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Identifying Purification and Storage Techniques for the Human Papillomavirus Type 16 Major Capsid Protein L1

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Expression of human papillomavirus (HPV) major capsid protein L1 in *Escherichia coli* produces L1 proteins that can self-assemble into pentamers, with 72 pentamers forming a capsomere. With effective purification and storage techniques, these L1 pentamers could provide a more economic alternative for manufacturing HPV vaccines. Various techniques were evaluated in order to develop the most efficient method for L1 protein purification. Primary purification of L1 protein fused to Glutathione S-transferase (GST) was accomplished by GST affinity chromatography. DEAE anion exchange chromatography and heparin affinity chromatography were then tested as secondary purification techniques. Results showed heparin chromatography removed L1 degradation products, while DEAE chromatography removed significant levels of endotoxin from the sample. Several storage methods, including ammonium sulfate precipitation and freezing, were also analyzed based on their ability to maintain L1 protein conformation. A concentration of 40% ammonium sulfate was determined to be the most effective at precipitating protein from solution. In addition, a conformation-specific antibody that recognizes the neutralizing epitope of L1 was used to analyze resuspended proteins and showed high levels of binding, indicating that L1 protein conformation was maintained. Further analysis may prove that this is an effective technique for protein storage during transport, which would improve vaccine cost-effectiveness.

Introduction:

Human Papillomavirus (HPV) is a non-enveloped, double-stranded DNA virus (1). It infects the mucosal squamous epithelium causing lesions, which can develop into cancer. There are over 90 different types of HPV, but not all of them are cancerous. Each HPV subtype contains a genome that encodes for two structural proteins that make up the viral capsid. L2 is a minor capsid protein that forms an internal complex with the virus chromatin (2). L1 is the major capsid protein that assembles into 72 pentamers, which associate to form the viral capsid. Infection by HPV is primarily dependent on the interaction of the L1 capsid protein with cell surface receptors on the host cell.

Expression of L1 recombinant capsid proteins can be induced in eukaryotic systems, such as yeast or insect cells, to form virus-like particles (VLPs). These VLPs consist of 72 pentamers of L1, which mimic the HPV capsid. Immunization with VLPs induces the production of neutralizing antibodies, which provide immunity against HPV infection (3). In addition, they lack a viral genome so they cannot cause infection. Merck and GlaxoSmithKline have developed vaccines using VLPs. GlaxoSmithKline's vaccine Cervarix™ targets HPV16 and HPV18, which cause over 70% of cervical cancers (4). Merck's vaccine Gardasil™ targets HPV16 and HPV18 as well as HPV6 and HPV11, which lead to benign genital warts.

Both of the currently available vaccines are effective in providing protection against their respective subtypes; however, there are several challenges that these vaccines do not address. Most importantly is vaccine availability. Cervical cancer is the second deadliest cancer among women worldwide with roughly 80% of cases occurring in less developed countries (4). The high rate of cervical cancer is mainly due to the lack of cancer pre-screening programs, such as PAP smears. Providing preventative treatment through vaccine administration would be the most cost effective system to reduce the

high rate of HPV related cervical cancers. Both Gardasil™ and Cervarix™ are expensive to manufacture because they are produced in eukaryotic organisms and require extensive purification (5). In addition, they are expensive to transport, as they need a cold chain.

Several techniques have been formulated to reduce the cost of HPV vaccine production. For example, a way to express HPV16 L1 in *Escherichia coli* has been developed. In addition, purification of isolated L1 capsid proteins can induce pentamer formation. These methods have the potential to lower the manufacturing costs, but for this to occur, further refinement is needed.

The research described in this paper addresses various purification techniques targeting HPV16 L1 for use in vaccine development. In addition, several storage techniques were evaluated based on their ability to maintain effective L1 protein conformation. This would allow for a more cost effective means to transport L1 pentamers for use in vaccines.

Materials and Methods:

Expression of HPV16 L1 Protein

E. coli transformed with a plasmid coding for HPV16 L1 with an N-terminal glutathione S-transferase (GST) was obtained from Dennis Macejak. The sample was streaked onto a Luria-Bertani (LB) plate containing the antibiotic ampicillin (amp) and incubated overnight at 37°C. One colony was used to inoculate 50 mL of Terrific Broth containing ampicillin (TB-amp) at 250 rpm overnight at 30°C. A 4 mL sample from the overnight culture was used to inoculate 500 mL TB-amp at 250 rpm at 37°C until the OD_{595} reached 4. To induce protein expression, 200 µL of 0.5 M isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture. The temperature was reduced to 25°C and shaken at 250 rpm until the OD_{595} reached 8. The cells were then harvested by centrifugation at 6000 rpm for 15 min at 4°C.

Cell Lysis

Pellets from 250 mL of culture were resuspended in 200 mL buffer L (50mM Tris [pH 8], 200 mM NaCl, 5 mM EDTA and 5% glycerol) containing 5 mM DTT, protease inhibitor cocktail tablets (Roche) and 1 mM PMSF. The cells were lysed by passing them through a French pressure cell at 2000 psig ATP, $MgCl_2$ and DNase were added to the lysate at final concentrations of 2 mM ATP, 10 mM $MgCl_2$, and 40 U/mL DNase. The lysate was rocked at room temperature for 1 hour. Urea was added to a final concentration of 3.2 M. The lysate was rocked for an additional 1 hr at room temperature and then dialyzed overnight at 4°C with two changes of 5 L buffer L with 5 mM DTT. Cellular debris was then removed through centrifugation at 12500 rpm for 20 min at 4°C. The supernatant was decanted, filtered at 0.2 µm and stored at 4°C.

GST Affinity Chromatography

The lysate was purified using FPLC (AKTA) with a GSTrap column (GE Healthcare). The lysate was injected onto the column at 0.5 mL/min, and 20 mL buffer L containing 2 mM DTT was used to wash the column. A second wash was conducted using 20 mL buffer L with 2 mM DTT and 0.01% Tween80. GST-L1 was then eluted using 20 mL buffer L with 2 mM DTT, 0.01% Tween80 and 10 mM reduced glutathione. Fractions 3-6, which showed the highest protein concentration, were saved for further analysis, including sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS establishes a negative charge after binding polypeptide chains, which allows proteins to be separated based on size.

Removal of GST tag

Cleavage of the GST tag was completed to isolate L1 alone. Fractions 3-6 from the

GSTrap column were combined. The GST tag was then cleaved off by adding 10 U thrombin per mg protein, and the sample was incubated overnight at 25°C. The cleaved sample was then run over the GSTrap column to separate purified L1 from the GST tag. The sample was injected onto the column as before, and the column was washed using 20 mL buffer L with 2 mM DTT and 0.01% Tween80. Fractions from the flow-through were collected. GST was then eluted using 20 mL buffer L with 2 mM DTT, 0.01% Tween80 and 10 mM reduced glutathione. All fractions showing high protein concentration were saved and analyzed via SDS-PAGE.

Western Blot

Western blot analysis was conducted to ensure proper identification of the isolated proteins. Both uncleaved and thrombin cleaved fractions from the GSTrap column were separated by 10% SDS-PAGE and then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore) by electroblotting at 60mA for 45min. The membrane was then blocked using Tris-buffered saline with Tween20 (TBS-T) with 5% milk. Each membrane was treated with either an anti-GST antibody (GE Healthcare) or an anti-L1 antibody (6) in TBS-T for 45 min, before washing three times with TBS-T for 10 min. Each membrane was then treated with the appropriate alkaline phosphatase-conjugated secondary antibody (Promega) for 45 min. The membranes were again washed three times with TBS-T for 10 min. Bands were detected with a 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) color development substrate (Promega).

DEAE Anion Exchange Chromatography

A DEAE column was used as a second purification step to remove endotoxin from the solution. Fractions 3-6 from the GSTrap column were combined and dialyzed into 500 mL buffer L with 75 mM NaCl at 4°C overnight. The buffer was changed to fresh buffer and dialysis continued for another 6 hours. The purified proteins were then run over a 1 mL DEAE column, and the flow-through was collected. The column was washed with 2 mL buffer L with 100 mM NaCl, and 1 mL fractions were collected. Three more washes were completed using 1 mL buffer L with 125 mM, 500 mM, and 1 M NaCl. All eluates were collected as well as a sample of beads from the column. Eluates were examined through SDS-PAGE and an endotoxin assay.

Endotoxin Assay

Natalie Meinerz generously completed an endotoxin assay to determine the endotoxin concentration in the fractions from the GSTrap column versus the eluates from the DEAE column. This assay was completed using Limulus Amebocyte Lysate (LAL) provided in an endotoxin assay kit (Lonza).

Gel Filtration Chromatography

A Superose column (GE Healthcare) was also evaluated as a second purification step. Fractions 3-6 from two GSTrap column runs were combined and placed in an Amicon® Ultra-15 centrifugal concentrator tube (Millipore). The sample was centrifuged at 4000 g in 10 min increments until the solution was concentrated from 0.475 mg/mL to 1.90 mg/mL. The concentrated solution was run over a Superose6 column at 0.5 mL/min. The column was washed with buffer L with 2 mM DTT. Fractions off the column were collected and analyzed through SDS-PAGE.

Heparin Affinity Chromatography

A HiTrap Heparin column (GE Healthcare) was also tested as a possible second purification technique. Fractions 3-6 from two GSTrap column runs were combined and dialyzed into buffer L with 75 mM NaCl. This salt concentration was optimized in a preliminary experiment. A previous trial using 200 mM NaCl did not allow a high level of GST-L1 protein binding to the column. Buffer L with 75 mM NaCl was also used to equilibrate the HiTrap Heparin column. The sample was

then injected onto the column, which was then washed. The bound proteins were eluted from the column using a linear gradient of increasing salt concentration, from 75 mM to 2 M NaCl. Fractions were saved from peaks showing high protein concentration and analyzed using SDS-PAGE.

A second experiment was conducted with slight modifications. Fractions 3-6 from two GSTrap column runs were combined, and the GST tag was cleaved off using thrombin as described previously. In this case, the cleaved sample was not run over the GSTrap column to remove the GST tag, but was placed directly onto the HiTrap Heparin column.

Precipitation of HPV16 L1

Various precipitation techniques were evaluated as possible means for protein storage.

Ethanol Precipitation

Ethanol was cooled at -20°C overnight before use. Purified GST-L1 (200 µg) from the GSTrap column was combined with 9x volume of ETOH. The sample was vortexed and incubated at -20°C for 2 hrs. The sample was then centrifuged at 14000 rpm for 10 min at 4°C to pellet the protein precipitate. The supernatant was decanted and disposed. The pellet was dried for 30 min at room temperature. Buffer L with 2 mM DTT (1x volume) was added to resuspend the pellet. The results were analyzed using SDS-PAGE.

Acetone Precipitation

The same technique described for ethanol precipitation was used for acetone precipitation, except 4x volume of acetone was added to 200 µg of GST-L1.

Ammonium Sulfate Precipitation

A saturated solution of ammonium sulfate [$(NH_4)_2SO_4$] was formulated using 5.5 g $(NH_4)_2SO_4$ dissolved in 10 mL distilled water. This solution was cooled to 4°C until crystals formed. The saturated $(NH_4)_2SO_4$ solution was then added to 200 µg GST-L1 in varying concentrations. Final concentrations of the four different samples were 20%, 30%, 40%, and 50% $(NH_4)_2SO_4$. Each sample was rocked at 4°C for 2 hrs. Samples were then centrifuged at 14000 rpm for 10 min at 4°C. The supernatant was decanted and stored for further analysis. Each pellet was then resuspended in 1x volume of buffer L with 2 mM DTT. Results were analyzed through SDS-PAGE and ELISA.

Freeze/Thaw Analysis

Protein stability after freezing and thawing was examined. A 200 µg sample of GST-L1 was stored at -80°C overnight. The sample was then thawed at 4°C. Results were analyzed through SDS-PAGE and ELISA.

Enzyme-Linked Immunosorbant Assay (ELISA)

Protein conformational stability after exposure to various storage techniques was examined by completing an ELISA. A carbon coating buffer was formulated using 150 mM sodium carbonate, 350 mM sodium bicarbonate, and 30 mM sodium azide, and the pH was adjusted to 9.6. Each well of a 96-well plate was coated with 50 µL of 2 µg/mL casein-glutathione conjugate in carbonate coating buffer and incubated overnight at 4°C. Each well was then washed three times with wash buffer containing 1xPBS and 0.05% Tween20. Wells were blocked for 1 hr at 37°C with wash buffer with 5% milk (block buffer).

Block buffer was mixed with GST-L1 to the desired concentration and serial dilutions were made (1 µg, 0.5 µg, and 0.25 µg/well). A no protein control was also included. Plates were then incubated for 1 hr at 4°C. Wells were then washed three times with 200 µL wash buffer. Either a mouse V5 antibody (7) or an anti-L1 antibody (50 µL of 1:1000 dilution) was added to each well and incubated for 1 hr at 25°C. Plates were washed three times using 200 µL wash buffer. A volume of 50 µL of a 1:5000 dilution of the appropriate secondary HRP-conjugated antibody was added to each well and incubated for 1 hr at 25°C. Each well was again washed three times with 200 µL wash buffer. TMB substrate (50 µL; Thermo Scientific) was added to each well and incubated at 25°C for

20 min. A volume of 50 µL of 2M H_2SO_4 was then applied to quench the reaction. The absorbance was read at 450 nm.

Results:

In order to attain the purest form of the L1 capsid protein, several different purification techniques were examined. It was demonstrated previously that fusion of L1 to an N-terminal GST provided an easier means of purification via GST affinity chromatography (8). To replicate these results, a plasmid containing a coding region for GST-16L1 was used. Once the protein was expressed as described in the methods, GST affinity chromatography was used as the primary purification technique before all experimental secondary purification techniques. The N-terminal GST bound to the glutathione beads within the column and allowed excess material to flow through. As buffer L with reduced glutathione was added to the column, the glutathione competed for binding, allowing GST-16L1 to be eluted from the column. Results from elution with reduced glutathione are shown in figure 1. GST-16L1 was eluted in fractions 3 to 6, which is observable by the distinct peak in figure 1.

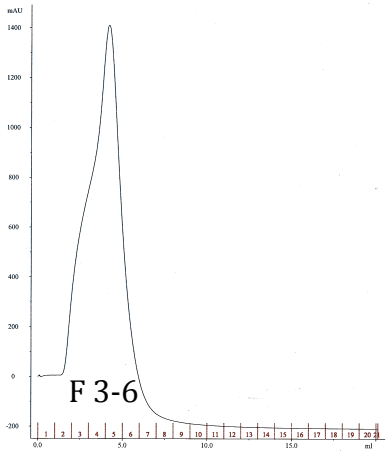


Figure 1. GST affinity chromatography. A graph displaying GSTrap elution with reduced glutathione. GST-16L1Δ was eluted in fractions 3-6.

Fractions 3-6 from the peak were analyzed along with the column flow-through and wash samples by SDS-PAGE (figure 2). A well-defined band at 78 kDa was visible in fractions 3-6 (lanes 4-7). This size corresponds to GST-16L1, which further shows that GST affinity chromatography is useful for purifying GST-16L1. A band of similar size is also present in the flow-through, indicating that some GST-16L1 did not bind to the column. There also were several other bands visible in fractions 3-6. These may be GST-16L1 degradation products or they could consist of other proteins.

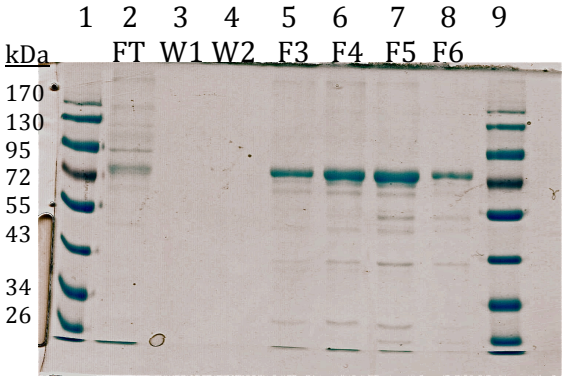


Figure 2. SDS-PAGE analysis. Lanes 1 and 9 contain the ladder. Lane 2 represents the column flow-through. Lanes 3 and 4 are column washes. Lanes 5-8 are fractions 3-6 from the column elution (figure 1). A strong band is seen at 78 kDa in fractions 3-6, which corresponds to GST-16L1.

The N-terminal GST is useful in purification techniques, but for vaccine development, it is desirable to have HPV16 L1 that is not fused to GST. In order to achieve this, thrombin, which has a cleavage site between the GST and L1 sequences, was added to the sample. The GST tag was then removed using a second round of purification on the GST column. Results from the GSTrap purification can be seen in Figure 3. The L1 capsid protein flowed through the column without binding. It formed a peak from fractions 3 to 12. The

GST tag was eluted with glutathione in fractions 24 to 26.

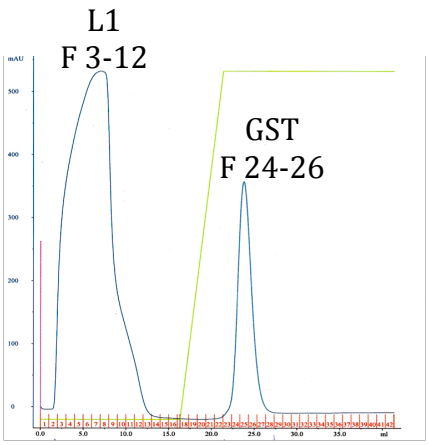


Figure 3. Cleaved GST and L1 purification by GST affinity chromatography. L1 protein flowed through in fractions 3-12. The GST tag was eluted in fractions 24-26.

Western blot analysis was then conducted to ensure proper identification of the isolated proteins. Results are depicted in Figure 4. Figure 4a shows a PVDF membrane that was treated with an anti-GST antibody. A defined band can be seen at 26 kDa, signifying the presence of cleaved GST (lanes 2 and 4). Very little GST was detected in the column flow through, indicating that most of the GST bound to the column.

Figure 4b shows the PVDF membrane that was treated with an anti-L1 antibody. Several bands are present in the first three lanes. These bands might have resulted from L1 degradation products. However, significant bands are present at 52 kDa and 78 kDa. The 78 kDa band corresponded to uncleaved GST-16L1, while the 52kd band corresponded to cleaved L1. In addition, no L1 capsid protein could be identified in lane 4, the second peak in Figure 3, which indicates that L1 was not lost during purification. This result, in addition to findings that most GST was removed from the sample containing L1, demonstrate that thrombin cleavage followed by GST affinity chromatography is an effective way to isolate the L1 capsid protein.

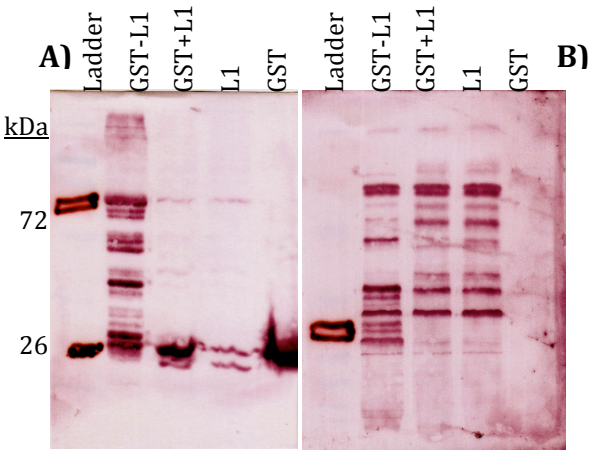


Figure 4. Western blot analysis of thrombin cleaved sample purified with GST affinity chromatography. **A)** PVDF membrane treated with an anti-GST antibody. **B)** PVDF membrane treated with an anti-L1 antibody.

In a parallel experiment, another second purification step was undertaken in order to remove endotoxin from the solution. Endotoxins are present in *E. coli* and can be very harmful to humans. If injected (i.e., in vaccine administration), they can cause adverse side effects, such as tissue damage or a strong immune response (9). Therefore, it is necessary to remove endotoxin from the solution. DEAE anion exchange chromatography was conducted as a means to purify the GST-16L1 product away from the endotoxin and possibly other contaminating proteins. Results are indicated in Figure 5. Many bands present in the initial sample input (lane 3), were not seen in fractions 2, 4, and 6 (lanes 4-6), but reappeared in the elution fractions (lanes 7-11). This indicates that degradation products or other protein impurities that were present in the original sample bound to the DEAE column and were removed from the solution (fractions 2-6, lanes 4-6).

To determine whether DEAE anion exchange chromatography removed endotoxin from the solution, an endotoxin assay was conducted. The amount of endotoxin in the original sample was compared with that of the purified flow-through 6. Results showed that the level of

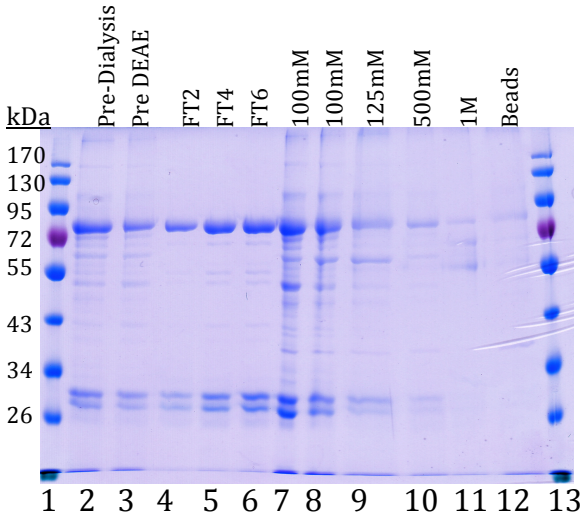


Figure 5. SDS-PAGE analysis of DEAE anion exchange chromatography. Lane 3 is the original column input. Lanes 4-6 were taken from the column flow-through. Lanes 7-11 are from the column elution with increasing concentrations of NaCl. Lane 12 shows any remaining material on the column beads. Bands at 78 kDa signify the presence of GST-16L1.

endotoxin in the solution decreased from 2.3 EU/ng to 0.02 EU/ng, a 100-fold decrease. Thus, purification with a DEAE column is an effective way to remove endotoxin. However, through this purification technique, some GST-16L1 was lost because it bound to the column and was eluted with the endotoxin, which decreased the percent yield to 82%.

Size-exclusion chromatography was also evaluated as a second purification step. A Superose6 column was utilized as described in the methods. A graphical analysis of the column flow-through (Figure 6) showed three distinct peaks. Comparison of each of these peaks against standard peaks revealed that the first peak had a molar weight (MW) of around 2 million daltons. The second peak had a MW of around 232,000 daltons and the third peak was too small to be compared against the standards.

Fractions from each peak were stored and analyzed via SDS-PAGE. The results in

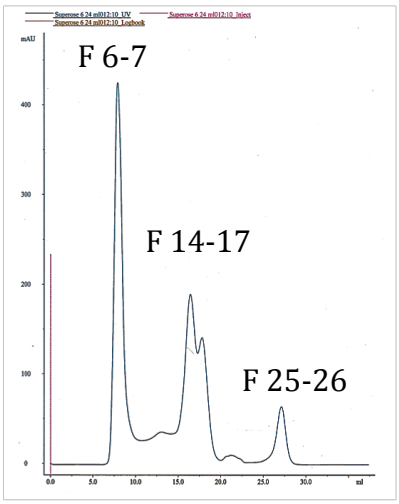


Figure 6. Size-exclusion chromatography with a Superose6 column. The peak from fractions 6-7 was around 2 million Daltons, the peak from fractions 14-17 was around 232,000 daltons and the third peak was too small to be evaluated.

Figure 7 show a defined band at 78 kDa in the first two peaks, indicating the presence of the GST-16L1 protein. The peak spanning fractions 25-26 did not show any protein products, which could have resulted from the products running off the gel as a result of their minute size. The presence of GST-16L1 in the first peak indicates that there were several aggregates in solution that flowed through the column in these first fractions. Thus, the second peak is most likely the desired isolate of GST-16L1. It is unclear whether size-exclusion chromatography was an effective means of purification. Based on the SDS-PAGE results, few other bands besides the 78 kDa GST-L1 can be seen, so there is little means for comparison. However, the percent yield was significant at 77%.

Heparin affinity chromatography was the final method examined as a second purification step. The use of this type of chromatography was based on the findings of Joyce and Giroglou that HPV infection requires interaction with cell surface proteoglycans, specifically heparin sulfate

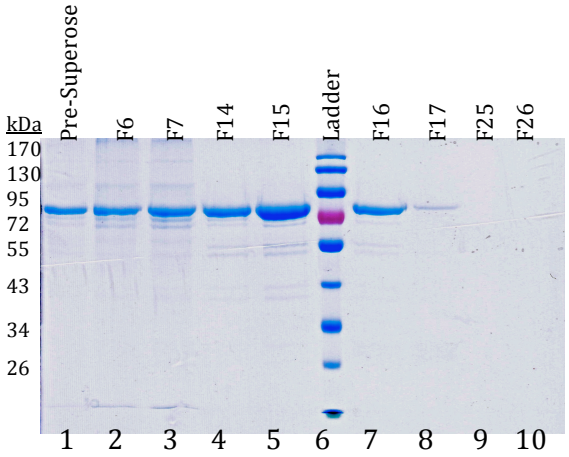


Figure 7. SDS-PAGE analysis of Superose6 column fractions. Lanes 2-3 are fractions 6 and 7 of the first peak (Figure 6). Lanes 4-5, and 7-8 are fractions 14-17 of peak 2. Lanes 9-10 are fractions 25-26 from peak 3. A 78 kDa band was seen in both peaks 1 and 2 showing the presence of GST-16L1.

side chains (10). Thus, samples of L1 can be purified based on their interaction with heparin. A HiTrap Heparin column was used as described in the methods to elicit this binding interaction. Figure 8 displays the results from an uncleaved GST-16L1 sample injection. Some sample flowed through the column without binding, as indicated by the first peak of Figure 8a. However, the majority of sample bound to the heparin column and was eluted with a salt gradient. Fractions from each peak were stored and analyzed via SDS-PAGE (Figure 8b). Lanes 3-4 were samples taken from the first peak. These lanes display a strong band at 78 kDa, indicating GST-16L1, but they also display several other bands, which could be degradation products or other contaminants. Lanes 5-6 show fractions taken from the second peak (Figure 8a). They also have a clear band at 78 kDa, but they lack many of the bands present in the first peak. Thus, many of the contaminants were removed from the solution. Although some GST-16L1 was lost through this purification technique, the percent yield was significant at 46%.

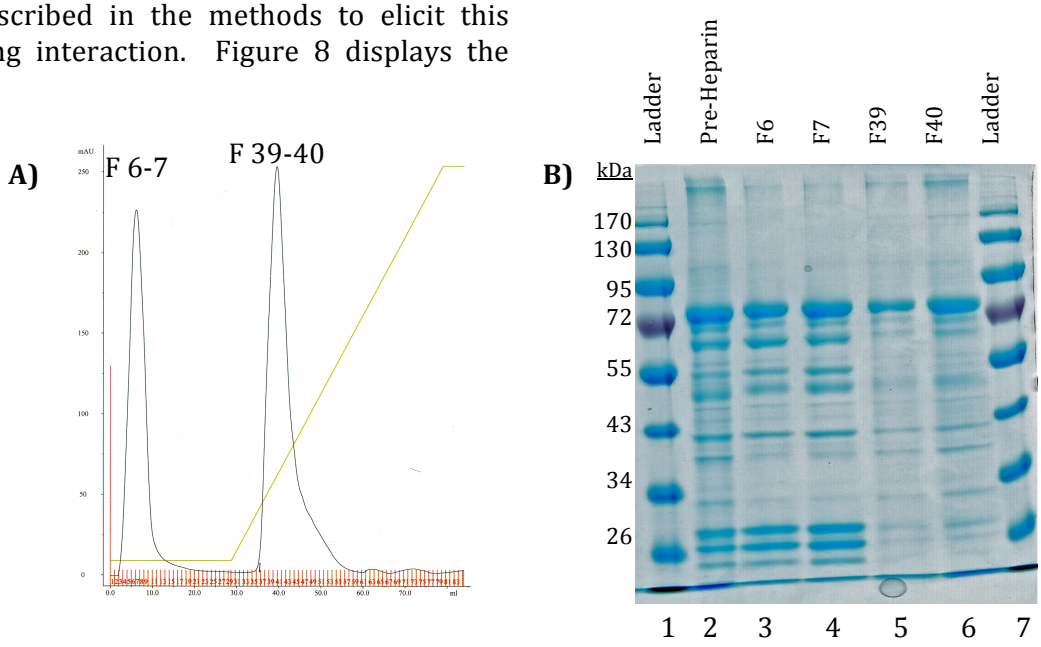


Figure 8. Results from GST-16L1 purified with heparin affinity chromatography. **A)** A graph showing the products and their various elution times. **B)** SDS-PAGE analysis of heparin column results. Lane 1 and 7 contain the ladder. Lane 2 shows the sample input, which was from an initial GSTrap column. Lanes 3-4 show fractions 6-7 taken from peak 1. Lanes 5-6 show fractions 39-40 taken from peak 2. The removal of impurities can be seen through the absence of bands from the original to the eluted sample.

Heparin affinity chromatography was repeated with a sample of thrombin-cleaved GST and L1. Results are depicted in Figure 9. The GST tag flowed through the column without binding. The L1 capsid protein bound to the column and was eluted with an increasing salt gradient, as seen in peak 2. Fractions from each peak were again analyzed via SDS-PAGE (Figure 9b). Lane 2 shows the original uncleaved sample of GST-16L1, and lane 3 shows the cleaved sample, which was purified with the HiTrap Heparin column. Lanes 4-5 show fractions 5 and 6 taken from peak 1. They have a well-defined band at 26 kDa, indicating the presence of GST. Lanes 7-8 represent fractions from the second peak and show a strong band at 52 kDa, signifying the presence of the L1 protein as well as some uncleaved GST-L1 remaining in the solution (78 kDa). In addition, lane 5 shows fraction 11 from the first peak. Interestingly, it contains some L1 capsid protein as well as several additional bands that are not seen in the eluates. When the L1 from this minor peak was combined with the second peak, the percent yield reached 46%, which is significant. Thus, it can be concluded that purification by heparin affinity chromatography proved to be effective in removing degradation products as well as in isolating L1 from the cleaved GST tag.

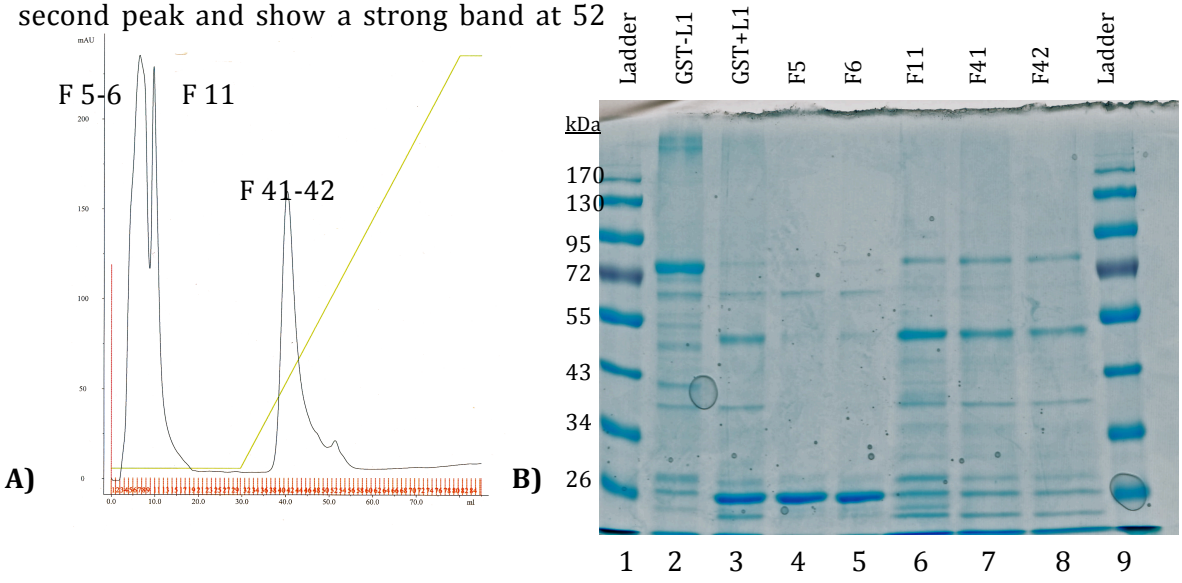


Figure 9. Results from a cleaved sample of GST and L1 purified with heparin affinity chromatography. **A)** A graph showing the products and their various elution times. **B)** SDS-PAGE analysis of heparin column results. Lane 1 and 9 contain the ladder. Lane 2 shows the original uncleaved sample. Lane 3 shows the cleaved GST and L1 prior to the HiTrap Heparin column purification. Lanes 4-5 represent fractions 5 and 6 taken from the first peak. Fraction 11 from the first peak is shown in Lane 6. Lanes 7-8 show fractions 41-42 taken from the second peak.

In addition to evaluating purification techniques for isolating GST-16L1, several storage techniques were analyzed. For vaccine development it is crucial to find an effective way to store GST-16L1. The storage method must not alter the L1 conformational epitope that provides immunity. The first storage technique that was tested was freezing and then thawing of a sample of GST-16L1. SDS-PAGE (Figure 10a) and ELISA (Figure 10b&c) were used to analyze this sample. SDS-PAGE showed few

differences between the original sample (lane 2) and the sample that underwent freezing and thawing (lane 3). Results from the ELISA were also evaluated. The ability of the sample to bind the V5 and L1 antibodies was compared before and after freezing. While the L1 and V5 antibodies reacted with L1 from the sample, results are inconclusive because they cannot be evaluated in the linear range. Thus, further evaluations need to be conducted. However, the percent yield revealed that 100% of the original sample was resuspended in solution.

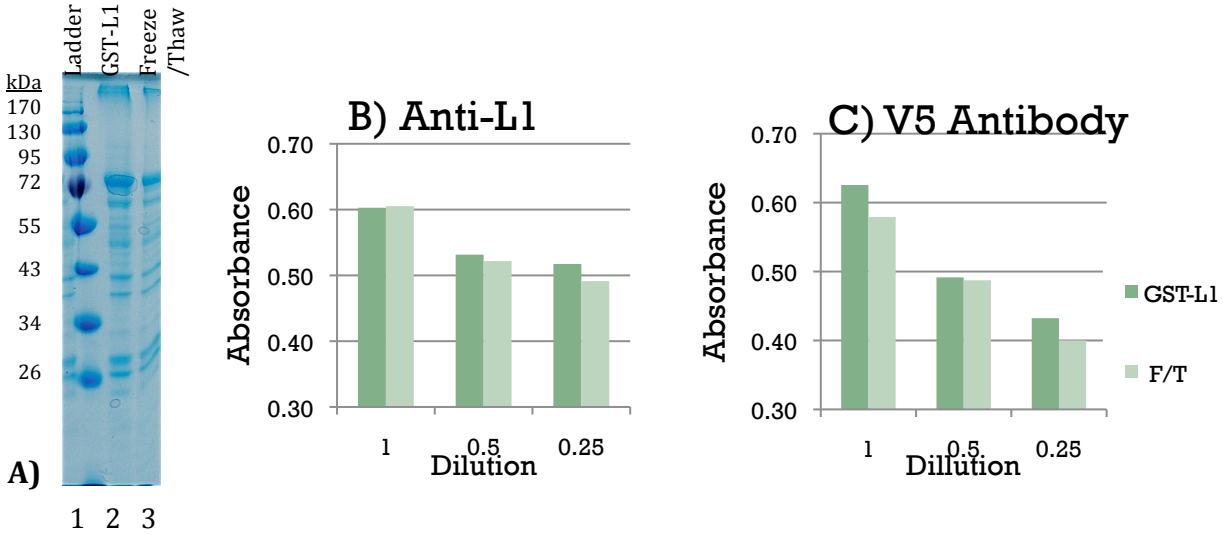


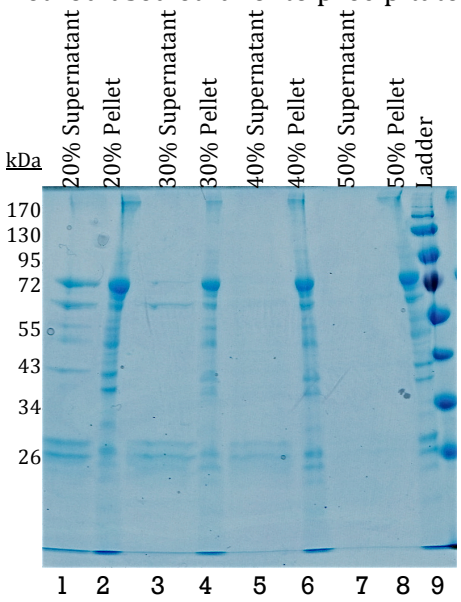
Figure 10. Results from freeze/thaw analysis. **A)** SDS-PAGE comparing the original sample (lane 2) with the sample that underwent freezing and thawing (lane 3). Ladder is shown in lane 1. **B)** ELISA with an anti-L1 antibody. **C)** ELISA with the V5 antibody.

Several precipitation techniques were also analyzed as potential storage methods. By precipitating proteins out of a solution, they can be stored in pelleted form. Ammonium sulfate has widely been used to for this purpose, but little research had been conducted on the precipitation of GST-L1 prior to this experiment. Four different samples were precipitated using varying concentrations of an ammonium sulfate solution as described in the methods. Results from SDS-PAGE analysis revealed that higher concentrations of ammonium sulfate solution, specifically 40% and 50%, allowed more sample to precipitate. This fact is evidenced by the decreased number of bands in the higher concentration supernatants, as opposed to 20% and 30% ammonium sulfate solution supernatants. Similar results have been obtained from a Bradford protein assay as well as percent yield values from each sample (results not shown).

ELISA analysis was conducted on each resuspended pellet (Figure 11b and 11c). Each sample was evaluated in parallel in order to determine trends. Like the analysis of freeze/thaw samples, ammonium sulfate precipitates were treated with either an anti-L1 antibody or the V5 antibody. Although the samples reacted with both the anti-L1 antibody and the V5 antibody, comparisons between the samples with respect to conformation and maintenance of the conformational L1 epitope could not be determined because the absorbance values for the amounts of protein used in this experiment were not in the linear range. Percent yield analysis revealed that 91% of L1 protein from the original sample was

precipitated by the 50% ammonium sulfate solution and 100% by the 40% ammonium sulfate solution.

Two other precipitation methods were evaluated in addition to ammonium sulfate. One method used ethanol to precipitate GST-L1 and the other used acetone. Results are depicted in figure 12. GST-16L1 did not precipitate out of solution with either acetone or ethanol, so results were inconclusive.



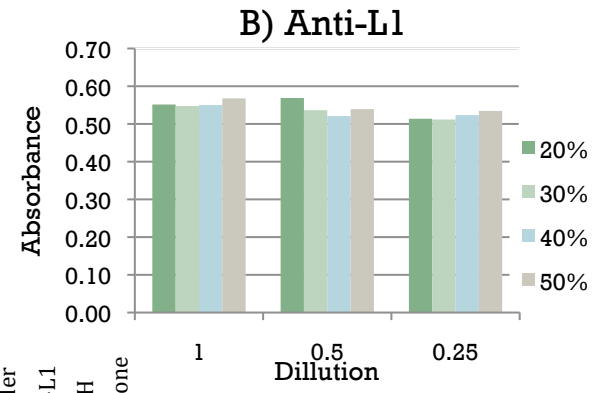
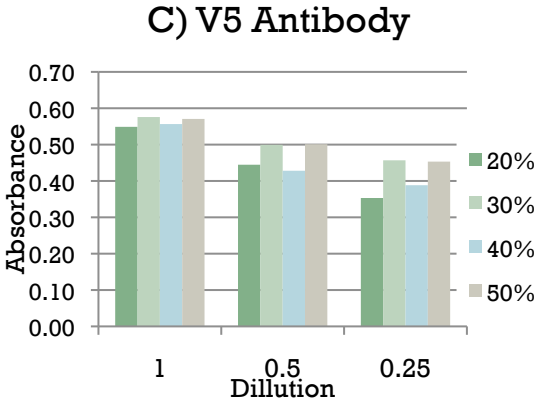


Figure 11. Results from ammonium sulfate precipitation. **A)** SDS-PAGE comparing the ability of various concentrations of ammonium sulfate solution to precipitate GST-L1. The ladder is shown in lane 9. **B)** ELISA with an anti-L1 antibody. **C)** ELISA with the V5 antibody.



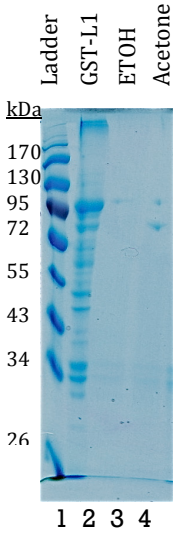


Figure 12: Ethanol and acetone precipitations. Lane 1 is the ladder, and the original sample is in lane 2. Resuspended ethanol precipitate (lane 3) and acetone precipitate (lane 4) lacked GST-16L1.

Discussion:

Through the course of these experiments, a great deal of valuable information was obtained, including the application of DEAE anion exchange chromatography to purify GST-16L1. DEAE had previously been shown to be an effective method for removing endotoxin from protein solutions. In a prior research experiment, over 75% of endotoxin was removed from a protein solution with only minor loss of product (11). This technique used has now been demonstrated to be effective in purifying GST-16L1, specifically.

In addition, our results also indicated a 99% decrease in endotoxin with only a small amount of protein loss. Because future vaccine research in our laboratory is directed towards using bacteria rather than eukaryotes for L1 protein expression, this method will be very helpful in ensuring a safe product for vaccine administration.

The use of heparin affinity chromatography was also demonstrated to be an effective purification technique. In a previous study, HPV11-L1 was shown to have a high affinity for heparin side chains. This acknowledgement was then used to purify VLPs with a HiTrap Heparin column. The VLPs showed significant binding to the column and were easily eluted with a salt gradient (12). Information from that experiment was applied to HPV16 L1 and produced similar results, suggesting that heparin affinity chromatography could be useful in purifying GST-16L1. In addition, applying a thrombin cleaved sample to the column showed that heparin affinity chromatography can also be used as a means for separating the L1 protein from the cleaved GST tag. This could provide an alternative to GST tag removal via GST affinity chromatography. Further analysis should be conducted in order to conclude if one method is superior over the other.

Through the course of this project, ammonium sulfate was used to precipitate GST-16L1. The greatest amount of precipitation was seen with 40% and 50% ammonium sulfate solution, with the highest being at 40%. Each sample also reacted with the V5 antibody, which demonstrated that precipitation maintained L1 protein conformation. A separate study showed results consistent with these. It was found that HPV16 L1 precipitated best with 45% ammonium sulfate solution after evaluating 40%, 45% and 50% (12). In addition, this previous study analyzed the use of precipitation for purification rather than storage. As a result, an ELISA analysis was not conducted. It would be useful to conduct an ELISA analysis after precipitation with 40%, 45%, and 50% ammonium sulfate solution because results shown in this paper were inconclusive. It would also be beneficial to further extend ELISA dilutions, so results would be in the linear range.

Further research on precipitation with ethanol and acetone would also be useful. Though this study was not able to precipitate GST-16L1 using either acetone or ethanol, it would be extremely valuable to develop a precipitation protocol that is effective. In conclusion, it is apparent that the purification and storage methods for HPV16 L1 are plentiful. These various techniques will allow for more efficient purification of HPV16 L1 and easier transport, which may lower the cost of vaccine production and allow a wider population to have access to this much needed vaccine. Optimizing the best combination of these protocols will only be accomplished through further analysis and understanding of each specific technique.

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