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TRANSIENT EXPRESSION AND PURIFICATION OF S2 PROTEIN FROM PORCINE EPIDEMIC DIARRHEA VIRUS IN PLANTS

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SUMMARY

Porcine epidemic diarrhea (PED), caused by the Porcine epidemic diarrhea virus (PEDV), is an acute, highly contagious disease of pigs of all ages, especially piglets under one week old, with the mortality rate reaching 95–100%. Developing an effective vaccine against PEDV in Vietnam is urgent. Spike protein containing S1 and S2 subunits is considered the main target for vaccine development, and the S2 subunit contains immunodominant neutralizing epitopes of PEDV. To date, the expression of S2 protein in plants has not been reported and evaluated. In this study, the gene encoding the S2 subunit of a PEDV strain belonging to genotype 2a was amplified, sequenced, and inserted in a pRTRA vector containing the GCN4pII (pII) motif. The plant expression cassette containing S2-pII was then inserted into the pCB301 vector. The *Agrobacterium tumefaciens* harboring the pCB301-S2-pII vector was transformed into *Nicotiana benthamiana* leaves via agroinfiltration. The accumulation level of S2-pII protein in tobacco leaves was semi-quantified by Western blot, accounting for approximately 86.7 mg/kg of fresh leaves and 1.47% total soluble protein, which was 294-fold higher than the accumulation level of S1-pII protein in our previous publication. S2-pII protein was successfully purified by immobilized metal affinity chromatography (IMAC). The oligomeric state of S2-pII protein was characterized by size exclusion chromatography (SEC). The S2-pII protein was determined to be a multimer protein with a high molecular weight. These results are the basis for more extended studies to develop a plant-based S2 vaccine against PEDV infection.

Keywords: S2 protein, porcine epidemic diarrhea virus, transient expression, plant, vaccine

INTRODUCTION

Porcine epidemic diarrhea (PED) is one of the most dangerous acute infectious diseases occurring in pigs of all ages, especially in piglets under one week of age, with the highest mortality rate of up to 95–100% (Hanke *et al*., 2017). PED is caused by the Porcine Epidemic Diarrhea Virus (PEDV), which belongs to the Coronaviridae family (Li *et al*., 2020). Since the outbreak in the UK and Belgium in the 1970s, the PED epidemic has been causing serious economic damage to the pig industry in many countries around the world, especially countries in Asia and America (Lee, 2015). In Vietnam, PED was first detected in some pig herds in southern provinces in 2008. After that, the PED epidemic quickly spread to many provinces in

Vietnam with an almost 100% mortality rate in piglets, causing high damage to the domestic livestock industry (Do *et al*., 2011; Nguyen *et al*., 2012). PEDV strains isolated in Vietnam have very diverse origins (Europe, Asia, and North America), belonging to subgroups G1b, G2a, and G2b (Than *et al*., 2020). Phylogenetic studies based on PEDV isolates in 11 provinces of Vietnam during the period 2015–2016 showed that the majority of PEDV strains isolated in Vietnam belong to genogroup G2 (Than *et al*., 2020).

Vaccination is considered an effective preventive strategy for controlling PED. To date, most PEDV vaccines in Vietnam are imported vaccines containing PEDV strains of genogroup G1, so they are not effective in protecting pigs against PEDV strains in the field. Therefore, the development of efficient, quick, and safe vaccines with a high protective effect against PEDV strains in the field is extremely urgent. Production of plant-based vaccines using transient expression technology is considered a promising strategy because of outstanding advantages such as being able to produce on an industrial scale with low cost, having a quick production time of only a few days, a high level of protein accumulation in plant cells, and avoiding contamination with viruses and bacteria causing diseases in animals and humans (Chen *et al*., 2013; Topp *et al*., 2016).

Among PEDV proteins, the S protein is considered to be the main target for the development of a subunit vaccine against PEDV (Li *et al*., 2017; Gong *et al*., 2018; Chen *et al*., 2018). The PEDV protein S is divided into the S1 region (amino acids 1–729) and the S2 region (amino acids 730–1383) (Xia *et al*., 2019). The S2 subunit contains four PEDV immuno-neutralizing epitopes, including amino acids 744–759, 747–774, 756–771 and 1371–1377, recognized by ten S protein-specific monoclonal antibodies, while the S1 subunit has only one immuno-neutralizing epitope consisting of amino acids 499–600 that is recognized by a monoclonal antibody (Okda *et al*., 2017). Therefore, developing a subunit vaccine against PEDV based on the S2 subunit is a potential strategy. Several studies have expressed partial or full S2 subunits in *E. coli* systems (Paudel *et al*., 2014; Zhao *et al.,* 2018; Sritun *et al*., 2021).

In previous studies, the COE or S1 antigens have been successfully expressed in plant systems using transient expression technology (Ho *et al*., 2020; Ho *et al*., 2021; Nguyen *et al*., 2021). The COE antigens were proven to induce an immune response in mice or protect pigs against PEDV infection (Ho *et al*., 2020; Nguyen *et al*., 2021; Ho *et al*., 2022). However, to date, the expression of S2 protein in plant systems using transient expression technology has not been reported.

Here, a plant expression vector containing a gene encoding the S2-fused GCN4pII motif was constructed. This vector was transformed into *Agrobacterium tumefaciens,* which was then infiltrated into *Nicotiana benthamiana* leaves. The expression of S2-pII protein was successfully detected by Western blot. Notably, S2-protein was expressed at a very high level in tobacco leaves. S2-pII protein was purified by immobilized metal affinity chromatography (IMAC), then purified and characterized by size exclusion chromatography (SEC). These results are the basis for further studies to develop a subunit vaccine against PEDV based on transient expression technology in plants.

MATERIALS AND METHODS

Construction of a cloning vector containing the gene encoding the S2-pII protein

The PEDV PS6 strain was isolated at the Phu Son farm, Dong Nai, Vietnam, and provided by the National Veterinary Joint Stock Company (Navetco). The S2 region was sequenced by the Institute of Biotechnology. The DNA fragment encoding the S2 protein (amino acids 730–1324) was amplified with a

specific primer containing *Bam*HI and *psp*OMI at the 5' end and then inserted into the cloning vector pRTRA-35S-SP-His-COE-GCN4pII-cmyc-KDEL (Ho *et al*., 2022) at the sites of *Bam*HI and *psp*OMI, using the enzyme T4 DNA ligase according to the manufacturer's instructions (Thermo Fisher Scientific). The recombinant pRTRA plasmid was transformed into the *E. coli* XL1 blue strain and placed on LB agar supplemented with the selective antibiotic carbenicillin 50 mg/L. To select *E. coli* clones carrying the recombinant plasmid pRTRA-35S-SP-His-S2-GCN4pII-cmyc-KDEL (abbreviated as pRTRA-S2-pII), the recombinant plasmid was digested with *Bam*HI and *psp*OMI. Finally, the gene encoding the S2-pII protein in the pRTRA-S2-pII plasmid was confirmed by sequencing according to Sanger's method using primers 35S-SQF (5'-CACTGACGTAAGGGATGACGC-3') and 35S-Term-R (5'-CTGGGAACTACTCACACA-3'). Nucleotide sequences were analyzed using BioEdit 7.0 and Lasergen 7 (DNAstarinc., Madison, WI, USA).

Construction of a plant expression vector containing the gene encoding the S2-pII protein

The pRTRA-S2-pII vector was digested with *Hin*dIII to obtain the expression cassette containing S2-pII. Vector pCB301 was also digested with *Hin*dIII and *SAP*. Next, the expression cassette containing S2-pII was inserted into the vector pCB301, then transformed into the *E. coli* XL1 blue strain and placed on LB agar supplemented with 50 mg/L kanamycin. The recombinant plasmid pCB301-S2-pII was selected by colony-PCR with primers 35SQF and 35S-Term and confirmed with *Not*I. Finally, the recombinant pCB301-S2-pII plasmid was transformed into the *A. tumefaciens* AGL1 strain by electroporation at 2.5 kV, 25 μF capacitance, and 400 Ohm resistance.

Transient expression of S2-pII protein in *N. benthamiana* leaves

The transient expression of S2-pII protein was performed in *N. benthamiana* leaves according to the procedure described previously (Ho *et al*., 2021). The *A. tumefaciens* strain carrying the recombinant pCB301-S2-pII vector and the *A. tumefaciens* carrying the vector containing the gene encoding the Hc-pro protein were grown in separate falcon tubes. Each contained 5 mL of LB supplemented with the selective antibiotics carbenicillin 50 mg/L, kanamycin 50 mg/L, and rifamycin 50 mg/L. Bacteria were shaken at 120 rpm/min for 14–16 hours at 28^{o}C. All of the above cultures were transferred to a 50 mL LB container and continued to culture for 14–16 hrs. Bacteria were collected by centrifugation at 4200 rpm/min for 15 min at 4°C. Bacterial residues of *A. tumefaciens* were dissolved in MES buffer (10 mM $MgCl_2$, 10 mM MES, pH 5.6) until OD_{600} reached 0.6. The bacterial suspension was used for leaf transformation on hydroponic *N. benthamiana* (7 weeks old from seeding) by vacuum suction for 1 min 30 seconds, 27 inches, and 0 atm. The tobacco plants, after transformation, were kept in the greenhouse to continue growing. The leaves of tobacco plants were collected 5 days after transformation. Whole leaves were collected and stored at -80 °C until analysis.

Evaluation of the expression level of S2-pII protein in *N. benthamiana* leaves by SDS-PAGE and Western blot

The expression level of the S2-pII protein in tobacco leaves was determined by SDS-PAGE and Western Blot according to the procedure described previously (Ho *et al*., 2021). Tobacco leaves were extracted in an SDS sample (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% (w/v), bromophenol blue, and 10% (v/v) glycerol), denatured at 95 ^{o}C for 10 min, and centrifuged at 13000 rpm for 30 min at 4 ^{o}C. Total soluble protein (TSP; 15–45 μg) was separated by SDS-PAGE (10% polyacrylamide), then transferred onto a nitrocellulose membrane overnight at 18 V. The membrane was blocked with 5% skimmed milk diluted in PBS before incubation with a monoclonal 6x-his tag antibody produced in mice (Thermo Fisher Scientific) for 1 h. The

membrane was washed three times with 0.5% milk mixed in PBS, with 5 min intervals between stages. Then, the membrane was incubated with anti-mouse IgG-HRP (Invitrogen) for 1 h. The presence of the S2-pII protein containing the 6x-his tag in the sample was detected by colorimetric reaction using DAB substrate (Thermo Fisher Scientific) in the presence of 0.04% H_2O_2. Western blot images were captured by using an Amersham™ Imager 680. The expression level of the S2-pII protein was relatively calculated based on a calibration curve constructed from signal bands of the SEC-purified H5-pII protein standard (50 ng, 100 ng, 200 ng, 300 ng, and 400 ng (Phan *et al*., 2017) using ImageQuant TL 8.0 software (Cytiva).

Purification of S2-pII protein by IMAC

The recombinant S2-pII protein fused the 6xhis-tag was purified by the IMAC method according to the procedure described in a previous publication (Ho *et al*., 2020). Tobacco leaves expressing the S2-pII protein were cooled in liquid nitrogen and homogenized in liquid nitrogen using a porcelain mortar. Tobacco leaves (80 g) were added to 240 ml of cold extraction buffer consisting of 20 mM sodium phosphate, 0.5 M NaCl (pH 7.4), 10% glycerol, and 1 tablet of complete protease inhibitor cocktail (Roche), then the mixture was homogenized using a commercial blender. The total soluble protein was separated by centrifugation three times (8,000 rpm/min, 30 min at 4°C), then mixed with 20 ml of Ni Sepharose Fast Flow (Cytiva). The mixture was stirred gently by magnetic stirring overnight at 4 °C. The mixture is packed into the chromatographic column. The column containing the mixture was washed twice, each time with 500 ml of cold wash buffer (20 mM sodium phosphate, 0.5 M NaCl, and 10 mM imidazole, pH 7.4). An elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole, pH 7.4) was then used to solubilize the recombinant protein from the column. The purified S2-pII protein was dialyzed overnight at 4°C using a Spectra/Por® Regenerated Cellulose cutoff membrane of 6–8 kDa (Spectrum) in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) at 4 °C. The purified S2-pII protein was concentrated on Pierce™ Protein Concentrator PES column, 10K MWCO, 5-20 mL (Thermo Fisher Scientific), then mixed with 50% glycerol and stored at -20°C.

Purification and characterization of the oligomeric state of S2-pII protein by SEC

The oligomeric state of the S2-pII protein was analyzed using an SEC-based method previously published (Ho *et al*., 2022). IMAC purified S2-pII (100 µg) protein was loaded to a Superose™ 6 increase 10/300GL column (GE Healthcare) and purified using an AKTA pure machine. The kit includes high molecular weight protein standards in the range of 75–2000 kDa (GE Healthcare) that were loaded and run under the same conditions to estimate the molecular weight of the S2-pII protein. The presence of S2-pII protein in SEC fractions was analyzed by SDS-PAGE and Western blot. SEC fractions containing the presence of S2-pII protein were collected and concentrated on a Pierce™ Protein Concentrator PES column, 10K MWCO, 5–20 mL (Thermo Fisher Scientific), then supplemented with 50% glycerol and stored at -20°C for further analysis.

RESULTS AND DISCUSSION

Construction of the cloning vector containing gene encoding the S2-pII protein

To construct the cloning vector, the gene encoding the S2 protein was amplified by PCR using specific primers. The electrophoresis result showed that the S2 protein-coding gene segment was successfully amplified with the correct theoretical size of 1.791 kb (Fig. 1A). Next, the gene encoding the S2 protein was inserted into the pRTRA-pII vector. Clones harboring the recombinant pRTRA-S2-pII plasmid were selected by colony-PCR and digested with *Bam*HI and *psp*OMI. The electrophoresis result of colony-PCR products using primers 35S-SQF

and 35S-Term-R showed the appearance of a band of approximately 2.27 kb, consistent with theoretical calculations in two clones number (No.) 1 and 6 (Fig. 1B). For further demonstration, plasmids No. 1 and 6 were digested with *Bam*HI and *psp*OMI. The results demonstrated that two bands with sizes of about 3.674 kb and 1.791 kb were obtained, which are consistent with theoretical calculations in plasmids No. 1 and 6 (Fig. 1C). The sequencing results of these plasmids once again confirmed that the two clones No. 1 and 6 carried recombinant pRTRA-S2-pII. A schematic diagram of the expression cassette carrying the gene encoding the S2-pII protein is shown in Fig. 1D.

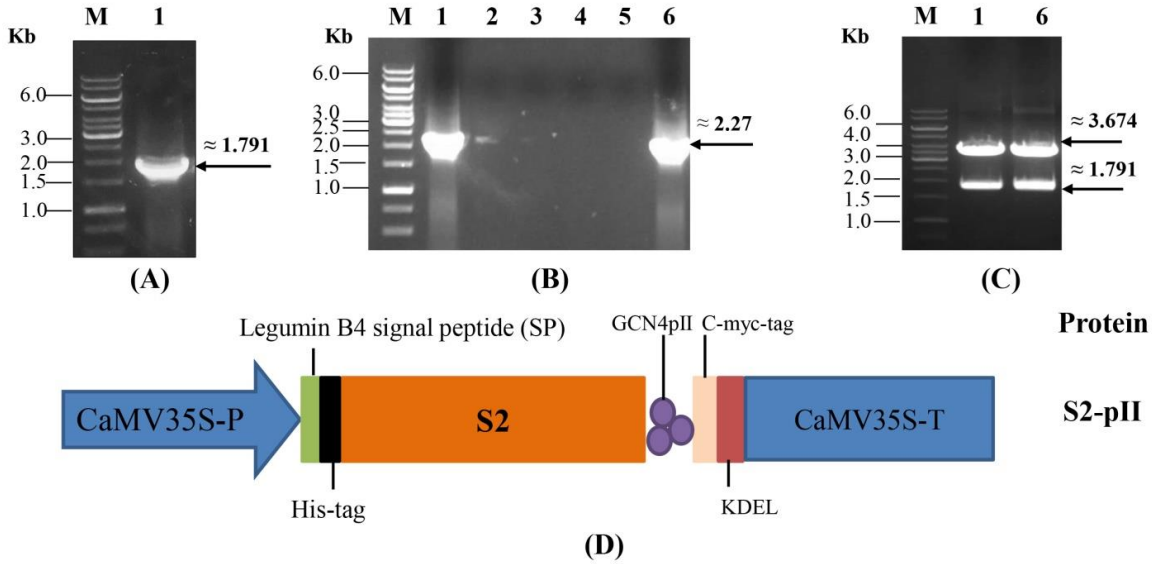

Figure 1. Construction of a cloning vector containing a gene encoding S2-pII. (A) Amplification of the gene encoding S2 of the PEDV PS6 strain; (B) Colony-PCR to select clones harboring recombinant pRTRA-S2-pII vector using primers 35SS-QF/35-Term-R; (C) Selection of recombinant pRTRA-S2-pII plasmid by digestion with *Bam*HI and *psp*OMI; (D) Schematic diagram of the expression cassette containing the gene encoding the S2-pII protein. CaMV35S-P: promoter of 35S Cauliflower mosaic virus, Legumin B4 signal peptide. KDEL: maintain proteins in the endoplasmic reticulum (ER), GCN4pII: multimeric motif, His-tag: 6xHis-tag, c-myc-tag, CaMV35S-T: terminator 35S Cauliflower mosaic virus.

Construction of a plant expression vector containing the gene encoding the S2-pII protein

The plasmid pRTRA-S2-pII No. 1 was digested with *Hin*dIII to obtain the expression cassette carrying the gene encoding S2-pII. This expression cassette was then inserted into the pCB301 vector. The results of colony-PCR and the *Not*I digestion showed that clone No.1 had the recombinant pCB301 vector with a size similar to the theoretical calculation (Fig. 2A, B). Therefore, the recombinant pCB301-S2-pII vector was successfully constructed. The plasmid pCB301-S2-pII No. 1 was transformed into an *A. tumefaciens* strain. Colony-PCR results showed that all four *A. tumefaciens* clones carried the plasmid pCB301-S2-pII (Fig. 2C). The diagram of the pCB301-S2-pII vector is shown in Fig. 2D. Thus, we have selected a clone of *A. tumefaciens* carrying the recombinant plasmid pCB301-S2-pII.

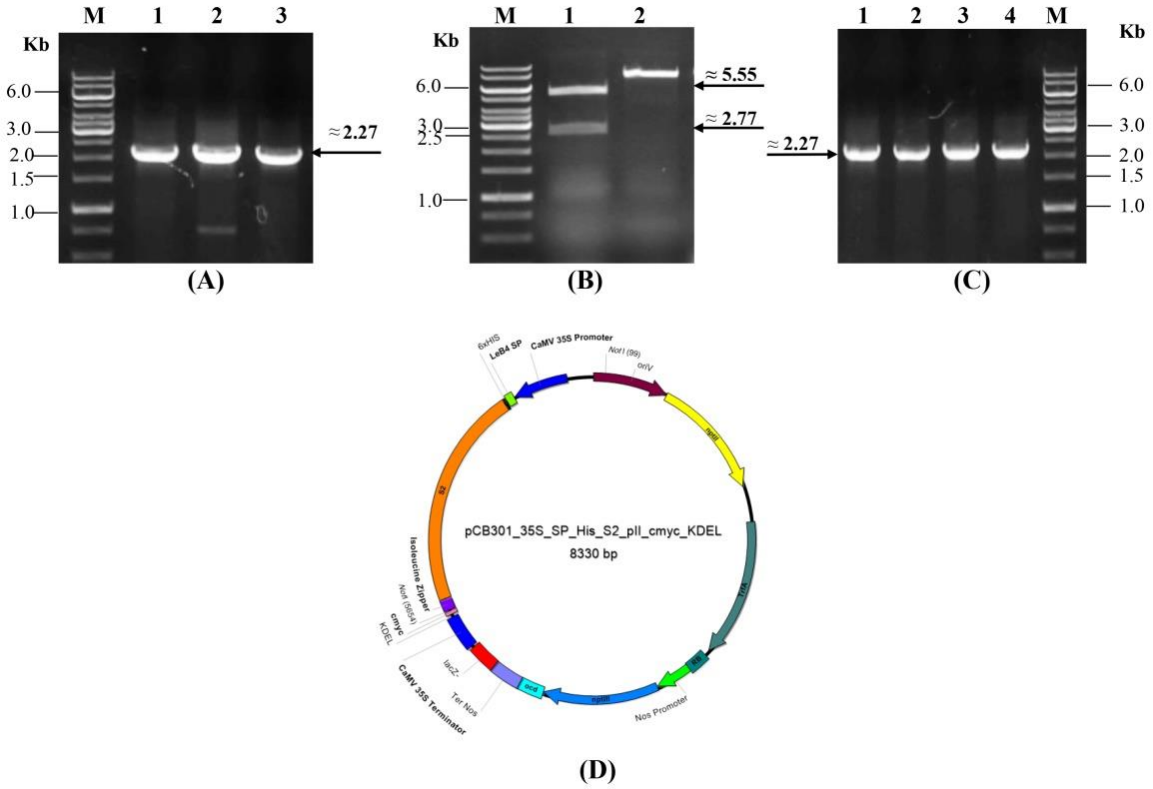

Figure 2. Construction of a plant expression vector harboring the gene encoding the S2-pII protein. (A) Selection of recombinant pCB301-S2-pII vector by colony-PCR using primers 35SS-QF/35-Term-R; (B) Selection of pCB301-S2-pII plasmid by digestion with *Not*I; (C). Selection of *A. tumefaciens* clones carrying the recombinant vector pCB301-S2-pII by colony-PCR; (D). Diagram of pcB301-S2-pII vector, marker: GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific).

Evaluation of the expression level of S2-pII protein in *N. benthamiana* leaves by SDS-PAGE and Western blot

Leaves were collected five days after the agro-infiltration for the detection of the expression of S2-pII protein (Fig. 3A). The expression of the recombinant S2 protein was assessed by Western blot using an anti-6x-his tag antibody. Western blot results showed that two bands were detected from the total soluble proteins extracted from the transformed leaves (Fig. 3B). The two bands detected were approximately 100 kDa and above 250 kDa. Therefore, the S2-pII protein was successfully expressed in tobacco leaves. The predicted molecular weight of monomeric S2-pII protein based on the calculation of the Lasergene software is 72.33 kDa; however, the actual molecular weight of monomeric S2-pII protein is more than 100 kDa. This could be explained by the influence of the N-glycosylation sites on the S protein (Makadiya *et al*., 2016), which affects protein migration and cleavage during SDS-PAGE electrophoresis. When expressing S2 protein in the *E. coli* system, if there is no N-glycosylation site on S2 protein, the molecular weight of S2 protein is about 90 kDa (Sritun *et al*., 2021). The natural S protein of PEDV exists as a homotrimer glycoprotein (Walls *et al*., 2016). A band (>250 kDa) was detected in tobacco leaf extracts expressing the S2-pII protein, which could be explained by the formation of an oligomerized form of the S2 protein upon fusion with the pII motif. The formation of oligomer protein structures has

also been reported in the fusion of the H5 protein of influenza A/H5N1 and the COE protein of PEDV with the pII motif (Phan *et al*., 2017; Ho *et al*., 2020; Ho *et al*., 2022).

The expression level of recombinant S2-pII protein in plants was quantified *via* the H5-pII protein (Phan *et al.*, 2017). A calibration curve was constructed from signal bands and known amounts of the H5-pII protein by using ImageQuant TL 8.0 software (Cytiva). The calibration curve used for the quantification of S2-pII protein is shown in Fig. 3C. The quantity calibration curve shows the relationship between raw volume (X) and calibrated volume (Y) of H5-pII protein bands. The expression level of the S2-pII protein in tobacco leaves was quantified at approximately 86.7 mg/kg of fresh leaves, accounting for 1.47% of total soluble protein. When compared with the expression level of S1-pII protein of 0.005% total soluble protein in our previous study (Ho *et al*., 2021), S2-pII protein had an expression level 294-fold higher than that of S1-pII protein in tobacco leaves. In addition, the expression level of S2-pII protein was two-fold higher than that of COE fused Poly-Fc of immunoglobulin G in tobacco leaves (0.74% of total soluble protein, Nguyen *et al*., 2021). However, the expression level of S2-pII protein was 1.36-fold lower than that of COE-pII protein in our previous study (2.01% total soluble protein, Ho *et al*., 2022).

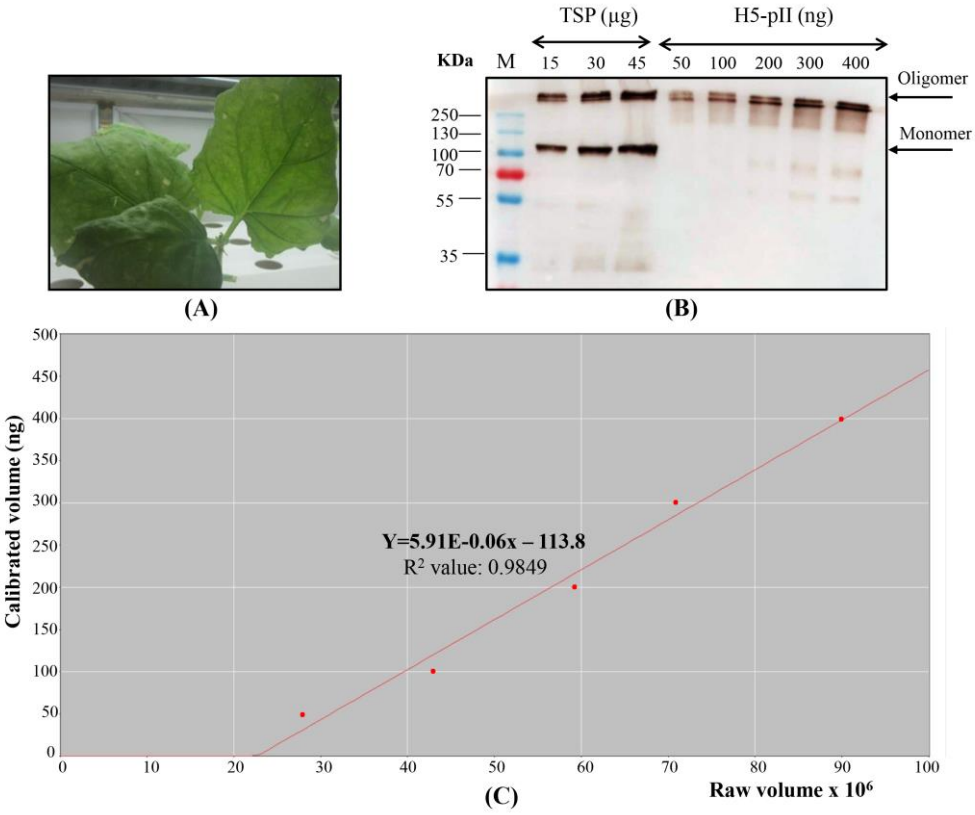

Figure 3. Evaluation of the expression of recombinant S2-pII protein in *N. benthamiana*. (A) *N. benthamiana* leaves after 5 days of transforming an *A. tumefaciens* strain carrying the vector pCB301-S2-pII; (B) Evaluating the expression of S2-pII protein by Western blot using an anti-6x-his tag antibody. The transformed leaf extract containing total soluble protein (TSP: 15, 30, and 45 µg) was electrophoresed on 4 –10% SDS-PAGE gel. H5-pII protein (50, 100, 200, 300, and 400 ng) was used to quantify S2-pII protein in leaf extract; (C) A calibration curve was constructed using ImageQuant TL 8.0 software (Cytiva) and used for the quantification of S2-pII protein. The amount of H5-pII protein was shown in red dots.

Purification of the S2-pII protein by Immobilized Metal Affinity Chromatography (IMAC)

The S2-pII protein was purified from the *N. benthamiana* leaves by the IMAC. Western blot analysis of S2-pII IMAC fractions using an anti-6x-his tag antibody showed that almost all S2-PII protein bound to Ni Sepharose Fast Flow (Fig. 4A). Coomassie blue staining analysis of S2-pII IMAC fractions showed that the purified S2-pII protein was relatively pure (Fig. 4B). After calculation, the IMAC-purified S2-pII protein has a purity of nearly 85%. Thus, we successfully purified S2-pII protein from *N. benthamiana* leaves by IMAC.

Purification and characterization of the oligomeric state of S2-pII protein by SEC

To characterize the oligomeric state of S2-pII protein, purified S2-pII protein and a high molecular weight protein kit (GE Healthcare) were applied to the SEC column. SEC diagrams of S2-pII protein and high molecular weight proteins (GE Healthcare) are shown in Fig. 5A. After purification of S2-pII protein, SEC fractions were collected and analyzed by Western blot. The Western blot result showed that S2-pII protein was presented on fractions 9 to 25, corresponding to a molecular weight in the range of 158 kDa to above 2000 kDa (Fig. 5B). S2-pII protein was mostly presented on fractions 15 to 23, corresponding to a molecular weight in the range of 440 kDa to above 669 kDa, which is higher than that of COE-pII protein. The high molecular weight of protein when fused to the pII motif characterized by SEC was also reported in the previous study (Ho *et al*., 2022). When characterized COE-pII protein by SEC, COE-pII protein was mostly detected in fractions 22 to 26, corresponding to a molecular weight of approximately 440 kDa (Ho *et al*., 2022). Therefore, the S2-pII protein is a multimer protein with a high molecular weight. After SEC purification, fractions 9 to 25 were collected for further studies.

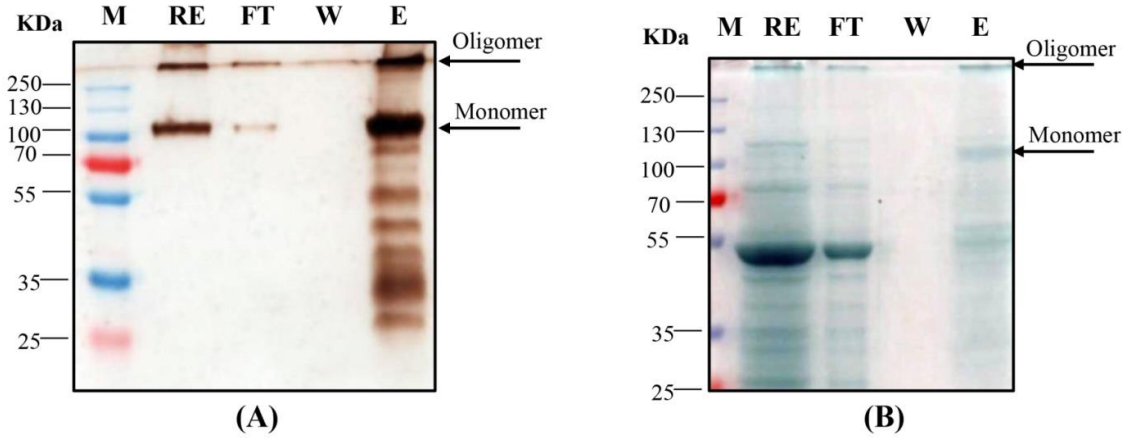

Figure 4. Purification of S2-pII protein by IMAC. (A) Evaluation of S2-pII protein purification by Western blot using an anti-6x-his tag antibody. RE: raw extract containing total soluble protein; FT: flow through; W: wash; E: purified S2-pII protein; (B) Evaluation of S2-pII protein purification by Coomassie blue staining using an anti-6x-his tag antibody. M: PageRuler™ Plus Prestained Protein Ladder (Thermo Fisher Scientific)

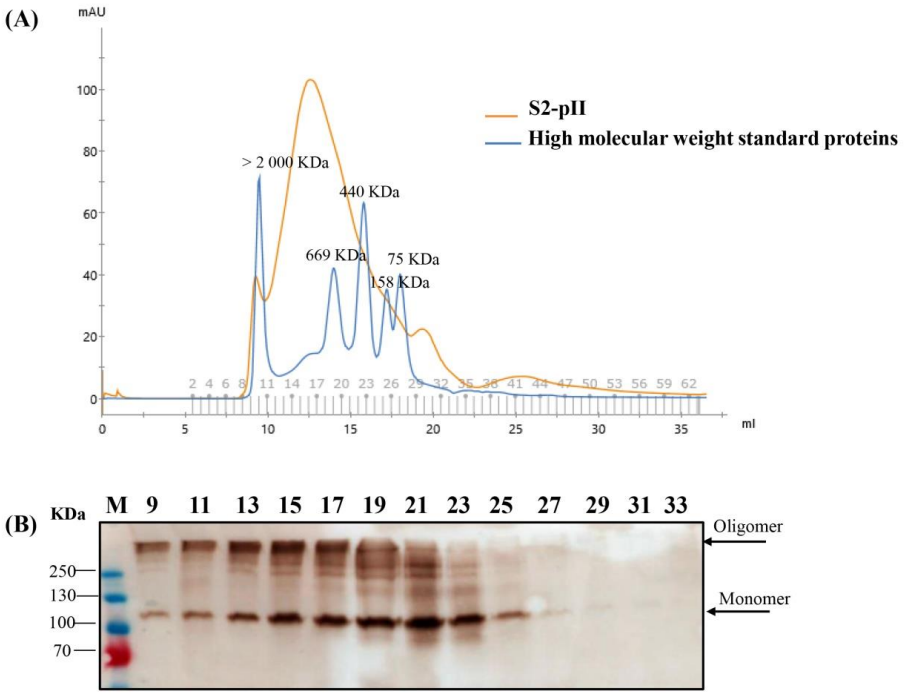

Figure 5. Purification and characterization of the oligomeric state of S2-pII protein by SEC. (A) SEC diagram of S2-pII protein and high molecular weight standard proteins (GE Healthcare) including Conalbumin (75 kDa), Aldolase (158 kDa), Ferritin (440 kDa), Thyroglobulin (669 kDa), and Blue Dextran 2000. (B) Detection of the presence of S2-pII protein in SEC fractions by Western blot, M: PageRuler™ Plus Prestained Protein Ladder (Thermo Fisher Scientific), 9-33: fractions after SEC purification of S2-pII protein

CONCLUSION

This is the first study about the expression of S2-pII protein from PEDV in *N. benthamiana* via agroinfiltration. In the current study, S2-pII protein was accumulated at a high level (approximately 86.7 mg/kg of fresh leaves) and was successfully purified by the IMAC. The oligomeric state of the S2-pII protein was characterized by SEC. The S2-pII protein was determined to be a multimer protein with a high molecular weight in the range from 158 kDa to above 2000 kDa. This is the premise for the development of plant subunit vaccines against PEDV strains in Vietnam.

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