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Isolation, cloning and optimization for expression of neutrophil gelatinase associated lipocalin (NGAL) in prokaryote

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KEYWORDS

Acute kidney injury; Neutrophil gelatinase associated lipocalin; Prokaryote expression.

ABSTRACT

Acute kidney injury (AKI) is a sudden loss of kidney function followed by a failure to maintain fluid, which is associated with increased mortality. Recently, neutrophil gelatinase associated lipocalin (NGAL) has been recommended as the first biomarker for prediction of kidney damage in the early phase. For medical applications of the NGAL protein, a large amount and expression system of this protein is required. Currently, the assay to determine NGAL levels is enzyme-linked immunosorbent assay (ELISA), which is accurate but inconvenient for clinical applications, because its long-time process takes about 4 hours and the antibody against human NGAL is expensive. Therefore, high-level expression of NGAL is required in order to develop a commercially NGAL strip test. The present study aimed to construct the recombinant plasmid, to optimize the expression of NGAL in Escherichia coli BL21(DE3) and Origami™B(DE3) strains. The host strain, IPTG concentration and induction time were also evaluated. NGAL gene was cloned in pET32a and transformed to E. coli BL21(DE3) and Origami™B(DE3). The construct of NGAL and the expression vector was successful. BL21(DE3) showed a high growth rate indicated by reaching the mid-log phase faster than Origami™B(DE3). However, both E. coli BL21(DE3) and Origami™B (DE3) showed no expression of NGAL. In conclusion, this study demonstrates the efficiency of the expression host to produce protein for development as a commercial strip test. The suitable system to express NGAL protein should be further investigated.

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Introduction

Acute kidney injury (AKI) is an abrupt reduction in kidney function followed by failure to maintain fluid, acid balance and electrolyte homeostasis. AKI is a serious condition globally and it is associated with high mortality in patients in an intensive care unit (ICU). Early diagnosis and treatment of AKI are important. Nowadays, AKI is typically diagnosed based on an elevation of serum creatinine (SCr). However, rapid change of kidney function cannot be indicated in real time by the level of SCr because the level of SCr can only be detected after kidney function has been destroyed beyond 50%[1]. SCr can also be affected by several factors such as age, muscle mass, diet, drugs and exercise[2]. The level of SCr has low sensitivity to detect kidney damage in an early diagnosis. Therefore, it is necessary to explore for highly sensitive biomarkers for early detection of AKI. Gaspari et al[3] reported that a protein called neutrophil gelatinase associated lipocalin (NGAL) was an alternative choice for the prediction of kidney injury in the early phase, instead of SCr. NGAL has potential for use in clinical applications because its level is rapidly increased into the blood and urine[4,5]. Taken together, NGAL is considered as an appropriate biomarker for diagnosis of AKI and also for prediction of clinical outcomes, including the need for initiation of dialysis and for the onset of mortality[6].

At present, measuring NGAL level is performed by enzyme-linked immunosorbent assay (ELISA), which has a high accuracy but is not suitable for clinical applications. The ELISA process for AKI diagnosis takes a long time, about 4 hours[7], with a high cost for the antibodies against human NGAL. Therefore, the development of a reliable, rapid, easy-to-use and cost-effective method for NGAL detection is needed. For medical applications of the NGAL protein, a large amount of NGAL protein is necessary. In the present study, the bacterial expression system is the method of choice for the expression of the recombinant NGAL protein, due to its simplicity, cost effectiveness, and capacity to produce high yield protein[8].

The aim of this study was to construct and produce recombinant NGAL protein by cloning in *Escherichia coli*. The optimal conditions for the expression host, *E. coli* BL21(DE3) and Origami™ B(DE3) strains, were compared. The IPTG concentrations and induction time were also evaluated.

Materials and methods

Neutrophil gelatinase associated lipocalin gene

Total RNA was isolated from colon carcinoma and extracted by reagent TRIzol (Invitrogen, USA) according to the manufacturer's instructions. Then, total RNA was used to generate the first-strand Complementary DNA (cDNA) using the cDNA synthesis kit (Invitrogen, USA), which was provided by the Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, Thailand and preserved at -80°C before use.

Escherichia coli and vectors

E. coli DH5α strain was used as a host for cloning plasmid. *E. coli* strains, BL21(DE3) and Origami™ B(DE3) were used as the expression host. The pGEM®-T easy vector (Promega, USA) was used as a vector for cloning and the pET-32a vector (Invitrogen, USA) was used as an expression vector.

Primers design

Two primers were designed for amplification of the NGAL gene fragment (GenBank accession number: EU644752.1) by polymerase chain reaction (PCR). The forward and reverse primers were extended with tails that contain *EcoR*I and *Xho*I-cutting sites respectively for subsequent digestion and cloning into a pET32a vector. In addition, special attention to primer design parameters such as length of 18-24 bases, 40-60% GC content, melting temperature (Tm) of 50-60°C have been considered. To reduce the likelihood of primer-dimer and secondary structure formation for all the primers, the oligonucleotide calculator was used (http://biotools.nubic.northwestern.edu/OligoCalc.html), and target specificity was also evaluated using BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). This primer set was also optimized for annealing temperature.

Amplification of NGAL gene

Complementary DNA (cDNA) was used as the template and the NGAL gene was amplified with specific primers using the polymerase chain reaction (PCR) system. The PCR parameters were set using a thermal cycler as follows: pre-denaturation at 94°C for 5 min; 35 cycles of heat denaturation at 94°C for 30 secs, primer annealing at 55°C for 30 secs, DNA extension at 72°C for 30 secs; and a final extension at 72°C for 7 min. The expected PCR product size was eluted from electrophoresis gel before being ligated into pGEM®-T easy vector. The identity of NGAL was verified by sequencing.

Construction of a plasmid expressing recombinant NGAL

Initially, the NGAL gene was amplified by PCR using the primer set F-*EcoR*I: 5'-GAATTCGTGC-CACTAGGTCTCCT-3' and R-*Xho*I: 5'-CTCGAGGC-CGTCGATACAC-3'. The resulting PCR product, with the predicted size and expression vector, was digested by the same restriction enzyme *EcoR*I/ *Xho*I using the double digestion method at 37°C for 3 hours and cloned into pET32a.

Preparation of recombinant NGAL

Protein expression was carried out in *E. coli* BL21(DE3) and Origami™ B(DE3) competent cells using the heat shock method. The following day, single colonies were selected on LB agar containing ampicillin and screened by colony PCR. The bacterial colonies were transferred onto an LB broth and cultured at 37°C with shaking at 180 rpm for 18 hours. Then, the recombinant plasmid was extracted from the bacteria using the Presto™ Mini Plasmid kit according to the manufacturer's instructions.

Optimization of expression condition

The positive colonies were inoculated in LB broth containing ampicillin and incubated at 37°C with shaking at 180 rpm until the cell density reached the mid log-phase of growth. Then, protein expression was induced with isopropyl ß-D-1-thiogalactopyranoside (IPTG). The concentration of IPTG was varied in each tube with final concentrations of 0.1, 0.3, 0.5, 0.7, and 1 mM. The time of induction was examined at different time intervals of 2, 4, 6, 12 and 24 hours. Then, the cells were harvested by centrifugation at 10,000g for 5 minutes and the expressed protein was investigated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weight of expressed recombinant NGAL was analyzed by SDS-PAGE. The cell pellets were prepared by mixing with 2X solubilizing buffer (0.5 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 0.5% (w/v) bromphenol blue, and 10% ß-mercaptoethanol), and then heating in boiling water for 5 minutes. The SDS-PAGE system contained two gels, including a 13% polyacrylamide gel as a separating gel and a 4% polyacrylamide gel as a stacking gel. Twenty µg Samples were loaded into the wells and separated at 150 volts until the tracking dye reached the bottom of the gel, continued by staining with Coomassie Brilliant Blue R-250 solution for 30 min. Then the gel was destained with destaining solution until the background cleared and protein bands appeared.

Results

Specific primer design

The sequence of the NGAL gene from the NCBI database (GenBank accession number: EU644752.1) as a template for designing new primers with cloning enzyme restriction sites for pET32a vector construction is shown in Figure 1A. The cloning enzyme at the restriction sites of these primers did not affect the template primer binding.

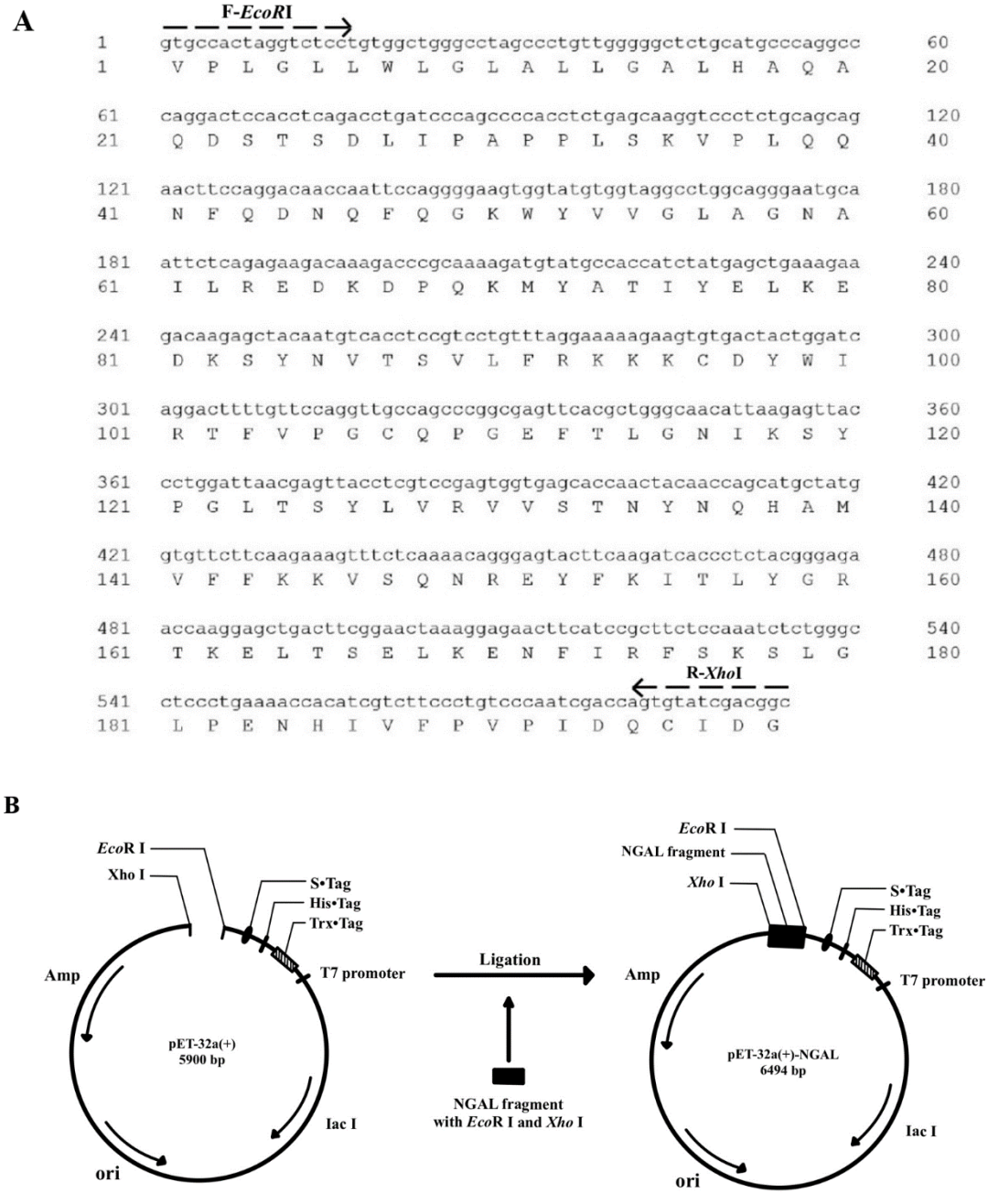


Figure 1 Characteristics of *E. coli* expression plasmid pET32a-NGAL.
(A) Sequence of the NGAL gene and the amino acids it encodes. Primer binding sites are indicated by a dotted arrow.
(B) Schematic of the recombinant plasmid construction containing the NGAL gene insert and key features required for subsequent protein expression and purification.

The optimal condition for NGAL gene amplification

The annealing temperature of the PCR reaction for primer was optimized for NGAL fragment amplification (Figure 2A). The results suggested that the optimal annealing temperature is 55°C, because the NGAL product from PCR showed an intense band at this temperature. The specific band of 594 bp appeared as expected (Figure 2B).

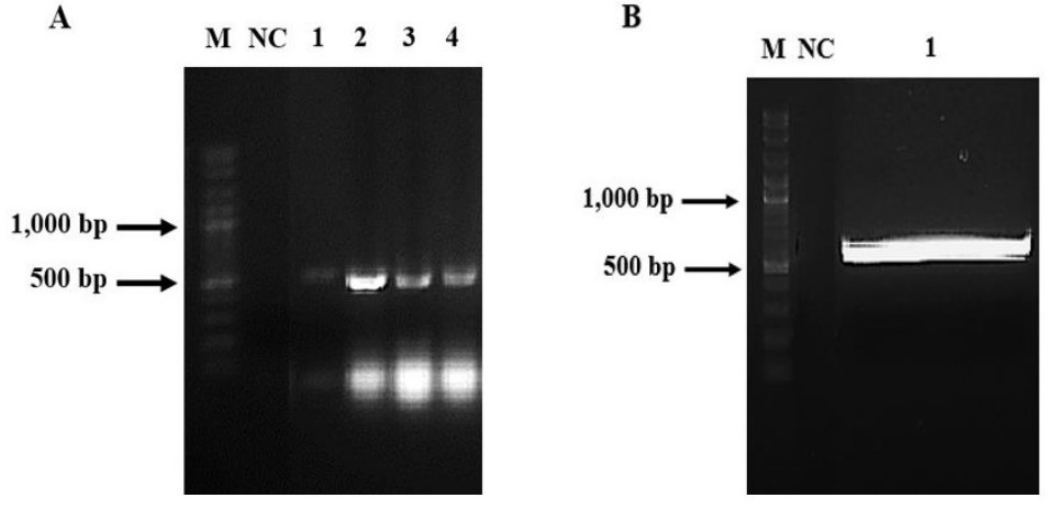


Figure 2 Agarose gel electrophoresis of NGAL product from PCR amplification
(A) Annealing temperature.
Lane 1 at 50°C.
Lane 2 at 55°C.
Lane 3 at 60°C.
Lane 4 at 65°C.
(B) Purified NGAL.
Note: Lane 1, NGAL purified from agarose gel electrophoresis amplification at 55°C; lane M, marker 100 bp DNA ladder; lane NC, negative control (PCR buffer without DNA template).

Construction of recombinant expression plasmid

The NGAL fragment, which was inserted in the pGEM®-T easy vector, was transformed into an *E. coli* DH5α competent cell. Then the pET32a vector and recombinant plasmid were prepared by digestion with a restriction enzyme. After that, the NGAL fragment and the pET32a vector were ligated between the *EcoRI*/*XhoI* cloning sites and transformed to *E. coli* BL21(DE3) and Origami™ B(DE3) competent cells (Figure 1B). The transformant colonies were selected for identification by colony PCR to ensure that the NGAL fragment carried the vector. The results showed that 10 positive clones of recombinant plasmid were transformed into *E. coli* BL21(DE3) competent cells, as shown in Figure 3A in lanes 1 to 10. This demonstrated that NGAL fragments at approximately 594 bp were obtained as expected. Ten transformant colonies were selected and screened for positive clones in *E. coli* Origami™ B(DE3) competent cells, only 7 clones contained the NGAL fragments as shown in Figure 3B (lanes 11 to 20). These results revealed that the recombinant processes were successful, because the NGAL fragments at approximately 594 bp were observed in lanes 11, 12, 14, 16, 17, 18 and 19 of agarose gel electrophoresis (Figure 3B).

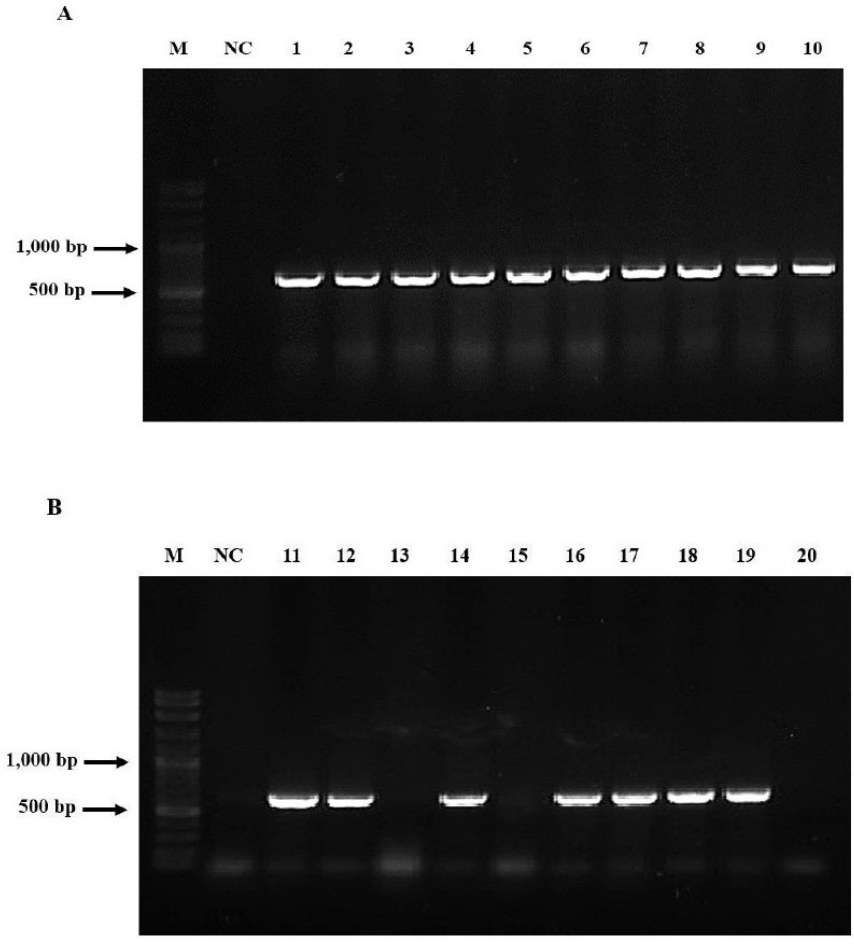


Figure 3 Agarose gel electrophoresis of positive clones for confirmation of the correct insertion of NGAL into competent cells.
(A) *E. coli* BL21(DE3) competent cell. *Note:* Lanes 1 to 10, expected clones of recombinant plasmid pET32a-NGAL.
(B) *E. coli* Origami™ B(DE3) competent cell. *Note:* Lanes 11 to 20, expected clones of recombinant plasmid pET32a-NGAL; lane M, marker 100 bp DNA ladder; lane NC, negative control (PCR buffer without DNA template).

SDS-PAGE analysis

The molecular weight of pET32a-NGAL, by SDS-PAGE analysis, was approximately 42.96 kDa, while the molecular weight of pET32a alone and NGAL were approximately 20.4 and 22.56 kDa, respectively.

To select the optimized protein expression condition, three parameters were studied for the expression of the proteins including the bacterial host, IPTG concentrations and induction times. The lowest IPTG concentration and lowest induction time were investigated. IPTG concentrations in ranges of 0.1, 0.3, 0.5, 0.7 and 1 mM, at a fixed incubation time of 24 hours, were performed. SDS-PAGE reveals that the bands of both *E. coli* BL21(DE3) and Origami™ B(DE3) competent cells (Figures 4A and 5A) were similar, with no over expression at all concentrations of IPTG. In Figures 4B and 5B, 1 mM IPTG was fixed at different induction times, ranging through 2, 4, 6, 12 and 24 hours. The results showed no over expression and no difference was observed in *E. coli* BL21(DE3) and Origami™ B(DE3) competent cells for all induction times when compared with the non-induced lane.

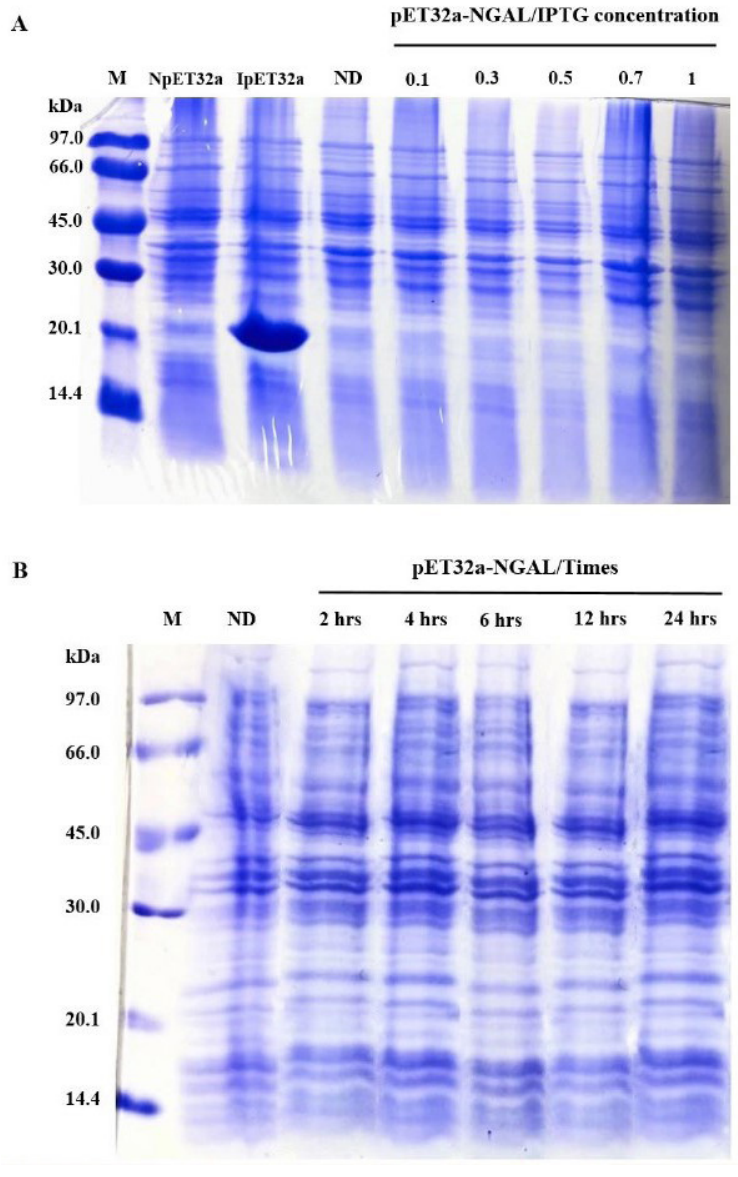


Figure 4 Optimization of IPTG concentration and induction time of recombinant NGAL in *E. coli* BL21(DE3) competent cell by SDS-PAGE.

(A) Optimization of IPTG concentration (0.1 - 1 mM), fixed incubation time of 24 hours.

(B) Optimization of induction time (2 - 24 hours), fixed IPTG 1 mM.

Note: Lane M, low MW protein marker; lane NpET32a, pET32a uninduced with IPTG; lane IpET32a, pET32a induced with 1 mM IPTG; lane ND, uninduced.

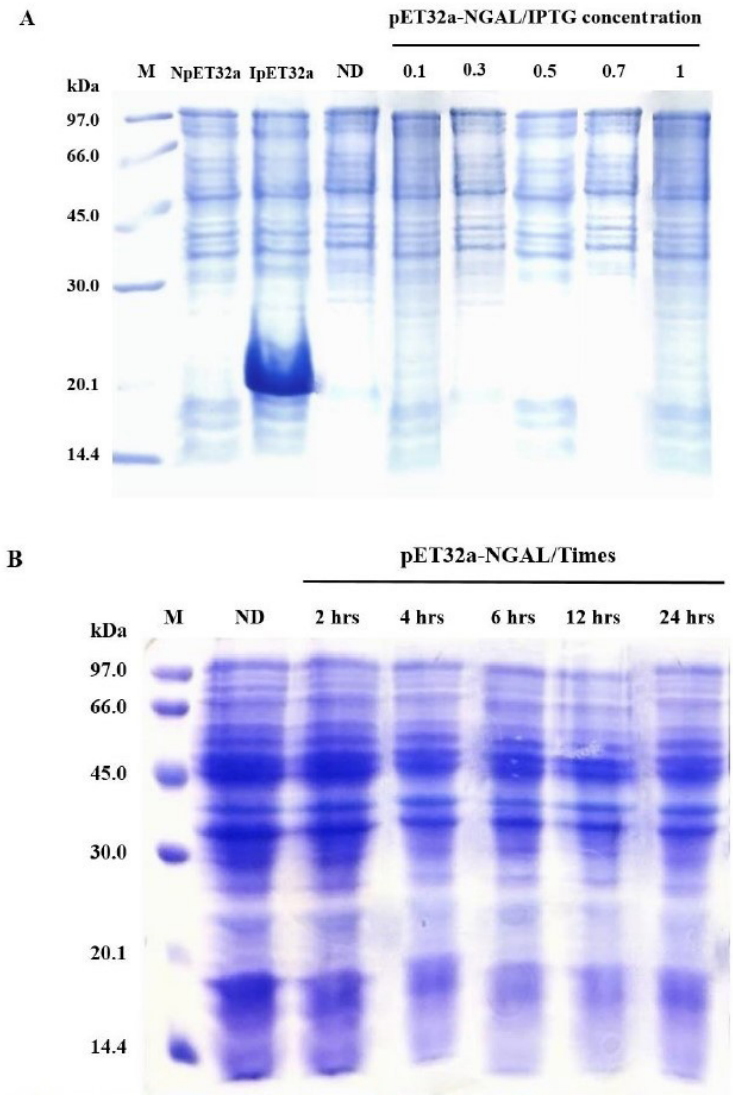


Figure 5 Optimization of IPTG concentration and induction time of recombinant NGAL in *E. coli* Origami™ B(DE3) competent cell by SDS-PAGE.
(A) Optimization of IPTG concentration (0.1 - 1 mM), fixed incubation time of 24 hours.
(B) Optimization of induction time (2 - 24 hours), fixed IPTG 1 mM.
Note: Lane M, Low MW protein marker; lane NpET32a, pET32a uninduced with IPTG; lane IpET32a, pET32a induced with 1 mM IPTG; lane ND, uninduced.

Discussion

NGAL plays an important role in several pathological conditions such as kidney injury, burn injury, bacterial infection and also in some types of human cancers such as colon cancer, rectal cancer, and pancreatic cancer[9]. It has been recommended as the first biomarker for the prediction of AKI[10]. NGAL has potential for use in clinical applications because the level of NGAL rapidly increases in the blood and urine during AKI[4]. ELISA is a standard method for assaying NGAL, but it is not convenient for clinical applications due to its high cost and time consuming. Developing a reliable and cost-effective detection for NGAL may help in the prediction of AKI.

To produce an NGAL detection kit, a large amount of NGAL is required. The expression system of NGAL is also necessary for developing commercial NGAL antibodies. Cloning is one of the suitable strategies for NGAL production. The bacterial expression system is a preferred choice for the expression of recombinant proteins due to its capacity to produce high yield protein at a low cost[8].

In the present study, three parameters were investigated, including *E. coli* host strain, IPTG concentration and induction time. The purification of NGAL carried out in the *E. coli* system was successful (Figures 3A and 3B). One of the popular prokaryotic expression systems is *E. coli* strain BL21. The NGAL was cloned to pET32a vector that contained a histidine tag (His-tag) and a thioredoxin tag (Trx-tag). Upon expression, these tags are integrated into the NGAL protein which facilitates protein purification (His-tag) and reduces protein aggregation (Trx-tag) in the bacterial system [11]. Therefore, we used pET32a as the expression vector system. In an attempt to express recombinant NGAL in *E. coli*, the recombinant plasmid was constructed. *E. coli* BL21(DE3) and Origami™ B(DE3) were cultivated and optical density (OD) at logarithmic growth phase was measured. The concentration of bacterial growth at OD_{600} was 0.6. pET32a-NGAL/ BL21(DE3) was induced at 3 hours and pET32a-NGAL/Origami™ B(DE3) was induced at 5 hours after bacterial inoculation, respectively. BL21(DE3) was considered to have higher efficiency to produce recombinant protein because the growth rate of BL21(DE3) was faster than the Origami™ B(DE3) strain. The possible explanation is that it may enhance amino acid synthesis rate because these strain recombinant protein productions can be driven by the very strong T7 promoter system[12]. Due to the mutations of trxB and gor genes in Origami™ B(DE3), it can increase the disulfide bond formation in the cytosolic fraction. However, these strains may increase protein production but sometimes decreases the solubility of protein[13]. After cultivations of both *E. coli* BL21(DE3) and Origami™ B(DE3) in the optimization of expression conditions, they were compared with negative control (pET32a without insertion) from which no over expression bands were observed (Figures 4A-B and 5A-B). In general, low temperature cultivation of *E. coli* enhances soluble protein expression[14]. Moreover, the high concentration of inducer can reduce the protein expression due to its toxic effects on cells. In an attempt to express NGAL in *E. coli*, using the specific epitopes of NGAL instead of cloning whole NGAL protein should be further investigated and codon optimization is recommended. It is important to note that all the factors including host, vector, concentration of IPTG, time and temperature should be considered and optimized in order to determine the appropriate conditions for the expression of the protein.

Conclusion

This study has successfully constructed the recombinant plasmid pET32a-NGAL. *E. coli* BL21(DE3) is considered to be more efficient to produce recombinant protein because its growth rate is faster than the Origami™ B(DE3) strain. Expression of recombinant NGAL in *E. coli* with this construct plasmid, expressing protein with optimal solubility and appropriately, should be further investigated. Other expression hosts, expression systems, and methods for the production of recombinant protein should also be studied.

Take home messages

The construction of the recombinant plasmid and the evaluation of the production of recombinant protein was compared between two expression hosts. This study demonstrates the efficiency of the expression host to produce protein for development as a commercial strip test in the future.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

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