Reverse staining method of polyacrylamide gels by imidazole-zinc salts for detection and purification of L1 capsid protein in *E.coli*

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ABSTRACT

The human papillomavirus L1 major capsid protein (HPV L1), the basis of the current vaccines, self-assembles into virus-like particles (VLPs). Herein, we describe the expression and purification of recombinant HPV16 L1 in *E. coli* system. The L1 protein was generated in a fused form using an inducible expression system. The recombinant GST-L1 fusion protein migrated as a 82 kDa protein in SDS-PAGE. The L1 proteins formed inclusion bodies which were purified by Zn^{+2} reverse staining of sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) as a sensitive detection method. In western blotting, the existence of a 82 kDa band for GST-L1 protein was confirmed by anti-HPV16 L1 monoclonal antibody Camvir 1. The purified protein fraction was concentrated by ultrafiltration and dialyzed against PBS. This study has implications for the development of L1 protein purification as well as chromatographic separation used by other studies. Indeed, we could present a simple method to purify L1 protein in *E. coli*.

Keywords: HPV; L1 protein; Reverse staining; *E. coli* expression system

INTRODUCTION

The high-risk human papillomaviruses (HPVs) are encountered in more than 99% of cervical tumors and HPV16 is found in approximately 50% of the cases [1, 2]. The HPV capsid is composed of two structural proteins, L1 and L2. The major capsid protein, L1, which has a relative molecular weight of 55 kDa by reducing SDS-PAGE, is intrinsically able to self-assemble into virus-like particles [3, 4]. A number of studies have reported successful expression of HPV L1 proteins in bacterial systems, e.g., *Salmonella typhimurium* [5], *Escherichia coli* [6-8], *Shigella flexneri* [9], *Lactobacillus casei* [10] and *Lactococcus lactis* [11]. In these systems, the L1 proteins were further purified by chromatographic methods. In this report, we describe the expression of HPV16 L1 in the form of insoluble aggregations (Inclusion bodies: IBs) in BL21 *E. coli*. Then, the permanent Zn^{+2} reverse staining method was used for the detection and purification of L1 fusion protein in polyacrylamide gel. This method is a "negative staining" with translucent proteins and an unclear gel background. The reverse staining using imidazole and zinc salts for protein detection in electrophoresis gels was originally introduced in 1990 [12, 13]. The method is based on the selective precipitation of a white imidazole-zinc complex all along the gel except in the protein bands [14]. Imidazole-zinc reverse stain is known for its high sensitivity, ease of use and cost-effective feature. It was later adapted to allow high-sensitivity negative detection of nucleic acids and bacterial lipopolysaccharides [12]. In addition, the sequencing of protein bands only detected by reverse staining on the gel but not by Coomassie blue staining [15].

MATERIALS AND METHODS

Expression of HPV16 L1 protein

The HPV16 L1 gene (accession no. AJ313179.1, kindly provided by Prof. Martin Muller, German Cancer Research Center (dkfz),

Germany) was cloned into the *Sma*I/*Not*I sites of pGEX vector [Invitrogen, received as a gift from Prof. Martin Muller]. The pGEX vector enables the expression of a fusion protein with a GST-tag at N-terminus. The *E. coli* BL21 strain was transformed with pGEX-L1 and grown at 37˚C in LB broth supplemented with ampicillin and chloramphenicol. For the production of recombinant GST-L1 (rGST-L1), the cultures were grown to an optical density of 0.8-1 at 600 nm. L1 expression was then induced by the addition of isopropyl-b-D-thiogalactopyranoside (IPTG) to a concentration of 1 mM. After a 2 h induction period, the cells were harvested by centrifugation at 8000 rpm for 10 min at 4°C. Protein samples were analyzed by SDS-PAGE in 12.5% (W/V) polyacrylamide gel followed by staining with coomassie brilliant blue.

Purification and immunoblot detection of L1 protein from inclusion bodies

A 500 ml induced culture of pGEX-L1 transformed *E.coli* BL21 was harvested by centrifugation as above described and the cells were washed with phosphate-buffered saline (PBS), pelleted by centrifugation and stored at -20°C until required. Once thawed, the cell pellet was re-suspended in lysis buffer containing 1 M Tris-Cl (pH= 8.0), 0.5 M EDTA and 5 M NaCl and incubated for 16 h at -20˚C. The insoluble material was collected by centrifugation at 10000 rpm for 15 min at 4°C. The insoluble pellet was then washed about four times. The inclusion body (IB) which is predominantly L1 protein was pelleted by centrifugation at 10000 rpm for 10 min at 4°C. The solubilisation process of isolated IBs was carried out at room temperature for 1 h with gentle agitation. An imidazole-SDS-Zn reverse staining method was used for further purification as described by Richard J. Simpson [13]. Fig. 1 shows the schematic model of reverse staining. Proteins in each collected fraction were analyzed by reducing SDS–PAGE and Coomassie blue staining. For further confirmation, cell lysates were electroblotted onto protran nitrocellulose transfer membrane (Schleicher and Schuell Bioscience, Dassel, Germany) and the L1 protein was immunodetected using the anti-HPV16 L1 monoclonal antibody Camvir 1 (Prof. Martin Muller, German Cancer Research Center (dkfz), Germany) in combination with the secondary antibody, peroxidase conjugated rabbit anti-mouse IgG (Sigma). The reaction signals were detected using peroxidase substrate 3, 3'-Diaminobenzidine (DAB, Sigma). Then, the purified protein fraction was concentrated by ultrafiltration and dialyzed against PBS. Protein concentration was measured using BCA assay kit (Pierce, Rockford, USA). The recombinant protein was kept at -70˚ C until use.

RESULTS

HPV16 L1 expression in E. coli

The induction of HPV16 L1 expression in IPTG-treated cultures of pGEX-L1-transformed *E. coli* BL21 was analyzed by reducing SDS–PAGE. As shown in Fig. 2, a protein of approximately 82 kDa mass accumulated in cell lysates of IPTG-treated cultures. This protein was not detected in un-induced cultures and pGEX vector as a negative control. Indeed, rGST-L1 and rGST migrated as a 82 kDa and 27 kDa protein in SDS-PAGE, respectively.

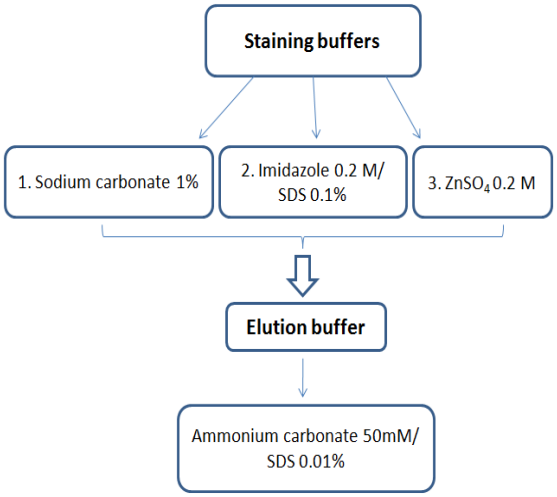

Figure1. Schematic model of reverse staining method

Purification of the insoluble L1 protein

The accumulation of prokaryotically expressed L1 in IBs facilitated its purification by the series of centrifugation and wash steps outlined under Materials and Methods. This resulted in a preparation that was observed by reducing SDS–PAGE as being partially pure (Fig. 3, lane 3). Reverse staining using Imidazole-$ZnSO_4$ resulted in further purification of L1. L1 was eluted with ammonium carbonate/SDS (Fig. 3, lane 4). The

identity of the purified protein as GST-L1 was confirmed by western blotting using the anti-L1 monoclonal antibody Camvir 1. The antibody reacted strongly to the 82 kDa protein, as well as to some lower molecular weight proteins which may result from the degradation of the full-length L1. Cell lysates from un-induced cultures were not recognized by this antibody.

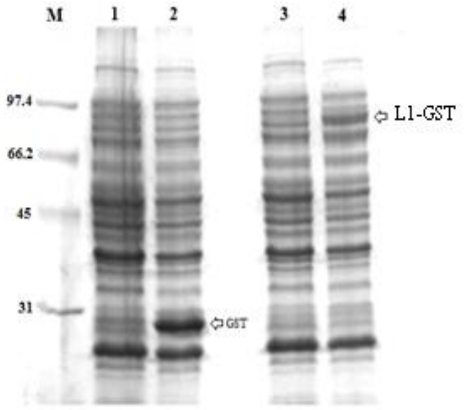

Figure 2. Analysis of the HPV16 L1 expression in *E. coli* BL21 cells from the recombinant expression vector pGEX-L1 by SDS–PAGE. The samples are the lysates of un-induced (lane 3) and induced (lane 4) cells. The pGEX vector was used as a negative control (lane 1: un-induced & lane 2: induced cells). The rGST-L1 and rGST migrated as a 82 kDa and 27 kDa protein in SDS-PAGE, respectively. Lane M: molecular weight markers.

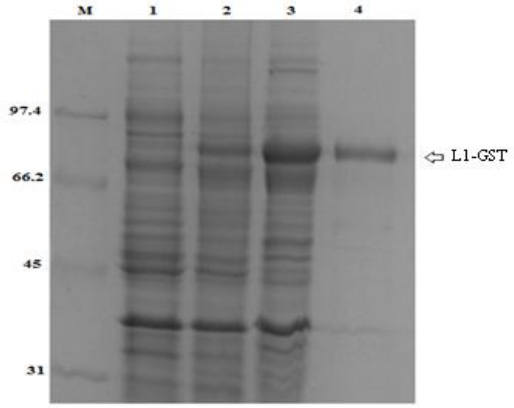

Figure 3. SDS–PAGE analysis of the protein contents in supernatant fractions obtained during the isolation of IBs from induced cultures of pGEX-L1-transformed *E. coli* BL21 (lane 3). The total cell extracts from un-induced cells or IPTG-induced cells were shown in lane 1 and 2, respectively. The purified HPV16 L1 in the fractions collected after reverse staining indicated in lane 4. Lane M: protein molecular weight marker.

DISCUSSION

The development of an efficient prophylactic vaccine against high-risk HPV types, especially HPV16, could greatly reduce the incidence rates of the disease associated with this virus. Clinical studies have demonstrated that either quadrivalent or bivalent vaccines composed of L1 VLPs have high efficacy against HPV infections. Recently, alternatives are sought for producing an effective and low-cost vaccine [16]. Several approaches for expressing the recombinant L1 from HPV16 have been tested using bacteria e.g., *E. coli*. Bacterial expression systems have proven to be limited in producing significant quantities of recombinant HPV 16 L1 due to inefficient purification and the risk of contamination with endotoxins. Herein, we showed a rapid protein elution from polyacrylamide gel bands at room temperature by reverse staining after preparation of HPV16 L1 in the form of insoluble inclusion bodies (IBs). An alternative procedure for preparing inclusion bodies was applied using salt reagents. IBs were isolated by centrifugation, washed with buffer and finally solubilized for further purification by reverse staining. Detection is accomplished by gel incubation in an imidazole solution, followed by incubation in a zinc salt solution to develop a negative stain pattern. The protocol described here gives a relatively straightforward purification strategy. The zinc/imidazole staining procedure for visualizing proteins in acrylamide gels is based on differential salt binding. The sensitivity of the method has been markedly improved by altering the composition of the precipitated salt to a complex of zinc and imidazole [17, 18]. Zinc-mediated protein fixation in the gel is fully reversible and the eluted biomolecules are neither chemically modified nor contaminated with organic dyes. This method can detect the bands corresponding to 5 ng of protein [14]. In the presence of SDS and 2-mercaptoethanol, the rGST-L1 migrated as a 82 kDa protein during poly acrylamide gel electrophoresis. The method used to purify L1 fusion protein has been scaled up for large scale production of this protein to obtain the necessary quantity required for a vaccine design against HPV.

CONCLUSION

The present study indicated the efficient production of L1 fusion protein in an *E.coli* system using IB isolation followed by reverse staining with Imidazole/Zinc.

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