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Expression and purification of a chimeric protein consisting of the ectodomains of M and GP5 proteins of porcine reproductive and respiratory syndrome virus (PRRSV)

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ABSTRACT

Porcine reproductive and respiratory syndrome (PRRS) is the most economically important infectious disease currently affecting the swine industry worldwide. In the US alone, it causes economic losses of more than 560 million dollars every year. Although killed-virus and modified-live PRRS vaccines are commercially available, the unsatisfactory efficacy and safety of current vaccines drives the impetus of developing novel PRRSV vaccines. To fulfill this purpose, we designed a chimeric protein consisting of the ectodomains of viral GP5 and M protein, the two most widely studied subunit vaccine targets, and expressed it in *E. coli*. An optimized purification/refolding process composed of immobilized metal ion affinity chromatography, dialysis refolding and anion exchange chromatography was developed to purify the chimeric protein from the inclusion bodies. This process could recover approximately 12 mg protein/l *E. coli* broth with near 100% purity and very low endotoxin level. In addition, the purified protein is antigenic, can bind to a cellular receptor for the virus (heparan sulfate), and can block virus infection of susceptible cells. Therefore, the chimeric protein is a promising subunit vaccine candidate against PRRSV.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is the most economically important infectious disease currently affecting swine industry worldwide. In the US alone, the economic losses caused by this disease amount to more than 560 million US dollars every year [1]. The clinical signs of PRRS include abortions and infertility at sudden onset, the birth of weak or dead piglets, severe pneumonia in neonatal and nursery pigs, reduction in growth performances, and increased mortality [2,3]. The causative agent of this disease is PRRS virus (PRRSV).

Along with equine arteritis virus (EAV), simian hemorrhagic fever virus (SHFV), and lactate dehydrogenase elevating virus (LDV), PRRSV is classified into *Arteriviridae* family within the genus *Arterivirus*, order *Nidovirales* [4]. This virus is an enveloped, linear positive-stranded RNA virus with an icosahedral capsid. Its 15-KB genome contains two large open reading frames (ORF1a and b) and a set of 6–9 ORFs downstream of the 1b gene [5]. Long nonstructural polyproteins, pp1a and pp1ab, are translated from ORF1a and ORF1b. The polyproteins are then co- or post-translationally cleaved into 14 functional nonstructural proteins (nsps) in a complex proteolytic cascade [6]. Viral minor structural proteins GP2a, 2b protein, GP3, GP4 are encoded by ORF2a, ORF2b, ORF3, and ORF4. Three major structural proteins of the virus GP5, M and N are derived from ORFs 5, 6 and 7, respectively [7].

M protein is the most conserved structural protein and the most potent T cell-stimulation antigen of the virus [7,8]. It contains a short N-terminal ectodomain followed by three transmembrane segments and a C-terminal endodomain [9]. GP5 protein possesses a putative signal sequence (aa 1–31), an ecotodomain (aa 32–60), three transmembrane helices (aa 61–125) and an endodomain (aa 126–200) [9]. Two immunologically important epitopes, epitope A and epitope B, have been identified within GP5 [10,11]. Epitope A (between aa 27 and 31) is immunodominant but non-neutralizing. It is thought to be a decoy epitope, because it is located seven amino acid residues ahead of the neutralizing epitope B and induces a strong non-neutralizing antibody response rapidly after infection [12]. In contrast, epitope B (between aa 37 and 45) is sequential, conserved among isolates, and not immunodominant. Neutralizing antibodies are mainly directed against epitope B of PRRSV GP5. However, the presence of the decoy epitope A and the sugars surrounding epitope B (glycan shielding) might cause the

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diminishment of the immune responsiveness against the adjacent neutralizing epitope [12].

M and GP5 form heterodimers on virion envelope. The M/GP5 heterodimer mediates PRRSV attachment to a cellular receptor, heparan sulfate during virus infection [13]. We hypothesize the ectodomains of M and GP5 proteins contribute to the attachment of the heterodimer to the cellular receptor. Therefore, by fusion expression of these two ectodomains, this chimeric protein may bind to the cellular receptor, heparan sulfate, and antibodies against it will presumably bind the M-GP5 heterodimer on virus surface and thus block virus' interaction with the heparan sulfate receptor. In addition, this protein has the major neutralizing epitope of the virus, epitope B, and does not have the adverse factor, the decoy epitope A. Therefore, this chimeric protein could be a potential vaccine candidate against PRRSV infection. Furthermore, because *E. coli* generally does not glycosylate proteins, the negative effects of glycan shielding on immunogenicity can also be avoided if *E. coli* is chosen as the expression system.

In this report, we expressed a chimeric protein (M/GP5-Ecto) consisting of the ectodomains of viral GP5 and M protein in *E. coli* inclusion bodies. A purification/refolding process was then developed to purify and refold the chimeric protein. The purified protein is antigenic, can bind to the cellular receptor for the virus (heparan sulfate), and can block virus infection of susceptible cells. The objective of the study is to obtain high quality, well-characterized refolded M/GP5-Ecto protein for use in vaccine development studies.

2. Materials and methods

2.1. Construction and transformation of pET-M/GP5-Ecto expression vector

The coding sequences of the M protein ectodomain and the GP5 protein ectodomain were artificially synthesized together with a flexible linker sequence $(GGGGS)_3$ between them and a 6 × His tag at the C-terminal by a commercial supplier (Genscript Corporation, Piscataway, NJ, USA). *E. coli* codon preference table was used in the gene synthesis for the maximal expression of the chimeric protein. The synthesized fragment was then subcloned into pET24b at *Nde* I and *EcoR* I site. The resultant plasmid was then transformed into competent BL21 (DE3) cells following the manufacturer's protocol (New England Biolabs, Ipswich, MA, USA).

2.2. Screening for M/GP5-Ecto protein expression

The recombinant *E. coli* cells were cultured in LB media supplemented with 50 μg/ml kanamycin. When OD_{600} of the cell cultures reached 0.6–0.8, IPTG was added to a final concentration of 1 mM and induced for 4 h. After fermentation, the cells were pelleted by centrifugation at 6000 × *g* for 15 min at 4 °C. The pelleted cells were lysed using B-PER bacterial protein extraction kit following the manufacturer's protocol (Thermo Scientific, Rockford, IL, USA). A small fraction of cell lysis suspension containing the soluble and insoluble cell components was collected for following SDS-PAGE analysis. The cell lysis suspension was then centrifuged at 15,000 × *g* for 15 min at 4 °C. The supernatant, the insoluble fraction, and the cell lysis suspension were analyzed by SDS-PAGE to determine the localization of M/GP5-Ecto protein in induced *E. coli* cells. The uninduced *E. coli* cells were processed in the same way as the induced cells and analyzed in parallel.

2.3. Preparation of solubilized inclusion bodies (IB)

The conditions for shaker-incubator were 37 °C and 250 rpm. Overnight incubated starter culture of the recombinant *E. coli* was used to inoculate 1 l of fresh LB media supplemented with 50 μg/ml kanamycin. When the OD_{600} of the cell cultures reached 0.6–0.8, IPTG was added to a final concentration of 1 mM and induced for another 4 h. After induction, the cells were harvested by centrifugation at 6000 × *g* for 15 min at 4 °C. The cell pellets were lysed using B-PER bacterial protein extraction kit following the manufacturer's protocol (Thermo Scientific, Rockford, IL, USA). The insoluble fraction was collected by centrifugation at 15,000 × *g* for 15 min at 4 °C, washed twice with inclusion body washing buffer (1% Triton X-100, 100 mM Tris–HCl, 10 mM 2-mercaptoethanol, pH 8), and then washed twice with DI water. The washed IB were incubated with IB solubilization buffer (50 mM Tris–HCl, 8 M urea, 0.5 M NaCl, 1 mM DTT, 30 mM imidazole, pH 7.9) at room temperature for 1 h with frequent vortexing. After centrifugation at 17,000 × *g* for 10 min, the supernatant was collected for the following purification steps.

2.4. Immobilized metal ion chromatography (IMAC)

Purification experiments were performed at room temperature using an ÄKTA™ purifier (GE Healthcare, Uppsala, Sweden). XK16/20 column was packed with 5 mL of Ni Sepharose 6 Fast Flow resin (GE Healthcare, Uppsala, Sweden). Prior to purification, the column was equilibrated with 10 column volumes (CV) of IMAC binding buffer (20 mM Tris–HCl, 6 M urea, 0.5 M NaCl, 30 mM imidazole, pH 7.9). A 25 ml of solubilized IB sample was applied onto the column at a flow-rate of 5 ml/min. After sample loading, the column was washed with 10 CV of IMAC binding buffer. Finally, the bound proteins were eluted with IMAC elution buffer (20 mM Tris–HCl, 6 M urea, 0.5 M NaCl, 300 mM imidazole, pH 7.9). The eluates were collected for the following dialysis experiments.

2.5. Refolding of M/GP5-Ecto protein by dialysis

The IMAC eluates were dialyzed against refolding buffers using Slide-A-Lyzer Dialysis Cassettes with a molecular weight cut-off of 3.5 K (Thermo Scientific, Rockford, IL, USA). The samples were first dialyzed twice, 8 h for each dialysis, against refolding buffer A (20 mM Tris–HCl, 1 mM DTT, pH 7.5). Then, the samples were further dialyzed twice against refolding buffer B (20 mM Tris–HCl, pH 7.5).

2.6. Anion exchange chromatography (AEX)

The refolded M/GP5-Ecto protein was polished by anion exchange chromatography. A C-column was packed with 1.5 ml of Q Sepharose Fast Flow resin (GE Healthcare, Uppsala, Sweden). After equilibration of the column with 10 CV of AEX binding buffer (20 mM sodium phosphate, pH 7.5), 5 ml of the refolded sample was loaded onto the column, followed by washing the column with 6 CV of the binding buffer. The bound proteins were then eluted using a NaCl gradient starting at 0 M and ending at 0.5 M. Finally, the column was regenerated by a buffer that contains 20 mM sodium phosphate and 1 M NaCl, pH 7.5.

2.7. SDS-PAGE and Western-blot

SDS-PAGE was performed using 4–12% polyacrylamide gel as described elsewhere [4]. In Western-blot experiments, two primary antibodies were used to detect the antigenicity of M/GP5-Ecto protein to anti-His-tag monoclonal antibody and PRRSV antiserum, respectively. The procedures for Western-blot using PRRSV antiserum were the same as described previously [14]. For Western-blot using anti-His-tag monoclonal antibody, the blot was probes with 1:2000 dilution of THE™ Anti-His mAb (Genscript Corporation, Piscataway, NJ, USA). After consecutive washing steps,

the membrane was incubated with 1:20,000 dilution of HRP conjugated goat-anti-mouse IgG antibody (Bethyl Laboratories Inc., Montgomery, TX, USA). After consecutive wash steps in TTBS, the blot was incubated in a 1:1 mixture of HRP luminal/enhancer solution and peroxide buffer (BioRad, Hercules, USA) for 5 min. Finally, the blot was visualized by a ChemiDoc XRS molecular imager (Bio-Rad, Hercules, USA).

2.8. Heparan sulfate binding assay

The ability of M/GP5-Ecto protein to bind heparan sulfate was tested by ELISA-based heparan sulfate binding assay. Polystyrene plates were coated with 10 μg/ml heparan sulfate in carbonate buffer (pH 9.5) at 4 °C overnight. Then the plates were blocked with 3% BSA in PBS buffer for 2 h at room temperature. Serially diluted M/GP5-Ecto samples were then added into wells of plates and incubated for 2 h at room temperature. After washing the plates four times using PBS buffer, 1:2000 dilution of THE™ Anti-His mAb (Genscript Corporation, Piscataway, NJ, USA) was added and incubated for 2 h. Following consecutive wash steps, HRP conjugated goat-anti-mouse IgG antibody (Bethyl Laboratories Inc., Montgomery, TX, USA) was added to the wells and incubated for another 2 h. After washing the plates four times with PBS buffer, 1:1 mixture of TMB (3,3,5,5-tetramethylbenzidine) peroxidase substrate and peroxidase solution B (Bethyl Laboratories Inc., Montgomery, TX, USA) was added to each well. After incubation for half an hour, 1 M phosphoric acid was added to stop the color development reaction. Finally, the plates were read on a plate reader (BioTek, Winooski, VT, USA) at 450 nm with a reference to 570 nm. The negative control (all assay components without M/GP5-Ecto protein) was included in every assay.

2.9. Virus blocking assay

To analyze the function of purified M/GP5-Ecto protein to block PRRSV infecting susceptible cells, an indirect immune-fluorescence test was performed as described elsewhere with some modifications [14]. The purified M/GP5-Ecto protein samples were added into wells of a plate coated with confluent MARC-145 cells. After incubation for 1 h, PRRSV dilutions with 2000 $TCID_{50}$/ml was added to each well and incubated for 15 min at 37 °C. The inoculums were then removed and 100 μl of fresh DMEM supplemented with 2% fetal bovine serum was added to each well. After incubation for 12 h at 37 °C in a humidified atmosphere of 5% CO_2, the plates were fixed with 80% acetone for 15 min at room temperature. After extensive wash with PBST, 50 μl of fluorescein isothiocyanate conjugated anti-PRRSV monoclonal antibody, SDOW17-F (Rural Technologies, Brookings, SD, USA), diluted 1:100 in PBST with 2% BSA was added to each well. After an hour of incubation, the cells were washed four times with PBS and the number of fluorescent foci in each well was counted. The virus inhibition rate was expressed as a ratio of reduced fluorescent foci number by a protein sample over the number of fluorescent foci of the negative control (all assay components without M/GP5-Ecto protein).

2.10. Endotoxin assay

To avoid introducing endotoxin contamination during the purification process, sanitation procedures were performed for chromatographic columns following the resin manufacturer's protocols; sterile filtration was performed for all the buffers; and samples are collected in sterile vessels. The endotoxin level of the purified protein was detected by ToxinSensor™ chromogenic LAL endotoxin assay kit following the manufacturer's instructions (Genscript Corporation, Piscataway, NJ, USA).

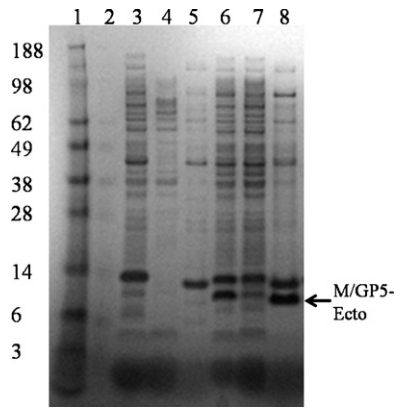

Fig. 1. Coomassie-blue-stained SDS-PAGE (4–12% polyacrylamide) for localization of M/GP5-Ecto protein expression in *E. coli*. (1) Marker; (2) 10 × diluted marker; (3) un-induced *E. coli* total protein; (4) un-induced *E. coli* soluble protein; (5) un-induced *E. coli* insoluble protein; (6) IPTG-induced *E. coli* total protein; (7) IPTG-induced *E. coli* soluble protein; (8) IPTG-induced *E. coli* insoluble protein.

2.11. Dynamic light scattering analysis

The size of the purified M/GP5-Ecto protein were analyzed by dynamic light scattering (DLS) using a Malvern Instrument Zetasizer Nano (Worcestershire, UK). Analysis was performed using the size standard operating procedure (SOP) for which the following parameters were used: material set to protein, dispersant set to water, taking two independent measurements of 30 runs each.

3. Results and discussion

3.1. Expression of M/GP5-Ecto protein in E. coli

Small-scale cultures of kanamycin-resistant clones were subjected to IPTG induction to identify clones capable of expressing M/GP5-Ecto protein. A typical induction experiment comparing the protein expression profiles of un-induced and induced *E. coli* is shown in Fig. 1. It is evident that IPTG induced the recombinant *E. coli* cells to express a unique 8 kDa protein in inclusion bodies, which is the expected molecular weight of M/GP5-Ecto protein (lane 8 of Fig. 1). The identified M/GP5-Ecto expressing clone was used for further experiments. We tried various strategies to express M/GP5-Ecto protein in a soluble form (lowering the incubation temperature, decreasing IPTG concentration, starting the induction at lower or higher OD_{600} and the combination), but none of them could effectively produce soluble M/GP5-Ecto. Therefore, we decided to develop a process to purify/refold M/GP5-Ecto protein from *E. coli* inclusion bodies.

3.2. M/GP5-Ecto protein purification/refolding process

The process we developed for the production of M/GP5-Ecto protein is schematically represented in Fig. 2. After fermentation and cell lysis, the inclusion bodies (IBs) were harvested and then solubilized in IB solubilization buffer containing 8 M urea. Since M/GP5-Ecto protein had a His-tag at its C-terminal, immobilized metal ion chromatography (IMAC) was chosen as the capture step. During IMAC chromatography, the target protein was purified under denaturing conditions. The elution peak was collected and dialyzed to refold the target protein. Finally, the target protein was polished by anion exchange chromatography to remove aggregates and remaining impurities.

Imidazole concentration in the binding buffer is the key factor that influences the efficiency of IMAC. Therefore, the optimal imidazole concentration of the binding buffer was scouted. We found

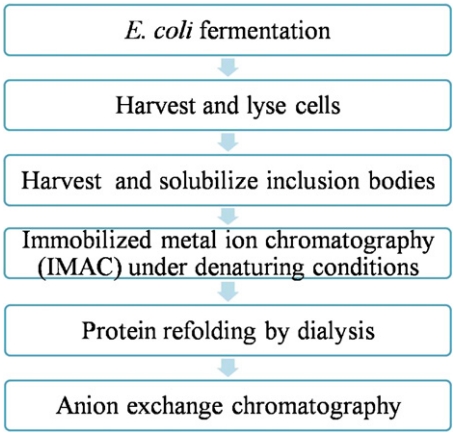

Fig. 2. M/GP5-Ecto protein purification/refolding process.

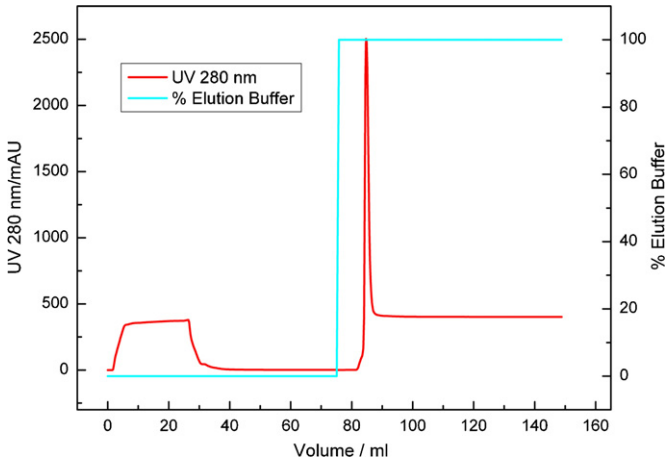

Fig. 3. Immobilized metal ion chromatogram of solubilized IB containing M/GP5-Ecto protein. Column: 5 ml of ml Ni Sepharose 6 Fast Flow resin packed in XK16/20 column; Binding buffer: 20 mM Tris–HCl, 6 M urea, 0.5 M NaCl, 30 mM imidazole, pH 7.9; elution buffer: 20 mM Tris–HCl, 6 M urea, 0.5 M NaCl, 300 mM imidazole, pH 7.9.

the binding buffer with 30 mM imidazole was optimal, which produced eluates with high purity and without significant loss of the target protein in the flow-through fraction (data not shown). Fig. 3 shows the IMAC chromatogram using 30 mM imidazole in the binding buffer. In this capture step, the majority impurity proteins of the inclusion bodies were removed in the flow-through fraction (Fig. 4, lane 3). The elution peak recovered contains M/GP5-Ecto protein and a few impurity proteins (Fig. 4, lane 4). The collected IMAC fractions were dialyzed against refolding buffers to refold the target protein. The refolding efficiency was about 56%. The refolded M/GP5-Ecto protein was then polished using Sepharose Q anion exchange chromatography (AEX). The chromatographic profile is presented in Fig. 5. The bound proteins were eluted in two peaks. Upon SDS-PAGE analysis it was found that the first peak recovers most of the target protein with a step yield of 76% and shows a single band on the silver-staining gel (Fig. 4, lane 6). The protein concentration of the pooled fractions for the first peak is 0.41 mg/ml. The second peak contains the target protein together with a few impurity proteins (Fig. 4, lane 7).

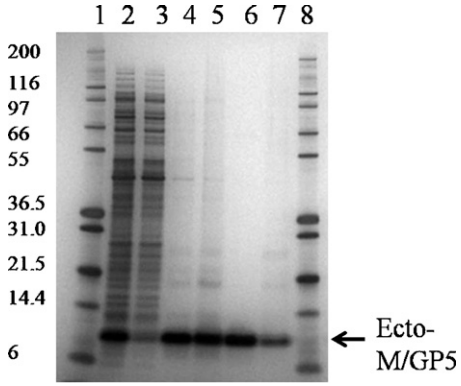

Fig. 4. Evaluation of M/GP5-Ecto protein purification/refolding process by silver-staining SDS-PAGE (4–12% polyacrylamide). (1) Marker; (2) solubilized inclusion bodies; (3) IMAC flow-through; (4) IMAC elution peak; (5) refolded sample; (6) AEX peak 1; (7) AEX peak 2; (8) marker.

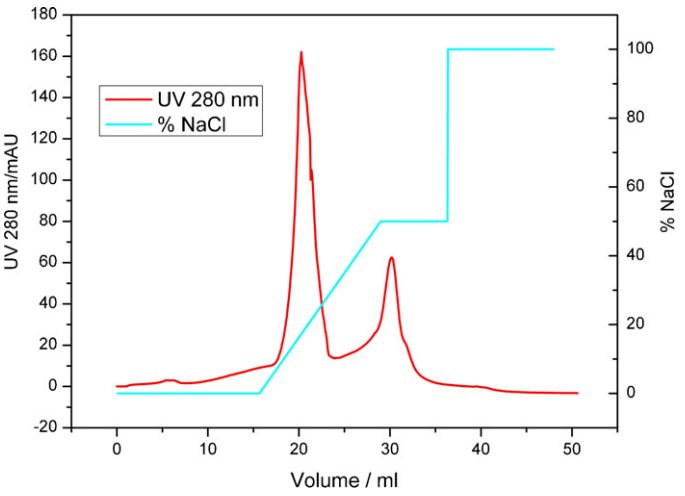

Fig. 5. Anion exchange chromatogram of refolded M/GP5-Ecto protein sample. Column: 1.5 ml of Q Sepharose Fast Flow resin packed in C column; binding buffer: 20 mM sodium phosphate, pH 7.5; elution buffer: 20 mM sodium phosphate, 1 M NaCl, pH 7.5.

3.3. *Characterization of refolded and purified M/GP5-Ecto protein*

The purified M/GP5-Ecto protein was characterized by various biochemical and biophysical methods.

The heterodimers formed by M and GP5 protein mediate PRRSV's attachment to heparan sulfate molecules on cell surface and initiate the process of the virus' entry into the target cells [15]. The ectodomains of M and GP5 protein are very likely involved in the attachment of the heterodimers to heparan sulfate receptor on susceptible cells. Therefore, we hypothesize that M/GP5-Ecto protein may have the function of binding heparan sulfate. To test this hypothesis, heparan sulfate binding assay were performed. Not surprisingly, M/GP5-Ecto protein recovered in the first AEX peak binds heparan sulfate in a concentration dependent manner (Fig. 6). However, the protein in the second AEX peak binds heparan sulfate significantly less than the protein in the first peak.

The ability of M/GP5-Ecto protein to inhibit PRRSV infection *in vitro* was analyzed by virus blocking assay. Similar to the results of heparan sulfate binding assay, M/GP5-Ecto protein in the first AEX peak blocks virus infection in a concentration dependent manner (Fig. 7). At a concentration of 500 μg/ml, M/GP5-Ecto protein significantly blocked PRRSV infecting susceptible cells with an inhibition rate of 90%. The protein recovered the second AEX peak had very limited effects on inhibiting virus infection.

Our results of heparan sulfate binding assay and virus blocking assay reveal that the first AEX peak recovered bioactive M/GP5-Ecto protein. This protein has the expected function of binding the cellular receptor, heparan sulfate, and can inhibit PRRSV infection *in vitro*. Probably, inhibition of infection by M/GP5-Ecto protein is

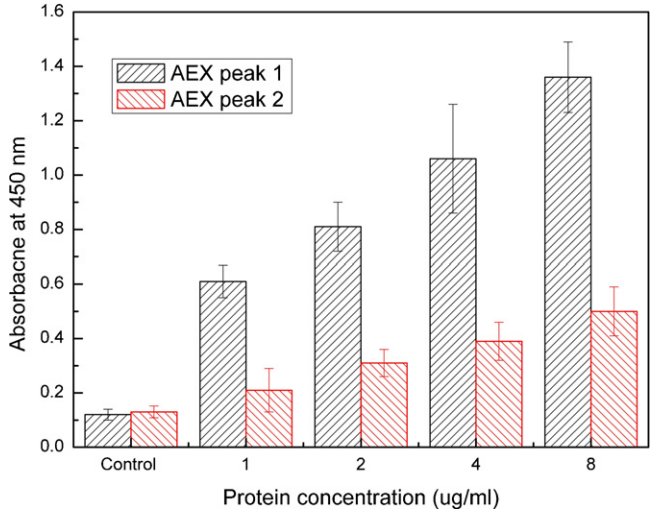

Fig. 6. Heparan binding assay of anion exchange chromatography (AEX) recovered protein.

resulted from its binding to the heparan sulfate receptor and thus sterically blocking the interaction between the cellular receptor and PRRSV particles. The inefficient attachment of the virus particles to the heparan sulfate receptor leads to the reduced number of virus-infected cells.

The second AEX peak contains the M/GP5-Ecto protein and a few impurity proteins. Although the M/GP5-Ecto protein in the second AEX peak was indistinguishable from that in the first peak based on SDS-PAGE analysis (Fig. 4), it showed significantly less binding activity to heapran sulfate as well as virus blocking ability. This indicates the second AEX peak recovered M/GP5-Ecto protein may have not been properly refolded. Thus, the M/GP5-Ecto protein recovered in the first AEX peak was chosen to be our vaccine component and was further characterized.

The antigenicity of the purified M/GP5-Ecto protein was analyzed by Western-blot. This protein reacted with both pig anti-PRRSV antiserum and anti-His-tag monoclonal antibody (Fig. 8). The homogeneity of the purified M/GP5-Ecto protein was further assessed by dynamic light scattering. The size distribution graph shows a single peak with a hydrodynamic diameter of 2.85 ± 0.46 nm (Fig. 9). This peak corresponds to a hypothetical

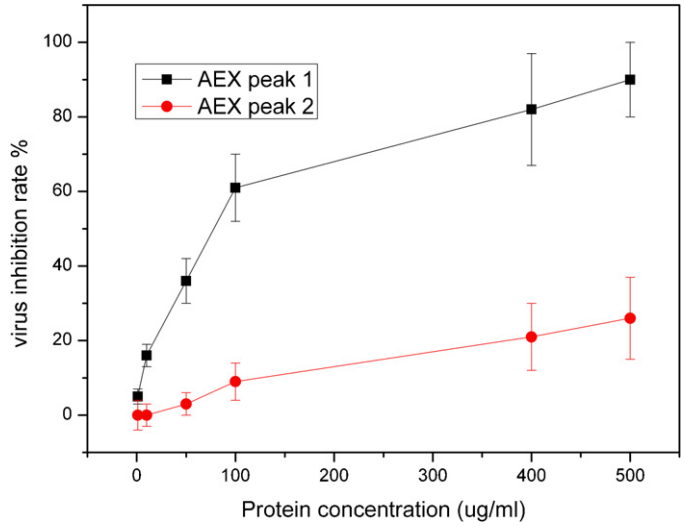

Fig. 7. Virus blocking assay of anion exchange chromatography (AEX) recovered protein.

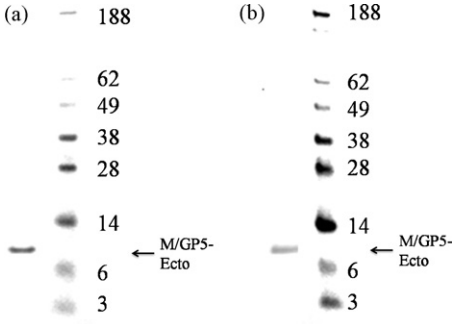

Fig. 8. Western-blot analysis of purified M/GP5-Ecto protein. (a) The primary antibody used was PRRSV antiserum. (b) The primary antibody used was anti-His-tag mAb.

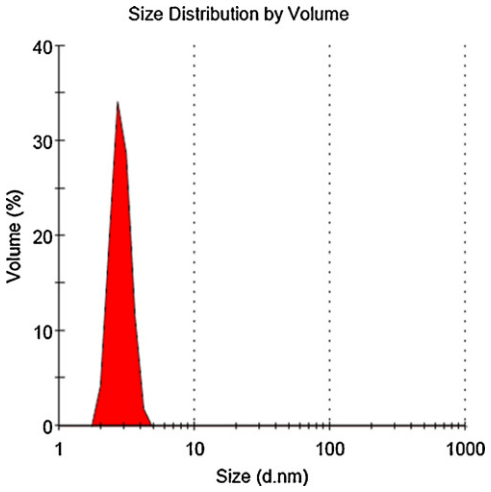

Fig. 9. Dynamic scattering analysis of purified M/GP5-Ecto protein.

globular protein of 7.71 kDa as calculated by the Zetasizer software. The results of dynamic light scattering indicate that the purified M/GP5-Ecto protein is homogenous in size, and once the protein is properly refolded, it is stable as a soluble protein.

Since endotoxin is highly toxic and can interfere with the immunogenicity of a vaccine [16], it must be controlled to a low level in a drug formulation. The threshold level of endotoxin for intravenous applications is set to 5 endotoxin units (EU) per kg body weight and hour by all pharmacopeias [17]. Singh and Malyala [18] calculated the endotoxin limits in drug formulations for animal study. Based on different administered doses, they calculated that the acceptable endotoxin level ranges from 6 to 2000 EU/mg [18]. We plan to administer this vaccine candidate at 10 μg/kg body weight. Thus, the limit is no more than 500 EU/mg protein. We monitored the endotoxin levels in the final product. The M/GP5-Ecto protein recovered in the first AEX peak had endotoxin levels of less than 10 EU/mg protein in all runs of the purification process (Table 1). For its vaccine application, no additional step is needed to further remove the endotoxin.

Table 1
Endotoxin levels of purified M/GP5-Ecto protein.

	Endotoxin level (EU/mg protein)
Run 1	8
Run 2	9
Run 3	6

4. Conclusion

We have expressed M/GP5-Ecto protein consisting of the ectodomains of M and GP5 protein of PRRSV and developed a purification/refolding process to produce biologically functional M/GP5-Ecto protein from *E. coli* inclusion bodies. The final concentration of the bioactive protein is 0.41 mg/ml. The purification/refolding process can produce approximately 12 mg M/GP5-Ecto protein from 1 l fermentation broth with an overall yield of 38%. The purified M/GP-Ecto protein has an endotoxin level far less than the regulatory requirement, is antigenic and homogenous. More importantly, it can bind to heparan sulfate and block virus infection of susceptible cells *in vitro*. Our findings further the understanding on biology of PRRSV, and this chimeric protein could be a valuable subunit vaccine for the prevention of PRRSV infection.

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