Study the effect of cloned pET-32a(+) plasmid by Lysostaphin gene against *Staphylococcus aureus*

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**Abstract**

Lysostaphin is a protein zinc metalloproteinase, extracted from *Staphylococcus simulans*, which disrupting peptide layer of *S. aureus*. In this study, Lysostaphin gene was detected in the *S. simulans* isolates. The molecular weight of the Lysostaphin gene is 750 bp. We were used the pET-32a(+) plasmid to cloning lysostaphin gene which transform to competent rubidium chloride E. coli DH5α for producing the lysostaphin protein. The lysostaphin protein from this gene which isolated from *S. simulans*, then used the expression of used to killed *S. aureus*, which has the thick layer of wall that is the very difficult bacteria response to treatment. The result was reported succeeded pET-32a (+) plasmid to expressed lysostaphin gene and gave lysostaphin protein with high quality and quantity. As well as the result was appeared the high accuracy of his tagging method in protein extraction and purification, and the quality and quantity more than other studies.

**Keywords**: Effect, Cloned, pET-32a(+), Lysostaphin, Gene

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**Introduction**

Bacteriocins are bacterial proteinaceous compounds, which have the activity of bactericidal against other bacterial spp., the bacteriocins have been proteolytic degradation (1). This bacterial proteinaceous compounds produced by gram positive bacteria. The bacterial proteinaceous can be divided into four classes, classes I and II bacteriocins are the most studied and have better clarified mode of action, since they possess prospect industrial and clinical applications (2). Treatment of staphylococcal infections has become difficult increasingly due to resistant strains colonization to some of the antibiotics (3). Staphylococci can be eliminated by lysostaphin from skin and nares of individuals increased risk infections *S. aureus* (4). It is binds to *S. aureus* and pentaglycine cross bridges cleaves within peptidoglycan, the cell wall envelope removing and osmotic rupture precipitating of staphylococci (5). Lysostaphin primarily active against coagulase positive *S. aureus* but some activity residual against coagulase negative *S. aureus*, it is requiring increased enzyme concentration and longer times incubation to be killed (6). *Lysostaphin* gene from the *S. simulans* isolates contain 1.5 kbp fragment of DNA (7). This gene was localized with in a *Hpa II / Hind III* fragment. The gene coding for preproenzyme which consist of three regions distinct: signal peptide at the end of aminoterminal, a series of tandem repeats and the active *lysostaphin* gene (7,8). Promoter of the gene have (−35) and (−10) at nucleotides regions (89-95) and (110-119), respectively (7).

There are a number of the reports of the lysostaphin expression promoter dodepeptidase. In many study, the r-lysostaphin produced from different pET vectors (9). One of the selective binding of the protein expressed is the His tags facilitate, it is affinity- nickel column. His tag sequences may be removed by protease optionally, another purification step requiring the tags which are no effect on the protein structure and function (10). Polyhistidines add by two ways. The first way by add repetitive histidine codons (CAT or CAC) to the PCR primers, as well as add
start or stop codon. The second technique by add a encoding His-tag sequences protein to the vector (11).

The aim of this study to expressed lysostaphin gene by pET-32a (+) plasmid and using the lysostaphin protein as bactericidal of S. aureus.

Materials and methods

Isolated genomic DNA from S. simulans according to Sambrook and Russel (12) with modification. This DNA used as a template for the amplification. The annealing temperature used in the PCR program was 53°C. The primer used in this study were Lys F1: (5’ gcg acg cat gaa cat tca 3’). Lys R1: (5’ ctt tat agt tcc cca aag aac 3’). The total volume of PCR reaction was 50μl (5μl 10X buffer, 8μl 1.25 mM of dNTPs, 2μl forward primer, 2μl reverse primer, 0.5μl Pfx, 5μl enhancer, 0.5μl DNA template, 1μl MgSO₄, and 26μl ddH₂O. The program of PCR technique consist of several stages: the first initial denaturation PCR program, 6min at 95°C, followed by 35 cycles 95°C for 45sec, 58°C for 45sec, then 72°C for 45sec. The last stage as 72°C for 6min, and then down to 4°C for 30min.

Then used primers have two restricted enzymes by add two restricted sits (Kpn I and EcoR I), one of them to the forward primer (Kpn I), the restrict sit for this enzyme (ggt acc) as well as start codon (atg), and the second one to the reverse primer (EcoR I), the restrict sit for this enzyme (gaa ttc) as well as stop codon (tca), and finally we are add codon of six histidine to the reverse primer in order to extract and purify the lysostaphin protein from cloning bacteria which have His tag sequences, the codon of one histidine (atg) add to revers primer, know, the last primers with two restricted sits (Kpn I and EcoR I) with start and stop codons and codon of six histidine are: Lys F2: 5’ GGT ACC ATG GCT GCA ACA CAT TCA 3’. Lys R2: 5’ GAA TTC TCA ATG ATG ATG ATG ATG CTT TAT AGT TCC CCA AAG AAC 3’.

Table 1 show the run of PET-32a(+) plasmid in the gel after cut by two restrict enzymes (Kpn I and EcoR I), then isolated and purified by gel extraction kit from the gel.

The PCR products from first PCR by Lys F1 and Lys R1 used as a template for sub PCR by using Lys F2 and Lys R2. The PCR product was run to electrophoresis, then the DNA from the gel was isolated and purified by the kit of DNA extraction. The extracted gene was cut by two restricted enzymes (Kpn I and EcoR I) as represented in the table 2, then the cut away gene cleaned from the few bp posts cut by these enzymes.

Clones were done to study the effect of PET-32a(+) plasmid, the cloning done between plasmid and PET-32a(+) cuts by EcoR I and Kpn I enzymes. Then ligated gene of lysostaphin digested by enzymes with PET-32a(+) digest by the same enzyme as described above, then transformed to rubidium chloride E. coli DH5α.

Colonies were replica-plated onto fresh (LB) Luria - Bertani solid medium, which containing 100μg/ml ampicillin, then incubated overnight at 37°C, then transformants bacteria confirmed by PCR, to detect successful cloning by colony PCR. This quick protocol which designed to screening the plasmids insert to the E. coli DH5α and this plasmid has the insert. which is called (constructive plasmid).

Table 1: Digestion of pET-32a(+) by Kpn I and EcoR I enzymes

<table>
<thead>
<tr>
<th>No. of Samples</th>
<th>No. of Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET-32A(+)</td>
<td>20μl</td>
</tr>
<tr>
<td>10X buffer</td>
<td>5μl</td>
</tr>
<tr>
<td>BSA</td>
<td>5μL</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>16μl</td>
</tr>
<tr>
<td>Kpn I</td>
<td>2μl</td>
</tr>
<tr>
<td>EcoR I</td>
<td>2μl</td>
</tr>
<tr>
<td>Total reaction</td>
<td>50μl</td>
</tr>
</tbody>
</table>

Table 2: Digestion of gene of lysostaphin by EcoR I and Kpn I enzymes

<table>
<thead>
<tr>
<th>Substance</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysostaphin gene</td>
<td>20μl</td>
</tr>
<tr>
<td>10X buffer</td>
<td>5μl</td>
</tr>
<tr>
<td>BSA</td>
<td>5μl</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>16μl</td>
</tr>
<tr>
<td>Kpn I</td>
<td>2μl</td>
</tr>
<tr>
<td>EcoR I</td>
<td>2μl</td>
</tr>
<tr>
<td>Total reaction</td>
<td>50μl</td>
</tr>
</tbody>
</table>

Determine the activity of cloning E. coli by lysostaphin gene to inhibit S. aureus by performed a standard disk diffusion assay. Prepare S. aureus and E. coli by culturing in the broth of nutrient to phase of exponential (OD₆₂₀ 0.1). Then inoculated S. aureus were in the Muller - Hinton Agar (MHA), as well as prepared and placed in discs from E. coli in the plates centre. Overnight, under aerobic condition, incubated at 37°C, determined the inhibition zones of culture.

Post determined the activity against S. aureus by cloning bacteria, we are extract and purify by His-tag method lysostaphin protein from cloning bacteria. This processing done by used the FF crude columns of HisTrap (Sweden), then determined the concentration of protein by Bio-Rad Protein Assay. By SDS-PAGE analysis, limited the purity of protein (13).
Results

The result of this study in the figure 1 showed the result of electrophoresis of the total genomic DNA of S. simulans. In addition, the figure 2 showed the gel electrophoresis of pET-32a(+) plasmid and lysostaphin gene, this figure showed digested plasmid by Kpn I and EcoR I enzymes and digested gene of lysostaphin by same enzymes, which appeared the band of the gene about 750 bp. While figure 3 showing the product of lysostaphin gene from PCR cloned by pET-32a(+) plasmid by colony PCR, this figure showing band about 750 bp.

Figure 1: Electrophoresis of total genomic DNA of S. simulans, lane 1 is the ladder, lanes 2,3,4 are the total genomic DNA.

Figure 2: Gel electrophoresis of pET-32a(+) plasmid and lysostaphin gene, lane 1 is the ladder, lanes 2,3,4,5 are gene of lysostaphin from pET-32a(+) plasmid, lanes 7,8 are lysostaphin gene amplified by first detection about 750 bp, lane 6 is negative control.

The antibacterial activity of transformed E. coli have pET-32a(+) plasmid with gene of lysostaphin insert against S. aureus (Figure 4A), while figure 4B showed the effect of E. coli non-transformed by gene of lysostaphin against S. aureus. Table 3 showed the protein of lysostaphin concentration from cloned bacteria with pET-32a(+) plasmid after extraction by column of His-tag, also showing in this table first lysostaphin protein concentration 1.113 more than the third concentration of BSA 0.893 and less than second concentration of it 1.773. SDS-PAGE analysis of proteins showed in figure 5 with standard techniques, this figure showed only one protein band, this figure recorded lysostaphin molecular weight about 27,000 Dalton.

Figure 3: PCR product of lysostaphin gene from cloned bacteria by colony PCR, lane 1 is the ladder, lanes 2,3,4,5 are gene of lysostaphin from pET-32a(+) plasmid, lanes 7,8 are lysostaphin gene amplified by first detection about 750 bp, lane 6 is negative control.

Figure 4: A. Effect of antibacterial activity of transformed E. coli by pET-32a(+) plasmid have lysostaphin gene against S. aureus, B. Effect of activity of antibacterial against S. aureus by non-transformed E. coli.
The antibacterial activity of transformed *E. coli* have pET-32a(+), which showed the effect against *S. aureus* by *E. coli* non-transformed, this result agreement with the previously studies (11,23), which recorded of effectiveness against *S. aureus* by transformed *E. coli* have lysostaphin active gene 750 bp.

SDS-PAGE analysis of proteins recoded the molecular weight of lysostaphin protein about 27,000 Dalton, this result matching by Mustafa; Trayer and Buckley (11,24).

The result of the present study showed that limited increasing in concentration of lysostaphin protein when used pET-32a(+) plasmid compared with other study (25), which recorded less then concentration of lysostaphin protein with other plasmid, this study recorded lysostaphin concentration from pTrc99a plasmid 1.012mg/ml which less than our study 1.113 mg/ml, as well as 0.622 mg/ml concentration by used pBAD30 plasmid.

In many study, the r-lysostaphin were produced from different pET vectors, pET28a is one of them which yield 22 mg, the other one is pET 23b which yield 20 mg, and pET15b, yield 11 mg of protein purified from one later of *E. coli* (9,26,27). Our study gave successful of expressed lysostaphin gene by pET32a vector in *E. coli*, this protein production are mature r-lysostaphin because the activity against *S. aureus* and it is a large quantity of r-lysostaphin, this match and alignment with our data (9). It is found many different systems for expression have been developed, but the proteins recombinact production in *E. coli* remains the most one used (28).

The activity of lysostaphin protein against *S. aureus* is because capability to lysis peptidoglycan of cell walls bacteria (24,29). The site of action of lysostaphin protein is the cross-bridge pentaglycine of the peptidoglycan (30). Staphylococcal species in general and *S. aureus* is one of them composed of five glycine (Gly) residues (31). One of the antistaphylococcal agents is the lysostaphin, which is unique among in bacterial kills, it is have ability to killing a large number of organisms in the *Staphylococcus* genus because of the glycine-glycine bonds existence (32,33). Lysostaphin enzyme consists of two domains, those are: N-catalytic terminal domain and a cell wall binding domain (34).

Lysostaphin protein is a bacteriolytic metalloprotease secreted from *S. simulans* biovar *staphylolyticus*. It is function is degrading the staphylococci cell wall of multiple species by pentaglycine crosslinks hydrolysis within peptidoglycan (35).

**Conclusion**

We are conclude from succeeded pET-32a(+) plasmid to expressed **lysostaphin** gene and expressed protein lysostaphin in high quality and quantity. As well as conclude from our result, high accuracy of His tag technique in protein extraction and purification.

**Discussion**

Mastitis is one of the most important economic disease of dairy cattle, the effects of this disease come from resistance to treatment and costs losses (14). It is regarded as the first common disease, which causes losses of antibiotics by treatment in dairy cattle and responsible for antibiotics increasing resistance (15).

Mastitis treatment is very restricted and as a cause disease propagation, *S. aureus* is regarded a main cause of mastitis in the ruminants (16,17). One of the studies for detection of capsulated *S. aureus* strains from bovine and buffaloes mastitis is Shamoon (18). *S. aureus* appears as a one of the twelve most health-threatening pathogens in the resistant bacterial list compiled by WHO which promotes new antibiotics development (19,20). Staphylococcal species and especially *Staphylococcus aureus* have developed resistance multidrug lead to serious risks of health then treatments complex, by using the novel and effective strategies development to kill these bacteria (21). To the present study gave a new strategy to treat this bacterial problem of *S. aureus*.

The result of the current study showed the size of gene of **lysostaphin** was 750 bp which accepted by (7,22,23).
Aknowledgement

We are Aknowedge the University of Basra, College of Veterinary Medicine, Public Health department for gave us all the facilities in the Public Health Lab.

Conflict of interest

Authors declares that they have no conflict of interest regarding publishing this review article.

References

دراسة تأثير كلونة بلازميد (+)
pET-32a(+) بجين اللايسوستافين ضد بكتيريا المكورات العنقودية الذهبية

جلال ياسين مصطفى
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الخلاصة
لايسوستافين هو بروتين من الخارصين الفلزي، ينتج من بكتيريا المكورات العنقودية نوع سميلنس، يعمل على تحطيم الجدار الببتيدي لبكتيريا المكورات العنقودية الذهبية. في هذه الدراسة تم عزل جين اللايسوستافين من المكورات العنقودية نوع سميلنس، وكان حجم الجين ما يقارب 750 قاعدة نيوترونية. استخدمنا بلازميد pET-32a(+) لклонة جين اللايسوستافين إلى بكتيريا الايشيريشيا القولونية المستقلة نوع (DH5α) لإنجاح بروتين اللايسوستافين من هذا الجين المكلف، والمعزول من المكورات العنقودية نوع سميلنس الذي استخدم تعبيره الجيني في ما بعد كمصداق لقتل بكتيريا المكورات العنقودية الذهبية أحد أصعب أنواع البكتيريا بالعلاج لسمك جدارها الخارجي. أظهرت النتائج نجاح بلازميد pET-32a-lys للتعبير عن جين اللايسوستافين وإنتاج بروتين اللايسوستافين ويكفاءته عالية، كما أظهرت النتائج دقة تقنية الاستخلاص والتنقية باستخدام عمود الهستين المتعدد، وكون كمية البروتين وكفاءتة اعلى من دراسات سابقة على بلازميدات أخرى.