Original Article

Cloning and expression of hepatitis E virus ORF2 as a vaccine candidate

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ABSTRACT

Introduction: Hepatitis E virus (HEV) is a fecal-oral transmitting virus which causes a chronic liver disease. ORF2 is an immunogen capsid protein of HEV that has been proposed to be used for Hepatitis E vaccine design. It is a 660-amino acid protein which includes an immunogenic region (residues 112-607). This protein has been expressed in complete and truncated forms, using different expression vectors such as pRSET-C, pMAL, pSG and baculovirus expression systems. *Escherichia coli* BL21 which is used as a host for protein expression was utilized as a host for pET26b vector in this study. We evaluated the expression of ORF2 as Hepatitis E vaccine candidate in presence of several IPTG concentrations. **Methods:** First, *orf2* gene was sub-cloned into a pET26b vector which adds a C-terminal His-tag to the coding sequence. The procedure was confirmed by gel electrophoresis and double digestion. Subsequently, the recombinant pet26b-ORF2 was transformed into *E. coli* BL21 cells for protein expression and the resulted recombinant protein was analyzed by Bradford assay, SDS-PAGE and Western blotting. **Results:** SDS-PAGE and Western blotting confirmed the proper protein expression while there was no significant difference among the expressions of protein in presence of different IPTG concentrations. **Conclusion:** The expression of HEV ORF2 protein was successfully performed in *E. coli* BL21 and it showed that ORF2 can be expressed in presence of different concentration of IPTG with no significant difference in protein expression. The produced recombinant protein could be used in further vaccine-related studies and also its expression can be studied at several different temperatures.

KEYWORDS: Hepatitis E virus, ORF2, Vaccine.

INTRODUCTION

Hepatitis E virus (HEV) is one of the five types of hepatitis viruses that lead to liver disease. HEV is a non-enveloped virus which belongs to *Hepeviridae* family and Orthohepevirus genus. It is a capped, polyadenylated, single-stranded and positive sense RNA with approximately 7.2kbp length. This viral RNA encodes 3 overlapping open reading frames (ORFs) [1, 2]. Hepatitis E infection caused by HEV is a water-borne disease and commonly emerges as a self-limiting disease that rarely leads to death, except in patients who take immunosuppressive drugs after graft transplantation [3]. It causes acute hepatitis in developing countries and areas with low level of hygiene while it is rarely reported in the developed countries [4]. People who had already chronic liver disease, pregnant women and older people show severe cases of acute hepatitis E [5-7]. HEV includes an antigenic capsid that stimulates the immune responses. ORF2 is a protein unit that participates in HEV capsid construction. It has the potential to stimulate immune response and has been proposed to be used in HEV vaccine research and design [8]. The molecular weight (Mw) of ORF2 is about 72 kDa and consists of 660 amino acids [8]. This antigenic protein has been expressed in a vast spectrum of hosts in various vectors. Insect cells has been used as an expression host for ORF2 with Baculovirus which have produced proteins with diverse Mw, from 30 to 100 kDa in which a 72kDa protein has been reported to be the most abundant [9].The ORF2 is expressed in other hosts as well. For instance, recombinant Baculovirus containing a full length *orf2* has been transduced into S10-3 human hepatoma cells and the

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over expression of the 72 kDa ORF2 has been achieved [10]. This protein has also been expressed at high levels by inserting the *orf2* in Semliki Forest Virus (SFV) replicon andBHK-21 cells as the host[11].Moreover, *Lactococcuslactis*, strain NZ3900 has also been used to overexpress ORF2 protein on the cells surface that could potentially be used as an oral vaccine against Hepatitis E infection[12].A particulate antigenic form of recombinant ORF2 capsid protein, designated as p239, was used as vaccine, licensed and launched in China recently [13].
In this study, we used pET26b as an expression vector in order to overexpress ORF2 with different concentrations of the inducer. The pET26b is a 5.4kb expression vector containing PelB sequence, for secretion of the expressed protein into the periplasmic space. It also adds a His-tag to the C-terminal of the protein of interest and uses a T7 promoter [14].

MATERIALS and METHODS

Cloning of *orf2*

The sequence of ORF2 (GenBank accession number M80581.1) was obtained from NCBI database. The recombinant pUC57-ORF2 vector containing *orf2* gene was propagated and extracted from *Escherichia coli* BL21.The *orf2* gene was excised from pUC57-ORF2 plasmid with *Eco*RI/*Not*I restriction enzymes and sub-cloned into the same restriction sites in pET26b, which added a C-terminal His-tag to the coding sequence in order to facilitate the protein purification. The ligated product was observed on agarose gel and the cloning confirmation was conducted with double digestion using *Eco*RI/*Not*I restriction enzymes. The digested fragment was excised and evaluated on 1.2% agarose gel.

Protein expression, extraction and purification

Recombinant pET26b-ORF2 was transformed into *E. coli* BL21 (DE3) for protein expression. The overnight culture was inoculated into fresh Luria-Bertani (LB) medium (10 g bactotryptone, 5 g yeast extract and 10 g NaCl per liter of water), with 50 µg/ml ampicillin and the culture was grown at 37˚C until the log phase reached. The expression of the recombinant protein was induced by 1, 0.6 and 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) concentrations, and the cells were grown in 37˚C for 3 h. The cells were then harvested by centrifugation at 6,000 g for 20 min. The cell pellet was suspended in PBS. While incubated on ice for 30 min, the bacterial cells were sonicated for 10 seconds at 200 W (10 times) using UP200Ht hielscher sonicator (Hielscher Ultrasonics, Germany) and the bacterial pellet was harvested by centrifugation at 10,000 g for 20 min. The soluble (supernatant) fraction was analyzed by SDS-PAGE and Bradford assay and the insoluble part (pellet) was re-suspended in lysis buffer (300mM NaCl and 50mM NaH2PO4). The supernatant was harvested and supplies were analyzed by SDS-PAGE. The expressed protein was purified by affinity chromatography using Ni-NTA system (QIAGEN, Germany). The amount of 2 ml of Ni-NTA resin was used for each 1 L of the supernatant, containing His-tag-ORF2 and incubated at 25°C for 1 h. After that, the mixture was loaded on the column (Merck Milipore, Germany). The washing buffer (40 mM Imidazol, 50 mM NaH2PO4, 500 mM NaCl and 4 M Urea) with pH 8 was applied. In order to elute the His-tag-ORF2 from the column, an elution buffer (500 mM Imidazol, 50 mM NaH2PO4, 500 mM NaCl and 4 M Urea) with pH 8 was added.

SDS-PAGE and Western blotting

The purified proteins were applied on 15% SAS-PAGE for analysis along with a protein size marker (GenScript Protein ladder, 5 to 270kDa). The proteins were then blotted to a PVDF membrane and anti His-tag antibody was used in order to detect the purified His-tag-ORF2 protein.

RESULTS

Cloning of *orf2* into pET26 expression vector

Following transformation of *E. coli* BL21 with pET26-ORF2, positive colonies were selected on LB Agar containing Kanamycin antibiotic by colony-touch PCR. PCR was carried out using designed primers and followed by electrophoresis on 1.2% agarose gel which revealed an amplicon with 755bp size for ORF2-His-tag (Fig. 1A). Moreover, for more confirmation, recombinant vector was digested by *Eco*RI/*Not*I and loaded on 1.2% agarose gel (Fig. 1B). Moreover, the integrity of pET26b-ORF2 construct was verified by amplification and nucleotide sequencing, using designed primers.

Protein expression analysis

Transformed *E. coli* BL21was inoculated into LB Broth and the protein expression was induced by 1, 0.6 and 0.4 mM IPTG, separately. Thereafter, periplasmic proteins were extracted and purified. The soluble fraction of the sonicated and centrifuged cell suspension was analyzed and confirmed by Coomassie brilliant blue staining and SDS-PAGE (using GenScript Protein ladder). The Mw of ORF2 was approximately27kDa.As shown in Fig. 2, different concentrations of IPTG had no significant effect on ORF2 expression.
In order to confirm the presence of the purified his-tagged ORF2 recombinant protein, Western blotting was conducted using an anti His-tag antibody conjugated with HRP which uniquely detected the expected recombinant protein with proper Mw (Fig. 3).

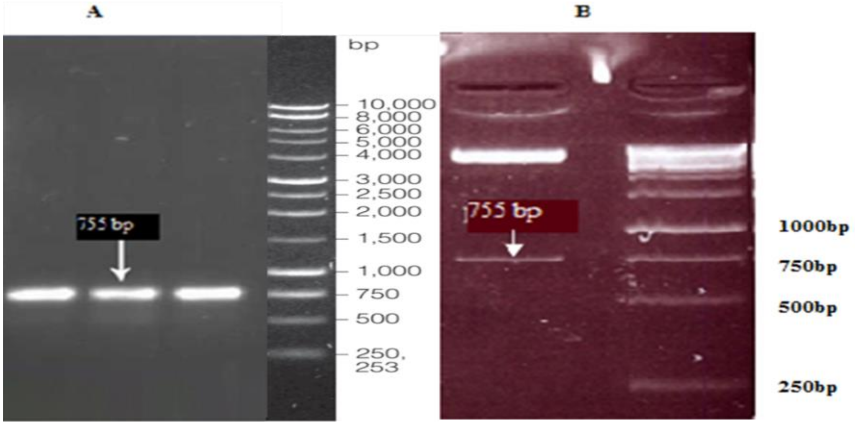

Fig. 1. A) Colony-touch PCR, confirming positive colonies harboring pET26-ORF2. B) Two distinct bands confirmed gene cloning in which the band with 755 bp is the digested out ORF2 coding fragment.

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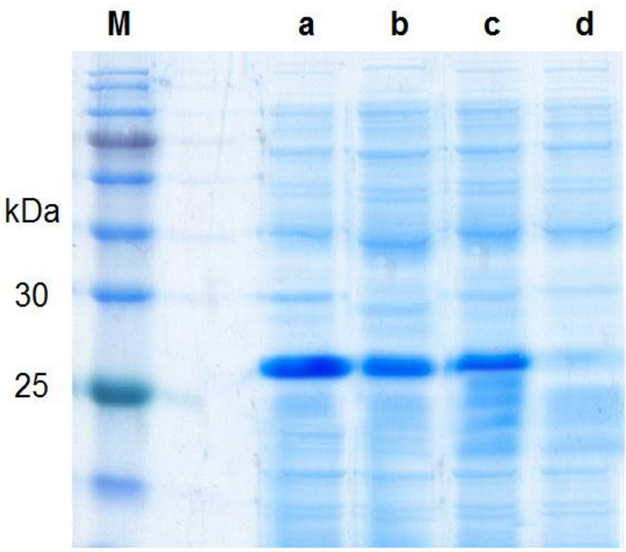

Fig. 2. Analysis of protein expression in different concentrations of IPTG by SDS-PAGE.The overexpressed protein, induced with 1 mM (a), 0.6 mM (b) and 0.4 mM (c) IPTG. Uninduced protein expression (d) demonstrates low level of protein expression. M depicts protein size marker.

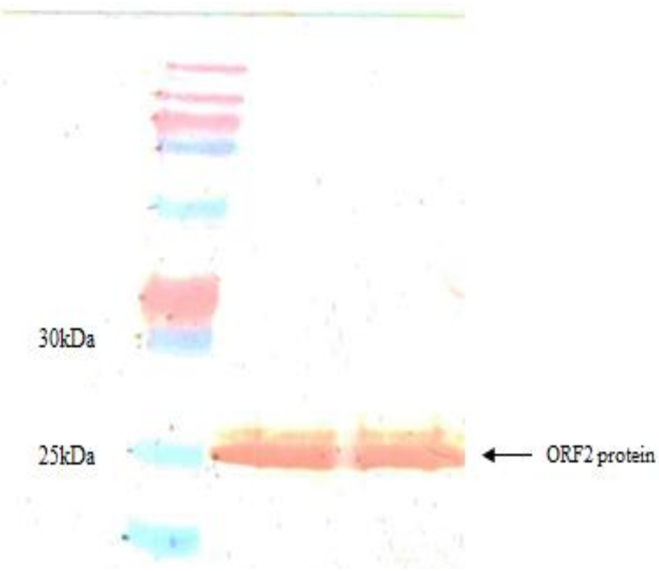

Fig. 3. Western blotting of the expressed proteins. The 26kDa blots confirmedORF2 expression that was specifically detected by the anti his-tag conjugated antibody.

DISCUSSION

Hepatitis causes inflammation of liver cells that may be symptomatic or asymptomatic. Viruses are the most common causes of hepatitis. Hepatitis E Virus (HEV) is one of the most prevalent sources of sporadic hepatitis.HEV was identified for the first time in 1980,afterwardsits genome was characterized in 1991 and it was recognized as a water-borne and fecal-oral transmitting infection in the developing and poor hygiene countries[15, 16]. Among 3 ORFs of its single-stranded positive sense RNA, ORF2 encodes its capsid protein and is essential for binding of HEV to the host cells and can stimulate the immune system for eventually protective antibody production[17].Therefore, HEV vaccine researches have focused onORF2-based vaccines[17].*In vitro* proliferation of HEV is hard to accomplish; thus, recombinant protein studies have been developed to support HEV vaccine designs. For instance, recombinant ORF2 has been expressed in a wide range of hosts such as vaccinia virus[18],tobacco[19] and Huh-7 cells[17].Moreover, *orf2* gene has been cloned and expressed in recombinant baculovirus in order to be used in diagnosis and immunogenic applications [20, 9]. Interestingly, transgenic tomato has also been used to express *orf2* in its fruits and leaves. The expressed ORF2 in this fashion can potentially stimulates the immune system while being used as an oral vaccine[19].

E. coli BL21 is one of the most commonly used expression hosts[17]. In this study, a recombinant His-taged-ORF2 protein was expressed in *E. coli* BL21. The protein expression was induced by different concentrations of IPTG, separately. The apparent expressions of the recombinant protein (with the theoretical Mw and pI of 27kDa and pI 5.11, respectively) were almost the same in presence of 1 mM IPTG and lower. The His-tag-ORF2 protein could be successfully purified by Ni-NTA system as a single band. SDS-PAGE, Western blotting and nucleotide sequencing confirmed the integrity of the expected overexpressed ORF2 protein. Therefore, following further optimizations in terms of time of incubation with the inducer and proper endotoxin removal procedures, this procedure can be used in subsequent HEV vaccine researches.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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