Original Article

Expression of recombinant S2 domain of Spike protein of Porcine epidemic diarrhea virus

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Abstract

Porcine epidemic diarrhea virus (PEDV) is the causative agent of severe watery diarrhea in suckling piglets. PEDV outbreaks have been consistently reported in Europe, Asian countries and Thailand. The recombinant protein, especially the spike (S) protein, from field strain PEDV could be used to develop a sensitive diagnostic test or an effective vaccine. In this study, the recombinant S2 domain protein from the PEDV Thai isolate was expressed using the *E. coli* expression system. The full length S2 domain of the S gene of the PEDV G2b Thai isolate was amplified using a PCR technique and was cloned into the pBAD Directional TOPO® expression vector. For the expression of the whole recombinant S2 domain protein, the optimum concentration of arabinose was 0.002% and the optimum induction time was 6 hours. Based on SDS-PAGE, the size of the whole recombinant S2 domain protein bands was approximately 90 kDa. The whole recombinant S2 domain protein showed specific interaction with mouse anti-histidine monoclonal antibody and rabbit polyclonal antibody against PEDV G1a. The ability of the whole recombinant S2 domain protein in this study to react with antibody induced by different PEDV genogroup (G1a) showed the potential for using this whole recombinant S2 domain protein to detect PEDV infection or to induce antibodies against different PEDV genogroups. According to this study, the whole recombinant S2 domain protein may be a useful tool for the development of a diagnostic test for PEDV or for a vaccine against PEDV.

Keywords: PEDV, prokaryotic expression system, recombinant protein, S2 domain

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Received: October 22, 2020.
Accepted: January 15, 2021.
doi: https://doi.org/10.14456/tjvm.2021.29

Introduction

Porcine epidemic diarrhea virus (PEDV) is the causative agent of severe watery diarrhea in suckling piglets and weight loss in fattening pigs. The high mortality of piglets causes severe economic loss to the swine industry throughout the world (Song *et al.,* 2015). PEDV is the positive sense, single-stranded RNA virus that belongs to the genus *Alphacoronavirus*, family *Coronaviridae*. PEDV has been divided into genogroup 1 (G1) and genogroup 2 (G2) (Lee, 2015). The amino acid changes at the N-terminal domain (residues 21–324) of the S gene (Deng *et al.,* 2016) located at the S1 domain are the key to differentiation between genogroups G1 and G2. The G1 genogroup can be divided into subgroups 1a and 1b, and the G2 genogroup also can be divided into 2a and 2b. PEDV outbreaks have been consistently reported in Europe and Asian countries since 1981 (Song *et al.,* 2015). The first PEDV outbreak in Thailand was reported in 2007 (Puranaveja *et al.,* 2009) and the Thai isolates belonged to both of G1 and G2 genogroups (Stott *et al.,* 2017).

Commercial vaccines have several problems such as low effectiveness against different genogroups (Li *et al.,* 2012; Wang *et al.,* 2013), no solid lactogenic immunity (Song and Park, 2012) and shedding of vaccine virus that reverses to being virulent (Opriessnig *et al.,* 2017). Low effectiveness has been reported of a vaccine for disease prevention due to the genetic differentiation between the PEDV vaccine strain and the field epidemic strain (Li *et al.,* 2012; Wang *et al.,* 2013). The spike (S) glycoprotein of PEDV is of considerable interest for vaccine development because it contains 2 domains that play a role in specific binding to the host cell receptor (S1) and fusing the envelope of the virus with the host cell membrane (S2) (Belouzard *et al.,* 2012). The neutralizing epitopes are distributed through both the S1 and S2 domains (Chang *et al.,* 2002; Cruz *et al.,* 2008; Sun *et al.,* 2007; Sun *et al.,* 2008). The whole S proteins from the G1 and G2 genogroups have been expressed using a eukaryotic expression system and also their different neutralization activity between the G1 and G2 genogroups has been reported (Wang *et al.,* 2016). S1 subunit vaccine has been also shown to have antigenicity but did not provide complete protection against PEDV infection (Makadiya *et al.,* 2016). Thus, further development is necessary of other parts of the S protein containing other neutralizing epitopes to provide better protection. The S2 domain has 3 neutralizing epitopes (^{753}YSNIGVCK^{760}, ^{769}LQDGQVKI^{776} and ^{1373}GPRLQPY^{1379}) (Cruz *et al.,* 2008; Sun *et al.,* 2008). This domain also contains the HR1 and HR2 regions at residues 978–1117 and 1274–1313, respectively (Okda *et al.,* 2017). Currently, there have been very limited studies on expression of the S2 domain. Only expression of the partial S2 domain (Paudel *et al.,* 2014) and the six peptides derived from Heptad Repeat Regions (HR1) and HR2 (Okda *et al.,* 2018; Zhao *et al.,* 2018) have been studied using the *E. coli* expression system. The results from these previous studies showed that the partial S2 domain had high immunogenicity and could induce neutralizing antibody (Paudel *et al.,* 2014). The HR2 peptides were also confirmed to be competitive inhibitors in PEDV *in vitro* infection assays and antibodies from HR2-immunized mice were shown to be capable of neutralizing PEDV infection (Zhao *et al.,* 2018). The S2 domain is more conserved than the S1 domain (Bosch *et al.,* 2016). However, there has been no reported study on the expression of the whole S2 domain. Thus, the aim of the current study was to express the full length S2 domain of the PEDV Thai isolate using the *E. coli* expression system and to characterize the immunological properties of the whole recombinant S2 domain protein.

Materials and Methods

Sample collection: Small intestinal samples from pigs naturally infected with PEDV were collected at the Kamphangsaen Veterinary Diagnostic Laboratory, Faculty of Veterinary Medicine, Kasetsart University, Nakhon Pathom, Thailand. Suspected PEDV samples were confirmed using routine reverse transcription polymerase chain reaction (RT-PCR) for PEDV. The PEDV-positive intestinal samples were kept at -80°C until use.

RNA extraction and reverse transcription polymerase chain reaction: Thirty milligrams of the PEDV-positive intestinal samples were mixed with 200 µl of 1xPBS and homogenized using a Bead Rupture 12 machine (Omni International). The homogenate was centrifuged at 11,000×*g* for 5 minutes. Subsequently, the supernatant was collected and subjected to RNA extraction using a Viral RNA Extraction Kit I (FavorPrep™) according to the manufacturer's instructions. For cDNA synthesis, 20 µl of reverse transcription reaction mixture were composed of 4 µl of 5x reverse transcription buffer, 2 µl of random hexamer, 2 µl of 10 mM dNTP, 1 µl of MMLV reverse transcriptase (Thermoscientific™) and 11 µl of RNA template. The reverse transcription reaction conditions consisted of enzyme activation at 25°C for 10 minutes, cDNA synthesis at 37°C for 1 hour and enzyme inactivation at 70°C for 15 minutes. For amplification of the full length S2 domain, RT-PCR was performed using forward (5' CACC ACG CCA TGT TCT TTT TCA GAG 3') and reverse (5'CTG CAC GTG GAC CTT TTC 3') primers. For PCR, the PCR mixture was composed of 10 µl of 5x Phusion HF buffer, 1 µl of 10 mM dNTP, 1 µl of each forward and reverse primer, 0.5 µl (2 U/µl) of Phusion Hot Start II DNA polymerase (Thermo Fisher Scientific, USA), 5 µl of cDNA template and 31.5 µl of distilled water to give a total volume of 50 µl. After initial denaturing at 95°C for 3 minutes, amplification was performed using 35 cycles at 95°C for 30 seconds, annealing at 56°C for 10 seconds, extension at 72°C for 1 minute and a final extension at 72°C for 10 minutes. The expected PCR products were 2,064 bp in size. The PCR products were analyzed using 1% agarose gel electrophoresis at 100 V for 30 minutes, visualized under ultraviolet illumination.

Cloning of the full length S2 domain of PEDV: The PCR products of the full length of the S2 domain of the S gene were purified and cloned into the pBAD Directional TOPO® expression vector (Invitrogen™, USA) according to the manufacturer's protocol. Briefly,

the TOPO® cloning reaction was composed of 1µl of purified PCR products, 1 µl of salt solution, 3 µl of sterile water and 1µl of pBAD Directional TOPO® expression vector. The reaction mixture was mixed, incubated at 22° C for 30 minutes and then transformed using One Shot® TOP10 *E. coli* (Invitrogen ™, USA). The positive clones were sequenced at First BASE Co., Ltd. (Selangor, Malaysia).

Nucleotide and amino acid analysis and phylogenetic construction of full length S2 domain: The nucleotide sequence of recombinant S2 domain plasmids (accession number MW423799) was subjected to BLASTN analysis and translated to amino acid using the Bioedit program, version 7.05 (Tom Hall, USA). The amino acid sequences were aligned with reference strains and the representative subgroup sequences of G1a, G1b, G2a and G2b using the ClustalW and BioEdit programs. The phylogenetic and molecular evolution tree was constructed from the full length S2 amino acid sequence obtained in this study and other sequences obtained from GenBank database (Table 1) using a Bayesian inference and Markov chain Monte Carlo (BMCMC) method with the mixed amino acid model analysis. The BMCMC model was independently run for each amino acid sequence on a total of 3 million generations sampling every 1,000 generations. The analysis was performed until the standard deviation of split frequencies was below 0.01 and the effective sample sizes (ESS) were greater than 200. The first 25% of trees were discarded as "*burn in*". The remaining trees were used to construct a consensus tree. The resulting tree was viewed using the FigTree v1.4.3 program.

Table 1 PEDV strains obtained from GenBank and GenBank accession numbers used for multiple alignment and phylogenetic study.

PEDV strain	Origin	GenBank accession no.	Subgroup
CV777	Belgium	AF353511	G1a
SM98	South Korea	GU937797	G1a
DX	China	EU031893	G1a
CH/S	China	JN547228	G1a
SD-M	China	JX560761	G1a
LZC	China	EF185992	G1a
Br1/87	UK	Z25483	G1a
DR-13	South Korea	JQ023161	G1a
83P-5-parent	Japan	AB548618	G1a
JS2008	China	KC109141	G1a
JS-2004-2	China	AY653204	G1b
LJB/03	China	DQ985739	G1b
CH/JLGZL	China	JQ638923	G1b
KNU-1406-1	South Korea	KM403155	G1b
OH851	USA	KJ399978	G1b
Ohio 126	USA	KJ645702	G1b
Indianna12.83	USA	KJ645635	G1b
GER/L00719/2014	Germany	LM645058	G1b
FR/001	France	KR011756	G1b
15V010	Belgium	KR003452	G1b
1-55ST0412	Thailand	KF724936	G2a
4-5RW-VCF0712	Thailand	KF724935	G2a
6-56ST0413	Thailand	KF724938	G2a
NPPED2008_2	Thailand	KC764952	G2a
SBPED0211_2	Thailand	KC764957	G2a
SBPED0211_3	Thailand	KC764959	G2a
SPPED0212_1	Thailand	KC764958	G2a
SPPED0212_2	Thailand	KC764956	G2a
PED0210-2	Thailand	KC764955	G2a
KNU-802	South Korea	GU180143	G2a
KNU-0901	South Korea	GU180144	G2a
KNU-1301	South Korea	KJ451036	G2a
Spk1	South Korea	AF500215	G2a
Chinju99	South Korea	AY167585	G2a
BP-2016	Thailand	LC496368	G2b
KNU-1303	South Korea	KJ451038	G2b
KNU-1401	South Korea	KJ451047	G2b
VN/JFP1013_1	Vietnam	KJ960178	G2b
IA2	USA	KF468754	G2b
Minnesota71	USA	KJ645706	G2b
Colorado	USA	KF272920	G2b
Kansas125	USA	KJ645701	G2b
CHGD-01	China	JX261936	G2b
AH2012	China	KU646831	G2b
BJ-2012-1	China	JN825712	G2b

Optimization for expression of the whole recombinant S2 domain protein: A single colony of recombinant *E. coli* was grown in 3 ml LB broth containing 50 µg/ml of kanamycin and shaken overnight at 200 rpm and 37°C. Subsequently, 100 µl of starter culture were subcultured in 10 ml LB broth containing 50 µg/ml of kanamycin and shaken at 200 rpm and 37°C until the optical density (OD_{600}) reached 0.6. To determine the optimal induction of the arabinose concentration for the induction of the whole recombinant S2 domain protein, 10-fold serial dilutions (final concentrations of 0%, 0.00002%, 0.0002%, 0.002%, 0.02% and 0.2%) of arabinose were added to LB broth for the induction of each inoculum for 6 hours at 37°C. To determine the optimum induction time of the whole recombinant S2 domain protein, 10 ml of the cultures were induced by 0.002% final concentration of arabinose, harvested every 2 hours at 0, 2, 4, 6 and 8 hours after induction and kept at 80°C for further verification using SDS-PAGE. The wild type TOP10 competent cells were used as a negative control. Samples (each 150 µg) of the extracted proteins were loaded in each lane and analyzed using 10% SDS-PAGE for 45 minutes at 150 V. The amount of protein was measured using a Nanodrop 2000 spectrophotometer (Thermoscientific ™, USA). Gels were stained with staining solution (0.01% Coomassie Brilliant Blue, 40% distilled water, 50% methanol and 10 % acetic acid) for 15 minutes and de-stained with de-staining solution (40% distilled water, 50% methanol and 10 % acetic acid). The expected size of the whole recombinant S2 domain protein was 90 kDa. Then, the whole recombinant S2 domain protein was purified using HisTrap™ FF 1 ml (Ni-NTA column) with an AKTA system (GE Healthcare, USA) according to the manufacturer's recommendations.

Preparation of polyclonal antibody against PEDV: Three New Zealand White rabbits were intramuscularly injected using commercially available PEDV vaccine (ProVAC ®, Korea) 3 times with 2 weeks apart. Five milliliters of whole blood were collected before vaccination each time and 1 week after each booster. Ten milliliters of whole blood was also collected at 2 weeks after the last booster. The obtained rabbit hyperimmune serum was used to test reactivity against the recombinant S2 proteins by western blot analysis.

Western blot analysis of the whole recombinant S2 protein: Both crude and purified whole recombinant S2 domain proteins were separated using 10% SDS-polyacrylamide gel and electro-transferred onto nitrocellulose membrane using the semi-dry method and Bio-Rad Tran-Blot SD Semi-Dry Electrophoretic Transfer Cells (BIORAD ®, USA) at 10 V for 45 minutes. The nitrocellulose membranes were incubated with blocking buffer (5% skimmed milk in 1x PBS) at 37°C for 1 hour. Subsequently, the membranes were incubated with either 1:5000 mouse anti-histidine (GE Healthcare, USA) or 1:50 rabbit anti-PEDV (SM98 vaccine strain; G1a) polyclonal antibody overnight at 4°C. After washing 3 times with 1x PBS-T for 10 minutes each time, the membranes were incubated with either 1:1000 goat anti-mouse IgG-HRP or goat anti-rabbit IgG-HRP polyclonal antibodies (Invitrogen ™, USA) at 37° C for 1 hour. The membranes were washed 3 times for 10 minutes each time with 1xPBS-T and subsequently incubated with DAB substrate (Thermo Fisher Scientific, USA) for 5-10 minutes at room temperature. Finally, the reaction was stopped by soaking the membranes with PBS-T and the positive reaction was visualized as a dark brown band on the nitrocellulose membrane.

Results

The full length S2 domain of the S gene of PEDV was successfully amplified and had a size of approximately 2 kbp (Fig. 1). According to the nucleotide alignment, the nucleotide sequencing result of the full length S2 domain was highly homologous to the PEDV Thailand isolate BP-2016 (99.81%). The amino acid substitutions among CV777, SM98, BP-2016 and the whole S2 domain in this study are shown in Table 2. The deduced amino acid of the whole S2 domain protein showed 3 epitopes (^{753}YSNIGVCK^{760}, ^{769}SQSGQVKI^{776} and ^{1373}GPRLQPY^{1379}). Compared to the CV777 reference strain and the SM98 vaccine strain, there were amino acids substitutions L769S for CV777 or P769S for SM98 and D771S for CV777or Y711S for SM98 which were located in the 2nd epitope of the S2 domain. However, the amino acid sequences of all 3 epitopes were similar to BP-2016. There were 30 and 27 amino acid substitutions along the whole S2 domain protein compared to the CV777 reference strain and the SM98 vaccine strain, respectively. Seven amino acid substitutions H978Y, R983K, Q 998P, E1021D, N1028K, S1049A and I1056V were detected in the HR1 region and one amino acid substitution R1303Q in the HR2 region compared to the CV777 reference strain. There were 4 amino acid substitutions, R983K, Q998P, E1021D and S1049A detected in the HR1 region and one amino acid substitution R1303Q in the HR2 region compared to the SM98 vaccine strain. However, the whole S2 domain protein had 2 amino acid substitutions, R983K and Q998P at the HR1 region compared to the S2 domain of the BP-2016 strain. Phylogenetic analysis indicated that the whole S2 domain amino acid sequence in this study was closely related to the BP-2016 strain that was grouped in G2b (Fig. 2).

The optimum conditions for the expression of the whole recombinant S2 domain protein was with an optimum concentration of arabinose of 0.002% (Fig. 3) and an optimum induction time of 6 hours (Fig. 4). As expected, the molecular weight of the whole recombinant S2 domain protein was approximately 90 kDa.

Western blot analysis indicated that the whole crude recombinant S2 domain protein reacted specifically with mouse anti-histidine monoclonal antibody (Fig. 5) and both of the whole crude and purified recombinant S2 domain proteins reacted specifically with the rabbit anti-PEDV polyclonal antibody (Fig. 6A and 6B).

Table 2 The amino acid substitutions of CV777, SM98, BP-2016 and the recombinant S2 domain

No.	Amino acid position	Strain (GenBank Accession number)			
		CV777 (AF353511)	SM98 (GU937797)	BP-2016 (LC496368)	Recombinant S2 (MW423799)
1	724	N	N	S	S
2	768	P	P	S	S
3	769[a]	L	P	S	S
4	771[a]	D	Y	S	S
5	779	M	T	T	T
6	811	V	A	A	A
7	848	V	V	A	A
8	896	G	R	R	R
9	964	A	A	V	V
10	968	L	I	F	F
11	970	T	A	A	A
12	978[b]	H	Y	Y	Y
13	983[b]	R	R	R	K
14	998[b]	Q	Q	Q	P
15	1021[b]	E	E	D	D
16	1028[b]	N	K	K	K
17	1049[b]	S	S	A	A
18	1056[b]	I	V	V	V
19	1172	D	A	A	A
20	1178	G	G	D	D
21	1179	D	E	E	E
22	1198	T	N	D	D
23	1205	L	F	F	F
24	1212	F	F	Y	Y
25	1220	S	S	G	G
26	1237	S	S	R	R
27	1242	D	D	E	E
28	1265	I	N	T	T
29	1270	P	P	S	S
30	1303[c]	R	R	Q	Q

[a] Located in second epitope (^{769}SQSGQVKI^{776}) of S2 domain
[b] Located in HR1 of Heptad Repeat Regions
[c] Located in HR2 of Heptad Repeat Regions

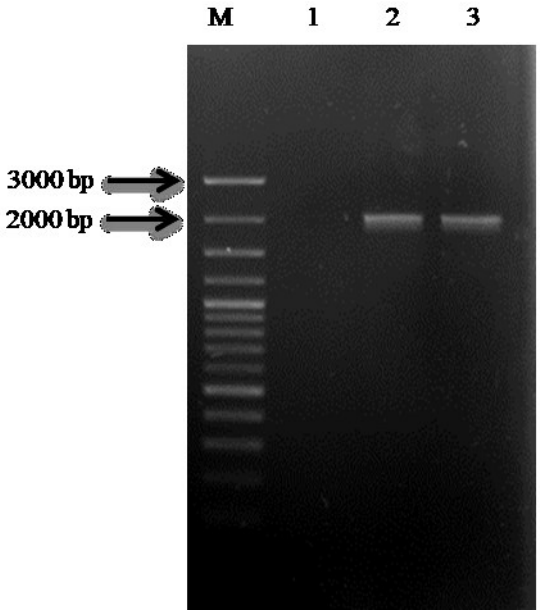

Figure 1 1% Agarose gel electrophoresis of PCR products of full length S2 domain of S gene of PEDV. Lane M = 100 bp +3k DNA ladder (AccuBand™, SMOBIO Technology, Taiwan), Lane 1 = negative control, Lane 2 = S2 domain (2,064 bp) and Lane 3 = positive control.

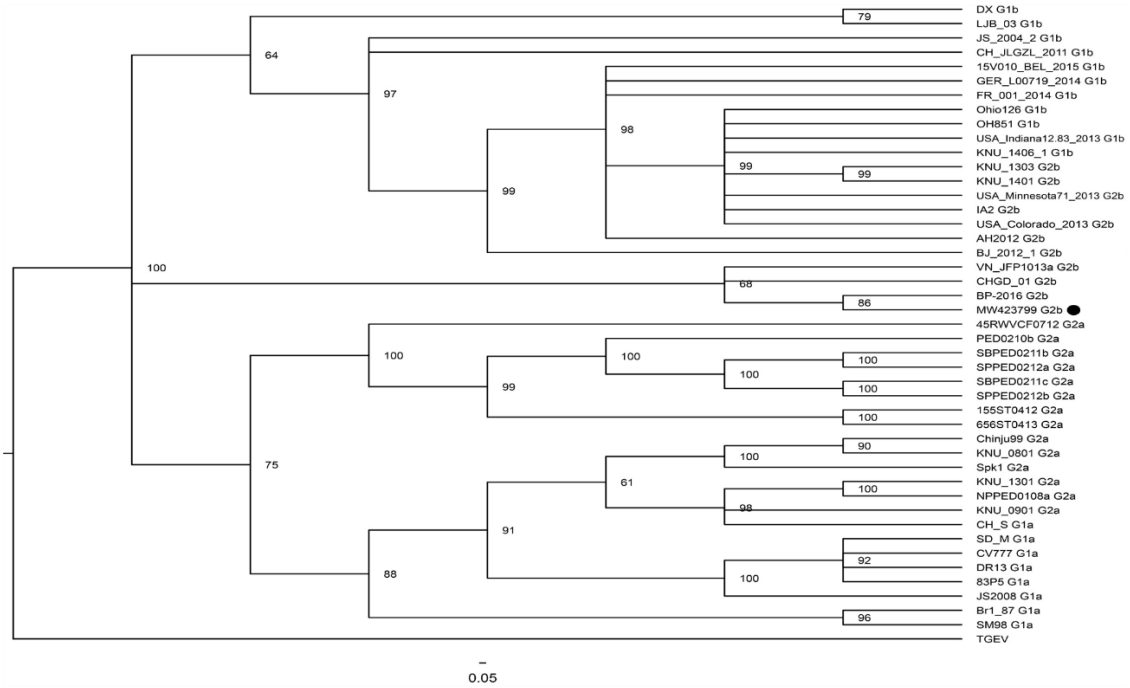

Figure 2 Bayesian phylogenetic analysis based on amino acid sequence of full length S2 domain protein (accession number MW423799) in this study and full length S2 domain protein of other representative PEDV strains. The tree was constructed using the Bayesian MCMC method consisting of 3 million generations with sampling every 1,000 generations in this study. Node values (percentages) indicate posterior clade probabilities. "●" indicates the amino acid sequences in this study.

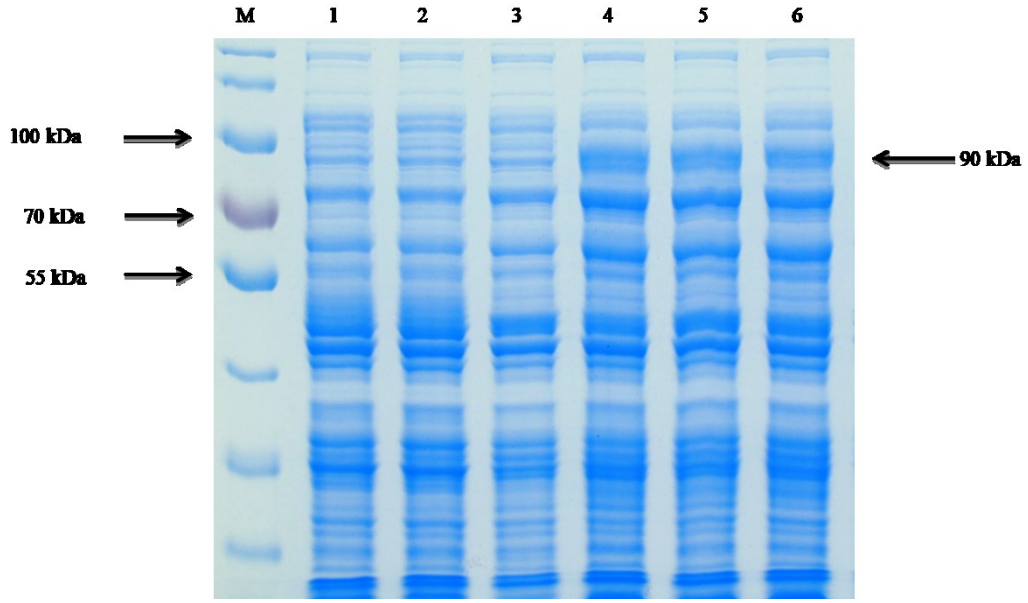

Figure 3 10% SDS-PAGE analysis of optimum arabinose concentration used for the induction of whole recombinant S2 domain protein expression. Lane M = protein marker (PageRuler™ Plus Pre-stained Protein Ladder, Thermo Fisher Scientific, USA) and Lanes 1-6 = 0, 0.00002, 0.0002, 0.002, 0.02 and 0.2% arabinose at 37°C for 6 hours, respectively.

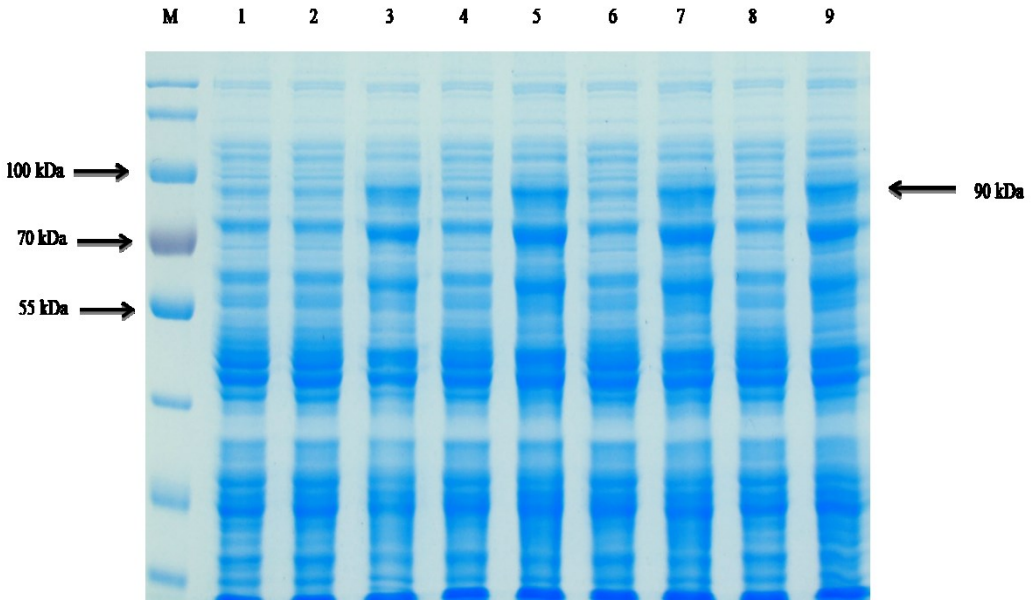

Figure 4 10% SDS-PAGE analysis of optimum induction time for the induction of recombinant S2 domain protein expression using 0.002% arabinose. Lane M = protein marker (PageRuler™ Plus Pre-stained Protein Ladder, Thermo Fisher Scientific, USA); Lanes 2, 4, 6 and 8 = protein from wild type *E. coli* induced for 2, 4, 6 and 8 hours respectively, and Lanes 1, 3, 5, 7 and 9 = protein from recombinant *E. coli* induced for 0, 2, 4, 6 and 8 hours, respectively.

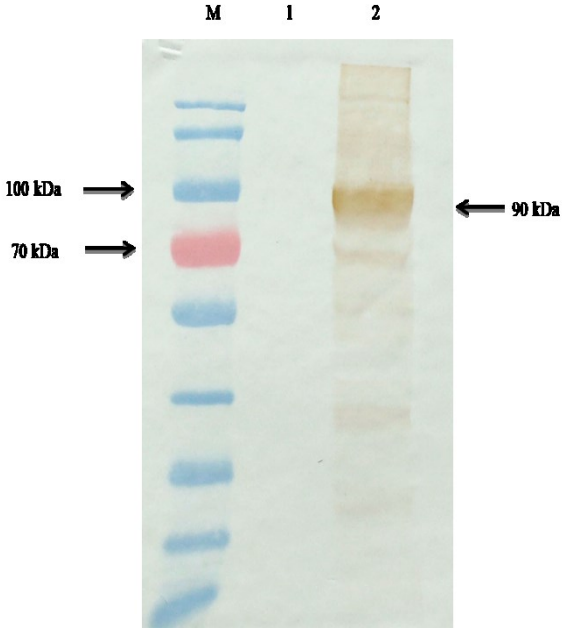

Figure 5 Western blot analysis of recombinant S2 domain protein using mouse anti-histidine monoclonal antibody. Lane 1 = crude protein from non-induced recombinant *E. coli*, Lane 2 = crude protein from induced recombinant *E. coli*, and Lane M = protein marker (PageRuler™ Plus Prestained Protein Ladder, Thermo Fisher Scientific, USA).

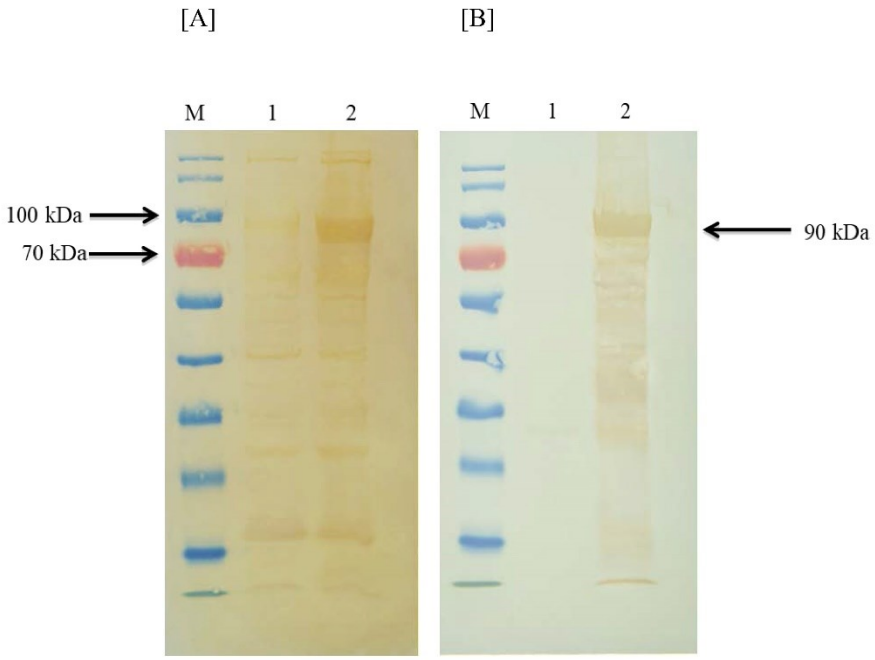

Figure 6 Western blot analysis of crude (A) and purified (B) recombinant S2 domain proteins using rabbit anti-PEDV polyclonal antibody. Lane 1 = crude (A) or purified protein (B) of non-induced recombinant *E. coli*, Lane 2 = crude (A) or purified induced recombinant *E. coli* (B), and Lane M= protein marker (PageRuler™ Plus Pre-stained Protein Ladder, Thermo Fisher Scientific, USA).

Discussion

The S2 domain of PEDV was successfully cloned and expressed. The sequence of the whole S2 domain protein amino acid had the highest similarity and was closely related to BP-2016, a local Thai strain, which was clustered in G2b. There was no amino acid substitution between the whole S2 domain protein and the BP-2016 isolate at all 3 epitopes.

The optimum conditions for the expression of the whole recombinant S2 domain protein in this study were 0.002% arabinose for 6 hours. Information on the expression of the recombinant S2 domain protein is very limited. Only the expression of smaller fragments of the S2 domain using the *E. coli* expression system have been reported, such as the 57 kDa truncated recombinant S2 protein (amino acid residue 838 -1383) (Paudel *et al.*, 2014) and the six peptides derived from HR1 and HR2 (Okda *et al.*, 2018; Zhao *et al.*, 2018). The partial S2 domain has been shown to have high immunogenicity and could induce neutralizing antibody (Paudel *et al.*, 2014) and HR2 peptides were confirmed to be competitive inhibitors in PEDV *in vitro* infection assays. The antibodies from HR2 immunized mice were capable of neutralizing PEDV infection (Zhao *et al.*, 2018).

There has been no report to date on the expression of recombinant S2 domain protein in other expression systems. Western blot analysis indicated that the whole recombinant S2 domain protein in the current study reacted specifically with rabbit anti-PEDV polyclonal antibodies. Even though these polyclonal antibodies were induced by injecting rabbits with the SM98 vaccine strain (G1a), the whole recombinant S2 domain protein in this study had no glycosylation. There were 27 amino acid substitutions between the recombinant S2 domain protein and the S2 domain of the SM98 vaccine strain, especially 2 amino acid substitutions P769S and Y771S at epitope ^{769}PQYGQVKI^{776} of the SM98 vaccine strain. However, the other 2 epitopes, ^{753}YSNIGVCK^{760} and ^{1373}GPRLQPY^{1379}, were conserved between the whole recombinant S2 domain protein and the S2 domain of the SM 98 vaccine strain. This result correlated with a previous study that reported expressed recombinant partial S2 domain protein (amino acid residue 790-1386) or carboxy-terminal region of the S protein containing only 1 epitope, ^{1373} GPRLQPY^{1379} using an *E. coli* expression system which reacted to antibody against PEDV (Liu *et al.*, 2019). In addition, the epitope ^{1373}GPRLQPY^{1379} has been shown to have high antigenicity and induced the highest neutralizing antibody to PEDV among other expressed fragments of S protein or full-length nucleoprotein in the same study (Cruz *et al.*, 2006; Cruz *et al.*, 2008; Paudel *et al.*, 2014).

Thus, the *E. coli* expression system was an inexpensive and high yield method for the expression of the whole recombinant S2 domain protein. Furthermore, the ability of the whole recombinant S2 domain protein of PEDV G2b to react with antibody induced by PEDV G1a showed the potential use of this whole recombinant S2 domain protein for the detection of PEDV infection or inducing antibody against different PEDV genogroups. However, the ability of the whole recombinant S2 domain protein in this study to induce neutralizing antibody against PEDV or detect antibody against PEDV in either the same or a different genogroup needs further study.

Acknowledgements

The authors thank the Veterinary Diagnostic Laboratory, Faculty of Veterinary Medicine, Kasetsart University, Kamphaengsaen Campus, Thailand and Assist. Prof. Dr. Sarawan Kaewmongkol, Faculty of Veterinary Technology, Kasetsart University for

providing the facilities used in this research. The research was supported by research graduate and graduate research assistant scholarships from the Faculty of Veterinary Medicine, Kasetsart University.

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