Journal of Chromatography B, 911 (2012) 43–48

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

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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ARTICLE INFO

Article history:
Received 14 August 2012
Accepted 10 October 2012
Available online 17 October 2012

Keywords:
Porcine reproductive and respiratory syndrome virus (PRRSV)
Vaccine
Heparan sulfate
Protein refolding
Protein purification

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.

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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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http://dx.doi.org/10.1016/j.jchromb.2012.10.014

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,

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