THE USE OF LABORATORY TECHNIQUES FOR THE PURIFICATION OF NSP4

A Senior Scholars Thesis

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ABDULHAMID FIRAS AL-DOURI

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

UNDERGRADUATE RESEARCH SCHOLAR

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Major: Biomedical Sciences

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Approved by:

Research Advisor: Judith Ball
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ABSTRACT

The Use of Laboratory Techniques for the Purification of NSP4. (May 2012)

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Rotavirus (RV), family reoviridae, is a deadly pathogen that affects young children and infants worldwide by causing severe watery gastroenteritis. Rotavirus Gastroenteritis (RGE) causes 55,000 to 70,000 hospitalizations, 205,000 to 272,000 emergency room visits, and 410,000 physician visits each year, with total direct and indirect costs estimated at approximately $1 billion annually. Rotavirus causes gastroenteritis by producing a viral enterotoxin called NSP4, which infects the mature enterocytes of the villus tip of the small intestine. It negatively affects intestinal disaccharides and Na^+ solute symports coupled with water transport. The NSP4 protein is quite complex due to the fact that it oligomerizes with other proteins and it has proven difficult to separate from the other proteins. Our goal is to purify NSP4 using a wide array of laboratory techniques which will assist in discerning the mechanisms of action of NSP4 and any possible ways of treating it. After running several samples through the gravimetric gel filtration column, we were successful in removing several of the impurities and increasing the purity of tNSP4. Through the use of Western Blot and Silver Stain

analyses, we were still able to see some bands that were likely contaminants and bands that appeared as multimers of the tNSP4. This means that there are still several unknown compounds bound to the tNSP4.

NOMENCLATURE

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

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CHAPTER I

INTRODUCTION

Rotavirus (RV), family reoviridae, is a deadly pathogen that affects young children and infants worldwide by causing severe, life-threatening gastroenteritis. This results in the death of about 600,000 children each year. In fact, nearly every child is infected with RV at least once by the time they are five years of age [5]. RV also is known to infect the elderly and adult immune compromised individuals. Rotavirus Gastroenteritis (RGE) causes 55,000 to 70,000 hospitalizations, 205,000 to 272,000 emergency room visits, and 410,000 physician visits in the USA each year, with total direct and indirect costs estimated at approximately $1 billion annually [6].

Rotaviruses are non-enveloped, triple-layered (VP2, VP6, VP7) virions with a single spike protein (VP4) and a genome of 11 double stranded, RNA segments. Six structural and six non-structural proteins are encoded by the segmented double stranded RNA genome. One of these proteins is nonstructural protein 4 (NSP4), our protein of interest. Rotavirus causes gastroenteritis by infecting the mature enterocytes of the villus tip of the small intestine [8]. It negatively affects intestinal disaccharides and Na^+ solute symports coupled with water transport and eventually strips the villus of absorbing cells. This results in a malabsorptive diarrhea accompanied by malabsorption of carbohydrates

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This thesis follows the style of European Journal of Physiology.

and their accumulation in the intestinal lumen, as well as the wasting of many nutrients.

NSP4 is encoded by gene 10 and is the first described viral enterotoxin [1]. Several experiments and studies have described the complexity and mystery of this multifunctional glycoprotein. The NSP4 toxin is translated on endoplasmic reticulum (ER)-bound ribosomes, stays in the ER to function in viral morphogenesis, then leaves the ER and travels to the plasma membrane (PM) by an unconventional secretory pathway that bypasses the Golgi apparatus [4,7]. Dr. Ball's laboratory also has demonstrated that NSP4 traffics to the PM by distinct transport kinetics that appears to be dependent on the cell type. However, regardless of the cell type, NSP4 (1)bypasses the Golgi and goes directly to the PM from the ER, (2) the C-terminus remains on the cell surface long enough to be detected by surface biotinlyation, and (3) it is subsequently secreted into culture media without disruption of the host cell [1-3]. Many attempts have been made to purify NSP4 to homogeneity, with limited success. Other laboratories have utilized multiple techniques and have achieved varied percentages of NSP4 purity. One of the difficulties in isolating NSP4 is the fact that it tightly binds and oligomerizes with a variety of viral and cellular proteins, as well as host lipids, including cholesterol. The goal of this study is to purify NSP4 that is expressed in yeast to homogeneity to gain a better understanding of the mechanisms of this enterotoxin. Our primary aim is to examine its interaction(s) with host cell molecules. We plan on accomplishing this by expressing NSP4 in the absence of other viral proteins and purifying the toxin through the use of several lab techniques, including gel filtration

chromatography, 2-Dimensional gels (SDS-PAGE and isoelectric focusing), and High Pressure Liquid Chromatography (HPLC). Successful completion of this project will play an important role in discerning the mechanism(s) of action of NSP4 and elucidate any possible ways to block its activity.

CHAPTER II

METHODS

Growing yeast

A strain of yeast called MAV 203 was grown on a YPAD media plate. A wooden applicator was used to streak the yeast across the plate. The plate was placed in a 37°C incubator and placed in a plastic bag with an opening (to prevent contamination). The yeast usually began to grow about a 3-5 days after streaking the plate.

Preparing the NSP4

A strain of yeast was transformed with the plasmid pYes-DEST52 containing truncated NSP4 and a *GAL1* promoter. The expressed tNSP4 only contained amino acids 150-175 . Expression of tNSP4 was ensured by allowing the yeast to reach log phase growth before it was harvested. A colony of yeast was picked and put in 10 mL of YPAD solution and 10 mL of 10% Galactose. The yeast were pelleted, the media was removed, and the yeast were frozen at -80°C.

Lysing of the yeast

The yeast were removed from the -80°C freezer and allowed to thaw on ice until a liquid yeast pellet had formed. The method used to extract the tNSP4 was by using a Chaps/Lyticase extraction method. This required the use of a Y-lysis buffer (Zymo Research), Chaps Lysis Buffer (composed of 10 mM Chaps, 0.1 mM EDTA, and 10 mM

TRIS), and protease inhibitors (Bio-Rad). Once the yeast had fully thawed, about 310 µL of the Y-lysis buffer was added to pellet, as well as 2.5 µL of lyticase. The mixture was mixed well by pipetting up and down several times. The mixture was incubated in a 37°C water bath for 75 minutes. Following the incubation the mixture was centrifuged at 1,500 RPM for 10 minutes at 4°C. The supernatant was discarded and 375 µL of the Chaps Lysis Buffer was added to the yeast pellet. The solution was mixed well with the pellet by pipetting up and down, sonicated for 5 minutes, and incubated on ice for 10 minutes. After incubation, the pellet was centrifuged at 3,000 RPM for 10 minutes at 4°C. The resulting supernatant was then saved and kept on ice.

Chaps Lysis Buffer again was added to the pellet, sonicated, centrifuged, and the supernatant was saved once again. This was done a total of 3 times to ensure maximum extraction of the proteins. The saved supernatant samples were spun using a microfuge at 13,000 RPM to pellet any remaining yeast. The supernatant is our sample of interest that contains the NSP4 and was stored in a -80°C freezer. Meanwhile, any leftover precipitate was discarded.

Dot blot

Once the yeast is lysed, it is important to make sure that the supernatant collected actually contains NSP4. Soak a piece of filter paper and nitrocellulose membrane (both about 7 cm X 13.5 cm) in 1X PBS for 5 minutes. Place the nitrocellulose membrane on top of the filter paper in the vacuum. Turn the vacuum on to suck up excess moisture

before adding about 20 µL of the supernatant collected to the nitrocellulose membrane. After allowing the membrane to absorb the supernatant for about 5 minutes, add 20 µL of 1X PBS to the membrane.

The membrane was then removed from the vacuum and soaked in a 5% dry milk/PBS (BLOTTO) solution (2.5 g of dry powdered milk in 50 mL of 1XPBS) for an hour to block nonspecific antibody binding. The membrane was then washed four times at 5 minutes each. The first and fourth washes are with 1X PBS, while the second and third washes are with 1X PBS and .0025% Tween surfactant. Once washing is completed, the membrane was exposed to the primary antibody "rabbit anti-NSP4 150-175" at a dilution of 1:5000 in 2.5% BLOTTO. The blot was incubated with the primary antibody for an hour at room temperature and while rocking or overnight at 4°C and while rocking. After the blot is probed with the primary antibody, it was washed four times in the same way it was previously washed. The membrane was then probed with the secondary antibody "Goat anti-rabbit IgG conjugated to horse rabbit peroxidase (Pierce)" at a dilution of 1:20,000 in 2.5% BLOTTO (Pierce) for an hour at room temperature while rocking. The membrane was once again washed four times like before. The next step required the membrane to be exposed to the Chemiluminescent HRP Substrate (Millipore), which binds to the HRP of the secondary antibody and catalyzes a reaction which generates visible light. 2 mL of the first solution (HRP Substrate Peroxide Solution) and 2 mL of the second solution (HRP Substrate Luminol Reagent) were mixed and added to the nitrocellulose membrane for about 5 minutes.

Excess fluid was removed from the membrane by dabbing it with a piece of filter paper. The membrane was placed in a film cassette, which blocks all light from entering. In a dark room, we reopened the cassette and placed a piece of Blue Basic Autorad Film (8”X10”) (BioExpress) to expose the film to the bound chemiluminescent substrate for 5 minutes. After 5 minutes, the film was developed. If the supernatant is NSP4 positive, then the film should have a black spot where the supernatant was added.

Enrichment of tNSP4 by gel filtration

Gel filtration is a method used to separate proteins on the basis of size or molecular weight. To begin, a large glass tube was filled with tiny porous beads of a specified molecular weight. The beads are made of a covalently cross-linked dextrose gel called Sephacryl (Sigma Aldrich). Following equilibration in the eluting solution Acetonitrile, the NSP4 protein sample was placed on top of the bed of Sephacryl beads and was allowed to flow through the beads. Larger proteins flow through faster, while smaller proteins flow slower due to their tendency to absorb further into the gel beads. In the end, the pull of gravity allows the protein to flow from one end of the column to the other.

As the extracted yeast sample containing NSP4 flows through the column, a mixture of the sample and our eluting solvent (20% acetonitrile) flows through the bottom of the column and is sent through a Sigma UA6 UV detector. The detector detects the protein concentration based on their absorption at 230 nm. A fraction collector is connected to

the UV detector, which allows the mixture to separate into different test tubes every 12 minutes. The UV detector also transmits the absorption to a reader that creates a graph that shows the change in concentration of protein. Once all the sample has flowed through, we collect the fractions which most likely contain NSP4 based on previous data and discard the rest.

Lyophilization

Lyophilization, sometimes known as freeze-drying, is a process used to dehydrate samples. This is an important step considering the protein samples collected are typically too dilute to analyze. The fractions collected from the gel filtration were pooled into 50 mL conical tubes, labeled., shell frozen at -80°C and added to the lyophilizer (Virtis). Generally, it takes about 2 days for the sample to completely dry. Afterwards, the dried samples are kept in a cool, dry jar with desiccant for later use.

Analysis of gel filtered samples

The lab techniques of silver stain and SDS-PAGE are used to determine which of the samples contain NSP4 and their purity. SDS-PAGE is prepared by mixing three solutions in a 200 mL flask. The first solution is 12.5 mL of 12.5% acyrlamide/bis (Next Gel, Sigma Aldrich). The second and third solutions are added to initiate polymerization and include 67.5 µL of a 0.1% solution of ammonium persulfate and 6.75 µL of TEMED (Bio-Rad). This mixture is gently stirred for less than a minute due to the fact that it begins to solidify quickly. The solution is quickly poured between 2 glass plates with a

plastic comb placed at the top to form distinct, individual wells and given about 30 minutes to polymerize. Two gels are prepared using this method, one will be used for a silver stain while the other will be transferred to nitrocellulose for Western blot.

The samples we obtained from the gel filtration and lyophilized are dissolved in 100 µL of 1X PBS and incubated on ice. The concentration of protein in each sample was quickly determined through the use of an instrument called the Nano-Drop which reads their absorbance at 230 and 280 nm. This was used to calculate the concentration of protein in µg/ µL. The concentrations of the samples were then used to estimate the required volume of sample needed for each well. Typically, 10 ug of protein (about 12 µL of protein sample) was prepared with about 7 µL of reducing buffer (1X SDS buffer). Each sample was boiled for 5 minutes and then centrifuged for about a minute. The samples, as well as a molecular weight maker, were then loaded into individual wells and the apparatus was connected to a power source with a constant 150V for about 1.5 hours.

Silver stain

The first gel undergoes silver staining utilizing the Pierce Silver Stain Kit. This kit is an ultrasensitive silver stain system used to detect the presence of small quantities of proteins in polyacrylamide gels. The gel was first placed in ultrapure water and washed twice for a total of 10 minutes and then fixed in a 30% ethanol and 10% acetic acid solution for a total of 30 minutes. The gel was then washed twice for 5 minutes each in

10% ethanol 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 one minute, and then washed twice for a minute each in ultrapure water. Once the gel was stained, it was incubated in developer working solution (.5 mL enhancer and 25 mL developer) for 2-3 minutes or until bands appear. A 5% acetic acid solution was used to stop the reaction.

Western blot analysis

The second gel was used for Western Blot analyses to detect the NSP4 in our sample and to note the size distribution of the expressed protein. The first step is to electro-transfer the separated proteins contained within the gel onto a nitrocellulose membrane. To do this, a "sandwich" of different layers must be created. Starting from the bottom, the "sandwich" is composed of a fiber pad (Bio-Rad), a filter paper (Bio-Rad), our gel, a nitrocellulose membrane (Bio-Rad), followed by another filter paper and a final fiber pad on top. The sandwich is soaked in transfer buffer (20% methanol, 20% 5X transfer buffer, and 60% ultrapure water). While making the sandwich, it is essential to make sure that no bubbles are between the layers by rolling out the bubbles with a tube, and that you do not touch the nitrocellulose membrane. Connect the transfer to the power source set at 300 mA for 1.5 hours.

After the transfer is completed, the membrane is removed and soaked in a 5% dry milk/PBS (BLOTTO) solution (2.5 g of dry powdered milk in 50 mL of 1XPBS) for an

hour to block nonspecific antibody binding. The membrane was then washed four times at 5 minutes each. The first and fourth washes are with 1X PBS, while the second and third washes are with 1X PBS and .0025% Tween surfactant. Once washing is completed, the membrane was exposed to the primary antibody “rabbit anti-NSP4 150-175” at a dilution of 1:5000 in 2.5% BLOTTO. The blot was incubated with the primary antibody for an hour at room temperature and while rocking or overnight at 4°C and while rocking. After the blot is probed with the primary antibody, it was washed four times in the same way it was previously washed. The membrane was then probed with the secondary antibody “Goat anti-rabbit IgG conjugated to horse rabbit peroxidase (Pierce)” at a dilution of 1:20,000 in 2.5% BLOTTO (Pierce) for an hour at room temperature while rocking. The membrane was once again washed four times like before. The next step required the membrane to be exposed to the Chemiluminescent HRP Substrate (Millipore), which binds to the HRP of the secondary antibody and catalyzes a reaction which generates visible light. 2 mL of the first solution (HRP Substrate Peroxide Solution) and 2 mL of the second solution (HRP Substrate Luminol Reagent) were mixed and added to the nitrocellulose membrane for about 5 minutes. Excess fluid was removed from the membrane by dabbing it with a piece of filter paper. The membrane was placed in a film cassette, which blocks all light from entering. In a dark room, we reopened the cassette and placed a piece of Blue Basic Autorad Film (8”X10”) (BioExpress) to expose the film to the bound chemiluminescent substrate for 5 minutes. After 5 minutes, the film was developed. The dark bands generated signify

where NSP4 is present. The results from the silver stain (shows all protein bands) and the Western blot (shows only the NSP4 bands was evaluated).

CHAPTER III

RESULTS

Yeast lysates containing tNSP4

After lysing all the yeast using the standard procedure mentioned in chapter 2, a dot blot was used to check for the presence of tNSP4 in the supernatant. The first attempt produced a dot blot that was positive for tNSP4. Despite this initial success, after retesting the supernatants several times a couple of weeks later, the dot blots all came back with no dots. Finally after several failed attempts, we readjusted the concentrations of the antibodies used in the protocol and repeated the experiment again from the beginning. Upon lysing the yeast and collecting the new supernatants, this dot blot (Figure 1) shows distinct, round black dots that signify the presence of tNSP4, but the background was high presumably due to insufficient blocking.

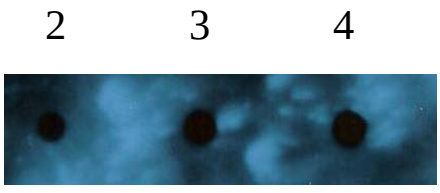

FIGURE 1-Dot Blot. Dot Blot done on 2/7/12 shows the presence of three dots (labeled 2, 3, 4 to correspond with the tubes).

Therefore, this part of the experiment presented the most challenges. The various failed attempts showed the importance of antibody concentration used when probing a dot blot or a western blot with an antibody. Using too much antibody will over-saturate the blot, while using too little will not result in any bands. The importance of blocking the blot for the specified time prior to probing with the primary antibody is also vital.

Gravimetric gel filtration

After acquiring supernatant that tested positive for tNSP4, it was time to begin purification of the expressed tNSP4. The initial and primary step was to pour and run a gel filtration column of specific molecular weight cut off. The goal was to purify the protein of interest from any contaminants that vary in molecular weight. As stated earlier, a constant flow of 20% acetonitrile was tried as one of the solvents. In addition, the supernatant was dissolved in 10M urea in order to remove some of the NSP4-interacting proteins from NSP4 molecules. The column was composed of GE Healthcare S-100 High Resolution Sephacryl beads of pore size 47 µm. After the column was equilibrated, the tNSP4-containing samples were carefully layered on the column meniscus. The sample was allowed to enter the column before additional 20% acetonitrile was added to facilitate the sample running through the column. A typical chromatograph is shown in Figure 2 when monitored at 280 nm, sensitivity of 0.1, and chart speed of 6. The column ran for about seven hours and fractions were collected to evaluate for the presence of tNSP4.

FIGURE 2-Gel Filtration Chromatograph. The major peak was resolved at about 3 hours into the experiment. 36 fractions were collected across the peak.

Spectrophotometer

Each fraction collected from the gel filtration column shell-frozen, lyophilized, and re-suspended in 100 µl of 1X PBS after being lyophilized. The fractions were then taken to the spectrometer to determine the concentration of tNSP4 in the fractions collected. Table 1 shows the concentrations of all the fractions collected from the gel filtration column. Note that the concentration is the highest for fractions 16-18 by a considerable margin. Fractions 16-18 also correspond to the peak seen in Figure 2. The other fractions approximately were the same varying between 0.17 – 0. 40 mg/ml (average of 0.443 when the major peak included; average of 0.244 when the major peak was excluded).

TABLE 1. Concentrations of fractions isolated after gel filtration column.

Fractions	Concentration (mg/ml)
1-3	0.20
4-6	0.21
7-9	0.17
10-12	0.19
13-15	0.24
16-18	**2.38**
19-21	0.31
22-24	0.40
25-27	0.33
28-30	0.26
31-33	0.26
34-36	0.36

Western blot

To verify which of the fractions contained the tNSP4 and its relative purity, the samples were tested by Western blot. Figure 3 shows the Western blot results. There is tNSP4 (15 kDa) present in fractions 16-18 which corresponds to the large peak in Figure 2. The

other lanes failed to show any tNSP4. It is unknown if this is due to the low quantity of protein in the samples or if tNSP4 was enriched in fractions 16-18. A silver stain was also done which showed our tNSP4 had several unknown compounds still bound to it.

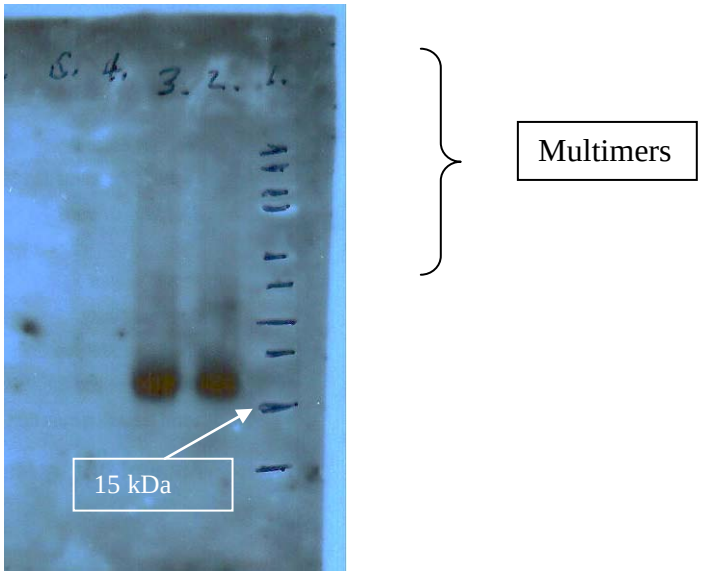

FIGURE 3-Western Blot. Western Blot was used to test for the presence of NSP4 in our fractions. This figure shows that the only tube that contains NSP4 is fractions 16-18. The arrow points out the tNSP4 at 15kDa. The area above the two bands are multimers that remain present.

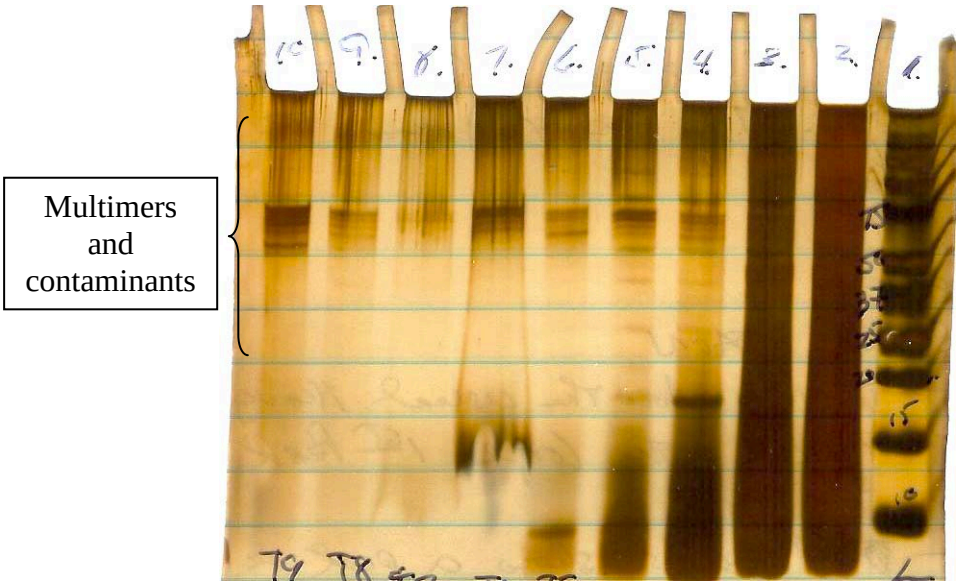

FIGURE 4-Silver Stain. Silver stain was performed on a polyacrylmide gel. A protein marker is shown in lane 1. This figure shows that the only tube that contains NSP4 is fractions 16-18 and confirms that there are still contaminants bound to our tNSP4.

CHAPTER IV

CONCLUSION

In summary, we transformed several colonies of yeast with the plasmid pYes-DEST52 and allowed it to reach log phase growth. Once the colonies grew and began producing the truncated version of NSP4, the yeast were harvested and then lysed using a Chaps/Lyticase extraction method. Centrifugation was performed to separate the supernatant from the pellet, and a dot blot was performed with the supernatant to ensure the expression of the recombinant tNSP4.. The dot blot (Figure 1) showed that the supernatant collected was positive for tNSP4.

After gathering several tNSP4-positive samples, a gravimetric gel filtration column was used to perform the first purification step. The column was prepared by pouring sephacryl beads of pore size 47µm carefully and slowly in order to avoid the introduction of bubbles, allowed to settle, and equilibrated in the sample buffer. The samples were introduced at the top of the column and allowed to run through the beads. Figure 2 shows there was a major peak of tNSP4 at approximately 3 hours into the run. The fractions eluting from the column were collected, dialyzed, and lyophilized. The lyophilized fractions were solubilized in water and tested for concentration using a nano-drop. The only fraction that had a significant concentration of tNSP4 was fractions 16-18, which corresponded to the major peak in Figure 2. As shown in Table 1, all the other fractions had low, insignificant concentrations of protein.

After running several samples through the gravimetric gel filtration column, we were successful in removing several of the impurities and increasing the purity of tNSP4. Through the use of Western Blot and Silver Stain analyses, we were still able to see some bands that were likely contaminants and bands that appeared as multimers of the tNSP4. This means that there are still several unknown compounds bound to the tNSP4 as Figures 3 and 4 show. Attempts were also made to use different solutions to separate the bound proteins with boiling prior to gel filtration but these attempts were not successful.

Therefore, our use of gravimetric gel filtration column was only marginally successful in that tNSP4 failed to release the cellular binding partners. Our next step is to use reverse phase High Pressure Liquid Chromatography (HPLC) for further purification. Instead of having the sample drip through beads by the use of gravity like in a gel filtration chromatography, HPLC introduces the sample under high pressures of up to 400 atm. In addition, HPLC is highly automated and very sensitive, it allows the use of a much smaller particle size for the column packing material. This provides a larger surface area for interaction between the stationary phase and the tNSP4 flowing past it. It also allows for gradients of the eluting buffer. This will allow a much better separation of the components of the mixture. Currently, we are in the process of acquiring sufficient ‘gel filtered’ sample for the HPLC analyses.

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