

Construction and expression of rabbit neutrophil peptide-1 gene in *Escherichia coli*

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Abstract Rabbit neutrophil peptide-1 (NP-1) is a prototypic rabbit α -defensin with a broad antimicrobial spectrum. The coding sequence of NP-1 was amplified and cloned into pET-31b(+) to construct an expression vector, pET31-NP1, which was transformed into *E. coli* BLR(DE3) pLysS for expressing the fusion NP-1 protein (fNP-1). The fNP-1 is downstream of a ketosteroid isomerase (KSI) and upstream of a (His)₆-Tag, as KSI-NP1-His₆. The optimal condition, cultivation in enriched LB medium and induction with 0.5 mM IPTG for 6 h, was determined for fNP-1 production. The fNP-1 was purified by Ni-NTA resin and cleaved by cyanogen bromide to release matured NP-1 peptide. The matured NP-1 peptide showed significant antimicrobial activities against clinical bacteria, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Staphylococcus aureus*. The application of this expression approach represents a potential method to produce NP-1 by fusion protein expressed in *E. coli* without cytotoxicity.

Keywords Antimicrobial activity · Fusion protein · Rabbit neutrophil peptide-1

Introduction

Defensins are endogenous, cysteine-rich, 3- to 4-kDa cationic peptides that exert antimicrobial activity through membrane permeabilization (Wimley et al. 1994; White et al. 1995; Gudmundsson and Agerberth 1999). Three subfamilies of defensins, including α -, β - and θ -defensin, exist among mammals (Ganz 2003). In humans, α -defensins are found in neutrophils (Lehrer et al. 1993), T and B lymphocytes (Agerberth et al. 2000). In rabbits, but not in humans, α -defensins are also prominent in pulmonary alveolar macrophages (Ganz et al. 1985). Rabbit neutrophil peptide-1 (NP-1), an Arginine-rich and cationic peptide with 33 amino acids (Fuse et al. 1993), is a prototypic rabbit α -defensin that is abundant in rabbit granulocytes and rabbit alveolar macrophages (Patterson-Delafield et al. 1981; Selsted et al. 1983), and it was first called MCP-1 (macrophage cationic peptide-1). Later sequencing proved that NP-1 from rabbit polymorphonuclear leukocytes (Selsted et al. 1985) and MCP-1 were identical. Rabbit NP-1 exhibits growth inhibition activity *in vitro* against a broad spectrum of pathogenic microorganisms including Gram-positive and Gram-negative organisms, as well as some virus pathogens (Sinha et al. 2003).

In order to investigate the possibility of pharmaceutical application of rabbit NP-1, a relatively large quantity of NP-1 needs to be produced economically. Recombinant technique has emerged as attractive cost-effective method and its application is expected to be widespread. In this study, we have been able to overexpress NP-1 fused to ketosteroid isomerase (KSI) by the plasmid pET-31b(+) in *E. coli* BLR(DE3)pLysS. The fusion strategy was employed in expression vector construction to avoid the toxic effect on host cell and produce the activated NP-1 after removing the fused proteins.

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Materials and methods

Strains, vectors and reagents

Escherichia coli strain JM109 was used as the host for cloning and the propagation of plasmids. *E. coli* BLR(DE3) pLysS (Novagen, Madison, WI, USA) was used as the expression host. Plasmid pCR2.1-TOPO (Invitrogen, Carlsbad, CA, USA) was used for subcloning of the PCR product of NP-1 gene, and pET-31b(+) (Novagen) was chosen for fusion protein expression. All the restriction enzymes and T4 DNA ligase (Promega, Madison, WI, USA) were used for the construction of recombinant vector. All chemical reagents were purchased from Sigma (St. Louis, MO, USA) or Merck (Darmstadt, Germany). The recombinant DNA techniques were exploited as described by Sambrook et al. (1989).

Synthesis and construction of a DNA fragment coding for NP-1

For the construction of a vector comprising a NP-1 gene encoding mature NP-1 peptides, the cDNA was prepared from white blood cells of rabbit as template DNA. A primer pair (NP1-F and NP1-R) were synthesized according to the sequence registered in GenBank (Accession number: M28883) and used to amplify the NP-1 gene fragment carrying restriction enzyme *Alw*NI recognized sites at its 5' and 3' termini. The amplified PCR DNA fragment was cloned to pCR2.1-TOPO, resulting in the vector, named pNP1-TOPO.

- (1) NP1-F: 5'-CAG ATG CTG GTG GTC TGT GCG TGC-3' (underlined sequences indicate the *Alw*NI recognized site)
- (2) NP1-R: 5'-CAG CAT CTG GCG GCG GCA GCA CA-3' (underlined sequences indicate the *Alw*NI recognized site)

Construction of NP-1 expression vector

The amplified NP-1 gene fragment was recovered from the pNP1-TOPO digested with *Alw*NI. Vector DNA, pET-31b(+), was also cleaved by the same restriction enzyme and the larger fragment was recovered. The recovered DNA fragments were ligated to construct the expression vector, pET31b-NP1. The complete sequence of the inserted NP-1 gene was confirmed by direct sequencing of the vector DNA.

Expression of the fusion protein

For expressing the fusion NP-1 protein (fNP-1), the expression vector pET31b-NP1 carrying the desired mature

NP-1 gene were purified and transformed into *E. coli* BLR (DE3)pLysS. A fresh bacterial clone of *E. coli* BLR(DE3) pLysS harboring pET31b-NP1, named BLR(DE3)/pET31b-NP1, was grown in enriched Luria-Bertani (LB) medium (1% Bacto-tryptone, 2% yeast extract and 0.5% NaCl, pH 7.2) supplemented with 100 $\mu\text{g ml}^{-1}$ ampicillin, 34 $\mu\text{g ml}^{-1}$ chloramphenicol and 0.2% (w/v) glucose at 37°C. When the cells were cultured to OD₆₀₀ at about 0.5, they were inoculated into 600 ml fresh enriched LB medium with the volume ratio of 5% and the fusion protein expression was induced by the addition of isopropyl- β -D-l-thiogalactopyranoside (IPTG).

Purification and cleavage of the fusion protein

After additional 6 h culture by the induction of IPTG, the cultured cells of BLR(DE3)/pET31b-NP1 were harvested by centrifugation at 8,000g for 20 min, then resuspended in 20 ml cooled of binding buffer (20 mM Tris-HCl, 0.5 M NaCl and 5 mM imidazole, pH 7.9) and disrupted by sonication. After centrifugation at 12,000g for 20 min, the insoluble pellet (inclusion body) was dissolved in 10 ml of binding buffer containing 6 M guanidine-HCl. Following the centrifugation to remove residual matter, the fusion protein containing (His)₆-Tag was purified using the pre-charged nickel-nitrilotriacetic acid (Ni-NTA) agarose affinity chromatography (Invitrogen). The fusion protein bound to the resin was washed by washing buffer (20 mM Tris-HCl, 6 M guanidine-HCl, 0.5 M NaCl and 40 mM imidazole, pH 7.9), and then eluted with elution buffer (20 mM Tris-HCl, 6 M guanidine-HCl, 0.5 M NaCl and 1 M imidazole, pH 7.9). The elution of fusion protein was collected and dialyzed at 4°C against deionized water for 24 h. The purified fusion protein was analyzed by 15% SDS-polyacrylamide gel electrophoresis and western blotting. The concentration of protein was analyzed by BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

The purified fusion protein was lyophilized and treated with 1 mg/ml CNBr (Sigma) fusion protein (in 70% formic acid) for 24 h at 25°C to release mature NP-1 peptide. The cleaved fusion protein was dissolved in 30 ml deionized water and rotated by rotary evaporator to remove the remaining CNBr. The released mature NP-1 peptide was analyzed by 4–12% NuPAGE gel (Invitrogen) and quantitated.

Western blotting analysis

Protein sample was resolved by electrophoresis and electro-transferred onto PVDF membrane (Amersham Biosciences, Piscataway, NJ, USA). The membrane was blocked with 5% (w/v) non-fat milk in PBS and incubated with rat anti-NP1 antisera in 1:1,000 dilution for 2 h. Following washing

with PBS containing 0.1% Tween 20, the blot was reacted with alkaline phosphate-conjugated anti-rat IgG (diluted 1:2,500). The protein bound enzymes on the membrane were visualized with BCIP/NBT Substrate Solution (PerkinElmer, Boston, MA, USA).

Assay of antimicrobial activity

Antimicrobial activity of the NP-1 was analyzed using radial diffusion assay as described previously with slight modifications (Lehrer et al. 1991). Clinical isolates, *E. coli* (ATCC 8739), *P. aeruginosa* (ATCC 10145), *B. subtilis* (ATCC 6633) and *S. aureus* (ATCC 6538P), were obtained from the American Type Culture Collection (Manassa, VA, USA) and used in this study. All bacteria were incubated overnight and diluted in 3% trypticase soy broth (TSB), and grown for 2.5–3.5 h (mid-exponential growth phase; 0.2–0.25 bacterial OD₆₂₀, around 4–8 × 10⁷ CFU ml⁻¹) at 37°C with shaking.

Following centrifugation (6,000 g, for 10 min), the bacterial pellets were washed with 10 mM PBS (pH 7.4) and resuspended in 10 ml of the same buffer. A volume containing approximate 1 × 10⁶ bacterial CFU was added to 10 ml PBS that contained 3 mg of powdered TSB, 1% w/v of agar, and 0.02% Tween 20. Bacteria-containing medium was poured into a 100-mm Petri dish to form a uniform layer 1 mm deep. A gel punch (3 mm inside diameter) was used to make evenly spaced wells. Samples of 5 µl of the CNBr-treated NP-1 or the control solvent was added to the wells. The plates were incubated for 3 h at 37°C and then overlaid with 10 ml of sterile agar consisting of 6% w/v solution of TSB and 1% agar. After incubation at 37°C for 10–16 h, the diameters of the clearance zones were measured.

Minimum inhibitory dose (MID), i.e., the minimum dose of NP-1 forming a detectable clear zone larger than the size of the central well (3 mm) under our experimental conditions, was determined from serial dilutions of the CNBr-treated NP-1 by plotting the known doses of a peptide that was added to the well versus the respective clearance zones. The regression equation was then used to determine the MID for each tested microorganism.

Results and discussion

Construction of expression vector

The NP-1 gene suitable for *E. coli* expression was cloned into pET-31b(+) successfully to construct the expression vector, pET31b-NP1. The construction strategy of pET31b-NP1 was shown in Fig. 1a and the inserted NP-1 DNA fragment within plasmid pET-31b(+) was verified (Fig. 1b). The constructed NP-1 gene was under the control of T7

promoter and fused with a downstream of ketosteroid isomerase (KSI) and an upstream of (His)₆-Tag to generate the fusion NP-1 protein as KSI-NP1-His₆ (Fig. 2) and the expressed fNP-1 is 18.8 kDa. There is (His)₆-Tag at the C-terminum of fNP-1, which serves as the later purification of fusion protein. There are also *Alw*NI recognized sites at the 5' and 3' termini of NP-1 gene. By the *Alw*NI, methionine residue could be translated and provided cyanogen bromide (CNBr) cleavage sites for releasing mature NP-1 peptide from the fusion protein.

Expression of fusion NP-1 protein

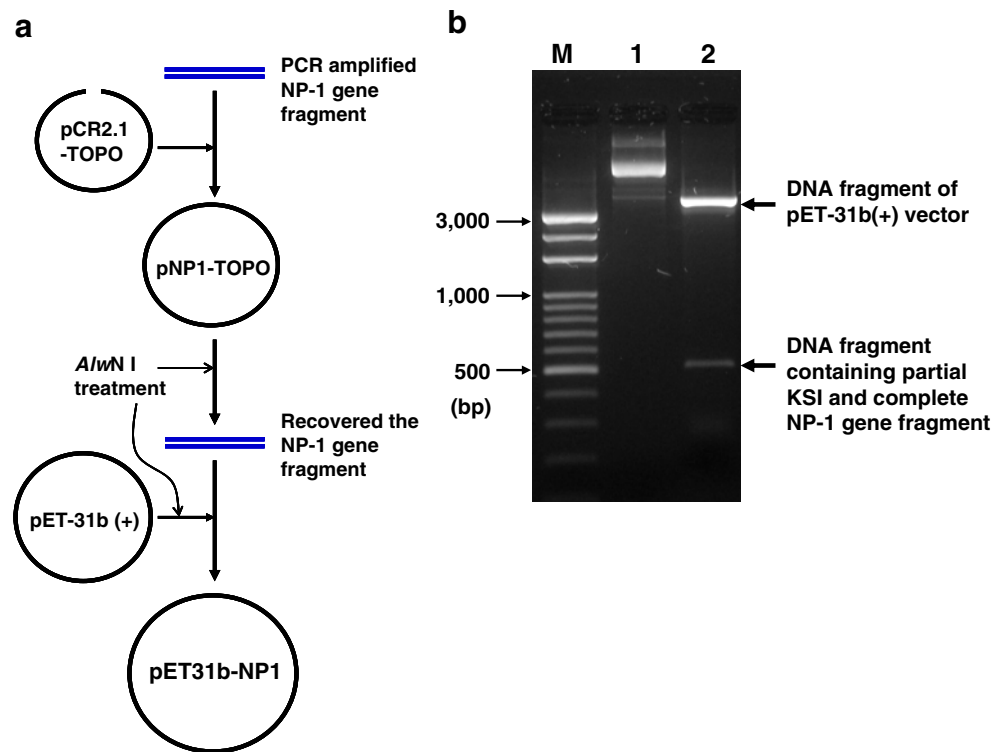
The constructed expression vector, pET31b-NP1, was transformed into *E. coli* strain, BLR(DE3)pLysS, to obtain a cloned *E. coli* strain, named BLR(DE3)pET31b-NP1, to produce the fusion protein, fNP-1. The *E. coli* BLR(DE3)/pET31b-NP1 was seeded and cultured in enriched LB medium. After 1 mM IPTG induction and SDS-PAGE analyses, there was an obvious foreign protein band with a molecular weight of 18.8 kDa, which is consistent with the predicted fusion protein, fNP-1 (KSI-NP1-His₆) (Fig. 3a). The fusion protein could be purified by Ni-NTA agarose resin and further confirmed by reacting with rat anti-NP1 antisera (Fig. 3b). Introduction of artificial fusion protein, such as KSI fusion partner (enhancing the formation of inclusion bodies), at the N- or C-terminus of fusion protein, is a popular way to facilitate purification of recombinantly produced proteins (Cipáková et al. 2006).

Induction effect of IPTG

For the expression vector, pET31b-NP1, the foreign protein expression was triggered by adding IPTG into the culture medium. Final IPTG concentration and induction time should be optimized because of its great contribution to fusion protein expression (Xu et al. 2006). In this work, IPTG concentration was examined from 0.3 to 1 mM and the expression of fNP-1 was analyzed in Fig. 4a. At 0.5 mM, the fNP-1 expression was the highest; therefore, further studies were all carried out under this condition.

In the process of fusion protein production in *E. coli* BLR(DE3)/pET31b-NP1, IPTG induction is the turning point between cell growth and fusion protein synthesis. Therefore, the effect of induction timing was evaluated by adding IPTG at different stages of growth phase. The production of fNP-1 was compared as indicated in Fig. 4b. With different induction timing, the concentration of protein varied in a wide range from 0.14 to 1.31 g l⁻¹, with the highest value observed when induced at 6 h, i.e., at the middle stage in exponential growth of cloned *E. coli*. Hence, induction at the middle stage of exponential growth was adopted.

Fig. 1 Construction of the fused rabbit NP-1 (fNP-1) expression vector. **a** Constructing flowchart of a pET-31b(+) vector with NP-1 gene fragment was present. **b** The RT-PCR amplified NP-1 DNA fragment cloned into pET-31b(+) vector was identified by agarose electrophoresis; lane M shows DNA marker with the molecular weight indicated, lanes 1 and 2 show the DNA of constructed vector without and with restriction enzymes, *NdeI* and *XhoI*, treatment, respectively



Antimicrobial activity of the fusion protein

The *E. coli* BLR(DE3)/pET31b-NP1 was cultured and induced by 0.5 mM IPTG for 6 h. The fNP-1 accounted for around 35% of total protein. Using KSI as a fusion partner, the fusion protein was expressed as insoluble protein and accumulated in inclusion bodies in *E. coli* (Park et al. 2009). Therefore, the inclusion bodies of *E. coli* cells were harvested for the purification of fNP-1. The fNP-1 was purified with Ni-NTA Resin. The purities of eluted fNP-1 were about 80%. Typically, the yield of fusion protein was 10–20 mg from 10 g of wet pelleted cells. After dialysis against deionized H₂O, the

purified KSI-NP1-His₆ fusion protein was cleaved with CNBr and the mature NP-1 was released.

To show that the expression approach used retaining the antimicrobial activity of the prepared NP-1, a radial diffusion assay was performed. The elution samples were administered to a panel of bacterial organisms. As shown in Table 1, the purified NP-1 provided high, dose-dependent antimicrobial effect against Gram-negative bacteria (*E. coli* and *P. aeruginosa*) and Gram-positive bacteria (*B. subtilis* and *S. aureus*). An antimicrobial peptide, rabbit NP-1, was successfully expressed in bacteria *E. coli* as a fusion protein in the present study. However, further work should be done

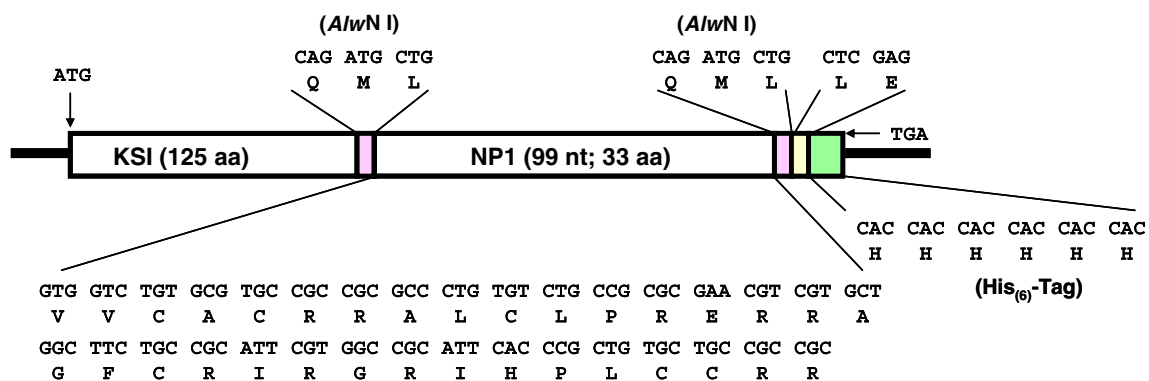


Fig. 2 Map and sequences of the fusion rabbit NP-1 (fNP-1) within pET31b-NP1 vector. In the expression vector of pET31b-NP1, NP-1 coding sequence was placed downstream of ketosteroid isomerase (KSI) gene and upstream of (His)₆-Tag sequence, KSI-NP1-His₆. Use of the *AlwNI* sites enabled unidirectional insertion of coding sequence

adjacent to a methionine residue and provided the cyanogen bromide (CNBr) cleavage site for releasing mature NP-1 peptide from the fusion protein. The fNP-1 gene was under the expressing regulation of T7 promoter. *aa* Amino acid residues, *nt* nucleotides

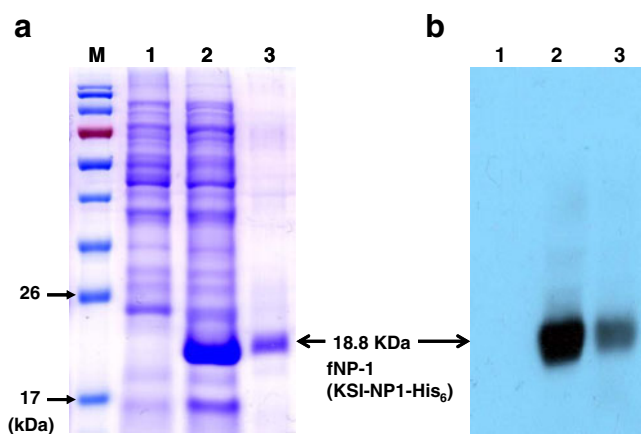


Fig. 3 Expression and verification of the expressed fNP-1. **a** SDS-PAGE analysis of the expressed fNP-1 was shown; lane M shows protein marker with the molecular weight indicated, lanes 1 and 2 show the total protein profile from the *E. coli* BLR(DE3)/pET3b-NP1, which were cultured in the enriched LB medium without and with IPTG induction, respectively. Lane 3 shows the purified fNP-1 by Ni-NTA resin. **b** Western blotting analysis of the expressed fNP-1. The samples were resolved on SDS-PAGE, transferred to PVDF membrane, incubated with rat anti-NP1 antisera, probed with alkaline phosphate-conjugated anti-rat IgG, and then visualized with BCIP/NBT treatment. The recognized fNP-1 is indicated by the arrow

to elevate the expression level of the NP-1 and to establish an effective purification process. Moreover, it is also worth testing the capacity of produced antimicrobial peptides against to drug-resistant strains (Cipáková et al. 2006).

Conclusion

In summary, this work presents the expression method that can be applied for the production of rabbit NP-1 in bacteria.

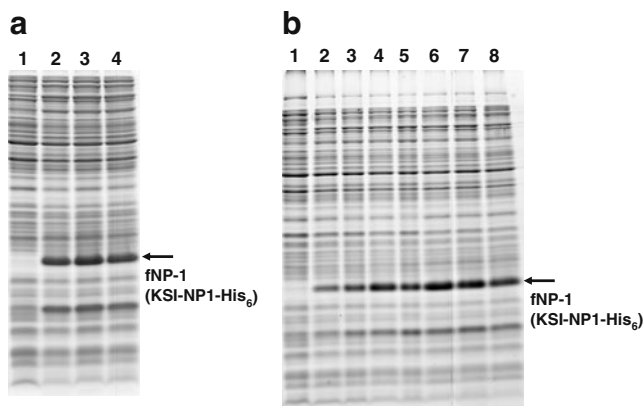


Fig. 4 Effect of IPTG concentration and IPTG inducing time on the fNP-1 expression level of cloned BLR(DE3)/pET31b-NP1. **a** Lanes 1–4 show the total protein profiles of the cultures treated with 0, 0.3, 0.5, and 1.0 mM of IPTG for 12 h. **b** Lanes 1–8 show total protein profiles of the cultures at 0, 1, 2, 3, 4, 5, 6, and 12 h after 0.5 mM IPTG induction. The expressed fNP-1 is indicated by the arrows

Table 1 Antibacterial activity of NP-1

Bacteria	Minimum inhibitory dose (MID) of NP-1 (ng) ^a
Gram-negative	
<i>E. coli</i> (ATCC 8739)	66±10
<i>P. aeruginosa</i> (ATCC 10145)	79±14
Gram-positive	
<i>B. subtilis</i> (ATCC 6633)	116±25
<i>S. aureus</i> (ATCC 6538P)	145±23

^a Experiments were repeated five times and the values presented are means ± SEM

The above results showed that KSI-NP1-His₆ fusion protein expression system was able to circumvent the toxicity of NP-1 on the host bacterial cells. Since produced mature NP-1 exhibited strong antimicrobial activity against tested microorganisms, we could conclude that this expression approach of producing fusion NP-1 provides a powerful tool for production of sufficient quantities of biologically active NP-1 for its further biological study.

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