EXPRESSION AND PURIFICATION OF NOVEL PEPTIDE-BASED RECOMBINANT PROTEINS TARGETING BREAST CANCER STEM CELLS

Istiqomah Agusta[1], Silvia Triwidyaningtyas[2], Budiman Bela[2,3], Mardiana Mardiana[†1], Septelia Inawati Wanandi[4,5]

[1]Doctoral Program in Biomedical Sciences - Faculty of Medicine Universitas Indonesia, [2]Research Center for Virology and Cancer Pathobiology (PRVKP) Faculty of Medicine, Universitas Indonesia, [3]Department of Microbiology, Faculty of Medicine, Universitas Indonesia, [4]Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Indonesia, [5]Molecular Biology and Proteomics Core Facilities - IMERI, Faculty of Medicine, Universitas Indonesia

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Corresponding Author
Name : Septelia Inawati Wanandi
Email : septelia.inawati@ui.ac.id

Abstract

Introduction: Peptides with specific affinity toward breast cancer stem cells (BCSCs), including GYSASRSTIPGK and GAIRIRLSEPLS, have gained interest in targeted cancer diagnostics; however, the low abundance of BCSCs often results in limited detection sensitivity. This study addresses this limitation by conjugating BCSC-specific peptides to self-assembling *Human Papillomavirus* (HPV16) L1 protein, resulting in virus-like particles composed of 72 pentamers that provide substantial signal amplification. In addition, the SpyTag–SpyCatcher system derived from the *Streptococcus pyogenes* CnaB2 domain provides a robust strategy to enhance peptide–protein stability. **Objectives:** This study aimed to express and purify three recombinant proteins as key components for assembling a BCSC-specific diagnostic construct: HPV16 L1–SpyTag, SpyCatcher–GYSASRSTIPGK, and SpyCatcher–GAIRIRLSEPLS. **Methods:** Recombinant genes encoding the target proteins were expressed in *Escherichia coli* (*E. coli*) BL21(DE3) following IPTG induction for 1–4 hours. Bacterial cells were harvested and lysed under native or denaturing conditions, and recombinant proteins were purified using Ni-NTA affinity chromatography. **Results:** SDS–PAGE analysis showed clear bands at the expected molecular weights for HPV16 L1–SpyTag (58.5 kDa), SpyCatcher–GYSASRSTIPGK, and SpyCatcher–GAIRIRLSEPLS (~15 kDa), with induction-dependent increases in band intensity. The SpyCatcher–peptide fusions were predominantly soluble and purified under native conditions, whereas HPV16 L1–SpyTag was largely insoluble and required denaturing purification. **Conclusion**: HPV16 L1–SpyTag and SpyCatcher–peptide fusions were successfully expressed and purified in *E. coli*, establishing a versatile platform with potential to enhance BCSC-targeted detection and treatment strategies in breast cancer**.**

Keywords: Breast Cancer Stem Cells, Recombinant Protein Expression, SpyTag–SpyCatcher System

Introduction

Breast cancer remains the most frequently diagnosed cancer and a leading cause of cancer-related mortality among women worldwide. In 2022, it accounted for an estimated 2.3 million new cases and more than 660,000 deaths globally. Asia bears the highest

absolute burden, and in Indonesia alone, over 66,000 new cases were reported, with most diagnosed at advanced stages associated with poor survival, resulting in an average of 64 breast cancer–related deaths per day.[2]

Breast cancer is increasingly recognized as a highly heterogeneous disease, recently characterized by multiple molecular subtypes based on hormone receptor and HER2 status: Luminal A (ER^+/PR^+, $HER2^-$, low Ki-67), Luminal B (ER^+, may be $HER2^+$ or $HER2^-$, higher Ki-67), HER2-enriched ($HER2^+$, ER^-/PR^-) and Triple-negative breast cancer (TNBC) ($ER^-/PR^-/HER2^-$). These molecular subtypes critically influence therapeutic effectiveness, metastatic ability, and clinical prognosis.[3] One of the proposed contributing key factors to breast cancer heterogeneity is the presence of breast cancer stem cells (BCSCs). BCSCs represent a small but biologically significant subset characterized by the capacities for self-renewal, differentiation, and long-term tumor propagation. BCSCs are thought to play a central role in malignant transformation and tumor stability, suggesting that selective targeting of BCSCs could enhance treatment efficacy and reduce relapse.[4]

Despite substantial variability in both the abundance and characteristics of BCSCs across different breast cancer cell lines and patient tumors,[5] Several molecular and phenotypic markers have been identified for BCSCs identification, such as CD44, CD24, and aldehyde dehydrogenase (ALDH). Various conventional techniques are commonly used to identify cancer stem cells (CSCs), including magnetic-activated cell sorting (MACS), fluorescence-activated cell sorting (FACS), flow cytometry, and Hoechst 33342 staining. [3,4] Nonetheless, these approaches present several limitations, including unintended cell activation, disruption of cellular biology, limited selectivity, and potential cytotoxic effects.[5] Moreover, since these BCSCs detection techniques often use samples of cancer cells that have detached from a primary tumor or metastatic lesion and entered the bloodstream, known as circulating tumor cells (CTCs), which are very low in patient samples, the effectiveness of CTC-based detection remains suboptimal.[6]

Recently, peptides for cell recognition, particularly in tumor-targeting applications, have attracted growing research interest. Peptides offer several advantages, including low molecular weight, high stability, minimal immunogenicity, low toxicity, and ease of synthesis and large-scale production.[7] Among the available strategies for identifying cell-specific peptides, the phage display peptide library technique is the most widely employed. It has been successfully applied to target various cancer cell types, including BCSC–specific peptides.[8]

To overcome the limited abundance of breast cancer stem cells (BCSCs) within tumors, enhancing the biological sensitivity of peptide-based targeting strategies is essential. One promising approach involves fusing BCSC-specific peptides to the L1 capsid protein of *Human Papillomavirus* (HPV16). The HPV16 L1 protein provides a multivalent display platform by self-assembling into virus-like particles (L1-VLPs) composed of 72 pentamers, enabling the simultaneous presentation of multiple targeting ligands, substantially

amplifying binding signals, and improving target recognition.[9] In addition, the stability and robustness of peptide-based detection systems can be further improved through site-specific bioconjugation strategies, such as the SpyTag/SpyCatcher system. This system forms a spontaneous and irreversible isopeptide bond, ensuring strong and stable peptide–protein linkage under diverse physiological conditions.[10]

In light of this, we designed a BCSC-specific diagnostic construct based on BCSC-specific peptides from a previous study, linked to L1 of HPV16 by the SpyTag/SpyCatcher system, produced the synthetic encoding genes, and cloned and transformed them into *E. coli* BL21 (DE3) for protein expression. This study aimed to express and purify three recombinant proteins, namely HPV16 L1–Spy Tag, Spy Catcher–GYSASRSTIPGK, and Spy Catcher–GAIRIRLSEPLS, as key components for assembling a construct.

Materials and Methods

Three *E. coli BL21 (DE3)* strains carrying recombinant plasmids encoding HPV16 L1–SpyTag, SpyCatcher–GYSASRSTIPGK, and SpyCatcher–GAIRIRLSEPLS, respectively, were employed for recombinant protein expression.

Protein Expression

Transformed bacterial strains were cultured overnight in 5 mL Luria–Bertani (LB) medium containing 100 µg/mL ampicillin at 37 °C with continuous shaking at 150 RPM (Bio-Rad). The overnight culture was subsequently transferred into Terrific Broth (TB) supplemented with 100 µg/mL ampicillin at a 1:20 inoculation ratio. The TB medium composition was 1% glycerol, 1.2% tryptone, 1.2% yeast extract, 0.34% KH_2PO_4, and 1.1% K_2HPO_4. Cultures were incubated at 37 °C with shaking (150 RPM, Bio-Rad) for 2 h before induction. Recombinant protein expression was induced using isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mM. Approximately 1 mL of culture samples was harvested at 1, 2, 3, and 4 h post-induction. All induction assays were performed at 37 °C. Key parameters for protein expression induction were strictly standardized across independent biological replicates to minimize batch-to-batch variability and improve reproducibility. These parameters include final IPTG concentration, starter culture-to-TB medium inoculation ratio, incubation temperature, pre-induction growth time, and shaking conditions.

Bacterial Cell Lysis

Bacterial pellets were lysed under native or denaturing conditions. For native lysis, pellets were resuspended in buffer A (500 mM Tris–HCl, pH 8.0; 100 mM NaCl; 1 mg/mL lysozyme), whereas denaturing lysis was performed using buffer containing 50 mM sodium phosphate, 6 M guanidine HCl, and 300 mM NaCl (pH 8.0). Lysis buffers were added at a

ratio of 10 mL per gram of wet pellet, and suspensions were incubated at room temperature for 1 h with gentle agitation (60 rpm, Bio-Rad). After centrifugation at 12,000 rpm for 10 min at 4 °C, the supernatants were subjected to Ni-NTA® (Qiagen) affinity purification according to the manufacturer's instructions.

Protein Purification

For native purification, the resin was washed with buffer containing 50 mM sodium phosphate, 500 mM NaCl, and 20 mM imidazole (pH 8.0), and recombinant proteins were eluted using 50 mM sodium phosphate, 500 mM NaCl, 250 mM imidazole, supplemented with 10% (v/v) glycerol (pH 8.0). Under denaturing conditions, the wash buffer contained 100 mM sodium phosphate, 100 mM Tris-Cl, and 8 M urea (pH 6.3), and proteins were eluted using 100 mM sodium phosphate, 10 mM Tris-Cl, 8 M urea, with 10% glycerol (pH 4.5–5.9). Purified proteins were analyzed using 12% SDS-PAGE to confirm expression.

SDS PAGE

The 12% resolving gel was prepared using Tris-HCl pH 8.8, 10% SDS, 10% APS, TEMED, 30% acrylamide, and distilled water, and allowed polymerize. The 4% stacking gel was then prepared with Tris-HCl pH 6.8, 10% SDS, 10% APS, TEMED, 30% acrylamide, and distilled water. Protein samples were mixed with 300 µL sample buffer, incubated at 4 °C, then heated on a heat block at 90–100 °C for 10 min and centrifuged at 12,000 rpm for 1 min. Protein markers were prepared by mixing the stained protein ladder with the sample buffer at a 1:3 ratio. Samples and molecular weight markers were loaded into the gel wells, and electrophoresis was carried out in 1× running buffer at a constant voltage of 120 V (maximum 400 mA) for 30–60 min, until the dye front reached the bottom of the gel. Following electrophoresis, the gel was stained in Page Blue® (Thermo Scientific) solution overnight, then destained until clear protein bands were visible. Gel images were documented using the Image Quant LAS-4000 system (GE Healthcare).

Results

Protein Expression

The three recombinant plasmids, each individually harboring genes encoding HPV16 L1–Spy Tag, Spy Catcher–GYSASRSTIPGK, and Spy Catcher–GAIRIRLSEPLS, were successfully transformed into *E. coli* BL21(DE3), a strain commonly used for recombinant protein expression.

HPV16 L1–Spy Tag, Spy Catcher–GYSASRSTIPGK, and Spy Catcher–GAIRIRLSEPLS protein induction were performed using 1 mM IPTG at different time durations to evaluate optimal expression conditions. SDS-PAGE analysis revealed a prominent protein band migrating between 48–63 kDa, which corresponds to the predicted molecular weight of the HPV16

L1–Spy Tag recombinant protein (HPV16 L1 size is 58,5 kDa, SpyTag is 13 amino acid residues) and 15 kDa corresponding to the expected size of Spy Catcher–GYSASRSTIPGK/GAIRIRLSEPLS) (Fig 1). Band intensity increased upon IPTG induction, confirming overexpression of the target proteins. Time-course analysis showed detectable expression as early as 1 h for HPV16 L1–SpyTag and 2 h for SpyCatcher–GYSASRSTIPGK/GAIRIRLSEPLS, with progressive increases at later time points. Densitometric analysis at 4 h indicated that HPV16 L1–SpyTag comprised approximately 10% of total cellular protein, while the SpyCatcher–peptide fusions accounted for 7–8% in *E. coli* BL21(DE3). These results collectively confirm efficient IPTG-induced expression of all target proteins in recombinant *E. coli* BL21(DE3).

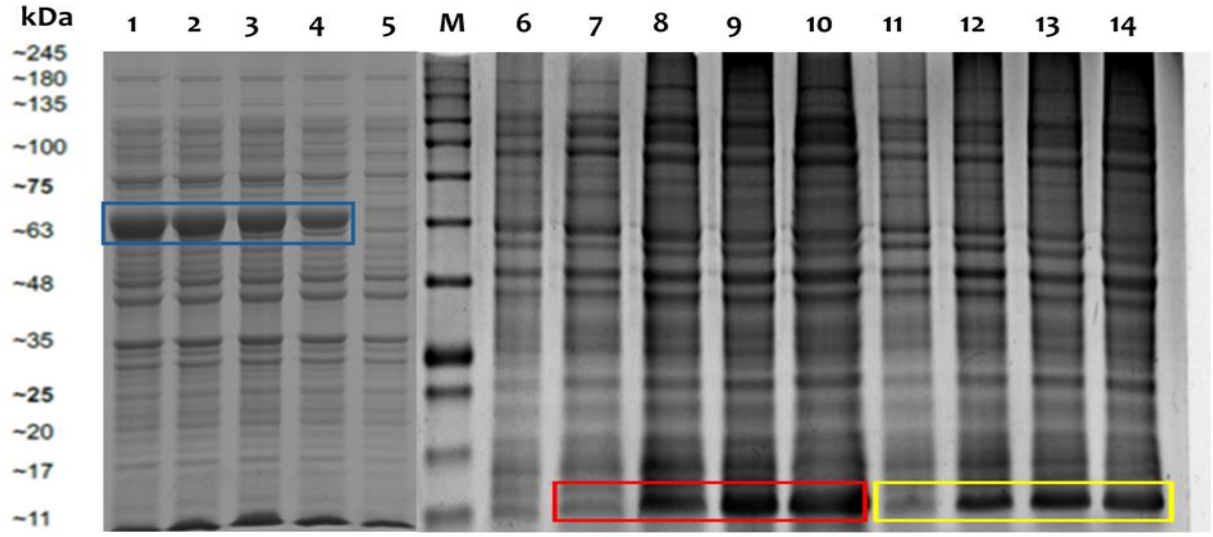

Figure 1. The expression of recombinant proteins in E.coli BL21 (DE3) induced with 1 mM IPTG and various lengths of induction time. M: marker, No, 1-4 HPV16 L1–Spy Tag expression was induced for 4,3,2,1 hour respectively, No, 7-10 and 11-14 were Spy Catcher–GYSASRSTIPGK (red box), and Spy Catcher–GAIRIRLSEPLS (yellow box) induction at 1, 2, 3, and 4 hour. No. 5, 6: wild type BL21 (DE3).

Protein Purification

Following lysis under native conditions, SDS-PAGE analysis revealed a strong band corresponding to the recombinant HPV16 L1–SpyTag protein (~58.5 kDa) predominantly in the pellet fraction (picture not shown). In contrast, when cells were lysed under denaturing conditions, a substantial portion of the protein was released into the supernatant (Figure 2). Purification under these conditions further confirmed successful recovery of the target protein, as indicated by the absence of detectable bands in the final (4th) wash fraction and the presence of the protein in the elution fractions: appearing clearly in fraction 1, increasing in intensity in fraction 3, and decreasing in fraction 5 (Figure 2).

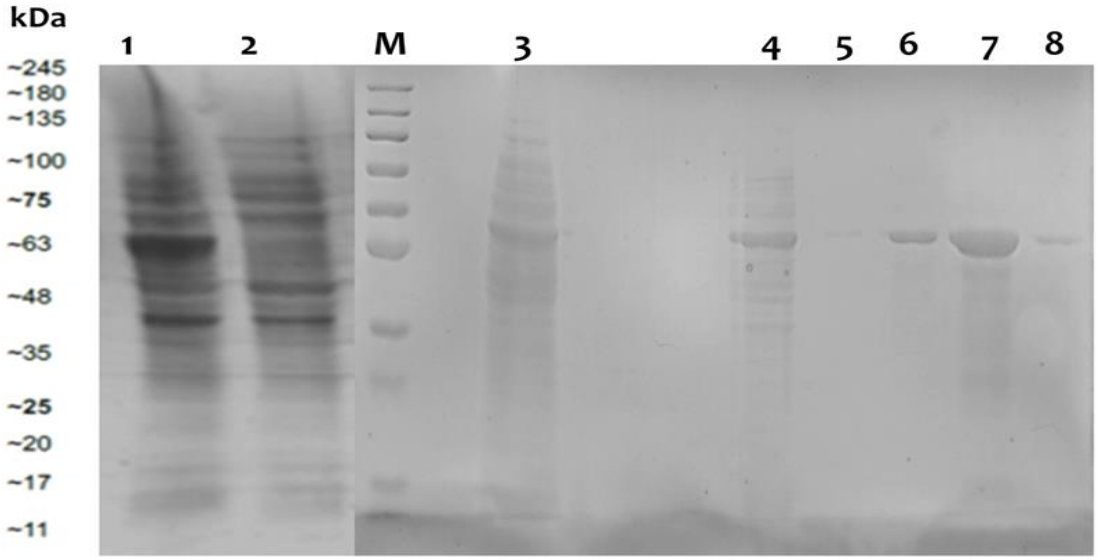

Figure 2. Cell lysis and purification of HPV16 L1–Spy Tag recombinant protein under denaturing conditions. M: marker, No. 1-2 were supernatant, and pellet produced by cell lysis, No. 4-5 were washing fraction 1 and 4, No. 6-8 were elution fraction 1,3 and 5, respectively. No. 3: HPV16 L1–Spy Tag expression after 4 hours IPTG induction.

In comparison, native lysis of *E. coli* BL21(DE3) expressing Spy Catcher–GYSASRSTIPGK and Spy Catcher–GAIRIRLSEPLS resulted in prominent protein bands of ~15 kDa in the supernatants, indicating better solubility of these constructs. Native purification also proceeded successfully, evidenced by the absence of a protein band in the fourth wash fraction and the consistent appearance of the target protein across elution fractions 1–5 (Figure 3). Although the purified protein yield was not quantified in this study, future experiments will include protein concentration measurements using the Lowry assay. Based on reported Ni–NTA purification efficiencies, the yield is anticipated to fall within the typical range of 1 to >10 mg/L of *E. coli* culture.

Protein Visualization using SDS PAGE

All recombinant proteins in this study were analyzed using 12% SDS-PAGE, with electrophoresis carried out at 120 V. For the high-molecular-weight proteins, HPV16 L1–SpyTag protein, gels were run for approximately 50–60 minutes, while the low-molecular-weight proteins, SpyCatcher–GYSASRSTIPGK and SpyCatcher–GAIRIRLSEPLS proteins required a shorter running time of about 40 minutes.

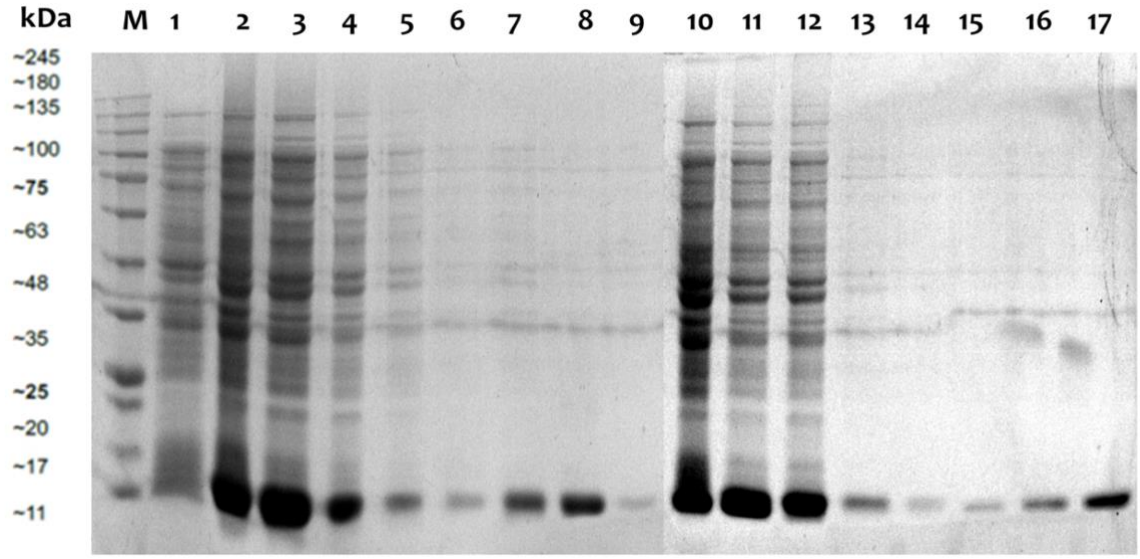

Figure 3. Cell lysis and native purification of SpyCatcher–GYSASRSTIPGK and SpyCatcher–GAIRIRLSEPLS recombinant proteins. M, marker; No. 1, wild-type *E. coli* BL21 (DE3). No. 2–9 correspond to SpyCatcher–GYSASRSTIPGK, and No. 10–17 correspond to SpyCatcher–GAIRIRLSEPLS. No. 2 and 10, induced protein expression; No. 3 and 11, soluble supernatant after cell lysis; No. 4 and 12, column flow-through; No. 5 and 13, wash fraction 1; No. 6 and 14, wash fraction 4; No. 7–9 and 15–17, elution fractions 1, 3, and 5, respectively.

Discussion

Recombinant proteins can be produced using a variety of expression systems in both eukaryotic and prokaryotic hosts; among prokaryotic platforms, *Escherichia coli* remains the most widely used and accessible system due to its rapid growth, ease of genetic manipulation, and low cultivation cost, making it a preferred choice for high-level recombinant protein expression.[11]

In this study, the recombinant HPV16 L1–SpyTag protein was successfully expressed in an *E. coli* BL21 (DE3) system, as evidenced by the appearance and increased intensity of a distinct ~58.5 kDa protein band on SDS-PAGE following induction with 1 mM IPTG, compared to the non-induced control. In many bacterial expression systems, induction of recombinant protein expression is achieved by adding isopropyl β-D-1-thiogalactopyranoside (IPTG), a non-metabolizable analog of allolactose that acts as a lactose analog that binds and inactivates the lacI repressor, thereby initiating transcription from the T7 promoter and activating downstream recombinant protein synthesis in BL21 (DE3) expression hosts.[12] The result aligns with previous reports in which HPV16 L1 protein was efficiently expressed in BL21 (DE3), confirming that the bacterial system is suitable for producing this capsid protein at high yield.[13] The addition of the 13-residue SpyTag peptide

at the C-terminus did not disrupt expression, indicating that the modification is structurally tolerated and does not impair translation efficiency. Overall, these findings suggest that the SpyTag fusion strategy is compatible with L1 capsid protein expression in bacteria and provides a promising platform for further assembly and conjugation studies.

As for the ~15 kDa SpyCatcher–GYSASRSTIPGK and SpyCatcher–GAIRIRLSEPLS fusion proteins, they were also successfully expressed in *E. coli*, induced by 1 mM IPTG. The expression of these proteins, which were synthetically designed and engineered *de novo* by incorporating breast cancer stem cell–targeting peptides into the SpyCatcher scaffold, represents an original outcome of this study. SpyCatcher is a protein partner of SpyTag that forms an irreversible isopeptide bond upon conjugation to another protein of interest in this study, HPV16 L1–SpyTag, thereby forming the SpyTag/SpyCatcher system. This system, which originated from the CnaB2 domain of the FbaB protein of *Streptococcus pyogenes*, offers a modular approach in bioconjugation strategy.[10] Both of its components can be genetically fused to any two proteins of interest, avoiding direct fusion's pitfalls.[14] Specifically for our study, the modular arrangement of BCSCs specific peptide – SpyCatcher – SpyTag- HPV16 -L1 was intended to enhance molecular stability between the HPV16 L1 protein and the peptides, which otherwise interact through an intrinsically weak covalent bond. To our knowledge, no previous reports have described SpyCatcher fused with BCSC-specific peptides GYSASRSTIPGK or GAIRIRLSEPLS, making this recombinant expression a novel contribution. The construct's stable expression in *E. coli* further supports its potential for scalable production and subsequent conjugation to HPV16 L1–SpyTag for targeted ligand display.

Recombinant proteins were purified using Ni-NTA® (Qiagen) resin. It is an affinity chromatography method using metal chelation, which selectively binds polyhistidine-tagged proteins via coordination with immobilized nickel ions, allowing efficient capture and elution of the target proteins under controlled conditions.[15,16] To facilitate recombinant protein purification, polyhistidine tags were genetically introduced by cloning the target genes into expression vectors encoding an N-terminal 6×His tag. Under native lysis conditions, the expressed HPV16 L1–SpyTag protein was predominantly retained in the insoluble fraction (pellet). These observations suggest that the expressed L1 protein mainly accumulated as inclusion bodies, a phenomenon commonly observed in high-level expression of viral capsid proteins in *E. coli*.[13] Proteins found in the soluble fraction of *E. coli* lysates decrease inversely with protein size (>100 residues).[17] High levels of recombinant protein expression in *E. coli* combined with a lack of ability to perform posttranslational modifications, leading to rapid translation and accumulation of partially folded or misfolded polypeptides, which often aggregate into inclusion bodies.[18]

To recover functional protein from inclusion bodies, chaotropic agents such as guanidine hydrochloride and urea are routinely employed to disrupt the hydrophobic and non-covalent interactions that stabilize these aggregates, resulting in complete release of the

denatured protein.[19] In this study, purification carried out under denaturing conditions using urea-containing buffers successfully yielded the target protein, demonstrating improved solubility. However, such treatment typically leads to loss of native protein conformation. Subsequent refolding of the solubilized protein is therefore necessary to restore its native structure and activity and is commonly accomplished by gradual removal of denaturants via dialysis or stepwise dilution into a native buffer.[20,21] The lower-molecular-weight proteins, SpyCatcher–GYSASRSTIPGK and SpyCatcher–GAIRIRLSEPLS fusion proteins, were successfully purified under native conditions, indicating that these constructs remained soluble in the bacterial cytoplasm without requiring denaturation. It also simplifies downstream processing and preserves their biologically relevant conformation.

Conclusions

Despite current HPV16 L1 based product: prophylactic HPV vaccines are predominantly manufactured using eukaryotic hosts such as yeast (*Saccharomyces cerevisiae*) and baculovirus-infected insect cells for its ability to yield properly folded and post-translationally modified L1 appropriate for VLP assembly, *E. coli* remains an attractive alternative expression system due to its rapid growth rate, simple genetic manipulation, low production cost, and suitability for large-scale protein production. All of our target proteins in this study were successfully expressed in *E. coli* BL21 (DE3) and purified in Ni-NTA® (Qiagen) under native (SpyCatcher–GYSASRSTIPGK and SpyCatcher–GAIRIRLSEPLS) and denatured conditions (HPV16 L1–SpyTag). The successful production of these proteins provides a critical foundation for the development of a sensitive diagnostic, therapeutic, and prognostic construct targeting breast cancer stem cells, thus improving the quality of breast cancer management.

Competing Interests

None declared

Acknowledgments

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