Development of subunit vaccines against porcine reproductive and respiratory syndrome virus (PRRSV)

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Abstract

Since emerging in Europe and the US, PRRS has spread globally and become the most significant infectious disease currently devastating the swine industry. In the US alone, the economic losses caused by this disease amount to more than 560 million US dollars every year. Modified-live PRRSV vaccines (MLV) are the most effective option currently available for the control of the disease. MLVs can confer solid protection against homologous re-infection and have significant effects in reducing viral shedding. But the vaccine efficacy varies upon heterologous challenge. None of the current vaccines are able to completely prevent respiratory infection, transplacental transmission, as well as pig-to-pig transmission of the virus. More importantly, the intrinsic risk of MLV vaccine to revert to virulent virus under farm conditions poses a great safety concern. The unsatisfactory efficacy and safety of current PRRSV vaccines drives the continuous efforts of developing a new generation of vaccines.

The strategy we focus on for novel PRRSV vaccine development is subunit vaccine. The reasons for choosing this strategy are: 1) subunit vaccines only contain the immunogenic fragments of a pathogen. Administration of such pathogen fragments eliminates the risk of pathogens reverting back to their virulent form as in the case of modified live vaccines. 2) Subunit vaccines have advantages in terms of vaccine production since a well-defined pathogen fragment can more easily be produced consistently.

To achieve of our goal of developing safe and efficacious subunit vaccines against PRRSV, three projects were completed. First, a scalable process for purification of PRRSV

particles from cell culture was developed. This process produced purified viral particles for ELISA and cell-based assays used in vaccine development. Second, a plant-made oral subunit vaccine against PRRSV was developed. Administration of the plant-made vaccine, the vaccinated animals produced virus-specific serum and intestine mucosal antibodies with neutralization activity, as well as cellular immune responses with a preference of virus-specific IFN-γ production. Since neutralization antibodies and virus-specific IFN-γ response are the crucial factors contributing to protection against PRRSV infection, the plant-made oral subunit vaccine strategy is an attractive strategy for developing a new generation of the vaccine to control PRRS disease. Third, a chimeric protein consisting of the ectodomains of viral M and GP5 proteins was expressed and purified. The protein product showed a single band on a silver-stained gel and contained an endotoxin level of less than 10 EU/mg protein. In addition, the purified protein showed expected bioactivities. It was antigenic, could bind to a cellular receptor for the virus (heparan sulfate), and could block virus infection of susceptible cells. Therefore, the chimeric protein is a promising subunit vaccine candidate against PRRSV.

Dedication

I dedicated this dissertation to my parents, Xingguo Hu and Huajin Wen.

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Chapter I: Introduction

In the late 1980s, the 'mystery swine disease', later renamed porcine reproductive and respiratory syndrome (PRRS), emerged in U.S. swine herds. A few years later, this disease appeared in Europe and has since become endemic at the global level. PRRS has clinical signs of 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 [1-3]. The cause of PRRS was first determined by researchers at the Central Veterinary Institute (Lelystad, the Netherlands) in 1991 when Koch's postulates were fulfilled with a previously unknown enveloped RNA virus (Lelystad virus) [4, 5]. Almost at the same time, a similar virus (VR-2332) was isolated in the US [6, 7]. Lelystad virus and VR-2332 are the prototype of type 1 (European) and type 2 (North American) PRRS virus (PRRSV), respectively.

PRRSV is classified into the *Arterviridae* family within the genus *Arterivirus*, order *Nidovirales*, along with equine arteritis virus (EAV), simian hemorrhagic fever virus (SHFV), and lactate dehydrogenase elevating virus (LDV) [8]. Mature PRRSV virions are enveloped and contain an icosahedral capsid with a 15-kb linear positive-stranded RNA genome encapsulated [9]. The genome is polycistronic, containing two large open reading frames (ORF1a and b) and a set of six to nine ORFs downstream of the 1b gene [10]. ORF1a and ORF1b constitute approximately 75% of viral genome and encode two long nonstructural polyproteins, pp1a and pp1ab, with expression of the latter depending on a ribosomal frameshift signal in the ORF1a/1b overlap region [11]. The pp1a and pp1ab replicase proteins are co- or post-translationally cleaved into 14 functional nonstructural proteins (nsps) in a

complex proteolytic cascade by four protease domains encoded in ORF1. To date, the nsps have been suggested to possess functions of proteases (nsp1α and nsp4), RNA polymerase (nsp9), helicase (nsp10), replicative endoribonuclease (nsp11), regulation of subgenomic mRNA synthesis (nsp1), and induction of replication-associated membrane rearrangement (nsp2 and nsp3) [12].

ORF2a, ORF2b, and ORF3-7 encode for viral structural proteins GP2a, 2b protein, GP3, GP4, GP5, M and N, respectively. Recently, a novel structural protein with 51 amino acids encoded by an alternative ORF5 (ORF5a) was indentified [13]. These structural proteins are translated from six subgenomic mRNAs that are synthesized as a 3'–coterminal nested set of mRNAs with a common 5'-leader sequence [11]. GP2a, GP3 and GP4 are N-glycosylated minor envelope proteins and 2b is a non-glycosylated minor protein. The major envelope protein GP5 and M form disulfide-linked heterodimers, which may be the basic protein matrix of the viron envelope [14]. The most abundant N protein is a highly basic protein and it is multimerized to form the icosahedral core structure of PRRS virions [15]. The three main structural proteins GP5, M and N are indispensable for both virion formation and viral infectivity, whereas the minor proteins (GP2a, GP3 and GP4) are essential only for infectivity [16]. GP5/M heterodimers, GP4, GP2a, and 2b protein have been suggested to play roles in PRRSV entry into susceptible cells. GP5/M heterodimers are involved in virus attachment and internalization through binding with cellular receptors heparan sulfate and sialoadhesin [17-19]. GP4 mediates interglycoprotein interactions and, along with GP2a, interacts with CD163, which is considered as a receptor for viral uncoating and genome release [20, 21]. The 2b protein is likely an ion-channel protein which facilitates

uncoating of virus and release of the genome in the cytoplasm [22].

Vaccination is the most effective method of preventing infectious diseases. Currently, killed-virus and modified-live PRRSV vaccines are used clinically to control PRRS. However, both types of vaccines are not satisfactory. Killed-virus vaccines are considered ineffective or of limited efficacy at best, even against homologous infection [23]. Although modified-live PRRSV vaccines can confer solid protection against clinical diseases induced by homologous infection, the vaccine efficacy drops significantly upon heterologous challenge [24, 25, 26]. In addition, there is a great safety concern on the intrinsic risk of modified-live PRRSV vaccine to revert to virulent virus under farm conditions. The reversion of a commercial modified-live PRRSV vaccine to a pathogenic phenotype in vaccinated pigs has been demonstrated [27,28,29,30], and several vaccine-like and vaccine-derived PRRSV isolates have been isolated and shown to cause diseases in pigs [31,32]. The unsatisfactory efficacy and safety of current PRRS vaccines drives the development of a new generation of PRRSV vaccines. Subunit vaccines, which eliminate the safety concern of virulent reversion of modified-live vaccines and allow the immune system to be focused on protective B cell or/and T cell epitopes in a particular viral protein, are the major focus.

The overall goal of this study is to develop safe and efficacious subunit vaccines against PRRSV infection. To achieve this goal, (1) a purification process was developed to produce native PRRSV particles for ELISA and cell-based assays used in vaccine development; (2) the use of transgenic plant tissues as an effective delivery system for oral subunit vaccines was studied; and (3) a chimeric protein consisting the ectodomains of viral M and GP5 proteins was expressed and characterized as a potential vaccine candidate against

PRRSV infection. This dissertation is composed of six chapters: general introduction, literature review, purification of PRRSV particles from infected cell culture, plant-made oral vaccine against PRRSV, expression and purification of a chimeric protein for PRRSV vaccine application, and general conclusions.

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Chapter II: Literature Review

Porcine reproductive and respiratory syndrome virus (PRRSV) vaccines: current status and strategies to a universal vaccine

Commercial vaccines

Since emerging in Europe and the US, PRRS has spread globally and become the most economically import infectious disease in swine industry [1]. In the US alone, the economic losses caused by this disease amount to more than 560 million US dollars every year [2]. Modified-live and killed-virus PRRSV vaccines are two types of vaccines currently available to control this disease. However, killed-virus vaccines are generally considered ineffective or of limited efficacy at best [1, 2, 64, 65]. Modified-live PRRSV vaccines (MLV) can confer solid protection against homologous reinfection (1, http://vetmed.illinois.edu/news/PRRSwhitepaper.pdf) and have significant effects in reducing viral shedding [66-68]. But the vaccine efficacy varies upon heterologous challenge [4-6, 69-72]. Both types of PRRSV vaccines are unable to completely prevent respiratory infection, transplacental transmission, as well as pig-to-pig transmission of the virus [51-53]. In addition, there is a great safety concern on the intrinsic risk of MLV vaccines to revert to virulent virus under farm conditions. In fact, the reversion of a commercial MLV vaccine to a pathogenic phenotype in vaccinated pigs has been demonstrated [7-10]. Many vaccine-like and vaccine-derived PRRSV isolates have been isolated and shown to cause diseases in pigs [11,12]. The unsatisfactory efficacy and safety of current PRRSV vaccines drives the continuous efforts of developing a new generation of vaccines.

Experimental PRRSV vaccines

Live attenuated PRRSV vaccine candidates

Despite the associated risks, live attenuated PRRSV vaccines are widely studied due to their immunogenic potential. Traditional multiple serial passage methodology has been used to develop attenuated vaccines against newly emerging pathogenic PRRSV. Tian et al. developed a live attenuated vaccine candidate against a highly pathogenic PRRSV by multiple serial passages in a continuous cell line, a process that is concomitant with a progressive loss of the virulent phenotype [76]. This vaccine candidate can protect piglets from lethal challenge of the homologous virus strain. Genetically engineering of wild-type strains are also employed to develop attenuated PRRS virus vaccines. Verheije and colleagues modified a parent stain (vABV437) by introducing two amino acid substitutions in GP2 (vABV688), or replacing the ectodomain of M with that of the murine lactate dehydrogenase-elevating arterivirus (vABV707), or deleting the six C-terminal amino acids of the N protein (vABV746), and thus developed three live attenuated PRRSV vaccine candidates [13]. They found that the vaccine candidates could reduce viraemia in pigs, but could not protect immunized pigs against infection. Only vABV707 was able to protect immunized pigs against homologous challenge and none of the vaccine candidates can protect against heterologous challenge. Wan Welch et al. constructed two replication-defective PRRSV vaccine candidates by genetically deleting ORF2 and ORF4 respectively and tested their efficacy [14]. Although they induced neutralization antibodies and reduced virus load, these vaccine candidates did not diminish clinical signs and even appeared to enhance pathology. Researchers also constructed PRRSV virus chimeras by

different combinations of a licensed modified live vaccine (Ingelvac® PRRS MLV) and a virulent field isolate (MN184) [15, 16]. Three of the tested chimeras were able to prevent lung consolidation to similar extent as traditionally prepared cell-passaged attenuated vaccines.

Recombinant vectors expressing PRRSV viral proteins as vaccine candidates

An alternative method of PRRSV vaccine development is using virus or bacterial vectors to express PRRSV viral proteins. Structural proteins, in particular GP3, GP5, and M protein are the targets for development of recombinant vector vaccines. Plana Duran and colleagues used baculovirus to express PRRSV (strain Olot/91) ORF2 to ORF7 individually, but only ORFs 2, 3, 5 and 7 were expressed. ORF 3 and ORF 5 gene products (GP3 and GP5) were identified as major candidates for vaccine development since they conferred 68.4 and 50% protection, respectively, as evaluated by the number of piglets born alive and healthy at the time of weaning [17]. The authors also found that the ORF7 gene product (N protein) is the most immunogenic protein of PRRSV, but the antibodies induced in sows are non-protective and may even interfere with protection [17]. The immunogenicity of GP3 was further characterized by using two recombinant adenoviruses, rAd-GP3 expressing complete GP3 and rAD-tGP3 expressing truncated GP3 in which amino acids 2-64 were deleted [18]. After mice immunized with the recombinants rAd-GP3 and rAD-tGP3, PRRSV-specific neutralization antibodies, T-cell proliferation responses and cytotoxic T responses were observed, although the production of neutralizing antibodies was delayed. Interestingly, the immune response elicited by rAd-tGP3 was stronger than that of rAd-GP3 [18]. The mechanism underlying this phenomenon remains unclear, but it might suggest some adverse

factors lies within the deleted sequence of GP3. Qiu et al. further characterized the immunogenicity of GP5 using a recombinant pseudorabies virus expressing GP5 protein. Compared to a commercial inactivated vaccine, the recombinant pseudorabies virus provided better protection against clinical diseases and pathogenic lesions [19]. Using canine adenovirus 2 as the expression vector and delivery vehicle, Zhou and colleagues also demonstrated the immunogenicity of GP5 and the potential of using the recombinant canine adenovirus 2 as a vaccine [20]. However, the authors did not evaluate the protection of recombinant canine adenovirus 2 expressing GP5 by virus challenge experiments. Bastos et al. tested the feasibility of using *Mycobacteriusm bovis* BCG for the expression of GP5 and M protein and the antigen-specific immune responses in mice [21]. Their results indicate that M and truncated GP5 protein can be expressed in this system, and mice inoculated with recombinant *Mycobacteriusm bovis* BCG can develop both antigen-specific humoral and cellular immunity. In the subsequent experiments using pig models, they confirmed that the recombinant *Mycobacteriusm bovis* BCG could confer pigs with partial protection against PRRSV infection [22].

To enhance host immune responses to PRRSV immunogens, researchers further developed recombinant vector vaccines expressing viral proteins as a fusion protein or co-expressing several viral proteins. Jiang and colleagues expressed GP5, M and their fusion protein in replication-defective adenovirus and characterized their immunogenicity in a mouse model [23]. The authors observed that mice immunized with recombinant adenovirus expressing GP5 and M fusion protein produced significantly higher titers of neutralizing antibodies, stronger lymphocyte proliferation responses, and anti-PRRSV CTL responses,

compared to mice immunized with a recombinant adenovirus expressing individual GP5 or M protein. Recombinant fowlpox virus co-expressing GP5 and GP3 was also evaluated as a vaccine candidate and the results indicated that the recombinant fowlpox virus conferred partial protection against PRRSV challenge as indicated by lower temperature, viraemia and virus load in bronchial lymph nodes of immunized pigs compared to control animals [24]. Jiang et al. demonstrated that recombinant adenoviruses (rAd) expressing a fusion protein of GP3-GP5 or GP3-GP4-GP5 could also enhance vaccinees' humoral and cellular immune responses [25]. Recently, Zheng and co-workers investigated the effects of expression patterns of GP5 and M proteins in the recombinant vaccinia virus ankara (rMVA) on inducing the immune responses [26]. They concluded that rMVA co-expressing GP5 and M could generate better humoral and cellular immune responses than rMVA expressing individual GP5 or M protein or the fusion protein. They suggested GP5 and M protein co-expressed in rMVA may partly mimic the formation and display of GP5/M heterodimers in native virions, thus leading to better vaccine efficacy.

DNA vaccine candidates

DNA vaccines are another strategy for PRRSV vaccine development. Pirzadeh and Dea identified GP5 as a candidate for subunit vaccines and tested the efficacy of DNA vaccination by immunization of pigs with a plasmid encoding GP5 [27]. DNA-vaccinated pigs were protected from generalized viraemia and typical macroscopic lung lesions and reduced the severity of interstitial pneumonitis and broncho-alveolitis. Jiang et al. used attenuated *S. typhimurium* as a delivery system for oral DNA vaccines containing ORF5 and they found the neutralization antibody titers in the sera of mice was at the same level as that

in mice injected with naked plasmid DNA containing ORF5 [28]. Fang and colleagues developed a DNA vaccine containing modified GP5 with enhanced immunogenicity by inserting a Pan Dr T-helper cell epitope (PADRE) [29]. Li and co-workers further enhanced the immunogenicity of the GP5-based DNA vaccine by mutating four potential N-glycosylation sites of the protein [30]. Similar to recombinant vector-based PRRSV vaccine development, the strategy of co-expressing several viral proteins was also used to enhance the immunogenicity of the DNA vaccine candidates. Jiang et al. reported that a DNA vaccine expressing GP5 and M protein simultaneously could form a GP5/M heterodimeric complex in transfected cells. The DNA vaccine induced significantly higher neutralization antibody titers and lymphocyte proliferation responses than those induced by DNA vaccines expressing GP5 or M individually [31].

Plant-made oral PRRSV subunit vaccine candidates

In recent years, plants have been identified to provide many practical, economic and safety advantages over conventional systems for production and delivery of subunit vaccines [32]. It is particularly true for a plant-made edible subunit vaccine that could provide a safe and convenient method for inducing both systemic as well as mucosal immunity, which is the first line of defense against epithelium-transmitted pathogens but seldom generated by most current vaccines administered by needle and syringe [33, 34]. The first reported plant-made PRRSV vaccine uses tobacco leaves expressing viral GP5 protein [35]. Pigs fed with the transgenic tobacco leaves could have developed specific mucosal and systemic responses against PRRSV, however, the neutralization antibody titers in the sera were low (1:4-1:8). The very low expression level of GP5 in tobacco leaves (0.011% of total soluble proteins)

could be one of the reasons for the very low neutralization antibody titer. To potentiate the immune responses of pigs to the transgenic tobacco produced PRRSV subunit vaccine, the researchers later expressed a fusion protein of GP5 and a mucosal adjuvant, *E. coli* heat-labile enterotoxin B subunit (LTB) in tobacco leaves. The immune responses of pigs administered with tobacco leaves producing the fusion protein were relatively higher than those administered with leaves producing GP5 only, although the differences were not statistically significant [36]. However, nicotine and other harmful chemicals in tobacco may prevent the use of transgenic tobacco leaves as a practical edible vaccine. In this perspective, other plants, especially natural food of pigs, such as potato and corn, are more suitable for production of PRRSV viral proteins and could be used as edible vaccines. The results of a recent study demonstrated that mice orally administered with transgenic potatoes expressing GP5 could also generate specific serum and gut-mucosal antibodies [37]. In our study, we produced PRRSV M protein in transgenic corn tissues and tested their efficacy as an edible vaccine [38]. After feeding mice the transgenic corn tissues, this edible vaccine could induce serum and intestine mucosal antigen-specific antibodies with neutralization activity, and cellular immune responses with a preference of IFN-γ production. Since neutralization antibodies and virus-specific IFN-γ response are the crucial factors contributing to protection against PRRSV, production and oral delivery of viral protein in transgenic plants is very attractive for the development of a new generation of PRRSV vaccines [38].

Adjuvants for PRRS vaccines

To date, adjuvants including cytokines, chemical reagents, and bacterial products have been examined to potentiate the immune response conferred by PRRS MLV vaccines, killed

vaccines, DNA vaccines, recombinant vector-based vaccines, and synthetic peptide vaccines. Some of them do increase the immunogenicity of the adjuvanted PRRS vaccines. The adjuvants for PRRSV vaccine development have been reviewed excellently by Charerntantanakul [39].

Heterogeneity of PRRSV

PRRSV is currently classified into two genotypes, type 1 (European) and type 2 (North American). Both genotypes are only 55-70% identical at the nucleotide level [40]. Even within each of the two major genotypes, PRRSV isolates have a high degree of genetic variability with 90% or less nucleotide homology of its GP5 coding region [41].

RNA viruses are prone to genetic drift caused by inaccurate RNA replication with calculated frequency of 10^{-3} to 10^{-5} nucleotide substitutions per site [42]. In fact, PRRSV exists as a quasispecies (a spectrum of mutants with related genotypes) on farms, and within individual animals, during natural infection [43]. This can cause persistent infection resulting from selection of mutants that escape neutralization antibodies or cytotoxic T lymphocytes (CTL) [3]. Negative selection, positive selection, and random sampling of these mutations are the driving force for quasispecies evolution [44]. RNA recombination evident by results of Yuan et al. [45] and van Vugt et al. [46] provides another mechanism for evolution of PRRSV. Strain evolution will result in an ever-broadening strain diversity [41, 45, 46]. The enormous diversity of PRRSV strains limits the value of almost all of the PRRSV vaccines tested. Developing a universal vaccine that can protect against field PRRSV stains that exhibit considerable genetic diversity becomes a major challenge for current vaccine development.

Strategies to a universal PRRS vaccine

For PRRSV infection, humoral immune responses represented by neutralization antibodies and cellular immune responses represented by virus-specific interferon-gamma-secreting cells have been correlated with protection [38]. Thus, an effective universal PRRS vaccine must invoke a strong response against diverse field strains from both T cells and B cells.

Multi-strain vaccines

The most straightforward strategy to a universal PRRSV vaccine is to develop a multivalent vaccines consisting of antigenically distinct strains, as in the case for poliovirus vaccines. However, because complementing strains have been poorly examined, it is still unknown whether a universal PRRSV vaccine containing a few prototypes strains can be formulated [74]. The results of a study showed that a multi-strain vaccine containing five attenuated strains of PRRSV is not more effective than a single-strain vaccine (both vaccines provided partial protection) against a virulent field strain of PRRSV unrelated to any of the strains used for vaccination [47]. Recently, Anderson et al. (2012) established a graph theory-based method to identify core viruses from a de novo constructed virus network derived from virus sequence data. The top-ranked sequence indentified by the method covered 48% of putative epitopes derived from known diversity of PRRSV. Obviously, this method will be very useful for selection of representative virus isolates and development of polyvalent vaccines against PRRSV [76].

Conserved-region vaccines

Another strategy to be explored for a broadly protective PRRSV vaccine is to use immunogens comprised of the most conserved region or a number of conserved regions of the viral proteome that are arranged in a chimeric protein. The purpose of this strategy is to incorporate B cell and T cell epitopes within conserved regions of the virus into vaccines. Vaccine-induced immune responses would thus have high probability of interacting with the circulating viruses. If a vaccinated animal were infected, the vaccine-elicited immune responses might have the potential to shift the immunodominance profile and focus the initial immune response on conserved regions of the virus, where mutations that facilitate immune escape would likely have a high fitness cost [48-51]. The viral conserved-region approach was encouraging in development of universal vaccines against influzena and HIV virus, two viruses notorious for genetic and antigenic variability. A universal influenza A vaccine based on a conserved domain of influenza A virus, the extracellular domain of the M2 protein (23 amino acids) successfully provided 90%-100% protection against a lethal virus challenge after mice were intraperitoneally or intranasally administered with hepatitis B virus core (HBc) protein displaying the vaccine antigen [52]. Letourneau and co-workers designed a universal HIV-1 vaccine by assembling the 14 most conserved regions of the HIV-1 proteome into a chimeric protein [53]. Each segment is a consensus sequence from one of the four major HIV-1 clades. BALB/c and HLA-A*0201mice could generate HIV-1 specific T cell responses when administered with the antigen delivered as DNA in a human adenovirus serotype 5 vector, and in a modified vaccine virus Ankara (MVA) vector. In addition, the antigen was able to prime CD8+ and CD4+ T cell to highly conserved epitopes in humans and those epitopes could induce memory T-cell responses in patients during natural

HIV-infection [53]. Obviously, for a PRRSV universal vaccine, identifying conserved regions of the viral proteome is the starting point for this strategy.

Polyepitope vaccines

Although PRRSV exhibits great genetic and antigenic variability, cross protection does exist, even between genetically distant type 1 and type 2 virus [41]. Common epitopes are believed to be involved in protection in different stains [54]. The polyepitope vaccine strategy is based on artificially designed minigenes, which are comprised of a string of conserved epitopes and expressed as polypeptides, or in DNA or viral vectors. The advantage of this approach is to allow the immune system of vaccinees to focus on rationally selected conserved B cell and/or T cell epitopes. This is particularly important for PRRS vaccine development, because adverse factors for generating protective immune responses are present within viral proteins, such as existence of immunodominant decoy epitope adjacent to the major neutralization epitope and glycan shielding of this neutralization epitope [55, 56].

Recognition of epitopes on viral proteins by recent studies facilitates this strategy. A linear B-cell epitope of type 2 PRRSV has been identified as the primary neutralization epitope (^{37}SHLQLIYNL^{46}) of viral GP5 protein [56, 57]. It is conserved among type 2 isolates and neutralization antibodies are mainly directed against this epitope [55-57]. In 2006, de Lima et al. identified two conserved B-cell linear epitopes within M protein by peptide ELISA [58]. Recently, two immunodomiant T-cell epitopes on GP5 of type 2 PRRSV have been recognized [59]. The epitopes (^{117}LAALICFVIRLAKNC^{131} and ^{149}KGRLYRWRSPVIIEK^{163}) are relatively conserved with at most two amino acids variation and can recall a strong IFN-γ response. Conserved T-cell epitopes were also indentified in

GP4 and N protein of type 1 PRRSV [60]. It will be interesting to test the protective efficacy of these epitopes and then make a decision on whether to include some of these epitopes in a universal polyepitope vaccine.

Apparently, the limited knowledge on protective and conserved B-cell and T-cell epitopes of PRRSV is still a barrier for us to design a polyepitope vaccine which can confer broad protection against various field stains. To expedite vaccine development by this strategy, bioinformatics tools may be used first to scan viral genomes for conserved and potential B cell and T cell epitopes. The predicted epitopes can then be *in vitro* screened by peptide ELISA (for B-cell epitope) and ELISpot or intracellular cytokine cytometry (for T-cell eiptopes). Following confirmation, the peptides that have strong ELISA signals or stimulate a T cell response can be considered as the building blocks of polyepitope vaccines. However, incorporation of B-cell epitopes into polyepitope vaccines should be cautious due to antibody dependent enhancement (ADE) of PRRSV [61, 62]. After determining the epitopes to be included, computationally engineering polyepitope vaccine rather than simply linking epitopes should be employed to enhance correct processing and to minimize unnatural junctional epitopes [63].

Conclusions

Development of effective vaccines against PRRSV has been a significant challenge. Through almost 20 years of extensive research in PRRSV vaccine development, it is evident that to combat PRRSV infections it is critical to develop universal vaccines that can provide broad protection against circulating filed strains. In order to develop successful universal vaccines, better understanding of PRRSV appears to be the key. Only through the advanced

knowledge of PRRSV immunobiology, will we be able to identify and use conserved regions or polyepitope vaccine strategy to develop universal PRRSV vaccines.

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Table 1 Recombinant vector-based PRRSV vaccine candidates

Expression / delivery system	Protein expressed	Animal model	Immune responses / vaccine efficacy	Reference
Baculovirus	GP3	Pregnant sows	Elicits serum antibody responses in sows; protects 68.5% of piglets from the disease at the time of weaning.	[17]
Baculovirus	GP5	Pregnant sows	Protects 50% of piglets from the disease at the time of weaning.	[17]
Baculovirus	N	Pregnant sows	Elicits strong serum antibody responses in sows; protects 16.6% of piglets from the disease at the time of weaning.	[17]
Adenoviruses	Complete GP3	mice	Elicits PRRSV-specific neutralization antibodies, T-cell proliferation responses and cytotoxic T cell responses	[18]
Adenoviruses	Truncated GP3 (deletion of aa 2-64)	mice	Elicits stronger neutralization antibodies, T-cell proliferation responses and cytotoxic T cell responses than that of complete GP3	[18]
Pseudorabies virus	GP5	Piglets	Protects piglets from clinical signs and reduces pathogenic lesions of the disease in spite of no detectable anti-PRRSV antibodies before challenge	[19]
Canine adenovirus 2	GP5	Piglets	Elicits virus-specific neutralization antibodies and lymphocytes proliferation responses	[20]
Mycobacteriusm bovis BCG	M and truncated GP5 (lacking the of first 30 NH_2-terminal residues)	Mice	Elicits antigen-specific serum antibodies, neutralization antibodies and IFN-γ production	[21]
Mycobacteriusm bovis BCG	M and truncated GP5	Piglets	Elicits antigen-specific serum antibodies, neutralization antibodies; Establishes partial protection against PRRSV infection as indicated by lower	[22]

			temperature, viremia and virus load in bronchial lymph nodes than control animals.	
Replication-defective adenovirus	GP5 and M fusion protein	Mice	Elicits stronger neutralization antibody and lymphocyte proliferation responses than mice immunized with adenovirus expressing GP5 or M alone	[23]
Fowlpox virus	GP3 and GP5 fusion protein with/without swine IL-18	Mice	Elicits virus-specific serum antibodies, neutralization antibodies, T-lymphocytes proliferation, and IFN-γ production in serum and T-lymphocytes; Establish partial protection against PRRSV infection.	[24]
Replication-defective adenovirus	GP3-GP5 or GP3-GP4-GP5 fusion protein	Mice	Elicits stronger neutralization antibody and cytotoxic T cell responses than mice immunized with adenovirus expressing a viral protein alone	[25]
Modified vaccinia virus ankara (MVA)	Co-expressing of GP5 and M under two promoters	Mice	Elicits stronger neutralization antibody responses, as well as greater IFN-γ and IL-2 production than MVA expressing individual GP5 or M protein or the fusion protein.	[26]

Table 2 DNA vaccine candidates against PRRSV

Coding sequence of viral protein	Delivery system	Animal model	Immune responses / vaccine efficacy	Reference
GP5	Plasmid	Piglets	Elicits anti-GP5 antibodies, neutralization antibodies, and specific blastogenic response; Protects from generalized viraemia and typical macroscopic lung lesions.	[27]
GP5 or truncated GP5 (deletion of the first 25 NH_2-terminal residues)	Plasmid or attenuated *Salmonella typhimurium aro*A	Mice	Elicits neutralization antibodies	[28]
Modified GP5 with a Pan DR T-helper cell epitope (PADRE) inserted between the neutralization epitope and the decoy epitope	Plasmid	Mice	Elicits enhanced neutralization antibodies, GP5-specific serum antibodies, and T-cell proliferative activities compared to the native GP5.	[29]
Modified GP5 with insertion of PADRE and mutation of four potential N-glycosylation sites	Plasmid	Mice and piglets	Elicits significantly enhanced GP5-specific serum antibodies, PRRSV-specific neutralization antibodies, IFN-γ level, and lymphocyte proliferation response.	[30]
GP5 and M	Plasmid	Mice and piglets	Elicits stronger neutralization antibody and lymphocyte proliferation responses than plasmid expressing GP5 alone	[31]

Table 3 Plant-made oral PRRSV subunit vaccine candidates

Expression / delivery system	Protein expressed	Animal model	Immune responses / vaccine efficacy	Reference
Tobacco leave	GP5	Pigs	Elicits PRRSV-specific serum IgG antibodies and neutralization antibodies, saliva IgA antibodies, and blastogenic response of peripheral blood mononuclear cells	[35]
Tobacco leave	Fusion protein of GP5 and *E. coli* heat-labile enterotoxin B subunit (LTB)	Pigs	Elicits relatively higher PRRSV-specific serum IgG antibodies and neutralization antibodies, saliva IgA antibodies, and blastogenic response of peripheral blood mononuclear cells compared to tobacco leaves expressing GP5 alone; Reduces viremia, tissue viral load and lung lesions in virus challenge experiments	[36]
Potato	GP5	Mice	Elicits antigen-specific serum IgG antibodies and intestine IgA antibodies.	[37]
Corn tissues	M	Mice	Elicits serum and intestine mucosal antigen-specific antibodies with neutralization activity, and cellular immune responses with a preference of IFN-γ production	[38]

Chapter III: Purification of porcine reproductive and respiratory syndrome virus (PRRSV) from cell culture using ultrafiltration and heparin affinity chromatography

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Abstract

Porcine reproductive and respiratory syndrome (PRRS) virus is the causative agent of the most significant infectious disease currently affecting the swine industry worldwide. Density gradient ultracentrifugation remains the most commonly used method for PRRSV purification. However, this technique has notable drawbacks including long processing time and limited processing volume in each run. To overcome these limitations, a scalable process was developed. PRRSV propagated in MARC-145 was released by three freeze/thaw cycles. After a low speed centrifugation step, the virus particles in the supernatant were concentrated twice by an ultrafiltration step. The ultrafiltration step concentrated the virions effectively with no detectable loss while some cultural/cellular proteins were removed. The virions in the ultrafiltration retentate were then applied to a heparin affinity column on a fast performance liquid chromatography unit. The combined ultrafiltation and heparin affinity chromatrography process removed more than 96% of cellular and medium proteins. During a step-wise elution strategy, the viral particles were eluted at two separate peaks recovering 27.5% and 25.4% of viral particles loaded onto the column with a purity of 194 and 3917 particles/μg protein, respectively.

Key words: PRRSV; virus purification; heparin affinity chromatography; membrane ultrafiltraion

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is the causative agent of PRRS, the most significant infectious disease currently affecting the swine industry worldwide [1]. In the US alone the economic losses caused by PRRS amount to more than 560 million US dollars every year [2]. PRRSV belongs to *Arterviridae* family with the genus *Arterivirus*, order *Nidovirales*, along with equine arteritis virus (EAV), simian hemorrhagic fever virus (SHFV), and lactate dehydrogenase elevating virus (LDV). The virus is an enveloped, linear positive-stranded RNA virus with a diameter of 45-70 nm [3]. Its 15 kb genome contains eight open reading frames (ORFs) as well as 5' and 3' untranslated regions. ORF1a and 1b comprise about 80% of the viral genome and are predicted to encode viral polymerase [4]. ORFs 2, 3 and 4 encode for three minor structural proteins GP2, GP3, GP4, respectively. Three major structural proteins GP5, M and N are derived from ORFs 5, 6 and 7, respectively [3]. The most abundant N protein is a highly basic protein. It is multimerized to form icosahedral core structure of PRRS virions [5]. GP5 is the major envelope glycoprotein, which is disulfide-linked with non-glycosylated M protein in native virions [6]. GP5/M heterodimers are likely to form the basic protein matrix of the viron envelop [7]. GP5 has been associated with the development of neutralizing antibodies and protection [8, 9] and M protein has been associated with the development of strong cellular immunity [10].

In scientific research, purified viruses are essential for obtaining information on chemical, physical, biological properties of viruses and producing antiserum for serological studies [11]. In addition, when viruses are used as prophylactic vaccines or as therapeutics through gene therapy, purification of virus is mandatory to prevent toxicity, inflammation or

immune response due to cellular and medium contaminants [12]. Establishment of virus purification strategy depends on virus properties as well as production characteristics. Density based ultracentrifugation methods [11, 13], size based ultrafiltration [14] and size exclusion chromatography methods [15, 16], charge based ion-exchange chromatography methods [17, 18], and specific interaction based affinity chromatography methods [19-22] have been investigated for the purification of various viruses.

Specifically, purification of PRRSV has traditionally been carried out by cesium chloride or sucrose density gradient ultracentrifugation [23-25]. However, this method is labor-intensive and difficult to scale-up and requires long processing time [19]. Additionally, density gradient centrifugation often gives variable degrees of purity and poor yield, and it is not serotype specific [18]. In recent years, liquid chromatography, which provides high level of purity and increased productivity, has become the method of choice for efficient virus purification [19, 26]. The adoption of selective chromatographic procedures was strongly encouraged over conventional virus purification techniques [19, 27]. However, to the best of our knowledge, no liquid chromatography procedure for the purification of PRRSV particles has been reported to date.

Heparin and the structurally related heparan sulfate belong to the family of glycosaminoglycans [19]. Recent work has identified heparan sulfate as one of two PRRSV receptors on macrophages. When the viruses were treated with heparan sulfate or heparin, an analogue of heparan sulfate, during inoculation [28 ,29], or when porcine alveolar macrophages (the primary target cells of the virus) were treated with heparinase I, an enzyme which destroys heparan sulfate, the PRRSV infection was significantly reduced [29].

Analysis of the attachment kinetics to macrophages revealed that PRRSV first binds to heparan sulfate, followed by an interaction with sialoadhesin, another PRRSV receptor on macrophages [30]. Since PRRSV binds to heparan sulfate in the course of infection it should be quite probable to purify PRRSV using a heparin affinity column. This new selective chromatography procedure should allow a high level of purification of PRRSV from native cell cultures. This study here describes a novel and scalable process for the purification of PRRSV. The PRRSV released by freeze/thaw cycles of the infected cells was concentrated by ultrafiltration. Then, concentrated viral particles were purified by heparin affinity chromatography.

Materials and Methods

Cell culture and virus propagation

Virus was propagated in the MARC-145 cell line generously provided by Dr. K. J. Yoon (Iowa State University College of Veterinary Medicine). The methods for cell culture and virus propagation were described elsewhere [31]. The virus stock was prepared by three consecutive freeze/thaw cycles of the infected cells when approximately 90% of the infected cells developed cytopathic effect (CPE).

Clarification and ultrafiltration

The virus containing cell lysate was clarified by low speed centrifugation at 5000×g for 20 min at 4 ^{o}C [32]. The clarified samples were collected into sterile containers. For ultrafiltration, a Macrosep® centrifugal device (Pall Corporation, East Hills, USA) with an Omega™ membrane with a cut-off of 300000 nominal molecular weight limit (NMWL) was

used. This device is made of two parts: the upper sample cup with the membrane and the lower filtrate collection tube. The Macrosep® centrifugal device is compatible with any fixed-angle or swinging bucket rotor that accepts 50 ml tubes. The membrane of this device was pre-washed twice with ultrapure water. Then, 12 ml of clarified sample was loaded into the upper cup of the centrifugal device and centrifuged for at 5000×g for 1 hr at 4 ^{o}C. For purification and buffer exchange, approximately 10 ml of binding buffer (0.1 M NaCl, 20 mM Tris-HCl, pH 7.5) was added to the ultrafiltration retentate and centrifuged at 5000×g for 1 hr at 4 ^{o}C. The last step was repeated twice.

Heparin affinity Chromatography

Virus purification experiments were performed at room temperature using an ÄKTA™ explorer 100 fast performance liquid chromatography (FPLC) system (GE Healthcare, Uppsala, Sweden). Prior to virus purification, the HiTrap™ Heparin HP (1 ml bed volume) column (GE Healthcare, Uppsala, Sweden) was equilibrated with 20 column volumes of binding buffer and the ultrafiltration retentate containing the virus was filtered through a Acrodisc® 0.45-μm PVDF membrane (Pall Corporation, East Hills, USA). A 3.5 ml of filtered ultrafiltration retentate was loaded onto the column. Elution was accomplished using a stepwise elution strategy included a wash step at 0.1 M NaCl in 20 mM Tris-HCl (pH 7.5), followed by two virus elution steps at 0.46 M NaCl and 2 M NaCl in 20 mM Tris-HCl (pH 7.5), respectively. After each run, the column was regenerated with 20 column volumes of 1.5 M NaCl in 50% ethanol. The flow rate used for adsorption and elution steps was 0.6 ml/min, and 2 ml/min for regeneration. The fractions of the chromatography process were concentrated with YM-10 Microcon® centrifugal filter device (Millipore Corporation,

Billerica, USA) when needed.

Protein assays

Total protein concentration was determined by the Bradford Protein Assay (BioRad, Hercules, USA) according to the manufacturer's instructions using bovine serum albumin (BSA) as the standard.

SDS-PAGE and Western blot

Polyacrylamide gels were run under reduced condition as described elsewhere [31]. For total protein analysis, the SDS-PAGE was stained with SimplyBlue™ SafeStain (Invitrogen, Carlsbad, USA) following the manufacturer's protocol. Gel images were captured by a ChemiDoc XRS molecular imager (BioRad, Hercules, USA) with EPI illumination. For Western blot, gels were transferred to a PVDF (BioRad, Hercules, USA) membrane in 1xNuPAGE transfer buffer (Invitrogen, Carlsbad, USA) supplemented with 10% methanol and 0.1% antioxidant (Invitrogen, Carlsbad, USA). After transfer, the membrane was incubated for 1 hr with gentle agitation in 5% (w/v) non-fat dry milk in TTBS buffer (20mM Tris-HCl, 0.5M NaCl, 0.1% (v/v) Tween 20, pH 7.5). The blot was probed with 1:300 dilution of PRRS antiserum (αPRRSV) (National Veterinary Services Labs, Ames, IA) in TTBS for 1 hr at room temperature. Following consecutive washing steps in TCBS buffer (20 mM citrate, 500 mM NaCl, 0.1% (v/v)Tween 20, pH 5.5), the blot was incubated at room temperature for 1 hr in a 1: 20,000 dilution Protein-G HRP conjugate (BioRad, Hercules, USA) in TCBS buffer. After consecutive wash steps in TTBS, the blot was incubated in a 1:1 mixture of HRP luminal/enhancer solution and peroxide buffer for 3 min. The blot was exposed in a ChemiDoc XRS molecular imager with the ChemiHi sensitivity

settting.

Quantitative RT-PCR

Quantitative RT-PCR was performed to determine the number of virus particles in a particular sample by Veterinary Diagnostic Laboratory at Iowa State University. The method was described by Prickett et al. [33].

Results

Virus recovery and concentration

The viruses were first released from the infected cells by three consecutive freeze/thaw cycles, and the cell lysate was clarified by low speed centrifugation to remove the cell debris. Low speed centrifugation resulted in clarified supernatants with a recovery of 96.4% of the virus (Table 1). Clarified supernatants were concentrated and partially purified by an ultrafiltration step, which also serves as a buffer exchange step. A 300 kDa membrane was chosen for concentrating PRRSV, which has a size of approximately 70 nm. The process took 3 hours to complete. No detectable virus was present in pooled permeates based on the western blot result (Fig. 2 (b)), and 82.3% of virus was recovered in the retentate (Table 1). These facts confirm that the virus was successfully retained by this 300 kDa NMWL membrane. Based on the protein assay, this ultrafiltration step also removes 24.5% of total proteins and the final concentration of protein was 1619 μg/ml (Table 1).

Heparin affinity chromatography

The ultrafiltration rententate (3.5 ml) was then subjected to FPLC heparin affinity chromatography under the conditions described in Materials and Methods. A flow-through

peak at 0.1 M NaCl, and two defined elution peaks at 460 mM NaCl and 2 M NaCl respectively, were observed in the chromatogram (Fig. 1). The quantitative analysis of each chromatography fraction is summarized in Table 1. At the virus adsorption step, most bulk proteins (88.3% of the total protein in the ultrafiltration retentate) were removed as they were present in the two flow-through fractions. However, 43.4% of viral particles were present in the two flow-through fractions. Bound viral particles were then eluted with increased salt concentration. Most heparin-binding proteins and 27.5% of viral particles were eluted at 460 mM NaCl. The purity of this fraction was 194 particles/μg protein. Interestingly, when using a stringent elution condition with 2 M NaCl, 25.4% of viral particles were recovered in another elution peak with a purity of 3917 particles/ μg protein.

Analysis of viral proteins

The fractions produced in the purification process were analyzed by SDS-PAGE (Fig.2 (a)) and western blot (Fig. 2 (b)). The most prominent bands in the Commassie stained gel appeared at 62 kDa. Past study suggests that the band corresponds to serum albumin precursor [31]. Although a 300 kDa NMWL of membrane was employed in the ultrafiltration step, most of the proteins with a molecular weight much less than 300 kDa were not removed during this step. However, the following heparin affinity chromatography step was very effective and removed most of the residual proteins. Probably due to the heterogenic nature of the virus, αPRRSV antiserum obtained from NVSL only reacted with two predominant viral structural proteins N (17 kDa) and GP5 (29 kDa), but the reaction with GP5 was weak. This antiserum also recognized a 50 kDa band that corresponds to a non-viral protein, as evident by identification of the same protein in uninfected cell culture (Fig. 2 (b) lane 10). The

ultrafiltration step provides valuable concentration of viral particles. GP5 and N was enriched in retentate while no detectable viral proteins were present in permeates (Fig. 2 (b)). Heparin affinity column captured viral particles, which were eluted as two separate elution peaks. At the first elution peak, virus particles were eluted together with most of the heparin-binding proteins. At the second elution peak, virus was highly pure and few faint bands of the cellular proteins could be detected on Commassie stained SDS-PAGE gels even when the sample was 10-fold concentrated. No viral proteins were detected in the regeneration fraction (Fig. 2 (b), lane 8).

Discussion

Traditionally, PRRS virus particles are purified by gradient ultracentrifugation after a precipitation/concentration step by polyethylene glycol. Concentration by fractional precipitation involves a temporal change of phase (liquid to solid) for viral particles, which in terms of molecular conformation may be sufficiently traumatic to physically damage the viral particles [27]. Ultracentrifugation has inherent limitations including long processing time and small processing volume in each run. The choice of novel downstream processing steps was therefore focused on methods that do not involve phase change and are easy to scale up. Membrane and chromatographic technologies have the desirable characteristics. In this report, ultrafiltration was employed as a concentration, buffer exchange and partial purification step. In this step, 82.3% of the virus particles were recovered. However, based on western blot results (Fig. 2 (b)), there was no detectable loss of virions in the permeate. The possible explanation for the reduced recovery is that some virions were adsorbed onto the membrane

and were not recovered. A desorption step after recovering the retentate may increase the virion recovery using a buffer with high salt concentrations, but this would defeat the purpose of getting the sample buffer conditioned for the following heparin affinity chromatography step. To test our hypothesis, an independent experiment was carried out. After recovering the retentate, 0.5 ml of desorption buffer A (2 M NaCl in 20 mM Tris-HCl, pH 7.5) or 0.5 ml of desorption buffer B (0.1% Tween 20 and 2 M NaCl in 20 mM Tris-HCl, pH 7.5) was used to repeatedly wash the membrane for several times. The SDS-PAGE and western blot results of the desorption solutions were shown in Fig. 3. It is evident that viral proteins (particularly the N protein) were detected in the desorption solutions by Western blot (Fig.3 (b)), and this result validates our hypothesis that the viral particles adsorb onto the membrane during the ultrafiltration process.

Adsorptive chromatography is the method for selective fractionation of bioproducts in large-scale [34]. Given the proposed specific interaction between PRRSV and heparin [29], heparin affinity chromatography was used to capture PRRS virus particles. Approximately 53% of the virus in ultrafiltration retentate was captured in this step. During elution of bound virus by increased salt concentration, two defined peaks occurred at different ionic strength. Interestingly, virus was present in both peaks and western blot results revealed no difference of viral protein patterns (Fig. 2 (b)). There is no obvious explanation for the fact that PRRS virions bound to heparin affinity column are eluted at different ionic strength. However, this phenomenon does not seem to be unique for PRRSV. Occurrence of two virus-containing peaks at different elution ionic strength was also observed when heparin affinity chromatography was used to separate VSV-G pseudotyped retrovector [19].

Although the large size of virions make virus inaccessible into the majority of pores in the gel matrix and thus restricts virus adsorption to the external surfaces of adsorbents, the binding capacity of the absorbents is sufficient considering the very low molarity of virus in suspensions. Approximate calculations of the theoretical capacity for spherical 70 nm diameter viruses (as PRRSV) packed as monolayer on the outer surface of absorbent particles with a diameter of 34 μm (as the heparin affinity resins used in this study) yield a value greater than 10^{13} particles per ml absorbents. The reported capacity for heparin affinity absorbents is more than 10^{9} particles per ml of settled absorbents [19, 35]. It is possible that due to the presence of cellular heparin-binding proteins that compete for the heparin binding sites on the absorbents, some viral particles fail to be captured and appeared in the flow-through. However, separate experiments were carried out with reduced loading, but the recovery and the presence of virions in the flow-through did not change significantly (data not shown).

The choice of heparin affinity chromatography as a PRRS virus purification step was based on recent results of the identification of heparan sulfate as a receptor on macrophages for PRRS virus attachment [28-30]. By using solubilized native virions, past studies also revealed that the viral structural M protein and disulfide-linked M-GP5 complex bind to heparin and concluded that M protein and M-GP5 complex contribute to PRRSV's attachment on the heparan sulfate receptor [29]. Considering there is direct interaction between heparin and viral structural proteins independent of envelope components derived from membrane of a specific packaging cell line, heparin affinity chromatography may be applied to purify PRRSV propagated in various cell lines. Additionally, as M protein is the

most conserved structural protein [36], this method may be extended to purify different PRRSV strains.

The presence of PRRSV virions in different fractions during heparin affinity chromatography suggests that different virus particles have different affinity with heparin. Since heparan sulfate is ubiquitously presented on cell surface as receptors for some viruses to bind and gain entrance to the to-be-infected cells [37], the varying affinity towards heparin may indicate the different infectivity of different PRRSV virions. It would be very interesting to develop purification processes to further purify virions from all three fractions, flow-through, elution peak 1 and 2, and to investigate the infectivity of those virions. The information gained may provide critical understanding on the relationship between virus surface structure and its infective ability and provide guidance for future vaccine design.

Conclusion

The goal of this project was to develop an easily operable and scalable process for the recovery and purification of PRRSV from cell culture. Using mainly a two step process, ultrafiltration and heparin affinity chromatography, PRRSV virions were purified while more than 96% of the medium and cellular proteins were removed. This is the first reported process for PRRSV purification, and our results also revealed the differential binding ability among virions, which may correlate to their respective ability of infecting cells.

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Table 1. Purification results at various stages of the process.

	Particles (per ml)	Protein conc. (μg/ml)	Volume (ml)	Particle number	Protein removal (%)[(a)]	Virus Recovery (%)[(b)]	Purity (particles/μg protein)
Virus stock	2.78E+04	917	12	3.34 E+05	0%	100%	30
Clarified sample	2.68E+04	893	12	3.22 E+05	2.6%	96.4%	30
UF retentate	5.29E+04	1619	5[(c)]	2.65 E+05	24.5%	82.3%	33
Flow-through frac. 1	1.00E+04	661	4	4.00 E+04	46.7%	21.6%	15
Flow-through frac. 2	1.01E+04	590	4	4.04 E+04	41.6%	21.8%	17
Elution peak 1	2.04E+04	105	2.5	5.10 E+04	N/A[(d)]	27.5%	194
Elution peak 2	2.35E+04	6	2	4.70 E+04	N/A[(d)]	25.4%	3917

(a): Protein removal is reported for each individual step, and it is calculated based on the total protein in the sample used in a particular step.

(b): Virus recovery is also reported for each individual step, and it is calculated based on the total virus particle number in the sample used in a particular step.

(c): Only part of the UF retentate (3.5 ml) was applied onto heparin-affinity column. Calculation of protein removal and recovery of chromatographic fractions (below the dashed line) was based on total protein in 3.5 ml of UF retentate and total viral particle number in 3.5 ml of UF retentate, respectively.

(d): Not available

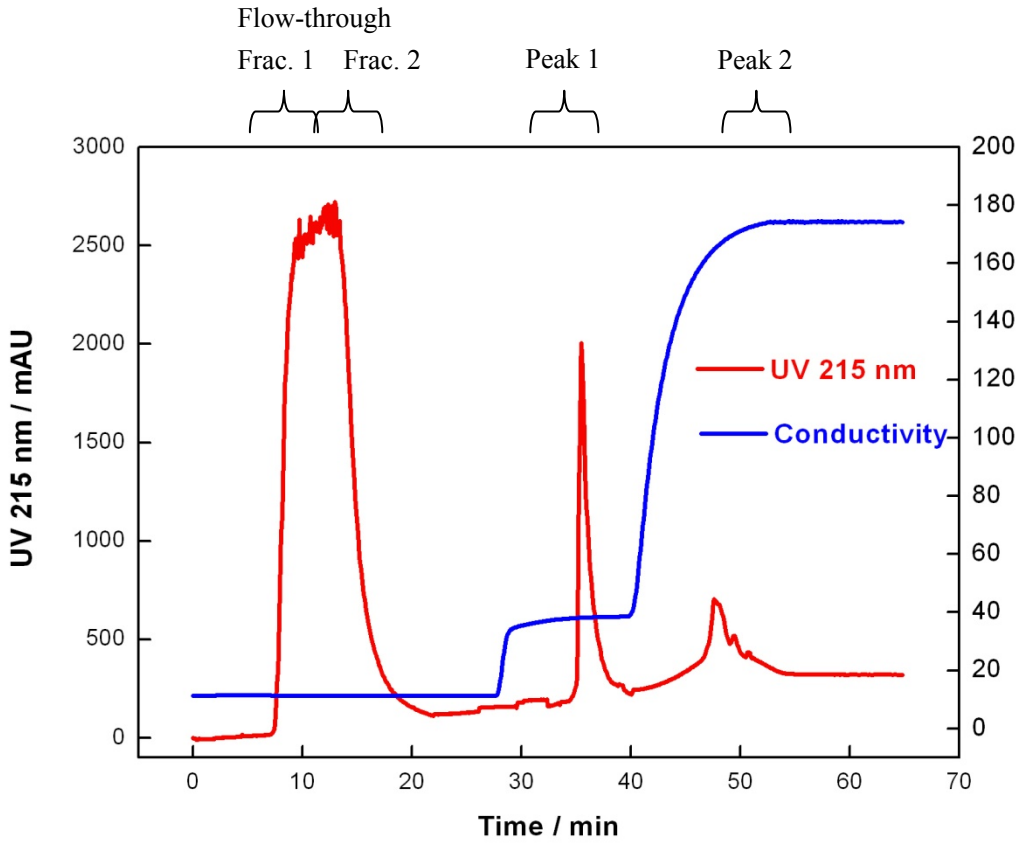

Fig. 1 Heparin affinity chromatography of 3.5 ml ultrafiltration retentate. A 3.5 ml of filtered ultrafiltration retentate was loaded onto the column. Elution was accomplished using a stepwise elution strategy included a wash step at 0.1 M NaCl in 20 mM Tris-HCl (pH 7.5), followed by two virus elution steps at 0.46 M NaCl and 2 M NaCl in 20 mM Tris-HCl (pH 7.5), respectively. The flow rate used for adsorption and elution steps was 0.6 ml/min.

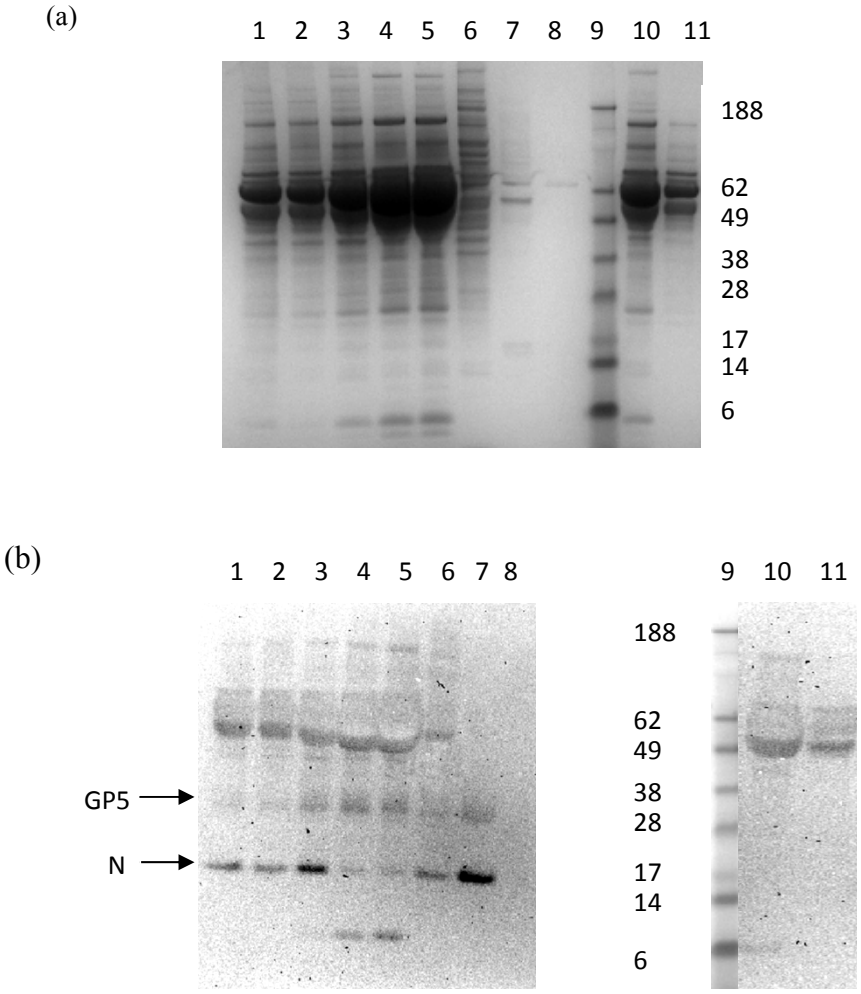

Fig. 2 (a) Commassie stained SDS-PAGE gel and (b) Western blot of fractions produced in the purification process. 1: virus stock; 2: clarified sample; 3: ultrafiltration rententate; 4: concentrated flow-through faction 1; 5: concentrated flow-through faction 2; 6: concentrated elution peak 1; 7: concentrated elution peak 2; 8: concentrated column regeneration fraction; 9: protein standard; 10: uninfected MARC-145 cell culture (negative control); 11: concentrated untrafiltation permeate.

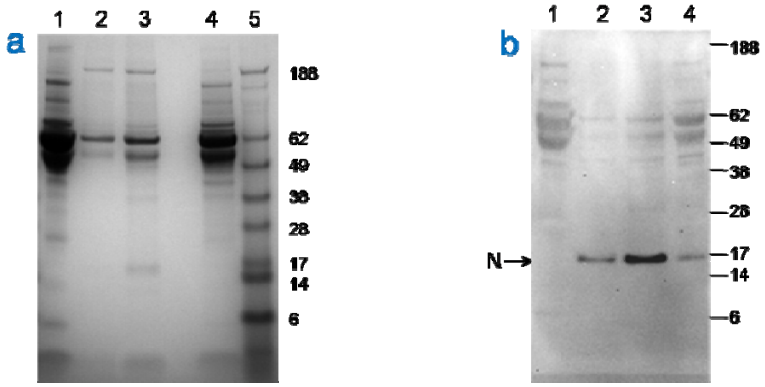

Fig. 3 (a) Commassie stained SDS-PAGE gel and (b) Western blot of desorption solutions. 1: negative control (uninfected MARC-145 cell culture); 2: desorption solution A (membrane washed with 0.5 ml of 2 M NaCl in 20 mM Tris-HCl, pH 7.5); 3: desorption solution B (membrane washed with 0.5 ml of 0.1% Tween 20 and 2 M NaCl in 20 mM Tris-HCl, pH 7.5); 4: positive control (virus stock); 5: protein standard.

Chapter IV: Immunogenicity study of plant-made oral subunit vaccine against porcine reproductive and respiratory syndrome virus (PRRSV)

(Reproduced with the permission of J. Hu, Y. Ni, B. A. Dryman, X. J. Meng, C. Zhang. Vaccine 30 (2012), 2068-2074)

Abstract

Currently, killed-virus and modified-live PRRSV vaccines are used to control porcine reproductive and respiratory syndrome disease (PRRS). However, very limited efficacy of killed-virus vaccines and serious safety concerns for modified-live virus vaccines demand the development of novel PRRSV vaccines. In this report, we investigated the possibility of using transgenic plants as a cost-effective and scalable system for production and delivery of a viral protein as an oral subunit vaccine against PRRSV. Corn calli were genetically engineered to produce PRRSV viral envelope-associated M protein. Both serum and intestine mucosal antigen-specific antibodies were induced by oral administration of the transgenic plant tissues to mice. In addition, serum and mucosal antibodies showed virus neutralization activity. The neutralization antibody titers after the final boost reached 6.7 in serum and 3.7 in fecal extracts, respectively. A PRRSV-specific IFN-γ response was also detected in splenocytes of vaccinated animals. These results demonstrate that transgenic corn plants are an efficient subunit vaccine production and oral delivery system for generation of both systemic and mucosal immune responses against PRRSV.

Key words: Plant-made vaccine; subunit vaccine; porcine reproductive and respiratory

syndrome virus; PRRSV; transgenic plants; transgenic corn

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a highly transmissible disease characterized by reproductive disorders in sows, and respiratory distress and mortality in young pigs [1]. Since suddenly emerging in the late 1980s in Europe and the US [2,3], PRRS has spread globally and become the most significant infectious disease currently devastating the swine industry [4]. In the US alone the economic losses caused by PRRS amount to more than 560 million US dollars every year [5].

Porcine reproductive and respiratory syndrome virus (PRRSV) is the causative agent of PRRS. This virus is an enveloped, linear positive-stranded RNA virus with a diameter of 45-70 nm [6]. It is classified into two distinct genotypes, type 1 (EU-like) and type 2 (North American-like) [7]. Due to quasispecies evolution and RNA recombination, PRRSV is genetically heterogeneous between and within each of the two major genotypes [8]. Regardless of its genotype, PRRSV has a very narrow cell tropism and infects almost exclusively pig monocytes and macrophages, and can induce prolonged viremia and establish persistent infections [9,10]. The virus is able to persist on infected farms through persistently infected carrier sows and is frequently reintroduced to farms after eradication [11,12].

Vaccination is the most effective method of preventing infectious diseases. Currently, killed-virus and modified-live PRRSV vaccines are used clinically to control PRRS. However, both types of vaccines have inherent drawbacks. Killed-virus vaccines are considered ineffective or of limited efficacy at best, even against homologous infection [12]. In a recent study assessing the efficacy of commercial PRRSV vaccines, the killed-virus vaccine failed to establish protective immunity [4]. Although modified-live PRRSV vaccines can confer

solid protection against clinical diseases induced by homologous infection, the vaccine efficacy drops significantly upon heterologous challenge [13,14,15]. More importantly, the intrinsic risk of modified-live PRRSV vaccine to revert to virulent virus under farm conditions poses a great safety concern. The reversion of a commercial modified-live PRRSV vaccine to a pathogenic phenotype in vaccinated pigs has been demonstrated [16,17,18,19], and several vaccine-like and vaccine-derived PRRSV isolates have been isolated and shown to cause diseases in pigs [20,21]. The unsatisfactory efficacy and safety of current PRRS vaccines drives the development of novel vaccines against PRRSV. Subunit vaccines, which eliminate the safety concern of virulent reversion of modified-live vaccines and allow the immune system to be focused on protective B cell or/and T cell epitopes in a particular viral protein, are the major focus.

PRRSV matrix (M) protein is one of the most abundant viral antigens on the viral envelope and is the most conserved structural protein of the virus with 78 to 81% amino acid identity between type 1 and type 2 strains [6,12,22]. M protein is involved in the entry of PRRSV into cells [10,23,24] and two neutralizing epitopes have been identified within this protein [25]. Moreover, M protein is the most potent T cell-stimulation antigen of PRRSV and is suggested to play a major role in cellular immunity [26]. Generally, through stimulating the secretion of cytokines, promoting phagocytosis mediated by macrophages, activating apoptosis induced by natural killer cells and cytotoxic T cells, cellular immunity can reduce or eliminate viral replication after infection has occurred and is particularly crucial in clearance of a persistent virus infection [27,28]. For PRRSV, particularly, cellular immunity represented by virus-specific IFN-γ production is correlated with protection against

reproductive failure in sows [29] and is attributed to the primary protective mechanism of a modified-live PRRSV vaccine [4]. Therefore, M protein is a highly promising candidate for development of a new generation of PRRSV vaccines. However, as a small protein, M protein (18-19 kDa) alone is poorly immunogenic. Adjuvants or delivery systems, or both, are therefore required to potentiate the immune responses induced by M protein.

Recent studies have shown that the plant system provides many practical, economic and safety advantages compared with conventional systems for production and delivery of subunit vaccines [30]. Particularly, it is true for plant-made oral subunit vaccines that could provide a safe and convenient method for inducing both systemic and mucosal immunity, the first line of defense against epithelium-transmitted pathogens [31,32]. By expressing viral or bacterial antigens in transgenic plants, plant-made oral subunit vaccines showed their ability to generate antigen-specific mucosal IgA and serum IgG in animals [33,34,35,36] and in human [37,38,39]. Several plant-made oral subunit vaccines also conferred immunized animals protection against pathogen challenge [33,34,40].

As an economical pig food, corn is the most practically convenient and cost-effective plant system for production of vaccines against diseases infecting pigs. In this study, we expressed PRRSV M protein in corn plant materials and tested its potential as an oral vaccine. Our results indicate that this novel vaccine can induce antigen-specific serum and intestine mucosal antibodies with virus neutralization activity, and cellular immune responses with a preference of IFN-γ production.

Materials and Methods

Construction of expression vector

To ensure correct and high-yield expression of PRRSV (VR-2385) M protein in transgenic corn, the coding sequence of M protein (Genbank accession no. U03040.1) was designed by using corn preferred codons, optimizing translation initiation and termination context and eliminating instability sequence and spurious mRNA processing signals [41,42,43,44,45,46,47,48]. The designed sequence was then artificially synthesized and cloned into plasmid pU57 by a commercial supplier (Genscript Corporation, Piscataway, NJ). The resultant plasmid was digested with restriction enzymes *XmaI* and *SacI*. After gel purification, the 580 bp *XmaI-SacI* fragment was subcloned into pAHC25 (kindly provided by Dr. Quail, Plant Gene Expression Center, University of California, Berkeley) by replacing its *gus* gene [49]. Thus, the M protein encoding sequence was under the control of corn ubiquitin promoter and *nos* terminator. The resulted plasmid pAHCM (Fig. 1) was used for biolistic transformation of corn callus. Plasmid pAHCM contains a *bar* gene coding for phosphinothricin acetyltranferase, which confers transformed plants resistance to bialaphos to facilitate selection. The fidelity and purity of the pAHCM was confirmed by restriction digestion, spectrum analysis and DNA sequencing.

Transformation of maize callus for M protein expression

Transformation of maize callus was performed by particle bombardment at the Plant Transformation Facility at Iowa State University. Transformation and selection method was described in past study [50]. Gold particles coated with plasmid pAHCM were bombarded into Hi II immature zygotic embryos using PDS 100/He biolistic gun (BioRad, Hercules, CA). After cultivation of the bombarded embryos on callus initiation media (4 g/L N6 salts, 1 ml/L

(1000×) N6 vitamin stock, 2 mg/L 2,4-D, 100 mg/L myo-inositol, 2.76 g/L proline, 30 g/L sucrose, 100 mg/L casein hydrolysate, 2.5g/L gelrite, 25 μM silver nitrate, pH 5.8) for 10 to 14 days, the calli were transferred to selection media containing 2 mg/L bialaphos (4 g/L N6 salts, 1 ml/L (1000×) N6 vitamin stock, 2 mg/L 2,4-D, 100 mg/L myo-inositol, 30 g/L sucrose, 2.5 g/L gelrite, 2 mg/L bialaphos, 5 μM silver nitrate, pH 5.8). The transgenic calli were selected, maintained and propagated by transferring callus pieces to fresh selection media every two weeks.

PCR analysis of genomic DNA

DNA was extracted following a standard protocol provided by Plant Transformation Facility at Iowa State University (http://www.agron.iastate.edu/ptf/service/Callus DNA extraction.pdf). PCR was run for 30 cycles on a Mastercycler® PCR device (Eppendorf, Hamburg, Germany). Using primers specific for M protein coding sequence (5'-CGACCGCACCTCAGAAAGTT-3' and 5'-TTGTCATTCGCAGCAATCGGAT-3'), an amplification of 355 bp PCR product was expected in the transgenic plants. A negative control of wild-type corn callus and a positive control of pACHM plasmid were included in each experiment. PCR samples were run on 2% agarose gel against a 50 bp DNA ladder (Promega, Madison, WI).

Protein extraction

About 200 mg of callus were collected into a microcentrifuge tube and 500 μl of chilled extraction buffer (PBS supplemented with 0.05% Tween-20, 2.5 mM 2-mercaptoethanol and protease inhibitor cocktail, pH 7.4) was added. The callus was ground into powder using a disposable plastic pestle (Kimble, Vineland, NJ) and then centrifuge at

12,000×g for 10 min. The supernatant was transferred to a new microcentrifuge tube. Total soluble protein (TSP) concentration in each sample was measured by Bradford method using BioRad protein assay reagent (BioRad, Hercules, CA).

Western-blot analysis to detect M protein in transgenic plants

SDS-PAGE and Western-blot was performed as described elsewhere with small modifications [51]. Briefly, five micrograms of TSP from each sample was heated at 70 ºC for 10 min and loaded onto 4-12% NuPAGE Novex® Bis-Tis Precast Gels (Invitrogen Carlsbad, CA) and separated by electrophoresis. The separated proteins were then transferred to a PVDF membrane (BioRad). The membrane was then blocked for 1 h at room temperature in 5% (w/v) non-fat dry milk in TTBS buffer (20 mM Tris-HCl, 0.5 M NaCl, 0.1% (v/v) Tween-20, pH 7.5), then probed with 1:280 dilution of PRRSV antiserum (National Veterinary Services Labs, Ames, IA) in TTBS for 1 h. After washing in TTBS for four times, the blot was probed with the secondary antibody (1:15,000 diluted goat-anti-pig IgG HRP conjugate). After consecutive wash steps in TTBS, the blot was incubated in a 1:1 mixture of HRP luminal/enhancer solution and peroxide buffer (BioRad) for 5 min. Finally, the blot was visualized by a ChemiDoc XRS molecular imager (BioRad).

ELISA

The expression levels of M protein in transgenic corn calli were estimated by ELSIA using purified bacterial M protein as standards. The ELISA plate was coated with 100 µl diluted protein extracts in PBS overnight at 4 ºC. PBST buffer (PBS with 0.05% Tween-20) was used for all dilutions and washes. Antibodies were used at dilutions similar to those in the Western blotting protocol. Wells were then loaded with 100 µl 1:1 mixture of TMB

peroxidase substrate and peroxidase solution B (Bethyl Laboratories Inc., Montgomery, TX) and incubated for 20 min at room temperature. The reaction was terminated by adding 100 µl of 1 M phosphoric acid. The plate was read on a plate reader (BioTek, Winooski, VT) at 450 nm.

Corn callus processing

Fresh calli were kept at -70 ºC overnight and then lyophilized for 72 h in a freeze dyer (VirTis, Gardiner, NY). The lyophilized plant tissues were then ground into fine powder using a mortar and pestle. The dried callus powder was stored at -70 ºC prior to use.

Animal immunization

Eight-week-old female BALB/c mice were randomly divided into a test group and a control group with eight mice in each group. In the test group, each mouse was administered of 30 mg of dried transgenic corn callus powder containing about 2.6 µg of M protein suspended in 0.5 ml PBS plus 0.1 ml of Sigma adjuvant system® (Sigma-Aldrich, St. Louis, MO) by gavage. In the control group, each mouse received 30 mg of dried non-transgenic corn callus powder in the same solution by gavage. Prior to each immunization, all mice were fastened overnight. Four doses of callus powder were administered to mice on day 0, 15, 30, and 45. All animals were housed separately to facilitate collection of individual fecal pellets. Samples of feces and blood (180 µl by means of cheek pouch) were collected before the first dose (pre-immune) and two weeks after each immunization. All animal experiments were conducted according to the protocols approved by Virginia Tech Institutional Animal Care and Use Committee (IACUC).

Determination of M protein-specific serum IgG

ELISA plates were coated with 3μg/ml of *E. coli* produced M protein in PBS and incubated overnight at 4 ºC. After consecutive washes with PBST and blocking with 5% non-fat dry milk for 2 h at room temperature, 10-fold diluted serum samples were added and incubated for 2 h. After four washes, goat-anti-mouse IgG-Fc HRP conjugate (Bethyl Laboratories Inc., Montgomery, TX) with a dilution of 1:20,000 was added and incubated for 2 h. The wells were then washed four times with PBST, and 1:1 mixture of TMB peroxidase substrate and peroxidase solution B was added to each well (Bethyl Laboratories Inc., Montgomery, TX). After incubation for 20 min, 1 M phosphoric acid was added and the plates were read at 450 nm on a plate reader (BioTek, Winooski, VT).

Determination of M protein-specific fecal IgA

Fecal samples were prepared by adding 10 ml of fecal extraction buffer (PBST with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) per gram of fecal pellets. Suspensions were thoroughly homogenized with disposable pestles (Kimble, Vineland, NJ). After incubating for 30 min at 4 ºC, suspensions were centrifuged for 10 min at 17,000×g. The supernatant was collected and measured by Bradford assay for TSP concentration. In ELISA analysis of fecal IgA, diluted fecal extraction samples containing 50 μg of TSP were applied onto the plates and the secondary antibody used was 1:20,000 diluted goat-anti-mouse IgA HRP conjugate. All the other procedures were the same as described in determination of M protein-specific serum IgG above.

Virus Neutralization assay

For fecal samples, fecal extraction buffer was first exchanged for DMEM medium using a centrifugal device with a 10-kDa molecular weight cutoff (Millipore, Billerica, MA).

Fecal and serum samples were then two-fold serially diluted in DMEM supplemented with 2% fetal bovine serum. Diluted serum samples (started at 1:2 dilution) or fecal samples (started at 0.5 mg/ml TSP) were mixed with an equal volume of PRRSV (VR-2385) at a concentration of 2000 $TCID_{50}$/ml and incubated for 1 h at 37 ºC. The mixtures were then transferred to a 96-well cell culture plate coated with confluent MARC-145 cells and incubated for another hour at 37 ºC. 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 22 h at 37 ºC 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, Inc.), diluted 1:100 in PBST with 2% BSA was added to each well. After an 1-h incubation, the cells were washed four times with PBS and the number of fluorescent foci in each well was counted. Neutralization titers were expressed as the highest dilution that showed a 90% or greater reduction in the number of fluorescent foci.

In vitro re-stimulation of virus-specific IFN-γ and IL-10 production

For evaluation of cellular immunity, splenocytes of mice were isolated and assayed by ELISA to detect the production of IFN-γ and IL-10 after *in vitro* re-stimulation by the virus. Two weeks after the final immunization, all mice were sacrificed and the spleens were collected aseptically. Spleen cell suspension was prepared by gently dissociating the tissue across a metal cell dispersing screen (Bioworld, Atlanta, GA). After centrifugation at 1,500 rpm for 5 min, the cell pellets were re-suspend in 1 ml plain RPMI-1640 (Invitrogen, Carlsbad, CA) and 5 ml red blood cell lysis reagent (155 mM NH4Cl, 10 mM KHCO3, 0.1

mM EDTA, pH 7.4) and then incubated for 5 minutes at room temperature to lyse red blood cells. After centrifugation, the pellets were re-suspended in 5 ml of complete RPMI-1640. The splenocytes were counted and plated at 10^7 cells/ml with 2,000 $TCID_{50}$ PRRSV (inactivated by UV rays and a one-hour incubation at 56 ºC), or with 10 μg/ml ConA (Sigma-Aldrich, St. Louis, MO) as a positive control. After incubation for 72 h at 37 ºC in a humidified atmosphere of 5% CO_2, the supernatants were collected and the production of IFN-γ and IL-10 were detected with commercial ELISA kits (R&D Systems, Minneapolis, MN) following manufacturer's instructions.

Statistical analysis

Results of serological assays, fecal sample assays and production of cytokines were compared using analysis of variance (ANOVA). A P-value of less than 0.05 was considered significant.

Results

Transformation of corn and molecular characterization of transgenic plants

The PRRSV (VR-2385) M protein coding sequence (Genbank accession no. U03040.1) was optimized, artificially synthesized, and subcloned into a monocot expression vector pAHC25 by replacing its *gus* gene. M protein coding sequence was under the control of corn ubiquitin promoter, known to provide strong and constitutive transgene expression in corn. The resultant vector pAHCM (Fig. 1) was used for stable nuclear transformation of corn Hi II immature zygotic embryos by particle bombardment. After regeneration and selection in the presence of bialaphos (a natural herbicide), twenty-three callus lines were considered

putative transformants. Eleven of the putative transformants could propagate fast and were screened by PCR, using their genomic DNA and primers specific for M protein coding sequence. Nine out of these were genetically transformed, as evidenced by a 355 bp expected PCR product, which were also detected for the positive plasmid DNA controls (Fig. 2). No products were detected in the non-transgenic callus controls.

Transgenic plant protein extracts were then analyzed by Western-blot to estimate M protein production and verify its antigenicity. As shown in Fig. 3, six of the PCR-positive callus lines expressed M protein, indicated by a specific 18 kDa band. Callus line H had the highest content of M protein as quantified by ELISA (5.1 μg M per gram of fresh callus, corresponding to 86 μg M protein per gram lyophilized callus), so it was chosen for oral administration to mice.

Serum immune responses

The mice in the test group (n=8) or in the control group (n=8) were orally administered with lyophilized transgenic plant powder or lyophilized non-transgenic plant powder, respectively, at days 0 (prime), 15 (first boost), 30 (second boost) and 45 (third boost). Biological samples were collected one day before the first dose (day 0) and two weeks after each administration. Serum immune responses from immunized animals were monitored by antigen-specific ELISA and virus neutralization assay. M protein-specific serum IgG in mice of the test group was detected after the primary immunization, gradually increased, and peaked after the third boost (Fig. 4). The ELISA results indicated that antigen specific IgG responses could be elicited by delivery of M protein in transgenic plant cells via the oral route. To test whether the elicited antibodies had the ability to neutralize PRRSV infectivity, virus

neutralization assays were conducted (Table 1). In the test group, mice developed detectable serum neutralization antibodies after the first boost and the titers increased to 6.7±2.1 after the third boost. None of the mice in the control group developed serum neutralization antibodies. The results also confirmed the presence of neutralization eptitopes within PRRSV M protein.

Mucosal immune responses

Intestine mucosal immune responses of mice were investigated by ELISA analysis of antigen-specific IgA in fecal samples (Fig 5). M protein-specific IgA in fecal extracts of the test group was statistically significant (P=0.02) to that of the control group after the primary dose and further increased after each boost. Interestingly, the antibodies in fecal samples of the test group also revealed neutralization activity, which was barely detectable after the first boost and peaked after the third boost (Table 1). No neutralization activity was detected in fecal samples from the control group.

Cellular immune responses

Cellular immune responses were evaluated by re-stimulated production of virus-specific IFN-γ and IL-10 by splenocytes of vaccinated animals (Fig. 6). Two weeks after the final immunization, the mice were sacrificed and spleen cell suspensions were prepared and re-stimulated with PRRSV antigens. The production of IFN-γ and IL-10 was quantified by ELISA. The mice in the test group produced significant PRRSV-specific IFN-γ at a concentration of 73 pg/ml, while production of IL-10 was barely detectable in both groups and statistically non-distinguishable between each group. The results suggested that the oral PRRSV subunit vaccine could elicit cellular immune responses with a preference

production of cytokine IFN-γ.

Discussion

Virus-specific antibody responses represent the humoral arm of adaptive immunity triggered during infection or vaccination. In present study, we demonstrated that humoral immune responses in mice can be elicited by oral administration of plant cells expressing PRRSV M protein. Antigen-specific serum IgG and intestine IgA were induced as early as two weeks after the primary immunization and peaked after the third boost (Fig. 4 and 5). The generated serum IgG response by our plant-made vaccine was comparable to the DNA vaccines and recombinant mycobacteria expressing PRRSV antigens [52,55]. In addition, the induced antibodies had neutralization activity (Table 1). Neutralization antibodies may be one of the factors contributing to protection against PRRSV [12]. However, neutralizing antibodies appear only at later post-infection (PI) times (sp. at periods equal or higher than 4 weeks PI) and killed-virus vaccines could fail to elicit neutralizing antibodies [53,54]. Interestingly, this plant-made subunit vaccine could induce neutralizing antibodies and the titer was slightly higher than that of mice inoculated by recombinant *Mycobacterium bovis* BCG or recombinant modified vaccinia virus ankara expressing PRRSV M protein [55,56]. This indicates plant cells are an efficient delivery system for induction of humoral immunity. By confocal analysis, Delputte et al. suggests neutralization antibodies induced by PRRSV inoculation may block infection through both a reduction in virus attachment and virus internalization [57]. Considering M protein is involved in PRRSV attachment and internalization mediated by heparin sulfate and sialoadhesin on porcine macrophage [10], the

neutralization antibodies elicited by this M protein-based vaccine may also function by inhibiting PRRSV entry. Generation of neutralization antibodies in the intestine mucus of vaccinated animals make this plant-made oral vaccine even more attractive, since the mucosal neutralization antibodies may directly block the entry of the virus, the first step of a virus establishing infection, thus preventing or reducing systemic spread.

For complex viruses with a large number of serotype (e. g. adenoviruses), rapid mutation rates (e.g., HIV) or the ability to form latency (e. g. EBV, CMV, HSV, VZV and HIV), the role of strong cellular immune responses in controlling and containing infection becomes crucial [27]. For PRRSV, particularly, cellular immune responses represented by production of virus-specific IFN-γ are important in the control of infections. The frequency of PRRSV-specific IFN-γ secreting cells has been correlated with protection against reproductive failure in sows during outbreaks of PRRS in commercial herds [29] and the primary protection mechanism of a commercial modified-live PRRSV has been attributed to its induced cellular immunity characterized by production of virus-specific IFN-γ [4]. The inhibitory effect of IFN-γ on PRRSV replication by blocking viral RNA synthesis via a dsRNA inducible protein kinase has been revealed as one mechanism for its protective function against PRRSV [58, 59]. In contrast, increasing IL-10 production is a feature in PRRSV-infected pigs and is suggested as one of the strategies used by PRRSV to modulate host immune responses and thus establish persistent infection [60]. In our study, a significant re-stimulated production of PRRSV-specific IFN-γ rather than IL-10 was observed in splenocytes from mice vaccinated with transgenic corn (Fig. 6). This indicates that the plant-made PRRSV subunit vaccine may confer desirable cellular immunity to vaccinated

animals.

Oral vaccines have a unique advantage of inducing antigen-specific mucosal immunity, which is seldom elicited by vaccines administered by needles and syringes [61]. The main protective function of mucosal immunity is the production of secretory anti-pathogen IgA antibodies and the associated mucosal immunologic memory. Generally, the effector mechanisms of secretory IgA includes, (a) performing immune exclusion in cooperation with innate mucosal defense mechanisms, (b) inhibiting colonization or invasion by pathogens, (c) inactivating viruses inside of epithelial cells and carrying pathogens and their products back to the lumen, thus avoiding cytolytic damage to the epithelium [62]. Regrettably, in practice it has often proven to be rather difficult to stimulate strong secretory IgA responses by oral-mucosal administration of antigens, particularly when using soluble protein antigens [63]. Excitingly, recent studies demonstrate that a strong antigen-specific intestine mucosal IgA response can be induced by oral administration of plant cells expressing bacterial or viral antigens [39, 40, 61, 64, 65]. Our results further confirm that plant cells are efficient mucosal delivery vehicles by showing that the plant-made oral PRRSV subunit vaccine stimulates antigen-specific IgA immune response after the primary immunization and further enhances the IgA antibody response following the boosters. It is speculated that the rigid plant cell walls can provide “bioencapsulation” to the antigen thus enabling the antigen to survive the full repertoire of degrading conditions of gastrointestinal tract such as acidic pH, proteases, and bile acids and to reach the gut-associated lymphoid tissues (GALT) in an intact and immunogenic state [35,66].

Using transgenic corn plants to produce an oral vaccine without any protein

purification and with minimal processing may provide a cost-effective alternative to current vaccine production strategies. In addition, since no known human or animal pathogens are able to infect plants, the plant-made vaccines can minimize the concern with potential viral or prion contamination. In this paper we show that M protein of PRRSV was orally immunogenic when produced and delivered in plant tissues. Excitingly, this plant-made oral subunit vaccine induced both humoral as well as cellular arm of antigen-specific systemic immune responses and mucosal immune responses. These results indicate that plant cell is an efficient vehicle to express and oral delivery PRRSV antigens. The two-layered protective immunity elicited by this plant-made oral vaccine encourages further study of the protective efficacy in swine using corn seeds regenerated from the transgenic callus and could lead to a much demanded safe, economical, and effective vaccine against PRRSV.

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Table 1 Neutralization antibody (NA) titers of serum and fecal samples from mice orally administered with transgenic corn callus. The titers were expressed as the highest dilution of serum or fecal antibodies that showed a 90% or greater reduction in the number of fluorescent foci. Antibody titers correspond to average titers ± standard deviation. No neutralization antibody was detected in mice receiving non-transgenic corn callus (titers were 0±0 for assays at all time points).

	Serum NA titer	Fecal NA titer
Prime	0±0	0±0
First boost	2.3±0.8	1.0±1.1
Second boost	4.0±2.2	1.7±0.8
Third boost	6.7±2.1	3.7±0.8

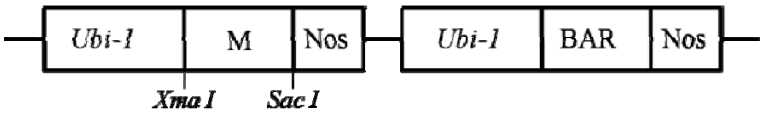

Fig. 1 Schematic diagram of plant transformation vector pAHCM. *Ubi-1*, corn ubiquitin promoter; M, PRRSV M protein; Nos, *nos* terminator; BAR, phosphinothricin acetyltransferase.

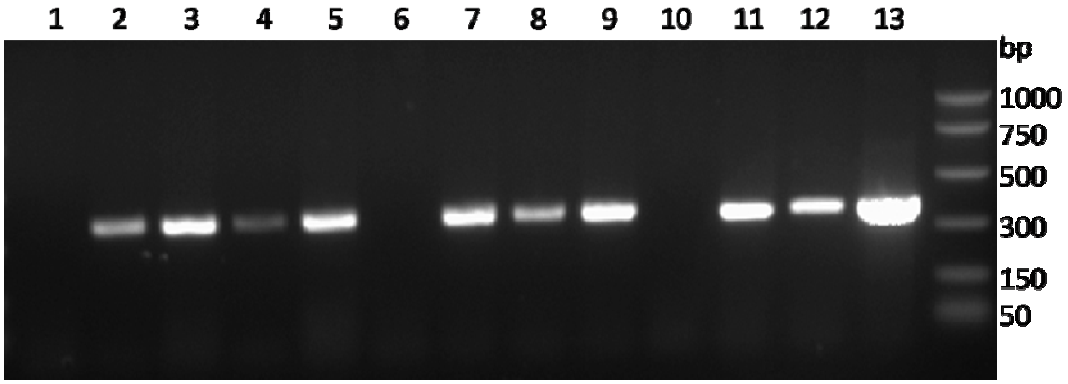

Fig. 2 PCR analysis of genomic DNA extracts from corn calli. 1, non-transformed corn callus; 2, callus line A; 3, callus line B; 4, callus line C; 5, callus line D; 6, callus line E; 7, callus line F; 8, callus line G; 9, callus line H; 10, callus line I; 11, callus line J; 12, callus line K; 13, plasmid pAHCM.

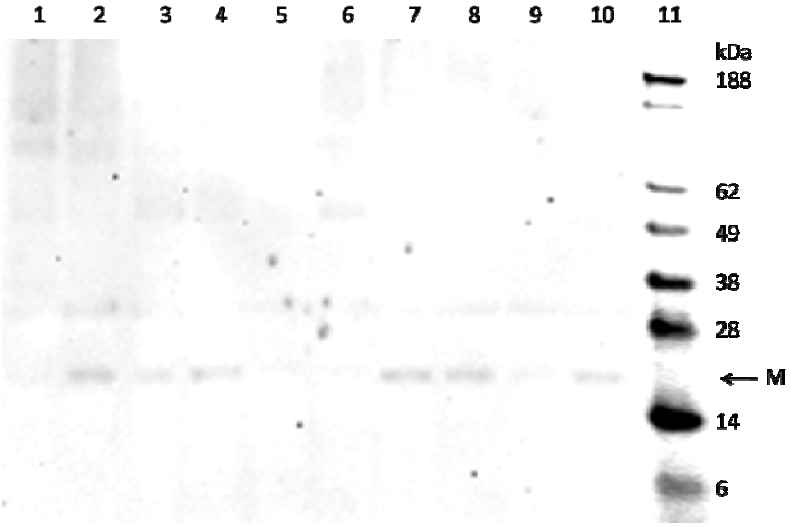

Fig. 3 Western blot analysis of M protein production in different transgenic corn callus lines. 1, non-transformed maize callus; 2, callus line A; 3, callus line B; 4, callus line C; 5, callus line D; 6, callus line F; 7, callus line G; 8, callus line H; 9, callus line J; 10, callus line K.

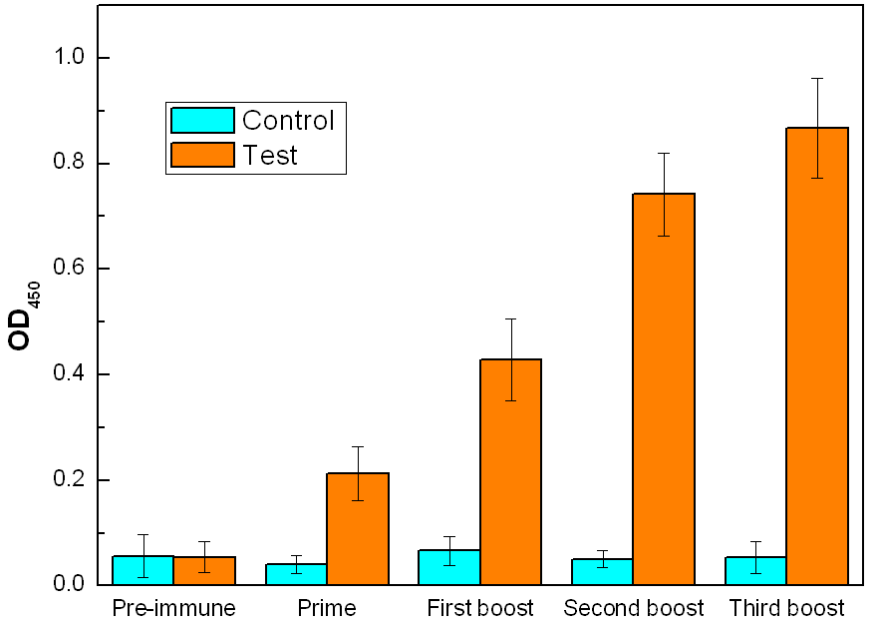

Fig. 4 Serum M protein-specific IgG antibody response of mice orally administered with transgenic (Test) or non-transgenic (Control) corn callus.

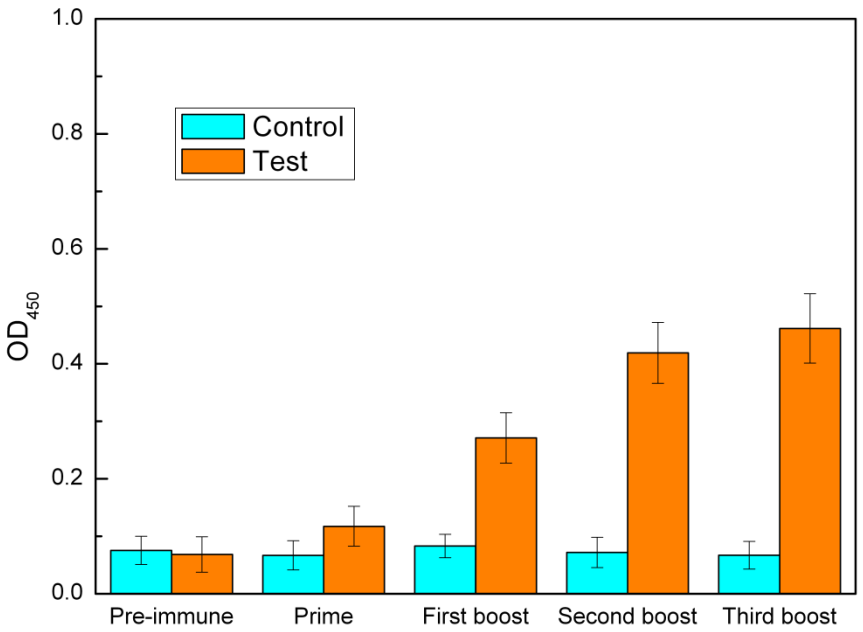

Fig. 5 Fecal M protein-specific IgA antibody response of mice orally administered with transgenic (Test) or non-transgenic (Control) corn callus.

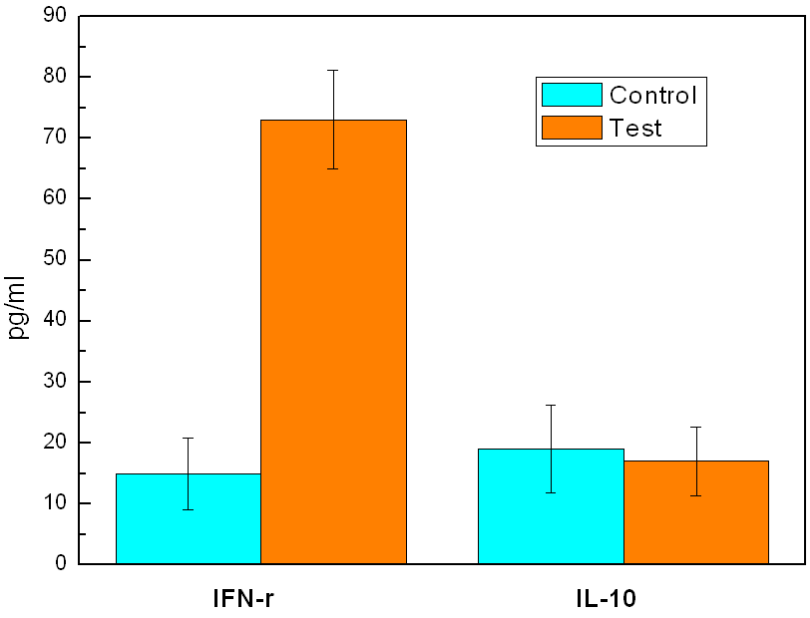

Fig. 6 Production of PRRSV-specific IFN-γ and IL-10 in splenocytes from mice orally administered with transgenic (Test) or non-transgenic (Control) corn callus.

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

J. Hu, Y. Ni, X. J. Meng, C. Zhang

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 / liter *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.

Key words: Porcine reproductive and respiratory syndrome virus, PRRSV, PRRS, vaccine, heparan sulfate, refolding, purification

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).

PRRSV is classified into *Arterviridae* family within the genus *Arterivirus*, order *Nidovirales*, along with equine arteritis virus (EAV), simian hemorrhagic fever virus (SHFV), and lactate dehydrogenase elevating virus (LDV) [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 six to nine ORFs downstream of the 1b gene [5]. ORF1a and ORF1b are translated into long nonstructural polyproteins, pp1a and pp1ab, which are co- or post-translationally cleaved into 14 functional nonstructural proteins (nsps) in a complex proteolytic cascade [6]. ORF2a, ORF2b, ORF3, and ORF4 encode for viral minor structural proteins GP2a, 2b protein, GP3, GP4. 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-stimulating 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 (aa1-31), 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 diminishment of the immune responsiveness against the adjacent neutralizing epitope [12].

M and GP5 form heterodimers on the 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 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 to 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. The pelleted cells were lysed using B-PER bacterial protein extraction kit following the manufacturer's protocol (Thermo Scientific, Rockford, IL, USA). The

suspension containing the soluble and insoluble cell components, the supernatant, and the insoluble fraction produced during cell lysis were collected and 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 ^{o}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 to 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 ^{o}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, 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, 1mM DTT, 30 mM imidazole, pH 7.9) at room temperature for 1h with frequent vortexing. After centrifugation at 17000×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 fast performance liquid chromatography (FPLC) system (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 3.5K (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 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 probed with 1:2,000 dilution of THE^{TM} 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 (BioRad, 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 ^{o}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, 1:2,000 dilution of THE^{TM} 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 ^{o}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 ºC in a humidified atmosphere of 5% CO_2, the plates were fixed with 80% acetone for 15 min at room temperature. After extensive washing 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

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).

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 of 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 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 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 of 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 second peak contains the target protein together with a few impurity proteins (Fig 4, lane 7).

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.

M/GP5 heterodimers 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 *in vitro* inhibit PRRSV infection was analyzed by virus blocking assay. Similar to the results of heparan sulfate binding assay, M/GP5-Ecto protein in the first AEX peak blocked 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 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 -M/GP5-Ecto protein and a few impurity proteins. Although 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, 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 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.

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 pharmacopoeias [17]. Malyala and Singh [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. 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.

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 purification/refolding process can produce approximately 12 mg M/GP5-Ecto protein from 1L 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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Table 1. Endotoxin levels of purified M/GP5-Ecto protein.

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

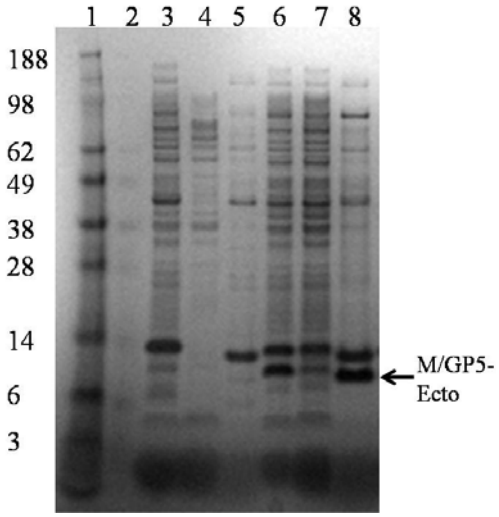

Fig. 1 Coomassie-blue-stained SDS-PAGE for localization of M/GP5-Ecto protein expression in *E. coli.* 1: marker; 2: 10×diluted marker; 3: Un-induced *E. coli* total protein; 4: Un-induced *E. coli* soluble protein; 5: Un-induced *E. coli* insoluble protein; 6: IPTG-induced *E. coli* total protein; 7: IPTG-induced *E. coli* soluble protein; 8: IPTG-induced *E. coli* insoluble protein.

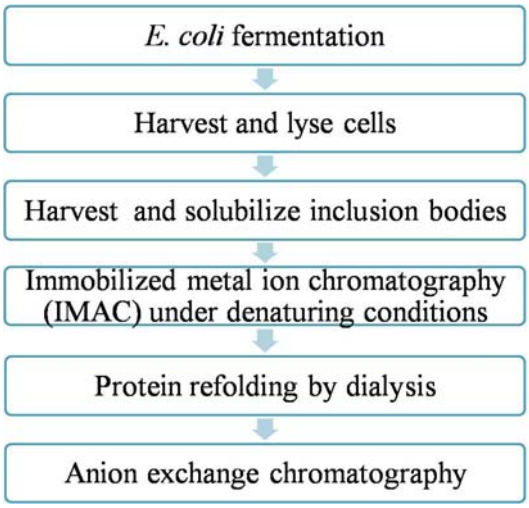

Fig. 2 M/GP5-Ecto protein purification/refolding process.

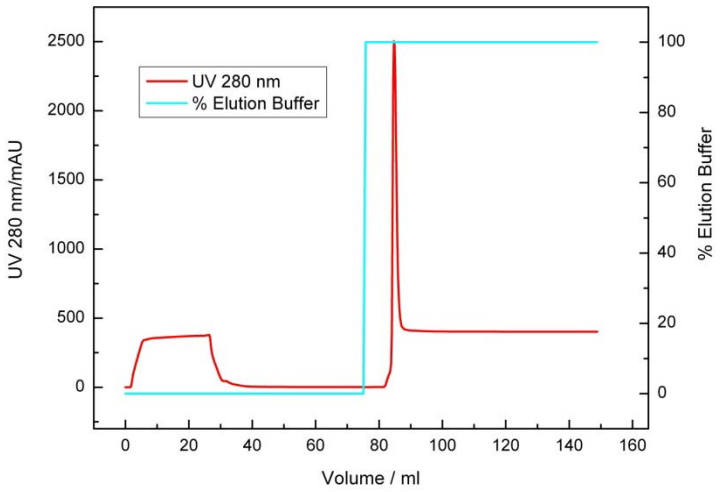

Fig. 3 Immobilized metal ion chromatogram of solubilized IB containing M/GP5-Ecto protein. Column: 5 ml of mL Ni Sepharose 6 fast flow resin packed in XK16/20 column; Binding buffer: 20 mM Tris-HCl, 6 M Urea, 0.5 M NaCl, 30 mM imidazole, pH 7.9; Elution buffer: 20 mM Tris-HCl, 6 M Urea, 0.5 M NaCl, 300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

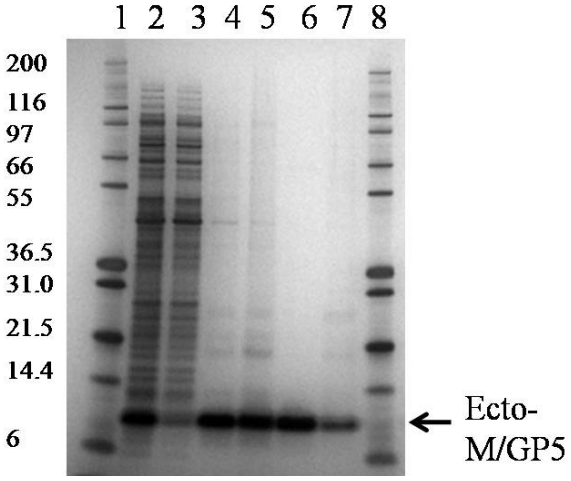

Fig. 4 Evaluation of M/GP5-Ecto protein purification/refolding process by silver-staining SDS-PAGE. 1: marker; 2: Solubilized inclusion bodies; 3: IMAC flow-through; 4: IMAC elution peak; 5: Refolded sample; 6: AEX peak 1; 7: AEX peak 2; 8: marker.

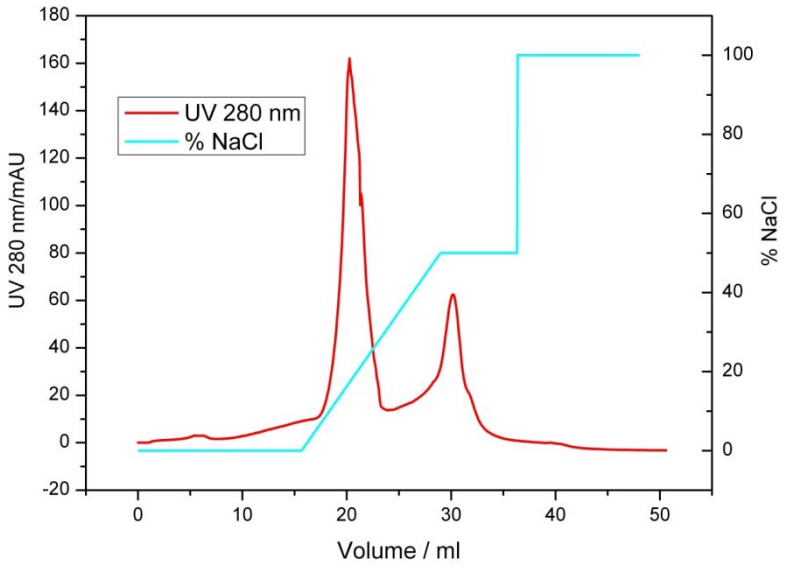

Fig. 5 Anion exchange chromatogram of refolded M/GP5-Ecto protein sample. Column: 1.5 ml of Q Sepharose fast flow resin packed in C column; Binding buffer: 20 mM sodium phosphate, pH 7.5; Elution buffer: 20 mM sodium phosphate, 1M NaCl, pH 7.5.

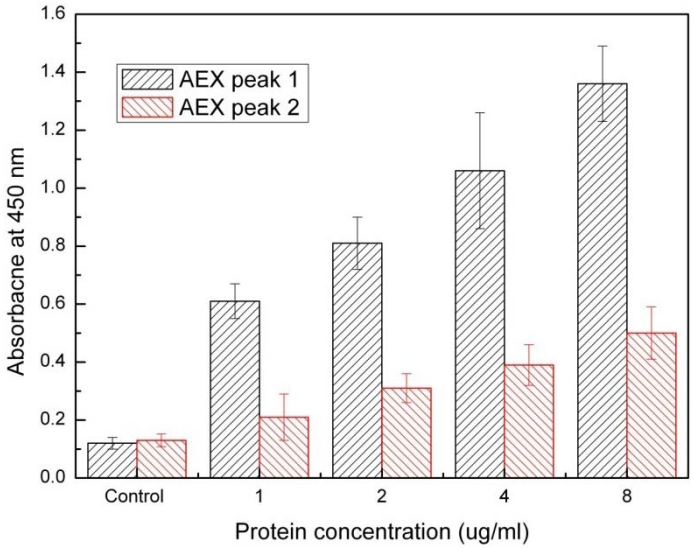

Fig. 6 Heparan binding assay of anion exchange chromatography (AEX) recovered protein.

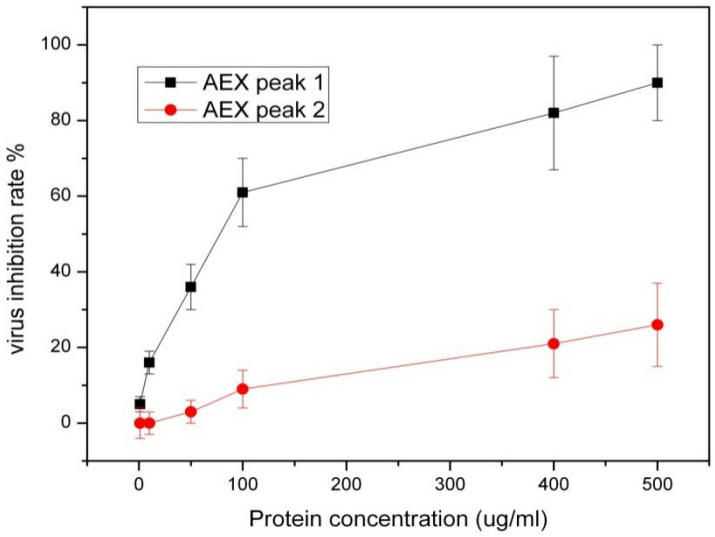

Fig. 7 Virus blocking assay of anion exchange chromatography (AEX) recovered protein.

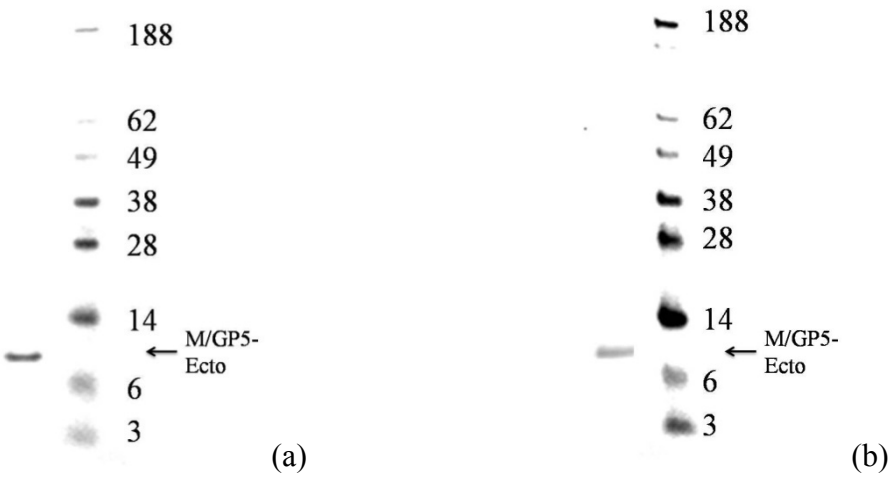

Fig. 8 Western-blot analysis of purified M/GP5-Ecto protein. (a) The primary antibody used was PRRSV antiserum; (b) The primary antibody used was anti-His-tag mAb.

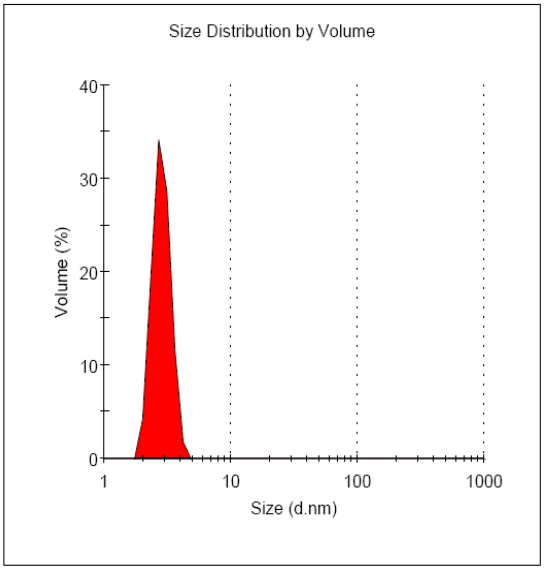

Fig. 9 Dynamic scattering analysis of purified M/GP5-Ecto protein.

Chapter VI: General Conclusions

Porcine reproductive and respiratory syndrome (PRRS) is the most significant infectious disease currently affecting the swine industry worldwide [1]. In the US alone, the economic losses caused by PRRS amount to more than 560 million US dollars every year [2]. Porcine reproductive and respiratory syndrome virus (PRRSV), an eveloped, single positive-strand RNA virus, is the causative agent of this disease. Although killed-virus vaccines (KLVs) and modified-live vaccines (MLVs) are available, the problems with the current vaccines including incomplete protection and reversion to pathogenic phenotypes encourage developing a new generation of PRRSV vaccines. In this dissertation, we used subunit vaccine strategy to develop novel PRRSV vaccines.

To facilitate PRRSV vaccine development, an easy operable and scalable process for purification of native PRRSV particles from infected cell culture was first developed. Conventionally, virus purification is carried out using density gradient ultracentrifugation, which is difficult to scale up and requires long process time. The process we developed mainly consisted of an ultrafiltration step and a heparin affinity chromatography step. The ultrafiltration step had multiple functions, including buffer exchange, sample concentration and partial purification. This step removed 24.5% of total proteins and recovered 82.3% of viral particles. The following heparin affinity chromatography purification step was inspired by the proposed specific interaction between PRRSV and heparan sulfate [3]. Approximately 53% of the virus in ultrafiltration retentate was captured in this step. This entire process removed more than 96% of the medium and cellular proteins and produced purified viral particles suitable for ELISA and cell-based assays used in vaccine development.

To explore economical solutions to produce PRRSV vaccines, we developed a plant-made oral subunit vaccine against PRRSV. Viral M protein was expressed in transgenic corn tissues at a level of 83 ng/mg dried corn tissues. The transgenic corn tissues were then directly fed to mice to evaluate the immunogenicity of the plant-produced antigen. Humoral immune responses in mice were elicited by oral administration of plant cells expressing PRRSV M protein. Antigen-specific serum IgG and intestine IgA were induced as early as two weeks after the primary immunization and peaked after the third boost. The generated serum IgG response by our plant-made vaccine was comparable to the DNA vaccines and recombinant mycobacteria expressing PRRSV antigens [4, 5]. More importantly, the induced antibodies had neutralization activity. Cellular immune responses were also induced, as demonstrated by a significant re-stimulated production of PRRSV-specific IFN-γ rather than IL-10 in splenocytes from mice vaccinated with transgenic corn tissues. Since neutralization antibodies and virus-specific IFN-γ are two key factors contributing to protection against PRRSV [6-8], the plant-made oral subunit vaccine showed promising vaccine efficacy. In addition, this oral vaccine can induce mucosal immune responses as evidenced by detection of intestine IgA antibodies in fecal extracts of vaccinated animals. Mucosal immune responses are the first defense line against epithelium-transmitted pathogen, which is, however, seldom induced by vaccines administered by syringes and needles. Generation of mucosal immune responses by administration of the vaccine makes plant-made oral subunit strategy even more attractive for development of novel PRRSV vaccines.

To identify a novel vaccine target, we expressed M/GP5-Ecto protein consisting of the ectodomains of M and GP5 protein of PRRSV in *E. coli* and developed a

purification/refolding process to produce biologically active M/GP5-Ecto protein from the inclusion bodies. The purification/refolding process produced approximately 12 mg M/GP5-Ecto protein from 1L fermentation broth with an overall yield of 38%. The purified M/GP5-Ecto protein had an endotoxin level far less than the regulatory requirement, was antigenic and homogenous. More importantly, the protein M/GP5-Ecto had expected bioactivities. It could bind to a cellular receptor for the virus, heparan sulfate, and could block virus infection of susceptible cells *in vitro*. Our findings further the understanding on biology of PRRSV, and this chimeric protein M/GP5-Ecto could be a valuable subunit vaccine for the prevention of PRRSV infection.

Reference

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