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ORIGINAL ARTICLE

Expression and purification of MERS-CoV envelope protein, an essential viroporin, using the baculovirus expression system

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ABSTRACT

Background and Objectives: The causative agent of Middle East Respiratory Syndrome (MERS) is a zoonotic Coronavirus (MERS-CoV) identified in Saudi Arabia in 2012. The envelope (E) protein of MERS-CoV is a small viral protein which plays several essential roles during virus replication. To facilitate study of the structure and function of the E protein, recombinant MERS-CoV E protein was expressed using the baculovirus expression system.

Materials and Methods: A recombinant E open reading frame including an 8-histidine tag at the amino terminus was designed and cloned into a baculovirus transfer vector. Following construction of a recombinant virus insect cells were infected and the expression of the E protein assessed by SDS-PAGE and Western blotting.

Results: Recombinant E protein, tagged at the N-terminus with a polyhistidine sequence, with a molecular mass of 10.18 kD was identified by Western blotting with an anti-His antibody. Following large scale infection E protein was released by detergent mediated lysis of infected cells and purified by Immobilized Metal Ion Affinity Chromatography (IMAC).

Conclusion: Purified full length recombinant MERS-CoV E protein can be isolated by IMAC and is suitable for further functional, biophysical or immunological studies.

Keywords: Middle east respiratory syndrome; Coronaviruses; MERS-CoV; Envelope protein; Baculovirus; Insect cells; Immobilised metal-affinity chromatography

INTRODUCTION

Coronaviruses (CoVs) are the largest RNA viruses, having a 27-31 kb positive sense single stranded genome (1, 2). CoVs can infect a wide range of animals and humans causing different types of pathology including respiratory, intestinal and neurological diseases. MERS-CoV is one of seven coronaviruses that infect humans including hCoV-OC43, hCoV-HKU1, hCoV-NL63, hCoV-229E, SARS and SARS-CoV-2. The first four members are responsible for mostly mild upper respiratory disease while the last two members and MERS-CoV account for zoonotic outbreaks and a recent pandemic (3, 4). MERS-CoV was first reported in Saudi Arabia in 2012 as a novel coronavirus belongs to betacoronaviruses lineage C (5, 6). It has since appeared in many other countries in the world including Korea, Jordan and Europe causing about 2591 laboratory confirmed cases and about 886 deaths, a case fatality ratio of 34.4%, mostly in patients with underlying diseases (http://www.emro.who.int/health-topics/mers-cov/mers-outbreaks.html). The clinical features of MERS-CoV are characterized by fever, dry cough,

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dyspnea, acute respiratory distress and multiorgan failure especially with comorbidities including diabetes, hypertension and cardiovascular and chronic renal diseases (7-9). Most of the human-to-human transmission of MERS-CoV has been shown to be by a nosocomial route (10). Index zoonotic cases may be associated with contact with dromedary camels as these have been reported as a MERS (11).

Alsalihi and her colleagues (2019) have reported MERS-CoV infection among Iraqi individuals and camels. Most of the Iraqi variants were grouped in clade B and were closely related to Saudi Arabia and Jordan variants in the phylogenetic tree (12).

The MERS-CoV genome is 30.1 kb with the 5' two thirds of the genome coding for 16 nonstructural proteins required for viral replication. The 3' third of the genome encodes four structural proteins including spike (S), envelope (E), membrane (M) and nucleocapsid protein (N) (5).

Among the structural proteins, the envelope protein plays an important role during virus assembly and packaging and has viroporin characteristics (13-15).

MERS-CoV E is a short protein consisting of ~82 amino acids which is expressed abundantly in infected cells but found at only low levels in the virus particle. MERS-CoV-E has only one transmembrane domain and is reported to form homo pentamers, like other coronavirus E proteins (16). The E protein has essential roles in many viral processes including assembly, intracellular trafficking and budding and deletion of E protein leads to virus attenuation (17). Currently there is no effective antiviral therapy or vaccine available for MERS-CoV and there is an urgent need for improved options to counter MERS-CoV infection. As E is central to virus replication, it may act as a target for drug development, however, high level expression and purification need to be demonstrated to enable further biochemical characterization.

Here, we show expression of recombinant E protein in insect cells utilizing the baculovirus expression system. Further we demonstrate that purification of E is possible using IMAC leading to a purified protein that may be used in the development of anti-MERS therapy or vaccine.

MATERIALS AND METHODS

Construct. The MERS-CoV envelope protein coding region from (GenBank accession number K9N5R3) was synthesized *de novo* (Integrated DNA Technologies, Coralville, IA, USA) and incorporated between the *NcoI* and *XhoI* sites of vector pTriEx1.1 (Merck Millipore, Burlington, MA, USA) with a polyhistidine tag at the N-terminus for the detection of expression by western blot. Positive plasmid DNA was purified from a cell culture pellet using a Miniprep kit (Thermo Fisher Scientific) following the manufacturer protocols. The concentration of the DNA was measured utilizing a NanoDrop spectrophotometer and clones were verified by DNA sequencing at Source BioScience.

Cell culture. The *Spodoptera frugiperda* 9 (Sf9) insect cell line (Invitrogen USA) was utilized for baculovirus amplification and protein expression. A monolayer or suspension of Sf9 cells were seeded and cultured at a cell density ranging between 2.5×10^5 / ml - 1×10^6 using Ex-cell serum free medium containing 1% penicillin/streptomycin, 2% foetal calf serum (FCS), at 27ºC with shaking for three-five days.

Production of recombinant baculovirus. For protein expression in insect cells, the recombinant baculovirus system was used. This system is characterized by the production of high levels of protein and its scalability if needed (18).

The flashBAC™ GOLD (FBG) baculovirus Expression System (Mirus Bio) was utilised for production of recombinant baculovirus stocks. The pTriEx 1.1 based plasmid was used with linearised FBG viral DNA to transfect Sf9 cells using the conditions advised by the vendor. After transfection of the cells with the transfection mixture, incubation at 27ºC for 5-6 days was done until cytopathic effect caused by the recombinant virus was visible. Recombinant virus was then passaged in Sf9 cells as required.

MERS-CoV E protein expression using recombinant baculovirus system. For smale scale protein production, 1×10^6 Sf9 cells were infected at a multiplicity of infection of 3 and incubated for five days at 27°C. The cells and media were collected and the presence of the MERS E protein was assessed by analysis of the pellet either by SDS-PAGE or by western blot with an anti-his antibody.

Immobilised metal-affinity chromatography (IMAC). An amino terminal histidine-tag was included in the designed E sequence for purification of

the recombinant protein by immobilised metal affinity chromatography (IMAC). Briefly, the cell pellet was resuspended in lysis buffer (50 mM Tris.Hcl, 500 mm NaCl, and 1% NP 40 pH 7.4) and sonicated for 10 minutes at intervals of 20 seconds with an amplitude of 80% (Sonics, Vibra Cell TM). The cell lysates were centrifuged at 10000 rpm at 4°C for 20 min. Then, the supernatant was filtered (0.22 µm) and the clarified lysate was applied to the IMAC column (Bio-Rad) which was pre-equilibrated with binding buffer (20 mM Imidazole, 500 mM NaCl, 0.02% Tween-20% in 20 mM PBS pH 7.4). After binding, the column was washed to an OD280 of zero and recombinant protein eluted using a 0-100% gradient of elution buffer (500 mm imidazole, 500 mm NaCl, 0.05% Tween-20 in 20 mm PBS pH 7.4). SDS-PAGE was utilized for analysis of the fractions and the positive recombinant E protein fractions were pooled and concentrated by spin-filtration.

SDS-PAGE. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used for protein separation using precast 4-12% Tris-Glycine gels (Invitrogen). The protein samples were combined with 2% ß-mercaptoethanol, half of the volume of 4 ×LDS loading buffer, and boiled for 10 minutes prior to loading. Electrophoresis was performed using MES running buffer for half an hour at 170 V. Gels were transferred to PVDF membranes for Western blot analysis or silver stained after electrophoresis in accordance with the manufacturer's instructions.

Western blot. Western blot analysis was performed for verification of MERS-CoV-E protein expression. Transfer of the SDS-PAGE gels was to Polyvinylidene difluoride membranes (Whatman) which had been pre-soaked in 100% methanol (Fisher) for about five minutes.

The gels and membranes were then transferred into 1× transfer buffer (25 mM Tris, 192 mM Glycine pH 8.3 with 20% methanol) using a semi-dry western blotting device, and electroblotted for 80 minutes at 35 V/150 mA. The membranes were then blocked for 1 hour in a solution containing 5% skimmed milk powder, 0.2% tween-20, and 1× TBS. Membranes were incubated for 60-minute with the primary antibody, such as Rabbit polyclonal 6× His tag Ab at 1: 10,000 in 1× TBST buffer and washed three times with TBST buffer for 5 minutes each on a platform rocker. A secondary antibody horseradish-peroxidase (HRP) conjugated goat anti-rabbit antibody (Dako) in a dilution of 1:10,000 in 1× TBST was added to the membranes and incubated for 60 minutes pursued by three times wash with TBST buffer on a platform rocker. According to manufacturer protocol a chemiluminescent reagent (Cyanagen) was used for visualization of protein expression and the membrane imaged using a Syngene G: BOX.

Determination of protein concentration. Protein concentration was determined using a series of dilution of bovine serum albumin (BSA) as a protein standard. BSA was diluted in a series ranging from 0.5 to 1.5 mg/ml. Volumes of 1, 5 and 10 µl from the BSA standards and samples of the recombinant E protein were analysed by SDS-PAGE as described, followed by staining with Coomassie brilliant blue and silver stain.

RESULTS

Cloning of the MERS-CoV- E gene. The E gene fragment was cloned into the pTriEx1.1 expression as described between the *NcoI* and *XhoI* restriction sites. DNA sequencing was used to validate the construction and to compile a plasmid map (Fig. 1) and a recombinant baculovirus was constructed as described.

E protein expression in insect cells. For verification of protein expression, a 6 well plate containing 1×10^6 Sf9 cells was infected with recombinant virus stocks at an MOI of 3 and incubated at 27ºC for 72 hours until cytopathic effect was verified using light microscopy. The infected cells were harvested and the cell pellets used for SDS-PAGE and western blot using antibodies to the His tag. Two individual virus isolates were analysed with mock infected cells as control. The western blot revealed an antibody reactive band at ~10 kDa consistent with the recombinant E protein that was absent from the mock infected sample (Fig. 2). One of the positive isolates was subsequently amplified to a larger high titre virus stock for infection at greater scale.

MERS-CoV-E purification using IMAC from insect cells. As a source of recombinant E protein was the goal of this work, the ability of the protein to be extracted and purified using the His tag present was examined following large-scale infection. The His tag allows Immobilized Metal Ion Chromatography

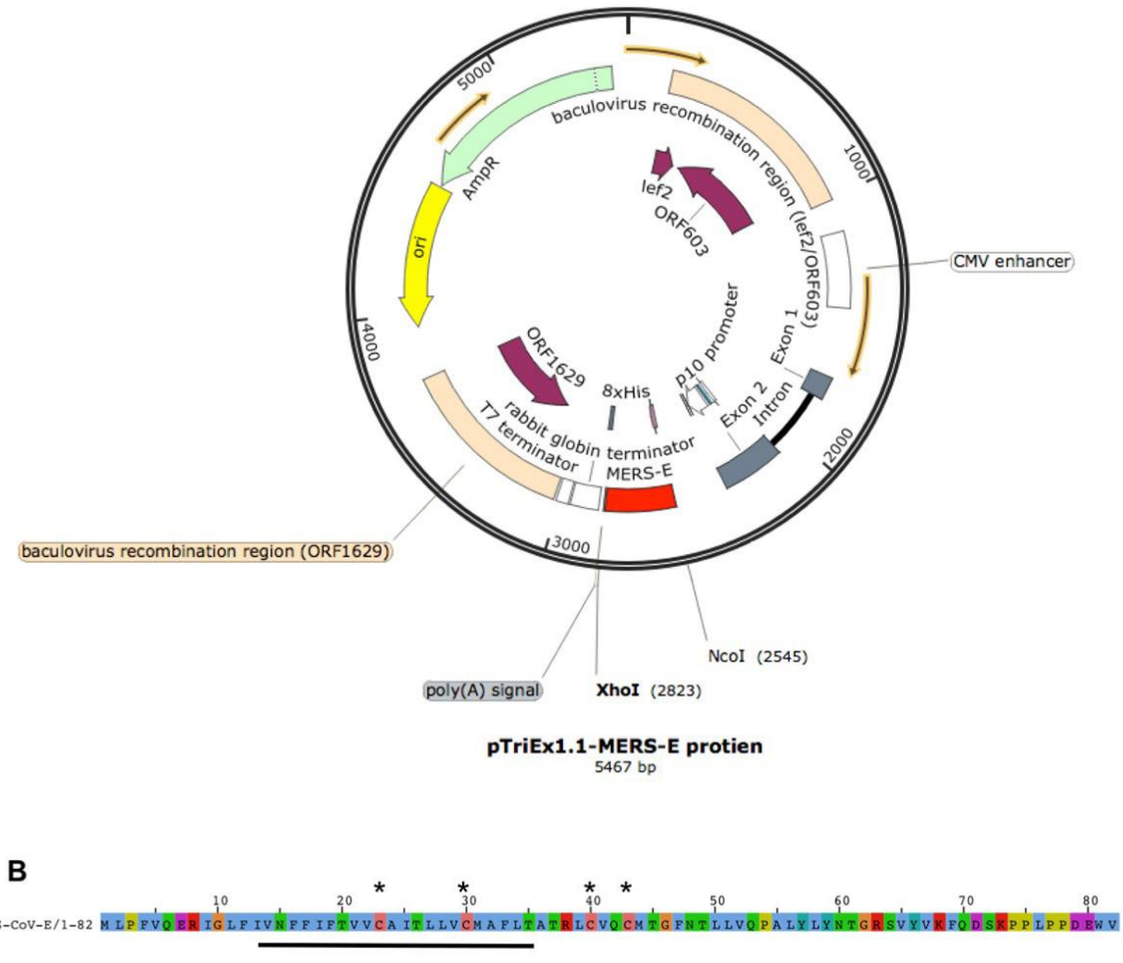

Fig. 1. A- Schematic representation of the pTrix1.1 vector containing the MERS-CoV-E construct. Key features of the plasmid are marked. B- The MERS-CoV E protein sequence. The single predicted transmembrane region is underlined and the cysteines indicated.

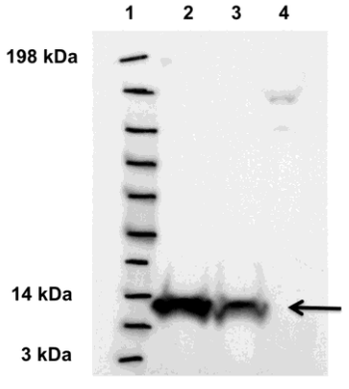

Fig. 2. Recombinant MERS-E protein expression in insect cells revealed by Western blot. A- Lane 1: See Blue™ Plus 2 Pre-Stained Protein Standard (Invitrogen), lane 2 and 3: Infection by two recombinant E viruses, lane 4: negative control (mock infected cells). The arrow indicates a molecular mass of ~10 kDa.

(IMAC), a form of affinity chromatography, as a single step purification method. In order to purify E protein, 500 ml of insect Sf9 cells at a density of 1.5 ×10^6 cells/ml were infected with 30 ml of recombinant E virus stock and incubated at 27°C for 72 hours on a rotating shaker. Cells were then collected by centrifugation at 4000 rpm for 20 min at 4°C and placed on ice. Cell lysis was achieved by resuspension in lysis buffer (50 mM phosphate buffer, 500 mM NaCl, 1% NP-40, pH 7.4) in the presence of a protein inhibitor cocktail (Roche), and followed by sonication on ice for 10 minutes. The lysate was subjected to centrifugation at 15,000 rpm for 20 minutes at 4°C and the supernatant were collected. Purification of MERS-CoV E protein from the lysate utilized a 5 ml prepacked IMAC column (GE) which was loaded with the cell lysate at a rate of 1 ml/min. The column was washed

with binding buffer (500 mM NaCl, 20 mM Imidazole, 20 mM PBS, 0.1% Tween-20 at pH 7.4) at a rate of 5 ml/min until the OD280 was at background level and the recombinant MERS-CoV E-His-tagged protein was eluted using a linear gradient (0-100%) of elution buffer over 5 column volumes at 2ml/min. The OD280 profile is depicted in Fig. 3. A small peak of non-specific protein elution is present in fractions 1-3, followed by a major single eluting peak in fractions 4-16. Alternate fractions of the elution were subjected to SDS-PAGE analysis, followed by silver staining and western blotting to determine the degree of purification (Fig. 4). Recombinant E eluted as a band of ~10kDa from fraction 5 onward but early fractions also contained several other proteins, possibly host derived membrane protein weakly associated with E. In fraction 11-19 however, recombinant E was the predominant band present. These fractions were concentrated by spin dialysis and re-analysed by SDS-PAGE next to known concentrations of BSA to determine specific concentration. E protein, together with a potential dimer at twice the molecular weight was assessed as 80% purity overall with a yield of ~0,5 mgs L^{-1}, sufficient for many downstream applications (Fig. 5).

DISCUSSION

The present study describes the expression and purification of recombinant MERS-CoV E protein using the baculovirus expression system. As a mem-

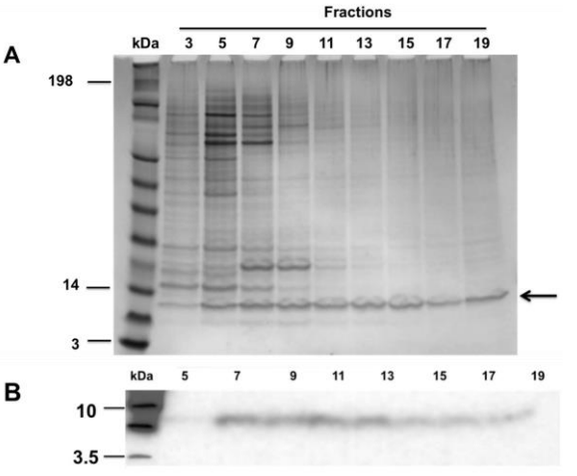

Fig. 4. Analysis of the elution profile for recombinant MERS-CoV-E following expression in Sf9 cells and IMAC purification. A. Silver stained SDS-PAGE. B. Western Blot with anti-His antibody. The fractions are indicated. Markers to the left of each panel are in kilodaltons.

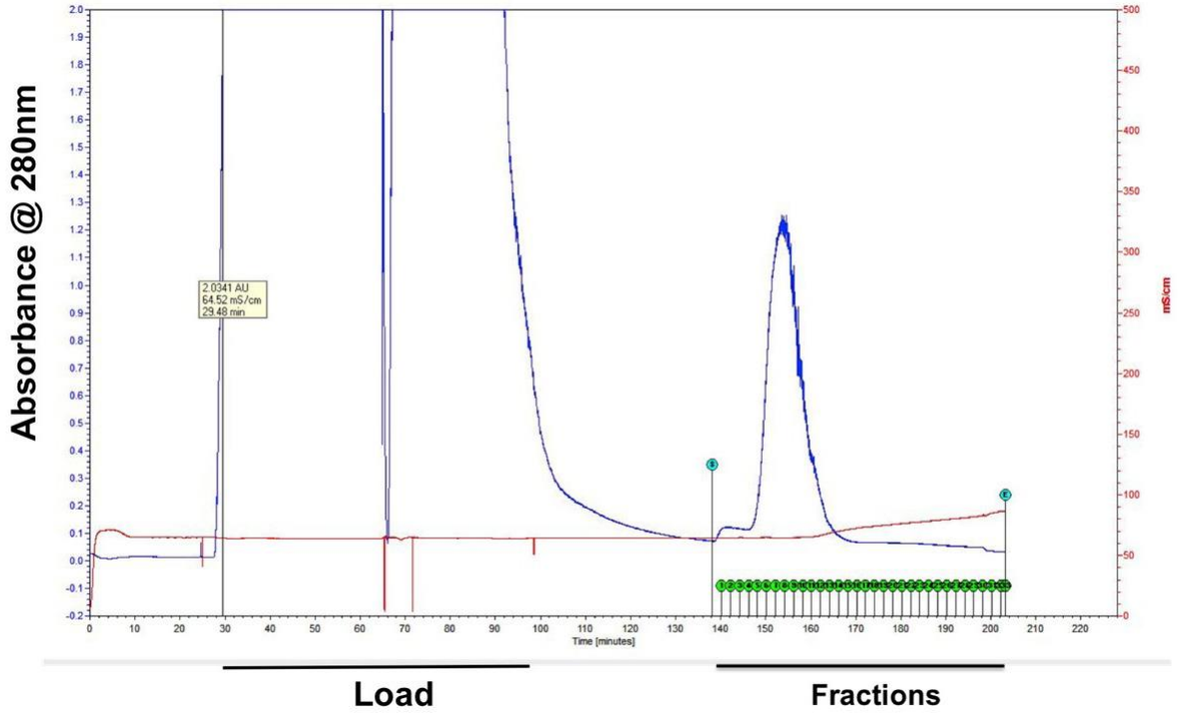

Fig. 3. MERS-CoV-E protein elution profile following IMAC affinity chromatography. The load is shown up to 100 min and the elution peak begins at fraction 3 – and extends over the subsequent 12 fractions. Fractions were of 2.5 min duration at a flow rate of 2 ml min^{-1} (5mls).

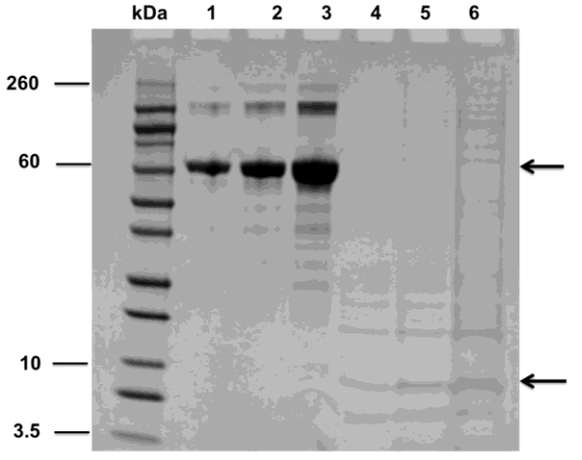

Fig. 5. Protein concentration assessment of MERS-CoV-E by SDS-PAGE next to a BSA standard. Marker is in kilodaltons (left of the gel). Lanes 1-3 represent 0.5, 1, 2.5 µg of BSA respectively. The arrows indicate a molecular mass of ~66.5 and 10.18 kDa for BSA and MERS-CoV-envelope protein respectively.

brane protein present at only low levels in the virus particle, E has been difficult to study biochemically. Previously we analysed MHV E protein function by using peptides and their ability to bind to giant unilamellar vesicles (GUVs) (19). Attempts to produce MERS E to date have largely relied on the expression of fusion proteins and the isolation of E following site specific proteolysis. Surya and colleagues used fusion to maltose binding protein (16) while the same design failed for Parthasarathy et al., who used fusion to the major outer membrane protein OmpA instead (20). Xu et al., expressed the SARS E protein as a fusion with glutathione-S-transferase (21) while Álvarez and colleagues also studied SARS E, but as a fusion to both the FLAG and HA tags and no large scale purification was reported (22). In our study E was only fused to a His tag located in the short pre-transmembrane domain, leaving the majority of the protein unmodified. Our finding that some fractions of the purified protein also contained various host proteins is consistent with the findings of Álvarez et al. who reported many host protein interactions with E as determined by mass spectrometry of the binding partners of E following "pull-down" with antibodies. Our data confirm that the baculovirus expression system is one of the most successful for the production of difficult-to-express proteins (23-26). The characteristics of the system we describe may play an indispensable role in studying further E biological processes (27), including vaccine development and other interventions (28).

CONCLUSION

MERS-CoV- E protein can be expressed and purified using the baculovirus expression system combined with IMAC to produce relatively pure E protein with reasonable yield. This method may be utilized for other coronaviruses membrane proteins, especially those considered as good vaccine or antiviral candidates.

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