Vietnam Journal of Biotechnology **22**(4): 641-654, 2024. DOI: 10.15625/vjbt-21878

OPTIMIZATION OF EXTRACTION AND PURIFICATION OF THE M PROTEIN FUSED GCN4pII MOTIF AND ELASTIN LIKE POLYPEPTIDE OF PORCINE EPIDEMIC DIARRHEA VIRUS FROM *NICOTIANA BENTHAMIANA*

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Received: 05.08.2024
Accepted: 11.11.2024

ABSTRACT

Porcine Epidemic Diarrhea (PED) has significantly impacted the swine farming industry in several countries worldwide, including Vietnam. Porcine Epidemic Diarrhea Virus (PEDV) has been proven to be the cause of PED. The COE and M proteins are potential candidates for subunit vaccine research against PEDV. The M protein, a largest envelope protein of PEDV, is highly conserved and plays a crucial role in virus assembly as well as in inducing the production of virus-neutralizing antibodies in the presence of complement. In a previous study, we constructed and expressed the M protein fused with the GCN4pII motif and Elastin-Like Polypeptide (M-pII-ELP) separately from the COE/G2a-pII protein in *Nicotiana benthamiana*. In this study, we assessed the co-expression of M-pII-ELP and COE/G2a-pII proteins in *N. benthamiana* using SDS-PAGE and Western blot. Next, we verified the assembly of virus-like particles (VLPs) by the M-pII-ELP protein alone through Transmission Electron Microscopy (TEM) analysis after ultracentrifugation with a sucrose gradient. Suitable buffers for the extraction and purification of M-pII-ELP protein using immobilized affinity chromatography (IMAC) were also selected. Results from SDS-PAGE and Western blot confirmed the co-expression of M-pII-ELP and COE/G2a-pII proteins in the plant; however, the expression of COE/G2a-pII protein was suppressed when co-expressed with M-pII-ELP. TEM analysis confirmed the formation of virus-like particles based on the assembly of the M-pII-ELP protein. Among the buffers tested for M-pII-ELP protein extraction, Tris-HCl buffer yielded the highest amount of M-pII-ELP protein. It was determined that the optimal imidazole concentrations for extraction and washing buffers in M-pII-ELP protein purification are 0 mM and 10 mM, respectively. These results lay the groundwork for further studies on developing plant-based subunit vaccines against PEDV.

Keywords: M protein, GCN4pII motif, Elastin like polypeptide, co-expression, virus like particle, *Nicotiana benthamiana*

INTRODUCTION

Porcine Epidemic Diarrhea (PED) has caused significant damage to pig-farming countries, especially in Asia, including Vietnam (Mai *et al*., 2020). In Vietnam, PED was initially identified in several swine farms in the Vietnam southern provinces in 2008, and it rapidly disseminated to numerous provinces across the country, resulting in the death of almost all piglets and inflicting a severe impact on the swine industry (Nguyen *et al*., 2012). Porcine Epidemic Diarrhea Virus (PEDV), the causative agent of PED, belongs to the Coronavirus family, Coronaviridae, and the genus Alphacoronavirus. Various PEDV strains were found in Vietnam, with most of these strains belonging to genogroup G2 (Than *et al*., 2020).

The COE-S protein, a key component of PEDV, is regarded as the primary target for developing a subunit vaccine against PEDV (Gong *et al*., 2018; Chen *et al*., 2018). In addition to the COE-S protein, the M protein is considered a potential candidate for production a potential vaccine that can protect against various PEDV strains due to high conservation across PEDV strains (Arndt *et al*., 2010). The M protein is the largest envelope component of the virus and plays a crucial role in the virus assembly process (Neuman *et al*., 2011). The M protein of PEDV encodes 226 amino acids and is a membrane-structured glycoprotein with a short amino-terminal end located outside the virus and a carboxy-terminal end inside the virus (Utiger *et al*., 1995). Moreover, the M protein is essential not only for virus assembly but also for inducing virus-neutralizing antibodies in the presence of complement (Saif, 1993). A comparison of PEDV strains reveals a 97.8-100% similarity in the genetic sequence encoding the M protein among different strains (Sun *et al*., 2014). Several studies have demonstrated that when the M protein, or M protein combined with E protein, or M, S, and E proteins are expressed in a plant system, they are capable of forming VLPs (Khamis, 2016; Peyret *et al*., 2021). Therefore, focusing on the M protein alone or in combination with the COE protein presents a promising pathway for vaccine development against PED.

GCN4-pII is an artificially designed trimerization motif, consisting of a core entirely formed by branched beta residues. GCN4-pII has been used to produce trimeric HA proteins of the H5N1 virus (Phan *et al*., 2013). GCN4-pII is utilized to promote the trimerization of target proteins, enhancing protein stability, and solubility, and potentially tripling the size of the target protein. Elastin-like polypeptide (ELP) is a type of polypeptide consisting of repeating VPGXG pentapeptides derived from elastin, where X is any amino acid except proline. ELP fusion can increase the accumulation of target proteins (Joensuu *et al*., 2010) and facilitate protein purification. In a previous study, the trimeric form of plant-based HA protein induced a stronger HA-specific immune response and neutralizing antibodies against H5N1 in mice compared to its monomeric form. Additionally, the data indicated that ELPylation did not affect the functionality or antigenicity of the HA trimer protein from *N. benthamiana* (Phan *et al*., 2013).

Agroinfiltration is considered a promising method for rapidly producing large quantities of antigenic proteins at low cost using plant systems, providing a timely response when an outbreak occurs (Topp *et al*., 2016). In earlier research, plant-derived

COE proteins were produced in *N. benthamiana* via agroinfiltration and were shown to elicit immune responses against PEDV (Nguyen *et al*., 2021; Ho *et al*., 2020, 2022a). The COE/G2a-pII protein elicited immune responses that protected swines against PEDV after virus challenge (Ho *et al*., 2022a). In addition, in a previous study, the expression of the M-pII-ELP alone in *N. benthamiana* was detected using SDS-PAGE and Western blot (Ho *et al*., 2023).

In this research, we conducted a co-transformation experiment of two Agrobacterium strains containing genes encoding the M-pII-ELP and COE/G2a-pII proteins in *N. benthamiana*, and the co-expression of these proteins was evaluated using SDS-PAGE and Western blot. The creation of VLPs by only M-pII-ELP protein was verified via TEM analysis after ultracentrifugation with gradient sucrose. Subsequently, appropriate buffers for the extraction and purification of the M-pII-ELP protein via IMAC were identified. These findings serve as foundation studies for developing a plant-based subunit vaccine against PEDV.

MATERIALS AND METHODS

Transient expression of M-pII-ELP protein and COE/G2a-pII protein in *N. benthamiana*

The M-pII-ELP protein was transiently expressed alone in *N. benthamiana* according to the protocol previously described (Ho *et al*., 2023). Briefly, the *Agrobacterium tumefaciens* suspension containing the gene encoding HcPro was mixed with the *A. tumefaciens* solution containing pCB301-M-pII-ELP vector until the OD_{600} reached a value of 1.0.

For the co-transient expression of M-pII-ELP and COE/G2a-pII proteins, the *A. tumefaciens* suspension containing the pCB301-M-pII-ELP vector (with an OD_{600} of 0.1 or 1.0) was mixed with the *A. tumefaciens* solution containing the pCB301-COE/G2a-pII vector (with an OD_{600} of 0.1) and the *A. tumefaciens* solution containing the gene encoding HcPro (with an OD_{600} of 1.0). The bacterial suspension was used for infiltration into 7-week-old hydroponic *N. benthamiana* leaves using a vacuum for 1 minute and 30 seconds. After infiltration, the *N. benthamiana* plants were returned to the greenhouse for continued growth. Four to five days after infiltration, leaves from the infiltrated plants were harvested kept at -80 °C for further experiments.

TEM analysis

To determine if VLPs were formed from the M-pII-ELP protein, total soluble proteins were extracted in VLP buffer (50 mM Tris-HCl, 140 mM NaCl, pH 7.5) and then concentrated using Pierce™ Protein Concentrators PES, 30K MWCO (Thermo Fisher™). A discontinuous sucrose gradient (30-60%) was added to the concentrated plant extract, and the solution was separated by ultracentrifugation at 70,000 rpm for 3 hours. The resulting fractions after ultracentrifugation were collected and examined by TEM. A droplet of the extract was placed on carbon grids and left to rest for two minutes. The grid was washed with three drops of water after remove the excess liquid. Finally, it was an addition of a drop of 2% uranyl acetate on the grid for one minute before removal. The grids prepared with protein samples were observed using a TEM, with measurements conducted on a

JEOL 1400 Flash microscope operating at 120 kV.

SDS-PAGE and Western blot

The presence of proteins in *N. benthamiana* leaves was analyzed using SDS-PAGE and Western Blot, following the protocol previously outlined (Ho *et al*., 2022b). Briefly, SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% (w/v) bromophenol blue, and 10% (v/v) glycerol) was added to ground leaves then mixture was heated at 95 °C for 10 minutes. The total soluble protein (TSP) was obtained after centrifugation at 13,000 rpm for 30 minutes at 4 °C. Samples were loaded on 4-10% polyacrylamide gel for SDS-PAGE. Next, proteins were moved to a nitrocellulose membrane. Western blotting was performed using an anti-6x-His tag antibody as the primary antibody, anti-mouse IgG-HRP (Invitrogen) as the secondary antibody, and DAB substrate (Thermo Fisher Scientific). The intensity of bands was analysed by Image J software.

Selection of extraction buffer to obtain M-pII-ELP protein

The M-pII-ELP protein detected leaves were frozen and ground using a porcelain mortar in liquid nitrogen. To extract the M-pII-ELP protein from tobacco leaves, three common extraction buffers were utilized. Buffer A contained 100 mM Tris-HCl, 15 mM NaCl, and 300 mM sucrose (pH 7.4). Buffer B consisted of 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH2PO4 (pH 7.4). Buffer C included 20 mM sodium phosphate, 0.5 M NaCl (pH 7.4). Different concentrations of Trition X-100 (0.5%, 1% and 1.5%) and Tween-20 (0.5%, 1% and 1.5%) were added to above buffer. Additionally, a crude extraction was performed in the SDS sample buffer, which was used as a positive control. One gram of ground tobacco leaves was mixed with buffer A, B, or C, and the crude extracts containing M-pII-ELP protein were obtained after centrifugation at 13,000 rpm for 30 minutes at 4 °C. The presence of M-pII-ELP in the plant extracts after extraction with different buffers was detected by SDS-PAGE and Western blot, as described above.

Purification of M-pII-ELP protein by IMAC

The M-pII-ELP protein fused a 6xHis tag was purified using IMAC with modifications from the protocol previously described (Ho *et al*., 2022). For small-scale, 20 grams of tobacco leaves were combined with 60 mL of chilled extraction buffer, composed of 100 mM Tris-HCl, 15 mM NaCl, 300 mM sucrose (pH 7.4) and different imidazole concentrations (0mM, 5 mM and 10 mM). A blender was used to emulsify the resulting mixtures. Four centrifugations (10,000 rpm for 30 minutes at 4 °C) were applied to collect soluble proteins. Next, 2 mL of Ni Sepharose Fast Flow resin (Cytiva) was combined with the supernatant and kept overnight at 4 °C. Mixture was then loaded into a chromatographic column. The 500 ml cold wash buffer (100 mM Tris-HCl, 15 mM NaCl, 300 mM sucrose (pH 7.4)) with different imidazole concentrations (10mM, 20 mM and 30 mM) was applied to column. The recombinant protein was then eluted from the column using an elution buffer (100 mM Tris-HCl, 15 mM NaCl, 300 mM sucrose, 500 mM imidazole, pH 7.4). A Spectra/Por® regenerated cellulose membrane with a 6-8 kDa cutoff (Spectrum) was used to change elution buffer of M-pII-ELP protein against PBS buffer. Finally, to

concentrate proteins, a Pierce™ Protein Concentrator PES column with a 10K molecular weight cutoff (MWCO) was employed.

For large-scale purification, optimized extraction (100 mM Tris-HCl, 15 mM NaCl, and 300 mM sucrose (pH 7.4)) and washing buffer (100 mM Tris-HCl, 15 mM NaCl, 300 mM sucrose, and 10 mM imidazole, pH 7.4) from small-scale purification were used to purify the M-pII-ELP protein. 100 grams of tobacco leaves were employed.

RESULTS AND DISCUSSION

Co-expression of M-pII-ELP protein and COE/G2a-pII protein in *N. benthamiana*

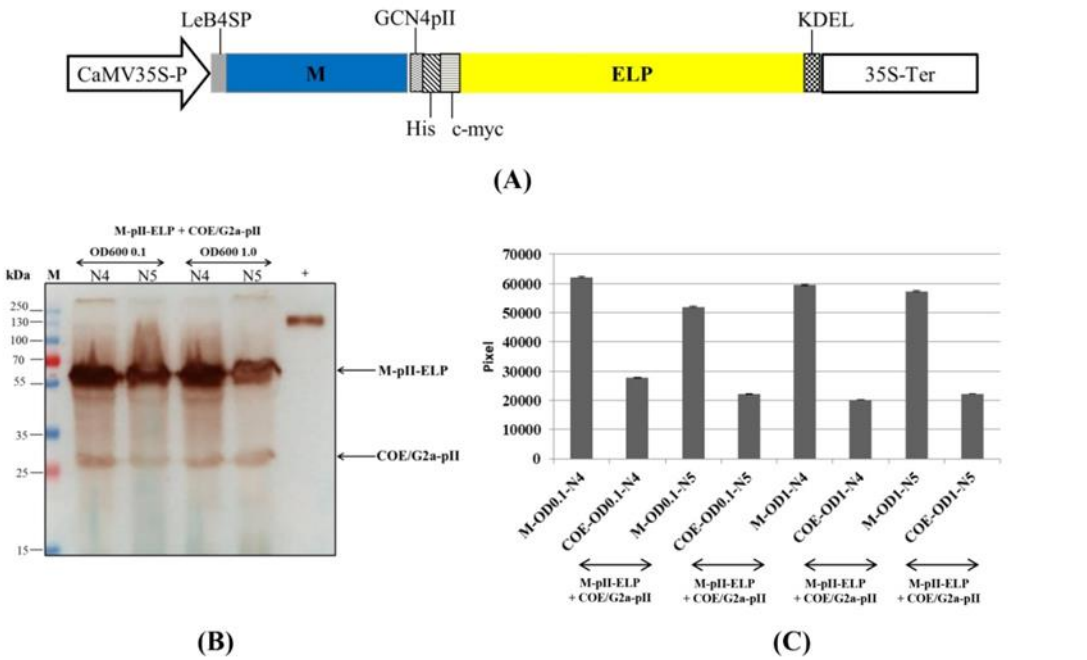

Figure 1. Detection of co-expression of M-pII-ELP protein and COE/G2a-pII protein in *N. benthamiana* leaves by SDS-PAGE and Western blot. (A) Diagram of the expression cassette carrying the gene encoding the M-pII-ELP protein. (B) Detection of co-expression of M-pII-ELP protein and COE/G2a-pII in *N. benthamiana* by Western blot using an anti-6x-His tag antibody. The *A. tumefaciens* suspension containing M-pII-ELP at an OD_{600} of 0.1 or 1.0 was mixed with *A. tumefaciens* suspension containing COE/G2a-pII at an OD_{600} of 0.1. Tobacco leaves were collected for Western blot analysis on days 4 (N4) and 5 (N5) after agro-infiltration. (+): positive control S1-6xHis tag (SARS-CoV-2). (C). Analysis of Western blot result by Image J software.

In previous studies, we constructed an expression vector carrying the fragment encoding the M-pII-ELP protein and COE/G2a-pII protein and created strains of *A. tumefaciens* containing the corresponding vector (Ho *et al*., 2022a; Ho *et al*., 2023). The expression cassette diagram including the gene encoding the M-pII-ELP antigen is presented in Figure 1A. In this study, we aim to evaluate the co-expression capability of M-pII-ELP and COE/G2a-pII proteins in *N. benthamiana*. After infiltration, the presence

of M-pII-ELP and COE/G2a-pII proteins in the tobacco leaves was detected using Western blotting (Figure 1B).

As expected, the presence of two bands on the membrane, approximately 70 kDa and above 25 kDa, indicates the successful co-expression of both proteins. Notably, the co-expression of M-pII-ELP and COE/G2a-pII proteins was stronger on day 4 after transformation compared to day 5. However, there was no significant difference in the co-expression of the two proteins when using the *A. tumefaciens* suspension mixture at OD_{600} values of 0.1 and 1.0 for transformation. Analysis of band intensity using ImageJ software revealed that M-pII-ELP accumulated approximately 3 times more in the leaves than COE/G2a-pII protein when two proteins were co-expressed (Figure 1C). In a previous study, semi-quantitative results indicated that COE/G2a-pII protein alone in *N. benthamiana* leaves was accumulated approximately 118 mg/kg of fresh leaves three days after agro-infiltration (Ho *et al*., 2022a). In contrast, the accumulation level of M-pII-ELP protein in tobacco leaves five days after agro-infiltration reached 243.4 mg/kg of fresh leaves, which is 1.98 times higher than that of COE/G2a-pII protein alone in *N. benthamiana* leaves (Ho *et al*., 2023). These findings demonstrate that the expression level of COE/G2a-pII protein significantly decreased when co-expressed with M-pII-ELP protein. It might be explained by the possibility that infiltrating all two genes together likely reduced the concentration of Agrobacterium carrying each construct or caused competition for cellular resources and subcellular localization conflicts. These factors may have resulted in lower accumulation of COE/G2a-pII protein. A similar phenomenon was also observed in a previous study, where the expression level of a protein was reduced when co-expressed with another protein (Khamis, 2016). Hence it would be better to achieve the stronger expression of M-pII-ELP and COE/G2a-pII proteins when each *A. tumefaciens* strain was transformed separately into *N. benthamiana* leaves. Therefore, we chose to express M-pII-ELP protein alone for further experiments.

VLP formation based on the assembly of M-pII-ELP protein in *N. benthamiana*

Previous studies on coronaviruses have shown that the M and E proteins, and sometimes N, play key roles in forming virus-like particles (VLPs) (Ho *et al*., 2004; Huang *et al*., 2004). In this study, the formation of VLP structures based on the assembly of M-pII-ELP protein alone in *N. benthamiana* leaves was assessed via TEM analysis after separating the plant extract containing M-pII-ELP protein using a discontinuous sucrose gradient (30-60%) through ultracentrifugation. As expected, VLP structures were detected in the bottom fraction (indicated by the red line) (Figure 2A). Circular particles were observed in this fraction containing the M-pII-ELP protein (Figure 2B) via TEM analysis. While native virions, excluding spike protein, typically measure slightly over 100 nm, M-ELP protein VLPs from PEDV was reported at around 50 nm in diameter and an envelope thickness approximately 9.3 nm (Khamis, 2016). The viral envelope of coronaviruses exhibits a distinct structural feature, where the M protein assembles into a lattice, creating a membrane with roughly double the thickness of standard biological membranes (4 nm) (Bárcena et al., 2009). In this study, an envelope thickness of VLPs close to 9 nm was observed.

In addition, the particle in Figure 2B was measured at 52.71 nm. This particle dimension and envelope thickness of the particles suggests that the VLPs are composed solely of the M-pII-ELP protein of PEDV.

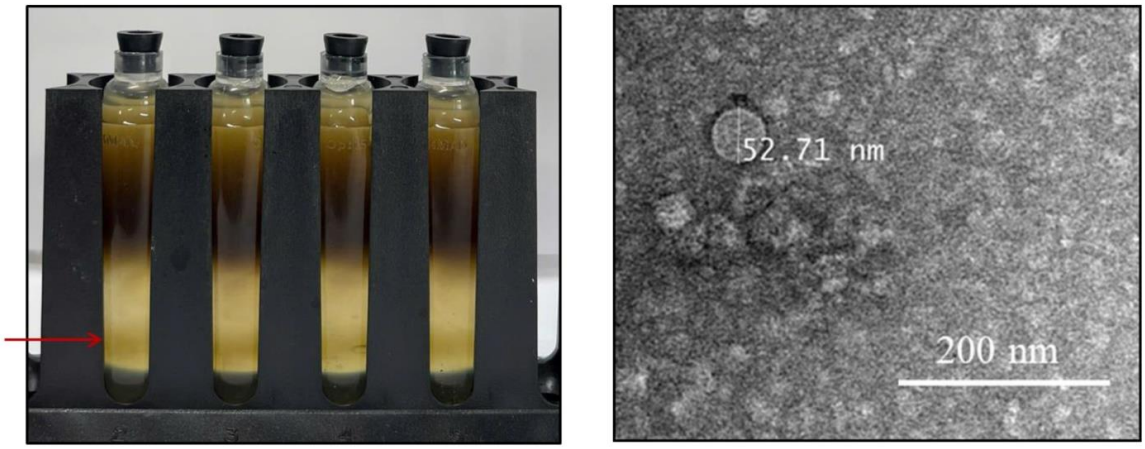

Figure 2. Confirmation of VLP formation was based on the assembly of M-pII-ELP protein in *N. benthamiana* as observed by TEM analysis. (A) The protein was isolated using VLP extraction buffer, and the extract was separated by ultracentrifugation through a 30–60% discontinuous sucrose gradient. The fraction containing M-pII-ELP protein is indicated by a red line. (B) TEM analysis of the resulting fraction (marked by the red line) after ultracentrifugation. The negatively stained grids were examined using a TEM, with analyses conducted on a JEOL 1400 Flash microscope.

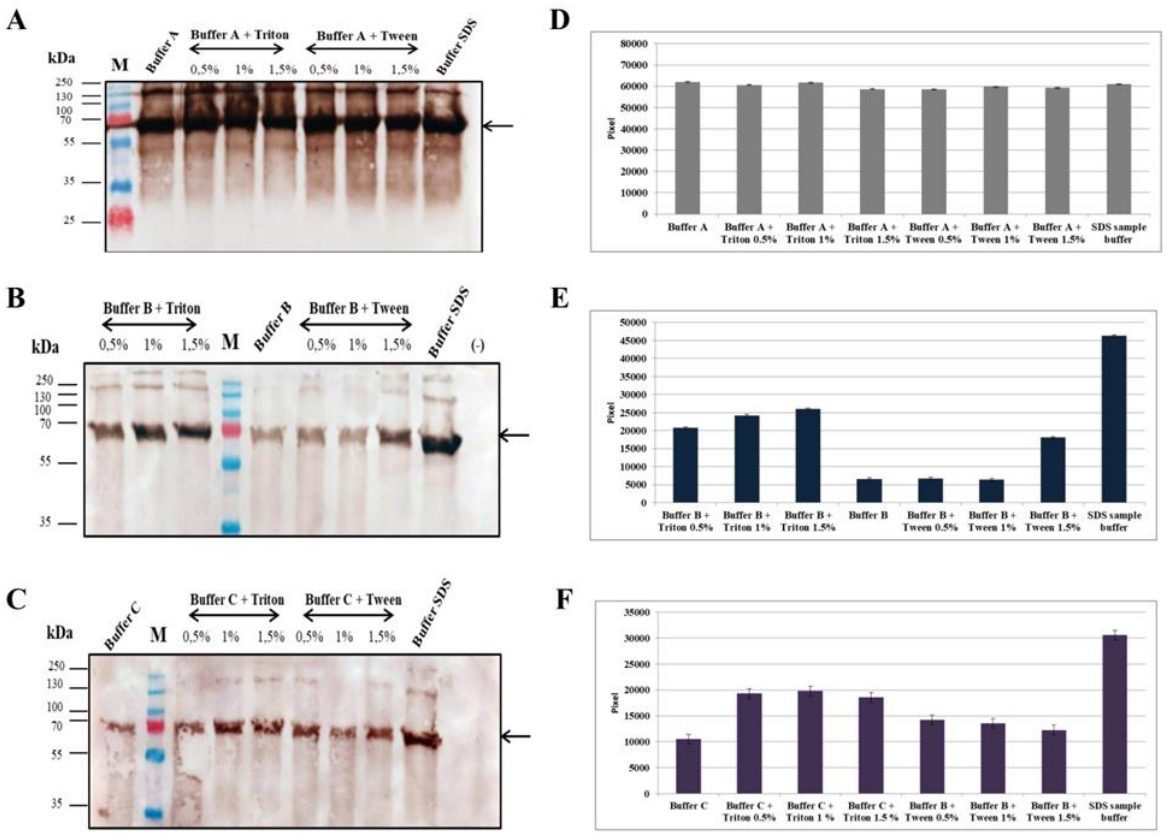

Figure 3. Detection of M-pII-ELP protein after extraction in various buffers was performed by SDS-PAGE and Western blot using an anti-His tag antibody. (A) Extraction of M-pII-ELP protein in Buffer A, containing 100 mM Tris-HCl, 15 mM NaCl, and 300 mM sucrose (pH 7.4). (B) Extraction of M-pII-ELP protein in Buffer B, composed of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 (pH 7.4). (C) Extraction of M-pII-ELP protein in Buffer C, which included 20 mM sodium phosphate and 0.5 M NaCl (pH 7.4). Varying concentrations of Triton X-100 (0.5%, 1%, and 1.5%) and Tween-20 (0.5%, 1%, and 1.5%) were added to these buffers. (D, E, F): Analysis of Western blot result by Image J software.

Selection of extraction buffer to extract M-pII-ELP protein from *N. benthamiana*

Three common buffers were used to extract M-pII-ELP protein from *N. benthamiana*. Additionally, the effects of Triton X-100 and Tween-20 in these extraction buffers on M-pII-ELP protein extraction were assessed. The presence of M-pII-ELP protein in the crude extract after extraction with these buffers was confirmed by Western blot (Figure 3A, B, C). The intensity of the bands was analyzed and compared using ImageJ software (Figure 3D, E, F). As shown in Figure 3D, E, F, when comparing the presence of M-pII-ELP protein in the crude extract after extraction with SDS-sample buffer as a positive control to that with the three buffers, a similar yield of M-pII-ELP protein was obtained with Buffer A, which contains 100 mM Tris-HCl, 15 mM NaCl, and 300 mM sucrose (pH 7.4). Furthermore, no significant difference in the extracted M-pII-ELP protein amount was observed when Buffer A was used with or without

detergents, such as Triton X-100 or Tween-20, at concentrations of 0.5%, 1%, or 1.5% (Figure 3A, D). In previous studies, Tris buffer was also chosen to extract several recombinant proteins expressed in *N. benthamiana* such as HA protein and M-ELP protein (Phan *et al*., 2013; Khamis, 2016). However, unlike in this study, there was a significant difference in extracted M-ELP protein amount between Tris buffer with and without Triton X-100 and Tween-20 in the previous study (Khamis, 2016).

In contrast, compared to the amount of M-pII-ELP protein in crude extract after extraction with SDS-sample buffer, a lower amount of M-pII-ELP protein was achieved with Buffer B (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4) and Buffer C (20 mM sodium phosphate and 0.5 M NaCl, pH 7.4). Although the yield of M-pII-ELP protein increased when Triton X-100 or Tween-20 was added to Buffer B or Buffer C, it remained lower than the amount obtained with the SDS-sample buffer (Figure 3B-F). Therefore, Buffer A without Triton X-100 and Tween-20 was selected for extracting M-pII-ELP protein from *N. benthamiana* leaves.

Selection of buffers for purification of M-pII-ELP protein by IMAC

IMAC is a widely used method for purifying histidine-tagged proteins (His-tagged proteins), typically employing a hexahistidine (His) sequence as the tag. This technique relies on coordination bonds formed between the His-tag and transition metal ions (such as Ni^{2+}, Co^{2+}, and Cu^{2+}) immobilized on column matrices (Bornhorst *et al*., 2000). Imidazole, a histidine analog, competes with the His-tag for binding to metal ions, making imidazole concentration a critical factor in IMAC purification (Lee *et al*., 2007). To purify M-pII-ELP protein by IMAC, the effect of imidazole concentration in the extraction buffer was first assessed. IMAC purification of M-pII-ELP protein was performed using different extraction buffers with 5 mM imidazole, 10 mM imidazole, or without imidazole, along with the same washing and elution buffers. After IMAC purification, the existence of M-pII-ELP protein was detected by Western blot (Figure 4 A, B, C). The intensity of bands on the membranes was analyzed by Image J software (Figure 4 D, E, F). As shown in Figure 4, M-pII-ELP protein was detected in all fractions collected after IMAC purification. Most of the M-pII-ELP protein in the crude extract, following extraction with buffer A without imidazole, was bound to the resin. In addition, the amount of M-pII-ELP protein in the flow-through fraction increased when the imidazole concentration in the extraction buffer increased at 5 and 10 mM, indicating that the amount of M-pII-ELP protein bound to the resin decreased. Additionally, based on the Bradford assay and Western blot results, the highest yield of M-pII-ELP protein was ultimately achieved in the eluted fraction when extraction Buffer A (100 mM Tris-HCl, 15 mM NaCl, 300 mM sucrose, pH 7.4) without imidazole was used for IMAC purification. Thus, Buffer A without imidazole was selected for extracting M-pII-ELP protein in IMAC purification.

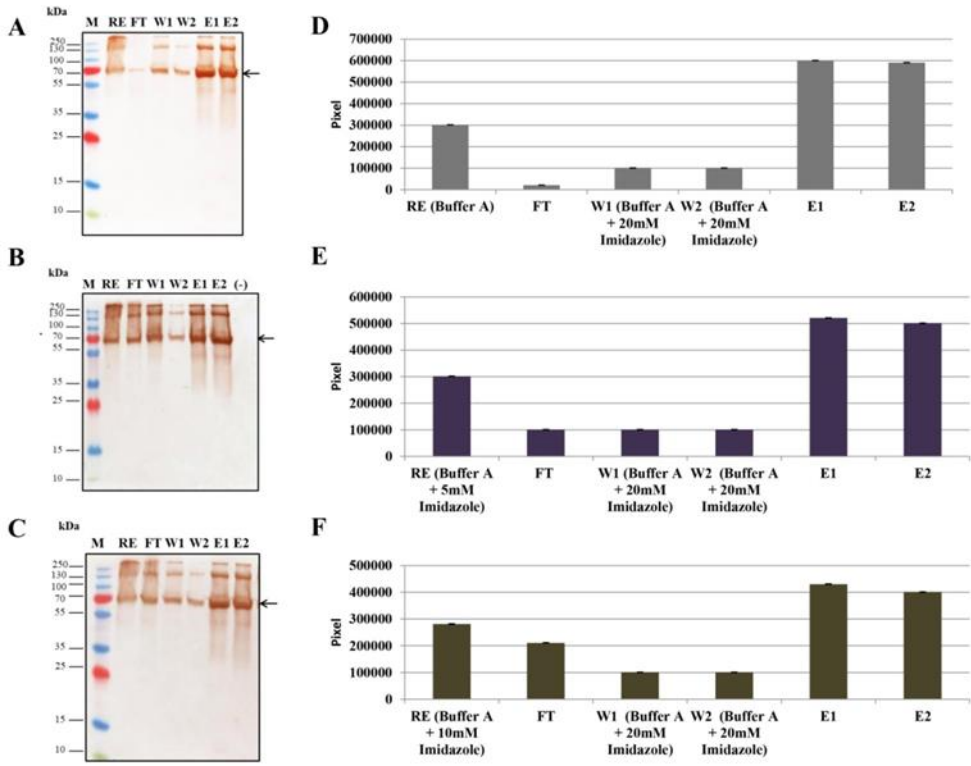

Figure 4. Detection of M-pII-ELP protein in fractions after IMAC purification using extraction buffer A with varying imidazole concentrations was performed by SDS-PAGE and Western blot with an anti-His tag antibody. RE: Raw extract; FT: Flow-through; W1, W2: Wash 1, Wash 2; E1, E2: Elution 1, Elution 2. (A) Buffer A containing 100 mM Tris-HCl, 15 mM NaCl, and 300 mM sucrose (pH 7.4) was used to extract protein. (B) Buffer A added 5 mM imidazole (pH 7.4) was used to extract protein. (C) Buffer A added 10 mM imidazole (pH 7.4) was used to extract protein. In all three purification protocols, the washing buffer (100 mM Tris-HCl, 15 mM NaCl, 300 mM sucrose, 20 mM imidazole, pH 7.4) and elution buffer (100 mM Tris-HCl, 15 mM NaCl, 300 mM sucrose, and 500 mM imidazole, pH 7.4) were used. (-): wild type crude extract. (D, E, F): Analysis of Western blot result by Image J software.

As shown in Figure 4, M-pII-ELP protein was also detected in the wash fraction after the column was washed with buffer containing 20 mM imidazole, indicating that the imidazole concentration in the wash buffer may need further optimization. Next, the effect of imidazole in the washing buffer on the IMAC purification of M-pII-ELP protein was assessed. IMAC purifications of M-pII-ELP protein were performed using Buffer A for protein extraction, along with different washing buffers (containing 10 mM, 20 mM, or 30 mM imidazole) and the same elution buffer. The presence of M-pII-ELP protein in fractions obtained after IMAC purification was detected by SDS-PAGE andWestern blotting with an anti-His tag antibody (Figure 5 A, B, C). The intensity of bands on the membrane was analyzed by Image J software (Figure 5 D, E, F). As illustrated in Figures 5A and D, a minor quantity of M-pII-ELP protein was also detected in the wash 2 fraction when a washing buffer with 10 mM imidazole was

used. Furthermore, M-pII-ELP proteins were detected in both wash 1 and wash 2 fractions when utilizing a washing buffer with 20 mM imidazole (Figure 5B, E). However, a larger amount of M-pII-ELP protein was observed in the wash 1, 2 fractions when using a washing buffer containing 30 mM imidazole (Figure 5C, F). Additionally, the purity of the M-pII-ELP protein in the eluted fraction after IMAC purification was assessed by SDS-PAGE, Coomassie staining, and Bradford assay (Figure 5G, H, I). As shown in Figures 5G, H, and I, the eluted fraction containing M-pII-ELP protein exhibited high purity when the washing buffer contained 10 mM, 20 mM, or 30 mM imidazole. However, a higher yield of purified M-pII-ELP protein was obtained with a washing buffer containing 10 mM imidazole compared to 20 mM or 30 mM. Therefore, the washing buffer containing 10 mM imidazole was selected for M-pII-ELP purification by IMAC. Similarly, a washing buffer with 10 mM imidazole was employed for COE/G2a-pII protein purification in a previous study (Ho *et al*., 2022).

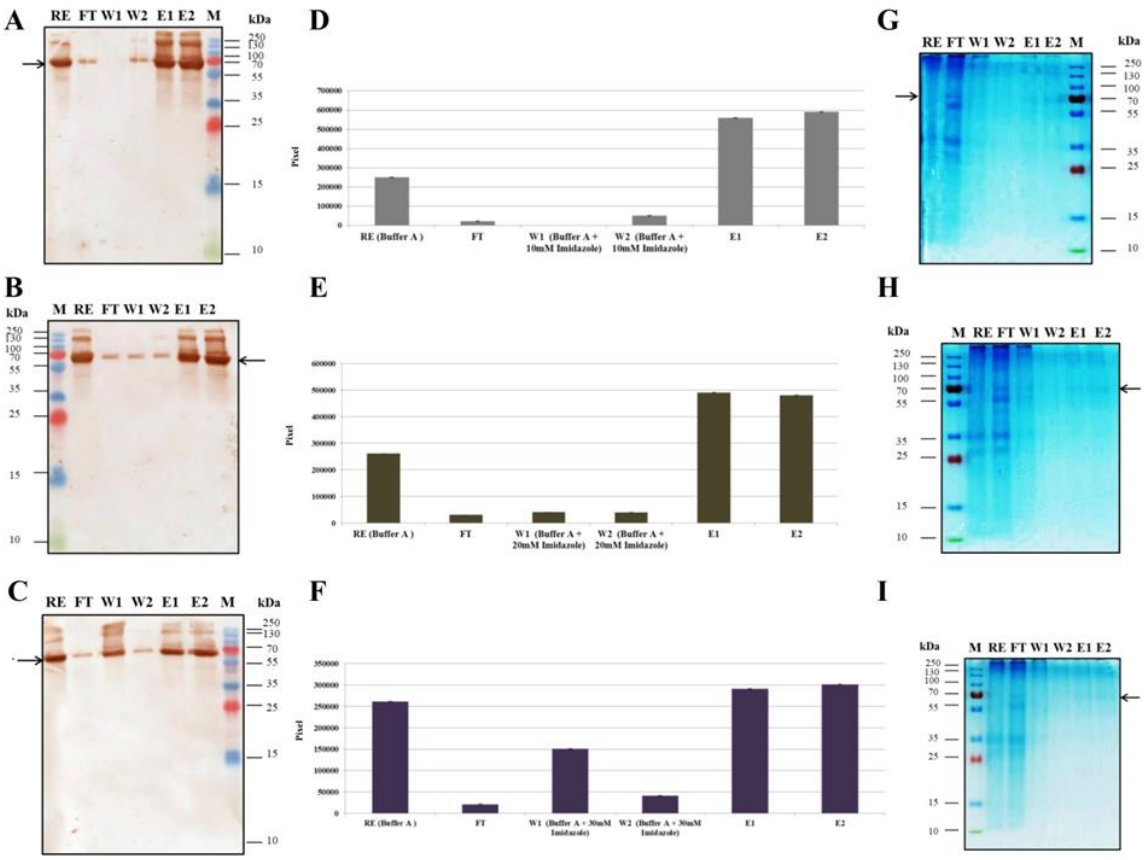

Figure 5. Detection of M-pII-ELP protein in fractions after IMAC purification, using washing buffers with different imidazole concentrations, was performed by SDS-PAGE and Western blot with an anti-His tag antibody and Coomassie blue staining. RE: Raw extract; FT: Flow-through; W1, W2: Wash 1,

Wash 2; E1, E2: Elution 1, Elution 2. (A, G) Washing buffer containing 100 mM Tris-HCl, 15 mM NaCl, 300 mM sucrose, and 10 mM imidazole (pH 7.4) was utilized to eliminate unbound proteins. (B, H) Washing buffer containing 100 mM Tris-HCl, 15 mM NaCl, 300 mM sucrose, and 20 mM imidazole (pH 7.4) was utilized to eliminate unbound proteins. (C, I) Washing buffer containing 100 mM Tris-HCl, 15 mM NaCl, 300 mM sucrose, and 30 mM imidazole (pH 7.4) was utilized to eliminate unbound proteins. In all three purification protocols, the Buffer A (100 mM Tris-HCl, 15 mM NaCl, 300 mM sucrose, pH 7.4) and elution buffer (100 mM Tris-HCl, 15 mM NaCl, 300 mM sucrose, and 500 mM imidazole, pH 7.4) were used. (D, E, F): Analysis of Western blot result by Image J software.

The optimized buffers were then used for large-scale purification of the M-pII-ELP protein. The existence of M-pII-ELP in the eluted fraction after purification was confirmed by SDS-PAGE, Western blotting, and Coomassie staining (Figure 6). As shown in Figure 6, M-pII-ELP protein was clearly detected in the eluted fractions. These data indicate that the M-pII-ELP protein was effectively purified from *N. benthamiana* leaves.

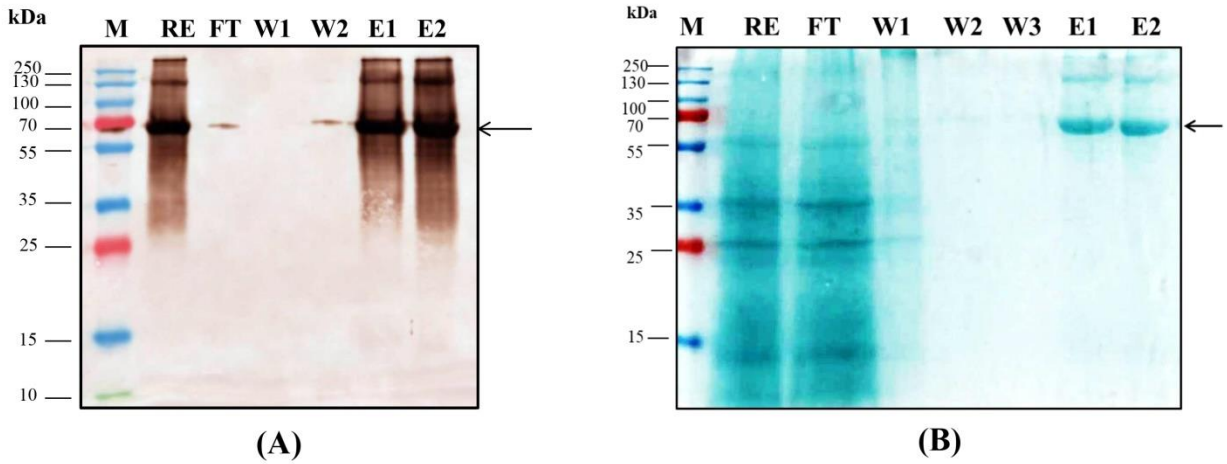

Figure 6. Detection of the presence of M-pII-ELP in fractions after large-scale purification using IMAC with optimal buffers by Western blot (A) and Coomassie blue staining (B). RE: Raw extract; FT: Flow-through; W1, W2: Wash 1, Wash 2; E1, E2: Elution 1, Elution 2. M-pII-ELP protein was extracted in buffer A, which contained 100 mM Tris-HCl, 15 mM NaCl, 300 mM sucrose (pH 7.4). The column was washed with a buffer containing 100 mM Tris-HCl, 15 mM NaCl, 300 mM sucrose, and 10 mM imidazole (pH 7.4). M-pII-ELP protein was then eluted using a buffer containing 100 mM Tris-HCl, 15 mM NaCl, 300 mM sucrose, and 500 mM imidazole (pH 7.4).

CONCLUSION

In this study, the co-transient expression of PEDV M-pII-ELP protein and COE/G2a-pII protein in *N. benthamiana* was firstly reported. In addition, the formation of a VLP structure based on the assembly of M-pII-ELP protein was firstly found. Moreover, optimized extraction and washing buffers for IMAC purification of M-pII-ELP protein were identified. This work lays the groundwork for the creation of subunit vaccines against PEDV from plants in Vietnam.

ACKNOWLEDGMENTS

This research was funded by the Institute of Biotechnology, Vietnam Academy of Science and Technology through the project: 'Optimization of extraction and purification conditions for the recombinant M protein of Porcine Epidemic Diarrhea Virus (PEDV) from plants', code: CSTE24-02. We thank Ms. Le Tra My for-plant preparation, Mrs. Nguyen Thi Thu Hien for project record support, and Pham Chi Mai for technical support in SDS-PAGE and Western blot.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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