Purification of the major envelope protein GP5 of porcine reproductive and respiratory syndrome virus (PRRSV) from native virions

By

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Porcine reproductive and respiratory syndrome virus (PRRSV) is the cause of a pandemic that has been devastating the U.S. and global swine industry for more than twenty years. PRRSV vaccine development is challenging due to virus heterogeneity. Evidence indicates that the major envelope protein, GP5, is the primary target for a subunit vaccine. In native virions GP5 primarily exists as a disulfide linked complex with the membrane protein, M, which also possesses immunogenic properties. Recent studies report that the GP5/M complex is a more significant vaccine candidate. Currently, no bulk purification methods have been reported for PRRSV proteins. The objective of this research was to develop a purification process for GP5 or GP5/M from native virions.

PRRS virions were isolated and concentrated through sucrose cushion ultracentrifugation and target envelope proteins were solubilized with Triton X-100 detergent for further processing. GP5/M was not consistently identified in samples and was therefore abandoned. GP5 was identified by Western blot throughout processing with a αORF5 antibody. Cation exchange chromatography (CEX) was utilized for partial fractionation of GP5, although the viral nucleocapsid protein, N, was a major impurity in CEX elution fractions. As a second chromatographic step, hydrophobic interaction chromatography (HIC) further purified GP5 by means of a two-stage elution scheme. Pure GP5 was eluted from the HIC resin in the second HIC elution stage by Triton X-100 displacement; however the protein is present as a homodimeric/tetrameric aggregate. This process will be useful in PRRSV vaccine development and the purified GP5 product could be used as much needed positive controls in animal studies.

Dedication

This thesis is dedicated to the memory of the 32 Virginia Tech students and faculty lost on April 16, 2007. To the faculty members: your contributions to Virginia Tech are endless and I thank you for making this university what it is today. And to the students: your passion for learning and impact in this world were only beginning to shine. May this work be a testament that you would have done more great things in life, simply because you were Hokies.

Attribution

Brad M. Matanin is the major contributor and writer of the manuscript in Chapter Three of this thesis. Co-author Dr. Chenming Zhang, Ph.D., Chemical Engineering, Iowa State University, Iowa 1999, Committee Chair provided advice, supervision, funding, and laboratory support. Co-author Dr. X.J. Meng, Ph.D., Immunobiology, Iowa State University, Iowa 1995, Committee Member provided advice, funding, and laboratory support.

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Chapter One

Introduction and Objectives

Porcine reproductive and respiratory syndrome (PRRS) is an economically important disease prevalent in swine producing countries around the world. It is estimated that annual PRRS costs amount to half a billion dollars in the United States alone [4]. Attempts to develop efficacious vaccines against the causative agent of PRRS, porcine reproductive and respiratory syndrome virus (PRRSV), have been met with challenges due to virus heterogeneity.

Previous studies have established that both GP5 and M proteins of PRRSV are associated with eliciting neutralizing antibodies, cellular immunity, and humoral immunity independently [1, 5-7]. Additionally, more recent studies have demonstrated enhanced cellular and humoral immune responses from vaccination with a recombinant GP5/M heterodimer antigen [2, 3].

GP5 and M form a disulfide-linked complex in native virions. Because of the potentials of GP5/M protein, individually or in complex form, in vaccine development, developing a process to purify these two proteins from native virions may have an impact in vaccine development. The purified proteins can also be used as much needed positive controls in animal studies for vaccine research.

The overall goal of this research was to develop a method for the isolation of native GP5 or GP5/M protein complex of porcine reproductive and respiratory syndrome virus from fractionated virions. The following objectives were pursued to achieve the aforementioned goal: (1) generate PRRSV in mammalian cell culture, (2) confirm the presence of target proteins in sample preparations, and (3) engineer a process for isolation of the target proteins. The specific questions to be answered are:

1. Is it possible to generate enough PRRS virions for native protein purification using established PRRSV culture protocol and laboratory resources?
2. Will target proteins retain biological activity during protein processing?
3. What are the effects of detergents on PRRSV proteins, specifically those associated with the viral lipid bi-layer?

4. What detergents are suitable for chromatography and what effect, if any, will detergents have on target protein purification?
5. Can the GP5/M disulphide linked complex be isolated and, if not, can GP5 and/or M be isolated using a general process?

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Chapter Two

Literature Review

2.1 Porcine Reproductive and Respiratory Syndrome

Porcine respiratory and reproductive syndrome (PRRS) is a multi-symptom disease characterized best by severe reproductive failure in sows and gilts and respiratory challenges from non-specific lymphomononuclear interstitial pneumonitis [8]. Both manifestations of the disease result in considerable economic loss in every swine-producing country around the world. Economic repercussions of the disease have been well documented in recent years. Studies have indicated increased mortality (1.9 to 10.2 %), reduced average daily gain (0.38 to 0.26 kg), increased treatment cost per pig (US$ 1.77 to 1.91), and reduced feed efficiency (1.77 to 1.91 kg feed per kg gain) as indicators of economic loss on an average large farm in North America [9]. Collectively these costs amount to approximately $560.32 million in losses to U.S. swine producers each year [31]. The infectious cause of PRRS is a positive-strand RNA virus known as porcine reproductive and respiratory syndrome virus (PRRSV) [8].

2.2 PRRSV

PRRSV is of the family *Arteriviridae* along with equine arteritis virus (EAV), simian hemorrhagic fever virus (SHFV), and lactate dehydrogenase elevating virus (LDV) [8, 11]. Evidence suggests that PRRSV was first discovered in U.S. domestic swine population in the late 1970s [31]. The first significant outbreak of the disease was documented in North Carolina in 1987 [18]. Since then, multiple strains have been identified on different continents that are each antigenically and genetically heterogeneous [27].

Mature PRRS virions consist of a positive single-strand RNA genome surrounded by the nucleocapsid protein; this core structure is enclosed in a lipid envelope [8, 11]. The viral genome contains eight open reading frames (ORFs) [8]. ORFs 1a and 1b encode proteins with replicase and polymerase activities. ORFs 2-7 of the PRRSV genome encode for six structural proteins. The major structural proteins of PRRSV are the 25

kDa glycoprotein (GP5), the 19 kDa membrane protein (M), and the 15 kDa nucleocapsid (N) protein [8]. The expression products of ORFs 2 and 4 are minor envelope glycoproteins (GP2 and GP4); the product of ORF 3 is a highly glycosylated nonstructural minor protein [8]. In virions and/or lysates of virus-infected cells, however, only the three major structural proteins can be consistently identified due to their relative abundance [16]. Figure 2.1 provides a schematic illustration of PRRSV.

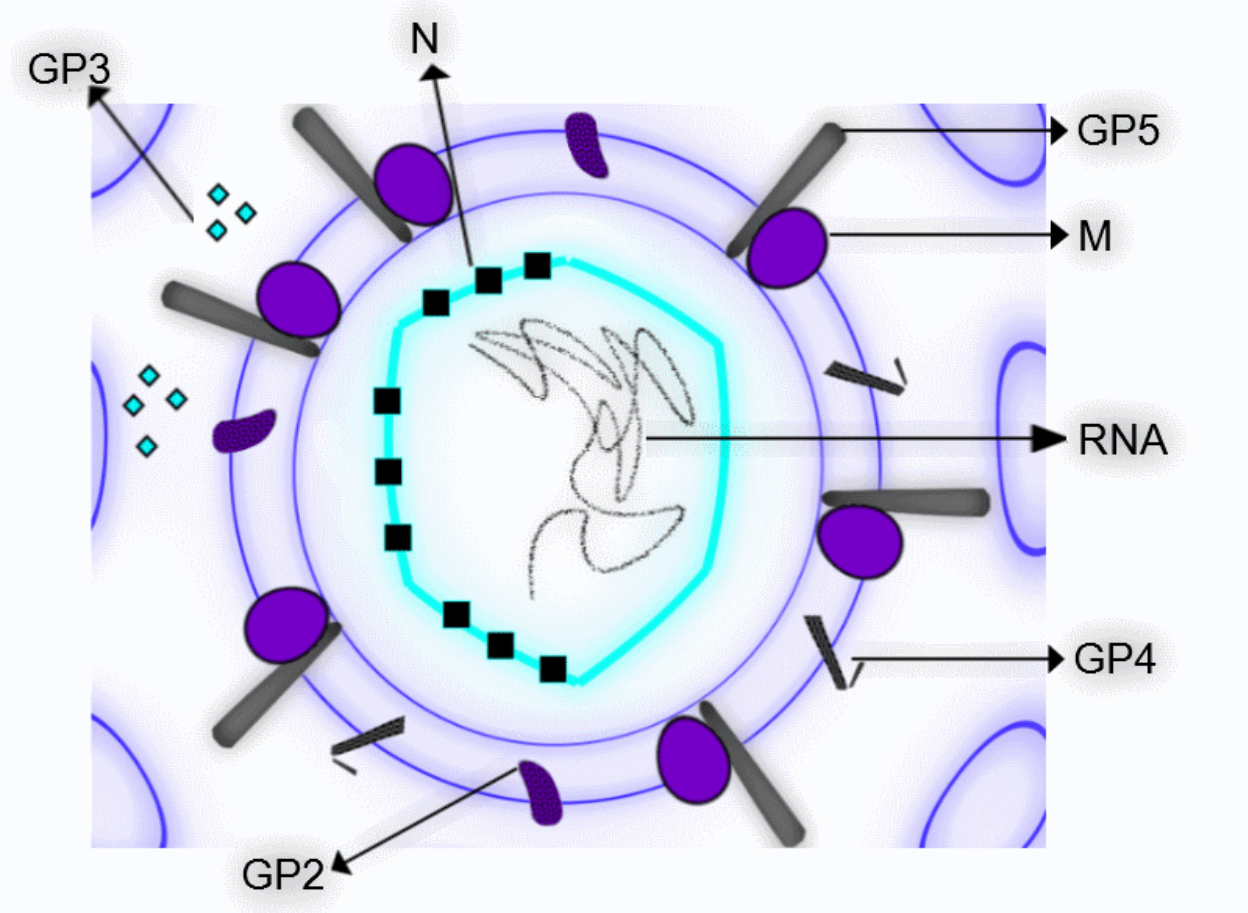

Figure 2.1 Schematic illustration of porcine reproductive and respiratory syndrome virus (PRRSV). GP5, M, GP4, and GP2 are lipid envelope associated as illustrated above.

Through multiple studies since discovery, much is known about the three major structural proteins of PRRSV. The N protein is highly basic and amounts to 20-40% of the protein content of the virion [8]. It is not N-glycosylated, although it contains potential N-glycosylation sites, and is exceedingly antigenic [28]. The M protein is also

non N-glycosylated. The M protein has three highly hydrophobic membrane spanning regions; it is almost entirely embedded within the lipid bi-layer of the virus. It may be the most abundantly expressed PRRSV protein on the basis of Western blot profiles probed with porcine convalescent antiserum [35]. In native virions and infected cells, the M protein forms a heterodimeric complex linked by disulfide bonds with the major structural glycoprotein, GP5. GP5 is closely associated with M and also possesses hydrophobic membrane spanning domains.

GP5 is the most heterogeneous structural protein of PRRSV; nucleotide sequencing discovered 88 to 99% amino acid identity among North American strains and 51-54% between North American and European strains [1, 8, 37]. The impetus behind GP5 variability is a hypervariable region near the N-terminal (aa 26 to 39), which can include between zero and three glycosylation sites. Positions 44 and 51, outside the variable region, represent glycosylation sites common to all North American strains [37]. Hence, the hypervariable region accounts for heterogenic glycosylation of GP5, which ranges between 2 and 5 N-linked glycosylation sites. This may explain the differences in GP5 molecular weight as each N-linked carbohydrate side chain adds approximately 2.6 kDa. Molecular weight heterogeneity is manifest on a Western blot as a diffuse band ranging in weight from 26-29 kDa [24].

Biological variations among PRRSV isolates translate to important antigenic variations. Studies have reported that antigenically, European isolates resemble each other, yet do not resemble U.S. isolates [45]. Serologic survey of field samples of multiple European and U.S. PRRSV isolates by immunoflourescence assay failed to identify positive for PRRSV in all cases, supporting that each can induce a unique antigenic immune response [27]. Pirzadeh et al. [37] reported differential reactivity of monoclonal antibodies between PRRSV strains; five monoclonal antibodies against GP5 of a Canadian isolate did not react with the European prototype strain. In another study, 3 U.S. isolates were found to differ antigenically from each other [30]. The extent of antigenic diversity among PRRSV strains is a hindrance to vaccine development; a vaccine based off of one strain will not protect against an antigenically different strain.

2.3 GP5 and M of PRRSV

In vivo, PRRSV infects macrophages of porcine heart, tonsil, and spleen tissues among others [8]. Propagation of US and European PRRSV isolates *ex vivo* has been reported in isolated porcine alveolar macrophages (PAMs) and blood monocytes [8, 26]. Moreover, two permissive monkey kidney cell line subclones, MARC-145 and CL2621, have been adopted as the primary means of *ex vivo* propagation of PRRSV in non-porcine cell cultures [8, 26]. PRRSV replicates in the cytoplasm of subjected cells once infected. Virions accumulate in the endoplasmic reticulum (ER) and/or the Golgi apparatus [8]. Extracellular virions are released through exocytosis [8].

GP5 and M form a disulphide linked heterodimeric complex in PRRSV virions. Evidence indicates oligomerization occurs directly after protein synthesis and the GP5/M complex is highly stable [24]. This is considered the start of virus assembly. PRRSV proteins from raw virions can be resolved on an SDS-PAGE gel and transferred to a nitrocellulose membrane under reducing or non-reducing conditions. Under reducing conditions, a membrane probed with antisera raised against GP5 will resolve a diffused band at 26 kDa; probed with antisera raised against M will resolve a more distinct band at 19 kDa. The GP5 band is diffused due to size variation as a result of heterogenic N-linked glycosylation [8, 22]. Under non-reducing conditions the disulphide link between GP5 and M is maintained. Either antisera will resolve a band at 40 kDa and 87 kDa: the GP5/M complex and possibly a complex dimer, respectively. No monomer bands were resolved under non-reduced conditions in this study. Similarly, as analyzed by SDS-PAGE, antiserums against either GP5 or M will immunoprecipitate both proteins from purified virions [24].

The presence or absence of N-linked oligosaccharides on the major structural PRRSV proteins (M, N, and GP5) has been established through endoglycosidase screening of proteins isolated from virions cultured in MARC-145 cells [24]. Each protein was digested with endo H and glyco F endoglycosidases to determine the presence and type of N-linked oligosaccharide. Endo H cleaves high-mannose oligosaccharides while glyco F cleaves all N-linked oligosaccharides and is not specific to one type of glycan [24]. Whether or not a protein is digested by a specific endoglycosidase can reveal its oligosaccharide profile. GP5 responds to both endo H and glyco F. When analyzed by Western Blot, GP5 is altered to a single species after glyco F digestion. Glyco F

treatment of GP5 reduces its molecular weight by 8 kDa on a Western blot. Assuming 2.6 kDa per carbohydrate side chain, this confirms that the three potential N-glycosylation sites on GP5 do host some type of N-linked glycan. Endo H partially digests GP5. Western blot of GP5 treated with endo H reveals an endo H resistant and an endo H sensitive species. Endo H sensitive species have high-mannose type oligosaccharides. The partial digestion of GP5 with endo H treatment suggests it is heterogeneously glycosylated. Therefore some GP5 molecules contain high mannose and complex type oligosaccharide residues while others have only complex N-linked oligosaccharide residues occupying the potential glycosylation sites. Heterogeneously glycosylated GP5 is more abundant than homogeneously glycosylated GP5. On the other hand, M and N proteins are not sensitive to endoglycosidase screening, indicating that they are not glycosylated [24].

Addition of high mannose N-linked oligosaccharides to GP5 occurs in the rough ER and further modification to complex type proceeds in the three regions of the Golgi Complex. Endo H resistant GP5 molecules are completely processed by subsequent reactions in the three differential cisternae, or processing regions, of the Golgi to contain only complex residues. Endo H sensitive GP5 molecules have both high-mannose and complex residues. High-mannose oligosaccharides most likely remain as a result of slight variations in protein folding which make regions of the oligosaccharide(s) unavailable for ensuing reactions in the Golgi pathway [21, 24]. The virus is released by exocytosis after GP5 is processed to its mature structure.

Furthermore, evidence strongly suggests that GP5/M oligomerization and glycosylation processing occur in series but are independent to each other. GP5/M heterodimers form first then GP5 is glycosylated. Hence, GP5 and M are linked independent of the heterogenic nature of GP5 glycosylation [24].

2.4 GP5 and M in PRRSV vaccine development

Vaccine development against PRRSV has been widely documented over the past decade [27]. In designing subunit vaccines against PRRSV, developing cellular and humoral immune response to viral polypeptides is of primary importance because both mechanisms of immunity are linked to protection against the virus [2, 8].

During infection, pigs primarily produce antibodies specific to the N protein [8]. Although the N protein is the most abundant structural protein and is highly antigenic, the correlation of anti-N specific antibodies with protective immune response is insignificant [2]. Because of this, the N protein is targeted for diagnosis of PRRS, not a subunit vaccine. Conversely, GP5 and M are more suitable vaccine candidates. M has been identified as the major PRRSV protein recognized by porcine T cells. Activation of antigen-specific T-lymphocytes enables the cellular immune system to destroy virus-infected cells. Hence, cell-mediated immunity (CMI) is central in protection against viral diseases [8]. Furthermore, GP5 elicits neutralizing antibodies in pigs, a critical aspect of humoral immunity [2, 36, 42].

Various research groups have developed experimental vaccines using recombinant adenovirus [12], recombininant baculovirus [20], and direct DNA immunization [36] to express GP5 to challenge the pig immune system. In general, neutralizing antibodies are weak or too slow to reach protective titers. In one study, DNA vaccination with ORF5 did reduce severity of interstitial pneumonitis in pigs but was insufficient to inhibit virus persistence [36]. PRRSV genetic heterogeneity throughout North America and the world, particularly in terms of GP5 variability, further limits the usefulness of these vaccines [27].

More recently, interesting results were obtained on the advantages of co-vaccination of GP5 and M proteins of PRRSV [16, 17]. Three different DNA vaccine constructs were created and subsequently administered to express GP5 alone, M alone, or GP5/M in vitro [17]. Results confirmed that co-expressed GP5 and M indeed formed heterodimeric complexes in transfected cells and induced significantly higher levels of neutralizing antibodies in mice and piglets [17]. Piglets co-immunized with ORF5/ORF6 DNA constructs had significant cellular and humoral immune responses after eight weeks [17]. Moreover, the results of this study suggest that the formation of GP5/M heterodimers could have a role in post-translational modification of GP5. GP5 antibody was better detected in ORF5/ORF6 co-immunized mice than mice immunized with ORF5 DNA constructs alone [17]. A similar study found that adenovirus expressing recombinant GP5/M fusion protein of PRRSV could elicit humoral and cell-mediated immune responses in mice [16]. Mice immunized with the recombinant GP5/M fusion developed

significantly higher titers (approximately 50-fold) of neutralizing antibodies 28 to 56 days post immunization than those immunized with recombinant adenovirus expressed GP5 or M alone [16].

Exploiting the natural configuration of GP5 and M for a vaccine is a viable course toward protection within the genetically diverse breadth of PRRSV. One thing that remains consistent among the varying strains and the continents is that GP5 and M proteins form a heterodimeric complex linked by disulphide bonds. GP5/M complex is significant in the virus life cycle [17, 24]. In the previously discussed study, Jiang et al. [16, 17] postulated possible explanations for enhanced neutralizing antibody response through the DNA vaccine co-expressing GP5 and M. They indicated that the process of GP5/M dimerization may better authenticate GP5 post-translational modification and conformational maturation by ensuring GP5 transports from the ER to the Golgi complex, a process necessary for N-glycan processing [8, 17]. Additionally, the authors reported that the M protein could provide a foundation on which GP5 forms conformation-dependent epitopes that are better situated to interact with T-cells to induce immune response [17]. Regardless of the reason, it is clear that the importance of the GP5/M heterodimer is significant and possibly universal among different strains. Further studies are needed to understand the role of GP5/M heterodimers in protective immune response among antigenically diverse strains and to develop a new generation of vaccines.

2.5 Propagation of PRRSV in mammalian cells

Synthesis and processing of GP5/M by native virions propagated in a permissive cell line can yield possible positive controls for vaccine investigation and model proteins for process development. It is well established that PRRSV replicates well in PAM cells; many other possible cell lines have also been screened [8, 23, 24, 26]. In one study, cell lines from monkey kidney, dog kidney, baby hamster kidney, porcine kidney, porcine fallopian tube, and swine testicle were screened for passage of Canadian PRRSV isolates [23]. None of the cells were permissive to PRRSV. Another study attempted propagation of US isolate VR-2385 in 24 different primary and continuous cell lines [26]. Among the cells screened, only PAM and cell line CRL 11171 were permissive to this

isolate [26]. Through further studies, two derivatives of the MA104 monkey kidney cell line have emerged as the primary hosts for PRRSV propagation *ex vivo*. MARC-145 and CL2621 are both highly permissive and well documented for propagation of North American isolates [8].

MARC-145 cells are cultivated in a common fetal bovine serum supplemented medium. Cells grow to confluent monolayers, at which point they are briefly washed then inoculated with PRRSV. PRRSV recognizes cell surface receptors via its viral attachment proteins, GP5 and M, and penetrates the cell [46]. Virus dissemination lasts 2-4 days post infection or until 90% of the cells show visible signs of cytopathic effect (CPE), commonly characterized by change in cell morphology, cell clumping, cell lysis, and cell detachment from the monolayer [8].

2.6 Isolation of PRRS virions

Isolation of PRRSV virions from solution is possible with isopycnic ultracentrifugation. Under a constant gravitational field biological molecules migrate according to their density, the speed of the centrifuge, and the density of the displaced fluid. Fluid density varies at intense gravitational force (100,000 × g) resulting in a density gradient. Within a density gradient separation occurs such that target biological molecules migrate to the layer of equal density. Studies have established the buoyant density of PRRS virions under isopycnic conditions in both cesium chloride and sucrose solutions [3, 8].

PRRS virion isolation from cell culture using a cesium chloride density gradient was developed to harvest viral RNA for PRRSV nucleotide sequencing [25]. PRRSV strain VR-2385 was propagated in CRL 11171 cells. The cell culture was harvested, and clarified virus suspension was loaded onto a solution of cesium chloride. PRRS virions were collected after overnight ultracentrifugation from an opalescent band equilibrated at 1.15 to 1.18 g/mL cesium chloride density [25]. Isopycnic ultracentrifugation with continuous sucrose gradients was used in another study to prepare concentrated and purified virions for Western immunoblot detection, immunoprecipitation, and pulse chase experiments [24]. The reported buoyant density of PRRS virions in sucrose is 1.13-1.15 g/mL [8].

Ultracentrifugation through a density cushion is another virion isolation technique similar to the isopycnic gradient method. Soluble molecules remain suspended in the supernatant while the cushion, a layer of dense fluid, acts as a filter for small insoluble waste fragments. Larger whole virions penetrate through the cushion and are pelleted on the bottom of the tube. This method was previously applied to concentrate and purify extracellular PRRS virions for Western immunoblot identification of viral proteins GP3, GP5, M, and N [14].

2.7 Detergent solubilization of membrane bound proteins

Significant to the shape and structure of PRRSV is the lipid bilayer, or the envelope, surrounding the nucleocapsid. Four proteins are closely associated with the envelope: M, GP5, GP2, and GP4 [8]. Once PRRSV is isolated, any method to selectively purify a membrane related protein must start with solubilization of that protein in an aqueous environment. There are several methods for protein solubilization. Amphiphillic detergents are the most widely used solubilization agents for protein purification and they also prevent aggregation. Solubilization properties vary by detergent, target molecule, and experimental conditions. Detergents are not the exclusive means of solubilization; organic solvents also play a role, however are usually avoided due to protein denaturing effects [10]. Several studies explain the effects of various detergents on viral membrane proteins with properties similar to PRRSV [6, 10, 15, 32, 34, 43]. Selecting the proper detergent is critical and the researcher must consider desired protein yield and functionality, and necessary downstream operations in process design.

Detergent selection according to the above criteria begins with careful consideration of their physical properties. Detergents are either ionic or non-ionic. In general, ionic detergents are more effective solubilization agents and usually dissociate protein complexes, but they may denature and compromise protein activity. Non-ionic detergents are less effective at dissociating protein complexes but provide a more stable environment for proteins. Consequently there exist two proportional tradeoffs (1) between separation or aggregation of proteins, and (2) between conservation of native conformation or partial denaturation [43].

The structure of a detergent molecule is characterized by a hydrophilic head group and a hydrophobic tail group which drives molecules into water soluble detergent aggregates called micelles. The hydrophobic tail, a hydrocarbon chain, of a detergent molecule groups with the tails of other detergent molecules, away from the aqueous environment. The hydrophilic heads are oriented on the outside of the micelle. Proteins interact with detergent micelles via hydrophobic interactions with the tail or hydrogen bonding or ion paring with the head. Micelle structure and ultimately protein-micelle interaction are dependent on hydrocarbon tail length, head surface area, and experimental conditions (pH, buffer ionic strength, temperature, and pressure) [10].

Protein-micelle chemistry should not affect the protein-resin interaction in chromatographic methods; the protein functional group involved in adsorption must remain accessible in the protein-micelle complex. In general, a suitable detergent is one that will not hinder protein-resin interaction. Micelle structure may determine whether a detergent/protein complex will interfere with chromatography resin chemistry by inducing non-specific interactions or preventing adsorption. For example electrical properties apparent with charged ionic detergent molecules may interfere with ion-exchange separation. Large micelles can be expected to interfere with size exclusion chromatography. Detergents with aromatic groups will absorb light at 280 nm, a spectral property that should be avoided when monitoring a chromatographic column is necessary [10].

Besides constraints apparent with chromatography, detergents may create restrictions with other operations in a separation process. Buffer components should be detergent compatible as well. For example carboxylic acid is a titratable functional group contained on bile acid type detergents. Acidic buffer conditions will cause these detergents to precipitate from solution, significantly increasing solution viscosity [10]. Hence pumping and filtration are rendered useless. The critical micelle concentration (*cmc*), the concentration at which micelles begin to form, establishes the relative ease of detergent removal. Low *cmc* detergents are easier to remove than high *cmc* detergents and are favored when detergent removal is necessary.

Choosing a detergent is more of an art than a science. No detergent is considered superior for solubilizing a broad range of proteins and for use with a broad range of

processes. Several detergents, however, are considered good starting points for virion solubilization screening and process development. Triton X-100, octylglucoside, CHAPS, sarkosyl, MEGA-9, and Brij 35 are effective at solubilizing viral membrane proteins for subsequent processing [7, 13, 34, 38, 41, 43, 44].

Amongst the many constraints associated with detergent use, many detergents have been successfully applied for viral protein purification. The effect of detergents on Sendai virus membrane protein structure and activity gives insight into detergent selection for protein solubilization from other lipid bilayer enveloped viruses similar to PRRSV [43]. Both PRRSV and Sendai viral membranes contain at least one major structural glycoprotein known to form a disulphide linked complex with another membrane spanning protein. Detergent effect was demonstrated on Sendai virions isolated by ultracentrifugation and disrupted by incubation with Triton-X 100 detergent. Triton-X 100 is a nonionic detergent with good solubilization properties that is known to interfere with chromatography column monitoring. For this reason it was used as a control solubilization agent then removed by a polyaromatic adsorbent. The separation and denaturation tradeoffs of various detergent additives were analyzed with size-exclusion HPLC along with biological activity assays. Non-aggregating and functionally active Sendai virus proteins could be eluted from the column with N-lauryl sarcosinate, an ionic detergent commonly called sarkosyl. Therefore this detergent best compromises the tradeoff discussed earlier. Nonionic octylglucoside did not prevent protein aggregation but maintained full protein functionality. Anionic sodium dodecyl sulphate (SDS) prevented aggregation but compromised immunological activity of one of the target proteins [43].

There is no single detergent that is guaranteed to be successful for any particular process. Trial and error by screening selected detergents at varying concentrations, buffer conditions, and incubation times, is the best way to select a detergent appropriate for a specific application.

2.8 Properties of PRRSV proteins

After virion isolation and detergent solubilization, further fractionation of PRRSV proteins is possible by exploiting differences in protein properties. Several physical

properties of PRRSV proteins are available to provide a reasonable starting point for process design. Table 2.1 identifies the molecular weight, glycosylation, and theoretical pI of the six PRRSV proteins (North American strains).

Table 2.1 Protein characteristics of PRRSV North American strains[a].

Coding area gene product	Protein	No. of aa residues	Predicted M_r (kDa)	Apparent M_r (kDa)	Glycosylation sites	Theoretical pI[b]
ORF2	GP2	265	29.4	27-29	2	9.94
ORF3	GP3	254	29.0	42-45	7	8.50
ORF4	GP4	178	19.6	31-35	4	8.45
ORF5	GP5	200	22.4	24-26	2-5	8.79
ORF6	M	174	19.1	19	1	9.83
ORF7	N	123	13.6	14-15	1	10.09

[a] Data was taken from [8].

[b] Data from Expassy Calculator (http://expassy.org) using nucleotide sequences as reported by [25, 29].

All PRRSV proteins are considered basic proteins based upon their theoretical pI, as determined by computer estimation based on their primary sequences. Of the three major structural proteins GP5, M, and N, only GP5 is glycosylated. GP5 contains both high-mannose and complex type N-linked glycans. M is linked via disulphide bonds with GP5 to form a 43 kDa heterodimer and evidence suggests the formation of an 87 kDa GP5/M complex dimer is possible [24].

Although the abundance of minor structural proteins in virion preparations is low such that SDS-PAGE identification is not possible, they should still be considered in process development. The minor PRRSV proteins GP2, GP3, and GP4 are all glycosylated. GP2 does not form homodimers nor does it link with any other viral protein [8]. GP2 and GP4 contain only complex N-linked glycans. GP3 is highly glycosylated, as evident by the difference between its predicted and apparent molecular weight. GP3 is weakly associated with the virus and believed to be restricted to the ER during synthesis. Hence GP3 is not detectable in purified virions because it is not released and not packaged as part of the virion; GP2 and GP4 are the only minor structural proteins embedded in the viral envelope [8].

2.9 Chromatographic techniques for detergent solubilized proteins

Certain chromatographic techniques have been successfully applied for the fractionation of detergent solubilized membrane proteins. Membrane bound proteins have the tendency to aggregate therefore a minimum amount of detergent is required in downstream operations after initial solubilization. The amount of detergent required is typically less than what is required for initial solubilization. When amphiphillic detergents are required at some point in chromatography, additional process variables must be considered. A good example is the effect of detergents on size exclusion, or gel filtration, chromatography. Gel filtration separates proteins based on size; smaller molecular weight particles diffuse more readily into the porous particles which retard their movement, while larger particles travel faster through the bed void. Detergent-protein interactions may result in a mixture of micelle types including detergent-protein micelles, detergent-lipid-protein micelles, or membrane fragments [10]. In addition, the incorporation of membrane proteins into micelle-protein complexes will considerably increase a protein's size, causing it to elute prematurely in a gel filtration operation. Both phenomena may decrease recovery and resolution and depend heavily on the detergent chosen for solubilization [10].

The effect of detergents on size exclusion HPLC of Sendai virus membrane proteins, as previously described, gives valuable insight. 0.1% SDS detergent added to the eluent buffer yields minimal separation of the integral membrane protein, F. Adding 4% SDS to the eluent buffer significantly improves resolution of pure F protein but denatures the HN membrane protein. Conversely, 0.1% octylglucoside additive maintained a single peak of aggregate proteins during elution which contained both F and HN proteins which were highly active [43].

Ion exchange chromatography (IEC) is another possible method for membrane protein fractionation. Like size exclusion, IEC protocols may be defected by detergent interactions as detergent molecules may carry certain charges. Therefore, ionic detergents should be avoided in favor of non-ionic or zwitterionic detergents when IEC is to be used for membrane protein separation [10]. Anion exchange HPLC was applied to isolate Sendai virus F protein from purified and detergent solubilized virions. F protein

was isolated by applying a linear NaCl gradient with a low percentage of non-ionic Triton X-100 [44].

Ion exchange was also applied for purification of membrane glycoproteins D (gD) of types 1 and 2 Herpes simplex viruses (HSV) [6]. Like GP5 of PRRSV, the D glycoproteins of HSV have multiple glycosylation sites and are potential vaccine candidates, making preserving native characteristics important. The glycoproteins of interest were expressed in baculovirus Sf21 insect cells and extracted from resuspended cell pellet in the presence of 4% non-ionic detergent pentaethyleneglycol monodecyl ether ($C_{10}E_5$). Glycoproteins were eluted during a two-step linear salt gradient. The first step eluted glycoprotein D with a salt gradient that included 0.005% $C_{10}E_5$; the second a salt gradient contained 0.1% $C_{10}E_5$. SDS-PAGE confirmed that some gD was eluted during the first step along with multiple impurities, while increasing detergent concentration for the second elution yielded only gD. Damhof et al. [6] suggested a novel explanation for selective elution in two steps: the low detergent concentration in the first elution causes hydrophilic proteins to elute with the salt gradient while micelle-gD complexes remain intact. During the following step higher detergent concentration selectively elutes hydrophobic gD protein by pulling it from the sorbent into solution micelles at a certain salt concentration. In a sense, gD is passed from stationary micelle to mobile micelle [6]. This study illustrates the importance and relevance of proper detergent concentration to the success of ion exchange processes.

Another study supports the significant effect that detergents have on separation of membrane proteins using ion exchange chromatography. The peptide transporter protein from detergent solubilized rabbit small intestinal brush border membranes was loaded onto a strong cation exchange column equilibrated with buffer containing either Triton X-100 or octylglucoside. With Triton X-100 in the equilibration and elution buffers the target protein was partially isolated. The presence of octylglucoside in both buffers resulted in complete separation of the peptide transporter protein (95% purity) [19].

Hydrophobic interaction chromatography (HIC) separates proteins based on interactions between hydrophobic parts of biomolecules and hydrophobic resin surfaces. Membrane proteins, in particular, bind tightly with HIC resins but so do the detergents used to solubilize them. The resulting interaction is difficult to predict due to interactions

between the resin and detergent-protein complex. This interaction was explored by separating Triton X-100 solubilized human brain tissue factor protein (TFP) using HIC [5]. TFP adsorbed to the column predominantly through its association with Triton X-100, although TFP is indeed hydrophobic. It followed that TFP was eluted only when the column was saturated with Triton X-100 in a low-salt buffer, or when propylene glycol, a polarity reducing agent, was applied [5]. Displacement elution of Triton X-100-protein complexes by Triton X-100 saturation of Phenyl Sepharose resins was successful for fractionating other membrane-associated proteins [19, 39]. This method has also been applied using other detergents for displacement [40].

Affinity chromatography is also a viable method for membrane protein fractionation that exploits highly specific biological interactions between target molecule and ligand. Antigen-antibody type affinity chromatography is applicable for viral membrane protein purification when specific antibodies are available. Target proteins bind tightly to the stationary phase and harsh eluents are often needed to reverse the bonds. Purity is between 1,000- and 10,000-fold [10]. When antibodies of a target protein are not available, immobilized lectin affinity is useful for fractionation of membrane bound glycoproteins. Lectins bind common protein carbohydrate groups including mannose, fucose, N-acetylglucosamine, and N-acetylgalactosamine [10]. The reaction is reversible through competitive elution with simple sugars, offering a gentler alternative to antigen-antibody elution and resulting better activity recovery. It may be possible to fractionate between glycosylated GP5 or GP5/M molecules by exploiting presumed differences in sugar modifications by applying a linear sugar elution gradient to a lectin affinity column. Lectin affinity could serve as a characterization tool in addition to serving as a separation medium.

Immobilized lectin affinity chromatography was applied for purification of glycoprotein G (gG) from native viral haemorrhagic septicaemia virus (VHSV). gG of VHSV has similar properties to PRRSV GP5; it is a virus glycoprotein contained in a lipid bilayer. Both GP5 and gG have 2-6 reported glycosylation sites [8, 34]. VHSV was harvested from infected cells, clarified, and precipitated with polyethylene glycol. gG was solubilized by sonication in the presence of CHAPS detergent, chosen because it best maintained gG's native trimeric structure. gG was loaded onto a Concanavalin A (ConA)

column and 50% of the soluble glycoprotein was recovered. The major drawback of ConA affinity chromatography reported was co-elution of residual unsolubilized glycoprotein/N-protein left as a result of mild detergent conditions, which were necessary for retaining gG activity. ConA was also reported to leak from the column because the ligand is not detergent compatible [34].

2.10 GP5/M purification

As a result of the recent challenges and successes associated with PRRSV vaccine development there remains a need for downstream processing methods that could consistently produce pure and functional GP5 or GP5/M proteins. Currently, no complete bulk purification methods have been reported for PRRSV proteins. A method for semi-purification of PRRS extracellular virions has been reported, yet no further processing of individual PRRSV proteins was mentioned [14]. Various studies have reported purification of other membrane-associated proteins from native virions or membrane fragments [4-7, 15, 19, 33, 43, 44]. However, it has yet been investigated whether or not such processes may be adapted and applied to purify GP5/M from PRRSV.

2.11 Summary

GP5 and M proteins of native PRRS virions have intrinsic biochemical properties that make them attractive positive controls for vaccine research. Specifically, the GP5/M heterodimer synthesized and processed during virus assembly may have a significant impact if isolated. GP5/M glycosylation and oligomerization are well documented and recent studies suggest that the association of both proteins is significant to inducing protective immunity [16, 17]. Propagation of PRRSV is possible in permissive cell lines as to provide a consistent stock for process development. Purification of native GP5 or GP5/M complex from native virions has not been established but may be possible by adapting virion isolation, detergent solubilization, and chromatographic methods based on known biochemical properties of PRRSV proteins.

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Chapter Three

Purification of the major envelop protein GP5 of porcine reproductive and respiratory syndrome virus (PRRSV) from native virions

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Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) is the cause of an economically important swine disease that has been devastating the global swine industry since the early 1990's. The current PRRSV vaccines are not very effective largely due to heterogeneic nature of the virus. The major envelope protein, GP5, exposes outside the virion, induces neutralizing antibodies, and thus is a primary target for developing a subunit vaccine. In this study, we report a process for purification of GP5 protein from native virions of PRRSV propagated in MARC-145 cells. PRRSV virions were first purified and concentrated through sucrose cushion ultracentrifugation. GP5 protein was subsequently solubilized with Triton X-100 detergent for further processing. Cation exchange chromatography (CEX) was utilized for partial fractionation of GP5, although the viral nucleocapsid protein (N) was a major impurity in CEX elution fractions. During a second chromatographic step, hydrophobic interaction chromatography (HIC) further purified GP5 protein by means of a two-stage elution scheme. Pure GP5 protein was eluted from the HIC resin in the second HIC elution stage by Triton X-100 displacement; however the protein is present as a homodimeric/tetrameric aggregate. This process may be useful in PRRSV subunit vaccine development.

Keywords: porcine reproductive and respiratory syndrome virus, PRRSV, protein purification, GP5

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS), caused by PRRS virus (PRRSV), is an economically important disease prevalent in swine producing countries around the world. It is estimated that annual losses associated with PRRS amount to half a billion dollars in the United States alone (Neumann et al., 2005). PRRS is best characterized by late-term abortions and stillbirths in sows and severe respiratory diseases in pigs (Dea et al., 2000; Meng, 2000). Several modified-live vaccines (MLV) against PRRS are available but they are not very effective against the diversified field strains of PRRSV. In some cases, reversion of MLV to a pathogenic phenotype has been reported (Botner et al., 1997; Storgaard et al., 1999). Attempts to develop more efficacious vaccines against PRRSV have been met with challenges largely due to virus heterogeneity (Meng, 2000).

The major envelope protein, GP5, elicits neutralizing antibodies in pigs, a critical aspect of anti-viral humoral immunity (Bautista et al., 1999; Pirzadeh and Dea, 1998; Weiland et al., 1999). GP5 is also the most heterogenic structural protein of the virus (Andreyev et al., 1997; Dea et al., 2000; Pirzadeh et al., 1998). In native virions from infected cells, GP5 forms a disulfide-linked heterodimeric complex with the membrane protein, M. It has been suggested that the M protein is recognized by porcine T cells and, along with GP5, plays a role in virus infectivity and assembly (Dea et al., 2000; Mardassi et al., 1996).

Previous studies have indicated that both GP5 and M proteins are important in eliciting protective immunity against PRRSV (Bautista et al., 1999; Pirzadeh and Dea, 1997; Pirzadeh and Dea, 1998; Weiland et al., 1999). More recently, it has been shown that there are enhanced cellular and humoral immune responses in animals vaccinated with a recombinant GP5/M heterodimer antigen (Jiang et al., 2006a; Jiang et al., 2006b). Because of the potential use of GP5 and M proteins, individually or as a complex, as a vaccine candidate, developing a process to purify these two proteins from native virions may aid the vaccine development and help understand the GP5 and M proteins interaction.

Methods for isolation and semi-purification of extracellular PRRSV virions were reported using cesium chloride isopycnic ultracentrifugation and ultracentrifugation

through a sucrose cushion (Benfield et al., 1992; Dea et al., 2000; Meng et al., 1994). Further purification of native GP5 and/or GP5/M proteins from isolated virions has not been reported but may be possible by adapting detergent solubilization and chromatographic methods based on known biochemical properties of PRRSV proteins. The objective of this study is to develop a process to purify GP5 and/or GP5-M heterodimer from native virions.

2. Experimental

2.1 Materials

The MARC-145 cell line, generously provided by Dr. K.J. Yoon (Iowa State University College of Veterinary Medicine), was used for PRRSV propagation. PRRSV strain VR-2385 (ISU-12) was originally isolated from a pig with severe PRRS (Meng et al., 1994). DMEM cell culture media, fetal bovine serum (FBS), Trypsin-EDTA, and Penicillin-Streptomycin were obtained from Invitrogen (Carlsbad, CA). Ultra purity grade sucrose was obtained from Bioworld (Dublin, OH). 1 x 3 ½ in. ultracentrifuge tubes were obtained from Beckman Coulter (Fullerton, CA). Triton X-100 detergent was obtained from Sigma (St. Louis, MO). Econo-Pac 10 DG disposable desalting chromatography columns were obtained from Bio-Rad Laboratories (Hercules, CA). Cation exchange and hydrophobic interaction resins were obtained from GE Healthcare (Waukesha, WI). Microcon and Amicon Ultra centrifugal filter units were obtained from Millipore Corporation (Bedford, MA). All other laboratory grade chemicals and salts were obtained from either Sigma or Fisher Scientific (Pittsburgh, PA).

All SDS-PAGE materials including NuPAGE 4-12% Bis-Tris mini gels, NuPAGE antioxidant, 4x LDS (lithium dodecyl sulfate) sample buffer, and 20x MES SDS running buffer (1 M 4-Morpholineethanesulfonic acid, 1 M Tris, 2% (w/v) SDS, 20 mM EDTA) were obtained from Invitrogen. Bio-Safe Coomassie stain was purchased from Bio-Rad and SilverQuest silver staining kit was obtained from Invitrogen. Western immunoblot products including Immun-Blot 0.2 µm polyvinylidene difluoride (PVDF) membranes, mini trans-blot filter paper, Immun-Star goat anti-rabbit (GAR)-HRP conjugate, Protein G-HRP conjugate Immun-Star HRP, non-fat dry milk, and HRP chemiluminescent kit (containing luminol/enhancer solution and peroxide buffer) were obtained from Bio-Rad.

NuPAGE transfer buffer (500 mM Bicine, 500 mM Bis-Tris, 20 mM EDTA) was obtained from Invitrogen. Polyclonal antiserum (αPRRSV) against PRRSV (NVSL strain) was obtained from National Veterinary Services Labs (NVSL, Ames, IA) and a mono-specific antibody (rabbit) against PRRSV GP5 (αORF5) was kindly provided by Dr. Serge Dea and Dr. Carl Gagnon (Université du Québec, Québec, Canada).

2.2 Preparation of PRRSV virus stocks in cell culture

U.S. PRRSV isolate ATCC VR-2385 was propagated in the highly permissive MARC-145 cell line. For cell passage, MARC-145 cells were grown to the monolayer (or 90% confluence) as observed by a light microscope. Cells were passaged every 3 to 4 days as needed for virus inoculation. Cell growth medium, DMEM enhanced with 10% FBS and 1 × antibiotics (10,000 units/mL penicillin G, 10,000 mg/mL streptomycin, 25 mg/mL amphotericin B), was removed from each flask and cells were incubated with 1 mL tripsin-EDTA (TE). TE was removed upon the first indication of cell detachment. Five milliters of cell growth medium was added to suspend cells; the suspension was aspirated and split over five flasks. Nine milliliters of cell growth medium was added to each flask and subsequently aspirated. Flasks were transferred to a humidified incubator (37°C, 5% CO_2) and incubated in the horizontal position for cell growth. Flasks were inoculated with PRRSV or used for further cell passage when 90% confluence was observed.

For virus culture, the 10% FBS-DMEM/PS cell growth medium was removed; cells were inoculated with 1 mL PRRSV stock thawed to 37°C. Flasks were incubated horizontally for 1 h (37°C, 5% CO_2). Nine milliliters of virus medium (2% FBS-DMEM) was then added to each flask and flasks were returned to the humidified incubator for 2 to 3 days. Virus culture continued until 90% of the cells showed visible signs of cytopathic effect (CPE) under a light microscope.

When CPE was observed the cell culture was terminated by freezing to -80°C. Two more consecutive freeze/thaw cycles from -80° C to 4°C were performed to lyse MARC-145 cells and release intracellular virions. The resulting virus stock solution was frozen at -80°C until use for process development or virus inoculation.

2.3 Process development for isolation of target proteins

GP5 was considered the primary target protein for purification while M and the GP5/M complex were considered secondary targets. The process for purification of GP5 was divided into three factions; (1) virus isolation, (2) detergent extraction and solubilization, and (3) chromatographic purification.

2.3.1 Virus isolation

The virus stock containing cell lysate was clarified by low speed centrifugation at 5,000 x g for 20 minutes at 4°C (Gonin et al., 1998). Purified PRRS virions were harvested from clarified supernatant by ultracentrifugation through a sucrose cushion. Approximately 25 mL of clarified supernatant was transferred to a 1 x 3 ½ in. polyallomer tube. Seven milliliters of ice cold 30% (w/v) sucrose prepared in 10 mM Tris-HCl, 1 mM EDTA, pH 7 buffer (TE_7) was gently under-laid. Polyallomer tubes were topped off with ice cold TE_7 buffer. Tubes were balanced and loaded into pre-cooled rotor buckets (rotor SW27). Virions were pelleted through the sucrose cushion at 28,000 rpm (Optima L-90K Ultracentrifuge, Beckman Coulter Inc.; Fullerton, CA) for 3 h at 4°C (Gonin et al., 1998). The resulting viral pellet was resuspended in 1 mL of TE_7 buffer. Virions were stored at 4°C until use.

2.3.2 Protein extraction and solubilization

Approximately 1.5 mL of purified PRRS virions was mixed with equal volume of solubilization buffer (5 mM Tris-HCl, 1 M NaCl, 1% (v/v) Triton X-100; pH 7). The mixture was mixed briefly vortexed and incubated for 25 minutes at room temperature. The sample was centrifuged at 17,000 × g for 30 minutes at 4°C. Three milliliters of supernatant was loaded onto a 10 DG disposable desalting column and subsequently eluted in 4 mL of cation exchange binding buffer A1 (50 mM NaPi, 0.03% (v/v) Triton X-100, pH 6.9).

2.3.3 Chromatography

All chromatographic experiments were carried out with an ÄKTA Explorer 100 (GE Healthcare, Uppsala, Sweden) fast-performance liquid chromatography (FPLC) system controlled by the Unicorn software (version 3.10).

For cation exchange chromatography (CEX), SP Sepharose™ Fast Flow resin was packed in an HR 5/10 glass column (GE Healthcare) to a 1 mL bed volume. The column was equilibrated with buffer A1 (50 mM NaPi, 0.03% (v/v) Triton X-100, pH 6.9), previously filtered and degassed. The flow rate used for all steps was 1 mL/min. Four milliliters of solubilized/desalted sample was applied to the column, the flow-through was collected, and the column was washed for 10 min. Bound proteins were eluted with a step or linear gradient of buffer B1 (buffer A1 + 2 M NaCl). One milliliter fractions were collected for analysis.

Phenyl Sepharose™ 6 Fast Flow (low sub) resin (GE Healthcare) was selected for hydrophobic interaction chromatography (HIC). HIC was performed using the same column type, bed volume, and flow rate as CEX. The column was equilibrated with buffer A2 (50 mM NaPi, 1.5 M ammonium sulfate, pH 7). Approximately 4 mL of CEX elution fractions were collected, pooled, concentrated 2 × with a centrifugal filter device, and loaded onto the HIC column. After flow-through fractions were collected a two-stage purification strategy was used for selective elution of GP5. First a step elution to buffer B2 (50 mM NaPi, pH 7) was applied for 20 min to elute unwanted proteins. This was followed by a second step elution with buffer B3 (B2 + 1% (v/v) Triton X-100), applied for 20 min to displace Triton X-100-protein complexes.

2.3.4 Analytical Methods

The purification process was analyzed with densitometric protein band analysis at critical control points around each major process operation. If needed, samples were concentrated with a centrifugal filter device.

SDS-PAGE

SDS-PAGE gels were run under both reduced and non-reduced conditions. Fifteen micro-liters of sample were mixed with 5 µL LDS sample buffer. Reducing conditions were achieved by addition of 2 µL of 500 mM DTT. The presence of DTT was reported

to resolves separate GP5 (25 kDa) and M (19 kDa), while omitting DTT resolves additional bands corresponding to GP5/M complex (43 kDa) and M/M complex (35 kDa) (Jiang et al., 2006b). Therefore DTT was included or omitted per experiment depending on the desire to view the GP5/M complex or GP5 and M individually.

Samples were vortexed, heated at 70°C for 10 minutes, then briefly centrifuged. 20 µL samples were loaded onto a 4-12% Bis-Tris NuPAGE gel in an X-Cell SureLock Mini-Cell (Invitrogen). The cathode buffer chamber was filled with 1 × MES running buffer supplemented with 500 µL of NuPAGE antioxidant for reduced conditions. The outer anode chamber was filled with 1 × MES running buffer for both reduced and non-reduced conditions. Gels were run for 200 V for 40 min. Ten micro-liters of Precision Plus protein standard (Bio-Rad) were run parallel to sample lanes.

For overall protein analysis SDS-PAGE gels were stained with Coomassie and/or silver stain. For Coomassie stain, gels were washed with deionized (DI) water and stained with BioSafe Coomassie. For silver stain, gels were washed with DI water and stained according to the SilverQuest fast staining protocol. Gel images were captured in a ChemiDoc XRS molecular imager with EPI white illumination and analyzed with Quantity One software.

Western Blot

PRRSV target proteins were analyzed by Western blot. Gels were transferred at 30 V for 60 min to a PVDF membrane in 1 × NuPAGE transfer buffer supplemented with 10% methanol, with or without 0.1% antioxidant depending on reducing conditions. After transfer the membrane was incubated for 50 min with gentle agitation in 5% (w/v) non-fat dry milk in TTBS buffer (20mM Tris-HCl, 0.5 M NaCl, 0.05 % (v/v) Tween 20, pH 7.4). The blot was incubated with gentle agitation overnight at 4°C in a 1:250 dilution of αPRRSV or a 1:25,000 dilution of αORF5 in TTBS buffer. Following consecutive wash cycles the blot was incubated for 1 h in a 1:15,000 dilution of Protein-G HRP conjugate for αPRRSV-probed blots or GAR-HRP for αORF5. After consecutive wash cycles the blot was incubated in a 1:1 solution of HRP luminol/enhancer solution and peroxide buffer for 5 min. PRRSV proteins were exposed in a ChemiDoc XRS molecular imager with the ChemiHi sensitivity setting.

3. Results and discussion

3.1 Identification of GP5 and M

The presence of target proteins was achieved by SDS-PAGE and Western blot at process control points. Fig. 1(c) shows GP5 appears as a diffuse band at 27-29 kDa on western blots probed with αORF5. αORF5 also reacts with a 60 kDa band, possibly a GP5 homodimer as seen in Fig. 5(c), lane 3. αPRRSV initially reacted with a 27-29 kDa band, a 19 kDa band, and a 43 kDa band in non-reduced blots (not shown). Past studies suggest that these bands correspond to GP5, M, and GP5/M respectively (Mardassi et al., 1996). When the western blot was prepared under reducing conditions for comparison we found no conclusive evidence that the 43 kDa band was GP5/M, although the band was not detected when DTT was included in sample preparation. Additionally, the 19 kDa doublet band was identified as N, not M, by LC/MS/MS analysis using MASCOT software (results not shown). As a result we could not confirm the presence of M and, consequently, the GP5/M complex in sample preparations.

αPRRSV antibody performance varied depending on the lot obtained from NVSL. When newer αPRRSV was obtained from NVSL the antibody failed to react with any protein other than N protein at 19 kDa. This is consistent with past studies which indicate that N is the most abundant and most antigenic PRRSV protein (Dea et al., 2000). However it is unclear why αPRRSV failed to react consistently with other viral proteins but we assume it is due to the heterogenic nature of the virus. Nevertheless, αORF5 consistently reacted with GP5 and proved valuable for process development.

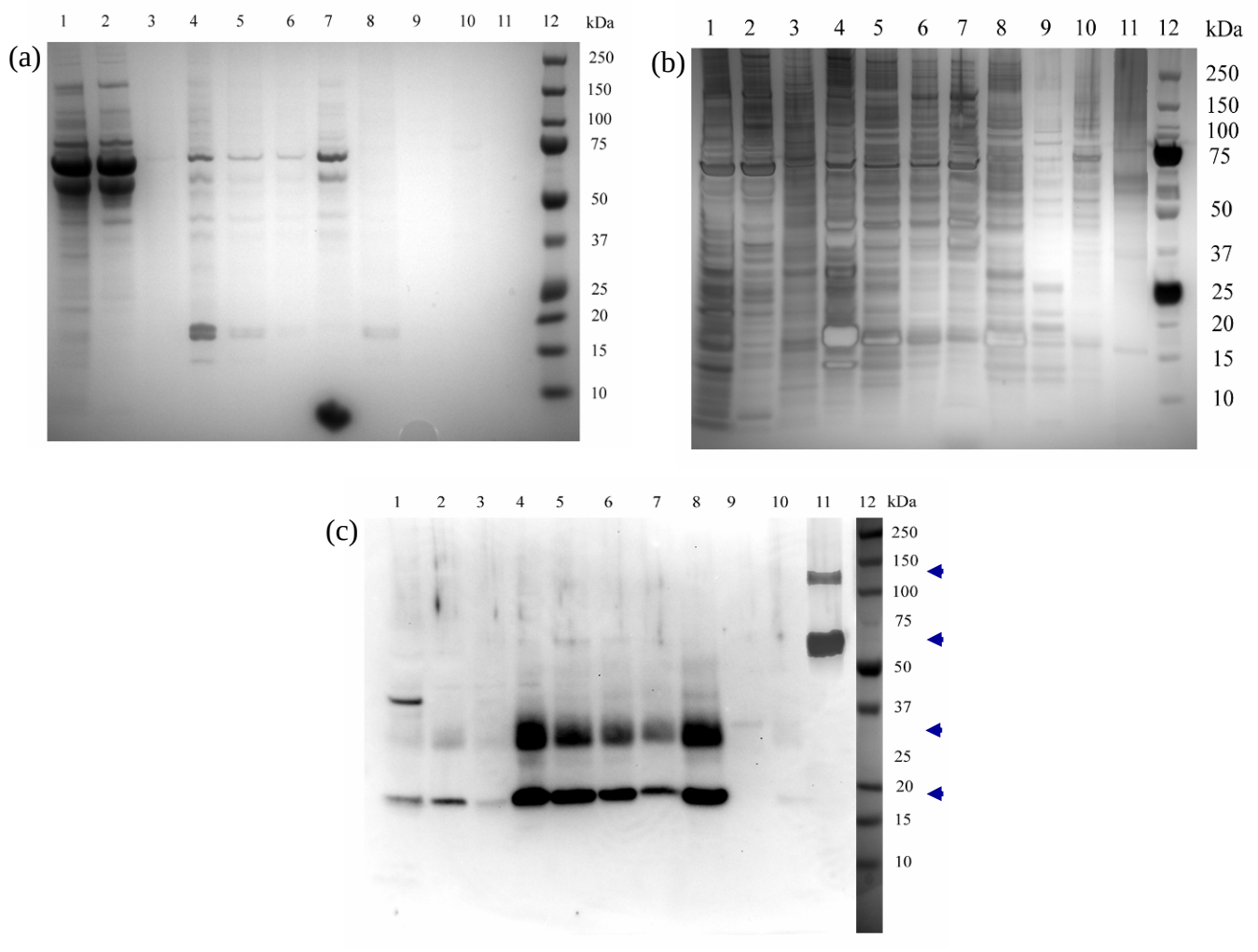

Figure 1. (a) Coomassie stained and (b) silver stained SDS-PAGE gel, and (c) Western blot (αGP5 and αPRRSV) of full process. L1: PRRSV infected cell culture, L2: clarified cell culture, L3: resuspended cell lysate pellet, L4: PRRS virions after sucrose cushion, L5: PRRS virions solubilized with 1% Triton X-100, L6: solubilized sample after NaCl removal, L7: pooled/concentrated CEX flow through fractions, L8: CEX step elution peak (conc. 2 ×), L9: HIC flow through fractions (conc. 4 ×), L10: HIC elution peak 1 (conc. 4 ×), L11: HIC elution peak 2 (conc. 10 ×), and L12: protein marker. Concentration of samples was achieved with YM-10 Microcon centrifugal filter devices (MWCO 10K).

3.2 Virus culture and isolation

In order to develop a process for purification of target proteins it was first necessary to determine an ideal working volume. Virus culture was carried out in T-25 flasks and each flask yielded 10 mL of virus stock. Attempts to produce greater volume of virus stock in T-75 flasks were unsuccessful as we found that virus titer did not significantly increase when flask size was increased (data not shown). As a result, 6 T-25 flasks (60 mL) were used for virus isolation during each process development run. Clarified virus stock was divided over 3 ultracentrifuge tubes for sucrose cushioning. Each viral pellet was resuspended in 1 mL buffer to yield a total of 3 mL for further processing; this was enough protein for the identification of target proteins with previously mentioned analytical methods, although sample concentration was often necessary.

Protein recovery during virus isolation is shown in Fig. 1. The most prominent bands in the Coomassie stained gel (lanes 1, 2) are serum albumin precursor (BSA), confirmed by LC/MS/MS, at 71 kDa from virus culture media. After low speed clarification of cell lysate extra-cellular virions are recovered in the supernatant (lane 2), as evident by identification of GP5 and N in the western blot. The resuspended pellet (lane 3) contains faint traces of GP5 and N. This may be due to the presence of intracellular virions remaining in lysed host cells. The sucrose cushion step provides valuable separation of PRRSV proteins from impurities. GP5 and N are enriched (lane 4) while BSA and other impurities are discarded with the supernatant. N is the most prominent band in fractionated virion samples run on SDS-PAGE and stained with Coomassie. GP5 content is too low for Coomassie identification although the Western blot indicates significant enrichment. When a cesium chloride density gradient protocol for PRRS virion fractionation, as described in (Meng et al., 1994), was attempted for comparison we found that the sucrose cushion protocol yielded higher recovery, was less time consuming, and was more efficient (data not shown).

3.3 Protein extraction and solubilization

Triton X-100 is a mild non-ionic detergent with good solubilization properties that will maintain disulfide linked complexes (Deutscher, 1990). GP5 was extracted from the viral lipid bi-layer and subsequently solubilized with a final concentration of 0.5% Triton

X-100 detergent and 0.5 M NaCl. GP5 was considered solubilized if it remained in the supernatant fraction of detergent samples ultracentrifuged at 100,000 × g, 1 h., and 4°C. We found that NaCl was necessary for initial solubilization with Triton X-100. Fig. 1 shows PRRSV proteins before and after detergent incubation (lanes 4, 5).

Other detergents including sarkosyl, octylglucoside, CHAPS, Brij 35, and sodium deoxycholate were screened (data not shown). We found that sarkosyl performed well in both solubilizing GP5 and maintaining its activity, however because it is anionic it interfered with chromatography. The other detergents mentioned either did not efficiently solubilize GP5, compromised GP5 activity as determined by western blot, and/or were too expensive for routine laboratory work. Triton X-100 performance was superior to other detergents in each regard.

3.4 Cation-exchange chromatography (CEX)

A concern with chromatographic purification of membrane-associated proteins is protein solubility in aqueous buffers and aggregation. Various studies have reported chromatographic purification of such proteins from native virions or membrane fragments by using detergents to affect solubility and prevent aggregation (Bisaccia et al., 1985; Carson and Konigsberg, 1981; Damhof et al., 1994; David and Morgan, 1988; Grun and Brinton, 1988; Kramer et al., 1990; Palker et al., 1987; Schlaf et al., 1996; Valpuesta and Barbon, 1988; Van Ede et al., 1989; Welling-Wester et al., 1988; Welling et al., 1983). Triton X-100 is well studied for membrane protein purification, specifically with hydrophobic interaction and ion-exchange chromatography methods (Carson and Konigsberg, 1981; Welling et al., 1983).

Cation-exchange chromatography was first performed using linear salt gradient elution to investigate target protein elution characteristics. PRRSV proteins are all basic at neutral pH but the theoretical pI of each does vary as evident in Table 1, and the difference enables ion exchange chromatography to be used for the seperation of these proteins, particularly GP5 and N. Triton X-100 was included in CEX buffers at a concentration slightly above its critical micelle concentration (*cmc*) *to* prevent aggregation of target proteins. The presence of Triton X-100 (< 0.1%) in

chromatography buffers does not impede ion-exchange adsorption kinetics, but may decrease resolution (Kramer et al., 1990).

Table 1. Protein characteristics of PRRSV North American strains[a].

Coding area gene product	Protein	No. of aa residues	Predicted M_r (kDa)	Apparent M_r (kDa)	Glycosylation sites	Theoretical pI[b]
ORF2	GP2	265	29.4	27-29	2	9.94
ORF3	GP3	254	29.0	42-45	7	8.50
ORF4	GP4	178	19.6	31-35	4	8.45
ORF5	GP5	200	22.4	24-26	2-5	8.79
ORF6	M	174	19.1	19	1	9.83
ORF7	N	123	13.6	14-15	1	10.09

[a] Data was taken from [2].

[b] Data from Expassy Calculator (http://expassy.org) using nucleotide sequences reported by [15, 31].

Fig. 3 shows GP5 and N are both recovered after de-salting. Fig. 3(a) shows that a majority of proteins do not bind to the CEX column at pH 6.9 and among them is the residual BSA not removed during sucrose cushioning. Both GP5 and N bind under the selected buffer conditions. PRRSV proteins elute during a 40 min linear salt gradient as part of two overlapping peaks shown in Fig. 2. SDS-PAGE analysis reveals a 19 kDa doublet in lanes corresponding to the second elution peak. A 27-29 kDa band is also evident in Lanes 7 and 8, also during the second peak. The 19 kDa band reacted with αPRRSV and the 27-29 kDa with αORF5 when probed on a western blot, Figs. 3(b, c). A 60 kDa band also reacted strongly with αORF5. LC/MS/MS analysis using MASCOT software confirmed the 19 kDa protein as N; the 27-29 kDa protein was identified as RPS4X, a 27.2 kDa ribosomal protein with pI of 9.95. We conclude that, because the MARC-145 cell line used is a monkey kidney cell derivative, the RPS4X peptide sequence closely resembles the sequence of a similar MARC-145 ribosomal protein and that this band represents a residual ribosomal protein from cell culture.

Upon further analysis of both peaks using αORF5-probed western blotting we found that dilute GP5 eluted over both during the gradient. We suspect that RPS4X simply overlaps with GP5 during the second peak, considering the fact that if the band at around 27 kDa was indeed all GP5, the corresponding band in the Western blot would be much

stronger. Poor resolution of GP5 may be attributed to the presence of 0.03% Triton X-100 in the elution buffer. Mixed protein-detergent micelles decreases the performance of cation-exchange resins used to fractionate detergent solubilized proteins (Kramer et al., 1990).

Cation-exchange is a valuable first step toward purifying GP5, but poor resolution resulted in low recovery over many fractions during gradient elution. Consequently, a step elution was applied to elute GP5 for further processing. Fig. 4 shows one major peak was recovered during a single step to 100% buffer B1. Western blot and SDS-PAGE analysis of this peak confirm promising GP5 enrichment and recovery, as evident in Fig. 1, lane 8. However other proteins (N included) and materials such as nucleic acids are contained in this fraction. On the other hand, the high salt buffer used for bulk CEX elution makes this fraction suitable for hydrophobic interaction chromatography.

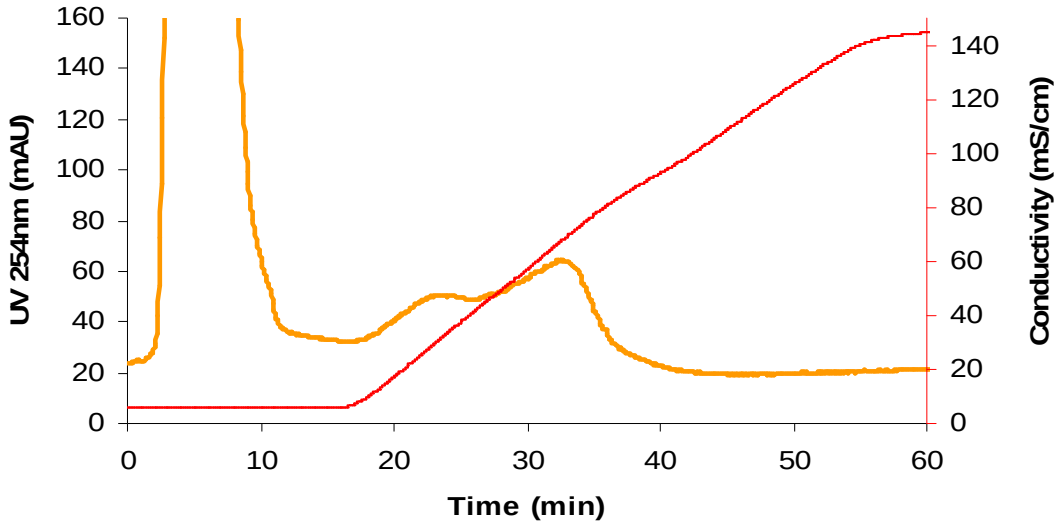

Figure 2. Cation-exchange chromatography of 3 mL PRRS virions solubilized with 1% Triton X-100 a de-salted. A 40 min. linear gradient from buffer A1 to buffer B1 (A1 + 2.0 M NaCl) was applied. GP5 monomer and dimer eluted over both peaks and N eluted as part of the second peak. Absorbance at 254 nm is reported due to interference of Triton X-100 with monitoring at 280 nm.

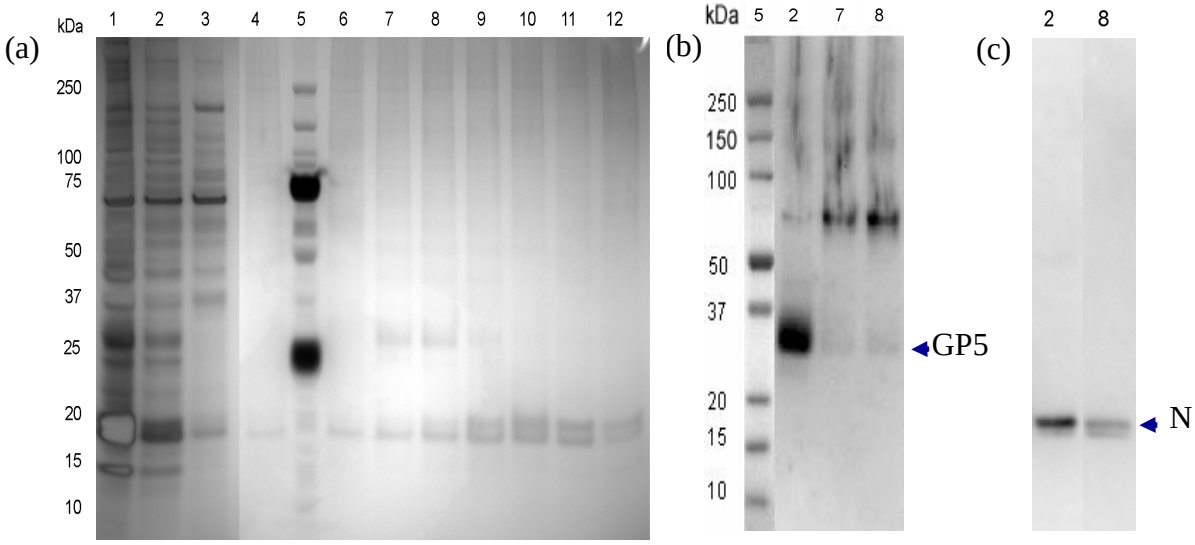

Figure 3. (a) Silver stained SDS-PAGE gel and Western blot (b) αGP5, (c) αPRRSV of CEX fractions recovered during linear gradient elution. L1: PRRS virions. L2: PRRS virions solubilized with Triton X-100 after NaCl removal. L3: pooled/concentrated flow through fractions. L5: protein marker. L4-L12: elution fractions from double elution peaks, shown in Fig. 2.

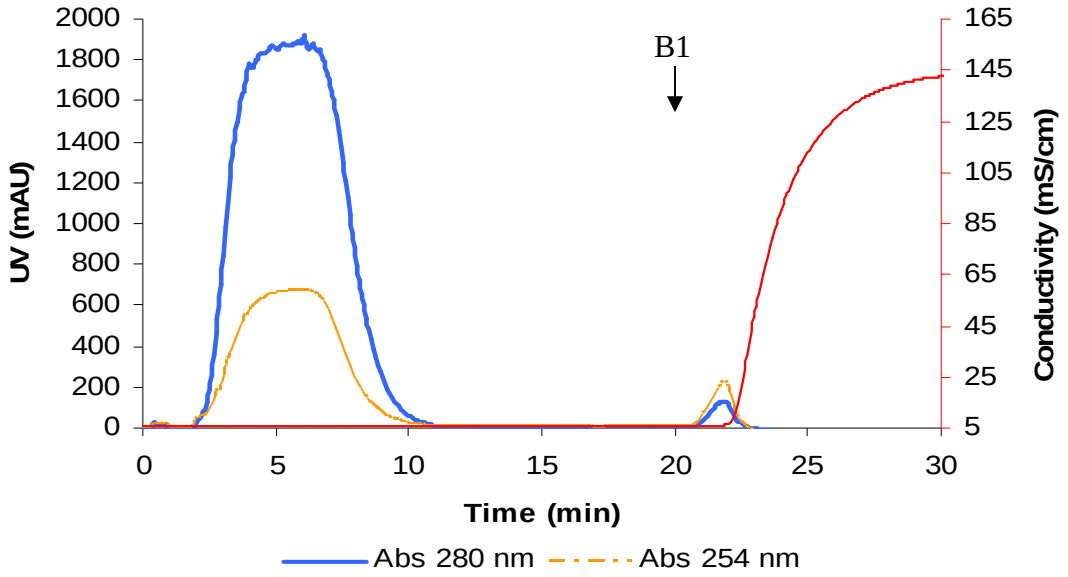

Figure 4. Cation-exchange chromatography of 3 mL PRRS virions solubilized with 1% Triton X-100 a de-salted. A step elution from buffer A1 to buffer B1 (A1 + 2.0 M NaCl) was applied and elution fractions were collected for HIC.

3.5 Hydrophobic interaction chromatography (HIC)

HIC was carried out to investigate if further fractionation of GP5 was possible. CEX step elution fractions were pooled and concentrated 2 × to 2 mL with an Amicon Ultra-4 (5k MWCO) device. Because the sample was not in an ammonium sulfate buffer, a concentration step was necessary to improve interaction of GP5 with the stationary phase by reducing the volume of non-ammonium sulfate buffer loaded. Attempts to increase binding efficiency by adding ammonium sulfate directly to CEX fractions resulted in precipitation.

For hydrophobic-interaction chromatography, Triton X-100 is known to bind to Phenyl Sepharose resin (Carson and Konigsberg, 1981). Evidence suggests that detergent solubilized proteins, such as GP5, may bind as part of a complex with Triton X-100 (Carson and Konigsberg, 1981). Because of this it was difficult to predict the GP5 elution. Elution of protein-detergent complexes from HIC usually occurs either by displacement, when the column is saturated with Triton X-100 in a low-salt buffer, or

when a polarity reducing agent, such as propylene glycol, is applied (Carson and Konigsberg, 1981).

A two stage step elution was applied to elute bound proteins. Fig. 5(a) shows the elution profile and corresponding analysis of HIC. Because it was difficult to predict GP5 elution behavior all fractions over both 20 min elution stages were collected and analyzed. High concentrations of ammonium sulfate and/or Triton X-100 interfere with SDS-PAGE protein migration so fractions were pooled, concentrated, and diluted with DI water. Fractions were then re-concentrated for analysis. Fig. 5(a) reveals three main peaks. The flow through peak contains the majority of the protein loaded, including traces of GP5 monomer and dimer as evident in Fig. 5(c). It appears that N is included in the flow through based on Fig. 5(b). However, when the corresponding western blot in Fig. 5(c) was probed with αPRRSV (not shown) we found that N does bind and is eluted during the first elution stage: lanes 6 and 7, Figs. 5(b,c). Upon application of buffer B2 a small peak first appears before the UV response increases to 230 mAU and plateaus slightly before the next stage. The change in buffers may have some effect on UV as Fig. 5(b) shows minimal proteins and other components are in lane 7, Fig. 5(c).

For the second stage, B3 was applied at 40 min. Buffer B3 causes significant UV interference at 280nm due to the high concentration of Triton X-100. The UV response lags until 48 min when a sharp peak appears, presumably just before the resin is saturated with Triton X-100 and the detergent desorbs. Lanes 10 and 11 in Figs. 5(b,c) show that this peak corresponds to selective elution of two aggregate GP5 forms: a dimer and tetramer judged based on the molecular weights of the bands at approximately 60 and 120 kDa, respectively. It appears that Triton X-100 desorbs from the Phenyl Sepharose at 50 min, at which point the UV jumps aggressively to 2,000 mAU. No protein was detectable in fractions collected after this point (lane 12, Fig. 5). Evidently, elution of Triton X-100 solubilized GP5 occurs upon competitive displacement by detergent molecules, as described by Carson and Konigsberg (1981). Repeated trials indicate that this elution behavior is consistent for GP5.

GP5 possesses two putative membrane-spanning domains and, like most membrane associated proteins, has a tendency to aggregate (Dea et al., 2000). Fig. 5 shows that, when analyzed with SDS-PAGE and western blot, GP5 resolves at its native molecular

weight of 27-29 kDa after CEX elution and before the HIC step. In the flow-through lane, unbound GP5 begins to appear primarily as a 54-58 kDa aggregate. The lanes corresponding to elution fractions collected during stage-two elution in HIC indicate that GP5 was predominantly entirely in aggregate form, Fig. 5(c), lane 10.

Nevertheless, this result is not unexpected. Aggregation is a common problem associated with HIC. Exposed hydrophobic groups on a protein such as GP5 are the driving force for aggregation in an aqueous environment (Baynes and Trout, 2004). Additives such as detergents dissuade aggregation. The exact reason why GP5 elutes from the HIC column as an aggregate is difficult to determine without extensive experimentation, but there are several possible explanations. Among them is the fact that native GP5 is most stable as a disulfide linked complex with M and that GP5/M may dissociate during HIC (Dea et al., 2000). When GP5 is displaced during the second elution stage, exposed hydrophobic groups on GP5, no longer joined with a detergent micelle and/or M, drive GP5 to form a dimer with itself. Triton X-100 is a mild non-ionic detergent that maintains the integrity of the GP5/M complex. Triton X-100 also adsorbs to the HIC resin. Therefore when GP5 is displaced slightly before the resin is saturated it may be in solution without detergent and/or without M, causing aggregation.

Attempts to resolve GP5 in its native state by re-solubilizing HIC elution fractions with Triton X-100 were unsuccessful. In the same regard, SDS detergent treatment during PAGE did not break up the aggregate therefore other additives, such as guanidinium chloride, urea, polymers, or amino acids, may be more appropriate (Baynes and Trout, 2004). Including arginine in HIC buffers may also improve column performance while preventing aggregation (Arakawa et al., 2007; Tsumoto et al., 2007).

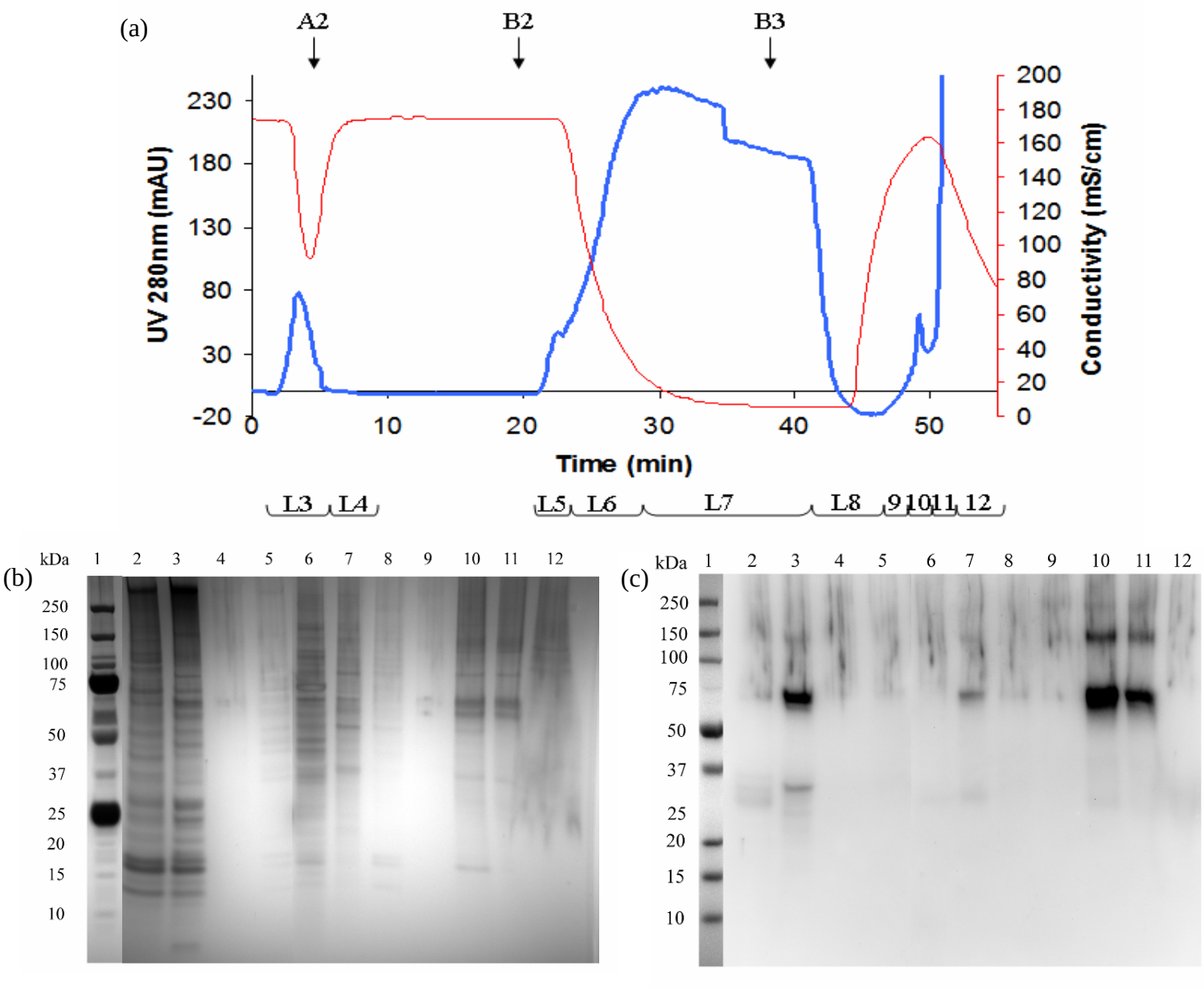

Figure 5. (a) Hydrophobic interaction chromatography of pooled CEX elution fractions. The chromatogram indicates the buffer used in the dual elution scheme (arrows). Fractions were pooled, concentrated, diluted with DI water, and re-concentrated (4 × to 10 ×) for SDS-PAGE and are indicated according to lane number. (b) Silver stained SDS-PAGE gel and (c) Western blot (αGP5) of HIC fractions. L1: protein marker, L2: fraction loaded, L3-12: as indicated in (a).

4. Conclusions

In this study, a process for fractionation of the major PRRSV envelope protein, GP5, from native virions was developed and analyzed. GP5 was identified as a diffuse band at 27-29 kDa via western immunoblotting with a robust mono-specific anti-GP5 antibody. Attempts to track the GP5/M heterodimer for purification were unsuccessful due to inconsistent reactivity of polyclonal anti-PRRSV pig sera obtained from NVSL.

PRRS virions propagated in MARC-145 cells were effectively concentrated and semi-purified from cell lysate and culture medium components (FBS) by ultracentrifugation through a sucrose cushion. Incubation of virions with Triton X-100 successfully disrupted the viral envelope and solubilized GP5 for further processing. Partial purification of GP5 by CEX in the presence of Triton X-100 is possible by applying a linear salt gradient. We were able to collect dilute GP5 over many fractions that also included impurities such the virus nucleocapsid protein. Attempts to improve CEX resolution were unsuccessful, possibly due to the presence of Triton X-100. GP5 contained in the CEX fractions bound to the HIC resin and was collected during displacement elution with Triton X-100 in a low-salt buffer. The final product appears to be a GP5 aggregate homodimer and homotetramer based on SDS-PAGE migration (54-58 kDa and 120 kDa) and strong reactivity with αORF5 in Western blotting.

GP5 aggregation during HIC may be prevented by certain additives and further investigation is necessary. Resolution of GP5 monomers may be essential if our pure product is to be used for vaccine development, although the aggregate product may be sufficient if it elicits desired immune responses. If so, this process will be expanded and used for purification of GP5 from many other PRRSV strains, in addition to VR-2385. We project that it will be possible to purify GP5 from other strains to produce a GP5 cocktail vaccine. Furthermore, process scale-up is possible although it will be necessary to explore alternatives to the sucrose cushion step, as it is a limiting factor. Finally, this process may have significance in determining the crystal structure of native GP5 if adequate amounts of GP5 can be produced for characterization.

Acknowledgement

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Chapter Four

Conclusions and Future Work

Conclusions

1. It is possible to generate enough PRRS virions for purification of native viral proteins. Virus culture methods used in this work are widely used by PRRSV researchers, therefore making this process easily adaptable for other groups.

2. A process for fractionation of the major PRRSV envelope protein, GP5, from native virions was developed. GP5 was identified as a diffuse band at 27-29 kDa via Western immunoblotting with a robust polyclonal mono-specific antibody against PRRSV ORF5. Attempts to identify the membrane protein, M, and GP5/M heterodimer and during purification were unsuccessful.

3. PRRS virions were effectively concentrated and semi-purified from cell lysate and culture medium components (FBS) by ultracentrifugation through a sucrose cushion. Incubation with Triton X-100 successfully disrupted the viral envelope and solubilized GP5 for further processing.

4. Partial purification of GP5 by cation-exchange chromatography (CEX) using SP Sepharose™ Fast Flow resin is possible by applying a linear salt gradient with a low concentration of Triton X-100. GP5 was collected over many fractions which also included impurities such as the virus nucleocapsid protein (N). Attempts to improve GP5 resolution were unsuccessful. CEX chromatography is a good first chromatographic step to fractionate GP5.

5. Hydrophobic interaction chromatography (HIC) was chosen as a second chromatographic step in the process. GP5 contained in CEX fractions bound to the HIC resin, Phenyl Sepharose™ 6 Fast Flow (low sub). A two-stage elution

selectively eluted pure, but aggregated, GP5 during the second stage. Displacement elution with Triton X-100 in a low-salt buffer achieved this separation.

6. The final product appears to be a GP5 aggregate homodimer and homotetramer based on SDS-PAGE migration (54-58 kDa and 120 kDa) and strong reactivity with αORF5 in Western blotting. These two proteins are purified to near homogeneity as estimated by SDS-PAGE.

7. Although the final GP5 product is in aggregate form, Western blot results do not entirely refute the possibility that the aggregate molecules are biologically active and could elicit the desired immune response.

8. The use of generic chromatography methods makes this process easily adaptable for purification of recombinant GP5. This process could have an impact in the continuing effort to develop a subunit PRRSV vaccine.

Future Work

1. It may be possible to denature and refold GP5 aggregate forms with solubilization agents such as guanidinium chloride, urea, polymers, or amino acids. Solubilization of aggregate GP5 should be attempted.

2. GP5 aggregation during HIC may be prevented by including additives such as arginine in the second elution buffer. The HIC step should be further investigated and optimized so that pure GP5 can be obtained in its native form.

3. Resolution of GP5 monomers may be essential if our pure product is to be used for vaccine development, although the aggregate product may be sufficient if it elicits desired immune responses. Aggregated GP5 obtained after HIC should be utilized in animal studies to determine its value.

4. This process should be expanded and used for purification of GP5 from many other PRRSV strains, in addition to VR-2385. We project that it will be possible to purify GP5 from other strains to produce a GP5 cocktail vaccine. Furthermore, process scale-up is possible although it will be necessary to explore alternatives to the sucrose cushion step, as it is a limiting factor.

5. This process may have significance in determining the crystal structure of native GP5 if adequate amounts of GP5 can be produced for characterization.

Appendix A

Protein identification

Appendix A presents results obtained from protein identification services performed at other facilities. Protein bands cut out of Coomassie stained SDS-PAGE gels were identified by analyzing mass spectrometry data with MASCOT bioinformatics software.

Cation exchange elution fractions that contained traces of the 27-29 kDa band and the 19 kDa doublet band, as identified on silver stained SDS-PAGE gels and Western blot probed with αPRRSV or αORF5, as described in Chapter 3, were pooled for analysis. Samples were concentrated with YM-10 Microcon centrifugal filter devices (MWCO 10,000) obtained from Millipore (Bedford, MA). Concentration was necessary for dilute CEX elution fractions to ensure target protein bands could be identified by Coomassie stain. Concentration factor of each sample is listed in Fig. A1.

All SDS-PAGE equipment was obtained as described in Chapter 3. 9-well gels were used in an effort to load a higher sample volume and ultimately more protein: 18.2 µL of sample was mixed with 7 µL LDS sample buffer and 2.8 µL of 50mM DTT. Samples were vortexed, heated at 70°C for 10 minutes, then briefly centrifuged. 28 µL samples were loaded onto a 4-12% Bis-Tris NuPAGE 9-well gel in an X-Cell SureLock Mini-Cell (Invitrogen). The cathode buffer chamber was filled with 1 × MES running buffer supplemented with 500 µL of NuPAGE antioxidant. The outer anode chamber was filled with 1 × MES running buffer and gels were run for 200 V for 40 min. Ten micro-liters of Precision Plus protein standard from Bio-Rad Laboratories (Hercules, CA) were run parallel to sample lanes. The gel was stained overnight with Bio-Safe Coomassie stain (Bio-Rad), de-stained with ultra-pure DI water, and presented for analysis at the Virginia Tech Mass Spectrometry Research Incubator lab (PI: Dr. Richard Helm) in a clean glass 'brownie' pan.

For analysis of the 27-29 kDa protein, the corresponding band from lane 6 (Fig. A1) was cut out of the gel with a sterile scalpel and sent to Alphalyse North America (Palo Alto, CA). This service was used after the Virginia Tech service could not yield results for this band using MALDI TOF/TOF technology.

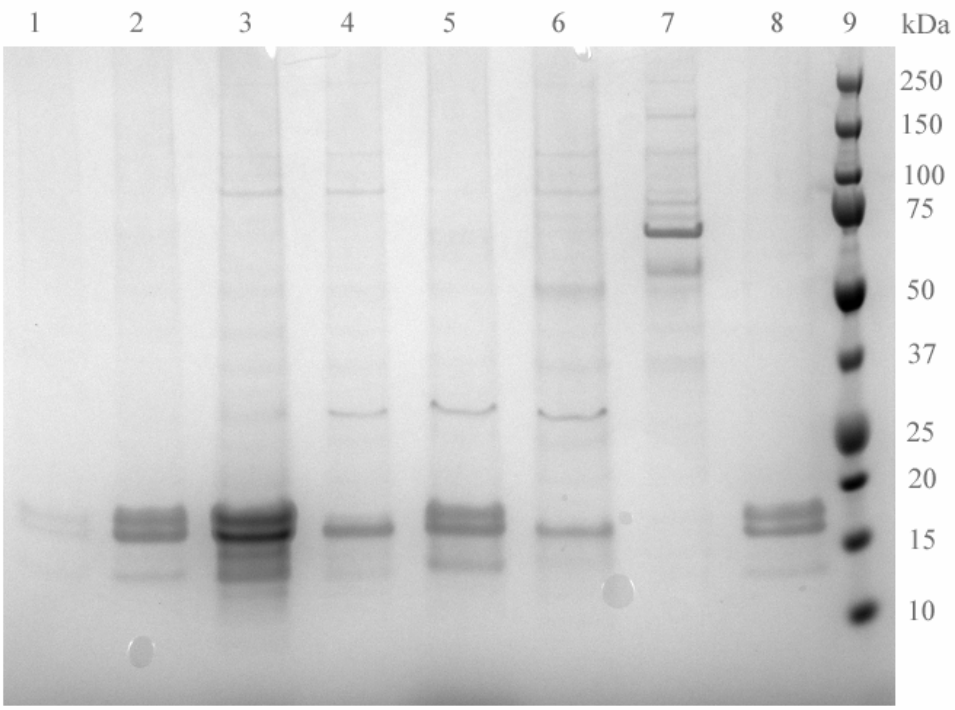

Figure A1. Coomassie stained gel presented for LC/MS/MS analysis. L1,8: Pooled CEX elution fractions from trailing end of the second elution peak resolved by a linear salt gradient as described in Chapter 3 (Conc. 120 ×). L2: Contents of L1 (Conc. 240 ×). L3: Pooled CEX elution fractions from trailing end of the second elution peak resolved by a linear salt gradient as described in Chapter 3 (Conc. 50 ×). L4-6: Pooled CEX elution fractions from leading end of the second elution peak resolved by a linear salt gradient as described in Chapter 3 (Conc. 30-50 ×). L7: PRRS virions solubilized with 1% Triton X-100. Concentration of samples was achieved with YM-10 Microcon centrifugal filter devices (MWCO 10K).

Analysis was performed as described in the analysis report obtained from each respective organization. The full reports are included at the end of this appendix.

Summary of Results

The upper doublet band appears at approximately 19 kDa on SDS-PAGE gels. It was identified as an isoform or post translational modification (PTM) of N. LC/MS/MS

analysis could not confidently identify the upper band as N. The lower doublet band was identified as N with full confidence. The molecular weight of N is typically 14-15 kDa on SDS-PAGE gels. It seems that the SDS-PAGE equipment used in this work may cause slight variation N migration on a gel. N is typically present as a disulfide-linked homodimer and, when run reduced as in Fig. A1, appears as two closely migrating double bands. Therefore we conclude that the upper and lower bands are both N.

The 70 kDa band from lane 7 was identified as serum albumin precursor (BSA) protein with full confidence. Lane 7 contains Triton X-100 solubilized PRRS virions from the sucrose cushion procedure outlined in Chapter 3. Fetal bovine serum was included in cell culture media. Results presented in Chapter 3 show that this impurity is primarily discarded with the supernatant after the sucrose cushion step. LC/MS/MS results indicate that some residual BSA remains in purified virions. However, BSA is an acidic protein and is subsequently removed in the flow through of CEX chromatography.

The 50 kDa band from lane 6 was confidently identified as ribulose 1,5-bisphosphate carboxylase-oxygenase (Rubisco). Rubisco is an abundant plant protein and exists in the CEX elution fractions pooled for lane 6 as a process related impurity. During this work the ÄKTA Explorer 100 system was also used for protein purification from a Tobacco. The presence of Rubisco in this sample indicates that the ÄKTA system was not properly cleaned at some point during process development.

The 27-29 kDa band was identified as RPS4X, a human ribosomal protein. It is likely that this protein is present in CEX elution fractions because its pI (9.95) is very near that of N (10.09). It is also likely that this protein is present from cell culture and was falsely identified as a human protein; this cannot be said with confidence, however. We can only postulate that, because the MARC-145 cell line used is a monkey kidney cell derivative, the RPS4X peptide sequence closely resembles the sequence of a similar MARC-145 ribosomal protein.

Results obtained from Virginia Tech service

Virginia Tech Mass Spectrometry Research Incubator

$LCMS^n$ Analysis

Sample Number(s): TW77C1-4

Submitted By: Brad Matanin

Submission Date: 02 Mar 2007

Contact Information: bmatanin@vt.edu

Principal Investigator: Mike Zhang

Analysis Goal: Confirmation of samples 1 and 2 as PRRSV membrane protein M. Identification of unknown samples 3 and 4.

Date(s) of Analysis: 5-6 Mar 2007

Type(s) of Analysis: Data dependent NSI $LCMS^n$ analysis on Thermo LCQ DecaXP of in-silico tryptic digests.

Technician: Tara Wiles

Conclusions: Samples 1 and 2 do not contain the PRRSV membrane protein M (see details below). Sample 3 contains RUBISCO and sample 4 contains BSA.

Estimated Technician Time: 3 hours

Estimated Instrument Time: 4 hours

Sample Prep for MS Analysis: Four $1mm^3$ spots were excised from each protein band. Samples were reduced with dithiothreitol and alkylated with iodoacetamide. An in-gel tryptic digest was performed overnight at 37°C with gel pieces overlaid with NH_4HCO_3.
The digest was halted with AcOH to pH~4 and peptides were extracted twice from the gel by vortex then sonication. The sample was dried down and resuspended in 20uL of 0.5% AcOH 5%ACN.

Sample Analysis by LC/MS/MS: Samples were analyzed utilizing an Ultimate capillary HPLC system by LC Packings interfaced with a ThermoFinnigan LCQ DecaXP ion trap mass spectrometer in NSI mode. A pressure bomb was implemented to load 1µL of sample onto a pulled tip 75ID capillary column packed with 4cm of Synergi 4u Hydro-RP (Phenomenex). Peptides were eluted over a linear gradient of 5% to 95% mobile phase B over 30 minutes at a flow rate of 100nL/min where mobile phase A was .5%AcOH and mobile phase B was MeCN with .5%AcOH. The most abundant peptides were collected twice and collision induced LC/MS/MS spectra was processed by Xcalibur version 1.2 software.

Database Searching and Protein Identification: MASCOT software was employed to analyze the tandem mass spectrometry peak lists. MASCOT search parameters included: maximum 1 missed cleavage, peptide mass tolerance of 1.5, fragment mass tolerance of 0.8, and a carbamidomethyl fixed modification. Protein identifications were considered significant and correct with manual verification of at least 3 peptides.

Search Parameters

Type of search : **MS/MS Ion Search**
Enzyme : **Trypsin** cuts C-term side of KR unless next residue is P
Fixed modifications : **Carbamidomethyl (C)**
Mass values : **Monoisotopic**
Protein Mass : **Unrestricted**
Peptide Mass Tolerance : **± 1.5 Da**
Fragment Mass Tolerance: **± 0.8 Da**
Max Missed Cleavages : **1**
Instrument type : **ESI-TRAP**
Data File Name : **C:\Inetpub\MASCOT\data\TW77C1\merge.mgf**
Database : **NCBInr 06Mar07**

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 55 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

Sample TW77C1 (~14kDa upper doublet band from lane 5)

Match to: **gi|198578** Score: **59**
ribosomal protein
Nominal mass (M_r): **17730**; Calculated pI value: **11.12**
Sequence Coverage: **5%**

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence	
26 - 34	474.48	946.94	946.59	0.36	0	IAFAITAIK	(Ions score 59)

NOTES: Not confident this protein is in the sample. The spectrum matches fairly well, but there is too low of an abundance of peptides matched for confidence. Please note that this peptide matched about 20 other organisms with the same protein.

Match to: **gi|1655596** Score: **59**
ribosomal protein L31 [Homo sapiens]
Nominal mass (M_r): **14084**; Calculated pI value: **10.54**
Sequence Coverage: **19%**

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence	
11 - 19	494.81	987.60	987.53	0.07	0	SAINEVVTR	(Ions score 44)
98 - 111	823.35	1644.68	1643.92	0.77	0	LYTLVTYVPVTTFK	(Ions score 16)

NOTES: same comments as above ribosomal protein.

Match to: **gi|74003971** Score: **233**
PREDICTED: similar to histone 1, H2ai (predicted) isoform 1 [Canis familiaris]
Nominal mass (M_r): **29091**; Calculated pI value: **10.68**
Sequence Coverage: **24%**
NOTES: Not confident this protein is in the sample. There were a lot of hits for histones from a large variety of organisms (mostly mammalian). I listed this one because it had the largest number of peptides matched, but the MW is way too high for the band sampled for the specific protein to be a correct match. However, some of the peptides matched may be correct. Most of the spectra for the histone hits throughout the Mascot results were not matched to peptide

sequences well enough for me to say they are a confident match, but some were decent. It is quite possible that histones are in the band sampled, but it cannot be stated with confidence from the results.

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence	
58 - 64	416.34	830.66	830.49	0.18	0	STELLIR	(Ions score 43)
124 - 129	715.38	714.37	714.40	-0.03	0	DIQLAR	(Ions score 21)
168 - 177	422.58	1264.72	1264.63	0.09	1	KESYSVYVYK	(Ions score 26)
168 - 177	633.47	1264.92	1264.63	0.29	1	KESYSVYVYK	(Ions score 42)
168 - 177	633.53	1265.04	1264.63	0.41	1	KESYSVYVYK	(Ions score 30)
169 - 177	569.54	1137.06	1136.54	0.52	0	ESYSVYVYK	(Ions score 16)
181 - 191	389.97	1166.88	1167.59	-0.70	0	QVHPDTGISSK	(Ions score 24)
221 - 226	662.98	661.97	663.36	-1.38	0	STITSR	(Ions score 9)
221 - 226	332.81	663.61	663.36	0.26	0	STITSR	(Ions score 18)
227 - 233	408.48	814.95	815.45	-0.50	0	EIQTAVR	(Ions score 26)
227 - 233	408.93	815.84	815.45	0.39	0	EIQTAVR	(Ions score 26)
234 - 242	477.42	952.82	952.60	0.23	0	LLLPGELAK	(Ions score 26)
243 - 250	414.70	827.39	827.41	-0.02	0	HAVSEGTK	(Ions score 16)

Match to: **gi|28566260** Score: **62**
nucleocapsid [Porcine reproductive and respiratory syndrome virus]
Nominal mass (M_r): **13740**; Calculated pI value: **10.21**
Sequence Coverage: **36%**
NOTES: Not confident this protein is in the band sampled. The lower doublet band had a much better hit for the same protein. This band may have had an isoform or PTM of the same protein but in low abundance. The spectra are not strong matches for the sequences matched.

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence	
36 - 39	447.69	446.68	446.26	0.42	1	SRGK	(Ions score 14)
73 - 97	1337.13	2672.24	2671.23	1.01	0	QLCLSSIQTAFNQGAGTCTLSDSGR	(Ions score 20)
98 - 113	944.27	1886.53	1885.97	0.56	0	ISYTVEFSLPTHHTVR	(Ions score 28)

Sample TW77C2 (~13kDa lower doublet band from lane 5)

Match to: **gi|28566268** Score: **191**
nucleocapsid [Porcine reproductive and respiratory syndrome virus]
Nominal mass (M_r): **13738**; Calculated pI value: **10.16**
Sequence Coverage: **55%**
NOTES: Fully confident this or a homologous protein is in the band sampled

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence	
48 - 64	665.13	1992.36	1992.01	0.36	1	KNPEKPHFPLATEDDVR	(Ions score 16)
49 - 64	622.36	1864.05	1863.91	0.14	0	NPEKPHFPLATEDDVR	(Ions score 32)
49 - 64	622.64	1864.90	1863.91	0.99	0	NPEKPHFPLATEDDVR	(Ions score 17)
73 - 97	1336.81	2671.61	2671.23	0.38	0	QLCLSSIQTAFNQGAGTCTLSDSGR	(Ions score 56)
73 - 97	891.75	2672.22	2671.23	0.99	0	QLCLSSIQTAFNQGAGTCTLSDSGR	(Ions score 26)
98 - 113	629.74	1886.20	1885.97	0.24	0	ISYTVEFSLPTHHTVR	(Ions score 29)
98 - 113	629.99	1886.94	1885.97	0.98	0	ISYTVEFSLPTHHTVR	(Ions score 55)
114 - 116	401.92	400.91	400.28	0.63	0	LIR	(Ions score 16)
117 - 123	632.13	631.12	631.32	-0.19	0	VTASPSA	(Ions score 17)
117 - 123	632.34	631.33	631.32	0.02	0	VTASPSA	(Ions score 10)

Proteins matching the same set of peptides:

gi|32441467 **Mass:** 13734 **Score:** 191 **Peptides matched:** 10

nucleocapsid protein [Porcine reproductive and respiratory

syndrome virus]

gi|515977 **Mass:** 13735 **Score:** 188 **Peptides matched:** 10
putative nucleocapsid protein

gi|836925 **Mass:** 13671 **Score:** 188 **Peptides matched:** 10
ORF7; putative nucleocapsid protein

gi|3015503 **Mass:** 13739 **Score:** 188 **Peptides matched:** 10
nucleocapsid protein [Porcine reproductive and respiratory syndrome virus]

gi|5001636 **Mass:** 13767 **Score:** 188 **Peptides matched:** 10
nucleocapsid protein [Porcine reproductive and respiratory syndrome virus]

gi|9630813 **Mass:** 13739 **Score:** 188 **Peptides matched:** 10
nucleocapsid protein N [Porcine respiratory and reproductive syndrome virus]

gi|12711603 **Mass:** 13797 **Score:** 188 **Peptides matched:** 10
structural protein N [Porcine reproductive and respiratory syndrome virus]

gi|20271255 **Mass:** 13653 **Score:** 188 **Peptides matched:** 10
nucleocapsid protein [Porcine reproductive and respiratory syndrome virus]

gi|28566224 **Mass:** 13737 **Score:** 188 **Peptides matched:** 10
nucleocapsid [Porcine reproductive and respiratory syndrome virus]

gi|28566254 **Mass:** 13709 **Score:** 188 **Peptides matched:** 10
nucleocapsid [Porcine reproductive and respiratory syndrome virus]

gi|33307235 **Mass:** 13683 **Score:** 188 **Peptides matched:** 10
nucleocapsid protein [Porcine reproductive and respiratory syndrome virus]

gi|38385777 **Mass:** 13738 **Score:** 188 **Peptides matched:** 10
N [Porcine reproductive and respiratory syndrome virus HN1]

gi|46561809 **Mass:** 13710 **Score:** 188 **Peptides matched:** 10
nucleocapsid protein N [Porcine reproductive and respiratory syndrome virus]

gi|60280984 **Mass:** 13767 **Score:** 188 **Peptides matched:** 10
GP7 envelope glycoprotein [Porcine reproductive and respiratory syndrome virus]

gi|56111968 **Mass:** 13692 **Score:** 187 **Peptides matched:** 10
nucleocapsid [Porcine reproductive and respiratory syndrome virus]

gi|119721024 **Mass:** 13727 **Score:** 186 **Peptides matched:** 10

N nucleocapsid protein [Porcine respiratory and reproductive syndrome virus]

Match to: **gi|12025520** Score: **177**
testis-specific histone 2a [Rattus norvegicus]
Nominal mass (M_r): **14275**; Calculated pI value: **11.02**
Sequence Coverage: **37%**
NOTES: Not confident this protein is in the sample. There were a lot of hits for histones from a large variety of organisms (mostly mammalian). I listed this one because it had the largest number of peptides matched. Some of the peptides matched may be correct. Most of the spectra for the histone hits throughout the Mascot results were not matched to peptide sequences well enough for me to say they are a confident match, but some were decent. It is quite possible that histones are in the band sampled, but it cannot be stated with confidence from the results.

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
22 - 30	472.92	943.82	943.52	0.30	0	AGLQFPVGR (Ions score 37)
22 - 30	472.97	943.93	943.52	0.41	0	AGLQFPVGR (Ions score 31)
34 - 36	401.92	400.91	400.28	0.63	0	LLR (Ions score 16)
83 - 89	425.94	849.87	849.52	0.36	0	HLQLAIR (Ions score 21)
83 - 89	426.01	850.00	849.52	0.49	0	HLQLAIR (Ions score 24)
83 - 96	565.20	1692.56	1691.90	0.67	1	HLQLAIRNDEELNK (Ions score 15)
90 - 100	651.02	1300.03	1299.68	0.36	1	NDEELNKLLGR (Ions score 35)
101 - 119	644.44	1930.28	1930.16	0.12	0	VTIAQGGVLPNIQAVLLPK (Ions score 43)
101 - 119	966.35	1930.69	1930.16	0.53	0	VTIAQGGVLPNIQAVLLPK (Ions score 50)
101 - 119	966.38	1930.74	1930.16	0.58	0	VTIAQGGVLPNIQAVLLPK (Ions score 25)

Match to: **gi|1655596** Score: **163**
ribosomal protein L31 [Homo sapiens]
Nominal mass (M_r): **14084**; Calculated pI value: **10.54**
Sequence Coverage: **27%**
NOTES: Not fully confident this or a homologous protein is in the sample. The spectra are not strong matches for the peptides matched despite the high ion scores.

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
11 - 19	494.78	987.54	987.53	0.01	0	SAINEVVTR (Ions score 66)
11 - 19	494.98	987.95	987.53	0.42	0	SAINEVVTR (Ions score 47)
98 - 111	823.05	1644.08	1643.92	0.17	0	LYTLVTYVPVTTFK (Ions score 58)
98 - 111	1645.82	1644.81	1643.92	0.90	0	LYTLVTYVPVTTFK (Ions score 7)
98 - 111	549.29	1644.83	1643.92	0.92	0	LYTLVTYVPVTTFK (Ions score 23)
112 - 121	1145.33	1144.32	1144.54	-0.21	0	NLQTVNVDEN (Ions score 18)
112 - 121	573.42	1144.83	1144.54	0.30	0	NLQTVNVDEN (Ions score 40)
112 - 121	1146.28	1145.27	1144.54	0.74	0	NLQTVNVDEN (Ions score 22)

Proteins matching the same set of peptides:

gi|4506633 **Mass:** 14454 **Score:** 163 **Peptides matched:** 8
ribosomal protein L31 [Homo sapiens]
gi|12859322 **Mass:** 14402 **Score:** 163 **Peptides matched:** 8
unnamed protein product [Mus musculus]
gi|27680589 **Mass:** 14324 **Score:** 163 **Peptides matched:** 8
PREDICTED: similar to ribosomal protein L31 [Rattus norvegicus]
gi|57043562 **Mass:** 14347 **Score:** 163 **Peptides matched:** 8

PREDICTED: similar to ribosomal protein L31 [Canis familiaris]

gi|73945409 **Mass:** 14439 **Score:** 163 **Peptides matched:** 8

PREDICTED: similar to ribosomal protein L31 isoform 2 [Canis familiaris]

gi|73956342 **Mass:** 14469 **Score:** 163 **Peptides matched:** 8

PREDICTED: similar to ribosomal protein L31 [Canis familiaris]

gi|74004054 **Mass:** 14444 **Score:** 163 **Peptides matched:** 8

PREDICTED: similar to ribosomal protein L31 [Canis familiaris]

gi|74007766 **Mass:** 14286 **Score:** 163 **Peptides matched:** 8

PREDICTED: similar to ribosomal protein L31 isoform 1 [Canis familiaris]

gi|82898755 **Mass:** 14326 **Score:** 163 **Peptides matched:** 8

PREDICTED: similar to ribosomal protein L31 isoform 1 [Mus musculus]

gi|94398865 **Mass:** 17836 **Score:** 163 **Peptides matched:** 8

PREDICTED: similar to ribosomal protein L31 [Mus musculus]

gi|109070885 **Mass:** 14377 **Score:** 163 **Peptides matched:** 8

PREDICTED: similar to ribosomal protein L31 isoform 2 [Macaca mulatta]

gi|109092282 **Mass:** 14415 **Score:** 163 **Peptides matched:** 8

PREDICTED: similar to ribosomal protein L31 [Macaca mulatta]

gi|109104161 **Mass:** 14390 **Score:** 163 **Peptides matched:** 8

PREDICTED: similar to ribosomal protein L31 isoform 1 [Macaca mulatta]

gi|109111958 **Mass:** 14451 **Score:** 163 **Peptides matched:** 8

PREDICTED: similar to ribosomal protein L31 isoform 3 [Macaca mulatta]

gi|109120744 **Mass:** 14400 **Score:** 163 **Peptides matched:** 8

PREDICTED: similar to ribosomal protein L31 [Macaca mulatta]

gi|109128995 **Mass:** 14492 **Score:** 163 **Peptides matched:** 8

PREDICTED: similar to ribosomal protein L31 isoform 1 [Macaca mulatta]

gi|109511077 **Mass:** 18321 **Score:** 163 **Peptides matched:** 8

PREDICTED: similar to ribosomal protein L31 [Rattus norvegicus]

gi|109512301 **Mass:** 17869 **Score:** 163 **Peptides matched:** 8

PREDICTED: similar to ribosomal protein L31 [Rattus norvegicus]

gi|109133243 **Mass:** 14427 **Score:** 161 **Peptides matched:** 8

PREDICTED: similar to ribosomal protein L31 [Macaca mulatta

Sample TW77C3 (~50kDa band from lane 6)

Match to: **gi|230921** Score: **255**
Chain L, Ribulose 1,5-Bisphosphate Carboxylase(Slash)oxygenase (Form III) (E.C.4.1.1.39)
Nominal mass (M_r): **53452**; Calculated pI value: **6.41**
Sequence Coverage: **24%**
NOTES: Confident this or a homologous protein is in the band sampled.

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence	
22 - 32	1406.65	1405.64	1405.68	-0.03	0	LTYYTPEYQTK	(Ions score 14)
22 - 32	703.94	1405.87	1405.68	0.20	0	LTYYTPEYQTK	(Ions score 31)
22 - 32	704.51	1407.01	1405.68	1.34	0	LTYYTPEYQTK	(Ions score 16)
33 - 41	511.33	1020.65	1020.52	0.13	0	DTDILAAFR	(Ions score 56)
195 - 213	724.28	2169.80	2168.98	0.82	1	GGLDFTKDDENVNSQPFMR	(Ions score 23)
218 - 227	631.17	1260.33	1260.62	-0.29	0	FLFCAEALYK	(Ions score 38)
218 - 227	631.45	1260.88	1260.62	0.26	0	FLFCAEALYK	(Ions score 26)
259 - 285	1512.25	3022.48	3021.45	1.04	0	ELGVPIVMHDYLTGGFTANTSLAHYCR	(Ions score 21)
340 - 358	1133.76	2265.50	2265.13	0.38	1	DITLGFVDLLRDDFVEQDR	(Ions score 24)
436 - 446	629.44	1256.86	1256.64	0.23	0	DLAQEGNEIIR	(Ions score 36)
436 - 446	629.46	1256.91	1256.64	0.28	0	DLAQEGNEIIR	(Ions score 35)
451 - 463	774.18	1546.34	1545.73	0.61	0	WSPELAAACEVWK	(Ions score 29)

Proteins matching the same set of peptides:

gi|475728 **Mass:** 52431 **Score:** 255 **Peptides matched:** 12

ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [Atropa belladonna]

gi|515239 **Mass:** 50039 **Score:** 255 **Peptides matched:** 12

Chain A, Ribulose-1,5-Bisphosphate CarboxylaseOXYGENASE (RUBISCO) (E.C.4.1.1.39)

gi|4262869 **Mass:** 53408 **Score:** 255 **Peptides matched:** 12

ribulose-1,5-biphosphate carboxylase/ oxygenase large subunit [Nicotiana tabacum]

gi|6093933 **Mass:** 52421 **Score:** 255 **Peptides matched:** 12

Ribulose bisphosphate carboxylase large chain (RuBisCO large subunit)

gi|7546555 **Mass:** 53146 **Score:** 255 **Peptides matched:** 12

Chain L, Crystal Structure Of Unactivated Tobacco Rubisco With Bound Phosphate Ions

gi|11182399 **Mass:** 52223 **Score:** 255 **Peptides matched:** 12

ribulose-1,5-bisphosphate carboxylase/oxygenase [Lycium chinense]

gi|11465965 **Mass:** 53378 **Score:** 255 **Peptides matched:** 12

ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [Nicotiana tabacum]

gi|28261725 **Mass:** 53404 **Score:** 255 **Peptides matched:** 12

ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [Atropa belladonna]

gi|81301574 **Mass:** 53378 **Score:** 255 **Peptides matched:** 12

ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [Nicotiana tomentosiformis]

gi|125859071 **Mass:** 52225 **Score:** 255 **Peptides matched:** 12

ribulose bisphosphate carboxylase large subunit [Lycium ferocissimum]

Sample TW77C4 (~70kDa band from lane 7)

Match to: **gi|1351907** Score: **1116**
Serum albumin precursor (Allergen Bos d 6) (BSA)
Nominal mass (M_r): **71244**; Calculated pI value: **5.82**
Sequence Coverage: **51%**
NOTES: Fully confident this protein is in the band sampled

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
66 - 75	582.47	1162.92	1162.62	0.30	0	LVNELTEFAK (Ions score 43)
66 - 75	582.47	1162.93	1162.62	0.31	0	LVNELTEFAK (Ions score 53)
76 - 88	732.39	1462.77	1462.58	0.19	0	TCVADESHAGCEK (Ions score 19)
89 - 100	474.03	1419.05	1418.69	0.37	0	SLHTLFGDELCK (Ions score 16)
89 - 100	474.08	1419.22	1418.69	0.54	0	SLHTLFGDELCK (Ions score 27)
89 - 100	710.68	1419.35	1418.69	0.67	0	SLHTLFGDELCK (Ions score 41)
161 - 167	464.39	926.76	926.49	0.28	0	YLYEIAR (Ions score 34)
161 - 167	464.45	926.88	926.49	0.40	0	YLYEIAR (Ions score 25)
168 - 183	682.46	2044.34	2044.02	0.32	1	RHPYFYAPELLYYANK (Ions score 41)
168 - 183	682.49	2044.43	2044.02	0.41	1	RHPYFYAPELLYYANK (Ions score 50)
169 - 183	945.16	1888.31	1887.92	0.39	0	HPYFYAPELLYYANK (Ions score 39)
169 - 183	630.51	1888.49	1887.92	0.57	0	HPYFYAPELLYYANK (Ions score 31)
184 - 197	874.60	1747.18	1746.70	0.49	0	YNGVFQECCQAEDK (Ions score 45)
184 - 204	830.08	2487.20	2486.10	1.10	1	YNGVFQECCQAEDKGACLLPK (Ions score 27)
198 - 204	758.28	757.27	757.42	-0.14	0	GACLLPK (Ions score 21)
249 - 256	922.50	921.49	921.48	0.01	0	AEFVEVTK (Ions score 22)
257 - 263	789.41	788.40	788.46	-0.06	0	LVTDLTK (Ions score 21)
257 - 263	789.48	788.47	788.46	0.01	0	LVTDLTK (Ions score 27)
257 - 263	395.45	788.89	788.46	0.43	0	LVTDLTK (Ions score 34)
286 - 297	722.32	1442.62	1442.63	-0.01	0	YICDNQDTISSK (Ions score 35)
286 - 297	722.75	1443.49	1442.63	0.86	0	YICDNQDTISSK (Ions score 13)
298 - 309	511.70	1532.06	1531.77	0.29	1	LKECCDKPLLEK (Ions score 20)
347 - 359	784.66	1567.30	1566.74	0.57	0	DAFLGSFLYEYSR (Ions score 61)
347 - 359	785.04	1568.06	1566.74	1.33	0	DAFLGSFLYEYSR (Ions score 46)
360 - 371	480.52	1438.53	1438.80	-0.27	1	RHPEYAVSVLLR (Ions score 49)
360 - 371	480.78	1439.31	1438.80	0.51	1	RHPEYAVSVLLR (Ions score 44)
360 - 371	480.92	1439.72	1438.80	0.92	1	RHPEYAVSVLLR (Ions score 32)
375 - 386	751.99	1501.96	1501.61	0.36	0	EYEATLEECCAK (Ions score 27)
375 - 386	752.05	1502.09	1501.61	0.49	0	EYEATLEECCAK (Ions score 45)
375 - 386	1503.82	1502.81	1501.61	1.21	0	EYEATLEECCAK (Ions score 31)
387 - 399	778.07	1554.12	1553.65	0.48	0	DDPHACYSTVFDK (Ions score 30)
387 - 399	519.11	1554.30	1553.65	0.66	0	DDPHACYSTVFDK (Ions score 30)
402 - 412	435.95	1304.81	1304.71	0.11	0	HLVDEPQNLIK (Ions score 38)
402 - 412	653.56	1305.11	1304.71	0.40	0	HLVDEPQNLIK (Ions score 42)
402 - 412	653.57	1305.12	1304.71	0.41	0	HLVDEPQNLIK (Ions score 34)
421 - 433	494.08	1479.21	1478.79	0.43	0	LGEYGFQNALIVR (Ions score 41)
421 - 433	740.72	1479.43	1478.79	0.65	0	LGEYGFQNALIVR (Ions score 33)
421 - 433	740.77	1479.53	1478.79	0.75	0	LGEYGFQNALIVR (Ions score 46)
437 - 451	820.49	1638.96	1638.93	0.03	1	KVPQVSTPTLVEVSR (Ions score 34)
437 - 451	547.47	1639.39	1638.93	0.46	1	KVPQVSTPTLVEVSR (Ions score 26)
437 - 451	547.56	1639.65	1638.93	0.72	1	KVPQVSTPTLVEVSR (Ions score 40)
469 - 482	575.84	1724.50	1723.83	0.68	0	MPCTEDYLSLILNR (Ions score 26)
499 - 507	569.94	1137.86	1137.49	0.37	0	CCTESLVNR (Ions score 27)

508 - 523	627.77	1880.29	1879.91	0.38	0	RPCFSALTPDETYVPK	(Ions score 25)
508 - 523	941.25	1880.48	1879.91	0.57	0	RPCFSALTPDETYVPK	(Ions score 34)
529 - 544	954.73	1907.45	1906.91	0.54	0	LFTFHADICTLPDTEK	(Ions score 36)
529 - 544	637.05	1908.12	1906.91	1.21	0	LFTFHADICTLPDTEK	(Ions score 22)
548 - 557	381.82	1142.44	1141.71	0.74	1	KQTALVELLK	(Ions score 50)
549 - 557	1014.43	1013.42	1013.61	-0.19	0	QTALVELLK	(Ions score 27)
549 - 557	507.96	1013.90	1013.61	0.29	0	QTALVELLK	(Ions score 47)
549 - 557	508.45	1014.88	1013.61	1.27	0	QTALVELLK	(Ions score 29)
569 - 580	700.48	1398.94	1398.69	0.26	0	TVMENFVAFVDK	(Ions score 44)
569 - 580	700.53	1399.05	1398.69	0.37	0	TVMENFVAFVDK	(Ions score 39)
581 - 597	964.60	1927.18	1926.79	0.39	1	CCAADDKEACFAVEGPK	(Ions score 25)
581 - 597	964.69	1927.37	1926.79	0.58	1	CCAADDKEACFAVEGPK	(Ions score 48)
581 - 597	643.54	1927.58	1926.79	0.79	1	CCAADDKEACFAVEGPK	(Ions score 33)
581 - 597	643.57	1927.68	1926.79	0.89	1	CCAADDKEACFAVEGPK	(Ions score 26)
598 - 607	501.88	1001.75	1001.58	0.18	0	LVVSTQTALA	(Ions score 55)

Results obtained from Alphalyse service

Protein Identification Report

Order 10883

Sample Matanin 4 Hit 1

Protein Information

Protein name:	RPS4X protein [Homo sapiens]
Alphalyse number:	ALPHA06633
GI-number:	gi|48376549
MW:	27243
pI:	9,95
Mascot score:	104
Sequence coverage:	33%

Analysis Information

- In-gel digestion, cleavage by Trypsin: cuts C-term side of KR unless next residue is P
- MS analysis method: MALDI-TOF peptide mass fingerprint and MALDI-TOF/TOF peptide sequencing
- Variable modifications: Carbamidomethyl (C),Oxidation (M)
- Database search program: Mascot version 2.1.03
- Peptide Tolerance: 60 ppm
- Allowed up to 1 miscleavage
- Database: NRDB (3946334 protein sequences)

Protein sequence

Matched peptides shown in bold underline

```
  1 DKLTGVFAPR PSTGPHKLRE CLPLIIFLRN RLKYALTGDE VKKICMQRFI
 51 KIDGKVRTDI TYPAGFMDVI SIDKTGENFR LIYDTKGRFA VHRITPEEAK
101 YKLCKVRKIF VGTKGIPHLV THDARTIRYP DPLIKVNDTI QIDLETGKIT
151 DFIKFDTGNL CMVTGGANLG RIGVITNRER HPGSFDVVHV KDANGNSFAT
201 RLSNIFVIGK GNKPWISLPR GKGIRLTIAE ERDKRLAAKQ SSG
```

Peptides used for identification

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
3 - 17	1564.80	1563.79	1563.85	-0.06	0	K.LTGVFAPRPSTGPHK.L
87 - 93	842.52	841.52	841.47	0.05	1	K.GRFAVHR.I
94 - 102	1078.59	1077.58	1077.57	0.01	1	R.ITPEEAKYK.L
115 - 125	**1215.66**	**1214.65**	**1214.65**	**0.00**	**0**	**K.GIPHLVTHDAR.T (Ions score 39)**
155 - 171	1798.82	1797.81	1797.81	0.00	0	K.FDTGNLCMVTGGANLGR.I Carbamidomethyl (C); Oxidation (M)
179 - 191	1506.78	1505.77	1505.77	0.00	1	R.ERHPGSFDVVHVK.D
211 - 220	**1167.67**	**1166.67**	**1166.66**	**0.01**	**0**	**K.GNKPWISLPR.G (Ions score 37)**

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Appendix B

Lectin affinity chromatography

During process development, lectin affinity chromatography was investigated as method for fractionating detergent solubilized GP5. Table 2.1 lists the reported number of glycosylation sites associated with each PRRSV protein. Of the three major structural proteins, GP5 is the only glycosylated molecule. It was hypothesized that a lectin affinity stationary phase would bind GP5 and provide valuable separation from non-glycosylated proteins.

GP5 is heterogeneously glycosylated as discussed in Chapter 2. Therefore it was worth exploring the possibility that lectin affinity could fractionate between glycosylated GP5 and/or GP5/M molecules by exploiting presumed differences in protein sugar modifications. This could be accomplished by applying a linear sugar elution gradient to a lectin affinity column. Lectin affinity could also serve as a characterization tool in addition to serving as a separation medium.

Two lectin affinity resins were purchased from GE Healthcare (Waukesha, WI). Lentil Lectin Sepharose 4B is an affinity ligand useful for purification of detergent-solubilized membrane glycoproteins and viral glycoproteins. The resin is compatible with certain detergents, so it is well suited for this work. Concanavalin A (Con A) Sepharose 4B is another lectin affinity ligand useful for similar reasons. However, Con A performance is compromised when detergents are necessary. This resin was also screened because it reportedly has more robust binding performance than Lentil Lectin.

Several experiments were designed to explore the worth of each resin for GP5 fractionation. Lectin affinity was first explored as a primary chromatographic step in the process, directly after PRRSV protein solubilization with detergents as outlined in Chapter 3. Experiments performed are outlined in Table B1. Resins were packed a C glass column (GE Healthcare) to a 3 mL bed volume.

Table B1. Summary of lectin affinity chromatography experiments designed for a first chromatographic step during process development.

Run No.	Resin	Sample	Equilibration (EQ)	Binding/wash (A1)	Elution (B1)	Result
1	LL	2 mL; 0.5% sarkosyl	20 mM Tris-HCl, 0.5 M NaCl, 0.5% sarkosyl, 1 mM $CaCl_2$ pH 7.4	20 mM Tris-HCl, 0.5 M NaCl, 0.5% sarkosyl, pH 7.4	0.3 M mannoside, 20 mM Tris-HCl, 0.5 M NaCl, 0.05% sarkosyl, pH 7.4	GP5 collected in flow-through, column leaching
2	LL	2 mL; 0.5% sarkosyl	20 mM Tris-HCl, 0.5 M NaCl, 0.5% sarkosyl, 1 mM $CaCl_2$, pH 7.4	20 mM Tris-HCl, 0.5 M NaCl, 0.5% sarkosyl, pH 8.3	0.3 M mannoside, 20 mM Tris-HCl, 0.5 M NaCl, 0.05% sarkosyl, pH 8.3	GP5 collected in flow-through, column leaching
3	LL	2 mL; 1.0% sodium deoxycholate	20 mM Tris-HCl, 0.5 M NaCl, 0.5% sarkosyl, 1 mM $CaCl_2$, pH 7.4	20 mM Tris-HCl, 0.5 M NaCl, 0.5% sodium deoxycholate, pH 8.3	A1 + 0.3 M mannoside	High sample viscosity clogged column
4	Con A	2 mL; 0.5% sarkosyl	20 mM Tris-HCl, 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, pH 7.4	Same as EQ	0.3 M mannoside, 20 mM Tris-HCl, 0.5 M NaCl, 0.5% sodium deoxycholate, pH 8.3	43 kDa and 19 kDa bands not present in flow through (identified by αPRRSV Western blot)
5	Con A	2 mL; 0.5% sarkosyl	20 mM Tris-HCl, 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, pH 7.4	Same as EQ	0.3 M mannoside, 20 mM Tris-HCl, 0.5 M NaCl, 0.5% sodium deoxycholate, pH 8.3	Same as Run No. 4. Leaching of Con A detected during run. Leaching was severe during cleaning cycle
6	Con A	4 mL; 0.5% sarkosyl	20 mM Tris-HCl, 0.5 M NaCl, 1mM $CaCl_2$, 1mM $MnCl_2$ pH 7.4	Same as EQ	0.3 M mannoside, 20 mM Tris-HCl, 0.5 M NaCl, 0.5% sodium deoxycholate, pH 8.3	Severe lectin leaching
7	LL	1.2 mL; 2% Triton X-100	20 mM Tris-HCl, 0.5 M NaCl, 1mM $CaCl_2$, 1mM $MnCl_2$ pH 7.4	Same as EQ	0.3 M mannoside, 20 mM Tris-HCl, 0.5 M NaCl, 0.5% sodium deoxycholate, pH 8.3	Some lectin leaching but not as severe as Con A, degraded peptides present in flow-through (identified by αPRRSV Western blot)

Table B1 shows that lectin affinity chromatography was not successful. Immobilized lectin proteins often leached from the column and contaminated samples. This was particularly severe for Con A when detergent was included in the sample or in chromatography buffers. GP5 did not bind to the column in most cases as it was often identified, via Western blot, in flow-through fractions.

The 43 kDa protein (possible GP5/M complex) and N were not detected in the flow through fractions of Run 4. Upon further investigation in Run 5, it was determined that severe Con A leaching compromised the benefit of any possible separation that could be taking place.

Ultimately, the use of lectin affinity chromatography as a first chromatographic step was abandoned. Consistent column leaching and complications from detergent use, such as high buffer viscosity and resin incompatibility, were among the reasons for this decision.

The use of lectin affinity as a second chromatographic step was briefly explored. As discussed in Chapter 3, GP5 and N elution from CEX overlapped and there remained a need to separate them. GP5 is glycosylated while N is not. Detergent concentration in CEX elution fractions was low (0.03% Triton X-100), therefore it seemed buffer conditions would not cause lectin leaching.

CEX chromatography was performed as discussed in Chapter 3. Briefly, PRRS virions were incubated with 1% Triton X-100, loaded onto the column, and target proteins were eluted with 2 M NaCl buffer containing 0.03% Triton X-100. Elution fractions were pooled for lectin affinity chromatography. Experimental conditions are summarized in Table B2.

Table B2. Summary of lectin affinity chromatography experiments designed for a second chromatographic step following CEX.

Run No.	Resin	Sample	Equilibration (EQ)	Binding/wash (A1)	Elution (B1)	Result
1	LL	CEX elution fractions; 0.03% Triton X-100	20 mM Tris-HCl, 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, pH 7.0	20 mM Tris-HCl, 0.5 M NaCl, 0.03% Triton X-100, pH 7.0	A1 + 0.5 M mannoside	N contained in flow-through. It was not clear where GP5 was. Lectin leaching contaminates elution fractions.
2	Con A	CEX elution fractions; 0.03% Triton X-100	20 mM Tris-HCl, 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, pH 7.0	Same as EQ	20 mM Tris-HCl, 0.5 M NaCl, 0.5 M mannoside, pH 7.0	Lectin leached throughout run. Leaching was severe during elution.
3	LL	CEX elution fractions; 0.03% Triton X-100	20 mM Tris-HCl, 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, pH 7.0	Same as EQ	20 mM Tris-HCl, 0.5 M NaCl, 0.5 M mannoside, pH 7.0	N in flow-through. Possible pure GP5 band at start of elution detected with SDS-PAGE. Band did not react with αORF5 in Western blot. Lectin leaching occurred.

As discussed in Table B2, the use of lectin affinity chromatography after CEX was unsuccessful for GP5 isolation. For run 1, Triton X-100 was present in the sample and included in the binding buffer. Although the concentration of Triton X-100 was low, this detergent still causes leaching of immobilized lectins from the Lentil Lectin column.

For run 2, CEX fractions were also loaded onto a Con A column and separation was attempted without the use of detergent in chromatography buffers. Significant leaching was still apparent due to the presence of Triton X-100 in the sample.

For run 3, the same buffer conditions used in run 2 were applied for the lentil lectin column. Evidence suggests that N does not bind to the lentil lectin resin under these conditions. However no conclusive evidence by Western blot (αORF5) identification was produced to indicate that GP5 binds and was collected during elution. Leaching was still evident with lentil lectin affinity chromatography when no detergent when Triton X-100 was included only in the sample. In conclusion, lectin affinity chromatography is not suitable as a second chromatographic step for further purification of GP5 after CEX chromatography.

Appendix C

CsCl density gradient for virion isolation

Cesium chloride (CsCl) isopycnic ultracentrifugation was explored as an alternative to sucrose cushion ultracentrifugation for isolation of PRRS virions. The procedure followed was adapted from Meng et al. (1994), listed in Chapter 2.

PRRSV infected MARC-145 cells were subjected to three freeze/thaw cycles and cell debris was clarified at 1,000 × g for 15 min. at 4°C. Virions were precipitated from solution during incubation with 7% polyethylene glycol 8000 supplemented with 2.3% NaCl, overnight at 4°C with gentle agitation. Precipitated virions were pelleted at 10,000 × g for 15 min. at 4°C and resuspended in TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 7.4). The buoyant density of PRRSV is 1.18-1.19 g/mL in CsCl. Dry CsCl obtained from Fisher Scientific (Pittsburgh, PA) was added to TE buffer to reach a final density of ρ_{2x} = 1.46 g/mL. The 2 × solution was mixed with equal volume of virus suspension for a final CsCl density of ρ_{1x} = 1.23 g/mL. The solution was loaded into an Ultra-Clear ½ x 2 in. ultracentrifuge tubes (Beckman Coulter, Inc., Fullerton, CA) set in a SW-50 swinging bucket rotor (Beckman). Tubes were spun at 34,000 rpm (100,000 × g) and 4°C for 18 h.

After ultracentrifugation, virions were isolated within the gradient and were harvested with a syringe. The sample was injected directly into a dialysis cassette (7,000 MWCO) and dialyzed overnight against several changes of TE buffer. Fig. C1 shows the effect of CsCl density gradient ultracentrifugation. It is important to note that the primary antibody used for the Western blot, αPRRSV, was from the first lot obtained from NVSL. This lot consistently reacted with GP5, as seen is Fig. C1, when CsCl density gradient experiments were performed.

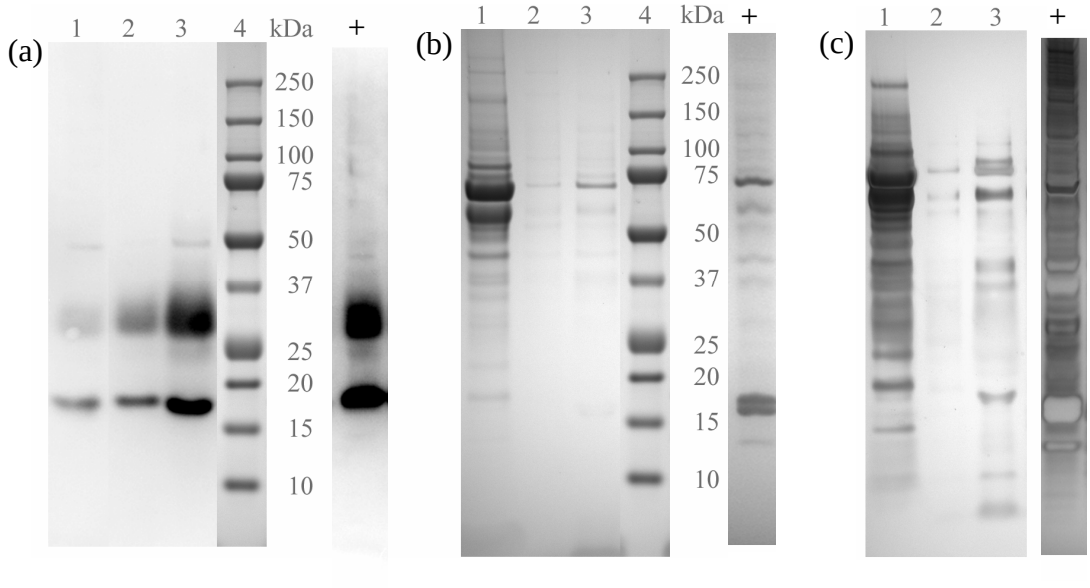

Figure C1. (a) Western blot (αPRRSV) and (b) Coomassie-stained and (c) silver-stained SDS-PAGE gel of CsCl density gradient procedure. L1: PRRSV cell culture. L2: PRRS virions isolated from the CsCl procedure outlined above. L3: contents of L2, concentrated 10 ×. L4: protein marker. +: PRRS virions isolated by sucrose cushion as described in Chapter 3.

As evident in Fig. C1, the CsCl density gradient method for isolation and concentration of PRRS virions yields desired results. GP5 is enriched while cell culture components, including BSA, are discarded. SDS-PAGE and Western blot results from sucrose cushion purified virions are included for comparison. The volumetric concentration factor for the sucrose cushion sample was 20 × and for the CsCl sample was 10 ×. Therefore although + is 2 × more concentrated than L2 on a volumetric basis, it is evident that the sucrose cushion protocol yielded higher protein recovery, although it is a bit less pure. In terms of efficiency, the sucrose cushion procedure took 4 h while the CsCl procedure took 48 h. The sucrose cushion procedure also consumed fewer laboratory resources such as dialysis supplies. It was concluded that the sucrose cushion alternative was clearly superior as a first processing step in terms of recovery and efficiency.

Appendix D

Detergent screening experiments

GP5 and M are both embedded in the lipid envelope that surrounds the nucleocapsid core of PRRSV. Once PRRSV was isolated, the lipid envelope was disrupted to release and subsequently solubilize target membrane proteins. This was achieved by detergent incubation. Multiple detergents were considered and screened at varying concentrations, buffer conditions, and incubation times. Triton X-100, octylglucoside, CHAPS, and sarkosyl were explored for target protein solubilization.

Octylglucoside, CHAPS, and 30% N-Lauroylsarcosine sodium salt solution (sarkosyl) were obtained from Sigma (St. Louis, MO). One hundred micro-liter aliquots of PRRS virions isolated by sucrose cushioning were mixed with equal volume of a 2 × detergent solution prepared in buffer (20 mM Tris-HCl, 0.5 M NaCl, pH 7.1). The final detergent concentration of each solubilization design is listed in Fig. D1. Samples were incubated at 4°C for 19 h with gentle agitation. Detergent incubation was terminated by ultracentrifugation at 47,000 rpm (100,000 × g) at 4°C for 1 h (TLA-55 rotor, Beckman Coulter). By definition, solubilized proteins remain in the supernatant after ultracentrifugation while insoluble components are recovered in the pellet. Supernatant samples were run on SDS-PAGE gels and probed with αPRRSV antibody in Western blotting to analyze solubilization results. The silver stained SDS-PAGE gels and Western blots are presented in Fig. D1.

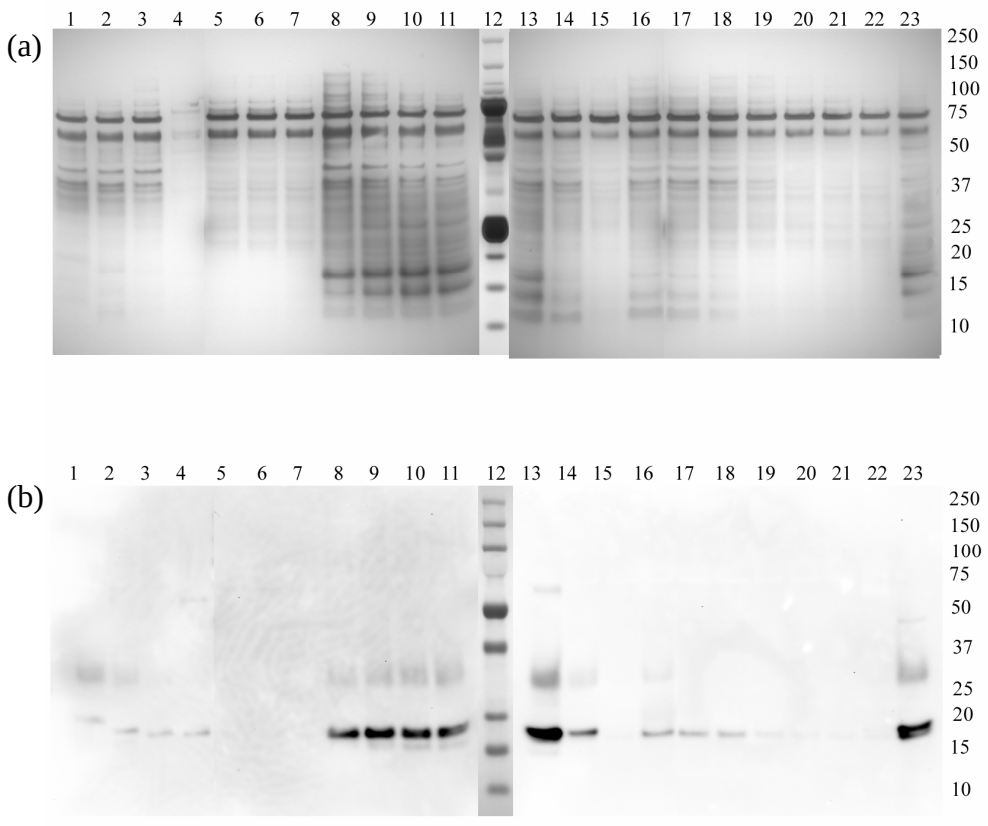

Figure D1. (a) Silver stained SDS-PAGE gel and (b) Western blot (αPRRSV) of supernatant fractions of detergent solubilized PRRS virions. L1-7: octylglucoside detergent screened at 2.5%, 1%, 0.5%, 0.25%, 0.1%, 0.05%, and 0.025% (v/v) respectively. L12: protein marker. L8-15: sarkosyl detergent screened at 2.5%, 1%, 0.5%, 0.1%, 0.05%, 0.025%, and 0.01% (v/v) respectively. L16-22: CHAPS detergent screened at 2.5%, 1%, 0.5%, 0.25%, 0.1%, 0.05%, and 0.025% (v/v) respectively. L23: PRRS virions purified by sucrose cushion.

Of the three detergents screened sarkosyl provided the best recovery of soluble protein. As apparent in Fig. D1(b), GP5 and N are effectively solubilized at concentrations between 0.05% and 2.5% (v/v) sarkosyl. GP5 and N activity is maintained as both proteins react with the Western blot primary antibody. Octylglucoside does not produce the desired solubilization effect at any concentration screened, although at high concentrations it may solubilize GP5 but not N. The effect of

CHAPS on PRRSV proteins is also mixed. N is partially recovered in supernatant fractions of samples solubilized with high concentrations of CHAPS but there is no sign of GP5 recovery. Compared to sarkosyl, both octylglucoside and CHAPS are less effective solubilizing agents for target PRRSV proteins. Sarkosyl was ultimately selected for initial process development. Sarkosyl is also significantly less expensive than octylglucoside and CHAPS detergents.

Sarkosyl is an ionic detergent. Although it provided robust solubilization of PRRSV proteins, its ionic properties were not compatible with chromatographic resins. Charged sarkosyl molecules are not suitable for ion-exchange chromatography. Sarkosyl should also be avoided when lectin affinity chromatography is employed because it may precipitate in the presence of divalent cations (Mn^{+} and Ca^{+}), which are required for binding. This may explain the poor results obtained with lectin affinity chromatography, as discussed in Appendix B.

Because the detergents presented in Fig. D1 either did not solubilize PRRSV proteins or complicated chromatography, Triton X-100 was explored as an alternative detergent. Triton X-100 was screened at a final concentration of 0.5% (v/v) with or without 0.5 M NaCl in the solubilization buffer. Samples were mixed with equal volume of 2 × detergent buffer (5 mM Tris-HCl, 1% Triton X-100, pH 7.0, ± 0.5 M NaCl) and incubated for 25 min. at room temperature. A short incubation time at room temperature was desired to improve overall process efficiency. After incubation samples were ultracentrifuged at 47,000 rpm (100,000 × g) at 4°C for 1 h (TLA-55 rotor, Beckman Coulter). Supernatant fractions were pulled off and residual pellet was resuspended in 2 × detergent buffer. SDS-PAGE and Western blot (αPRRSV) results are presented in Fig. D2.

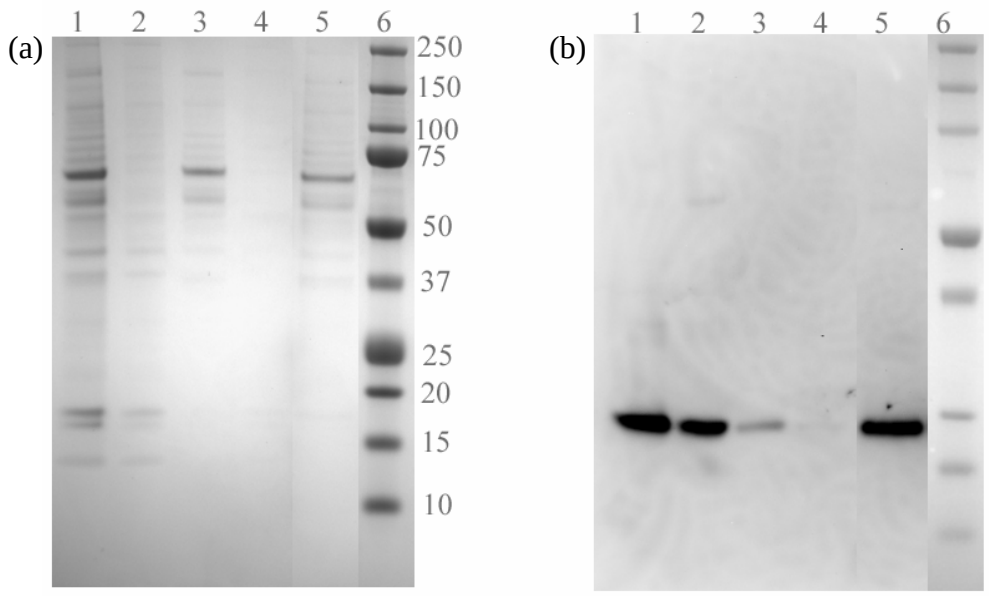

Figure D2. (a) Coomassie stained SDS-PAGE gel and (b) Western blot (αPRRSV) of supernatant fractions of Triton X-100 solubilized PRRS virions. GP5 did not react with αPRRSV in this blot. L1: PRRS virions purified by sucrose cushion. L2: resuspended pellet after 0.5% Triton X-100 treatment with no NaCl. L3: supernatant after 0.5% Triton X-100 treatment with no NaCl. L4: resuspended pellet after 0.5% Triton X-100 treatment with 0.5 M NaCl. L5: supernatant after 0.5% Triton X-100 treatment with 0.5 M NaCl. L6: protein marker.

Fig. D2 shows that 0.5 M NaCl is necessary to achieve the desired solubilization of PRRSV proteins. Soluble proteins are recovered in supernatant fractions in the presence of NaCl while without NaCl proteins are recovered in resuspended pellet fractions. It is worth noting that GP5 is not visible in Fig. D2(b) because αPRRSV antibody response varied as discussed previously in this thesis. It can be assumed, based on results presented in Chapter 3 (Fig. 1), that GP5 is recovered in the soluble supernatant fraction shown in lane 5 above. The detergent incubation scheme presented for Triton X-100 was utilized in the final process design.

Appendix E

FPLC chromatography methods

The chromatography methods presented in Appendix E were created using the Unicorn software (version 3.10) and carried out on an ÄKTA Explorer 100 FPLC chromatography system (G.E. Healthcare).

Table E1. FPLC method used for the cation-exchange experiment outlined in Chapter 3 (40 min. linear elution gradient from 0 – 2 M NaCl).

```
Method: C:\UNICORN\Local\fil\default\method\Brad\02172007 CEX Run.m02
Main method:
¤ Main
 0.00  Base Time
 0.00  Flow 1 {ml/min}
 1.00  ColumnPosition Position5
 11.00  InjectionValve Inject
 11.00  Fractionation 18mm 1 {ml} FirstTube Volume
 11.00  Message "Sample Loading" Screen
 11.00  SampleFlow 1 {ml/min}
 13.5  Message "Add 6 mL binding buffer to sample" Screen
 20.00  SampleFlow 0 {ml/min}
 20.00  InjectionValve Load
 25.00  Gradient 100 {%B} 40 {base}
 25.00  Set_Mark "0 M NaCl"
 45.00  Set_Mark "1.0 M NaCl"
 65.00  Set_Mark "2.0 M NaCl"
 75  PumpAInlet A2
 75  Gradient 0.00 {%B} 0.00 {base}
 85  End_Method
```

Table E2. FPLC method used for the cation-exchange step in the final CEX-HIC process, as outlined in Chapter 3 (2 M NaCl step elution).

Method: C:\UNICORN\Local\fil\default\method\Brad\04182007 CEX Run Step 2M elution .m02
Main method:
¤ Main
0.00 Base Time
0.00 Flow 1 {ml/min}
0.00 ColumnPosition Position5
0.00 InjectionValve Inject
0.00 Fractionation 18mm 1 {ml} FirstTube Volume
0.00 Message "Sample Loading" Screen
0.00 SampleFlow 1 {ml/min}
7.50 Message "Add 6 mL binding buffer to sample" Screen
21.00 SampleFlow 0 {ml/min}
21.00 InjectionValve Load
31.00 Gradient 100 {%B} 0 {base}
31.00 Set_Mark "2.0 M NaCl"
51.00 Gradient 0.00 {%B} 0.00 {base}
51.00 FractionationStop
51.00 PumpAInlet A2
71.00 PumpAInlet A1
91.00 End_Method

Table E3. FPLC method used for the hydrophobic-interaction step in the final CEX-HIC process, as outlined in Chapter 3 (two-stage elution).

Method: C:\UNICORN\Local\fil\default\method\Brad\04142007 HIC Run Dual Elution.m02
Main method:
¤ Main
0.00 Base Time
0.00 Flow 1 {ml/min}
0 BufferValveA1 A12
0 PumpBInlet B2
0 ColumnPosition Position6
1.00 InjectionValve Inject
1.00 Fractionation 18mm 1 {ml} FirstTube Volume
1.00 Message "Sample Loading" Screen
1.00 SampleFlow 1 {ml/min}
2.50 Message "Add 6 mL binding buffer to sample" Screen
7.00 SampleFlow 0 {ml/min}
7.00 InjectionValve Load
20.00 Gradient 100 {%B} 0 {base}
20.00 Set_Mark "Elution 1"
40.00 Gradient 0.00 {%B} 0.00 {base}
40.00 BufferValveA1 A13
40.00 Set_Mark "Elution 2"
61.00 PumpAInlet A2
81.00 PumpAInlet A1
81.00 FractionationStop
101.00 BufferValveA1 A12
121.00 End_Method

Table E4. FPLC method used for lectin affinity chromatography experiments summarized in Appendix B.

Method: C:\UNICORN\Local\fil\default\method\Brad\04022007 LentilLectin C Column 3 ml bed.m02
Main method:
¤ Main
0.00 Base Time
0.00 Flow 0.75 {ml/min}
0.5 ColumnPosition Position4
2.50 Fractionation 18mm 1.00 {ml} FirstTube Volume
2.50 InjectionValve Inject
2.50 SampleFlow 0.50 {ml/min}
17.50 SampleFlow 0.00 {ml/min}
17.50 InjectionValve Load
37.50 Gradient 100 {%B} 0.00 {base}
67.50 Gradient 0.00 {%B} 0.00 {base}
67.50 End_Method

Table E5. FPLC method used to clean lectin affinity resins (Lentil Lectin and Con A) after experiments summarized in Appendix B.

Method: C:\UNICORN\Local\fil\default\method\Brad\04022007 Cleaning Cycle for Lentil Lectin with line flush.m02
Main method:
¤ Main
0.00 Base Time
0.00 BufferValveA1 A13
0.00 PumpBInlet B2
0.00 Gradient 50 {%B} 0.00 {base}
0.00 Flow 4 {ml/min}
11.00 Flow 0.75 {ml/min}
11.00 Gradient 0 {%B} 0.00 {base}
12.00 ColumnPosition Position4
12.00 Set_Mark "1. pH 8.5"
33.00 Gradient 100 {%B} 0.00 {base}
33.00 Set_Mark "1. pH 4.5"
53.00 Gradient 0 {%B} 0.00 {base}
53.00 Set_Mark "2. pH 8.5"
73.00 Gradient 100 {%B} 0.00 {base}
73.00 Set_Mark "2. pH 4.5"
93.00 Gradient 0.00 {%B} 0.00 {base}
93.00 Set_Mark "3. pH 8.5"
113.00 Gradient 100 {%B} 0.00 {base}
113.00 Set_Mark "3. pH 4.5"
133.00 Gradient 0.00 {%B} 0.00 {base}
133.00 BufferValveA1 A11
133.00 Set_Mark "EQ Buffer"
163.00 End_Method

Vita

Brad M. Matanin was born on January 28, 1983 in Monroeville, Pennsylvania. He obtained a Bachelor of Science degree in Biological Systems Engineering with the bioprocess engineering option in May, 2005. Brad completed his Master of Science degree in Biological Systems Engineering in May 2007 under the guidance of Dr. Chenming Zhang. He was awarded the '2006-07 Most Outstanding Master's Student Award' by the Biological Systems Engineering Department and at the college level for both the College of Agriculture and Life Sciences and the College of Engineering at Virginia Tech.