Protein Expression and Purification 126 (2016) 77–83

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Production of soluble truncated spike protein of porcine epidemic diarrhea virus from inclusion bodies of *Escherichia coli* through refolding

Da-Chuan Piao [a], Yoon-Seok Lee [b], Jin-Duck Bok [b], Chong-Su Cho [a], Zhong-Shan Hong [c], Sang-Kee Kang [b, **], Yun-Jaie Choi [a, c, *]

[a] Department of Agricultural Biotechnology, Research Institute of Agriculture and Life Sciences, Seoul National University, Seoul, 151-921, Republic of Korea
[b] Institute of Green-Bio Science & Technology, Seoul National University, Pyeongchang-gun, 232-916, Republic of Korea
[c] Department of Animal Science, Tianjin Agricultural University, Tianjin, 300-384, People's Republic of China

ARTICLE INFO

Article history:
Received 3 May 2016
Received in revised form
29 May 2016
Accepted 30 May 2016
Available online 31 May 2016

Keywords:
PEDV **S** protein
Inclusion bodies
Solubilization
Refolding
Immunogenicity
Subunit vaccine

ABSTRACT

The emergence of highly pathogenic variant porcine epidemic diarrhea virus (PEDV) strains, from 2013 to 2014, in North American and Asian countries have greatly threatened global swine industry. Therefore, development of effective vaccines against PEDV variant strains is urgently needed. Recently, it has been reported that the N-terminal domain (NTD) of S1 domain of PEDV spike protein is responsible for binding to the 5-*N*-acetylneuraminic acid (Neu5Ac), a possible sugar co-receptor. Therefore, the NTD of S1 domain could be an attractive target for the development of subunit vaccines. In this study, the NTD spanning amino acid residues 25-229 (S25-229) of S1 domain of PEDV variant strain was expressed in *Escherichia coli* BL21 (DE3) in the form of inclusion bodies (IBs). S25-229 IBs were solubilized in 20 mM sodium acetate (pH 4.5) buffer containing 8 M urea and 1 mM dithiothreitol with 95% yield. Solubilized S25-229 IBs were refolded by 10-fold flash dilution and purified by one-step cation exchange chromatography with >95% purity and 20% yield. The CD spectrum of S25-229 showed the characteristic pattern of alpha helical structure. In an indirect ELISA, purified S25-229 showed strong reactivity with mouse anti-PEDV sera. In addition, immunization of mice with 20 μg of purified S25-229 elicited highly potent serum IgG titers. Finally, mouse antisera against S25-229 showed immune reactivity with native PEDV S protein in an immunofluorescence assay. These results suggest that purified S25-229 may have potential to be used as a subunit vaccine against PEDV variant strains.

1. Introduction

Porcine epidemic diarrhea (PED) is one of the most economically important enteric diseases of swine, which is characterized by severe diarrhea, vomiting, dehydration and death, with a mortality rate of up to 90% in neonatal piglets [1]. The disease was first reported in England in 1971 [2]. Subsequently, the disease has spread to Europe and most of the Asian swine raising countries, and severely affected swine industry especially in Asia [3,4]. During years 2010–2015, PED outbreaks with more severe clinical symptoms caused by new variant strains have been reported in China, the United States, South Korea and many other swine raising countries, causing substantial economic losses in the global swine industry [5–12]. Therefore, development of effective control measurements, such as vaccines and antiviral drugs, are urgently needed to combat newly emerged variant strains in PEDV-vulnerable countries.

Porcine epidemic diarrhea virus (PEDV), the causative agent of PED, was first identified in 1978 [13]. The virus has an approximately 28 kb in length, positive-sense, single-stranded RNA genome with a 5′ cap and a 3′ polyadenylated tail [14]. The virus possesses four structural proteins including 150–220 kDa spike (S)

Abbreviations: PEDV, porcine epidemic diarrhea virus; IBs, inclusion bodies; IPTG, isopropyl D-1-thiogalactopyranoside; OD, optical density; DTT, dithiothreitol; TBS, Tris-buffered saline; HRP, horseradish peroxide; CD, circular dichroism.

* Corresponding author. Department of Agricultural Biotechnology, Research Institute of Agriculture and Life Sciences, Seoul National University, Seoul, 151-921, Republic of Korea.
** Corresponding author.
E-mail addresses: kangsk01@snu.ac.kr (S.-K. Kang), cyjcow@snu.ac.kr (Y.-J. Choi).

http://dx.doi.org/10.1016/j.pep.2016.05.018
1046-5928/

glycoprotein, 7 kDa envelope protein, 20–30 kDa membrane protein and 58 kDa nucleocapsid proteins [15]. The PEDV S protein is a type I transmembrane glycoprotein which can be divided into S1 (aa 1-789) and S2 (aa 790-1383) domains [16]. The former is responsible for the cellular receptor binding and the latter is required for membrane fusion of the virus [17]. It has been reported that the C-terminal domain of S1 domain is responsible for binding to the porcine aminopeptidase N (pAPN), a functional cellular receptor for PEDV entry [18]. Furthermore, previous studies have also elucidated that like some of the other coronaviruses, the N-terminal domain (NTD) of the S1 domain could bind to the 5-*N*-acetylneuraminic acid (Neu5Ac), a possible sugar co-receptor for PEDV [19].

Although the exact function of the S1-NTD in the PEDV infection has not been determined yet, comparison of amino acid sequence of S protein between PEDV prototype and variant strains revealed that most of the amino acid mutations are mainly concentrated within the S1-NTD [4,6]. Subsequently, it has been confirmed that the PEDV CHGD-01 variant strain exhibits stronger sugar-binding activity than the CV777 prototype strain, suggesting that the mutated amino acids may participate in sugar binding, and the authors also proposed that the enhanced sugar-binding activity of the variant strains may be responsible for the recent PED outbreaks [19]. Furthermore, in an indirect ELISA based on NTD (tSc, aa 25-225) of PEDV S1 domain showed positive correlations between OD values of pig sera and virus neutralization titers [20]. Therefore, the NTD of PEDV S1 domain may have potential to be used as a subunit vaccine against PEDV variant strains.

To date, various expression systems have been used to produce the S protein of PEDV [21–24]. In spite of absence of post-translational modification such as glycosylation, expression of recombinant proteins in *Escherichia coli* has many significant benefits over other expression systems in terms of cost, ease-of-use and scale [25]. However, recombinant protein overexpression in *E. coli* often leads to the insoluble aggregates known as inclusion bodies (IBs). Although IBs generally consist of biologically inactive proteins, correctly folded soluble proteins can be obtained from IBs by complete denaturation followed by refolding *in vitro* [26]. The purified IBs are commonly solubilized by high concentration of chaotropic agents such as 6 M guanidine hydrochloride or 8 M urea, and refolding of the solubilized protein is initiated by removal of denaturants either by dialysis or dilution [27]. Finally, the refolded proteins can be purified by various chromatographic purification steps.

In this study, we expressed the NTD of PEDV S1 domain spanning amino acid residues 25-229 (S25-229) in *E. coli*. However, expression of recombinant S25-229 resulted in formation of IBs. To recover soluble S25-229 from IBs, we firstly optimized buffer conditions for efficient solubilization of purified S25-229 IBs. Subsequently, solubilized S25-229 was refolded by simple dilution method. The refolded S25-229 was directly purified by one-step cation exchange chromatography with high purity and yield. Purified S25-229 was characterized by circular dichroism spectrometer for the conformation of secondary structure. The reactivity of S25-229 with mouse anti-PEDV sera was determined by indirect ELISA. Finally, the immunogenicity of soluble S25-229 was evaluated through *in vivo* mouse immunization.

2. Materials and methods

2.1. Vector construction

The nucleotide sequence of S protein of PEDV K14JB01 strain (GenBank accession No. KJ623926) was used as a reference to synthesize S25-229 (nt 73-687) gene. The codon-optimized S25-229 gene was synthesized (Genscript, USA) and cloned into a pET28a (+) vector (Novagen, USA) using *BamH*I and *Xho*I restriction enzyme sites. The resulting expression plasmid, pET28/S25-229, was then verified by nucleotide sequencing.

2.2. Protein expression

The expression plasmid was transformed into the *E. coli* strain BL21 (DE3). A single colony of recombinant cells were picked and grown overnight in 5 ml LB broth supplemented with 30 μg/ml kanamycin. For the expression of recombinant protein, overnight culture was inoculated into 500 ml fresh LB media and shake-cultured until optical density at 600 nm reached 0.6. Culture was then induced by adding 0.3 mM isopropyl D-1-thiogalactopyranoside and incubated for 4 h at 37 °C. Cells were harvested by centrifugation at 4500 rpm for 7 min.

The recombinant protein expression was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot. Cells were lysed by sonication and centrifuged at 14,000 rpm for 5 min at 4 °C. The supernatant and pellet were collected separately. The pellet was dissolved in alkaline buffer (50 mM Tris-HCl, pH 12.5), and protein samples were separated by SDS-PAGE comprising 15% separating gel and 5% stacking gel. Gels were stained with Coomassie brilliant blue R250 solution, and recombinant protein was detected by immunoblotting. Briefly, after transferring the proteins to nitrocellulose membrane, blocking was performed with 5% skimmed milk for 1 h at room temperature. The recombinant protein was probed with anti-His monoclonal antibody (Abcam, UK) in TBST (TBS containing 0.1% Tween 20) at 1: 1000 dilution. The HRP-conjugated goat anti-mouse IgG secondary antibody (Santa Cruz, USA) was used at 1: 5000 dilution. Detection was carried out using an ECL detection kit (GE Healthcare, Sweden).

2.3. Isolation of IBs

E. coli cell pellet (2.28 g wet cell weight) obtained from 500 ml culture was resuspended in 25 ml of lysis buffer (50 Tris-HCl, pH 8.0) supplemented with 1 mg/ml lysozyme and incubated for 20 min at 37 °C with shaking. Then, 10 mM $MgSO_4$ and 0.1 mg/ml Dnase I were added to the cell lysate and incubated for 30 min at 37 °C. The cell suspension was then sonicated (VCX 500 sonicator, Sonics, USA) on ice at amplitude of 40% for 1 min with on/off pulse intervals of 5 s. The sonicated cell lysate was centrifuged at 10,000 rpm for 10 min at 4 °C. The pellet was resuspended in 25 ml of lysis buffer containing 0.1% Triton X-100 and incubated for 1 h at room temperature. Then, the pellet was collected by centrifugation and washed 3 times in 25 ml deionized water to remove detergent. Finally, purified IBs (75 mg total protein) were lyophilized and analyzed by SDS-PAGE.

2.4. Solubilization and refolding

Six different buffers in the presence of 8 M urea were used to solubilize purified S25-229 IBs: 20 mM Tris-HCl (pH 7.5), 20 mM HEPES (pH 7.0), 20 mM Bis-Tris (pH 6.5), 20 mM sodium acetate (pH 5.5), 20 mM sodium acetate (pH 4.5) and 20 mM sodium acetate (pH 4.5) containing 1 mM dithiothreitol. The purified S25-229 IBs (7.5 mg protein) were dissolved in 5 ml of each solubilization buffer and incubated for 1 h at room temperature. The IBs dissolved in 20 mM Tris-HCl (pH 12.5) buffer containing 8 M urea was designated as 100% dissolved control. Solubilization efficiency was estimated by the ratio of concentration of IBs dissolved in each buffer and 100% dissolved control.

The solubilized proteins were centrifuged at 14,000 rpm for 10 min at 4 °C. Then, supernatant was 10-fold flash diluted with