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Purification of the Bovine Papillomavirus Type 1 L1 Capside Protein from Escherichia Coli

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Abstract

I completed the honor's thesis project for two reasons; in order to gain research experience with molecular biology techniques that will serve as the foundation for future doctoral work; and due to the significant implications of papillomavirus infection in both human and animal populations, it is important to formulate a vaccine that is both immunogenically effective and cost-efficient so that susceptible populations can be protected from infection. The thesis project was to determine if bovine papillomavirus type 1 (BPV1) L1 capsid protein could be expressed and purified from *Escherichia coli*. Using several molecular biology techniques including; molecular cloning, gene expression, ion exchange chromatography, ammonium sulfate precipitation, size exclusion chromatography and electron microscopy, I found that recombinant BPV1 L1 protein could be expressed from *E. coli* and subsequently purified to yield L1 capsomeres. These finding suggest that BPV1 L1 capsomeres can be incorporated into a vaccine preventing infection of BPV1 in susceptible populations.

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Papillomaviruses (PVs) are double-stranded DNA viruses that infect squamous or mucosal epithelia. Infection by PV presents a range of epithelial lesions that are generally benign, but can transform to malignant growths. Over 100 PV types have been characterized and can infect a wide range of mammals, including humans. While human papillomaviruses (HPV) have gained notoriety due to their link to cervical cancer, (2) other PVs, such as bovine papillomaviruses (BPV), have significant agricultural and veterinary importance. BPV is the causative agent of epithelial lesions in bovine populations (3).

BPV infection of commercial livestock is of great financial importance in the agricultural and veterinary sectors. BPV infection of cows causes hyper-proliferative, benign lesions in mucosal and cutaneous tissue, which typically regress without medical treatment. However, BPV-induced lesions can transform to become malignant squamous cell carcinomas (4). There are 11 verified serotypes of BPV; specific serotypes, BPV1 and BPV2, can cross the species barrier and infect equine populations. Infection by BPV in horses and donkey's causes aggressive, but benign, cutaneous lesions, termed sarcoids, the association of BPV with sarcoid represents the only example of a papillomavirus capable of cross-species infection (5).

Sarcoids present as single or multiple epithelia lesions (*i.e*. they can occur at multiple anatomical locations) and typically originate at sites of previous injury and subsequent scarring. Unlike papilloma's developed from BPV infection of cows, sarcoids persist, are resistant to therapeutic interventions and often reappear following surgical excision (6). Depending upon the location and severity of the lesions, sarcoids can severely limit the mobility of the animal.

The etiologic link between BPV and equine sarcoid was first described after horses, inoculated with purified BPV, developed transitory sarcoid-like lesions (7). Subsequent investigations showed the presence of BPV DNA in sarcoid tumors from horses and donkeys using southern blot analysis. PCR technology has facilitated

detection of BPV1 or BPV2 DNA in 100% of lesions analyzed. Non-sarcoid lesions or tissue samples from animals without lesions showed no trace of BPV DNA (6). Despite lack of BPV DNA detection within the host cells, skin swabs of uninfected horses living near to BPV positive cows were found to be positive for BPV DNA (6). Additionally, tissue samples acquired from horses with early stage dermatitis contain episomal BPV1 genomes and expression of early viral genes (8). Within lesions, BPV DNA exists in the nuclei of infected cells as multiple episomal copies. Viral early and late genes have been shown to be transcribed in tumors, supporting the hypothesis of viral involvement in sarcoid development, however, infectious virions have not been isolated from sarcoids, suggesting that equines are non-permissive hosts for BPV or that the concentration of virions is too low for detection (9).

The significant impact of BPV is primarily within animal related industries. Horse racing, in particular, relies on the mobility and performance of the animal in order to assess its worth. Often, successful horses can be worth hundreds of thousands of dollars with potential gains in the millions correlated to stud fees. BPV infection resulting in appearance of sarcoids can limit the mobility of the animal, compromising its ability to perform. Loss of performance can represent a significant economic loss. Reciprocally, animal-based labor is the foundation of agricultural based economy in developing countries. Loss of animals to BPV infection can have devastating consequences. Due to the significant impact of BPV in both bovine and equine populations, there is a need for an effective prophylactic intervention that can be produced cost efficiently due to the economic variability of applicable markets.

The PV virion (capsid) has a non-enveloped icosahedral structure of 55 nm diameter made up of 72 capsomeres. The capsid is made up of two proteins, L1, the major structural protein, and L2, the minor protein (10). The capsid contains the circular double-stranded viral DNA of approximately 8 kb associated with cellular nucleosomal proteins (10). The viral genome (Figure 1) is divided into three parts;

approximately two thirds of the genome codes for the early proteins E1 to E7, approximately one-third codes for the structural proteins L1 and L2, and the remainder is mostly noncoding and contains the *cis* elements necessary for viral DNA replication and transcription.

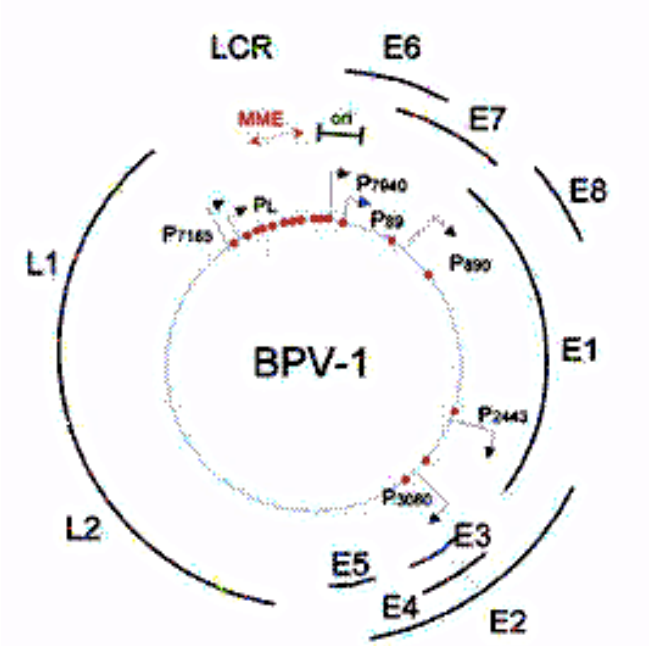

Figure 1. BPV-1 genome comprised of the early region encoding for the non-structural proteins responsible for viral replication, the late region encoding the major and minor structural proteins and the long control region containing the origin of replication, promoter sequences and enhancer elements.

Virus-like particles (VLPs) are empty PV particles, without the viral genome, that are formed by expression of L1 alone or L1 and L2 (11, 12). They are assembled in eukaryotic cells (*i.e*. yeast or insect), display remarkable structural and antigenic similarity to virions, and can induce high titers of antibodies in vaccinated individuals (13-15). These observations have led to the use of VLPs in vaccines against several HPV serotypes. Currently, there are two VLP vaccines against HPV available for commercial use. However, cost is a critical issue for VLP vaccination due primarily to the use of a eukaryotic expression system and requirement of cold-chain storage to maintain stability.

L1 capsomeres (Figure 2) are potential low cost alternatives to VLPs because they can be expressed in *Escherichia coli* (*E. coli)* and subsequently purified in high yields (16-18). Deletion of the N-terminal 10 amino acids (NΔ10) and the C-terminal 29 amino

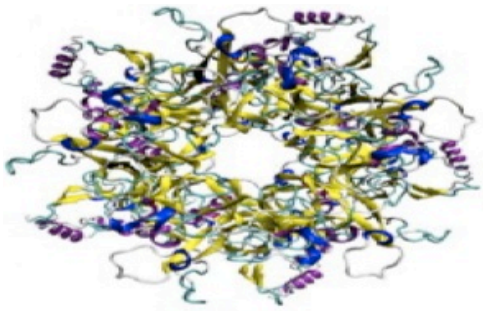

Figure 2. PV L1 capsomere; homopentameric protein complex composed of L1 monomers.

acids (CΔ29) from L1 enhances the yield of capsomeres by removing the invading arms necessary for interpentameric interactions required for capsid assembly (16, 18). Morphologically, capsomeres display identical type-restricted neutralizing epitopes as seen on VLPs. These epitopes are critical in eliciting a strong neutralizing antibody response. In one study, vaccination of dogs with GST-fused canine oral papillomavirus (COPV) L1 capsomeres protected against experimental oral challenge with COPV (19). Inoculation with COPV L1 was shown to be completely protective without an adjuvant using only 400 ng capsomeres or 50 ng VLPs. Additionally, serum immunoglobulins from the COPV L1 vaccinated dogs provided protection against COPV challenge in naïve recipients. Similar results were demonstrated in a more recent study, when GST-fused HPV 16 L1 capsomeres induced high-titers of neutralizing antibodies in mice and were subsequently completely protected from infection when challenged intravaginally with HPV 16 (20).

Since BPV1 has been linked as the causative agent of equine sarcoid and has significant veterinary and agricultural impact, a low cost vaccine against BPV1 is desirable. The purpose of the current investigation is to express BPV1 L1 capsomeres using a recombinant *E. coli* expression system. Successful extraction and purification of L1 capsomeres is the first step in vaccine formulation. Reasons for utilizing L1 capsomeres in formulating a vaccine against BPV are primarily cost related. Due to economically areas that BPV impacts, providing a cost-efficient vaccine will enable its implementation in broader markets and hopefully decrease the impact of BPV in veterinary and agricultural sectors.

Materials and Methods

Cloning BPV1 L1 into pETac vector. The mutant BPV1 L1 coding sequences with N-terminal and C-terminal deletions were generated as previously described (21). Briefly, full-length BPV1 L1 DNA was obtained from GeneScript as a codon-optimized gene for bacterial expression. To generate a truncated L1 fragment, a

portion of the L1 gene was PCR amplified with forward primer; 5'-AGTAGTCATATGACCCTGTATCTGCCGCCG-3', (NΔ9) containing an *NdeI* restriction enzyme site at the initiator methionine codon and a reverse primer; 5'-ATCTCGAGTTATTAGGCCCGGCGCCCTGTTG-3', (CΔ29) containing an *XhoI* restriction enzyme site.

The PCR-amplified fragment (BPV1 NΔ9CΔ29 L1) was purified with a QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's protocol. PCR-amplified BPV1 NΔ9CΔ29 L1 (BPV1 L1) and the expression vector, pETac, were digested with restriction enzymes, *NdeI* and *XhoI*. The restriction-digested products were then visualized by DNA gel electrophoresis and gel-purified using QIA Gel Purification Kit (Qiagen). The gel-purified, *NdeI-XhoI* digestion product, BPV1 L1, was ligated into the *NdeI-XhoI* restriction digested pETac vector to yield, pETac BPV1 NΔ9CΔ29 L1, placing BPV1 L1 downstream of the pTac promoter sequences. The presence of the pETac BPV1 NΔ9CΔ29 L1 plasmid (pETac BPV1 L1) was confirmed by DNA gel electrophoresis.

BLR (DE3) competent cells (Novagen) were transformed with ligation reaction product by heat shock at 42°C and then incubated in manufacturer supplied medium for 40 min. Cells were then plated on LB- Kan plates and incubated overnight at 37°C. A mini-preparation of cell growth culture was performed to confirm the presence of BPV1 L1 DNA in each of the colonies obtained. The correct BPV1 L1 DNA insert was verified by sequence analysis. A single colony of transformed bacteria was inoculated into 3 ml of Terrific broth (TB) medium (1.2% Tryptone, 2.4% yeast extract, 0.5% glycerol, 17 mM KH_2PO_4, and 72 mM K_2HPO_4), containing 100 μg/ml kanamyacin (Kan) and grown for 6 hours at 37°C to an optical density at 595nm (OD_{595}) of 4.0. The temperature was decreased to 25°C and 15 μl of 0.5 M isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture which was then grown to an OD_{595} of 8.0. Time point samples were taken at 0, 1, 2, and 3 hr to monitor protein expression. A sample of the mini-preparation was

combined with 50% glycerol to prepare a stock solution of transformed cells and stored at -70°C for future use.

Cell Growth Culture and Expression. 20 µl of BPV1 L1 positive stock solution was plated onto an LB-Kan plates and incubated overnight at 37°C. A single colony was harvested and grown for 6 hours at 37°C in 3 ml of TB. 500 µl of the 6 hour culture was then used to inoculate 50 ml of TB containing 100 µg/ml kanamycin and grown overnight at 37°C. The overnight culture was then used to inoculate 500 ml of fresh TB/Kan media and the culture was grown at 37°C to an OD_{595} of 4.0. The temperature was decreased to 25°C and 200 µl of 0.5 M IPTG was added to the culture which was then grown to an OD_{595} of 8.0. The cells were harvested by centrifugation at 6000 rpm for 15 min at 4°C, the supernatant discarded and the cell pellets were then stored at -20°C.

QHP Ion Exchange Column. The HiTrap Q-column (QHP column) (GE Healthcare) was prepared for ion exchange chromatography by initially washing with five column volumes of distilled H_2O (dH_2O), followed by priming with five column volumes of buffer B (10 mM Tris-HCl pH 8.0, 5% glycerol, 1.0 M NaCl, 5mM DTT). Prior to application of supernatant obtained from the whole cell lysate, the column was washed with five column volumes of buffer L (50mM Tris-HCl pH 8.0, 0.2 M NaCl, 1mM EDTA). The flow through from the supernatant application was collected as a partially purified solution.

For anion exchange chromatography by elution over an increasing salt gradient, the column was washed with five column volumes of dH_2O, followed by priming with buffer B. Following priming, the column was washed with five column volumes of buffer A (50 mM Tris-HCl pH 8.0, 5% glycerol, 250 mM NaCl, 5 mM DTT) before supernatant was applied for elution. Fractions obtained from elution were analyzed for BPV1 L1 presence and protein concentration.

Superose 6 Size Exclusion Column. The Superose 6 size-exclusion column was prepared for analysis of concentrated BPV1 L1 by washing with two column

volumes of dH_2O followed by an additional two column volumes of buffer L before the sample was injected for analysis.

L1 Protein Purification. The frozen bacterial pellet was resuspended into 200 ml of buffer L with 5 mM DTT and 1 complete protease inhibitor cocktail tablet (Roche). Bacteria were lysed by homogenization using a Panda homogenizer (GEA Niro Soavi) at 800-1000 barr. The lysate was passed through twice to ensure complete lysis.

Homogenized lysate was centrifuged at 22,000 x *g* for 30 min at 4°C and the supernatant was applied to the QHP column previously prepared for affinity chromatography, using an AKTA FPLC operating at 5.0 ml per minute. The flow through (QFT) was collected and the column was washed with four column volumes each of buffer L and buffer B. The washes with buffer L were collected and combined with the QFT.

L1 protein was precipitated by addition of $(NH_4)_2SO_4$ to 30% saturation to the QFT from the QHP column. Precipitation of the QFT was allowed to proceed for 6 hours with stirring at 4°C. The precipitated protein was collected by centrifugation at 13000 x *g* for 30 min at 4°C. The pellet was resuspended in 50 ml of buffer A and resolubilized by with high pressure (400-600 barr) using the Panda homogenizer. The resuspended pellet was passed through the apparatus twice to ensure complete resolubilization. Insoluble material was removed by centrifugation at 13000 x *g* for 30 min and the supernatant was collected.

The conductivity of the supernatant was equilibrated to match that of buffer A, then applied to a QHP column and eluted over an increasing salt gradient. Eluted protein in peak fractions of BPV1 L1 were combined and concentrated for further analysis. BPV1 L1 concentrate was applied to a Superose 6 column at 0.5 ml per min. Resulting peak fractions were concentrated and stored at 4°C.

Protein Quantification. Protein concentration for samples was determined using a Bradford Assay (Bio-Rad) and compared against a BSA standard curve.

Coomassie Stain and Western Blot. Samples were analyzed by 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and stained for total protein with Coomassie blue or analyzed by Western blot. For Western analysis, gels were transferred onto a PVDF membrane and the membranes were blocked in 5% milk/TBST (TBS/0.05% Tween 20) overnight at 4°C with rocking. The milk solution was removed and the membrane was rinsed with TBST before being incubated with a 1:10,000 dilution of anti-BPV L1 (MAB837;Millipore) primary antibody for 1 hour at room temperature. The membrane was washed with TBST three times for 10 minutes each rinsed with TBST and blocked with 5% blocking solution for 30 minutes at room temperature. Blocking solution was removed and membrane was rinsed with TBST before incubation with goat anti-mouse IgG conjugated to alkaline phosphatase (Promega) for one hour at room temperature. Western blots were developed using Nitro blue tetrazolium (NBT) and 5-Bromo-4-chloro-3-indolyl phosphate (BCIP).

ImageJ Analysis. ImageJ was used to estimate the amount of BPV1 L1 protein as a percent of the overall protein retained at specific steps of extraction and purification. Percent values of BPV1 L1 protein content were calculated based on area and pixel value statistics of Coomassie stained gels. Gels were analyzed on a lane-by-lane selection and data obtained was used to determine amount of protein retained through extraction and purification procedures for overall BPV1 L1 yield value.

Results

Purification of the BPV1 NΔ9CΔ29 L1 after expression in *E. coli.* The truncated BPV1 L1 coding sequence was cloned into the vector pETac to yield pETac BPV1 L1, ligated product was confirmed by DNA gel electrophoresis (Figure 3). Gene expression was induced from the pTac hybrid promote r by addition of IPTG to the growth culture of recombinant *E. coli*.

Samples were taken at each step during purification and were analyzed by SDS-PAGE. SDS-PAGE gels were used in either Coomassie staining techniques or Western blot analysis with anti-L1 mouse antiserum. Protein expression after induction was monitored by OD_{595} readings and samples were taken at 0, 1, 2 and 3-hour time-points demonstrating an increasing protein concentration (Table 1 and Figure 4).

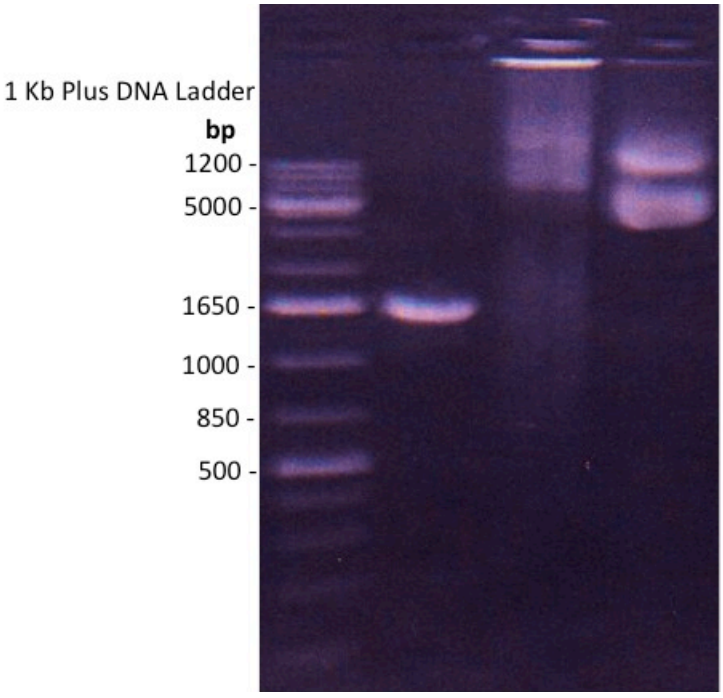

Figure 3. DNA gel electrophoresis of restriction enzyme digestion of PCR-amplified BPV1 L1 DNA insert and pETac cloning vector and ligation product, pETac BPV1 L1 cloning vector. Lanes: (1) 1 Kb Plus DNA Ladder; (2) BPV1 L1; (3) pETac BPV1 L1; (4) pETac.

Time Point	0 hr	1 hr	2 hr	3 hr
OD_{595}	4.38	5.51	6.96	8.32

Table 1. Optical density readings of cell culture growth post IPTG induction of pETac promoter sequences.

The cells were harvested by centrifugation once the OD_{595} reached 8.0. The whole cell lysate was applied to the QHP column for partial purification using ion exchange chromatography. Under the loading buffer conditions, BPV1 L1 was found in the QFT of the QHP column (Appendix I and Figure 5). The QFT was treated with 30%

ammonium sulfate (16.4 g $(NH_4)_2SO_4$ per 100 mL) and allowed to precipitate overnight. Although some BPV1 L1 was found in the supernatant (30% S), the majority was retained in the pellet (30% P) (Appendix I and Figure 5).

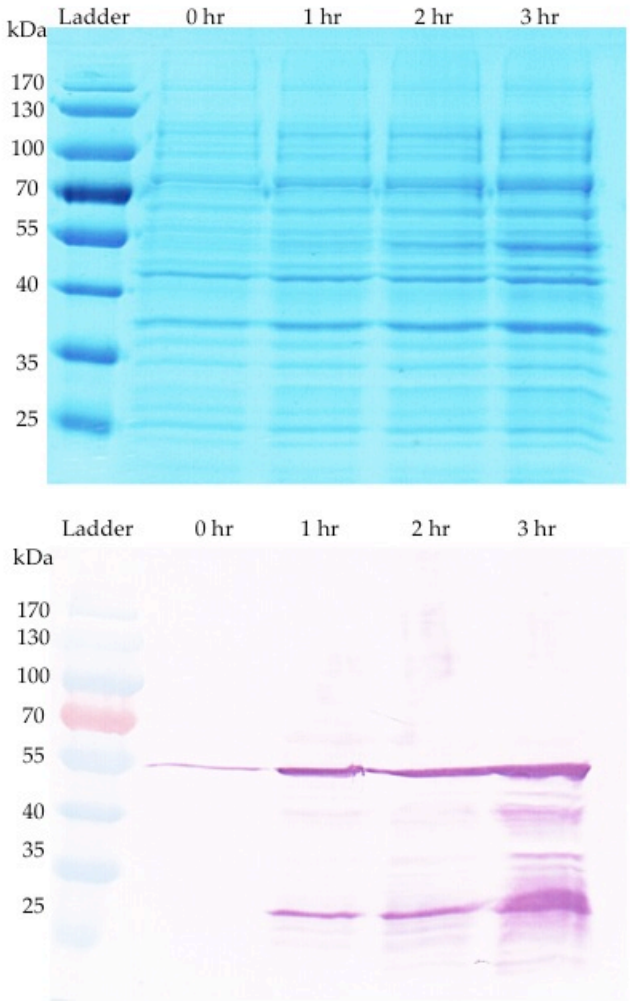

Figure 4. Representative coomassie stained gel and western blot analysis of time point samples monitoring BPV1 L1 gene expression post IPTG induction of pETac promoter sequence.

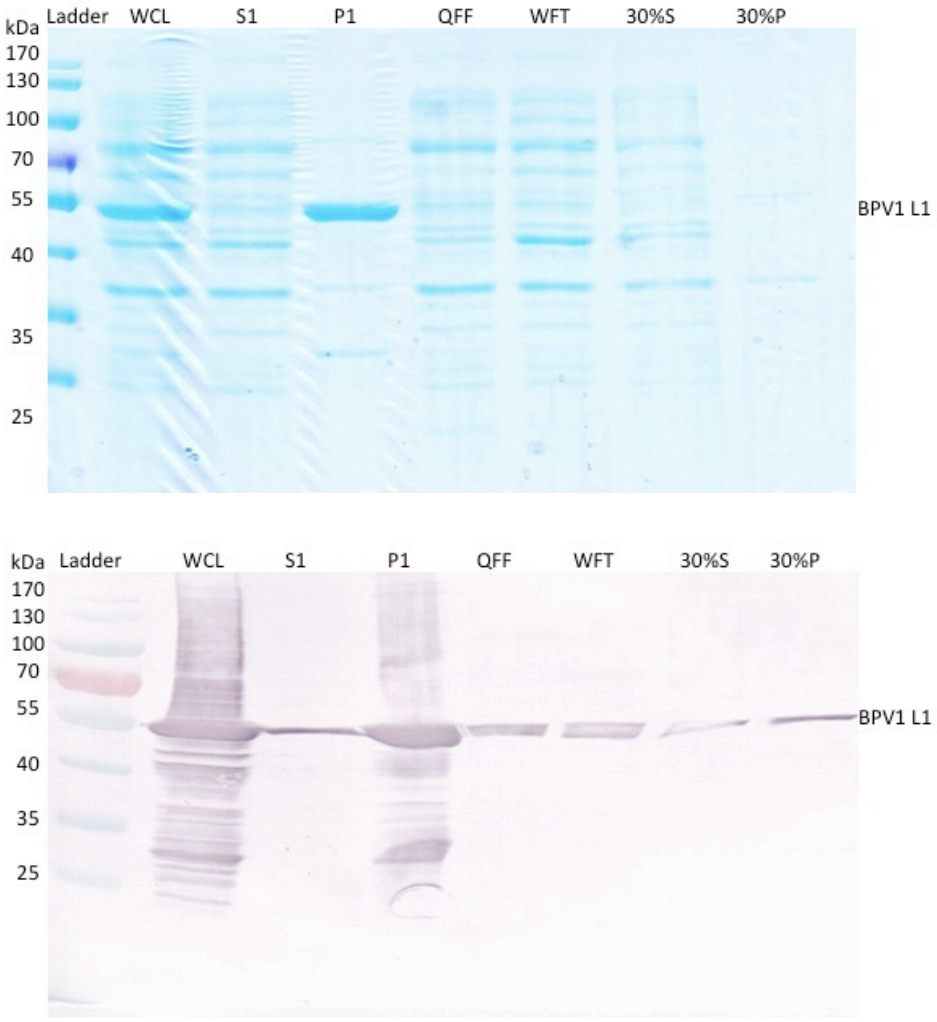

Figure 5. Representative coomassie stained gel and western blot analysis of lysate and partial purification ion exchange chromatography of BPV1 L1 protein. Lane: (1) Ladder; (2) Whole cell lysate (WCL); (3) Supernatant from WCL centrifugation; (4) Pellet from WCL centrifugation; (5) QHP column flow through; (6) Wash flow through; (7) 30% $(NH_4)_2SO_4$ supernatant; (8) 30% $(NH_4)_2SO_4$ pellet.

The overnight precipitate was resolubilized and applied to a QHP column for ion exchange chromatography with elution over an increasing salt concentration gradient. The FPLC tracing of OD_{280} (Appendix II) from ion exchange chromatography indicated peak fractions that were analyzed for BPV1 L1 presence and protein concentration. Fractions 15-18 contained peak concentrations of BPV1 L1 and were combined and concentrated (Appendix I and Figure 6).

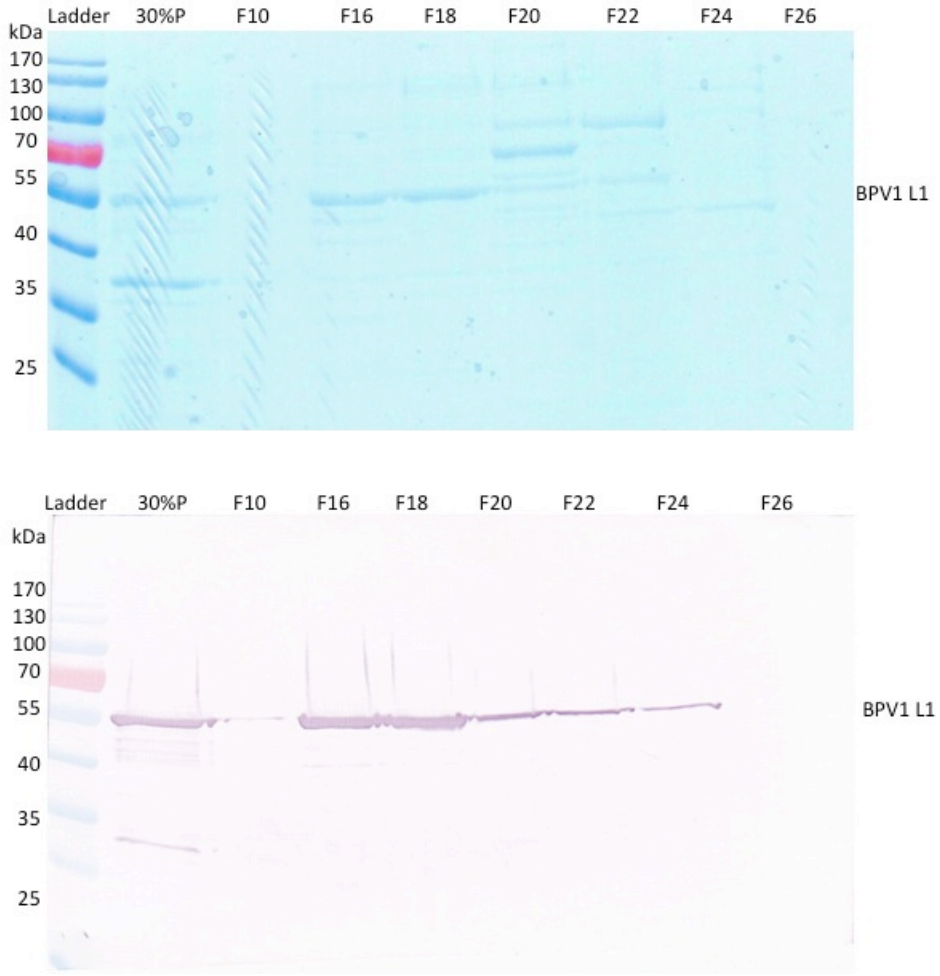

Figure 6. Representative coomassie stained gel and western blot of elution profile peak fractions from ion exchange chromatography of BPV1 L1 protein. Lanes: (1) Ladder; (2) 30% $(NH_4)_2SO_4$ pellet; (3) fraction 10; (4) fraction 16; (5) fraction 18; (6) fraction 20; (7) fraction 22; (8) fraction 24; (9) fraction 26.

A sample of the concentrated fractions was applied to the Superose 6 column for analysis. Based on the elution profile of known protein standards (data not shown), the FPLC tracing of OD_{280} (Appendix III) was consistent with elution profile of capsomere-structured complex in peak fractions 15-17 (Figure 7).

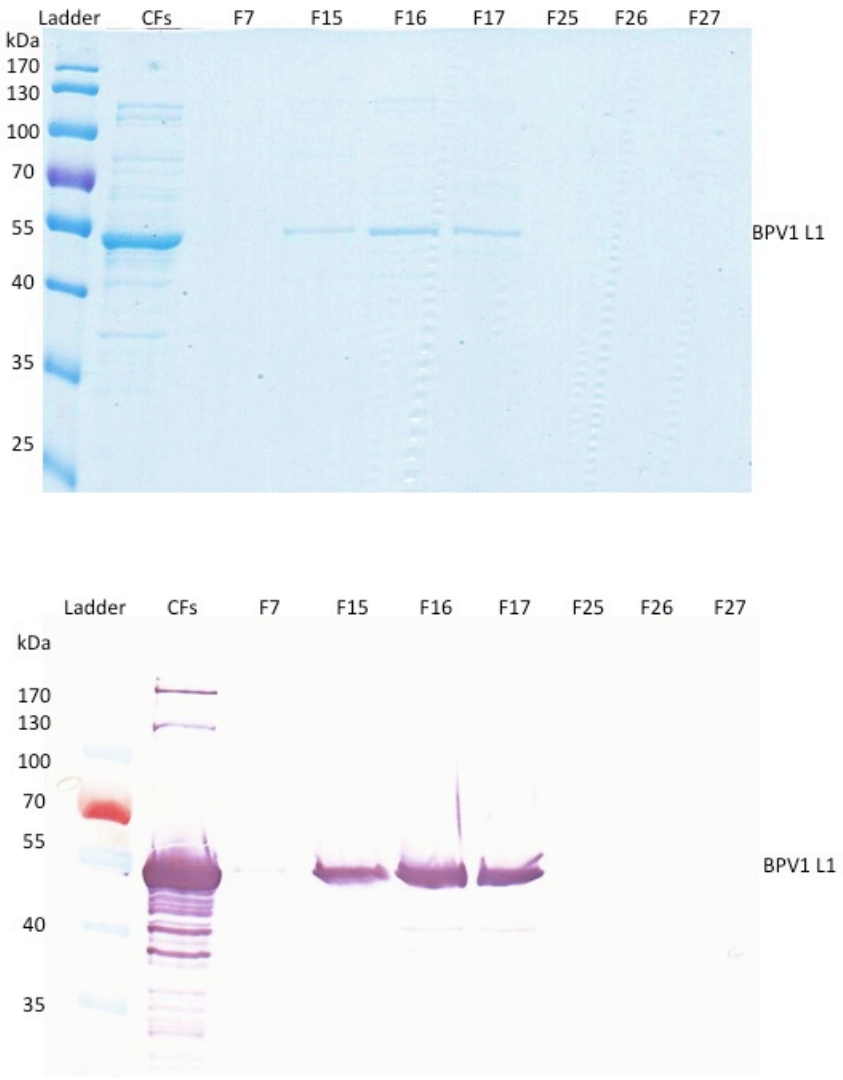

Figure 7. Representative coomassie stained gel and western blot of elution profile peak fractions from size-exclusion chromatography of concentrated BPV1 L1 protein. Lanes: (1) Ladder; (2) Combined BPV1 L1 fractions (15-18); (3) fraction 7; (4) fraction 15; (5) fraction 16; (6) fraction 17; (7) fraction 25; (8) fraction 26; (9) fraction 27.

The Superose 6 fractions were combined and concentrated and a sample was prepared for electron microscopy using 400 mesh copper grids with negative staining with 1% uranyl acetate to visually confirm BPV L1 pentamer formation (Figure 8). Coomassie stained gels were analyzed by ImageJ to estimate that the overall yield of BPV1 L1 was 1.5 mg/L of *E. coli* expression culture.

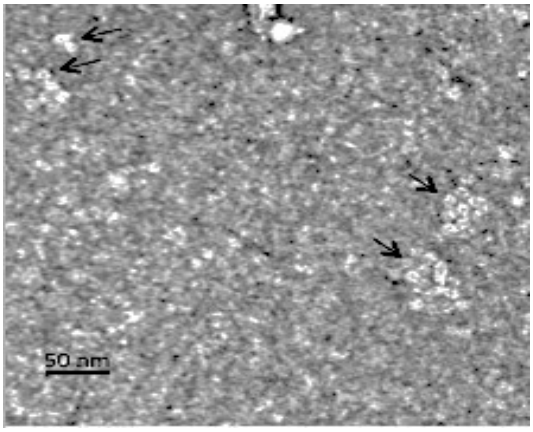

Figure 8. Electron micrograph of BPV1 L1 capsomeres purified from an *E. coli* expression system. Photograph by Kimberly Erickson.

Discussion

We have expressed and purified the BPV1 L1 protein using a recombinant *E. coli* expression system. The purified protein is soluble and forms capsomeres (Figure 8) but does not demonstrate the ability to self-assemble into higher capsid structures. These findings suggest that the truncated BPV1 L1 sequence effectively prevents capsomere-capsomere interactions and that the protein extracted can be further tested for immunogenic properties.

In order to obtain better yields of BPV1 L1 protein, future studies should investigate using different concentrations of $(NH_4)_2SO_4$ or multiple $(NH_4)_2SO_4$ precipitations. By analyzing the precipitate of variable amounts of $(NH_4)_2SO_4$, we determined that 30% $(NH_4)_2SO_4$ facilitated removal of the bulk of impurities while conserving the most BPV1 L1 by analyzing the precipitate of variable amounts of $(NH_4)_2SO_4$ (Appendix IV). However, a 25% $(NH_4)_2SO_4$ precipitation also resulted in a sample that lacked major impurities, although the precipitate contained less BPV1 L1. A strategy involving multiple precipitations may also be advantageous. For example, using a higher percentage of $(NH_4)_2SO_4$ prior to QHP column purification by elution over an increasing salt gradient, combining resulting peak fractions, followed by a

precipitation using a much lower percentage of $(NH_4)_2SO_4$ may assist in the removal of impurities.

In our investigation, we were only able to obtain yields suitable for scientific inquiry (1.5 mg/L of *E. coli* expression culture). The bulk of the BPV1 L1 protein failed to be extracted from the bacterial pellet during cell lysis (Figure 5). Further analysis of the Coomassie stained gels using ImageJ, enabled us to determine approximate BPV1 L1 yield (Appendix I) at specific steps of the extraction and purification procedures. ImageJ analysis indicated only 11% of BPV1 L1 was extracted from the original pellet. Additionally, the ion exchange chromatography done by elution over an increasing salt gradient indicated that BPV1 L1 did not elute in a concentrated peak, as seen in HPV-16 capsomeres (data not published); rather it elutes as a broad peak over several fractions (15-24) (Figure 6). Aggregation or mis-folding likely contributed to elution of BPV1 L1 over several fractions. Due to contamination by other proteins, only fractions 15-18 were utilized in subsequent purification steps and BPV1 L1 quantity data was not determined for discarded fractions. Despite our lack of success in obtaining substantial yields of BPV1 L1, we were able to successfully isolate BPV1 L1 capsomeres after size exclusion chromatography (Figure 7-8). As expected, aggregates eluted first from column, followed by BPV1 L1 capsomeres and finally smaller impurities (Appendix III). Interestingly, there was little or none BPV1 L1 aggregation as indicated by the Figure 7 Western blot (F7, lane 3), and that fractions 15-18 from the ion exchange chromatography step did in fact contain BPV1 L1 capsomeres.

To optimize the *E. coli* expression system, future investigations should test potential methods of protein extraction in order to obtain higher yields of BPV1 L1 protein. A previous investigation (21) demonstrated moderate success in extracting more HPV-16 L1 protein from the bacterial pellet using a buffer containing 1.0 M NaCl followed by resolubilization using high pressure. Additionally, increasing batch volume and utilizing fed batch fermentation or high-density growth media (23) have

been demonstrated to yield higher amounts of bacteria; however, these methods were beyond the scope of this investigation.

As stated previously, the impact of BPV1 in both the veterinary and agricultural industries requires the implementation of a cost efficient vaccine. The ability to purify the BPV1 L1 protein in a soluble and non-denatured form now permits further studies to test whether the recombinant BPV1 capsomeres are capable of inducing a neutralizing-antibody response as seen in similar studies involving HPV. Adjuvants can assist in eliciting a stronger innate immune response to vaccination and potentially lead to longer lasting humoral immunity. Unlike human-specific vaccines, the constraints on adjuvant utilization are less confining in veterinary medicine. While aluminum based adjuvants are implemented in several animal vaccines, future studies focused on the immunogenic properties of BPV1 L1 capsomeres should consider the usage of alternative adjuvants that may elicit stronger immunogenic responses.

For example, quil A is an adjuvant that is already widely used in veterinary medicine and has been integrated into several antiviral vaccines for cattle, pigs, horses, and cats, e.g., equine influenza virus and canine parvovirus. Quil A is partially purified mixture of saponins that act as an immunomodulator, capable of inducing strong TH1 and Th2 responses, as well as, cytotoxic T-lymphocytes. Saponin safety, however, is thought to be route of administration dependent. IV injections have been shown to result in toxicity, likely due to hemolysis. Local inflammatory reactions can occur with Quil A but can be avoided with the combination of cholesterol-containing liposomes (24).

The usage of alternative adjuvants may have human implications as well. Implementing different adjuvants with capsomere vaccines could result in a number of advantageous responses (i.e. longer lasting immunity) that would be desirable in human vaccines. Investigating the interactions between adjuvants, capsomeres and the immune system in animals will further elucidate our understanding of

immunomodulation and may facilitate more effective vaccination strategies for humans and animals in the future.

In this investigation we examined the potential of expressing, extracting and purifying BPV1 L1 capsomeres from an *E. coli* expression system. Given the pandemic nature of BPV1, capability of cross-species contamination and the economic impact in susceptible animal based markets, the need for a cost effective prophylactic vaccine is strong. The potential of a capsomere vaccine to be an effective intervention for BPV1 is great due to cost-efficiency of the *E. coli* expression system, and the strong immunogenic responses associated with capsomere inoculation. Future studies will need to investigate the efficiency of other methods designed to obtain higher yields of protein, in addition to determining the immunogenic properties of BPV1 L1 capsomeres in animal models.

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Appendix I. Tables 2 and 3.

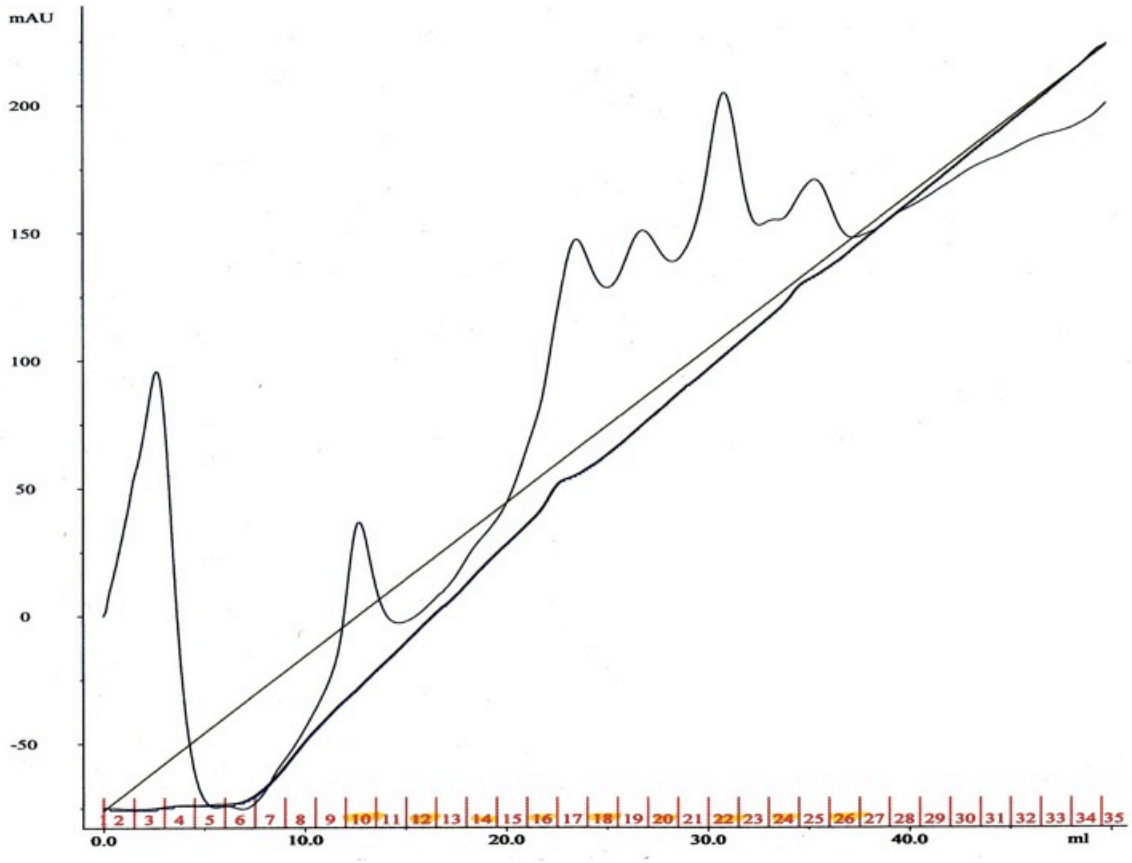
Table 2. Protein quantification and imageJ qualitative analysis lysate followed by partial-purification using ion exchange chromatography, precipitation of QFT with 30% $(NH_4)_2SO_4$ and elution profile peak fractions of resolubilized precipitate by ion exchange chromatography.

Sample	Total Protein	% BPV1 L1	Total BPV1 L1
WCL	366.0 mg	9.13%	33.42 mg
S1	108.0 mg	3.51%	3.78 mg
P1	256.0 mg	11.58%	29.64 mg
QFT	102.0 mg	1.5%	1.53 mg
WFT	10.6 mg	NA	NA
30% AS S	90.6 mg	NA	NA
30% AS P	22.0 mg	5.03%	1.11 mg
F16	0.08 mg	9.19%	0.01 mg
F18	0.09 mg	1.92%	0.002 mg

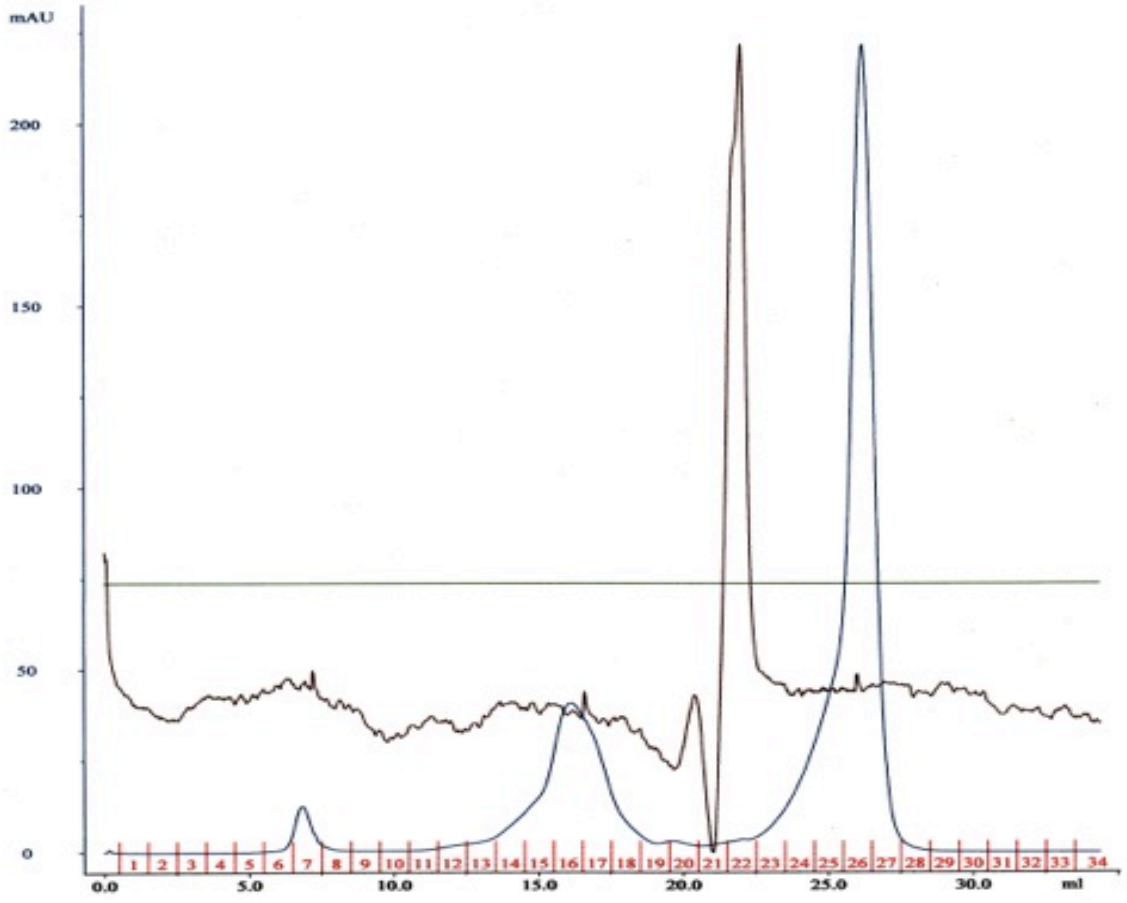
Table 3. Protein concentration quantification and imagej analysis of variable amounts of $(NH_4)_2SO_4$.

Sample	Total Protein	% BPV1 L1	Total BPV1 L1
BP QFT	1.78	1.5%	0.03 mg
20% AS P	0.71	15.5%	0.11 mg
25% AS P	0.86	21.5%	0.18 mg
30% AS P	1.06	19.05%	0.20 mg
35% AS P	1.08	NA	NA

Appendix II. Elution profile of analyte solution containing BPV1 L1 protein in ion exchange chromatography using a NaCl gradient.



Appendix III. Elution profile of size exclusion chromatography of BPV1 L1 protein concentrate sample; compared to elution profile of protein standards (data not shown) confirming BPV1 L1 protein had formed capsomere-complexes.



Appendix IV. Coomassie stained gel of variable amounts of $(NH_4)_2SO_4$ precipitation of BPV1 L1 protein.