasmid by Gibson assembly. Then, a DNA fragment containing the *uhpT* promoter and the N-terminal signal peptide sequence of the b-hemolysin (Hlb) was amplified separately and joined together by splicing by overhang extension PCR (Figure 2) which was cloned between the N-terminal signal peptide sequence and C-terminal 6x histidine sequence.

Constitutive expression of target gene in *S. aureus* expression host strain lacking the UhpT

To verify the induction of target gene expression by extracellular G6P under the control of *uhpT* promoter, we generated *S. aureus* RN4220 harboring a bioluminescent reporter plasmid in which the *uhpT* promoter was cloned into a promoterless bioluminescent LuxABCDE operon. When *S. aureus* RN4220 strain harboring a bioluminescent reporter plasmid was cultured in CY broth supplemented with G6P, the bioluminescent signal rapidly increased, peaked at 4 hours, and then rapidly decreased (Figure 3). These results suggest that induction of the target gene by G6P is temporal due to the rapid metabolism of G6P by *S. aureus*. To prevent uptake of extracellular G6P by *S. aureus*, we generated *S. aureus* RN4220 lacking the *uhpT* gene (D*uhpT*) by homologous recombination. When *S. aureus* RN4220 D*uhpT* strain harboring a bioluminescent reporter plasmid was cultured in CYP broth supplemented with G6P, the bioluminescent signal was sustained for 18 hours. These results indicate that a disruption of the *uhpT* gene prevents metabolism of extracellular G6P which constantly activates HptARS system, resulting in constitutive expression of target gene under the control of the *uhpT* promoter suggesting *S. aureus* RN4220 D*uhpT* strain is an ideal expression host strain to induce target gene expression by G6P.

Expression of staphylococcal cytotoxins by inducible expression vector system

To demonstrate that inducible expression vector system can produce a large quantity of target protein in response to G6P, the staphylococcal *lukE* gene was amplified and cloned into the inducible expression vector system and transformed to *S. aureus* RN4220 D*uhpT* strain. When cultured in CY broth supplemented with 2% G6P (w/v), the LukE was highly expressed as indicated by a distinctively thick protein band, corresponding to the expected molecular weight (32.7 kDa) which was absent in the culture from CY broth without G6P. A highly pure recombinant LukE was obtained by nickel affinity chromatography. These results demonstrated that the newly established inducible expression system successfully produce a large quantity of target protein which could be easily purified by affinity chromatography.

To verify the recombinant protein expressed in this system is highly pure and biologically active, we performed a cytotoxicity assay. Since LukE is an S component of the bi-component leukotoxins, the LukE alone is not biologically active and it requires a F-component of leukotoxin for active cytotoxicity [13]. When bovine leukocytes were incubated with the recombinant LukE alone, no detectable cytotoxicity was observed. By contrast, when both LukE and LukD were present together, strong cytotoxicity indicated by propidium iodine signal was observed (Figure 4). It is noteworthy that *S. aureus* RN4220 D*uhpT* strain also produces several F-component of leukotoxins including LukD. These results indicated that the F-component of leukotoxins naturally expressed by *S. aureus* RN4220 D*uhpT* strain were not present as contaminates in the LukE purified by affinity chromatography.

Discussion

Protein expression system in *E. coli* is the most common host for cloning and protein expression including biomedical products. One of the major limitations of *E. coli* expression systems is contamination by LPS. In humans, LPS induces secretion of proinflammatory, cytokines, inhibition of cell growth, and hyperactivation of immune cells resulting in endotoxic shock or even death [14]. Therefore, LPS must be removed from FDA-approved biologics which requires extensive and expensive procedures [15]. Furthermore, overly expressed recombinant proteins often form inclusion bodies, resulting in loss of function [16].

Protein expression system in Gram positive bacteria have been considered as an alternative approach due the lack of LPS in the Gram positive cell wall and efficient secretion of proteins by the N-terminal signal peptide sequence [17]. However, large scale protein production has been a challenge due to the lack of efficient inducible protein expression vector system. To tackle this unmet need, we designed and constructed an inducible and secretory expression vector and host system in *S. aureus* for large-scale recombinant protein production. We utilized the bacterial Hpt system which senses extracellular hexose phosphate such as G6P and highly activates the *uhpT* gene promoter to promote hexose phosphate uptake [9]. However, G6P is a highly metabolizable sugar and quickly deprived by bacterial metabolism, resulting in temporal activation of the *uhpT* gene promoter. To overcome this problem, we generated an expression host *S. aureus* strain lacking UhpT so that extracellular G6P cannot be metabolized by the bacteria and remains sustained in the media to constitutively activate the HptARS system, thereby

activating the *uhpT* gene promoter. This allows for expression of a large amount of target gene protein as demonstrated in Figure 4.

For simple purification of target protein, we designed the expression vector system to clone the target gene in-frame fused between the N-terminal signal peptide sequence of Hlb gene and a C-terminal 6x histidine residue sequence for secretion and purification by affinity chromatography. This greatly simplifies the purification process to harvesting culture supernatant by centrifugation, followed by filter sterilization and affinity chromatography. This excludes the need for disruption of bacterial cells in the purification step and prevents contamination of the expressed protein with bacterial components, which will greatly curtail the costs and time required for protein purification.

To test the purity of recombinant protein expressed and purified from inducible protein expression vector system established in this study, we chose staphylococcal LukE for testing. As forementioned, the LukE is a S component of the bi-component leukotoxin which requires both S and F component for active cytotoxicity [12]. Since *S. aureus* RN4220 D*uhpT* strain naturally produces both S and F component of the bi-component leukotoxins, the LukE purified from inducible protein expression vector system alone could be cytotoxic if there is contamination with F component of the bi-component leukotoxins naturally expressed by *S. aureus* RN4220 D*uhpT* strain. Our results showed that the LukE alone did not show any cytotoxicity while it was highly active only in the presence of recombinant LukD, the F component of the bi-component leukotoxin. Since bovine leukocytes are highly sensitive to LukE/D at less than 5 nM [18], these results suggest that the LukE purified in this study is highly pure.

In conclusion, our newly established inducible protein expression system is highly efficient to generate a large amount of highly pure and LPS-free recombinant protein from Gram positive bacteria which is useful for production of FDA-approved biologics. Since the hexose phosphate system is also conserved in *Lactococcus* and *Streptomyces* [19, 20], we are currently developing inducible protein expression system in these bacterial species.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

All authors agree to publish the manuscript.

Competing interests

The authors declare that they have no conflict of interest.

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Authors' contributions

JP and KS are responsible for project planning and experimental design; SY, NP, and JR performed most of the experiments; JR and JT provided technical advice. SY, KS, JT, and JP analyzed the data and wrote the paper. All authors read and approved the final manuscript.

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Not applicable.

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Tables

Table 1. Primers used in this study

Primer name	Sequence (5’ to 3’)
MCS_HisF	GAAGCTTGCGGCCGCACTCGAGCACCACCACCACCACACTGAGCTAG
MCS_HisR	CTCAGTGGTGGTGGTGGTGGTGCTCGAGTGCGGCCGCAAGCTTCTGCA
UhpTpF_EcoRI	TTTAGAATTCGGATGGTTAGGTAAAATG
UhpTpR	CTCTGTCACCTCAATCATTTTCG
UhpTp_HlbF	GATTGAGGTGACAGAGATGGTGAAAAAAACAAAATCC
HibsigR_BamHI	AATTGGATCCGGCTTTGGCACTATTGTCAGTA
LukEF_BamHI	ATATGGATCCAATACTAATATTGAAAATATTGGTG
LukER_XhoI	AATTCTCGAGATTATGTCCTTTCACTTTAA
UhpTUpF_SalI	ATTCGTCGACAAGGCATTCCATTATCGG
UhpTUpR_MluI	ATGTACGCGTGAATCCTAAGTATCGGCC
UhpTDnF_EcoRI	CTTTGAATTCGCTAATGTATTCTTCCATGG
UhpTDnR_SmaI	TTCTCCCGGGCATAGAAAGCAACGATTCCT
Lux_UhpTpR_BamHI	CAAAGGATCCCATCTCTGTCACCTCAAT

Figures

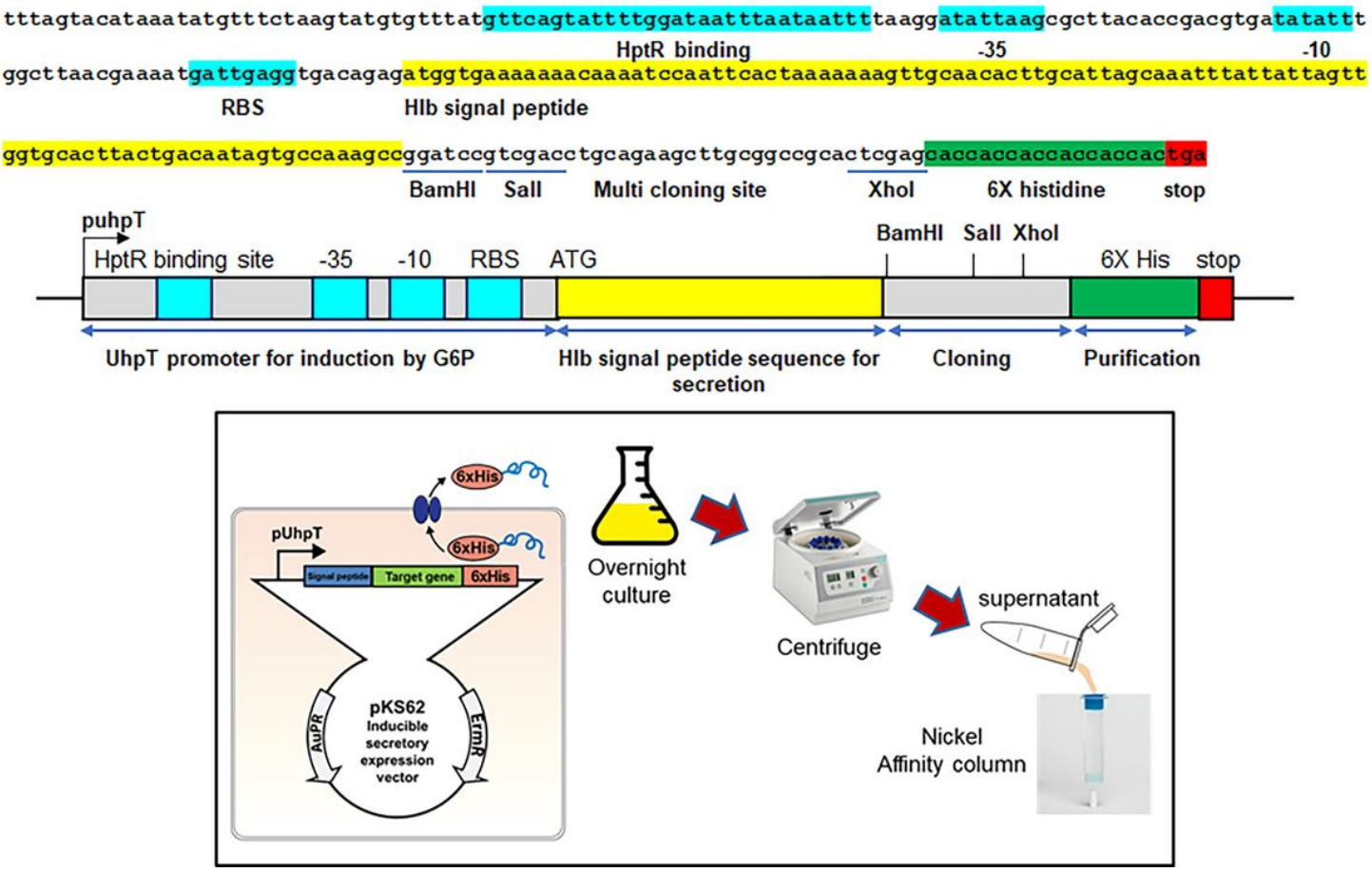

Figure 1

Schematic illustration of inducible protein expression vector system and expression and purification process

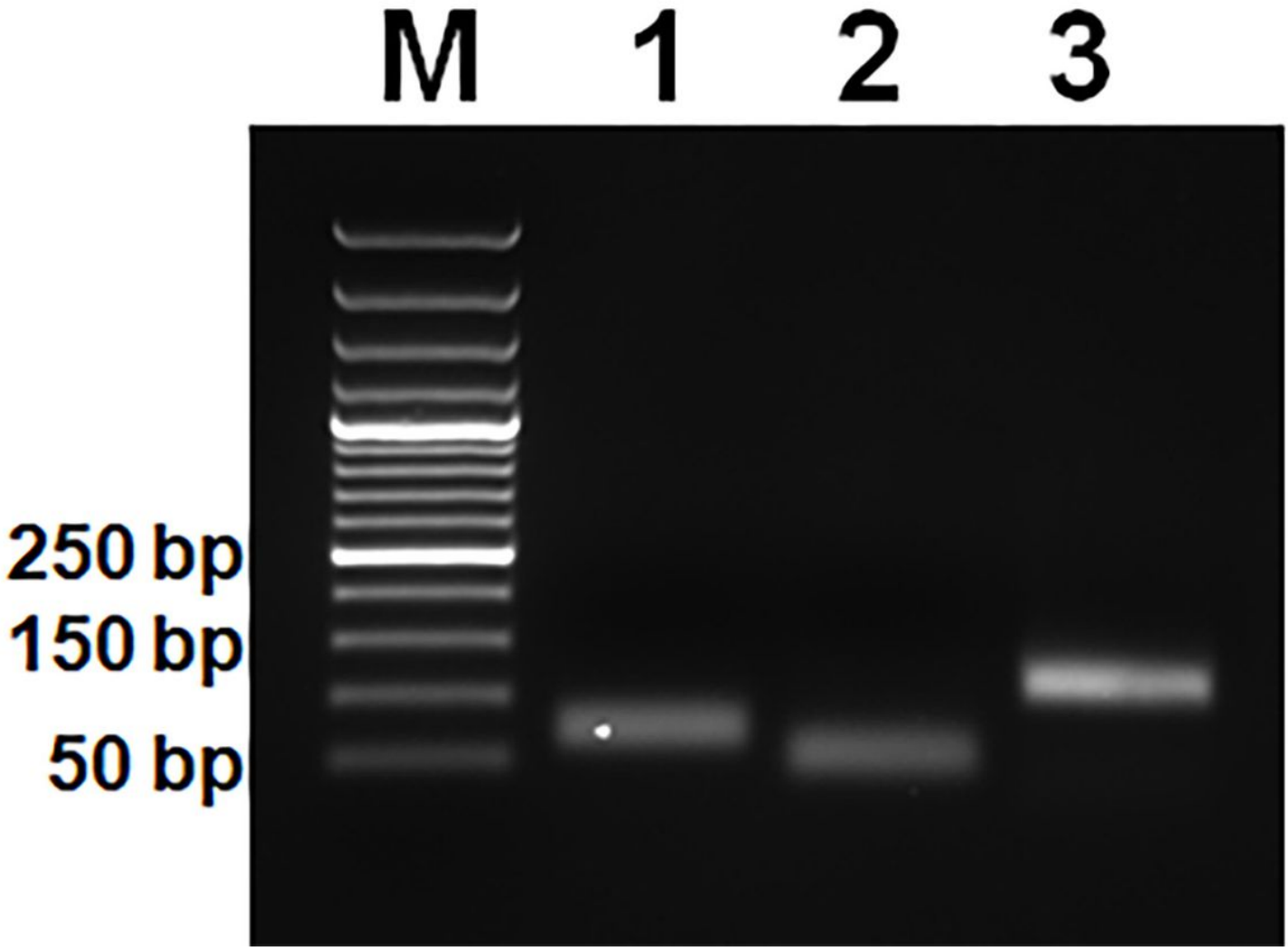

Figure 2

Fusion of the uhpT gene promoter and the N-terminal signal peptide sequence of the hlb gene by SOE PCR The DNA fragment containing the uhpT gene promoter (lane 1) and the N-terminal signal peptide sequence of the hlb gene (lane 2) was separately amplified by PCR, and then joined by SOE PCR (lane 3). M: Fermentas GeneRuler 50 bp DNA ladder

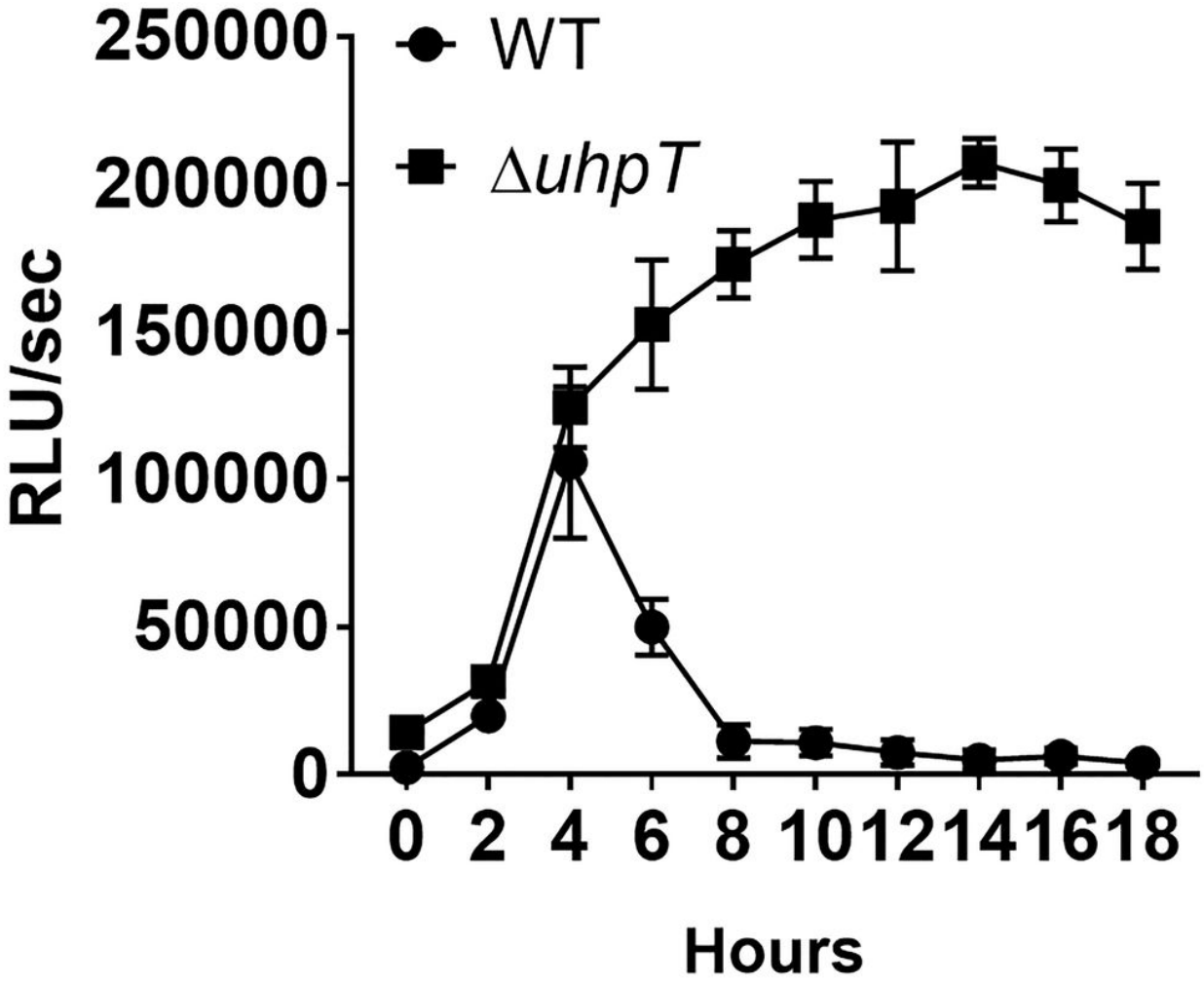

Figure 3

Activation of the uhpT gene promoter by G6P S. aureus RN4220 wild type (WT) and uhpT strains harboring bioluminescent reporter plasmid pLuxABCDE was cultured in CY broth supplemented with G6P. Activation of the uhpT gene promoter indicated by induction of bioluminescent signal was measured using Cytation 5 (BioTak). Data shown are the mean ± SEM combined from three independent experiments.

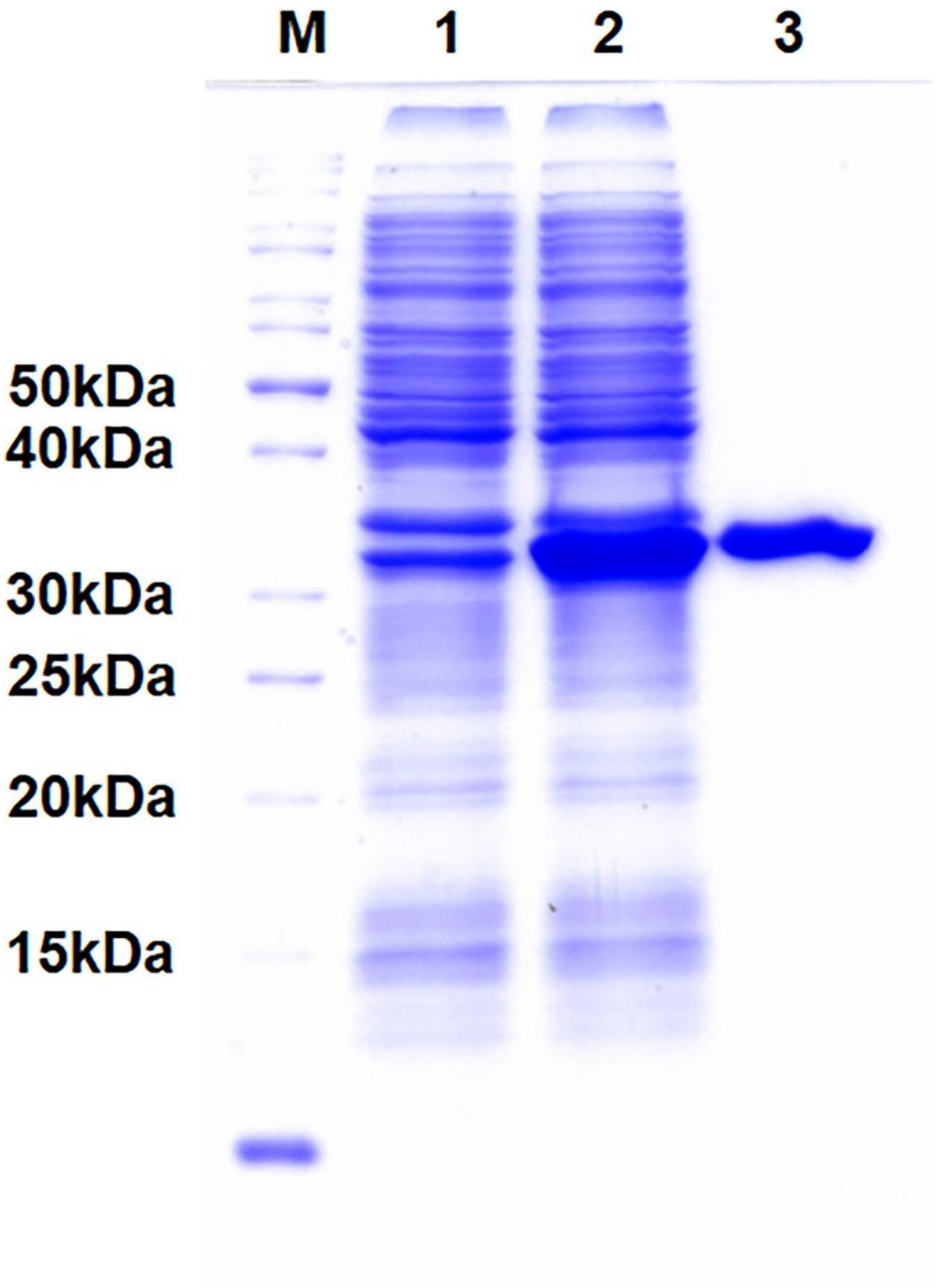

Figure 4

Expression and purification of LukE by inducible protein expression vector system (A) A DNA fragment encoding LukE was amplified from S. aureus strain RN4220 and then cloned into BamHI/XhoI site in the inducible protein expression vector system established in this study. Constructed plasmid was electroporated to S. aureus RN4220 uhpT. (B) S. aureus RN4220 uhpT harboring inducible protein expression vector system cloned with LukE was cultured in CY broth without or with supplementation of

G6P (2%, w/v) for 18 hours at 37 oC. Culture supernatants and purified LukE were analyzed by SDS-PAGE. M: Protein marker, lane 1: culture supernatant from CY broth without G6P, lane 2: culture supernatant from CY broth with G6P, lane 3: purified LukE

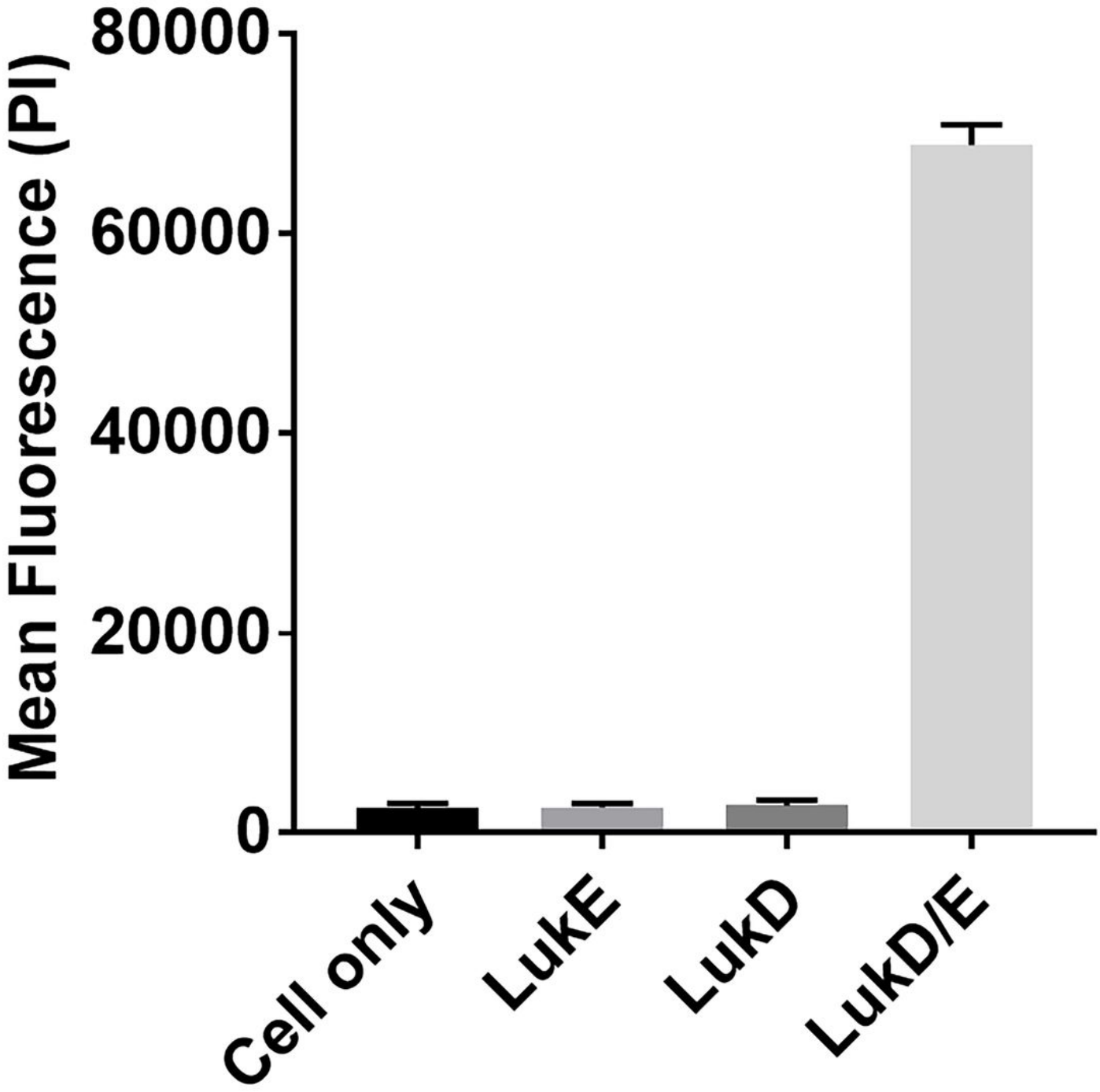

Figure 5

Biological activity of recombinant LukE expressed and purified from inducible protein expression vector system Bovine leukocytes (1×106 cell/mL) were incubated with recombinant LukE (1 µg), LukD (1 µg), or

both LukE/LukD (1 μg each) for 30 min at 37oC. Cytotoxicity as indicated by incorporation of propidium iodine (PI) to the cellular DNA was measured using Cytation 5. Data shown are the mean ± SEM combined from three independent experiments.