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

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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.

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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

45 ml of ice-cold refolding buffer (20 mM sodium acetate, pH 4.5, 1.2 M urea and 5% sucrose) and incubated with gentle rotation on a rotator (Finepcr, South Korea) for 4 h at 4 °C. The refolded solution was centrifuged at 14,000 rpm for 10 min. The refolding yield (%) was estimated from the following formula.

$$\text{Refolding yield } (\%) = (\text{Cr}\cdot\text{V}/\text{Ci}\cdot\text{V})\cdot 100\%,$$

where Ci = concentration of initial protein, Cr = concentration of refolded protein and V = volume of protein solution. In this equation we assumed anything soluble as refolded protein.

2.5. Purification of S25-229

The refolded S25-229 protein was purified by strong cation exchange chromatography using HiTrap SP FF, 1 ml column (GE Healthcare, Sweden) on an FPLC ÄKTA explorer 10 chromatography system (GE Healthcare, Sweden). The column was pre-equilibrated with 10 column volume of equilibration buffer (20 mM sodium acetate, pH 4.5, 2 M urea and 5% sucrose). The supernatant was loaded onto the column and the column was then washed with 10 column volume of equilibration buffer. The bound proteins were eluted using linear gradient of elution buffer (20 mM sodium acetate, pH 4.5, 2 M urea, 5% sucrose and 1 M NaCl). Protein concentration was determined by BCA assay kit (Pierce, USA) using bovine serum albumin as a standard. The purified S25-229 protein was analyzed by SDS-PAGE and western blot.

2.6. Circular dichroism (CD) spectroscopy

The eluted S25-229 was buffer-exchanged into PBS using 10 K ultra-0.5 centrifugal filter (Millipore, Germany). The CD spectra of purified S25-229 protein (0.5 mg/ml in PBS) were recorded using Chirascan-plus spectrometer (Applied Photophysics, UK). Spectra were collected from 190 nm to 260 nm using a cylindrical quartz cell with 1 mm path length. Each spectrum was scanned five times, and the average spectrum was plotted.

2.7. Indirect ELISA

The reactivity of purified S25-229 with mouse anti-PEDV immune sera was tested by an indirect ELISA. Briefly, the 96-well Immuno-plate (SPL, South Korea) was coated with 0.1 μg/well of purified S25-229 in carbonate buffer (pH 9.6) at 37 °C for 1 h and blocked with 1% bovine serum albumin in PBS for 1 h at room temperature. Sera were 10-fold serial diluted and reacted with antigen for 1 h at room temperature. HRP-conjugated goat anti-mouse IgG secondary antibody (Santa Cruz, USA) diluted at 1: 5000 in PBST was added to the wells and incubated for 1 h at room temperature. Plates were incubated with TMB substrate solution (Sigma, USA) for 15 min in the dark and the reaction was stopped by adding 0.16 M sulfuric acid solution. Absorbance at 450 nm was measured in an Infinite 200 PRO microplate reader (Tecan, Switzerland).

2.8. Mouse immunization

Six-week old C57BL/6J mice were purchased from Samtako (Osan, Korea). The experiment was performed in accordance with the guidelines for the care and use of laboratory animals under the approval of animal ethics committee at Seoul National University (SNU-130415-1). Mice were immunized subcutaneously with 20 μg of purified S25-229 in the presence of Freund's complete adjuvant (Sigma, USA) and boosted once in the presence of Freund's incomplete adjuvant after 3 weeks. Sera were collected from non-immunized and immunized mice at 0, 21 and 42 days after first immunization. Serum antibody levels were detected by indirect ELISA according to the method described in indirect ELISA section using 1000-fold diluted sera.

2.9. Immunofluorescence assay

Vero cells were maintained in α-MEM media supplemented with 5% heat-inactivated FBS and 1% antibiotic-antimycotic agent, and cultivated on coverslips placed in 35 mm confocal dish (SPL, Korea) for the immunofluorescence assay. After cells reached confluence, PEDV SM98 strain was inoculated onto the cell monolayer at multiplicity of infection (MOI) of 1 and incubated for 1 h at 37 °C. Subsequently, serum-free α-MEM media containing trypsin (2.5 μg/ml) was added and the culture was incubated for 3 days at 37 °C. Then, cells were fixed with 4% paraformaldehyde for 10 min at room temperature. Cells were subsequently blocked with 1% bovine serum albumin in PBS for 1 h at room temperature and then incubated with mouse anti-S25-229 immune sera for 1 h at room temperature. Then, cells were incubated with biotinylated goat anti-mouse IgG antibody at a dilution of 1: 200 (Vector, USA) followed by streptavidin-Alexa Fluor 488 (Invitrogen, USA) at a dilution of 1: 500. Finally, cells were counterstained with Hoechst 33342 dye (Invitrogen, USA) for 1 min, and cell staining was visualized using a confocal laser scanning microscopy LSM710 (Carl Zeiss, USA).

3. Results and discussion

3.1. Confirmation of recombinant S25-229 expression and IBs purification

Soluble expression of truncated S protein (tSc, aa 25-225) of PEDV CV777 prototype strain was reported in *E. coli* [20]. Here, we cloned and expressed NTD S25-229, an equivalent part of aa 25-225 of CV777 strain, of PEDV K14JB01 variant strain (Korean field strain isolated in 2013 [28]) in *E. coli* for our vaccine study following expression strategy of above report. The expression plasmid, pET28/S25-229, producing recombinant S25-229 protein (containing 205 amino acids, spanning amino acid region 25-229 of S protein) was constructed and transformed into the *E. coli* BL21 (DE3) strain. After IPTG induction for 4 h at 37 °C, non-induced and induced bacterial cells were lysed by sonication. Then soluble and insoluble protein fractions were separated and analyzed on 15% SDS-PAGE under non-reducing condition. However, unlike previous study, the recombinant S25-229 with a molecular mass of ~24 kDa was mainly observed within the insoluble fractions in both non-induced and induced cells (Fig. 1A, lane 2 and 4). Various IPTG concentrations, induction temperatures as low as 23 °C, and induction timings were tested, but did not affect the insoluble nature of S25-229 (data not shown). Approximately 30% of the total cell protein was S25-229. The expressed recombinant S25-229 was detected by western blot using an anti-His monoclonal antibody (Fig. 1B). In addition, western blot result showed an extra band of ~50 kDa within the insoluble fractions, which suggests the formation of dimers of S25-229 (Fig. 1B, dotted arrow). IBs were purified from bacterial cells with a yield of approximately 150 mg per liter of induced culture (Fig. 1C).

3.2. Solubilization and refolding of S25-229 from IBs

Generally, solubilization of IBs is achieved by the use of high concentration of chaotropic agents, such as urea and/or guanidine hydrochloride (Gdn-HCl) [30]. Although Gdn-HCl is generally preferred due to its superior chaotropic properties, we chose urea

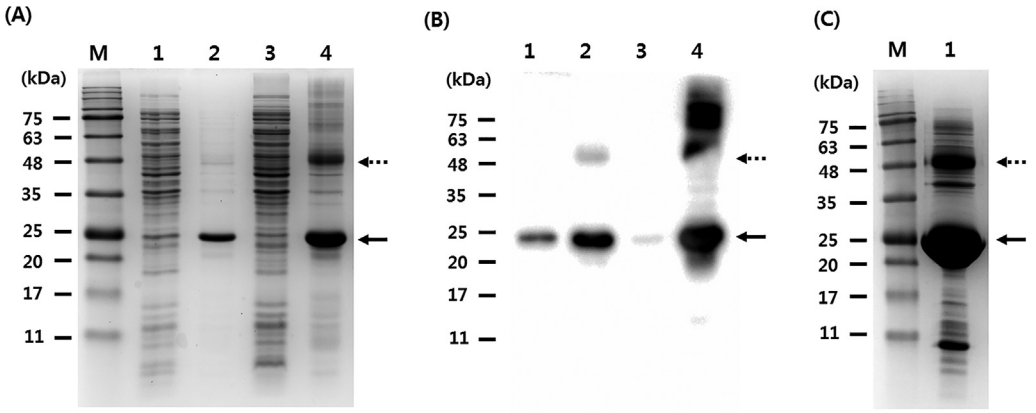

Fig. 1. Recombinant S25-229 expression and IBs purification. (A) SDS-PAGE analysis of recombinant S25-229 expression. Lanes: M, protein molecular weight marker; 1, supernatant of uninduced cell lysate; 2, pellet of uninduced cell lysate; 3, supernatant of induced cell lysate; 4, pellet of induced cell lysate. (B) Western blot analysis of recombinant S25-229 proteins using anti-His monoclonal antibody. Lanes: 1, supernatant of uninduced cell lysate; 2, pellet of uninduced cell lysate; 3, supernatant of induced cell lysate; 4, pellet of induced cell lysate. (C) SDS-PAGE analysis of purified IBs. Lanes: M, protein molecular weight marker; 1, purified IBs. Solid arrows indicate S25-229 monomer and dotted arrows indicate dimeric form of S25-229.

in the economic perspective for the solubilization of S25-229 IBs. In this study, 20 mM Tris-HCl (pH 7.5) buffer containing 8 M urea was initially used to solubilize the S25-229 IBs, but the solubilization efficiency was only 21% even in the presence of 8 M urea (Table 1). It has been reported that solubilization of basic fibroblast growth factor (bFGF) IBs was pH-dependent even in the presence of 8 M urea [29]. Therefore, we decided to check the role of pH on the solubilization efficiency of S25-229 IBs. As the theoretical isoelectric point (pI) of S25-229 was determined as 8.87, optimization experiments were carried out using solubilization buffers with pH values of <7.5. As shown in Table 1, the solubilization efficiency was improved from 21% to 81% as pH lowered from 7.5 to 4.5.

Because there was still some portion of the solubilized S25-229 retained as insoluble aggregates, S25-229 IBs were solubilized in the presence of reducing agent. The addition of 1 mM DTT to 20 mM sodium acetate (pH 4.5) buffer containing 8 M urea improved the solubilization efficiency from 81% to 95%, suggesting that intramolecular or intermolecular disulfide bonds may at least partially contribute to the formation of S25-229 IBs. Although the formation of disulfide bonds in the native PEDV S1-NTD is unknown, S25-229 protein contains 6 cysteines, and therefore, intramolecular or intermolecular disulfide bonds may be formed in S25-229 IBs. The solubilization yield was calculated to be 95% and about 7.1 mg of solubilized protein could be obtained from 7.5 mg of IBs (Table 2).

Dilution of the solubilized protein directly in the refolding buffer is one of the most widely used methods in the laboratory-scale refolding studies because of the simplicity [25]. In the present study, solubilized S25-229 IBs were 10-fold flash diluted into 45 ml of ice-cold refolding buffer (20 mM sodium acetate, pH 4.5, 1.2 M urea and 5% sucrose) and incubated with gentle rotation on a rotator for 4 h at 4 °C. Around 6.3 mg of protein was recovered from solubilized S25-229 IBs with step yield of 89% (Table 2).

3.3. Purification of S25-229

Because IBs are normally enriched of highly pure target proteins with purity levels up to 80%, the formation of IBs could be advantageous in the downstream purification process [30]. Indeed, previous studies have achieved one-step chromatographic purification of various recombinant proteins, such as ovalbumin, human growth hormone and human granulocyte colony stimulating factor [31–33]. In this study, refolded S25-229 was purified by strong cation exchange chromatography using HiTrap SP FF column on a FPLC ÄKTA explorer 10 chromatography system (Fig. 2A). Elution fractions were pooled and analyzed by SDS-PAGE (Fig. 2B). Pure S25-229 protein was obtained with purity more than 95% and overall yield of 20% using one-step chromatography run (Table 2). Purified protein was confirmed by western blot using anti-His monoclonal antibody (Fig. 2C). The low yield (23%) of cation exchange chromatography may be partially explained by the multimer formation of this protein. In SDS-PAGE and western blot (Fig. 1), dimeric band is clearly seen. However, this band disappeared after chromatography (Fig. 2B and C). We assumed that anything soluble is folded, structured and stable protein in refolding step. Considering that S25-229 monomer and dimer in Fig. 1C of purified IBs comprises over 80%, 77% loss in chromatography step indicates that refolded S25-229 proteins are soluble but in various structures including dimer and these be removed by tight binding to the column.

3.4. CD spectra of purified S25-229

The elution fractions were pooled, buffer-exchanged into PBS and the CD spectra of S25-229 were recorded to analyze the secondary structure. The CD spectrum of S25-229 showed the

Table 1
Solubilization of the S25-229 IBs with 8 M urea in various buffers.

Solubilization solutions	Solubilization efficiency (%)
20 mM Tris-HCl, 8 M urea, pH 7.5	21
20 mM HEPES, 8 M urea, pH 7.0	21
20 mM Bis-Tris, 8 M urea, pH 6.5	25
20 mM sodium acetate, 8 M urea, pH 5.5	43
20 mM sodium acetate, 8 M urea, pH 4.5	81
20 mM sodium acetate, 8 M urea, 1 mM DTT, pH 4.5	95

Table 2
Summary of S25-229 protein yields.

Steps	Protein (mg)	Purity (%)	Step yield (%)	Overall yield (%)
IBs[a]	7.5	≥80	100	100
Solubilization	7.1	≥80	95	95
Refolding	6.3	≥80	89	83
CE chromatography	1.5	≥95	23	20

[a] 75 mg of inclusion body proteins were obtained from 2.28 g wet cells harvested from 0.5 liter *E. coli* culture. An aliquot of 7.5 mg IBs were used as a starting material for purification.

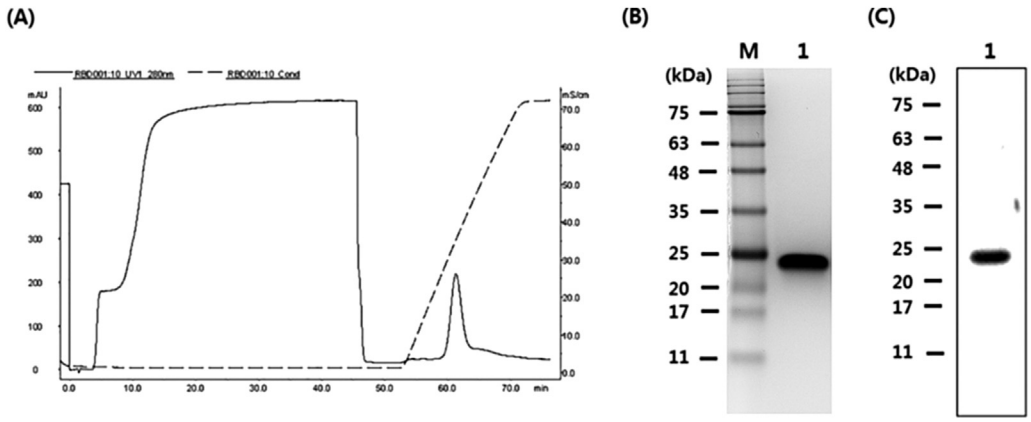

Fig. 2. Purification of refolded S25-229 by one-step cation exchange chromatography. (A) FPLC chromatogram of S25-229 purification loaded on a Hitrap SP FF column. (B) SDS-PAGE analysis of purified S25-229 after cation exchange chromatography. Lanes: M, protein molecular weight marker; 1, purified S25-229. (C) Western blot analysis of purified S25-229 using anti-His monoclonal antibody. Lanes: 1, purified S25-229.

characteristic pattern of alpha helical structure with bands at 208 nm and 222 nm (Fig. 3). However, since there was no commercial standard sample available for the CD analysis, we could not compare the secondary structure between refolded S25-229 and a standard.

3.5. Reactivity of S25-229 with mouse anti-PEDV sera

The reactivity of purified S25-229 with mouse anti-PEDV sera was tested by an indirect ELISA. The 96-well plates were coated with 0.1 μg/well of purified S25-229. PEDV negative sera were served as negative control. As a result, the purified S25-229 showed dominant reaction with mouse anti-PEDV sera as compared to the PEDV negative sera (Fig. 4). These results are consistent with previous research on the development of indirect ELISA based on tSc (aa 25-225) for the detection of PEDV infection in pigs [20].

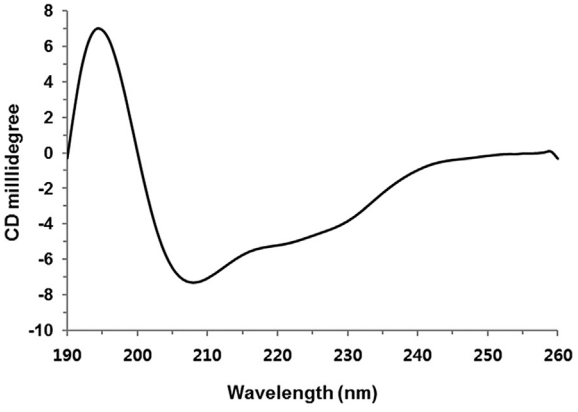

Fig. 3. CD spectra of purified S25-229.

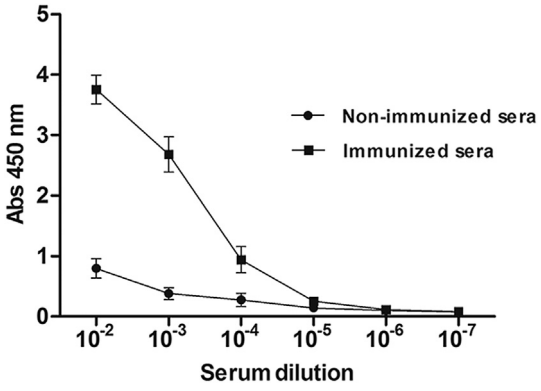

Fig. 4. Reactivity of recombinant S25-229 with mouse anti-PEDV immune sera in an indirect ELISA. Error bars indicate the standard deviations of the means.

Moreover, previous report also demonstrated that there were positive correlations between indirect ELISA results and virus neutralization titers, thus suggesting that the NTD of PEDV S1 domain may have potential to be used as an effective subunit vaccine against PEDV [20].

3.6. Mouse immunization of purified S25-229

To determine the immunogenicity of S25-229, C57BL/6J mice were immunized subcutaneously with 20 μg of purified antigen for two times at 3-weeks intervals. Sera were collected at 0, 21 and 42 days after first immunization from non-immunized and immunized mice. The 96-well plates were coated with S25-229 and reacted with corresponding mouse sera diluted at 1: 1000 for the detection of antigen specific serum IgG level. The ELISA result showed that S25-229 elicited strong humoral immune response

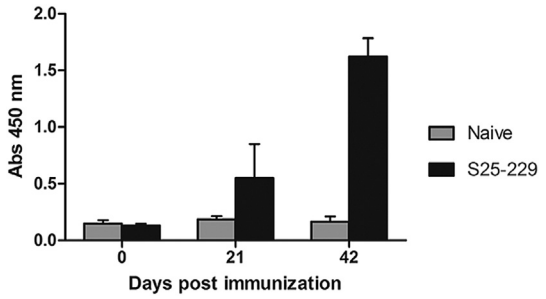

Fig. 5. Antigen specific serum IgG level of mice immunized with S25-229. Mice (n = 5) were immunized subcutaneously with 20 μg of purified S25-229 for two times at 3-weeks intervals. Sera were collected from non-immunized and immunized mice at 0, 21, 42 days after first immunization. Serum antibody levels were detected by indirect ELISA using 1000-fold diluted sera. Error bars indicate the standard deviations of the means.

IBs in *E. coli*. Therefore, our research was aimed to recover soluble S25-299 from IBs. Because our attempt to solubilize purified S25-229 IBs in 20 mM Tris-HCl (pH 7.5) buffer containing 8 M urea showed low solubilization efficiency, the solubilization condition was further optimized. We found that lowering the pH and adding reducing agent were helpful to improve the solubilization efficiency. Subsequently, solubilized S25-229 IBs were refolded by simple dilution method. Refolded S25-229 was 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 S protein of PEDV in an immunofluorescence assay. The results suggest that purified S25-

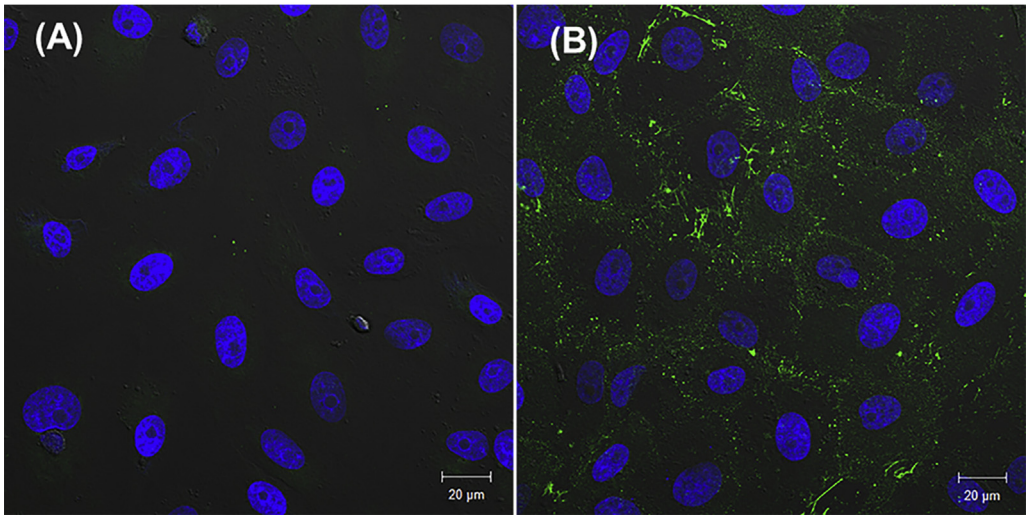

Fig. 6. Immunofluorescence assay of PEDV-infected Vero cells with mouse antisera against S25-229. (A) Assayed with non-immunized mouse sera (B) Assayed with S25-229 immunized mouse sera. Bound anti-S25-229 antibody was detected by biotin conjugated anti-mouse IgG secondary antibody and streptavidin Alexa Fluor 488 conjugate (green). Finally, cells were counter stained with Hoechst 33342 dye (blue) and analyzed by confocal laser scanning microscopy (CLSM). Images are representative of three independent experiments (S25-229 immunized mouse sera, n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

after second injection (Fig. 5). Therefore, these data together with indirect ELISA results indicate that the S25-229 has excellent immunogenicity, thus could be considered for using as a subunit vaccine candidate against PEDV.

3.7. Immunofluorescence assay (IFA)

To further determine whether mouse sera raised against S25-229 could react with native PEDV S protein, an immunofluorescence assay was performed. Vero cells were infected with PEDV SM98 at multiplicity of infection (MOI) of 1. After incubation for 3 days, cells were reacted with terminal mouse S25-229 antisera diluted at 1: 2000. As shown in Fig. 6, fluorescence signal was observed in the PEDV-infected cells treated with S25-229 antisera. In contrast, no fluorescence signal was observed in the cells treated with control sera obtained from non-immunized mice. The results also confirmed that the S25-229 was able to induce specific antibodies with binding ability to the native S protein of PEDV.

4. Conclusion

In this study, expression of S25-229 was mainly accumulated as 229 may have potential to be used as a subunit vaccine for the prevention of PEDV. Moreover, the method presented in this work provides an easy way to produce soluble recombinant S25-229 from bacterial IBs.

Author contributions

DCP designed the research, performed the experiments and wrote the manuscript. YSL and ZSH helped to interpret data. JDB, CSC, SKK and YJC helped to revise the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no competing interests.

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