Original Article

Production of HPV16-L1 Through BL21/pET32a Expression System

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Abstract
Background: The human papillomavirus (HPV) main capsid protein L1 is naturally capable of self-assembly as virus-like particles (VLPs). There are different recombinant protein expression systems, such as bacteria, yeast, insect, plant, and mammalian cells, for the generation of VLP-based candidate vaccines targeting various pathogens. In this study, we produced the HPV16-L1 protein by BL21/pET32a expression system, and VLP production was confirmed.
Methods: The recombinant plasmid pET32/L1 was transformed into *Escherichia coli* BL21 and selected with ampicillin. The positive clones containing the recombinant plasmid pET32/L1 were assessed by restriction endonucleases *HindIII* and *XhoI* and nested polymerase chain reaction (PCR). The expression of HPV16-L1 fusion protein in *Escherichia coli* BL21 was identified by SDS-PAGE and western blotting. Electron microscopy was used to evaluate VLP formation.
Results: A codon-optimized L1 gene was expressed in BL21 under the control of the T7/lac promoter. Purification of L1 protein was achieved after Ni NTA chromatography. The 60 kDa protein was detected in the lysates of BL21, recognized as HPV16- L1 protein by western blotting. The VLPs were confirmed using electron microscopy.
Conclusion: In this study, we established an efficient recombinant *E. coli* expression system for the production of HPV 16- L1 protein. The generated L1 protein was correctly self-assembled into VLPs. Therefore, BL21/pET32a as a prokaryotic expression system is a potent tool for HPV16-L1 VLP production.
Keywords: HPV, VLP, Vaccine, BL21, pET32

Introduction

Human papillomaviruses (HPVs) are known as etiologic agents of warts and malignant tumors. Persistency in HPV infection can result in carcinogenesis, and most HPV-induced tumors can be attributed to HPV16 and 18 (1). Every year, almost 630 000 cancer cases (4.5% of total cancers) are related to HPV, 83% of which are cervical cancer. Approximately, 75% of individuals with sexual activity are exposed to HPV in their lives (2).

The HPV capsid is composed of two structural proteins, L1 and L2. The HPV main capsid protein L1 is naturally capable of self-associating as virus-like particles (VLPs) (1,3). Using VLPs caused a massive development in subunit vaccines with enhanced immunogenicity (4). VLPs have the size and morphology of viruses (~22-200 nm), depending on the specific viral proteins involved (5). To date, two VLP structure-based HPV vaccines have been approved by the FDA for human use (6).

There are different recombinant protein expression systems, such as bacteria, yeast, insect, plant, and mammalian cells, for the generation of VLP-based candidate vaccines targeting various pathogens, as well as non-infectious diseases (7). Several approaches for expressing the recombinant HPV16-L1 have been tested using bacteria, e.g., *Salmonella typhimurium*, *Escherichia coli*, *Shigella flexneri*, *Lactobacillus casei,* and *Lactococcus lactis* (8).

Although yeast and baculovirus expression systems can produce HPV16 VLPs with high immunogenicity, some defects, such as considerable technical difficulties, high cost, and low yield, widely limit the use of these systems, especially in poor countries and regions (5,9). Therefore, looking for a new and more efficient approach to preparing HPV-L1 protein is necessary to reduce the cost of the HPV vaccine and expand its availability (10). A bacterial expression system might be a reasonable choice to solve this problem because of its high level of protein expression and rapid growth in a relatively inexpensive culture medium (11,12). In this study, we produced HPV-L1 protein through BL21/pET32a expression

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system, and VLPs were self-assembled.

Material and Methods

HPV16 L1 expression plasmid

The pET32a was kindly provided by Dr. Razavi (Tarbit Modares University). The recombinant plasmids were transformed into *E. coli* BL21 under the control of the T7/lac promoter and the transformation was confirmed through enzymatic digestion by *HindIII* and *XhoI* and nested polymerase chain reaction (PCR) using L1-specific primers (13). After that, transformed colonies were cultured using Luria-Bertani (LB) medium with 50 g/mL kanamycin and 80 g/mL ampicillin. The LB-broth was shacked at 37 °C to reach an optical density (OD) of 0.6. The 1.5 mM isopropyl-D-1-thiogalactopyranoside (IPTG) was added and the culture was incubated at 20 °C for 12 hours. After centrifugation at 12 000 g, the pellet was collected and suspended in 50 mM phosphate buffer (PB, pH 7.0). Then, cells were forwarded to chromatography.

Protein purification

The histidine tag (His-tag) embedded in the recombinant protein has a high affinity for nickel and facilitates its purification. The L1 protein with an N-terminal His-tag was extracted through Ni-NTA affinity chromatography (Merck, Germany). Briefly, the elucidated supernatant was loaded three times on the Ni-NTA column with a 0.5 mL/min flow rate. The washing of the column was performed with buffer B (400 mM NaCl, 50 mM Tris, 30 mM imidazole, pH 8.0). To optimize the purification condition, different concentrations of imidazole including 70, 150, 300, and 500 mM was tested and the best conditions (300 mM) were selected to obtain the highest amount of soluble recombinant protein. Then, the recombinant HPV-L1 was carefully eluted with buffer C (400 mM NaCl, 50 mM Tris, 300 mM imidazole, pH 8.0) and forwarded to SDS-PAGE and western blot.

SDS-PAGE and western blot analysis

SDS-PAGE was done through the Laemmli method (7). The specimens were diluted (1:10) in SDS-loading buffer (pH 6.8, 1 M Tris–HCl, 10% SDS, 50% glycerol, 0.5% bromophenol blue, 1 M b-mercaptoethanol), warmed for 10 minutes and centrifuged at 10 000 g for 5 minutes . Then, the specimens were loaded to electrophoresis gel to be separated using 10% polyacrylamide gel and also stained with Coomassie brilliant blue (Pierce). Moreover, for western blotting, another gel electrophoresis was done and transferred to nitrocellulose membranes. The Tris-buffered saline (TBS) with 2% bovine serum albumin was applied as a membrane-blocking solution. The membrane was suspended in TBS for 1.5 hours at room temperature, washed, and floated in an anti-HPV16-L1 monoclonal antibody (Invitrogen, Germany) overnight at room temperature. Through Western blotting, bands were detected using the horseradish peroxidase enzyme conjugated to an anti-mouse antibody (Biogen, Iran) for two hours at room temperature and by floating in 3, 3', 5, 5' tetramethylbenzidine.

Electron microscopy

To assess VLP formation, the purified L1 protein (60 to 90 μg) was dialyzed in a Slide-A-Lyzer dialysis chamber (Pierce) against alkaline buffer (0.01% Tween 80, 55 mM HEPES [pH 7.4], 0.5 M NaCl) and acidic buffer (0.01% Tween 80, 40 mM sodium acetate [pH 5.6], 1 M NaCl) for 2 hours at 25 °C with buffer exchange. The samples were added to carbon-coated grids, and after adsorption on it, negative staining using 2% uranyl acetate was performed. The grids were allowed to be dried, and then, observation of VLPs was performed with Zeiss EM 109 transmission electron microscope at Tarbiat Modares University.

Results

Expression of HPV16 L1 protein in E. coli

The HPV16 L1, which was codon-optimized, was expressed in BL21 using a T7/lac promoter. The transformed recombinant HPV16-L1 was verified through PCR and L1-specific primers as demonstrated in Figure 1.

L1 purification and characterization

The HPV16-L1 purification succeeded after Ni-NTA chromatography. The yield of purified recombinant protein was 0.7 μg/μL. After lysing using lysis buffer, the recombinant L1 was detected in the sediment in large quantities, demonstrating the protein retains as inclusion bodies. Through sample loading, some of the L1 protein was absorbed into the column, and the rest
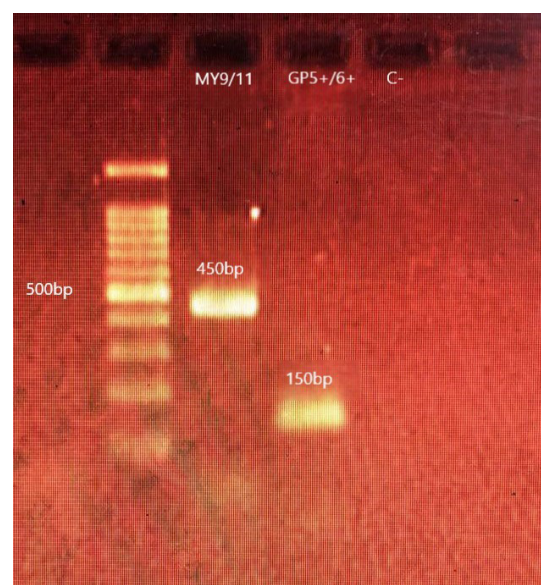

Figure 1. Confirmation of cloning by Nested PCR. 1: Ladder, 2: first round reaction, 3: second round reaction, 4: negative control

was detected in the flow through. The washing procedure was effective in eliminating weaker absorbed proteins. The high-salt eluted protein was a single protein with approximately 60 kDa, which was precisely identified by a monoclonal antibody against L1 (HPV16). By an anti-HPV16-L1 monoclonal antibody, a 60-kDa protein bond was detected in the lysates of BL21, by the western blot (Figure 2). Other molecular mass bands were also observed. These bands were L1 degradation products since they were not observed in total extracts of empty *E. coli*. The HPV16-L1 band did not appear in the negative control (*E. coli* transformed with empty pET-32a vector, Figure 2). Purified VLP formation was then assessed by electron microscopy. Several particles were detected in the process, and the VLPs seemed more clear and regular (45–50 nm) (Figure 3).

Discussion

The production of an effective prophylactic vaccine for high-risk HPV types, particularly HPV16, can significantly decrease the incidence rates of HPV-related cancers. Usually, the majority of prophylactic viral vaccines are built by live, inactivated, or attenuated viruses (13). Still, some concerns about possible risks in utilizing the vaccines comprising viral oncogenes have resulted in efforts for the development of the subunit vaccine (14). Therefore, according to VLP immunogenicity character, the present HPV vaccines are based on L1 VLPs (15).

Since the main immunogenic loops are exposed on the L1 protein, the HPV16-L1 protein is an immunogenic protein that leads to neutralizing antibody release. Also, in the production of L1 VLP as a candidate vaccine, codon optimization can suggest a chance to reduce costs due to higher expression ability, the omission of the assembly phase, and rapid-appropriate purification. Hence, using the same process for the other types of HPV is expected because of the high conservation of L1 among different types of HPV (16).

The L1 capsid protein is hard to produce in *E. coli* in a soluble condition. However, prokaryotic expression systems are a popular choice for recombinant protein expression because of the high expression of heterologous proteins, fast growth, easy culturing, and low production costs. One strategy to increase the L1 expression in *E. coli* is to fuse different tags or proteins.

Given that HPV-16 L1-His protein harbors 12 cysteines in its main sequence, the *E. coli* strain was used as the host due to its capacity to support the expression of proteins with disulfide bonds (15). HPV-16 L1-His production was stimulated in *E. coli* after administration of IPTG, and in the present study, it was revealed to be consistent with previous data on the expression of variants of HPV-16 L1 protein in the *E. coli* strain BL21 transformed with plasmid pET-32a-L1.

In studies conducted by Lai and Middelberg and Choe et al, metal chelate affinity chromatography was utilized for the purification of HPV-16 L1 protein attached to a histidine tag through its carboxyl end. In the present study, the L1-His extract in the lack of reducer compounds made it possible to purify it directly using IMAC-Ni2+, with a purity of ~90%, similar to that obtained by Lai and Middelberg, and it was mainly influenced by the co-purification of a 60 kDa fragment of L1-His protein (17, 18).

The aim of protein purification is not only to remove contaminants and excess compounds but also to increase the protein concentration and transfer it to an environment where it is stable and ready for consumption. In the first stage of purification, a band of recombinant protein with other proteins was observed in the binding
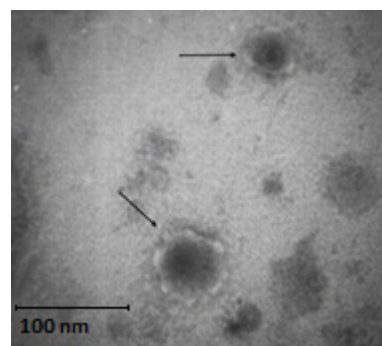

Figure 3. Morphology of VLPs by electron microscopy

Figure 2. SDS-PAGE. (A) Lysed bacteria, 1: Ladder, 2: BL21 (DE3) expressing L1, 3: Crude, 4: 4-hour sample with 1.5 mM IPTG; (B) Purification of L1, 1: Ladder, 2: Pellet, 3: Flow-through, 4-8: washed, 9: Ladder 10-13: The elution of L1 recombinant; (C) Western blot, 1: Ladder, 2: negative control, 3: HPV L1.

solution, while no protein band was observed in other columns. The cause of this issue can be inappropriate binding of the resin to the recombinant protein, low effectiveness of the resin, or short incubation time of the resin with the sample. To solve this problem, different concentrations of imidazole in lysis and washing buffers, increasing the incubation time with resin, reducing the washing time with washing buffer, and increasing the concentration of imidazole in the buffer solution, were tried separately. Finally, by removing imidazole from the lysis buffer, reducing the concentration of imidazole in the wash buffer, increasing the incubation time of the resin and sample to an overnight period at 4 °C, and increasing the concentration of imidazole in the buffer solution to 300mM, L1 protein was purified. One of the objectives of this study was to form VLPs for developing a vaccine. The proper formation of VLP requires proper folding of L1 capsomers; however, other involved factors cannot be ignored. The ionic impact of the environment on the protein is noted. Numerous studies have confirmed the role of high concentrations of NaCL and acidic pH in the formation of papillomavirus VLPs (9,10). Studies show that the C-terminal domain of the L1 protein plays an important role in capsid stability as well as the interaction of capsomers in the assembled capsid, and the cysteine sequences involved in disulfide bonds are located in the C-terminal domain (16). Removal of a small portion of the C-terminal region (24 amino acids) does not interfere with VLP formation, but the removal of larger components (44 amino acids) prevents VLP formation (19). Dialysis of the purified sample and removal of the buffer at pH 8, followed by the addition of a high-salt buffer (0.5-1 M NaCl) at acidic pH (6.5-4.5), are effective in VLP formation (20), however at very low pH (approximately 4) no aggregation is seen (21). In this study, after the purification of the L1 protein, the purified sample was suspended in a salt buffer with a pH of 5.6 to investigate the role of pH in the formation of disulfide bonds and the formation of VLP. Investigating two samples containing protein purified with two alkaline and acidic buffers and by electron microscopy confirmed the role of acidic pH in the formation of VLP particles.

Conclusion

In summary, we developed an efficient recombinant *E. coli* expression system to produce HPV 16 L1 protein, which showed great potential as a candidate for HPV vaccine development. The produced L1 proteins were correctly assembled into HPV VLPs themselves, making *E. coli*-expressed L1 VLPs a potential HPV vaccine for large-scale production.

Authors' Contribution

Conceptualization: Abdolvahab Moradi.
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Methodology: Sanaz Jahandideh.
Project administration: Abdolvahab Moradi.
Resources: Emad Behboudi, Abdolvahab Moradi.
Software: Emad Behboudi.
Supervision: Abdolvahab Moradi.
Validation: Abdolvahab Moradi.
Visualization: Emad Behboudi.
Writing–original draft: Emad Behboudi.
Writing–review & editing: Emad Behboudi, Abdolvahab Moradi.

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