

Original Article

Recombinant Production and Affinity Purification of the Mature Form of Staphylolysin (LasA) Protein in *E.coli*Behnaz Rahmani^{1,2}M.Sc., Hossein Zarei Jaliani^{2*}Ph.D., Akram Astani¹Ph.D.,
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A B S T R A C T

Article history

Received 20 Jul 2017

Accepted 3 Dec 2017

Available online 31 Dec 2017

Key words

LasA

Methicillin-resistant

Staphylococcus aureus

Staphylolysin

Background and Aims: Enzybotics are probably the future line of weapons against drug resistant bacteria. They lyse bacteria with the new mechanisms with few likelihood of generating resistance. LasA, which is secreted from *Pseudomonas* strains degrades *Staphylococcus aureus* (*S. aureus*) cell wall and has the potential to use against drug resistant *S. aureus* infections.**Materials and Methods:** Codon-optimized gene of the mature form of the LasA protein was ordered. The gene was double digested with NcoI and XhoI restriction enzymes and sub-cloned into pET28a(+) digested with the same enzymes. Recombinant construct was introduced into BL21 DE3 cell. Expression of the gene was induced by 0.2 mM isopropyl β -D-1-thiogalactopyranoside and recombinant protein was affinity purified by Ni-NTA mini-column. The staphylolytic activity of the recombinant LasA protein was evaluated on Methicillin-resistant *S. aureus* (MRSA) by disk diffusion.**Results:** Fragment of the LasA gene encoding mature form of the LasA protein was introduced into pET28a(+) expression vector. C-terminal his-tagged recombinant LasA protein was produced in BL21 DE3 *E. coli* cells. Over 50% purity has been achieved by affinity purification of the LasA protein from the total cell lysate. The yield of purified protein was 5.4 mg.l⁻¹. Growth of MRSA was completely inhibited by dilutions of recombinant his-tagged LasA.**Conclusions:** The present study shows that the mature form of the LasA can be expressed in *E. coli* BL21 DE3 cells. C-terminal his-tagged form of the mature LasA protein has staphylolytic activity against MRSA and so it can be a promising therapeutic agent.

Introduction

One of the major human pathogens associated with numerous clinical infections is *Staphylococcus aureus* (*S. aureus*) [1]. With the aid of the several virulence factors, this opportunistic pathogen causes severe diseases in human, including skin and respiratory infections [2]. Growing nosocomial and non-nosocomial health care problems of *S. aureus* infections is alarming all around the world. The prevalence of methicillin resistant *S. aureus* (MRSA) strains has also been increased during the last decades [3]. Antibiotic abuse is probably the main cause of the increase in drug resistance of bacteria. Enzybiotics, which have been introduced for decades, but ignored for the use in therapeutics until lastly, are enzymes with the ability to lyse bacteria. They are generally endolysins encoded by phages and kill bacteria when added exogenously [4, 5]. LasA, also called staphylolysin, is produced from *Pseudomonas aeruginosa*. Its gene structure [6, 7] and nucleotide sequence [8, 9] has been discovered from *Pseudomonas aeruginosa* PAO1 for 3 decades. LasA is produced as pre-proprotein and secreted as a 21 kDa mature protein from *Pseudomonas* strains after processing [10]. It has been demonstrated that Gly-Gly-Ala peptide bonds in elastin and bacterial cell wall can be a substrate for LasA. This protease belongs to the zinc metalloendopeptidases and degrades specifically *S. aureus* peptidoglycan [11]. LasA is implicated in several processes related to *Pseudomonas* infections, including shedding of the cell surface syndecan-1 [12, 13], elastin

degradation in connective tissue by elastolytic activity [14, 15], etc. In the present study, the mature form of the LasA protein was produced as recombinant C-terminal his-tagged form in *E. coli* and staphylolytic activity of this mature form of the protein was evaluated on MRSA by disc diffusion.

Materials and Methods

All chemicals were from molecular biology grade (Sigma or Merck Inc.).

Molecular Cloning

Coding sequence of catalytic domain of LasA gene (Uniprot Code: P14789), was codon optimized and chemically synthesized by Pishgaman Gene Transfer Co. pUC57-lasA was received and introduced into CaCl₂ competent DH5 α bacteria by 90 seconds of 42°C heat shock. One of the transformants cultured and the plasmid was extracted by the QIAprep spin miniprep kit. Purified plasmid (pUC57-lasA) and pET28a(+) expression vector were double digested by *NcoI* and *XhoI* restriction enzymes and purified from agarose gel slices by vivantis GF-1 gel recovery kit. LasA gene fragment was sub-cloned into pET28a(+) expression vector by incubation in the presence of T4 DNA ligase for 1 h at 37°C. DH5 α competent bacteria were transformed with expression cassette pET28a-lasA and the recombinant construct was purified from subculture of the transformants the day after. Colony- polymerase chain reaction (PCR) of the DH5 α transformants was performed with universal primers T7 promoter and

T7 terminator as follows; T7 promoter sequence: GAAATTAATACGACTCACTATAG, and T7 terminator sequence: GCTAGTTATTG CTCAGCGG. PCR program steps were as follows: an initial step of 95°C for 5 min., (95°C 30 seconds, 56°C 30 seconds, 72°C 40 seconds) for 30 cycles, and a final extension of 72°C for 10 min. Purified pET28a-lasA construct from a selected DH5 α transformant was used for the transformation of competent *E. coli* BL21 (DE3) bacteria. PCR in all steps was performed using a Labnet MultiGene™ Optimax Thermal Cycler.

Expression induction and purification

As mentioned above, *E. coli* BL21 (DE3) bacteria was used for the production of recombinant lasA protein. A colony of BL21 DE3 transformants harboring the pET28a-lasA construct was cultured in LB medium containing kanamycin antibiotic (50 $\mu\text{g} \cdot \text{ml}^{-1}$), at 37°C. An aliquot of this culture was used for inoculation of a 100 ml LB medium. After shaking, the culture at 120 rpm up to the OD^{600 nm} of approximately 0.6, induction of the recombinant protein expression was induced by addition of 0.2 mM Isopropyl β -D-1-thiogalactopyranoside. Bacteria were harvested by centrifugation at 5,000 rpm for 3 min. after overnight induction. Bacteria pellet was resuspended in a lysis buffer (25 mM HEPES, pH 7.0, 600 mM NaCl, 10 mM 2-mercaptoethanol, 10% glycerol). Lysis of the bacteria was performed by 15 seconds periods of bead-beating for 10 times, with at least 30 seconds of intervals incubation on ice. Lysate of the bacteria was centrifuged for 15 min. (12,000 rpm at 4°C) and the supernatant was loaded on

a mini-column filled with Ni-NTA agarose column (Qiagen Inc.) to purify the recombinant his-tagged lasA protein. Equilibration buffer and wash buffer was the same as the lysis buffer. Elution Buffer was also the same except for the 300 mM imidazole to elute recombinant protein. Eluted fractions were analyzed by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis for the presence of protein, and the fractions contained the protein band corresponding to desired molecular weight were dialyzed against the imidazole free and lower NaCl molarity (50 mM) buffer lacking 2-mercaptoethanol reducing agent. Spectrophotometry in all steps of the present study was performed using a Unico™ 2100 spectrophotometer.

Disk diffusion

A colony of MRSA was resuspended in sterile phosphate buffered saline until 0.5 McFarland turbidity standard provided. Before placing the disks, entire surface of each plate was covered with the staphylococcus suspension. Plates were air dried for 15 min. and overnight incubation was performed at 37°C. Sensitivity to recombinant lasA protein was determined with disks loaded with 6 μg recombinant lasA protein. Halo diameter in LasA-treated plates was significantly higher than the control ($p < 0.05$). This study was approved by Ethics Committee of Shahid Sadoughi university of medical sciences.

Results

Molecular Cloning

pUC57 plasmid harboring gene fragment encoding the mature form of the LasA protein was double digested with NcoI and XhoI

restriction enzymes. Gene fragment was recovered from agarose gel slices and ligated into double digested pET28a(+) expression vector. As shown in fig. 1, colonies have the insert gene between T7 promoter and terminator. One of the transformants was cultured and the extracted recombinant construct pET28a-lasA was introduced into BL21 DE3 cells.

Expression induction and purification:

Transformed *E. coli* BL21 (DE3) bacteria was

cultured and the expression of the recombinant LasA was induced by adding 0.2 mM IPTG to the culture media. As shown in fig. 2, the expression that was induced in all colonies tested. Size of the expressed recombinant LasA protein was the same as predicted to be about 21 kDa.

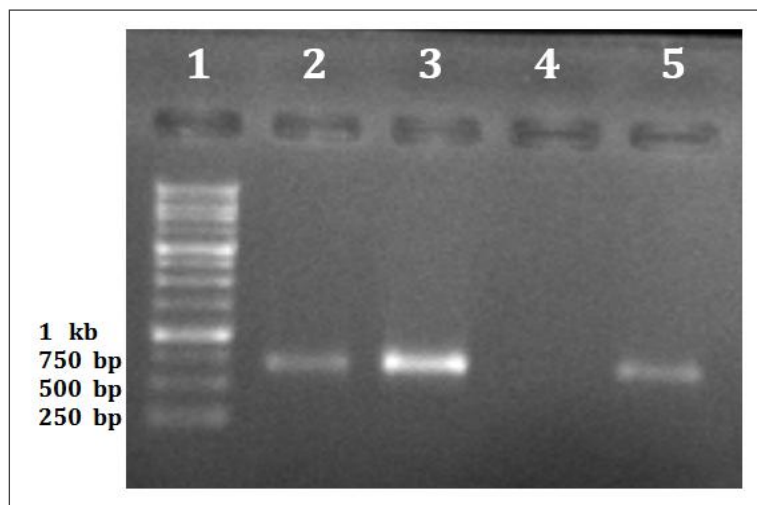


Fig. 1. Colony-PCR. Colony-PCR of the DH5α transformants was performed with universal primers T7 promoter and T7 terminator. Lane 1; 1 kb ladder, lane 2-5; PCR products of the colonies 1-4.

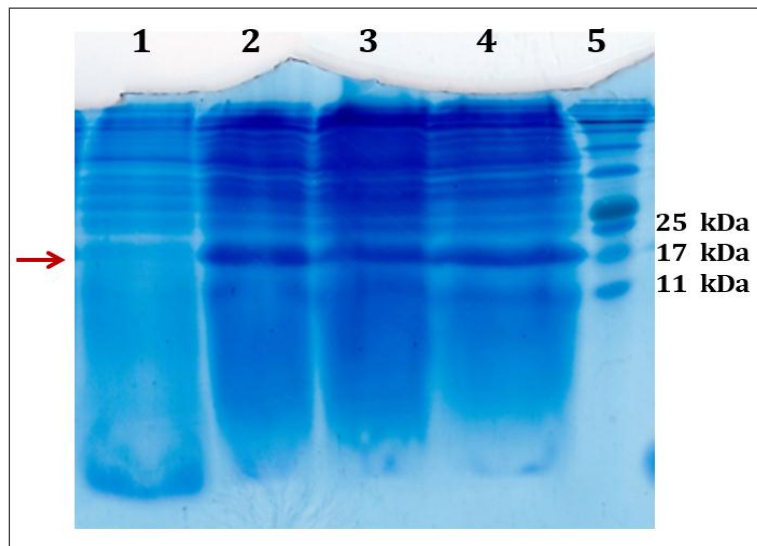


Fig. 2. Expression induction of the lasA gene. lasA protein was induced by 0.2 mM isopropyl β-D-1-thiogalactopyranoside in pET28a as C-terminal his-tagged fusion. Lane 1; BL21 DE3 cells containing pET28a-lasA construct before induction. Lanes 2-4; BL21 DE3 cells containing construct induced by 0.2 mM isopropyl β-D-1-thiogalactopyranoside clones 1, 2 and 3. Lane 5; pre-stained protein size marker (Sinaclon cat no. PR911641).

C-terminal his-tagged recombinant LasA was affinity purified by passing the supernate of the induced culture through a mini-column filled with Ni-NTA agarose resin. As shown in fig. 3, many protein bands were eliminated during the affinity purification. The purity of the recombinant LasA protein was calculated to be about 59% (ImageJ software) [16].

Disk diffusion

Several disks were loaded with dialyzed recombinant LasA protein and the inhibition of the growth of MRSA bacteria was tested with disk diffusion method. Clear and sharp halos around the disks loaded with recombinant LasA protein obviously showed that C-terminal his-tagged recombinant LasA protein inhibited the growth of MRSA bacteria (Fig. 4).

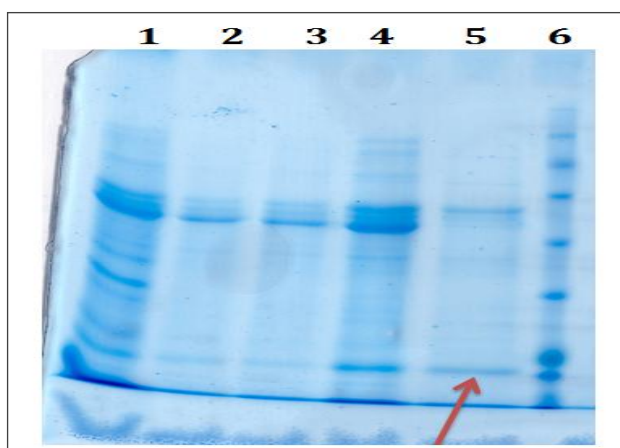


Fig. 3. His-tagged lasA protein purification. Affinity purification of the recombinant lasA protein was performed using mini column filled with Ni-NTA agarose resin. Lane 1; pellet of the lysate of the induced bacteria, lane 2; soup of the lysate before passing through column, lane 3; flowthrough, lane 4; first drops of the wash, lane 5; first fraction of the elution, lane 6; pre-stained protein size marker (Sinaclon cat no. PR911641).

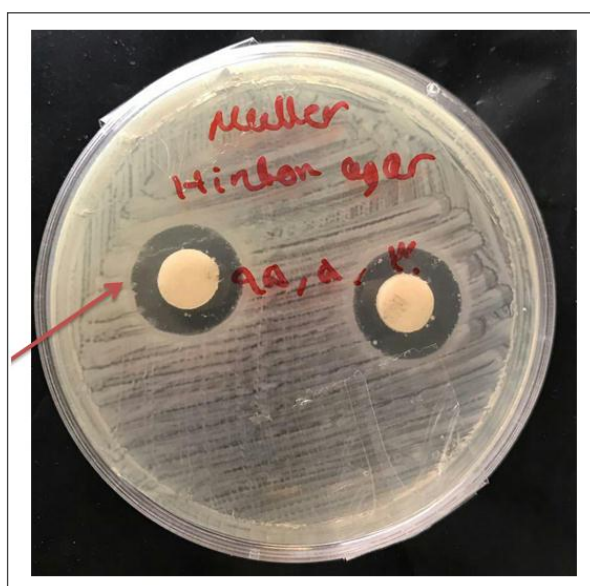


Fig. 4. LasA staphylolytic activity assessment by disk diffusion. Disk diffusion testing of purified recombinant lasA protein staphylolytic activity. The zone of inhibition (clear halo) around the disks loaded with lasA is sharp.

Discussion

In the new era of antibiotic abuse, emergence of resistant bacteria is seen worldwide. Bacterial infections, which had not been serious problems for decades after the discovery of antibiotics and their use in medicine, have been again one of the most severe threats of healthcare.

Overuse, inappropriate prescribing and extensive use of antibiotics in agriculture are among the main causes of prevalence of bacteria with resistance to various antibiotics [17, 18]. By the new developments in recombinant technology, the next decade is likely to witness a considerable rise in using enzybiotics to overcome the problem of antibiotic resistance [5, 19]. Until 1980s, in the literature “staphylolysin” usually referred to a substance with anti-staphylococcal activity. There were no details about the exact nature of the molecule or molecules, which are responsible for the activity. There are several examples of using the term even in 1930s-1940s without knowing even that the substance is a protein. In 1987, the nucleotide sequence of the LasA gene was determined and it was shown to have elastase activity. LasA protein is secreted from *pseudomonas aeruginosa* strains and hydrolyzes the glycine peptide bonds present in *S. aureus* peptidoglycan [6-9].

Efficacy of the LasA protein has also been examined in animal models. In an animal model of experimental keratitis caused by *S. aureus*, Barequet et al. have shown that LasA protein extracted from *pseudomonas*

aeruginosa strain FRD2128 has therapeutic effects against *S. aureus*. In their study, both methicillin sensitive and resistant strains of *S. aureus* were sterilized from infected cornea of rabbits [20]. They also examined the efficacy of the *pseudomonas aeruginosa* LasA protein in the treatment of endophthalmitis created by methicillin-resistant *S. aureus* in rat model. In addition to the effectiveness of the LasA protein, there were no adverse effects in the animal model of endophthalmitis [21].

Previous works have only focused on the using LasA protein extracted from *pseudomonas aeruginosa* culture supernatants. We have presented here the recombinant mature form of LasA produced in *E. coli* to have anti-staphylococcal activity. Codon-optimized gene encoding the mature fragment of LasA was cloned into the pET28a(+) and the protein was expressed in *E. coli* BL21 DE3 under the control of T7 promoter. Mature form of the LasA protein was expressed and purified by affinity chromatography using Ni-NTA column. The purity of the recombinant protein can be higher than that of achieved by the present study. It was probably due to the weak binding of the c-terminal his-tagged LasA protein to the Ni²⁺-loaded resin. When a low amount of imidazole (10-20 mM) was added to the lysis buffer to inhibit binding of the background proteins, binding of the recombinant LasA was largely inhibited. By the way, the purity and yield of the LasA protein produced in this study was sufficient to evaluate its staphylolytic activity.

Purified LasA showed significant growth inhibitory effect on methicillin-resistant *S. aureus* in disk diffusion tests. However, lysate of the induced BL21 DE3 bacteria lacked staphylolytic activity in disk diffusion method (data not presented here). This was probably due to the presence of 2-mercaptoethanol reducing agent, as it opened disulfide bonds and there were two disulfide bonds in LasA protein. Nevertheless, after purification and dialysis to remove the extra salts and 2-mercaptoethanol present in lysis buffer, the staphylolytic activity of the purified LasA protein can be pursued. Generally, c-terminal his-tagged recombinant LasA protein can be a promising agent against antibiotic resistant *S. aureus* strains.

Conclusions

The present study shows that the mature form of the LasA can be expressed in *E. coli* BL21 DE3 cells. C-terminal his-tagged form of the mature LasA protein has staphylolytic activity against MRSA and so it can be a promising

therapeutic agent. Since rising increase of the prevalence of antibiotic resistant strains of bacteria, existing therapeutic strategies relied on using conventional antibiotics appears to be ineffective. Enzybiotics may be the next footstep in fighting against antibiotic resistant bacteria. Recombinant LasA protein, which is produced in the present study, can be a hopeful strategy against resistant *S. aureus* strains, which are among the serious complications associated with nosocomial and community infections.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This work was supported by a grant from the Deputy of Research and Technology, Shahid Sadoughi University of Medical Sciences and was written based on a data set of a medical microbiology MSc thesis registered in faculty of Medicine, Shahid Sadoughi University of Medical Sciences. We thank personnel and faculty members of the clinical biochemistry department at Shahid Sadoughi university of medical sciences for kindly providing the projects of the protein engineering laboratory with facilities, materials and their guidance.

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