CIBTech Journal of Pharmaceutical Sciences ISSN: 2319–3891 (Online)
An Open Access, Online International Journal Available at http://www.cibtech.org/cjps.htm
2016 Vol.5 (2) April-June, pp.11-16/Noi et al.
Research Article

THE EFFICIENT PURIFICATION METHOD FOR HIGH RECOVERY OF RECOMBINANT S1 DOMAIN OF THE PORCINE EPIDEMIC DIARRHEA VIRUS SPIKE PROTEIN FROM RECOMBINANT *E. COLI*

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ABSTRACT
Porcine epidemic diarrhea virus (PEDV) is an enteric disease in swine caused by an alpha coronavirus. Development of inexpensive and simple culture media is always favorable for commercial production of recombinant proteins in *E. coli.* The high-level expression of eukaryotic proteins in E. coli often leads to formation of insoluble inclusion bodies (IBs) in the cytoplasm or periplasm. Recovery of active material from IBs is often difficult and involves two general steps: protein solubilization in a denaturant and protein refolding. On a commercial scale, reducing the number of protein purification steps is practical and economical because each purification step not only increases the final product but also causes successive yield losses of the recombinant protein. In this research, we developed an efficient and scalable procedure for production and purification of recombinant S1 domain of the Porcine epidemic diarrhea virus spike protein (rPEDV-S1) of E. coli. This process includes: an optimized batch culture with LB and glucose 10g/l with expression level 45%, cell harvesting, cell lyses with high pressure homogenizer, two steps washing, IB solubilization, refolding, and finally protein purified by FPLC with cation exchanger column. By the using of the new developed method, of 1.7 g l-l PEDV-S1 was produced in each batch, 650 mg pure of recombinant protein was obtained with recovery yield about 45% and purity over than 98%. According to available data this is one of the highest yield and production level of the purified recombinant protein that has been reported for recombinant S1 domain of the Porcine epidemic diarrhea virus spike protein which is expressed in E. coli.

Keywords: *PEDV-S1, Escherichia Coli, Inclusion Bodies, Purification, Solubilization*

INTRODUCTION
Porcine epidemic diarrhea virus (PEDV) is an envelope, positive-stranded RNA virus which readily infects pigs, resulting in highly contagious porcine epidemic diarrhea (Debouck and Pensaert, 1980). The S protein of PEDV is a class I membrane glycoprotein consisting of two subunits: the N-terminal S1 and the C-terminal S2. The S1 protein was previously shown to have protective activity in piglets (Oh *et al.*, 2014). Development of inexpensive and simple culture media is always favorable for commercial production of recombinant proteins in E. coli. Many of the efforts aimed at increasing recombinant protein production in bacterial strains have been directed to maximizing the biomass production with the high cell density cultivation method and little is known about the effects of media composition on the expression of recombinant proteins (Sørensen and Mortensen, 2005). Hence, in this study, at first the effects of medium composition on the production of recombinant S1 domain of the Porcine epidemic diarrhea virus spike protein (rPEDV-S1) was investigated in batch culture; and then a simple and cost-effective downstream process for the economical production of PEDV-S1 is developed.
The high-level expression of eukaryotic proteins in E. coli often leads to formation of insoluble inclusion bodies (IBs) in the cytoplasm or periplasm. Inclusion bodies are dense but porous particles of aggregated protein that usually only one or a few different proteins are inside the IB, and no ribosomal components or nucleic acids are resent and they are held together by non-covalent hydrophobic or ionic bonds. IBs

have some native-like protein structures, but also have an increased amount of non-native β-sheet (Carrio and Villaverde, 2002). Three Factors that contribute to inclusion body (IB) formation: 1) Inclusion bodies form most often as a result of over expression of a non-native protein. 2) Hydrophobic proteins form IBs more readily. 3) Non-native proteins with disulfide bonds are prone to form IBs because the disulfide bridge cannot form in the cytosol Accidental oxidation can also lead to improper disulfide formation (Ditta *et al.,* 1980; Carrio and Villaverde, 2002). Culture conditions such as temperature, pH, and nutrient supply play a very important role in controlling the partition of the recombinant protein into soluble and insoluble fractions. Recovery of active material from (IBs) is often difficult and involves two general steps: protein solubilization in a denaturant and protein refolding (Carrio and Villaverde, 2002). In general, proteins expressed as inclusion bodies are solubilized by the use of high concentrations of chaotropic solvents rPEDV-S1.

The rPEDV-S1 is produced by E. coli, the formation of disulfide bonds is either incorrect or inhibited because the reducing environment of bacterial cytosol, and it is accumulated in the form of IBs. Earlier reports indicate that the levels of rPEDV-S1 expressed in E. coli was at moderate to high levels (10–35%) (Weber *et al.,* 1972; Donovan *et al.,* 1996), and the yield of the final product was very poor and far from acceptable. This is probably because of unproductive downstream process technologies like isolation of protein IBs with low purity and recovery, misfolding, aggregate formation and unoptimized conditions of protein refolding, and chromatographic processes (Guan and Dixon, 1991). Therefore, in this research has been tried to develop a well-organized and scalable process for recombinant PEDV-S1 from E. coli batch cultivation.

MATERIALS AND METHODS

Microorganism and Vector System

E. coli BL21 (DE3) (Novagen, Inc.) was used as the host for PEDV-S1 expression. This strain was transformed with a commonly available plasmid, pRSET-A inducible expression vector (Novagen,Inc.), in which the PEDV-S1 gene (Department of Veterinary medicine, National Pingtung University of Science and Technology, Pingtung, Taiwan) was inserted into the NotI and NdeI sites. Host cells were transformed with the plasmid using the calcium chloride procedure. Transformed cells were spread on several LB agar plates containing 100 mg/l ampicilin.

Media and Solutions

LB (Luria-Bertani) medium was used for plate cultivation of E. coli strain BL21 (DE3) and M9 medium was used for preparation of seed culture. M9 medium was used for preparation of seed culture. The M9 modified medium consisted of 10 g glucose, 15 g K2HPO4, 7.5g KH2PO4, 2 g Citric acid, 2.5 g (NH4)2SO4, 2 g MgSO4.7H2O, and 1 ml trace element solution per liter. The trace element solution contained 2.8 g FeSO4.7H2O, 2g MnCl2 4H2O, 2.8 g CoSO4.7H2O, 1.5 g CaCl2.2H2O, 0.2 g CuCl2.2H2O, and 0.3 g ZnSO4.7H2O g per liter in 1M HCl. Batch cultivations were simultaneously carried out in two 2 l bench-top bioreactors with the working volume of 1 l (Babaeipour *et al.,* 2010).

Batch Cultivation

Batch culture was started by adding 100 ml of an overnight-incubated seed culture (OD600= 0.7-1) into the bioreactor containing 900 ml of medium. The pH was controlled at 7±0.05 by the addition of 25% (w/v) NH4OH or 3 M H3PO4. Dissolved oxygen was controlled at 30-40% of air saturation by controlling both the inlet air and agitation rate. Foaming was controlled by adding silicon-antifoaming reagent. In batch culture, cells were induced by the addition of IPTG (1mmol/l) when initial DCW (2.2 g DCW/l) reached a considered level (Kim *et al.,* 2004). Then, the production phase continued until the growth ceased. All batch fermentations were performed twice.

PEDV-S1 Purification

Cell Lysis and IB Recovery: The fermented broth was centrifuged at 4°C and 8000 g for 30 min and the obtained pellet was washed twice with 50 mM phosphate buffer pH 7.4.

The wet cells (50 g) were suspended in 200 ml of lysis buffer. The lysis buffer composition was 50 mM Tris–HCl containing 1 mM EDTA, 1 mM PMSF. The cells were broken by passing the medium through a

homogenizer three times (NIRO-SOAVI) at 800 bar. The cells were cooled to 4°C between each pass. The cell homogenate was centrifuged for 30 min at 6000g at 4°C, the supernatant was discarded and the inclusion bodies recovered.

IBs Washing: The IBs pellet obtained in previous step was resuspended in wash buffer and incubated 40 min and recovered by centrifugation at 25–28°C for 30 min at 8000 g. The washing protocol is 2.5 g l-1 Triton X-100 in 50 mM Tris–HCl pH 8.0 containing 5mM EDTA and 1 mM PMSF. In the second washing, the IBs pellet was resuspended in wash buffer and incubated 40 min and recovered by centrifugation at 25–28°C for 30 min at 8000 g. The washing protocol is 1 M urea.

IB Solubilization and Refolding: Washed inclusion bodies were dissolved in 30m M Tris–HCl, pH= 8, containing 6 M urea,1 mM EDTA and 100 mM GSH, The solution was incubated at 25-28 °C for 45 min and spun down at 10,000 g for 30 min to get rid of insoluble cell debris and recovered by centrifugation at 25–28°C for 30 min at 8000 g. and then solubilized inclusion bodies was refolded by refolding buffer that the refolding buffer protocol is 30 mM Tris–HCl (pH =7.5), 2 mM GSSG, 20 mM GSH, 1 mM EDTA, 3M Urea and incubated 12 hours at 4°C. After completion of refolding, the protein pH was adjusted with 2 M citric acid and centrifuged at 10,000 g for 20 min at 4°C.

Anion Exchange Chromatography: The pH of Refolded protein was adjusted to 5-6 by adding 2 M Acetic acid and then loaded in mono Q column in FPLC (SYKAM-S2100). The column temperature and flow rate were maintained at 20 °C and 1 ml/min respectively throughout the process. The column was equilibrated with 3 bed vol. of 25 mM sodium acetate buffer (pH= 4.5). The refolded protein sample was directly loaded on to the column at the same flow rate. The column was extensively washed with 3 beds vol. of the same buffer but with 1 M NaCl.

SDS-PAGE

In the reduced SDS-PAGE 10% gel and three standards of PEDV-S1 (Neupogen®, Roche, Germany), and a molecular weight marker (#SM0431, Fermentas) were used. Sample buffer [0.5 M Tris pH 6.8, 50% (v/v) glycerol, 100 g l-1 SDS, 20 g l-1 bromophenol blue and 50 g l-1 2-mercapto ethanol (2-ME) was added to three samples and standards before boiling for 5 min. The samples were loaded on to the gel and ran at a constant voltage of 120 V for 100 min. Gels were stained with Coomassie brilliant blue R250 (Weber *et al.,* 1972; Donovan *et al.,* 1996).

Western Blotting

For confirmation of PEDV-S1 band in gel, western blotting with polyclonal antibody His-tag was performed. Separated proteins on the SDS-PAGE gels were transferred into a poly-vinylidene fluoride (PVDF) membrane (Roche Diagnostic, Germany) for recognizing the exact existence of PEDV-S1. PVDF sheet was blocked with 3% BSA in TBS-T solution (20 mM Tris-HCl, pH 7.5, 150 mm NaCl, and 0.05% Tween 20). Then, anti-PEDV polyclonal antibody was added at a dilution of 1:500 in TBS-T solution for 1 h. A second incubation with HRP anti-rabbit (1:1000) in TBS-T was carried out and the third incubation of 5-10 min was done with Diaminobenzine (DAB) solution. (0.5 mg/ml DAB, 0.1% H2O2) (Burnette, 1981).

RESULTS AND DISCUSSION

Purification of PEDV-S1

Cell Lysis and IB Isolation

The rPEDV-S1 IBs in the cytoplasm of E. coli were isolated by lysing the bacterial cells. The efficiency of cell lysis was experiential at different sonication pulses of 5, 10, 15 and 20 s and at a different homogenizer pressure (600, 800. 1200 bar) and at different times. The highest IB recovery was observed by passing the medium through a homogenizer three times at 800 bar, and it is better to use homogenizer in industrial process.

Cell lysis was measured by plating the lysate suspension on LB-agar followed by colony counting. IB recovery was measured by the Bradford method. This condition was chosen as the optimum for further work. Thus, optimization of the early stages of the downstream process will have an impact on the overall process yield and final product purity.

IB Washing

The IBs separated after homogenizer was found to be contaminated with endotoxins, host DNA and HCPs. Impurities interfere with refolding and significantly affect the process yield and purity. Two-step wash procedure eliminates endotoxins, proteins and DNA of the host cell. Removal of these impurities and obtaining the IBs with high recovery and purity was demanding. Therefore, optimization of detergent concentration has effect on the efficiency of IB recovery.

We use different concentration of different detergents; finally we found that Triton X-100 was used to solubilize the bacterial cell wall components that infect the inclusion body preparation. Also, sodium deoxycholate to remove any residual cell debris particles, especially lipopolysaccharides units that contribute to the unacceptable levels of endotoxins in protein preparations from E. coli and urea are better than others.

IB Solubilization and Refolding

Key to the development of an efficient and cost-effective denaturant-based solubilization step is the determination of the minimum amount of denaturant needed to solubilize the protein and to allow for full bioactivity recovery in the refolding step.

Because a method has been described for PEDV-S1 extraction from IBs produced in E. coli used high amount of detergent and chaotropic agent, and hence additional steps had to be in use to eliminate these agents, so we optimize the amount of denaturant use in solubilization. Since rPEDV-S1 is hydrophobic in nature and conductivity sensitive, the combination of denaturant/reducing agent has an impact on solubilization and refolding. Hence, we use different concentration of urea (3, 6, 8 M) in different pH (6, 8, 12) (Figure 1).

The key to refolding is to remove enough denaturant to allow the protein to fold properly, but keep enough denaturant in the refolding buffer to allow proteins to fold/refold several times until attains the proper conformation protein.

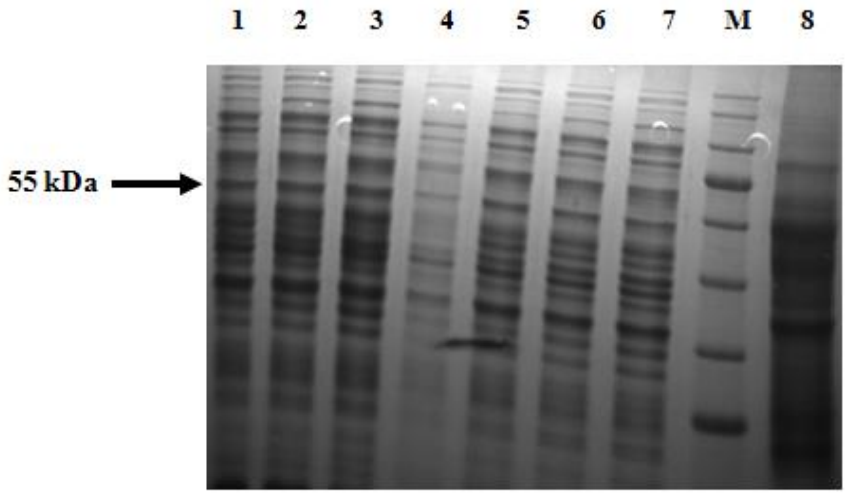

Figure 1: SDS-PAGE (10%) Use Different Concentration of Urea in IB Solubilization; Lane-M: Molecular Weight Marker (#SM0431, Fermentas), Lane-2: rPEDV-S1 in Water and Add Loading Buffer, Lane -3: rPEDV-S1 in 8 M Urea - pH 12, Lane- 4: rPEDV-S1 in 3 M Urea - pH 12, Lane-5: rPEDV-S1 in 8 M Urea - pH 8, Lane-6: rPEDV-S1 in 8 M Urea - pH 6, Lane-7: rPEDV-S1 Pellet in 8 M Urea - pH 6, Lane-8: rPEDV-S1 in 8 M Urea - pH 8 - 0.1 M Cystein

Anion Exchange Chromatography

The ion-exchange column is primarily useful for the removal of endotoxins, nucleic acids, HCPs and product-related impurities.

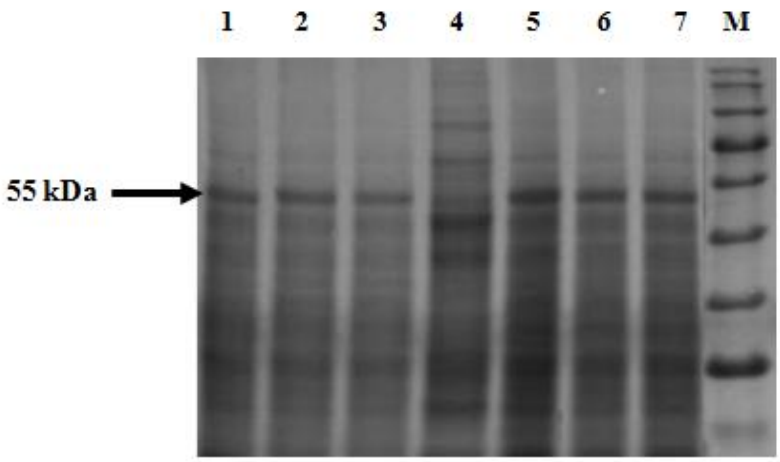

Figure 2: The Purity of the rPEDV-S1 at Different Process Stages was Observed by SDS-PAGE gel; Lane-M: Molecular Weight Marker (#SM0431, Fermentas), Lane-1-3: Purified rPEDV-S1, Lane-4: Negative Control, Lane-5: Refolded rPEDV-S1, Lane-6: rPEDV-S1 Protein Inclusion Body Pellet, Lane-7: rPEDV-S1 after Two-Step Wash

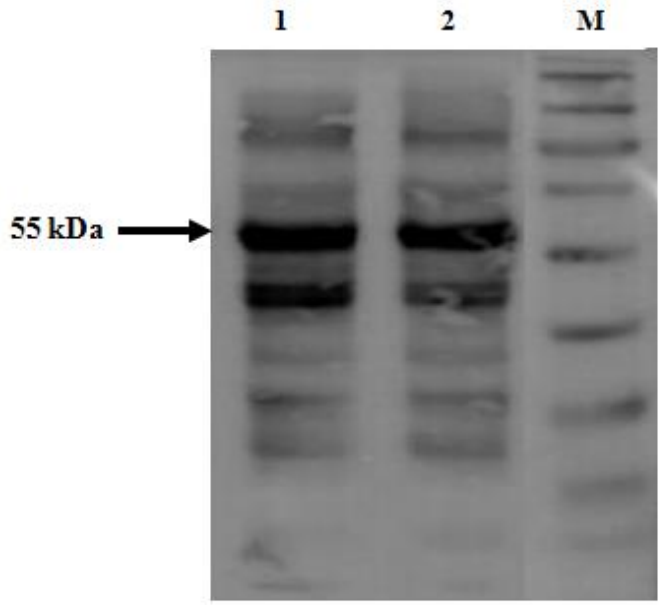

Figure 3: Western Blot of Purified rPEDV-S1 Showing a Single Protein Bond; Lane-M: Molecular Weight Marker (#SM0671, Fermentas), Lane 1, 2: Purified rPEDV-S1

In this study we use FPLC (SYKAM-S2100) with mono Q Column. The results obtained determine improvement of the method for effective removal of impurities and high recovery of protein with a monomer peak purity of > 97 %. In the present study we envisage a simple, fast, reliable and reproducible process for higher yields and a quality product with high purity.

The SDS-PAGE gel electrophoresis shows that the purity of the purified rPEDV-S1 by IEC is over than 98%. The final step of the purification showed a single band of 55 kDa, similar to reference standard. The same general pattern was revealed when the sample shown in Figure 3 was transferred from SDS-PAGE

to a nitrocellulose membrane and immunodetected with PEDV-S1 specific antiserum, confirming a typical profile (Figure 2, Figure 3).

In the present study, we developed an efficient and scalable procedure for production and purification of recombinant S1 domain of the Porcine epidemic diarrhea virus spike protein (rPEDV-S1) of E. coli. By the using of the new developed method, 2.2 g l-1 rPEDV-S1 was produced in batch cultivation with recovery yield about 45% and with purity over than 98%. The process established in this study may be functional in the recovery of other proteins expressed in E. coli as cytoplasmic IBs.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Albert L. Charles for logistical assistance.

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