PURIFICATION OF THE ROTAVIRUS NSP4 ENTEROTOXIN AND DISCERNMENT OF KEY HOST-CELL INTERACTIONS THAT CONTRIBUTE TO NSP4 SECRETION FROM THE CELL

A Senior Scholars Thesis

by

RENE EDUARDO AGUIRRE

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2011

Major: Biomedical Sciences

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Director of Honors and Undergraduate Research: Sumana Datta

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ABSTRACT

Purification of the Rotavirus NSP4 Enterotoxin and Discernment of Key Host-Cell Interactions that Contribute to NSP4 Secretion from the Cell. (April 2011)

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Rotavirus (RV) is a viral pathogen that infects everyone worldwide. It initially infects the enterocytes of the small intestine causing severe diarrhea in affected patients, primarily the young. RV nonstructural protein 4 (NSP4) was the first identified enterotoxin associated with RV infections. Our main focus was to understand how this protein interacted with infected host-cell molecules, thus inducing disease symptoms. We utilized a yeast expression system that had been transformed with a plasmid to express a truncated form of NSP4 (tNSP4) which still possessed toxic characteristics. Before we could study key interactions of the enterotoxin, isolation and purification of NSP4 was required. Purification was done by the use of gel filtration (GF) chromatography, and the use of an affinity column to complete the final purification steps. Our samples were analyzed for purity by protein quantitation, SDS-PAGE, Silver Stain, and Western Blot. Once purified, the tNSP4 was to be chemically linked to magnetic beads, which were then to be incubated with RV infected cell lysates to 'capture'

interacting host-cell molecules. The bound cellular proteins will be excised from 2-D gels and submitted to the TAMU Mass Spectroscopy Core for sequencing. A BLAST search of the recovered sequences against available databases will identify the interacting host-cell proteins.

DEDICATION

I would like to dedicate this thesis to all of whom were unfortunate enough to suffer the ultimate consequence of Rotavirus infection, as well as to those individuals that have experienced the terrible symptoms associated with this disease. I also would like to dedicate this to all friends and family of severely affected Rotavirus patients. My work over this past year is devoted to all of those committed research scientists that have brought us to a better understanding of how this virus affects humans and their ideas for future viral therapies against this pathogen.

ACKNOWLEDGMENTS

I would like to thank all of my family and friends for the support they have given me while I worked on my research. I would also like to express my gratitude to Dr. Judith Ball for all her help and the encouragement she has given me over the past year. Without the guidance of Dr. Ball, I would not have been intrigued to begin research on the effects of the Rotavirus. She has assisted me from the basic introduction to laboratory experience to the collaboration of a thesis based on her research. I also express great appreciation for all the help Jerry Ball has given me this past year. Without his help and his expertise, I would have no knowledge of all the ins and outs of every experimental technique I performed to complete my project. Lastly, I would like to express my thanks to Krystle Yakshe; she sacrificed much of her time and greatly assisted me through all of the perplexing steps that were involved in my project.

NOMENCLATURE

BLOTTO	Bovine Lacto Transfer Technique Optimizer
C	Celsius
DLP	Double Layered Particles
EDTA	Ethylenediaminetetraacetic Acid
ER	Endoplasmic Reticulum
g	Gram
HPLC	High Pressure Liquid Chromatography
HR	High Resolution
HRP	Horseradish Peroxidase
kD	Kilodalton
L	Liter
µB	Microbars
µg	Micrograms
µL	Microliters
M	Molar
mL	Milliliters
nm	Nanometers
NSP4	Non-Structural Protein 4
PBS	Phosphate Buffered Saline
PM	Plasma Membrane
RNA	Ribonucleic Acid

RPM	Revolutions Per Minute
RV	Rotavirus
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TEMED	Tetramethylethylenediamine
TRIS	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet
V	Volt
Y	Yeast

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CHAPTER I

INTRODUCTION

Rotavirus (RV) is a viral pathogen that infects all children worldwide, inducing a life threatening, dehydrating diarrheal disease similar to the stomach flu (5, 9, 10). Typically by the age of five, nearly every child has been infected with RV at least once (26). This pathogen also has had a major impact on elderly patients (6, 14) and those that are immunocompromised (7, 14, 15). Infection by RV is responsible for about 600,000 infant deaths annually (2, 20).

RV is characterized as having a double-stranded RNA genome, which belongs to the family Reoviridae. Upon infection, viral particles infect and eventually kill the enterocytes lining the small intestine, resulting in a malabsorption of nutrients (8, 11). However, this is not the only means by which symptoms occur. Other mechanisms have been proposed including the replacement of absorbing enterocytes with non-absorbing, immature crypt-like cells, resulting in a net loss of epithelial surface area (19, 22), and activation of the enteric nervous system (16). Another method of infection was also recognized upon identification of the RV non-structural protein 4, and its toxic effects on infected enterocytes (3). Due to the number of proposed mechanisms of infection, it is likely that the pathophysiology of RV is attributed to more than one method (2).

This thesis follows the style of Journal of Virology.

Rotavirus non-structural protein 4 (NSP4), encoded by gene 10, was the first viral enterotoxic agent identified as a cause of gastroenteritis (3). The presence of NSP4 results in a Ca^{2+}-dependent transepithelial secretion (27). NSP4 was initially identified as a glycoprotein vital to RV morphogenesis, by acting as an intracellular receptor to double-layered particles (DLP's) (18, 25). This unique morphology is characterized by the tendency for newly made sub viral particles to bud into the endoplasmic reticulum (ER) and obtain a transient lipid envelope from it (1).

Dr. Ball's Laboratory has shown that the NSP4 toxin, which was traditionally classified as a resident ER glycoprotein, and traffics to caveolae (a plasma membrane microdomain) by an unconventional, golgi-bypassing, secretory pathway (13, 23). They also revealed that NSP4 directly interacts with caveolin-1 (a caveolae structural protein), cholesterol, and a soluble immunophilin complex (17, 21, 23-24).

Recent work in Dr. Ball's laboratory has shown that NSP4 traffics to the plasma membrane by distinct transport kinetics, which is dependent on cell type (12, 13). However, regardless of the cell type, NSP4 (i) bypasses the Golgi *en route* to the PM, (ii) remains on the cell surface long enough to be detected by surface biotinylation and NSP4 C-terminal-specific antibody probes, and (iii) is secreted into culture media without disrupting the host cell.

According to other reports, there have been many inconsistencies. Some showed the secretion of NSP4 required different modifications of the released toxin (4, 28). Our data show the intact, native NSP4 is released from both RV-infected and NSP4-transfected cells. These data

suggest modifications of the toxic protein are not required for its release. We hypothesize that intracellular molecules that interact with NSP4 post infection are critical to the secretory transport from the ER.

Our plan is to provide valuable insights into the secretory transport mechanism of this novel toxin. Our primary aim is to study which host-cell molecules directly interact with NSP4. To accomplish this goal, we first would need to purify and isolate our target protein (NSP4) with the use of conventional, gravimetric, and high-pressure liquid chromatography (HPLC) techniques. Secondly, we would utilize our purified truncated protein to discern NSP4 interactions with host- cell proteins that likely contribute to NSP4 intracellular transport and release from the cell by employing direct binding assays. Successful completion of these experiments could potentially disclose important targets for future vaccines and antiviral therapies.

CHAPTER II

METHODS

Preparation of NSP4

Yeast-2-hybrid

Previously, a Saccharomyces cerevisiae strain, MAV203, was transformed with a pYes-DEST52 plasmid containing truncated NSP4 and *GAL1* promoter and enhancer sequences. The tNSP4 expressed in this yeast-2-hybrid only contained amino acids 150-175. The yeast were grown to log phase growth to ensure expression of tNSP4. The yeast were pelleted, the media was removed, and the yeast were frozen at -80°C.

Extraction of the yeast

Several frozen samples of the prepared yeast-2-hybrid that expressed tNSP4 (truncated NSP4) were processed for the extraction of tNSP4 and all other cytosolic components. The method by which we extracted the tNSP4 was by a Chaps/Lyticase extraction.

The reagents used were a Y-lysis buffer (Zymo Research), chaps lysis buffer (10 mM Chaps, 0.1 mM EDTA, and 10 mM TRIS), and a protease inhibitor. The tNSP4 expressed yeast was thawed on ice until a liquid yeast pellet had formed. Once thawed, about 625 µL of the Y-Lysis Buffer was added to the thawed yeast pellet. 5 µL of lyticase was added to the pellet. The mixture was then mixed and stirred for about 5 minutes with a glass stirring rod. It was important to stir the solution efficiently to ensure maximum extraction. Once stirred, the yeast was incubated in a 37ºC water bath for 75 minutes. The pellet was placed in a

centrifuge at 1,500 RPM for 10 minutes. This step was performed at 4ºC. The supernatant was discarded.

750 µL of the Chaps lysis Buffer was added to the pellet. The yeast/buffer mixture was stirred and re-suspended with a glass stirring rod for 5 minutes. This was followed by a 10-minute incubation in ice. After incubation, the pellet was spun in a centrifuge at 3,000 RPM for 10 minutes at 4ºC. This time, the supernatant was saved as our "raw" sample. The raw sample was kept on ice.

Another 750 µL of Chaps Lysis Buffer was added once again to the remaining pellet, stirred, centrifuged, and the supernatant was saved once again. These steps were repeated about 3 times to ensure maximum extraction. Note that only the steps involving the 750 µL of Chaps lysis buffer were repeated 3 times, and the 625 µL of Y-Lysis Buffer and lyticase was added only during the initial step.

After extraction from yeast, samples were separated into 1 mL aliquots. Each aliquot was centrifuged at 13,000 RPM to pellet out any remaining yeast. The supernatant was kept as the "raw" sample of unpurified tNSP4. The leftover precipitate (mainly yeast cells that were accidently not separated during extraction) was discarded. The samples were stored in a -80 ºC freezer until used for filtration.

Purification of tNSP4

Gravimetric gel filtration

Each 1 mL raw sample was taken through gel filtration chromatography for the initial purification step. This type of chromatography is utilized to separate proteins, peptides, and oligonucleotides on the basis of size or molecular weight (i.e. size exclusion). To prepare the column, a large glass tube is filled with tiny porous beads of specified molecular weight

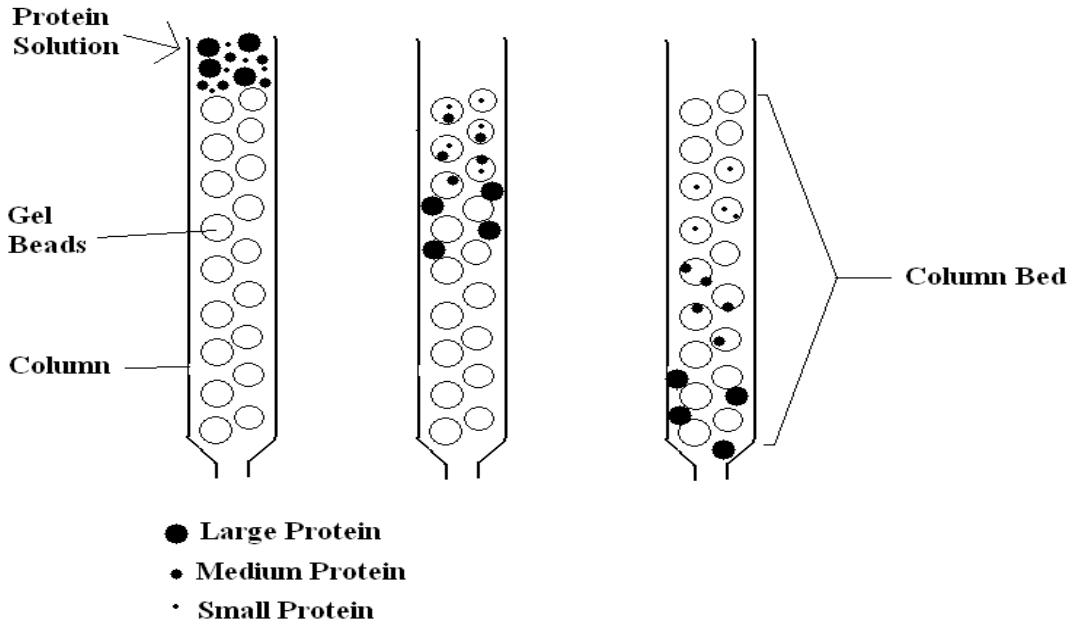

FIG. 1. Schematic Represenation of Separation by Gel Filtration. The protein solution is placed on top of the bed of gel (Sephacryl) beads. As the protein sample flows through, larger proteins proceed faster through the bed, whereas small proteins proceed much slower through the bed due the tendency for smaller biomolecules to "absorb" further into the gel beads. Illustration by Rene Aguirre.

separations. These beads are made up of a covalently cross-linked dextrose gel called Sephacryl (Sigma Aldrich). We specifically utilized Sephacryl 100 HR, which separates molecules from 1 kD to 100 kD. Each molecule diffuses into the beads to a greater or lesser extent, dependent on size. The smaller molecules diffuse into the beads much further and into more of the pores within the beads than the larger molecules. This effect causes smaller molecules to flow much more slowly through the column than larger molecules. (FIG 1) This step is called "gravimetric" gel filtration because gravity is the only means by which the molecules migrate from one end of the column to the other.

As the sample runs through the column, the mixture of the sample and our eluting solvent (20% acetonitrile) flows through the bottom of the column through a tiny tube that sends the mixture through a UV detector (Isco). The UV detector detects protein concentrations based on absorption at 230 nm. After the mixture flows through the UV detector, a fraction collector allows the mixture to separate into different test tubes at 12 minute intervals. The UV detector simultaneously transmits the absorption to a reader that creates a graph and shows the change in concentration known as a chromatogram. (FIG. 2)

Since each tube collects the sample for 12-minute intervals, and the chromatogram reads at the set speed, we can deduce which collecting tubes should be saved for additional analyses. This initial purification step yields several fractions to analyze for tNSP4. Note that we only collect the fractions that most likely contain tNSP4, and the rest are discarded. Our judgment

on which fractions actually contain tNSP4 is based on previous experiences with the same gel filtration method and positive absorbance. Upon completion of this step, the molecules and proteins within the raw sample have been partially separated by size.

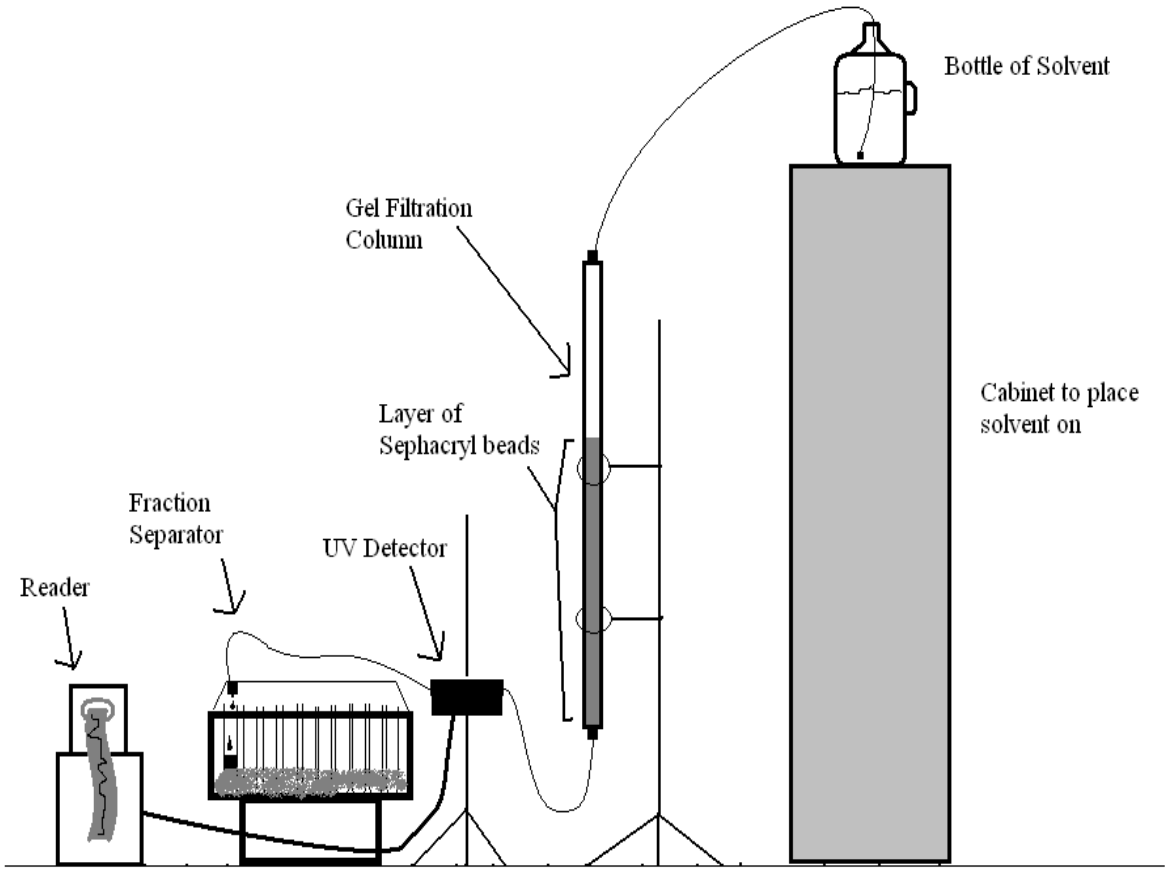

FIG. 2. Schematic Representation of Gel Filtration. The eluting solvent originates from the gallon bottle which is placed at a higher level than the top of the column. A tube is inserted to create a siphon so that a continuous flow of solvent flows onto the column. The raw sample and solvent flow through the layer of Sephacryl beads then out the bottom of the column to the UV detector. The fractions are then collected and separated by the fraction collector. The UV detector then translates absorbance readings from the UV detector and displays absorbance on a chromatogram. Illustration by Rene Aguirre.

Protein quantitation

Preparations of the column

The first step in gravimetric gel filtration is preparation of the column. The beads were swollen in water overnight at 4ºC and then poured as a slurry into the glass column. Care was taken to avoid bubbles and the beads from drying out. Once the beads were settled, we exchanged the water for 20% acetonitrile and stored the column at 4ºC. A pinch of sodium azide was added to the column to assure no bacteria grows in the column. About 200 mL of 20% acetonitrile was kept in the column to keep the beads from drying out. Before the column could be used for gel filtration, it must be removed from the cold room and be brought to near room temperature to ensure maximum separation.

Separation by gel filtration

The raw sample was removed from the freezer and thawed on ice. The sample was then transferred to a small test tube and an equivalent volume (1 mL) of 5 M urea and 20% acetonitrile (eluting buffer) were added. The now 3 mL volume of the diluted sample was ready to overlay on the top of the beads and was kept on ice until used to further complete the preparation steps.

The UV detector and reader must be given about 30 minutes to warm up. The fraction collector was loaded with clean glass collecting tubes on ice. The tubing that runs through the UV detector was also flushed out with our 20% acetonitrile solvent to clean out any impurities before separation. The gel filtration column still had a head of 30-40 mL of 20% acetonitrile which was removed using a vacuum pump prior to use. It was important not to disturb the

Sephacryl bed volume or the top layer of beads. The valve at the bottom of the column was opened to allow for any extra solvent to drain. We brought the level of the solvent to slightly above the level of the top layer of Sephacryl beeads (meniscus). Once the level of solvent was appropriate, we added the entire sample to the column in one step.

The sample was allowed to slowly enter the beads. Before the top layer began to dry out, we started the flow of 20% acetonitrile through the column. We had prepared about 2 L of 20% acetonitrile and used a siphon tube to begin the solvent flow. We placed the bottle of solvent at a higher level to ensure a continuous flow. Depending on the Sephacryl beads, the flow of solvent was usually about a 0.5 mL per minute, varied from 0.4 mL per minute to 0.6 mL per minute. We then began the timer, the collecting tube apparatus, and the reader. Once the column was running, the flow rate was checked at multiple times. If the flow rate was too high or too low, it was likely due to a problem with the beads, which can produce inaccurate results. To check the flow rate, the column was allowed to run for 12 minutes, and the volume of the sample that was collected during the same period was measured. To ensure a steady flow of solvent and an equal volume entering and leaving the column bed, the amount of the solvent "head" (deepness of the solvent on top of the beads) was monitored and remained steady throughout the run. It is usually a good idea to keep about 2 to 3 inches of head on top of the beads to ensure the top layer of Sephacryl beads do not dry out, as this could pose a major problem and ruin the column.

No protein was eluted from the column during the first 2 hours of solvent flow. Peaks on the chromatogram were monitored, which indicated where the protein was eluted from the

column. In other words, the higher the UV absorption, the greater the protein concentration, and the higher the peak. The column generally ran for a total of 8 to 9 hours before the entire sample was eluted. This allowed the column to be reused in the future. The first peak was usually a large peak and appeared right after the 12th tube (144 minutes). The tNSP4 typically elutes from the column on the negative slope of the first peak and continues to elute for several hours. Samples were saved from the start of the initial first peak to the start of the final major peak at about the 6-hour mark. This ensured that all of the tNSP4-positive samples were saved for analysis. The final peak usually includes biomolecules such as lipids or smaller proteins that we discarded. Three smaller peaks usually follow the final major peak. Once the third smaller peak has eluted, you are safe to stop running the column. To stop the solvent flow onto the column, the valve at the bottom was shut off and sealed. About a 6 inch head of 20% acetonitrile was placed on the top of the beads to ensure that it did not dry out and was returned to the cold room.

Optimization of solvents

We ran several gravimetric gel filtration runs. Our typical solvent used was a constant flow of 20% Acetonitrile (eluting buffer) and a 1 mL equivalent (of raw sample) of 5 M urea. The 1 mL of 5 M urea was mixed together with the raw sample and not continuously flowing through the column as the eluting buffer does. However, we attempted to make small changes in the composition of the 1 mL equivalent of 5 M urea to other chemicals to look for any improvement in purification.

Our first attempt at modifying our solvent was replacing the 1 mL equivalent of 5 M urea with a 10 M urea solution and ran several gravimetric gel filtration runs with this solvent. The urea solution was then replaced with a 1 mL equivalent of 8 M guanidine-HCL. We also attempted at removing the use of urea and guanidine all together, and used a 1 mL equivalent of 10% SDS solution. SDS is a known surfactant and can help in breaking off any lipids that our bound to NSP4. We also attempted to run the column with a 20% acetonitrile + 1% SDS solution, pH of 3.5.

Table 1. Comparison of Different Solvents.

Experiment	Constant Eluting Solvent	1 mL equivalent added to raw sample
1	20% Acetonitrile	5 M Urea
2	20% Acetonitrile	10 M Urea
3	20% Acetonitrile	8 M Guanadine
4	20% Acetonitrile	10% SDS
5	20% Acetonitrile + 1% SDS, pH 3.5	10% SDS

Lyophilization

Lyophilization (also known as freeze-drying) is a dehydration process that works to remove all water, and other volatile solvents (e.g. acetonitrile) from the sample. This step is critical since our protein samples that were collected are too dilute to analyze. The resultant dry protein sample can then be diluted in a smaller volume.

To prepare the samples saved from gel filtration, the lyophilization samples were pooled into 50 mL conical tubes, since the tube can only be filled to 1/3 of the volume. Our samples were labeled according to the aliquot of raw sample, which solvent was used (20% acetonitrile), the type of Sephacryl used (S-100 HR), and the tube collection number. Once all samples had

been transferred into 50 mL conical tubes and labeled, they were shell frozen at -80ºC and added to the lyophilizer. Generally the samples were lyophilized at -82ºC and a vacuum of less than 70 μB. The samples remained on the lyophilizer for about 2 days or until dry. Once complete, the dried samples were kept in a cool, dry (under dessication) environment for later use.

Affinity purification

The purpose of affinity purification is to finalize the purification of tNSP4 samples that have been enriched using gel filtration by gravity. Affinity columns work by allowing biomolecules to separate based on highly specific molecular interactions. Such connections include the interaction between an antibody and an antigen, or a substrate and an enzyme. In our case, we utilized the particular interaction between tNSP4 and NSP4-specific antibodies. As the protein sample flows through the column, antibodies in the column attach to the tNSP4. Once all other protein has eluted from the column, tNSP4 can then be removed from the highly specific antibody interactions. This allows only tNSP4 to be released from the column resulting in a pure sample of tNSP4. The affinity column was previously prepared using 5 mL of Rabbit-Anti-$NSP4_{150\text{-}175}$.

Preparation of the column

The column had to be cleaned with deionized H_2O, ultrapure water, and a 70% ethanol solution. Due to the small size of the column and the gel-like matrix, gravity would not have much of an effect in pushing the sample through the column. Therefore, a peristaltic pump was used in helping the cleaning solvents run through the column smoothly.

Once the column was clean, about 10 bed volumns (50 mL) of a 10 mM solution of TRIS, pH 7.5, was run through the column followed by 10 bed volumes of 0.1 M Acetate, 0.5 M NaCl, pH 4.5. Lastly, 10 bed volumes of our coupling buffer 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3, was run through the column. The acetate solution and the coupling buffer solution were run 3 times (i.e. acetate, coupling buffer, acetate, coupling buffer, acetate, coupling buffer). The flow of these solutions was a second cleaning step for the affinity column. Finally, 10 mM TRIS, pH 7.5, was run through the column until the pH of the eluted solution was 7.5.

Running the affinity column

Samples that had been previously purified with gel filtration were now ready to undergo affinity purification. According to Western Blot analyses and silver stains of previous samples from gel filtration, several samples with positive tNSP4 readings were pooled, dialyzed with 0.1 M ammonium bicarbonate, and lyophilized. These samples tested positive for tNSP4 right at about the 50 kD mark. This meant that any tNSP4 in the sample could have been a trimer, tetramer, or pentamer of tNSP4 or was bound to some other protein.

Once the affinity column was ready for use, the sample was placed on the column. The sample contained the lyophilized 50 kD tNSP4 that had been diluted in 500 µL of 10 mM TRIS, pH 7.5. The entire diluted sample was placed onto the column and was circulated for at least 3 times (i.e., once the eluted sample came out of the column, it was replaced back onto the column for a re-run). This insured maximal binding of tNSP4. The pump was set to a flow rate of about 1 mL/min.

Once the sample had been run through the column at least 3 times, the column was then washed with 10 mL TRIS, pH 8.8. The column was flushed until the outflow pH had risen to 8.8. The proteins that are bound by base-sensitive interactions were eluted by passing 10 bed volumes of freshly prepared 100 mM triethylamine, pH 11.5, through the column. The eluate was collected in a 50 mL conicle containing an equal volume of Tris, pH 8.0. The affinity column was then washed with 10 mM TRIS, pH 7.5, until the pH of the outflow reached 7.5. It was then ready to be stored at 4ºC for later use.

The eluate was then dialyzed using a 5-15 mL Slide-A-Lyzer dialysis cassette (Thermo Scientific) in 0.1 M Ammonium bicarbonate at 4ºC. The ammonium bicarbonate was changed every 4 hours for about 1 day until the sample was ready. The dialyzed sample was then ready to be lyophilized.

Sample trials

The tNSP4 rich samples from previous gel filtrations were aliquoted to use for different affinity column runs. Our first run we used one aliquot that weighed approximately 0.0015 g. For our second attempt at running a sample through the affinity column, we utilized 0.0030 g of tNSP4 rich sample. Recall that both samples were dissolved in 500 µL of 10 mM TRIS, pH 7.5.

Analysis of the gel filtered and affinity filtered Samples

Once all of the samples have been lyophilized they were ready to undergo analyses to test for the presence of tNSP4. The steps in protein analyses include SDS-PAGE, followed by Silver Staining, and Western Blot. After all the steps in analyses have been completed, we will know which of the samples contain tNSP4 and the relative purity.

SDS-PAGE

Gel-preparation

The gel used in electrophoresis has three primary components in its formulation. The first component is 12.5 mL of the acrylamide solution, 12.5% acrylamide/bis (Next Gel, Sigma Aldrich). The second component is 67.5 µL of a 0.1% solution of ammonium persulfate, which acts to initiate polymerization, and the third component is 6.75 µL of TEMED (BIO-RAD) also acts to initiate polymerization. These components are gently mixed for a minute or two, but not so long for the mixture to begin to solidify. The solution was poured between 2 glass plates with a plastic comb placed at the top to form distinct sample wells and given about 30 minutes to polymerize. Running buffer (0.05 M TRIS, 0.1% SDS, and 0.38 M glycine) was added to the top of the plates to ensure the gel does not dry out during or after polymerization. Two identical gels were prepared.

Sample preparation

Each dry sample was dissolved in 100 µL of 1X PBS and put on ice to keep cold. To determine the concentration of protein in each sample, a Nano-Drop (spectrophotometric device) was performed so that an equal concentration of each sample could be loaded into the

gels. The absorbance reading at 230 and 280 nm corresponds to the protein in our sample, which was used to estimate the concentration of protein in µg/µL as described above. Optimally, there was one strong peak at 230 nm. A nano-drop analysis was done for each sample that underwent SDS-PAGE.

Two identical tubes for each sample were prepared for SDS-PAGE separation. One of the samples was used for Silver Staining once the SDS-PAGE was completed. The other was used for transferring to a nitrocellulose membrane (BIO-RAD) for Western Blot analyses.

To prepare each sample for SDS-PAGE, the concentrations obtained from the nano-drop were used to estimate the required volume of sample needed. 12 - 15 µg of protein was prepared for each gel, generally in 3 - 5 µL of reducing buffer. For example, if the concentration of one sample was 5µg/µL, then about 2.5 - 3 µL of sample was used to accommodate the 12 - 15 µg of total protein.

Running the SDS-PAGE

Each sample was placed in boiling water for 5 minutes and then spun at 10,000 RPM in a microfuge for 2 minutes. Then the samples were loaded into the individual well, and the entire gel apparatus bathed in Laemlli running buffer. The proteins were separated by applying a constant 150 V for 1 hour and 20 minutes. Molecular weight markers were used in each gel.

Silver stain

Our first gel underwent Silver Staining employing a Silver Stain Kit (Pierce). This kit is a rapid and ultrasensitive silver stain system which detects the presence of proteins in polyacrylamide gels.

First we placed our gel in ultrapure water and washed it twice for 5 minutes each. Our gel was then fixed in a 30% ethanol and 10% acetic acid solution twice for 15 minutes each. The gels were then washed twice for 5 minutes per wash in a 10% ethanol solution followed by two 5 minute washes in ultrapure water. The gel was sensitized in the sensitizer working solution (50 μL sensitizer with 25 mL ultrapure water) for 1 minute, and then washed twice for 1 minute each in ultrapure water. The gel was stained for 30 minutes in our stain working solution (0.5 mL enhancer with 25 mL stain). After the gel was stained, it was washed twice for 20 seconds each in ultrapure water, and then incubated in developer working solution (0.5 mL enhancer and 25 mL developer) for 2 - 3 minutes or until the bands appeared. The reaction was stopped with a 5% acetic acid solution and then mounted for drying.

Western Blot analyses

Our second gel went through Western Blot analysis. This was performed so that we could detect tNSP4 in our sample.

Transferring the second gel to a nitrocellulose membrane

Our initial step was to create a sort of "sandwich" of different layers to transfer our gel to a nitrocellulose membrane (BIO-RAD). A fiber pad (BIO-RAD) was used as our bottom layer, on top of that filter paper (BIO-RAD) was added and then our gel was placed on top of the filter paper. A nitrocellulose membrane was then placed on top of the gel. This was followed by another filter paper and a final fiber pad on top. The sandwich was then soaked in our transfer buffer (20% methanol, 20% 5X transfer buffer, and 60% ultrapure water). 5X transfer buffer is a solution of 60.6 g TRIS and 288 g of glycine quantity sufficient to 3,200 mL. It was crucial to make sure that there were no bubbles in between the layers, and that you did not touch the nitrocellulose. To check for bubbles, after the layers were placed, we rolled out the bubbles with any small plastic tube. The entire transfer apparatus was bathed in a liter of transfer buffer and the gel was transferred to the nitrocellulose membrane for 2 hours under constant 350 milliamps.

Preparation of nitrocellulose membrane for analysis

Following the electro transfer, the nitrocellulose membrane, within the sandwich, that already had the gel transferred to it was removed from the bath of transfer buffer. The membrane was removed from the layered "sandwich" and then placed in a small plastic tub. A solution of 10% BLOTTO was added to the membrane and allowed to rock for 1 hour to block nonspecific antibody binding. We made sure to flip the membrane upside down and right side up numerous times to assure complete coverage by the 10% BLOTTO. The BLOTTO was used to block all of the non-specific sights on the membrane.

After the membrane was exposed to the 10% BLOTTO for an hour, the membrane was then rinsed 4 times at 5 minutes each. The first rinse was with 1X PBS, the second and third rinse was with 1X PBS and .0025% Tween (surfactant), and the fourth rinse was with 1X PBS. Once the membrane had been rinsed, it was the exposed to our primary antibody (rabbit anti-NSP4 $_{150\text{-}175}$ diluted at1:5,000 in 2.5% BLOTTO) (Pierce) for one hour at room temperature and while rocking. Alternatively, the membrane was incubated with primary antibody at 4°C overnight. We made sure that the membrane was flipped over numerous times to ensure a complete coating by the primary antibody. After an hour the membrane was washed 4 times like the previous washings.

The membrane was then exposed to our secondary antibody (Goat anti-rabbit diluted at 1:20,000 in 2.5% BLOTTO) (Pierce) for an hour while rocking. We also flipped the membrane numerous times to make sure it is completely coated with the secondary antibody. After the hour passed, the membrane was once again washed four times like before. After this step, it was now time to expose the membrane to the Chemiluminescent HRP Substrate (Millipore). Chemiluminescent detection uses an enzyme with a highly sensitive substrate to catalyze a reaction which generates visible light. The first component (HRP Substrate Peroxide Solution) and the second component (HRP Substrate Luminol Reagent) were mixed together and the nitrocellulose membrane was coated with this chemiluminescent chemical for 8 minutes. The membrane was then dabbed on filter paper to somewhat dry the membrane so it could be developed on film.

The membrane was placed in 20 X 25 cm Extremity Cassette (OU PONT) which blocked all light from entering in between two clear sheets. In a dark room, we re-opened the cassette and placed Blue Basic Autorad Film (8" X 10") (BioExpress) and was exposed to the bound chemiluminescent substrate for 5 minutes. After 5 minutes, the film was then developed and the film (where exposed to light) produced dark bands. These bands signified where tNSP4 was present.

CHAPTER III

RESULTS

Purification

Gravimetric gel filtration

After multiple trials of Gel Filtration Chromatography and the use of different solvents, our experiments showed changes in purification by the use of different solvents. Note that a constant flow of 20% acetonitrile was always used as one of the solvents. The 1 mL equivalent of 5 M urea was altered to look for changes. To recall, these were the different solvents (coupled with a constant flow of 20% acetonitrile) that were tried using gel filtration: a 1 mL equivalent (of raw sample) of 5 M urea, a 1 mL equivalent of 10 M urea, a 1 mL equivalent of 8 M guanidine, a 1 mL equivalent of 10% SDS with constant flow of 20% acetonitrile + 1% SDS, and a 1 mL equivalent of 5% SDS with a constant flow of 20% acetonitrile + 1% SDS, pH 3.5. Note that for two of our solvent trials, we used a 20% acetonitrile solvent + 1% SDS (for constant flow).

5 M & 10 M urea with 20% acetonitrile as constant flow

After studying the chromatogram, silver stain, and positive results from Western Blot, the 5 M urea seemed to keep any tNSP4 eluting from the column around the first large peak on the chromatogram. This indicated that any positive samples were very dirty (i.e. contaminated with various other proteins and biomolecules) and had no separation from various other proteins. This was confirmed with the analysis of the silver stain.

The 10 M solution of urea seemed to have similar effects on the elution of tNSP4 and also appeared only on the first peak. According to our results from the Western Blot, it showed that there still was not any separation from various other proteins resulting in very dirty purified tNSP4 positive samples. This was again confirmed by the silver stain. (FIG. 3)

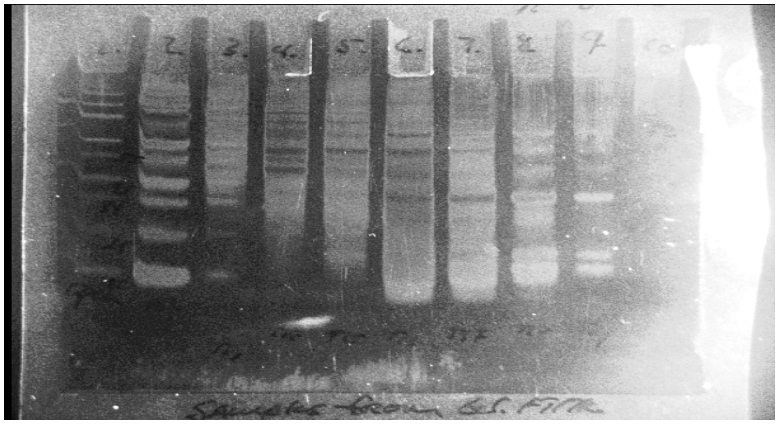

FIG. 3. Silver Stain Image of Impure tNSP4 from Gel Filtration with 10 M Urea. Protein marker is in lane 2. This image shows how contaminated samples of tNSP4 are from this silver stained gel. Typically if a sample is positive, only one positive band will appear on the western blot. This image clearly shows the high number of other bands that appeared in every sample informing us that the sample was very contaminated with other proteins.

Under normal circumstances, the wanted tNSP4 would have a molecular weight of close to 15 kD, however there were bands that showed up on the Western Blot at 30 kD and 50 kD. This meant that the tNSP4 was either bound to some other protein present in the sample, or that it was bound to other tNSP4 proteins. There was no band at around 15 kD on the Western Blot.

8 M guanadine with 20% acetonitrile as constant flow

One major difference with the use of guanidine was the first major peak. This peak normally (when using urea) showed up as 1 sharp peak; however it was broken up into multiple peaks. This is helpful giving in that it gives more separation of other proteins which could result in cleaner samples.

The 8 M guanidine solution also had a different effect on the purification of tNSP4 through the gel filtration column. After analysis of the Western Blot and chromatogram, it appeared that the tNSP4 eluted from the column in the first peak (split up) just as the different concentrations of urea resulted in. However, the only difference was that tNSP4 positive samples emerged on the latter part of the peak. Thus, the tNSP4 came out slightly later. Yet, analysis of the silver stain still showed that the samples were very contaminated with other proteins. The Western Blot still showed no bands at about 15 kD. The only positive bands showed up at 30 kD or greater and still meant that the tNSP4 was still bound to other proteins.

10% SDS with 20% acetonitrile + 1% SDS as constant flow

With the trials of SDS, the first peak was just one sharp peak. It did not split it up as the guanidine did. However, according to the Western Blot and chromatogram, it seemed that the SDS seemed to be breaking apart oligomers of tNSP4. Our bands of positive tNSP4 showed up on the chromatogram closer to 15 kD, but still slightly larger. There was still the possibility that tNSP4 was bound to something else. (FIG. 4)

Results also showed that the tNSP4 was eluting from the column a lot further and on the tail end of the first peak. This is because larger biomolecules elute from the column before smaller biomolecules do. Analysis of the silver stain showed that the samples on the tail end of the first peak were much cleaner than previous trials with different solvents. (FIG. 5)

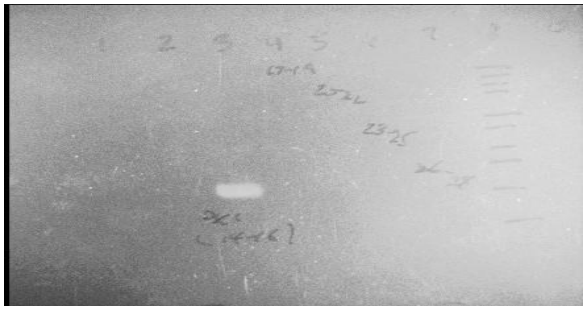

FIG. 4. Western Blot of Same Samples from FIG. 5. Protein marker is in lane 8. The tNSP4 positive band appears in the 3rd lane, which corresponds to the 3rd lane in FIG. 4. Lanes 4-7 all tested negative and showed no positive tNSP4 bands on the Western Blot.
10% SDS with 20% acetonitrile + SDS as constant flow (pH 3.5)

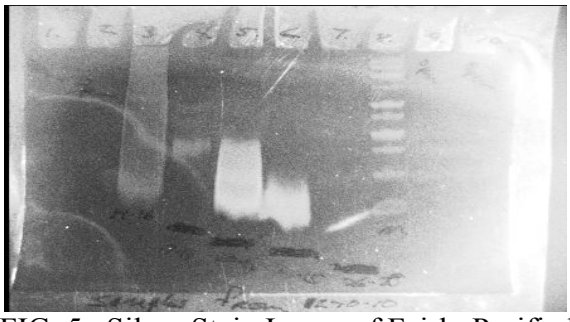

FIG. 5. Silver Stain Image of Fairly Purified tNSP4 from Gel Filtration with 10% SDS. Protein marker is in lane 8. The sample only in the 3rd well tested positive for tNSP4. A lot of contaminants are present in the 3rd lane. All of the other well indicated that larger proteins were eluting from the column a lot more separated from smaller proteins. The samples in wells 4-6 tested negative for tNSP4

According to the chromatogram, this trial seemed to break up the first peak. The Western Blot showed that the tNSP4 was pushed after the first initial peak. Since the tNSP4 was coming out slightly later, we concluded that the oligomers were indeed breaking up since larger biomolecules come out before smaller biomolecules in the gel filtration column. Analysis of the silver stain also showed that these samples were much cleaner than previous trials with different solvents.

Table 2. Comparison of different solvents including effect on separation

Experiment	Constant Eluting Solvent	1 mL equivalent added to raw sample	Effect on Separation (Low 1 - 2 - 3 - 4 High)
1	20% Acetonitrile	5 M Urea	1
2	20% Acetonitrile	10 M Urea	2
3	20% Acetonitrile	8 M Guanadine	2
4	20% Acetonitrile	10% SDS	3
5	20% Acetonitrile + 1% SDS, pH 3.5	10% SDS	4

Affinity purification

Two separate trials were run through the affinity column. As previously described, we utilized several purified tNSP4 positive samples that contained tNSP4 bands right at about the 50 kD mark. Typically, tNSP4 would have a molecular weight right around 15 kD.

For the first run, 0.0015 g of sample were used. Due to the exceedingly small amount of sample that was experimented with, our yield of affinity purified tNSP4 was very low and barely noticeable. Our base-sensitive interactions were separated into one tube, while the acid-sensitive interactions were separated into another conical tube. The samples were dialyzed and lyophilized for analysis. Due to the small amount of yield from the affinity

column, we were forced to pool the acid wash and the base wash in order to have enough sample to verify by Western Blot analyses and silver stain.

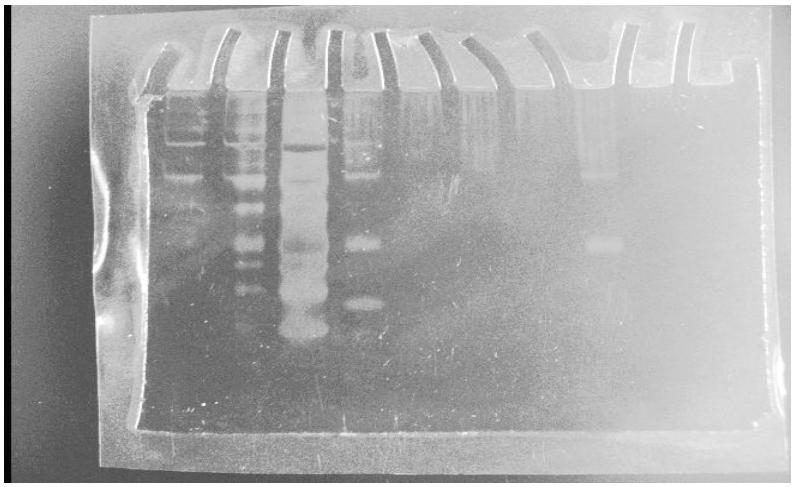

FIG. 6. Silver Stain Image of Affinity Purified tNSP4. Protein marker is in the 3rd lane (left to right). Disregard spill-over from markers in lane 1 and 2. Lane 4, 6, and 8 contain different samples from affinity purified tNSP4. Western Blot gave unclear results showing that there were only tiny amounts of tNSP4 in all 3 samples. The 3 lower bands in lane 4 are contaminants that did not test positive for tNSP4. Lane 6 contained the purest tNSP4, while the two lower bands in lane 8 did not test positive for tNSP4. Samples in lane 4 and 6 are contaminated.

For our second run, 0.0030 g of sample were used to run through the affinity column. A larger amount of sample was recovered once it was run through the affinity column. Following dialysis and lyophilization of both base and acid washes, it was evident that due to the higher amount of yield, a Western Blot and a silver stain could be performed with each. After the samples were analyzed, it was clear that the amount of tNSP4 that was recovered

from the affinity column was very miniscule. The silver stain also showed that two of our samples in fact did contain other protein that was not tNSP4 due to the presence of other bands that did not test positive. (FIG. 6)

According to nano-drop analysis, protein concentrations within the affinity purified samples were exceedingly low. There was not a high enough yield of tNSP4 to continue with the chemical linking of tNSP4 to magnetic beads for the final experiment of the proposed project to work.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Previously, a colony of yeast was transformed making it capable of producing a truncated form of Rotavirus nonstructural protein 4. This was proved by performing a Western Blot of the raw sample that was extracted from the yeast cells.

After several aliquots were run through the gravimetric gel filtration column, we succeeded in drastically increasing the purity of tNSP4. This was verified by Western Blot and Silver Stain analyses. Several attempts at changing the eluting solvent were made to observe any increases in protein separation. 20% acetonitrile was our primary eluting solvent used for each run. Different concentrations of urea, guanidine, and SDS were added to the eluting buffer. The SDS had the greatest effect on protein separation. SDS helped separate the eluting of tNSP4 from most other proteins in the raw sample which resulted in several samples that had fairly clean tNSP4 bands on the silver stain and Western Blot. These samples were then utilized in the affinity column for the final purification step.

Several clean tNSP4 samples that were purified with the help of SDS were pooled, dialyzed, and lyophilized and prepared to be purified by affinity. The affinity we utilized was previously made with beads that were bound to Rabbit-Anti-$NSP4_{150-175}$. Two separate trials of affinity purification were performed. The difference between the two trials was that twice as much sample was used in the second trial. After analysis of the Western Blot and silver stain, it was obvious that the affinity purified samples still tested positive for tNSP4.

However, the samples were contaminated with another protein that was at the 30 kD mark which did not test positive for tNSP4.

Prior to affinity purification, the samples were tested for concentration by a nano-drop analyzer. It was now evident that even with the concentration of protein that was contained in our samples that were purified by affinity, there would not be a high enough yield of purified tNSP4 to utilize magnetic beads that would be chemically linked to the tNSP4 for further discernment of interactions with host-cell molecules.

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