

HPV16 E7-CT (gp96) fusion protein: Molecular cloning, expression and purification of a recombinant 6xHis-tagged protein in *E. coli*

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ABSTRACT

The development of a therapeutic vaccine against human papillomavirus (HPV) is important for the control of cervical cancer. E7 is the major transforming protein produced in cervical cancers, and therefore represents potential tumor-specific antigen that could be the target of immunotherapy for cervical cancer. Among different vaccine strategies, protein-based vaccines are capable of generating CD8+ T cell responses in vaccinated animals and humans. Recently, development of novel strategies that enhance protein vaccine potency is important for generation of effective cancer vaccines and immunotherapies. Heat shock proteins (HSPs) including Gp96 have been shown to act as potent immuno-adjuvant to enhance antigen-specific tumor immunity. Therefore, the HSP-based protein vaccines can be administered by fusing antigens to HSPs, *in vitro*. It has been known that the HSP fragments (e.g., N-/or C-terminal regions) as mini-chaperones are better choice for immunization. The most straightforward method to produce large amounts of recombinant protein suitable for a vaccine is to clone the gene into a prokaryotic expression vector and produce the protein in *Escherichia coli*. In current study, we describe cloning of the HPV16 E7 gene linked to C-terminal fragment of gp96, identification and purification of the resultant E7-CT (gp96) fusion protein for next usage as a potential vaccine candidate protein against HPV in a pre-clinical trial. The recombinant E7-CT (gp96) migrated as a 51 kDa protein in SDS-PAGE. In Western blot experiment, the existence of a 51 kDa band for rE7-CT (gp96) was confirmed by rabbit anti-His as well as mouse anti-HPV16 E7 monoclonal antibodies. The protein of interest was both in the insoluble and the soluble fraction; therefore, purification was performed under denaturing and native conditions by affinity chromatography on Ni-NTA resin using 6xHis-tag.

Keywords: HPV; E7; Gp96; cervical cancer; protein expression; *E.coli*

INTRODUCTION

Human papillomavirus (HPV) belongs to the *Papillomaviridae* family and is a small virus formed by 72 pentameric capsomeres, 55nm in diameter [1]. Human papillomavirus (HPV) has been recognized as the primary cause of cervical cancer, and HPV DNA can be detected in 90% of all cases [2]. HPV 16, which is the most prevalent strain, has been detected in >50% of cervical

cancer patients [3]. The HPV 16 genome is composed of three regions: 1) early genes (E1, E2, E4, E5, E6, and E7) that encode proteins responsible for transcription, replication and interaction with the host's genome; 2) late genes (L1 and L2) encoding mainly capsid proteins responsible for the differentiation of HPV types [4]. The gene products of two HPV16 early open reading frames (ORF), E6 and E7, are together sufficient to immortalize human keratinocytes *in*

vitro [5]. E7 is the major transforming protein produced in cervical cancers, and therefore represents potential tumor-specific antigen that could be the target of immunotherapy for cervical cancer and pre-cancer [5]. Among different vaccine types, the protein/peptide vaccines appear to be the most promising and effective [6]. The major advantage of protein-based vaccines is that in principle they offer a high level of safety. HPV-specific therapeutic vaccines based on proteins have been shown to be well-tolerated in humans, without the occurrence of significant adverse effects [7, 8]. On the other hand, the weak induction of cell-mediated immunity by these vaccines may present a major problem to induce an effective anti-tumor response in cervical cancer patients. Therefore, efforts have been made to enhance their potency through the use of adjuvants or specific antigen-delivery strategies [9]. Currently, the efficiency of protein vaccines has been increased through fusion with heat shock proteins (HSPs) [10]. Up to now, several HSP family members including Hsp70, Hsp90 and Gp96 have been utilized in cancer vaccine research. Gp96, an endoplasmic reticulum (ER) molecular chaperone, has been determined as a potent adjuvant for eliciting immune responses in vaccine development against different disorders [11-14]. Gp96 contains an N-terminal signal sequence, characteristic of the ER-targeted proteins and a carboxyl terminal KDEL sequence (Lys-Asp-Glu-Leu), which is a retention/retrieval signal from the golgi to the ER. The gp96 fragments (i.e., N- and C- terminal of gp96) as mini-chaperones are better choice for immunization [15, 16].

Since HPV cannot be propagated in tissue culture to purify oncoproteins, therefore, it will be based on production of recombinant proteins in viral or bacterial expression systems. Currently, the most straightforward method to produce large amounts of recombinant protein suitable for a vaccine is to produce the protein in *Escherichia coli*. In this study, HPV16 E7 gene fused to C-terminal fragment of gp96 was cloned into the expression vector pQE30. The recombinant protein of E7-CT (gp96) was expressed and

purified in *E. coli* for HPV vaccine design in future.

MATERIALS AND METHODS

Generation of the recombinant E7-CT (gp96) protein [rE7-CT (gp96)]

The C-terminal fragment of gp96 gene was obtained from the pQE30 vector containing the coding sequence of CT (gp96) protein [previously prepared by Bolhassani *et al.*, 2009; Ref. 17]. For obtaining pUC-E7-CT (gp96) construct, the DNA encoding the C-terminal fragment of gp96 [~ 768 bp] was cloned into the *Sma*I and *Sac*I sites of pUC-E7-NT (gp96) [previously provided in Molecular immunology and Vaccine Research lab]. Two pairs of primers (MWG Biotech GmbH, Germany) were designed (the *Sma*I and *Sac*I restriction sites have been shown in bold). The forward and reverse primers for amplifying the CT (gp96) [CT1 & CT2] was designed as follows:
CT1: 5' CGG CCC GGG GAG TCC TCT CCA TTT GTT GAA 3'
CT2: 5' AT GAG CTC GGT ACC CAG TTC ATC TTT TAC ATC 3'

PCRs were performed by PCR thermal cycler (Techne, USA) under standard conditions (95°C for 1 min; 63°C for 1 min and 72°C for 1.5 min; 30 cycles) and the products were separated on a 0.8 percent agarose gel. The bands corresponding to the expected PCR products size were gel purified (QIAquick gel extraction kit protocol, QIAGEN), digested with *Sma*I and *Sac*I and ligated into a similarly digested expression plasmid pUC-E7-NT (gp96). The ligation mixture was used to transform *E.coli* DH5 α strain. Plasmid DNA [pUC-E7-CT (gp96)] was purified from recombinant clones by an alkaline lysis method (Qiagen Plasmid Mini-Kit) verified by restriction enzyme digestion and sequenced using the dideoxy chain termination method on an automated sequencer. After confirmation of construct, the E7-CT (gp96) gene was excised from the pUC-E7-CT (gp96) vector by *Bam*HI and *Sac*I, gel purified and sub-cloned into the pQE-30 vector (Qiagen), which enables the expression of a fusion protein with a 6xHis-tag at N-terminus. The *E. coli* M15 strain was

transformed with pQE-E7-CT (gp96) and grown at 37°C in LB broth supplemented with 100 µg/ml ampicillin and 25 µg/ml kanamycin. For the production of recombinant E7-CT (gp96), the cultures were grown to an optical density of 0.6–0.8 at 600 nm and protein expression was induced with 1 mM IPTG for 2, 4 & 18 h at 37°C. Protein samples were analyzed by SDS-PAGE in 12.5% (W/V) polyacrylamide gel (SDS gel apparatus; BioRad), followed by staining with coomassie brilliant blue. For western blot analysis, the proteins resolved on the gel were transferred onto protran nitrocellulose transfer membrane (Schleicher and Schuell Bioscience, Dassel, Germany). The membrane was pre-equilibrated with TBST solution [10 mM Tris-HCL (pH 7.4), 150 mM NaCl, and 0.1% Tween 20] containing 2.5% bovine serum albumin (BSA) overnight and then reacted with anti-His antibody (QIAGEN) and/or mouse anti-HPV16 E7 (monoclonal antibody, USBiological) for 2 h at room temperature. After three washes with TBST, the membrane was incubated with anti-mouse IgG-HRP (1:2000, Sigma) for 1.5 h at room temperature. The immunoreactive protein bands were visualized using peroxidase substrate 3, 3'-Diaminobenzidine (DAB, Sigma).

Purification and assay of the recombinant protein

The recombinant E7-CT (gp96) protein was purified by affinity chromatography [fast protein liquid chromatography (FPLC)] on Ni-NTA resin column (nickel-nitrilotriacetic acid) using 6xHis-tag according to the manufacturer's instructions (Qiagen protocol). Purification of recombinant E7 protein was done under native and denaturing conditions. Briefly, the recombinant protein can be purified by Ni-NTA chromatography based on the interaction between a transition Ni^{2+} ion immobilized on a matrix and the histidine side chains. Following matrix washing, 6x Histidine-tag fusion protein is eluted by adding free imidazole or EDTA or by reducing the pH. The purified proteins showed higher concentrations in native method than denaturing, but with additional bands. Therefore, in native method, an imidazole-SDS-Zn reverse staining method was

used for further purification. Then, the purified protein fraction was concentrated by ultrafiltration (Amicon) and dialyzed against PBS (dialysis membrane, MWCO: 3.500 kDa, Spectrum). Protein concentration was measured using BCA assay kit (Pierce, Rockford, USA). The recombinant protein was kept at -70°C until use.

RESULTS

Cloning of E7-CT (gp96) in *E. coli* expression system

For preparation of the E7-CT (gp96) fusion gene, the C-terminal of gp96 [~768 bp] was generated by PCR and inserted into the cloning vector pUC-E7-NT (gp96). Plasmid DNA sample [pUC-E7-CT (gp96)] was purified by an alkaline lysis method and then sequenced. Then, the E7-CT (gp96) gene sub-cloned into the expression pQE-30 vector, which enables the expression of a fusion protein with a 6xHis-tag at N-terminus. Figure 1 shows the DNA analysis of E7-CT (gp96) using PCR and digestion by the appropriate restriction enzymes (*Bam*HI/*Sac*I).

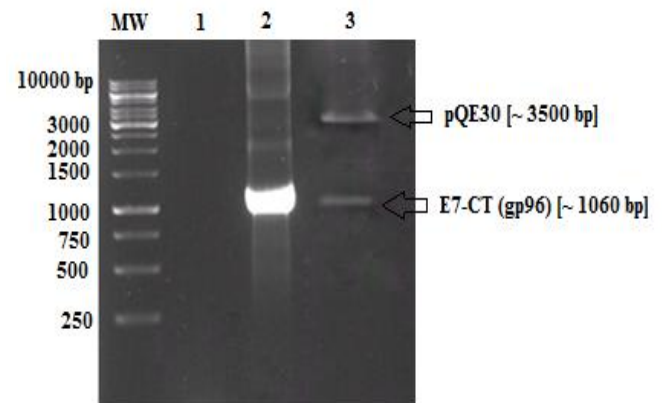


Figure 1. The confirmation of PCR (2) and Digestion (3) of E7-CT (gp96) fusion gene [~1060 bp] in pQE30; Molecular weight marker (1 kb, fermentase co.) is indicated as MW in figure. H₂O was used as a negative control in PCR (1).

Expression and purification of the recombinant E7-CT (gp96) protein

The recombinant E7-CT (gp96) protein was expressed and purified in *E. coli*. The rE7-CT (gp96) migrated as a 51 kDa protein in SDS-PAGE (Figure 2A). Western blot analysis was

performed using anti-His/or anti-E7 antibody to ensure the proper expression of rE7-CT (gp96). A specific band with expected size (51 kDa) was detected in the induced bacterial lysate (Figure 2B), although a few non-specific bands were also observed in both samples before and after IPTG induction with anti-His antibody. The additional bands were disappeared using anti-E7 monoclonal antibody due to further specificity (Figure 2B). Purification of rE7-CT (gp96) was done under denaturing and native conditions using affinity chromatography as shown in Figure 3. Purified samples were also confirmed by western blot analysis (Figure 2B). The obtained recombinant protein using native method was further purified by reverse staining method [data not shown]. Total protein concentrations of the samples were measured using a bicinchoninic acid (BCA) protein assay kit for next experiments.

DICSSUSSION

Therapeutic vaccines targeting E6 and E7 may provide the best option for controlling HPV-associated malignancies. Most studies focus on E7, because it is more abundantly expressed and better characterized immunologically. Furthermore, its sequence is more conserved than that of the E6 gene [18]. Among different vaccine strategies, protein-based HPV vaccines have been tested in pre-clinical/or clinical trials. Clearly, generation of humoral and cellular immune responses is influential parameters for designing ideal protein vaccine. The potency of HPV16 E7 peptide/protein-based vaccines may be further enhanced through the use of adjuvants e.g., heat shock proteins (HSPs) [19, 20]. Heat shock proteins have been described as important immuno-stimulatory molecules to enhance antigen-specific tumor immunity. It was demonstrated that the ability of gp96 to facilitate cross-presentation of chaperoned antigen by interacting with CD91 which leads to specific potent T cell response has been conserved between the amphibian *Xenopus* and mammals [15]. However, Heat shock proteins-based

vaccines are a novel approach with a promising role in cancer therapy.

In current study, the recombinant HPV16 E7 linked to C-terminal of gp96 was expressed and purified in *E.coli* expression system. In general, production of proteins, whether for biochemical analysis, therapeutics or structural studies, requires the success of three individual factors: expression, solubility and purification. Although, a number of expression hosts are available for protein production, the standard in the field still remains *E. coli*. Indeed, *E. coli* has significant benefits of cost, ease of use and scale, all of which make it suitable to use ways to generate heterologous proteins. Improving the generation of recombinant proteins in *E. coli* commonly involves changing some of the expression conditions: factors such as temperature, changes in the *E. coli* expression strain, different promoters or induction conditions, and co-expression of molecular chaperones and folding modulators [21]. Nowadays, there are a number of common fusion tags that are used to increase the efficiency of protein purification in *E. coli*, e.g., hexa-histidine (His6) tag.

Herein, the HPV16 E7-CT (gp96) gene was cloned into the bacterial expression vector (pQE-30). Electrophoresis analysis of IPTG-induced cell lysates showed the presence of a prominent protein band that was not detectable in non-induced cell lysates. In the presence of sodium dodecyl sulphate and 2- mercaptoethanol, the E7-CT (gp96) protein migrated as a 51 kDa protein during poly acrylamide gel electrophoresis. Based on SDS-PAGE analysis, the expression of E7-CT (gp96) protein was found to be almost disturbed between soluble and insoluble fractions. Therefore, the purification of rE7-CT (gp96) could be done using affinity chromatography under native and denaturing conditions. The method used to purify E7-CT (gp96) 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.

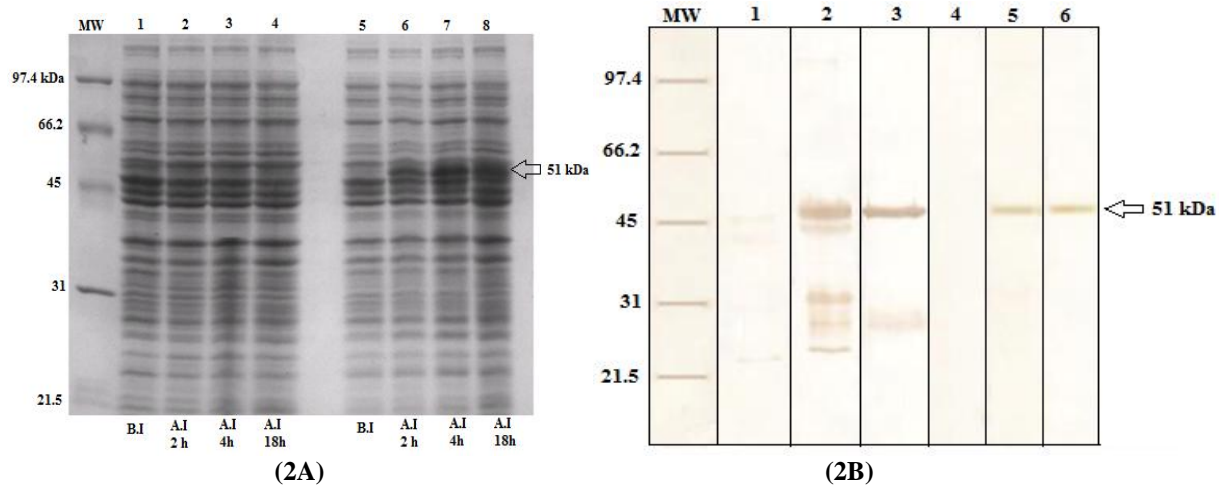


Figure 2. Expression of E7-CT (gp96) protein in *E. coli* expression system: (A) The E7-CT (gp96) gene was cloned into the pQE-30 expression vector. Analysis of recombinant protein was done by SDS-PAGE and Western blotting. The recombinant HPV16E7-CT (gp96) protein was run on a polyacrylamide gel electrophoresis (12.5%) and stained by coomassie blue. The weight of the molecular mass markers (MW: Premixed protein molecular weight marker: 14.4-97.4 kDa) is indicated as MW in figure. Lysate of un-induced (B.I./Before Induction) and isopropyl-beta-D-thiogalactopyranoside (IPTG, 1 mM) induced culture of *E. coli* (A.I./After Induction) in different times (2, 4, 18 hr) are shown on line 1-4 for empty vector (pQE30) and 5-8 for pQE-E7-CT (gp96), respectively. (B) Western blot analysis for the recombinant E7-CT (gp96) protein using anti-His antibody (1: B.I., 2: A.I., 3: purified protein) and anti-E7 antibody (4: B.I., 5: A.I., 6: purified protein). The existence of 51 kDa band for rE7-CT (gp96) is shown.

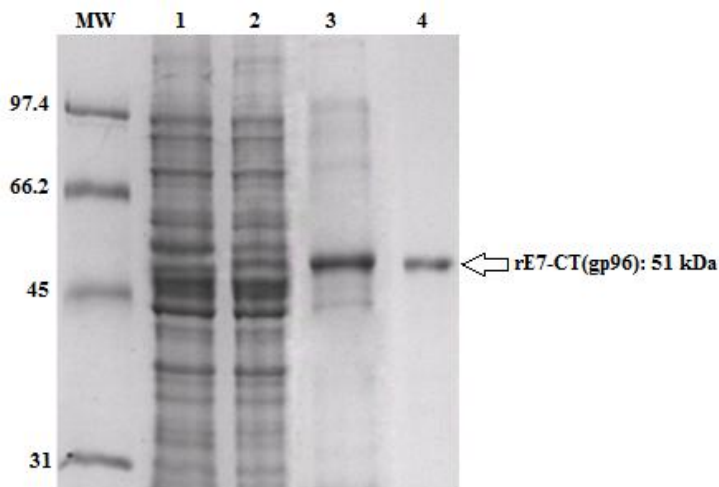


Figure 3. Purified recombinant protein by affinity chromatography using Ni-NTA resin: SDS-PAGE electrophoresis of lysate of un-induced (lane 1) and IPTG (1 mM) induced (lane 2) culture of *E. coli* and the recombinant protein purified by affinity chromatography using Ni-NTA resin under denaturing (lane 3) and native (lane 4) conditions.

CONCLUSION

The present study indicated the efficient production of E7-CT (gp96) protein in a

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prokaryotic expression system. This recombinant protein will be used for vaccine design against HPV in future.

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