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Original Article

Bac to Bac System Efficiency for Preparing HPV Type 16 Virus-Like Particle Vaccine

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Abstract

Today, the human papillomavirus **(**HPV) L1 protein is the main target in the construction of prophylactic HPV vaccines. The production of virus-like particles (VLPs) that closely resemble the natural structure of the HPV16 virus and induce high levels of virus-neutralizing antibodies in animals and humans is facilitated by the expression of HPV16-L1 protein in eukaryotic cells. The Bac-to-Bac system has been previously used to produce high levels of recombinant proteins. In this study, we utilized this expression system to generate HPV16-L1 VLPs in *Spodoptra frugipedra* (Sf9) insect cells. The wild-type L1 gene of papillomavirus type 16 was selected from Gene Bank and placed in bacmid structure after codon optimization using pFast Bac vector. The recombinant baculovirus containing HPV-16/L1 gene was then provided using the Bac-to-Bac system. It should be mentioned that the vector was transfected into the Sf9 cell. The cells were then lysed and the expression of L1 protein was revealed by SDS-PAGE and confirmed by Western Blot. The L1 purification was performed through Ni-NTA chromatography. The VLP formation of papillomavirus L1 protein was visualized by transmission electron microscopy. The expressed recombinant L1 was ~60 KD on SDS-PAGE which was identified in western blot by a specific anti-L1 monoclonal antibody. The electron microscopy confirmed the assembly of VLPs. Results of this study showed that the production of this protein at the industrial level can be optimized using a baculovirus/Sf9 system. The characteristics and advantages of this system are promising and it is a suitable candidate for protein synthesis.

Keywords: Baculovirus, HPV, L1, Protein purification, Sf9, Vaccine

1. Introduction

High-risk human papillomaviruses (HPV) are the causative agents of several cancers worldwide (1). The HPVs are a heterogeneous group of tissue-specific oncoviruses, consisting of more than 150 different types (2). They are classified into low- and high-risk types, according to their oncogenic characteristics. High-risk HPV types, including 16, 18, 31, 33, 45, 52, and 58, are the main agents involved in cervical cancer (3). High-risk HPV types with the transmission route of intercourse are responsible for 99.7% of cervical cancer cases. Among the high-risk types, HPV16 is the most related to cervical carcinoma and is identified in half of cervical cancers (4).

Therefore, HPV16-L1 is the main target in the construction of prophylactic HPV vaccines. The capsid of HPV16 is comprised of major (L1) and minor (L2) proteins and assembled by 72 capsomers of L1 protein (55-60KD) laid out in an icosahedral form (5). The approved vaccines of HPV harbor virus-like particles (VLPs) that are spontaneously assembled from L1 capsid proteins which cause type-limited protection against mucosal HPV infections that induce cervical, anogenital, and oropharyngeal carcinomas or condylomas (6, 7).

High prices of confirmed vaccines limit the availability of each vaccination or Pap screen prophylaxis in developing countries which have more than 80% of cervical cancer in the world (8). Therefore, studies on different approaches to reducing costs are still ongoing. The HPV16-L1 expression in eukaryotic expression systems leads to the assembly of VLPs that have the normal structure of HPV and induce excessive titers of neutralizing antibodies in animal models and patients (9). The VLP induced by HPV16-L1 is widely recognized as the most potent target for HPV16 prophylactic vaccines, and the FDA has confirmed that the HPV16 VLP vaccine can be produced for global vaccination programs (10).

The baculovirus/insect cell expression system has been extensively utilized for VLPs production and is a promising platform for the generation of viral antigens and vaccine products. It has been proven to be safe, easily manageable, and straightforward to scale up. Moreover, it has advantages, such as high expression yield, well protein folding, and preservation of post-translational modifications. Although it has been found that some insect cell lines, other than *Spodoptra frugipedra* (Sf9), can produce more recombinant proteins, they also release more proteases that can degrade the target protein. Therefore, the Sf9, with its higher cell density, faster growth rate, and greater tolerance to osmosis and pH, is probably the most widely used insect cell line in recombinant protein production (11, 12). In the present study, the Bac-to-Bac baculovirus expression system was used to produce HPV16-L1 VLPs in Sf9 insect cells.

2. Materials and Methods

2.1. Production of Recombinant pFastBacHTa

The gene encoding of HPV16-L1 protein (GenBank No. AIQ82846.1) was optimized according to the codon usage in Sf9 in order to induce some perversion. It should be mentioned that it was manufactured by GenScript (Nanjing, China). The HPV-L1 was cloned to the pFastBacHTa (pFB) plasmid for the baculovirus expression system (Invitrogen, USA) that facilitated the expression of the viral L1 capsid protein. The recombinant construct was confirmed by restriction enzyme analysis using *HindIII*+*SacI* restriction cleavage. The process of provision of competent cells transformed bacteria *Escherichia coli* DH10Bac, prepared DNA, and electrophoresis in agarose gels for cloning confirmation was based on guidelines (13).

2.2. Provision of Recombinant Bacmid

In this study, recombinant pFB-HPV L1 was transformed into DH10Bac competent cells carrying a bacmid with a mini-attTn7 target site, using lacZ gene disruption. The transformed DH10Bacs were cultured on specific Luria-Bertani agar supplemented with a range of antibiotics (gentamicin 7 µg/mL, tetracycline 10 µg/mL, kanamycin 50 µg/mL), X-gal, and IPTG at 37 °C for 48 h. Recombinant bacmid colonies were detected by their distinctive white color (Figure 1B). Purification of the bacmid with high molecular weight was carried out using the alkaline lysis method as per the Bac-to-Bac baculovirus expression system manual (Invitrogen, USA). The effectiveness of transposition was confirmed through polymerase chain reaction (PCR) testing using L1-specific primers (14).

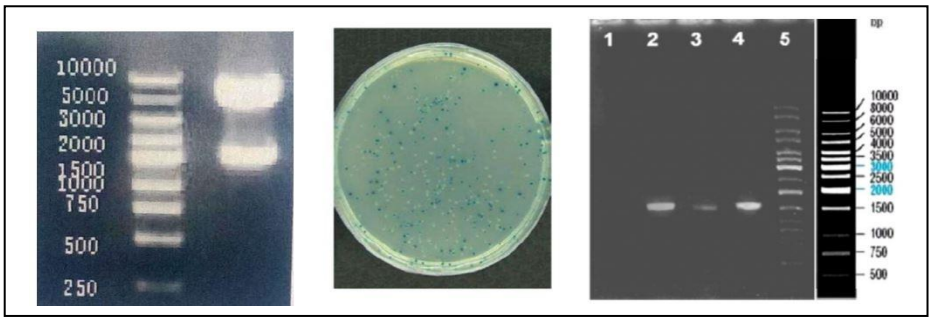

Figure 1. A: The confirmation of recombinant pFast HTa-L1 by restriction enzyme analysis. **B:** Recombinant bacmid formed colonies. **C:** L1 confirming by PCR

2.3. Transfection of Sf9 Cells with Recombinant Bacmid DNA

Spodoptera frugipedra cells were acquired from Tarbiat Modares University in Tehran, Iran, and cultured in insect cell culture medium of Grace, supplemented with heat-inactivated fetal bovine serum (GIBCO, Invitrogen, Germany) and 50 µg/ml of streptomycin and penicillin at 27 °C. The transfection of the isolated recombinant bacmid DNA into Sf9 cells was carried out using Lipofectamine, a cationic lipid, according to the instructions of the manufacturer.

For each transfection, 2×10^5 cells were seeded in a 6-well plate and incubated to proliferate for 1 day. The bacmid DNA (4 µg) and Lipofectamine 2000 (5 µl) were separately diluted in a medium (240 µl), mixed, and applied to the cells. The transfection process took 4-6 h at 27 °C. The baculovirus proliferation was monitored for 72 h using cytopathic effect analysis. Effective transfection was confirmed by the successful observation of cytopathic effects.

2.4. Purification of HPV16-L1 Protein

The histidine Tag (His-tag) with a high affinity for nickel was inserted in the L1 recombinant protein to facilitate its purification. The viral protein with an N-terminal His-tag was purified by Ni-NTA affinity chromatography (Merck, Germany). In short, the elucidated supernatant was loaded on the Ni-NTA column with a 0.6 ml/min flow rate. Buffer B (400 mM NaCl, 50 mM Tris, 30 mM imidazole, and pH 8.0) was used for washing the column. Afterward, the recombinant HPV-L1 was carefully eluted with buffer C (400 mM NaCl, 50 mM Tris, 300 mM imidazole, and pH 8.0).

2.5. HPV16-L1 Protein Verification in Sf9 Cells

The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and western blotting were performed to confirm HPV16-L1 protein expression in the Sf9 cells. The transfected Sf9 cells were harvested 72 h post-infection, washed with cold PBS, suspended in cell lysis buffer, and sonicated. The resulting proteins were run on a 12.5% acrylamide gel and stained with Coomassie blue (0.25%) or transferred to a nitrocellulose membrane. The membrane was blocked with blocking buffer (5% milk in tris-buffered saline) and exposed to 1:100 dilution of anti-HPV16-L1 monoclonal antibody (Abcam, USA) overnight. This was followed by horse radish peroxidase-conjugated anti-mouse antibody (Biogen, Iran) and TMB substrate to produce immunoreactive bands (Figure 2).

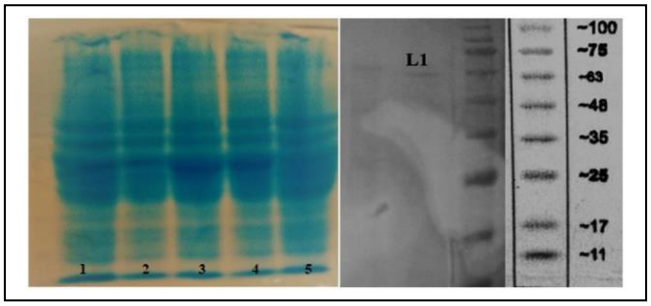

Figure 2. Left: SDS-Page after 48 and 72 hours in lane 3 and 4, respectively. **Right:** Western blot: L1 band is showed

2.6. Virus-Like Particles Confirmation

This study utilized electron microscopy to confirm the production of VLPs. The Sf9 cell extract was fixed in a solution containing 2% paraformaldehyde and 0.1% glutaraldehyde in PBS. The cells were washed with PBS and subsequently fixed using 1% osmium tetroxide. The specimens were then processed at different grades of alcohol and infiltrated with propylene oxide before being embedded in Epon 812. After sectioning with a diamond knife on an ultramicrotome (Leica), the sections were stained with uranyl acetate, mounted on 200-mesh grids, and observed with an electron microscope (HU12) at Tehran University in Tehran, Iran. This methodology confirmed the production of VLPs in the Sf9 cells.

3. Results

3.1. Production of Recombinant pFastBacHTa

In this study, the full gene of HPV16-L1 was cloned into the pFastHTa plasmid, resulting in the expected 4,800 bp vector and 1,590 bp fragment containing the

inserted gene. Figure 1 displays the resultant bands. Additionally, to verify the accuracy of digestion and electrophoresis results, PCR was performed on recombinant bacmid DNA containing HPV16-L1 using specific L1 primers, as demonstrated in figure 1. This ensured that the transformation and cloning process was successful and yielded recombinant bacmid DNA with the HPV16-L1 gene.

3.2. Generation of HPV16-L1 Bacmid DNA

Successful transformation of the recombinant plasmid pFast HTa-L1 was achieved in DH10Bac competent cells. The resulting recombinant bacmid held the HPV16-L1 gene insert and was later used for the transfection of Sf9 cells. After 48 h, Sf9 cells that had undergone transfection with the recombinant bacmid demonstrated swelling, rounding, and detachment from the surface of the flask, while the proliferation and morphology of non-transfected cells were typical. These observed changes in transfected cells serve as evidence of effective transfection and successful production of the HPV16-L1 gene in the Sf9 cell line.

3.3. Expression and Identification of HPV16-L1 Protein

In this study, the yield of purified recombinant L1 protein was 35 mg/L. The extracted L1 protein from transfected Sf9 cells was observed as a distinct band of approximately 60 kDa in size as demonstrated through SDS-PAGE (Figure 2). The molecular weight of HPV16-L1 protein is well-established, and the observed band aligns with its recognized value. Additionally, the presence of the L1 protein was confirmed using an anti-HPV16-L1 monoclonal antibody (Figure 2), indicating that the protein of interest was successfully expressed in the Sf9 cells.

3.4. Distinction of Virus-Like Particles through Electron Microscopy

The production of VLPs derived from the expression of the recombinant HPV16-L1 protein in Sf9 insect cells was confirmed. The VLPs were visually distinguishable from non-transfected Sf9 cells following infection with the recombinant baculovirus harboring the HPV16-L1 gene. Analysis through electron microscopy displayed simultaneous assembly of the expressed protein into proper VLP morphology, with a size range of 45-55 nm. This particular phenomenon is illustrated in figure 3, which provides evidence of successful VLP production from the recombinant HPV16-L1 protein expression in the Sf9 insect cell system.

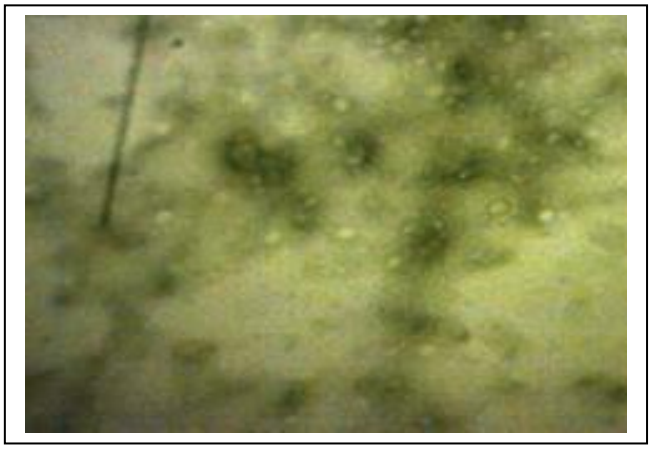

Figure 3. VLPs in electron microscopy

4. Discussion

The papillomavirus is known to cause more than 90% of cervical cancer cases, which is the fourth most common cancer among women worldwide and also one of the leading causes of death in women (15). Among papillomaviruses, types 16 and 18 are the most prominent which have a high risk of leading to cervical cancer and are transmitted through sexual contact (16). The capsid proteins of HPV, encoded by its L1 and L2 genes, are highly immunogenic and stimulate the humoral and cellular immune systems well (17). Based on this feature of these proteins, which also have the property of spontaneous aggregation and produce VLP, two types of vaccines, called Gardasil and Cervarix, have been prepared and approved by the World Health Organization and the FDA in more than 100 countries (18).

This vaccine is used to prevent cervical cancer, but can also prevent cancers caused by papillomaviruses. According to the protocol of the World Health Organization, these vaccines are administrated to

people aged 9-27 years old (19). One of the reasons for the lack of acceptance of this vaccine in middle-income countries is the high cost of its production process (20).

In the production of recombinant protein, the amount of production, the cost of the production process, the availability of the system, and most importantly, the accuracy and specificity of the produced protein are very important in terms of structure and function (21). The prokaryotic system is widely used for gene expression and the production of many heterologous proteins due to its simplicity, inexpensiveness, and availability (22). Various problems prevent efficient expression in prokaryotes, such as lack of post-translational processes, presence of pyrogen compounds in the bacterial wall, inactive and insoluble protein production, inability to provide biological activity, degradation by proteases, and instability of the produced protein (23). Therefore, some heterologous proteins need eukaryotic systems to be produced.

Baculoviruses have been used as a suitable tool for gene replacement for many years, such as the heterologous genes of many different organisms, including fungi, bacteria, animals, and viruses, especially their immunogenic genes in the genome of baculoviruses. The recombinant baculovirus-insect cells system has been widely used due to its safety in contrast to most mammalian virus systems in use and its large genome that is capable of accommodation of large exogenous genes. Recombinant baculovirus can grow and propagate in insect cell lines or larvae of several insect species. Moreover, it is available for further processing, modification, or targeting to appropriate cellular destinations and acting as authentic counterparts.

The resulting recombinant virus replicates in the appropriate cells and is capable of the expression of the localized gene and production of the target protein. These viruses proliferate in single-layer cultures as well as floating cultures of insect cells. The main example of this family is AcMNPV, which has the most applications as a recombinant virus in different studies (24).

MultiBac is an advanced baculovirus/insect cell protein expression system, which has been developed to express multiprotein complexes with several hitherto-inaccessible targets for research and industrial production (25). The innovation of a 2nd-generation Tn7-based system (Bac-2-the-Future), revealed that the new system is well-suited to various cloning systems and led to similar or higher titer and protein productivity in comparison to the currently existing systems (26).

SmartBac, which is a new baculovirus system, was also established for large-scale protein expression (27). Recently, the FlexiBAC system has been also introduced with advanced expression of both cytosolic proteins and secretory proteins which need proteolytic maturation. The complexity of this system makes it enable to reduce cloning steps and simplify protein production (28).

Previously, the baculovirus system was used in the production of several vaccines. For example, a baculovirus system was utilized for the production of an engineered vaccine for Porcine Circovirus type 2 and Mycoplasma (29). Furthermore, in a study, Hantaan VLPs were successfully formed by co-expression of Hantaan nucleocapsid protein and glycoproteins (Gn and Gc) in Sf9 cells for vaccine production. Moreover, in the aforementioned study, it was demonstrated that the VLPs can induce protection against viruses in animal models (30). In another study, CRISPR-Cas9 vectors showed capacity in protein glycosylation alteration in the baculovirus-insect cell system (31). Furthermore, the VLPs can be stored at –20 °C for a long time (>6 months) without losing their biological functions, which is a favorable feature for the VLP vaccine.

In this study, the Bac-to-Bac system produced by Invitrogen was used. Briefly, the Sf9 cell line was used to make recombinant baculovirus and produce recombinant L1 protein. The HPV-16 L1 gene was optimized and cloned in the pFastbac vector and DH10bac bacteria in baculovirus. Moreover, this gene

was confirmed by PCR and specific primers. The expression of the cloned gene in Sf9 cells was examined and confirmed by SDS-PAGE. Evaluation of the L1 gene or protein expression was confirmed by Western blotting using specific antibodies to this protein. Afterward, VLP purification, consisting of HPV-16 L1 gene expression, was performed in the Sf9 cell. Moreover, the VLP formation in the Sf9 cell was evaluated and confirmed by electron microscopy.

A long-term goal to accelerate the manufacturing capability of the baculovirus expression systems (BES) for VLPs should be to revolutionize in order to improve protein quality (such as optimization of folding, enhancement of glycosylation, and prevention of degradation) and stabilize protein quantity over longer periods. Although the BES has been broadly adopted for the basic construction of VLPs in different laboratories, upstream and downstream processing issues should conform to industry or, at least, basic research standards. In addition, different strategies for production and purification may affect the quality of VLPs to a great extent. In this section, many bioprocess considerations for the production of VLPs were briefly discussed.

This study showed that the production of this protein at the industrial level is optimized using a baculovirus/Sf9 system. The characteristics and advantages of this system made it a suitable candidate for large-scale protein synthesis. This expression system may be one of the main factors in the vaccine industry in the future.

Authors' Contribution

Conceive and design of the experiments: A. M.; writing of the paper: E. B. and A. M.; performance of the experiments: H. R.; Read and confirm of final version of article: all of authors; Revise: E. B., A. M.

Ethics

This research project has received the confirmation of the Ethics Committee of Golestan University of Medical Sciences with the code of IR.Goums.REC.1396.1

Conflict of Interest

The authors declare that this research was conducted in the absence of any relationships that could be construed as a potential conflict of interest.

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