IN VITRO ASSEMBLY OF BACTERIOPHAGE LAMBDA PROCAPSID TO ENABLE PAYLOAD ENCAPSULATION FOR TARGETED DRUG DELIVERY

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The ability to structurally engineer viral nanoparticles makes them a useful platform for targeted drug delivery. The procapsid of bacteriophage lambda is one such structure. This hollow icosahedral structure can be made from multiple copies of three major capsid proteins: gpE (structural), gpNu3 (scaffolding), and gpD (stabilizing), and is amenable to payload encapsulation to allow transportation and delivery of therapeutics. An effective method for encapsulation must be found, because expression of gpE, gpD, and gpNu3 in bacterial cells results in self-assembly of the fully-formed procapsid. Therefore, if bacteriophage lambda procapsid can be assembled *in vitro,* the structure may be able to form around a payload in solution. In this study the preliminary steps to *in vitro* assembly were completed by expressing and purifying gpE. Via western blot analysis, it was found that gpE-GST can be expressed via induction of an inducible plasmid, and purified via GST-affinity chromatography.

Introduction

Systemic application of many pharmaceuticals can have adverse effects on the recipient of the medication. For example, chemotherapeutic drugs are currently designed to target all rapidly-dividing cells, so the cancerous cells within the body are destroyed. Although this method is often successful at destroying the cancer and increasing the likelihood of patient survival, the cytotoxicity of the drug is associated with many harmful side effects, such as hair loss, weakness, cutaneous eruption, and injury to the lungs, liver, and blood vessels (Baldo, B.A.). These devastating side effects can be diminished via the implementation of targeted drug delivery. Targeted therapeutic drug delivery has a wide host of clinical applications, including, but not limited to, chemotherapeutics. It is advantageous because it not only mitigates unnecessary side effects by directly targeting the clinical issue, but it also has economic benefits in that it requires less of the drug to be used, as less medication will travel to unaffected regions of the body.

Many years of research into viral assembly pathways and modification has illuminated the possibility of using viral nanoparticles as vehicles for targeted drug delivery. These are attractive platforms because they are composed of biological material, are not toxic to humans, and are susceptible to chemical and genetic modification. Specific attention has been given to bacteriophages, viruses that infect bacteria, as these phages constitute a large class of viruses and their assembly pathways are well understood. Bacteriophages structurally consist of a core of genetic material, either DNA or RNA, which is packaged into a pre-assembled proteinaceous shell (the capsid). The capsid of the phage can be either filamentous or icosahedral in form, and it is this isolated structure, void of any viral nucleic acid, that is modified to be used as a vehicle for targeted drug delivery (Catalano, C.E.). This procapsid structure is amenable to chemical

modification because the side chains of specific surface residues can be used for addition reactions. This enables specific ligands to be bioconjugated to its exterior surface for the purpose of targeted delivery within the body. Additionally, its hollow nature allows it to be internally modified to encapsulate a therapeutic payload. Because the procapsid is susceptible to interior encapsulation, allowing transport and delivery of a drug, as well as exterior modification, allowing targeted delivery of said drug, the structure is an ideal platform for targeted drug delivery.

One such bacteriophage that has been given significant amounts of attention is bacteriophage lambda. The icosahedral procapsid of bacteriophage lambda is made up of two major proteins, gpB (portal protein) and gpE (structural protein), while gpD (stabilizing protein) is sometimes included as it is thought to aid in the stability of the structure. gpNu3 is the protein that provides the scaffold to allow for procapsid assembly, but is subsequently degraded by the protease gpC and is thus not included in the final procapsid structure. During viral capsid assembly, approximately twelve copies of gpB assemble into a ring like structure that will become the portal ring used for nucleic acid translocation. It is thought that this ring is used to nucleate the formation of the fully-formed capsid, thus, following its formation, gpNu3 forms the scaffold, around which 415 copies of gpE organize to assemble the capsid. gpC then degrades gpNu3 and both proteins exit the structure (Figure 1). The fully assembled icosahedron acts as the shell that protects the genetic material of a functional phage (Catalano, C.E.).

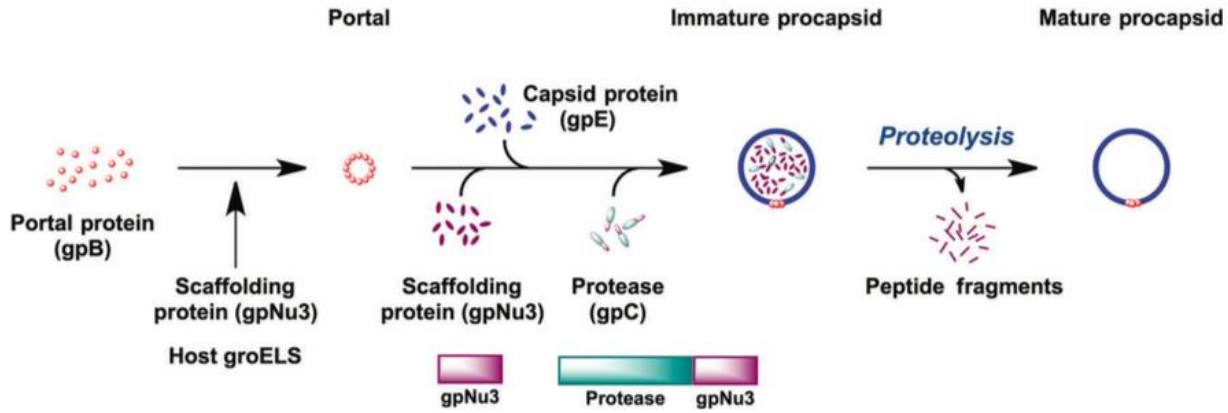


Figure 1: Bacteriophage lambda procapsid assembly pathway. gpB forms the portal to nucleate assembly, allowing gpNu3 to form the scaffold around which gpE assembles to form structural procapsid. gpC then degrades gpNu3 scaffold (Catalano, C.E.).

In order to be used as a therapeutic delivery system, bacteriophage lambda procapsid is expressed and assembled in an isolated fashion, void of any other viral component, including DNA, sheath, and tails. Many studies have shown that this procapsid structure is able to be externally modified to allow for exterior ligand attachment by taking advantage of basic reaction chemistry, such as cysteine-maleimide and lysine-NHS-ester reaction chemistry. Methods for drug encapsulation are less understood. Because expression of all necessary capsid proteins inside the same bacterial cell results in self-assembly of the procapsid within the cell, insertion of

a desired cargo is difficult. One hypothesis is that if the procapsid is allowed to assemble *in vitro* by combining the individual biochemical components necessary for assembly in solution, then the procapsid could assemble around a cargo of interest, assuming the cargo is included in the reaction mixture.

Each of the required capsid proteins can be expressed fused to a GST-tag in order for purification via affinity fast protein liquid chromatography (FPLC). In this study, a potential method for drug encapsulation is explored by performing the preliminary steps of *in vitro* bacteriophage lambda procapsid assembly. The major capsid protein, gpE, is successfully expressed and purified fused to a GST tag. Cleavage of the GST tag will allow for purification of isolated gpE, which can be used to assemble the procapsid in solution.

Materials & Methods

Preparation #1: Expression and Purification of gpE-GST:

One liter of 2xYT media was prepared and supplemented with 15 mL of 1M sodium phosphate buffer, 5 mL of 1M glucose, 1 mL of 1000x ampicillin, and 1 mL of 34 mg/mL chloramphenicol. This media was inoculated with a mini culture of BL21 *E. coli* cells containing the plasmid that encodes gpE-GST expression. The inoculated bacterial culture was incubated in a shaking incubator at 37℃ until the optical density reached 0.782, at which point the expression of gpE-GST was induced via addition of 1 mL of 1M IPTG. Following induction, the culture was incubated at 37℃ in the shaking incubator for 2 additional hours. The bacterial cells were then spun down for 15 minutes in an SLA-3000 rotor at 5000 xg and 4℃. The cell pellet was kept and stored at 4℃ for 2 nights. Following this incubation, the cell pellets were resuspended in 20 mL 1X procapsid buffer (50 mM Tris, 10 mM $MgCl_2$, 100 mM NaCl, pH 7.4) and 36 μL of DNase was added to the resuspension. This solution was then sonicated 10 times (10 seconds on, 10 seconds off), in order to lyse the cells. Cellular debris was spun down for 25 minutes in an SA-600 rotor at 7650 xg and 4℃. The supernatant contained the protein of interest (gpE-GST), so the pellet was discarded and the supernatant was stored at 4℃ for approximately 2 weeks. It was thought that during this 2 week period, the majority of gpE-GST in the lysate may have been degraded by protease activity, so PIC was added to the sample in order to salvage what protein may remain, and a second preparation of gpE-GST was started. However, further purification and analysis was performed on preparation #1. Fast protein liquid chromatography was used to purify the gpE-GST from the lysate. 7 mL of the crude lysate was run over a GST affinity column, allowing the protein of interest to bind, and approximately 4 mL of purified gpE-GST was eluted from the column using elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0). The eluted gpE-GST was separated into 2 fractions, one at 27 minutes in vial 0 (hereafter referred to as sample 1) and one at 29 minutes in vial 1 (hereafter referred to as sample 2), which were then subject to downstream analysis. SDS-PAGE analysis was used to confirm the presence of gpE-GST in each sample.

Preparation #2: Expression and Purification of gpE-GST:

The exact procedure from preparation #1 was followed, except that the lysate was only stored at 4℃ for 1 day, not 2 weeks. The elution of the desired protein yielded approximately 4 mL of purified gpE-GST, separated into 5 fractions: vials 0, 1, 2, 3, and 4. Vials 0-3 were eluted at 24 minutes and combined into one sample (hereafter referred to as sample 3) and the fraction in vial 4 was eluted at 26 minutes (hereafter referred to as sample 4). SDS-PAGE was used to confirm the presence of gpE-GST in each sample.

SDS-PAGE Analysis of Protein Samples:

SDS-PAGE analysis was used to confirm the presence of gpE-GST in the samples from preparation #2. Because no bands were seen on the SDS-PAGE gel that was run following preparation #1, samples from preparation #1 were concentrated and included in the gel on which the samples from preparation #2 were run. The gel contained a total of 8 samples: pre-induction and post-induction controls, concentrated samples 1 and 2 (preparation #1), unconcentrated samples 3 and 4 (preparation #2), and concentrated samples 3 and 4 (preparation #2). The samples that were concentrated were done so using 10K MWCO filter units spun in a tabletop Eppendorf centrifuge at 15,000 xg. Each sample was concentrated approximately 5-fold.

Western Blot Analysis of Protein Samples:

Upon completing the SDS-PAGE analysis of each eluted FPLC sample, it was evident that most samples contained purified protein. In order to determine the identity of the proteins in each sample, two of the concentrated samples (samples 1 and 4) were run identically on two different western blots, one incubated with an α-GST primary antibody, and the other with an α-gpE antibody. On each blot four samples were loaded: sample 1 with and without heat denaturant, and sample 4 with and without heat denaturant.

Cleavage of GST from gpE using HRV 3C Protease:

HRV 3C protease was used in an attempt to cleave the GST tag from the capsid protein gpE in sample 1 and 2 (preparation #1). Cleavage reactions were prepared by combining 10 µL HRV 3C 10X reaction buffer, 4 µL HRV 3C protease, and 86 µL of protein sample. Both reactions were incubated overnight at 4℃ in stationary Eppendorf tubes. The next day, GST spin columns were used to purify the gpE protein from the GST tag and protease. The spin column was first spun in a tabletop Eppendorf centrifuge at 3200 rpm for 2 minutes in order to remove storage buffer, and then equilibrated with 200 µL of wash buffer. The prepared protein solution (100 µL cleavage reaction + 100 µL wash buffer) was added to the resin bed of the spin column and was incubated at room temperature on tube rotator for 45 minutes. Following this incubation, the columns were spun at 3200 rpm for 2 minutes. The flow through was collected because it contained the purified protein of interest (gpE), as the GST tag and protease should stay attached to the resin bed.

Western Blot Analysis in Order to Confirm GST Cleavage:

In order to determine if the GST tag had been successfully cleaved from gpE, two identical western blots were run, one incubated with an α-GST primary antibody, and the other with an α-gpE antibody. The two blots were each loaded with 1 of each of the following samples, for both samples 1 and 2 (preparation #1): the protein sample before the addition of protease (pre-protease control), the protein sample containing the protease, and the protein sample after the removal of the protease and GST tag (after purification via the spin column).

Results

Preparation #1: Expression and Purification of gpE-GST:

The desired protein fusion, gpE-GST, was successfully expressed and purified in preparation #1 as seen by the tall, narrow peak at 30 minutes on the FPLC chromatogram (Figure 2). Although it was thought that the sample may have been subject to degradation because the lysate was stored for upwards of 2 weeks, FPLC analysis confirms that a purified protein, likely gpE-GST, was successfully purified and eluted from the column. The sample was eluted into two fractions, one in vial 0 at 27 minutes (sample 1), and the other in vial 1 at 29 minutes (sample 2). The large right shoulder is a result of the absorptive properties of reduced glutathione, which was used in the elution buffer to remove the gpE-GST from the column beads.

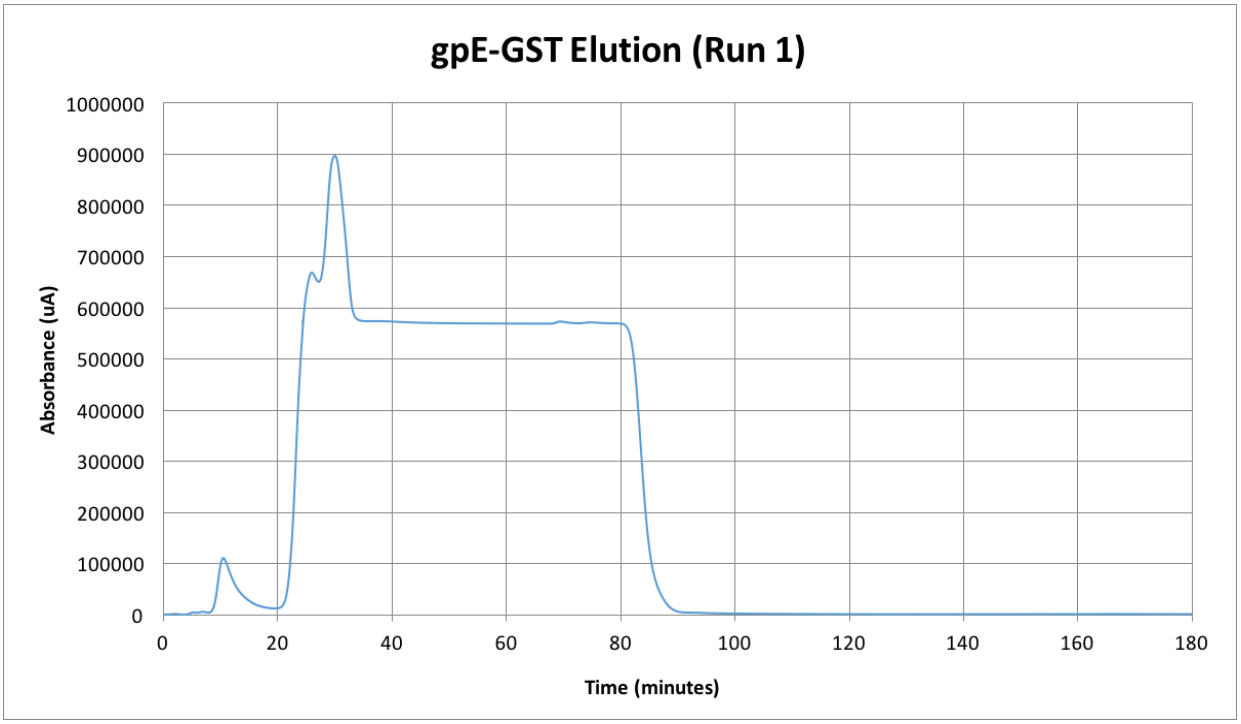


Figure 2: The FPLC chromatogram of the first preparation displays the elution of gpE-GST at approximately 30 minutes. The large, narrow peak confirms the presence of the purified protein within the sample.

Preparation #2: Expression and Purification of gpE-GST:

The desired protein complex, gpE-GST, was successfully expressed and purified in preparation #2 as seen by the tall, narrow peak at 25 minutes on the FPLC chromatogram (Figure 3). FPLC analysis confirms that a purified protein, likely gpE-GST, was successfully purified and eluted from the column. The sample was eluted into two fractions, one in vial 0-3 at 24 minutes (sample 3), and the other in vial 4 at 26 minutes (sample 4).

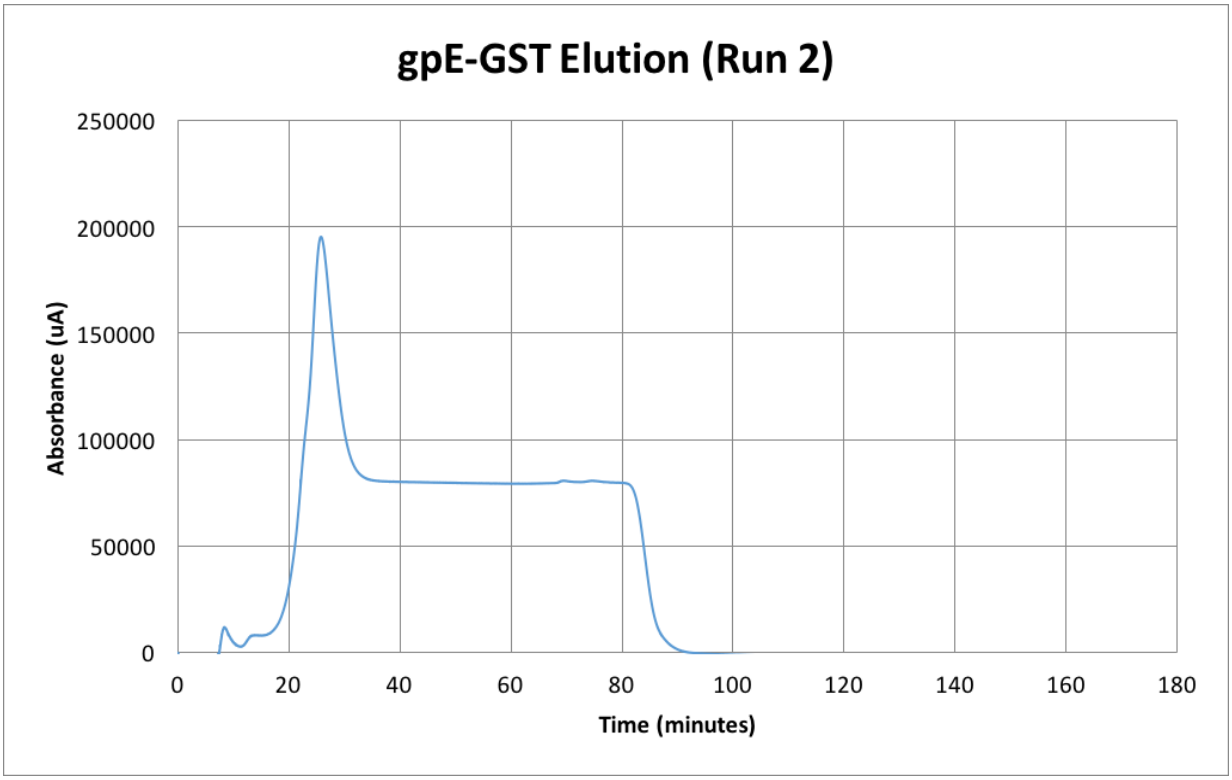


Figure 3: The FPLC chromatogram of the second prep displays the elution of gpE-GST at approximately 25 minutes. The large, narrow peak confirms the presence of the purified protein within the sample.

SDS-PAGE Analysis of Protein Samples:

An SDS-PAGE gel confirms the presence of gpE, GST, and gpE-GST in the FPLC fractions (Figure 4). The samples loaded into each lane are as follows: pre-induction control (lane 1), post-induction control (lane 2), concentrated sample 1 (lane 3), concentrated sample 2 (lane 4), unconcentrated sample 3 (lane 5), unconcentrated sample 4 (lane 6), concentrated sample 3 (lane 7), and concentrated sample 4 (lane 8). Significant singular bands can be observed in lanes 3, 5, 7, and 8. Each of these lanes demonstrates a band around 26 kDa, which is thought to be GST. Lanes 5, 7, and 8 show a light band around 37 kDa, which may be gpE. Lane 3 exemplifies a light band around 63 kDa, which is thought to be the gpE-GST fused proteins.

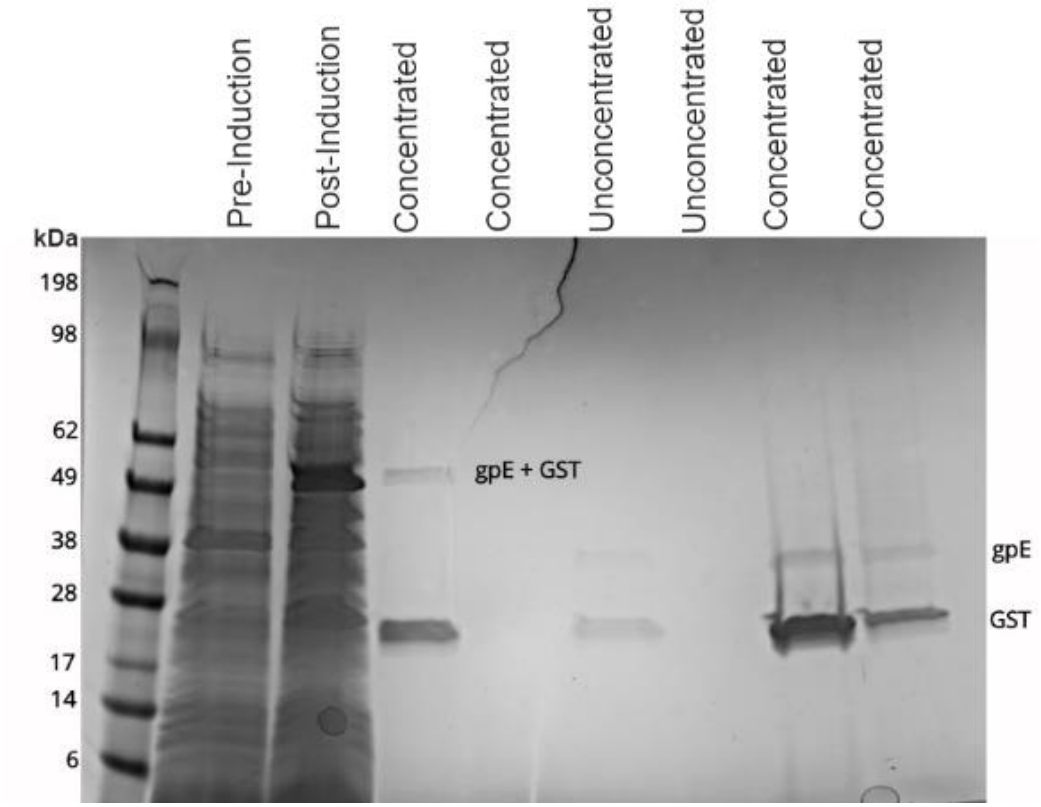


Figure 4: SDS-PAGE representing gpE-GST protein samples after elution through the FPLC. Lanes 1 and 2 demonstrate pre-induction and post-induction controls, respectively. The thick bands seen around 63 kDa in the post-induction sample confirms expression of the desired gpE-GST fusion protein, and correlates to the band at the same location in lane 3. Lanes 3 and 4 exemplify concentrated preparation #1 elution fractions, whereas lanes 7 and 8 exemplify concentrated preparation #2 elution fractions. Lanes 5 and 6 demonstrate unconcentrated sample of preparation #2 elution fractions. Banding suggests that GST and gpE are present in lanes 3, 5, 7, and 8.

Western Blot Analysis of Protein Samples:

Two western blots were completed prior to exposing each sample to protease to determine whether the banding pattern seen on the SDS-PAGE was the expected fused proteins, gpE-GST. This was done by running duplicates of two different samples (sample 1 and 4) on two

identical western blots, and exposing one to an α-GST primary antibody and the other to an α-gpE primary antibody (Figure 5). Duplicates of each sample were run on each blot because one duplicate of each sample was exposed to heat (95°C for 10 minutes), while the other was not exposed to any heat. This was done in order to determine if temperature-dependent denaturation was responsible for the separation of gpE and GST that is seen on the SDS-PAGE. The samples loaded into each lane are as follows: concentrated sample 1 exposed to heat (lane 1), concentrated sample 1 without heat (lane 2), concentrated sample 4 exposed to heat (lane 3), and concentrated sample 4 without heat (lane 4). When exposed to the primary α-GST antibody, sample 1 (lanes 1 and 2) depicts two visible bands (Figure 5a), suggesting this sample contains GST. It is thought that for this blot the proteins may not have transferred to the membrane efficiently, because it looks as though some sample from lane 1 shifted into the lane containing the ladder, making the banding pattern look messy. Sample 4 (lanes 3 and 4) exemplifies no bands when exposed to the α-GST antibody (Figure 5a). When exposed to the primary α-gpE antibody, the sample 1 (lanes 1 and 2) demonstrates two distinct bands (Figure 5b), suggesting gpE is present in this sample. Similarly, sample 4 (lanes 3 and 4) exemplifies no bands when exposed to anti-gpE (Figure 5b).

(a) (b)

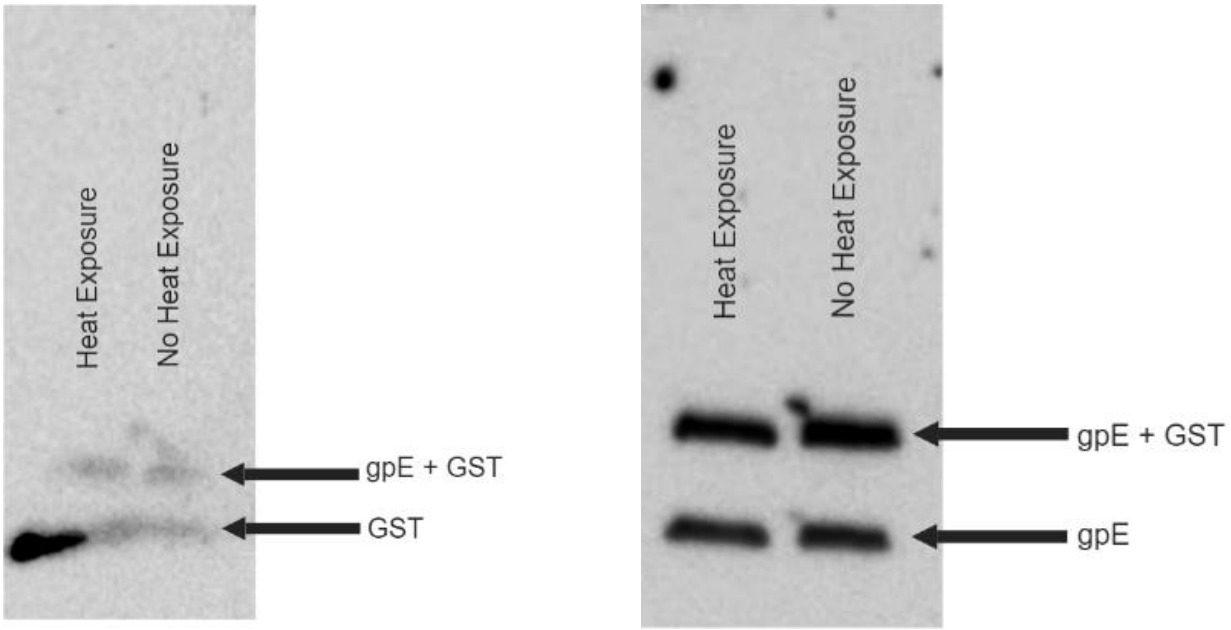
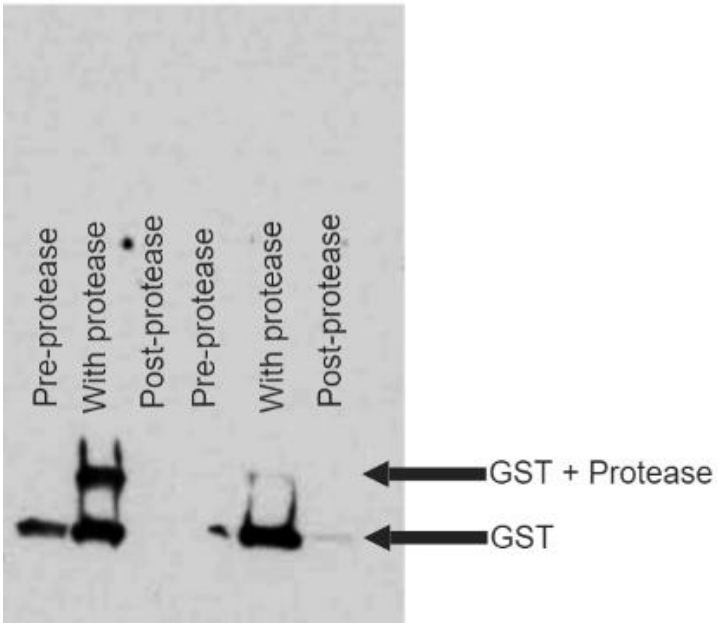


Figure 5: Western blot depicting GST (a) and gpE protein (b) using α-GST and α-gpE antibodies, respectively. Lane 1 and 2 exemplify sample 1 (preparation #1), with exposure to heat (lane 1) and without exposure to heat (lane 2). Lane 3 and 4 exemplify sample 4 (preparation #2), with exposure to heat (lane 3) and without exposure to heat (lane 4). Because the blots were run identically, the two bands in lanes 1 and 2 are thought to be identical samples. They both contain gpE and GST, which suggests that the gpE-GST fused proteins are present in sample 1 (preparation #1).

Western Blot Analysis in Order to Confirm GST Cleavage:

Western blot analysis was completed after exposure to protease to determine whether cleavage of GST from gpE and purification of gpE via spin column filtration was successful

(Figure 6). Because the expressed gpE and the HRV 3C protease are both fused to a GST tag, an α-GST antibody can be used to detect both of these proteins in each sample. This is necessary in order to determine whether the protease and GST tag from the gpE are filtered out of the solution via the spin column after cleavage. The samples loaded into each lane are as follows: sample 1 prior to exposure to protease (lane 1), sample 1 with addition of protease but before spin column purification (lane 2), sample 1 flow-through after spin column filtration (lane 3), sample 2 prior to exposure to protease (lane 4), sample 2 with addition of protease but before spin column purification (lane 5), and sample 2 flow-through after spin column filtration (lane 6). Prior to addition of protease (lane 1 and 4), a single band is observed, suggesting only the GST from the original protein expression is present. With addition of the protease, but before spin column (lane 2 and 5), two bands are observed, suggesting the GST from the original gpE protein expression and the GST on the protease are present. After purification via spin column (lane 3 and 6), no bands are noted, which was the expected result as GST was filtered out of the flow-through.



An identical western blot was run and exposed to an α-gpE antibody using the same samples as detailed in Figure 5. However, no bands were observed on this blot and thus no conclusion can be made regarding the presence, cleavage, or purification of isolated gpE.

Figure 6: α-GST western blot analysis of preparation #1 elution fractions prior to exposure of protease, with addition of protease, and after spin column purification. Lane 1 and 4 exemplify preparation #1 elution fractions prior to addition of the protease. Lane 2 and 5 exemplify preparation #1 elution fractions with addition of the protease but prior to spin column purification. Lane 3 and 6 exemplify preparation #1 flow-through after spin column purification. Banding suggests that the spin columns successfully filter out any GST-tagged protein in the sample.

Discussion

The goal of this study was to determine whether gpE could be expressed and purified fused to a GST tag, followed by gpE purification after GST cleavage. An inducible plasmid was used to express the fused proteins, while a series of centrifugations and fast protein liquid chromatography (FPLC) were used to purify gpE-GST. Based on the large, clean peak present on

the FPLC chromatograms from both preparation #1 and #2 (Figure 2 and 3, respectively), it was concluded that gpE-GST was successfully expressed and purified in both preparations. SDS-PAGE was used to confirm the presence of gpE-GST in each sample from both preparations. Originally, a gel was run with unconcentrated samples from preparation #1 (samples 1 and 2), but no bands were seen. Following preparation #2, a gel was run (Figure 4) that included concentrated samples from preparation #1 (samples 1 and 2), as well as unconcentrated and concentrated samples from preparation #2 (samples 3 and 4). Pre-induction and post-induction samples from preparation #1 were also included in this gel, and suggested that expression of gpE-GST was successful because a strong band can be seen in the post-induction control, that is not present in the pre-induction control, around 63 kDa. This is the expected size of the fused proteins, as gpE is known to be 37 kDa and GST is known to be 26 kDa. Surprisingly, the only FPLC elution sample that shows this same band is sample 1, which also shows a band around 26 kDa, suggesting separated GST is also present in the sample. Unconcentrated sample 3, concentrated sample 3, and concentrated sample 4 also show bands around 26 kDa and 37 kDa, suggesting separated GST and gpE may be present in these samples.

In order to verify the identity of the proteins within the bands of the SDS gel, two samples that displayed unique banding (samples 1 and 4) were subject to western blot analysis (Figure 5). Two identical blots were run: one was incubated with an α-GST primary antibody, and the other with an α-gpE primary antibody. The samples were each subject to both heat denaturant after sample preparation (95°C for 10 minutes) and no heat, hence the 4 distinct lanes on each blot. The banding pattern suggests that sample 1 contains gpE and GST in the same bands, suggesting the fused gpE-GST are present in this sample. The fact that two bands are present in each lane suggests that either dimerization of the fused proteins is occurring, or GST, gpE, and gpE-GST are all present in the sample, which would also result in two bands on each blot. The same banding pattern is seen in both lanes of sample 1, which suggests that the heat denaturation process has no effect on the composition of the sample. No bands were seen in the lanes for sample 4, suggesting that any gpE or GST in this sample is at such low concentrations that it was unable to be detected by the primary antibody, and thus the fluorescent secondary antibody.

Because it was confirmed that gpE-GST was present in sample 1, this sample was subject to GST cleavage and spin column purification in an attempt to purify the isolated gpE protein. Sample 2 (preparation #1) was also subject to GST cleavage and spin column purification. This was accomplished by adding HRV 3C protease to each sample and purifying the cleaved gpE by allowing it to flow through a GST spin column (which will bind the cleaved GST and GST-tagged protease). Two identical western blots were run to analyze this process; one was incubated with an α-GST primary antibody, and the other with an α-gpE primary antibody (Figure 6). Both samples were run before addition of protease as a negative control, with the addition of protease, and after spin column purification (flow-through). No banding was seen on the blot incubated with α-gpE antibody. This suggests that a methodological error led to inconclusive results, as the negative control on this blot was the same sample in lanes 1 and 2 on

the original western blot (Figure 5), on which banding is seen. On the blot incubated with an α-GST antibody, the expected banding was observed. For sample 1, there was a single band in the negative control (lane 1), representing the GST tag, two bands in the sample with protease (lane 2), representing the GST tag and the protease, and no bands in the flow through (lane 3), as GST is filtered out via spin column purification. For sample 2, there is a portion of a band in the negative control (lane 4), most likely suggesting presence of a GST tag, a dark band and a light band in the sample with protease (lane 5), representing the GST tag and the protease, and no bands in the flow through (lane 6). Because no bands were seen on the α-gpE blot, no conclusion can be made regarding the cleavage of GST from gpE and purification of gpE. However, results from the α-GST blot suggest that the spin columns are effective at filtering out GST and the protease from the sample.

Based off of the sequential results from this study, expression and purification of gpE fused to GST was successful. However, it is inconclusive whether the HRV 3C protease was capable of cleaving the GST-tag from gpE protein because no gpE bands were seen on the α-gpE western blot. However, GST presence was visualized, and it was determined that GST spin column purification was an effective method for removing GST (and the protease) from the sample. Since the results of the western blot of preparation #1 elution fractions exposed to α-gpE primary antibody were inconclusive, further studies could incorporate using similar methodology to confirm presence of gpE after before, during, and after protease cleavage. Determination of gpE removed from the GST tag would indicate that purification of isolated gpE is possible. Given these circumstances, the same methodology as used to purify gpE could be performed for other capsid proteins (gpD and gpNu3). If purification of each protein were to be successfully completed, each of these proteins may be combined in solution to assess the possibility and efficiency of *in vitro* procapsid assembly. FPLC and native gel analysis could confirm the assembly of fully formed bacteriophage lambda procapsid.

References

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