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ORIGINAL ARTICLE

Expression of Recombinant pET22b-LysK-Cysteine/Histidine-Dependent Amidohydrolase/Peptidase Bacteriophage Therapeutic Protein in *Escherichia coli BL21 (DE3)*

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Abstract
Objectives: Bacteriophage-encoded endolysins are a group of enzymes that act by digesting the peptidoglycan of bacterial cell walls. LysK has been reported to lyse live staphylococcal cultures. LysK proteins containing only the cysteine/histidine-dependent amidohydrolase/peptidase (CHAP) domain has the capability to show lytic activity against live clinical staphylococcal isolates, including methicillin-resistant *Staphylococcus aureus* (MRSA). The aim of this study was to clone and express LysK-CHAP domain in *Escherichia coli BL21 (DE3)* using pET22b as a secretion vector. The pET22b plasmid was used, which encoded a pelB secretion signal under the control of the strong bacteriophage T7 promoter.
Methods: The *E. coli* cloning strains DH5α and BL21 (DE3) were grown at 37°C with aeration in the Luria-Bertani medium. A plasmid encoding LysK-CHAP in a pET22b backbone was constructed. The pET22b vector containing LysK-CHAP sequences were digested with NcoI and HindIII restriction enzymes. Cloning accuracy was confirmed by electrophoresis. The pET22b-LysK plasmid was used to transform the *E. coli* strain BL21. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1mM to induce T7 RNA polymerase-based expression. Finally, western blot confirmed the expression of target protein.
Results: In this study, after double digestion of pEX and pET22b vectors with HindIII and NcoI, LysK gene was cloned into two HindIII and NcoI sites in pET22b vector, and then transformed to *E. coli DH5*α. Cloning was confirmed with double digestion and analyzed with agarose gel. The recombinant pET22b-LysK plasmid was transformed to *E. coli BL21* and the expression was induced by IPTG. The expression was confirmed by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting method. Observation of a 28.5 kDa band confirmed LysK protein expression.
Conclusion: In the present study, LysK-CHAP domain was successfully cloned and expressed at the pET22b vector and *E. coli BL21 (DE3)*.

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1. Introduction

Lysins are phage-encoded peptidoglycan hydrolases, which, when applied exogenously as purified recombinant proteins to Gram-positive bacteria, bring about rapid lysis and death of the bacterial cell [1,2]. Unlike antibiotics, phage lysins can be used to selectively target specific pathogenic bacteria without affecting the surrounding commensal microflora. They are reported to have a narrow host range similar to that of their phage producers, rendering them generally either species [3,4] or genus specific [5,6]. The potential of these enzymes as novel therapeutics against a number of Gram-positive pathogens on mucosal surfaces and in systemic infections has previously been demonstrated [7−16]. In relation to bacterial resistance, no strains have yet been identified with resistance to phage lysins [1,3,14]. It has been suggested that these enzymes have evolved to target specific molecules in the host peptidoglycan that are essential for cell viability, making resistance an unlikely event [17,18]. In general, *Staphylococcus aureus* phage lysins display a multidomain modular structure comprising a C-terminal cell wall binding domain and two N-terminal catalytic domains. Examples include LysK, MV-L, phi11, and LysH5 [15,19,20]. Native lysins of *S. aureus*, with the exception of MV-L, have typically shown poor expression, insolubility, and low activity when generated as recombinant proteins in a heterologous host [21−23].

However, a number of lysins have been reported, which consists of two catalytic domains including those of *S. aureus* (LysK, Phi11, and MV-L), where endopeptidase activity is a common feature [5,15,24]. Of all lysins reported to date, the streptococcal phage lysin PlyC is particularly unique, as it displays a multimeric modular structure consisting of two distinct gene products designated PlyCA (50 kDa heavy chain) and PlyCB (8 kDa light chain) [25]. The C-terminal binding domain of the majority of lysins is responsible for attaching the enzyme to its specific substrate in the bacterial cell wall via noncovalent binding of carbohydrate ligands [26]. A recent study on the crystal structure of the pneumococcal phage lysin Cpl-1 in free and choline-bound states suggested that the choline-binding domain assists in the correct positioning of the N-terminal catalytic domain [27]. Although it appears that the C-terminal domain is necessary for lytic activity of some endolysins [14,23,26], this is not always the case. A number of enzymes have shown an increased lytic activity upon removal of the binding domain [28−30]. For example, when LysK was truncated to its N-terminal endopeptidase domain, cysteine/histidine-dependent amidohydrolase/peptidase (CHAP), it had a two-fold higher lytic activity than the native enzyme [30]. It is possible that the C-terminal binding domain in the native enzyme may be limiting the potential activity of the N-terminal lytic domain by only allowing it to configure and function when bound to its target in the cell wall [30,31]. In contrast to lysins against Gram-positive pathogens, the enzymes associated with Gram-negative phages are often globular single-module enzymes as in the T7 lysin (lysozyme) [32,33]. LysK has been reported to lyse live staphylococcal cultures. LysK proteins containing only the CHAP domain has the capability to show lytic activity against live clinical staphylococcal isolates, including methicillin-resistant *S. aureus* (MRSA). The aim of this study is to clone and express LysK-CHAP domain in *Escherichia coli BL21 (DE3)* using pET22b. In the current research, we used pET22b because particular interest for the expression of disulfide bonded proteins is a family of pET vectors containing the N-terminal pelB secretion signal, which directs synthesized polypeptides to the *E. coli* periplasm [34]. Disulfide oxidoreductases and isomerases located in the *E. coli* periplasm catalyze the formation of disulfide bonds, enabling the accumulation of properly folded soluble protein and making the periplasm an ideal compartment for expression of certain therapeutic proteins. [35]. Construction of a single-domain protein for therapeutic purposes is desirable as well as facilitating protein production; this may decrease the probability of a significant immunogenic response. In addition to the main study, we would like to assess extracellular secretion property of pET22b in the Luria-Bertani (LB) medium.

2. Materials and methods

The *E. coli* cloning strains DH5α and BL21 (DE3) were grown at 37°C with aeration in the LB medium. When appropriate, the LB medium was supplemented with ampicillin (100 μg/mL) for plasmid selection. The growth media for these bacteria were purchased from Himedia Company, Mumbai - India. A plasmid encoding the C-terminally 6xHis-tagged recombinant protein LysK-CHAP in a pEX backbone (MWG-Biotech, Ebersberg, Germany) was constructed. Overexpression of proteins was performed in *E. coli* BL21 (DE3) (Invitrogen, Carlsbad, CA, USA) cultured at 37°C in modified LB medium (15 g/L tryptone, 8 g/L yeast extract, and 5 g/L NaCl) [36] supplemented with 100 μg/mL ampicillin for plasmid selection and maintenance. Vectors, pEX and pET22b, containing LysK-CHAP sequences were digested with NcoI and HindIII restriction enzymes, and then the sticky vectors were ligated by T_4 ligase enzyme. Cloning was confirmed by double digestion. Cloning accuracy was confirmed by electrophoresis.

2.1. Protein expression

The pET22b-LysK-CHAP plasmid was used to transform the *E. coli* strain BL21, which allows IPTG-regulated expression of T7 RNA polymerase. For

confirming the expression, 5 mL cultures were grown in an air shaker (250 rpm) at 37°C in the LB medium containing 100 mg/mL of ampicillin. At OD_{600} = 0.5, IPTG was added to a final concentration of 1mM to induce the T7 RNA polymerase-based expression. IPTG induction was done at a lower temperature (30°C), which has been shown to enhance LysK protein expression. Finally, western blot confirmed the expression of target protein.

3. Results

In the current study, cloning was confirmed with double digestion and analyzed with agarose gel; the presence of an 809-bp band confirmed cloning of the LysK gene (Figure 1).

Observation of a 28.5 kDa protein band confirmed LysK-CHAP protein expression (Figure 2).

Although protein expression was confirmed in this study, we could not detect any protein in the LB medium. This means that most of the protein has been secreted in the preplasmic area or inside of the bacterial cell.

4. Discussion

In the present study, LysK-CHAP domain was successfully cloned and expressed at the pET22b vector and *E. coli BL21 (DE3)*. This kind of lysin was previously studied by several scientists [15,19,20], but in our study for assessment of extracellular secretion of protein of LysK-CHAP domain, the domain was cloned in the pET22b vector and transformed into *E. coli BL21 (DE3)* for expression of the desired protein. According to our mentioned data, protein was expressed by the vector but should be scaled up for optimum production. In this study no sign of protein was detected in the LB medium but Cytoplasmic protein expression was confirmed by SDS-PAGE and western blotting.

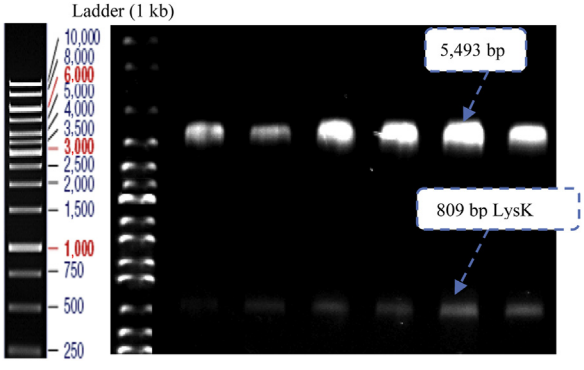

Figure 1. Cloning confirmation of LysK in pET22-vector.

To our knowledge, only a few phage endolysins, such as LysK, phi11, MV-L, and LysH5, have been reported to lyse live staphylococcal cultures [15,19,20]. LysK has a modular structure similar to the structure of these endolysins, with two catalytic domains, a CHAP domain and a central amidase-2 domain (*N*-acetylmuramoyl-L-alanine amidase), as well as a C-terminal SH3b cell-binding domain. In the current study, we also synthesized the CHAP domain containing 165 amino acids. According to another study [37], this sequence has a strong antibacterial potential. The potential of these enzymes as novel therapeutics against a number of Gram-positive pathogens on mucosal surfaces and in systemic infections has previously been demonstrated [7–16]. In relation to bacterial resistance, no strains with resistance to phage lysins have yet been identified [1,3]. It has been suggested that these enzymes have evolved to target specific molecules in the host peptidoglycan that are essential for cell viability, making resistance an unlikely event [17,18]. In general, *S. aureus* phage lysins display a multidomain modular structure comprising a C-terminal cell wall binding domain and two N-terminal catalytic domains. Examples include LysK, MV-L, phi11, and LysH5 [5,15]. Native lysins of *S. aureus*, with the exception of MV-L, have typically shown poor expression, insolubility, and low activity when generated as recombinant proteins in a heterologous host [5]. However, their modular structure has enabled the construction of truncated (CHAP) and chimeric versions of lysins (ClyS and P16-17) [16,22] to help circumvent these problems. Scientists previously showed that the activity of CHAPK against live *S. aureus* including MRSA was twofold higher than that of the native enzyme LysK [30]. To date, the multidomain MV-L lysin from phage MR11 and the chimeric two-domain lysin ClyS are the only anti-staphylococcal lysins that have been evaluated *in vivo* [15,16]. In this study, LysK proteins containing only the CHAP domain was expressed. Construction of a single-domain protein for therapeutic purposes is desirable, as well as facilitating protein production; this may decrease the possibility of a significant immunogenic response. Unlike antibiotics, intact endolysins are large proteins that are capable of stimulating a humoral immune response, especially when they are used intravenously. Most endolysins have a modular organization with a conserved N-terminal catalytic domain and a more diverse C-terminal cell wall binding domain [37,38]. However, several *S. aureus* phages produce endolysins with two catalytic domains at their N terminus, such as those from phages K, f11, and fMR11. These lytic enzymes present a CHAP domain followed by an amidase-2 domain (*N*-acetylmuramoyl-L-alanine amidase), where the CHAP domain seems to be most effective in inducing lysis [5,15,24]. The LysK-CHAP domain provides a valuable functional

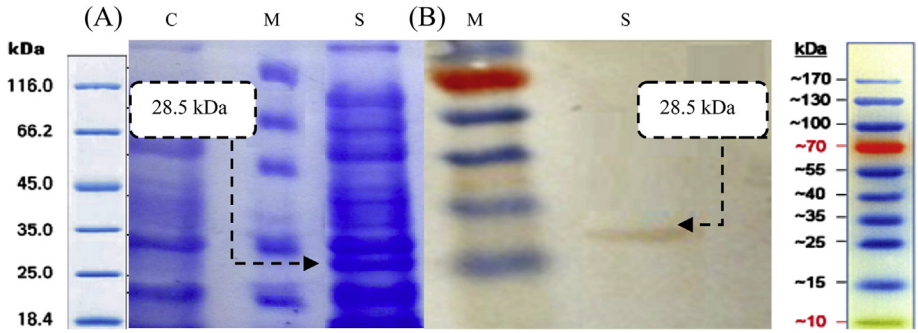

Figure 2. Analysis of LysK-CHAP protein expression with two methods. (A) LysK-CHAP protein expression was analyzed with SDS-PAGE. (B) LysK-CHAP protein expression was analyzed with Western blot. C = control; CHAP = cysteine/histidine-dependent amidohydrolase/peptidase; SDS-PAGE = sodium dodecyl sulphate-polyacrylamide gel electrophoresis; M = protein Marker; S = protein sample.

unit for domain-swapping studies. It would be interesting to investigate if a chimeric protein with the LysK-CHAP domain and a different substrate-binding domain would have an altered spectrum of inhibition, since it has been demonstrated that the CHAP domain alone has the same spectrum of inhibition in all the strains tested. Environments such as hospitals and nursing homes with a high number of MRSA infections can benefit considerably from exploitation of the CHAP domain of LysK.

In the current study, we synthesized LysK-CHAP domain and cloned it to the pET22b as a preplasmic secretion vector that enabling to express a lytic protein. Expression of a lytic protein in the preplasmic area of pET22b promises to be a suitable method for the production of lytic proteins for further applications.

Conflicts of interest

All authors declare no conflicts of interest.

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