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Expression and Characterization of a Bacterial Lectin Towards Development of Anti-Adhesion Molecules

Quinn Nyberg
Department of Chemistry and Biochemistry
The University of North Carolina Asheville
One University Heights
Asheville, North Carolina 28804 USA

Faculty Advisor: Dr. Caitlin McMahon

Abstract

Antibiotic resistance is a rapidly growing concern, and it is becoming increasingly important to develop new ways to treat bacterial infections. Anti-virulence strategies focus on disarming bacteria rather than killing them, putting less selective pressure on the bacteria, and making it less likely bacteria will become resistant to the treatments. One anti-virulence target is the formation of biofilms, which is facilitated by lectins on the surface of bacteria binding to carbohydrates on the host cell. By inhibiting lectin-mediated adhesion, bacteria will not be able to aggregate and bind to host cells, effectively making the bacteria less virulent. In some strains of *Escherichia coli,* this process involves the bacterial lectins F17G and GafD binding to the carbohydrate N-acetyl-β-D-glucosamine (GlcNAc) on the host epithelial cells. To test GlcNAc derivatives as competitive inhibitors of the GafD lectin, an expression and purification protocol for GafD was performed and optimized. This purification method relies on separating the periplasm from the cytoplasm, as well as using either the expressed 6x-histidine tag on GafD for cobalt (II) affinity, or functional GlcNAc binding affinity for purification. Then, conditions for multiple types of assays were optimized to evaluate binding, including an

enzyme-linked immunosorbent competitive binding assay (ELISA) and surface plasmon resonance (SPR). These tools will enable the evaluation of molecules as inhibitors of bacterial adhesion for anti-virulence and increase understanding of the GafD protein and its ligand binding.

Introduction

Antibiotic resistance is a growing concern for the modern world. Scientific development has not matched the pace of bacterial mutation and horizontal gene transfer due to immense selective pressure from the misuse and overuse of antibiotics.[1,2] Other than the evolutionary pressure placed on the bacteria, antibiotics can have other negative health effects. Antibiotics can kill the bacteria that live on and in the human body that are vital for bodily function, weakening the immune system and leaving the person vulnerable to further infections.[2] Treatment options for those at higher risk of these dangerous side effects of antibiotics are needed, as well as alternative treatments for multi-drug resistant infections.[1]

A report on 2019 data by *The Lancet* estimated 1.27 million deaths globally are directly attributed to an antibiotic resistant infection, and 4.95 million deaths are associated with an antibiotic resistant infection.[3] The bacteria can increase resistance to antibiotics through many cellular mechanisms, including but not limited to secreting enzymes to destroy the antibiotic, forcing the antibiotic out with efflux pumps, or mutating the antibiotic target in either the entry site on the membrane or within the cell.[2] Bacteria can also become more resistant through utilization of virulence factors. Virulence factors are defined as the processes in which bacteria can harm the host or increase the size and/or severity of the infection.[1] Virulence factors are specific to the bacterial strain, but some common groups of virulence factors include toxins, secretion systems, regulatory systems, and adhesins, amongst others.[1]

Anti-virulence treatment strategies aim to specifically target the virulence factors of pathogenic bacteria.[1] This is a relatively new strategy for treating bacterial infection, and there are very few anti-virulence drugs approved by the Federal Drug Administration, and even fewer on the market for infection treatment.[1] One candidate for anti-virulence strategies is multi-drug resistant *Escherichia coli*, particularly the carbohydrate-specific adhesins, or lectins, present on *E. coli*. These lectins are responsible for the adhesion of the bacteria to the host cell that eventually results in biofilm formation (figure 1).[4] A biofilm is a thin, membrane-like, polysaccharide matrix that bacteria form around itself to act as a barrier surrounding the colony of cells to make themselves less vulnerable to antibiotics.[1] *E. coli* begins biofilm formation via the lectins' bacterial fimbriae binding to carbohydrates that exist on the outer membrane of epithelial cells.[4] By binding to carbohydrates in different tissues, *E. coli* can cause many different types of illness, but

two commonly seen are diarrhea-associated illness, as well as urinary tract infections.[5] As the infection grows, the cell colony will begin building a biofilm around itself as a virulence factor, as shown in figure 1. By inhibiting the first step in this process, adhesion, the bacteria would not be able to attach themselves to the host cells, in turn preventing biofilm formation.

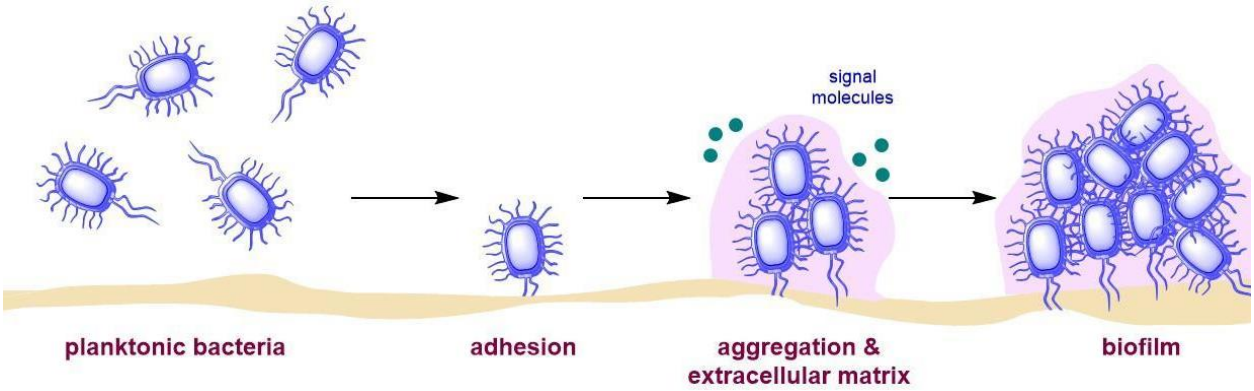


Figure 1: Process of biofilm formation (figure courtesy of Dr. Caitlin McMahon)

One example of this is in uropathogenic *E. coli* (UPEC), which expresses the lectin FimH. The lectin selectively binds to the monosaccharide mannose on urinary tract epithelial cells. [6] Located on the tip of the type 1 fimbriae on *E. coli*, the FimH lectin mannose binding site is responsible for the adhesion of the bacterial cell to the host cell.[6] Several groups, dating back to the late 1970's, have synthesized mannose derivatives that successfully inhibit adhesion to the host cell.[7] The mannose derivatives compete with naturally occurring mannose on the host cell membrane and successfully bind to the lectin before it can adhere to the host.

The McMahon laboratory is specifically investigating the F17G/GafD adhesin lectin found on G-fimbriae expressing enteropathogenic *E. coli* (EPEC), characterized by diarrhea-associated illness.[4] The difference between the lectins is that GafD is found in human infections and F17G is found in livestock infections and requires a fimbrial chaperone for infection, though both serve the same purpose in EPEC adhesion.[5] These lectins are part of the G fimbriae, a protein complex which extend from the surface of EPEC, and are found at the tip of said fimbriae as exposed receptor-binding sites.[4] These exposed receptor-binding sites, which bind selectively with N-acetyl-D-glucosamine (GlcNAc), allow the bacteria to bind to the brush borders found on intestinal epithelial cells.[5]

Others in the McMahon laboratory are currently developing a library of GlcNAc derivatives to test their inhibitory activity against purified GafD. Our overall goal is to create a reversible inhibitor that fits in the binding pocket and binds more strongly than natural GlcNAc. However, to be able to test specific binding and inhibition efficacy, purified GafD protein is needed. GafD has 179 amino acid residues in its sequence, 22

of which are predicted to be a periplasmic messenger signal sequence.[5] In sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), it migrates to around 19 kDa, which is slightly lower than to be expected for the length of the sequence.[4]

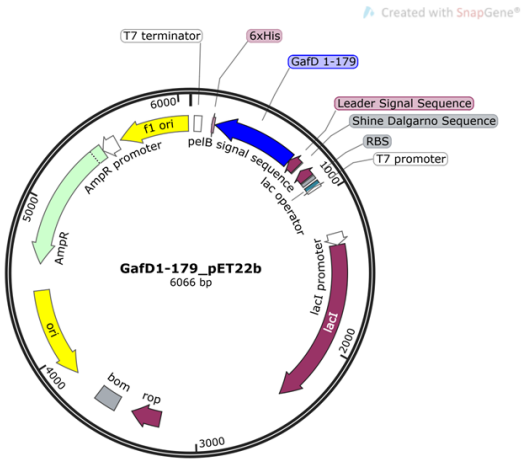


Figure 2: Plasmid Map of GafD1-179_pET22b

Fimbrial adhesins such as GafD are notoriously difficult to isolate to a usable stock due to their propensity for aggregation, and GafD has been expressed for this purpose very few times.[5] By utilizing the native periplasmic expression, we aim to isolate the protein without harsh methods such as cell lysis, which can be damaging to the protein structure.[5] Therefore, the first goal was to develop an optimized procedure for growth, isolation, and purification of *E. coli* GafD. Secondarily, enzyme linked immunosorbent assay (ELISA) conditions and surface plasmon resonance (SPR) were determined to test lectin binding to GlcNAc. Currently, we have created and isolated a plasmid for GafD expression with an encoded 6x-Histidine tag (Fig. 2). The plasmid retains the periplasmic messenger signal. The plasmid was used for expression in BL21 *E. coli,* and the protein was purified using His-tag-Co^{2+} affinity or GlcNAc affinity. To establish a binding curve for GafD to GlcNAc, ELISA conditions were designed and optimized. The initial ELISA allows for a baseline to be established for the affiniy between GafD and GlcNAc by evaluating binding at increasing concentrations of GafD. Using these optimized affinity assays and protocols, the inhibitor derivative library will be tested as competitive inhibitors of GlcNAc binding.

Experimental

General Methods & Instrumentation

All media and glassware were autoclaved or filtered under sterile conditions before use. Biological sterile technique was used. Luria broth (LB) was used as the growth media and was prepared with 0.75 mg/mL of ampicillin to select for plasmid-containing colonies. Plates were grown in a Barnstead Lab-Line incubator at 37 °C, while liquid cultures were grown in a New Brunswick Excella E25 Incubator Shaker Series at 37 °C and shaking at 250 rpm, with flasks covered with aluminum foil. All protein used was grown using BL21 *E. coli* modified with the plasmid GafD1-179_pET22b (Fig. 2) to have ampicillin resistance and to express GafD to the periplasm. Optical density was assessed using a PASCO wireless colorimeter and turbidity sensor 598-929. For high

volume centrifugation, a Thermo Scientific Sorvall Legend XTR Centrifuge was used, while small volume centrifugation utilized an Eppendorf Centrifuge 5420.

For fast protein liquid chromatography (FPLC), the BioRad NgC Chromatography System was used with a Cytiva HiTrap TALON crude 1 mL column. Protein purity was assessed via sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the BioRad Mini-PROTEAN Tetra System, BioRad PowerPac Basic, and BioRad any-kD mini-PROTEAN precast gel cassettes. Protein concentration was determined using both a Thermo Scientific NanoDrop Lite Spectrophotometer and gel-based quantification using SDS-PAGE and ImageJ software for analysis. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed at 130 V for approximately 60 minutes, with samples prepared with a 7.5 µL of protein sample and 2.5 µL of a 10:1 solution of 4x Laemmli:1 M DTT.

All ELISAs were performed on uncoated 96-well Thermo Scientific Nunc Maxisorp plates. Laminin was purchased from Corning. To read ELISA output, a BioTek Synergy HTX microplate reader was used. To complete localized surface plasmon resonance (SPR), an OpenSPR from Nicoya was utilized. For biotin-streptavidin coupled SPR, all materials were purchased from Nicoya, aside from running buffer, which was prepared in the laboratory, and β-D-GlcNAc-PEG3-Biotin, purchased from Sussex Research.

Expression of the Plasmid

To create a stock of purified GafD, the BL21 *E. coli* with the modified plasmid was grown overnight on a LB agar petri dish. One colony was identified and scraped using a pipette tip. The colony was introduced to two flasks with 25 mL of broth and were grown overnight at 37 °C and shaking at 250 rpm. To scale up the production, 10 mL of the sample were added to large flasks with 1.0 L of broth, creating four 1.0 L samples. The samples were grown at 37 °C and shaking at 250 rpm until the optical density at the orange wavelength (OD_{600}) was 1.0 absorbance units, which typically takes around 3-4 hours. Once it had reached the desired OD_{600}, 1 mL of 100 mM isopropylthiogalactopyranoside (IPTG) was added to induce protein expression for 2 hours.[6]

Periplasm Preparation

The samples were then centrifuged at 4°C and 4200 rpm for 28 minutes. The supernatant was decanted, and an accurate mass of the pellet was determined. On ice, the pellet was resuspended using cold 20 mM Tris with 20% w/v sucrose at an amount of 4 mL/g of pellet. The resuspended samples were transferred to conical tubes. Next, 40 µL/g of 0.5 M EDTA and 40 µL/g of 15 mg/mL lysozyme were added and allowed to incubate on ice, rocking, for 40 minutes.[4,9] After the allotted time had passed, 30 µL/g of 2.5 M $MgCl_2$ was added. Finally, it was centrifuged at 4°C and 10,000 rpm for 20

minutes. To remove EDTA before further purification, dialysis was performed using 3 L of 1x phosphate buffered saline (PBS) plus 250 mM NaCl. The periplasm containing supernatants were split into dialysis tubing, around 1 mL/cm tube, and were set to stir gently for 72 hours at 4 °C, with the PBS being replaced once daily.

Protein Purification

Two purification methods were both attempted, one utilizing fast protein liquid chromatography (FPLC) and the other using GlcNAc-agarose beads in a small gravity column.

FPLC

After syringe filtering the sample (~30 mL, 0.22 µm PES syringe filter), it was applied at a rate of 2 column volumes (CV)/minute after the column was equilibrated with the running buffer, PBS. After the sample was applied, the column was washed with a 4 CV gradient of 100% PBS to 15% of the elution buffer (PBS + 300 mM imidazole). Following this, the lectin was eluted using 100% elution buffer in 1 CV fractions.

Following the elution of the protein, FPLC elution fractions 2 & 3 were injected into ThermoScientific Pierce Slide-A-Lyzer Dialysis cassettes. The cassettes were added to 1.0 L of storage buffer (phosphate or tris buffer solution) and dialyzed for around 12 hours at 4 °C, changing the PBS every 4 hours. The sample was then removed and stored at 4 °C for use.

GlcNAc-Agarose Gravity Column

A GlcNAc-agarose gravity column method was used to further purify the lectin and act as a preliminary function test of GafD. First, 1 mL of GlcNAc-agarose (Sigma Aldrich) beads were added to a 10 mL gravity column and was then washed with 10 mL of 1x PBS to equilibrate the column. Next, 1 mL of the sample was applied, then washed with 10 mL of PBS. The lectin was then eluted in 1 mL fractions using a 5% w/v GlcNAc solution in PBS. The purity was assessed via SDS-PAGE before the protein-containing fractions were combined and desalted via the same dialysis method as the FPLC and stored at 4 °C for use.

Protein Loss Assessment

Solutions containing 1 mg/mL of either BSA or lysozyme were prepared to act as a control to assess protein loss through syringe filtering. GafD samples were undiluted at a concentration of around 0.3 mg/mL. The solutions were measured using a Nanodrop Lite spectrophotometer before and after filtering.

Gel-based Quantification

To quantify protein concentration while avoiding UV interference from imidazole, a gel-based quantification method was developed.[10,11] A BSA (bovine serum albumin) calibration curve was created by making serial dilutions of a 0.613 mg/mL BSA stock solution and applying it to a gel alongside the purified GafD fractions.[10,11] BSA is a widely used standard for protein concentration analysis due to its stable structure, known characteristics, and abundance.[10] As per the standard SDS-PAGE protocol, to create both the protein and BSA samples, 7.5 µL of the protein/BSA sample was combined with 2.5 µL of sample buffer solution (10:1 4x laemmli sample buffer: 1 M DTT). The samples were then heat shocked for 5 minutes at 95 °C before being centrifuged for 30 seconds at 12,000 rpm. The samples were then applied to the gel and it was run for 45 minutes at 130 V. The gel is then stained with Coomassie Blue, and bands were quantified using ImageJ.[10,11]

Bradford Assay

To determine the concentration of the expressed & purified GafD, a Bradford assay was performed. A BSA standard calibration curve was created to act as a reference for protein concentration at a total amount of 50 µL per well. Next, a 0.213 mg/mL GafD stock solution was added to the well at various dilutions, ranging from 25% to 100% GafD stock. Bradford reagent was used, and 200 µL was added to each sample well. It was allowed to incubate for 5-10 minutes before being read at A_{595} twice.

GafD/GlcNAc Binding Curve Assay

Laminin was used as an immobilized ligand to coat the plate. A 1.0 mL solution of 25 µg/mL laminin in 1x Tris buffer solution (TBS) was prepared, and 50 µL was added to the plate in the first two columns. Two wells were also prepared with the laminin solution as a control. The plate shook at 400 rpm for 1 hour at room temperature. The plate was washed with TBS for 3 x 5 minutes before blocking with TBS-T + 5% BSA (5 mL TBS, 5 µL Tween-20 (0.1%), 250 mg BSA) for 1 hour. The plate was washed again with 50 µL of TBS-T (0.1%) 3 x 5 minutes. The plate was then incubated for 2 hours while shaking with 50 µL GafD (0.213 mg/mL) serial dilutions in TBS-T ranging from 100 µg/mL to 0.01 µg/mL. As another control, 50 µL of the 100 µg/mL GafD solution was added to two empty wells. Once the time had elapsed, the plates were once again washed 3 times with TBS-T for 5 minutes each. The antibody, the anti-His tag horseradish peroxidase conjugate, was diluted 1:10,000 from the stock and 0.1% BSA was added as well. It was allowed to incubate while shaking for 2 hours with 50 µL of the antibody solution. The plate was washed 3 times with TBS-T for 5 minutes before it was developed. Acting as a color change indicator with the HRP, 70 µL of TMB was added to each well and

was allowed to develop for around 5 minutes. Finally, 70 µL of 2 M H_2SO_4 was added before it was read at A_{450} twice.

Surface Plasmon Resonance

To assess the binding affinity of the purified GafD protein to immobilized GlcNAc, SPR was utilized. The SPR was modified with a 50 µL loop (standard flowrates halved to accommodate) and operated at 22 °C. The running buffer was 1x filtered and degassed PBS with 0.1% BSA and 0.05% Tween (PBS-T+BSA). All dilutions for SPR were created with PBS-T+BSA as well. Nicoya materials were used for all parts of the SPR procedure aside from buffers and GafD. A gastight Hamilton syringe was used to measure the biotinylated GlcNAc and GafD. Results were visualized using TraceDrawer software.

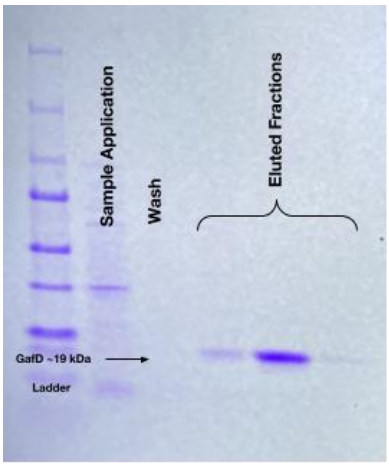
The OpenSPR Biotin Sensor was prepared per Nicoya procedure. To begin the coupling process for the biotin nanoparticle chip, 100 µL of 10 mM HCl was injected into both channels at a flow rate of 75 µL/min after a flushing cycle with the running buffer and air. Once it stabilized after 5 minutes and the loop had been flushed, 100 µL of 0.05 mg/mL streptavidin was injected into both channels before 100 µL of 0.05 mg/mL biotinylated GlcNAc was injected into just channel 2, both steps at a flow rate of 10 µL/min. Finally, to assess the binding affinity, the 100 µg/mL protein sample was injected into both channels at the same flow rate, followed by dissociation.

Results & Discussion

Expression and Purification of the Plasmid

GafD was successfully expressed and purified, and the methods of purification have been optimized to increase efficiency and yield. Purity was accomplished through two purification methods and verified via SDS-PAGE gel (Fig 3). The GafD that was purified using FPLC provided protein fractions that were higher in protein concentration in comparison to the amount from the gravity column (Fig 3a). The gravity column proved to be time consuming, and the subsequent desalt column caused more product to be lost (Fig 3b). To improve usable protein concentration for ELISAs and SPR tests, Sartorius low molecular weight spin concentrating tubes are being used.

a)



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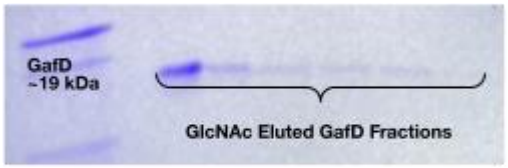

Figure 3: SDS-Page gel of GafD purified via a) FPLC b) GlcNAc-agarose beads gravity column.

Protein Concentration Determination

When comparing the protein concentrations obtained from Co^{2+} affinity chromatography and those obtained after the imidazole was desalted through size-exclusion chromatography, it became clear that protein was being lost during the purification process, specifically when it was applied to the FPLC. Following a control FPLC run using BSA as the protein, it was determined that protein was not being lost on the FPLC, but prior to sample application when it is syringe filtered. This is most likely due to the previously mentioned propensity for adhesins such as GafD to aggregate.[5] Assessment of both PES and PVDF 0.22 µm syringe filters was completed with BSA, lysozyme, and GafD. While the 1 mg/mL lysozyme and BSA solutions maintained their concentrations independent of filter material or maximum volume, GafD recovered approximately 85% of the initial concentration independent of filter material or maximum volume. Due to this, FPLC size exclusion-based filtration was abandoned in favor of dialysis to desalt the protein to minimize the protein loss.

To assess the concentration of the expressed protein and to optimize the accuracy of concentration measurements, three methods of protein concentration determination were compared: Nanodrop spectrophotometry, gel-based quantification, and the Bradford assay. Although the Nanodrop Lite is an acceptable method of protein concentration determination, the eluting agent used in FPLC, imidazole, absorbs in the same wavelengths as aromatic amino acids, which is what the Nanodrop Lite measures.[10] Within the fractions that contain the highest concentration of protein, the elution gradient has not yet reached 100%, resulting in an unknown imidazole concentration that presumably varies each time. Due to this, an accurate blank for the Nanodrop Lite is impossible to determine, resulting in inaccurate protein concentrations.

Gel-based quantification has proved to be useful in preserving sample volume and avoiding UV interference from imidazole, however the ImageJ software has proven to be slightly subjective when establishing the area under the curve, relying on self-defining the area (Fig. 4).[10,11] In the literature used to develop the gel-based quantification, it was established that due to the Coomassie staining, each gel creates a unique colorimetric profile that requires a new calibration curve.[11] Completing a Bradford assay could circumvent the issues faced with Nanodrop and gel-based quantification however, it uses almost 27x the volume of sample than gel-based quantification and is more sensitive to pipetting error. Based on the results of the protein concentration determination methods, it can be said that the current expression and purification procedure will reliably yield 0.3 mg/mL purified GafD (Fig. 5, Table 1).

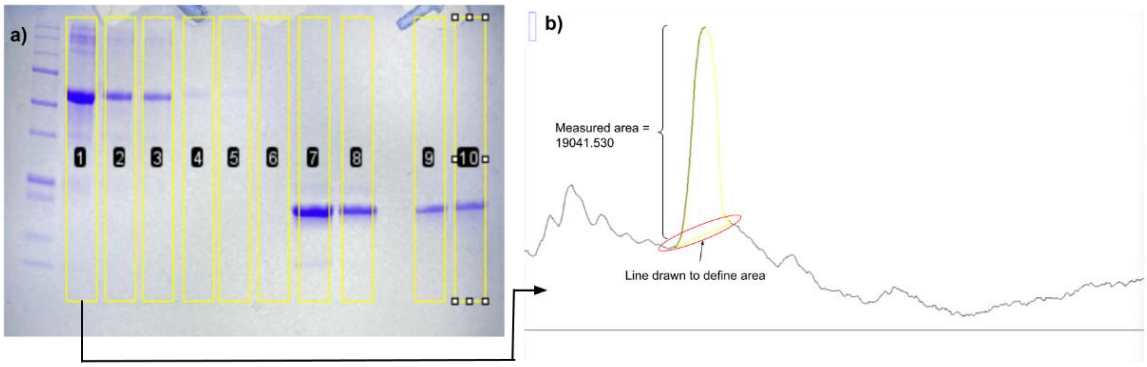

Figure 4: Example of how area under the curve is determined during gel-based quantification utilizing ImageJ densitometry methods, measured to 3 decimal places.[10,11] a) Image of gel used for quantification. b) Densitometric lane profile of lane 1 (0.613 mg/mL BSA) in figure 4a, measured to 3 decimal places, where the x-axis represents molecular weight, and the y-axis represents the optical density lane profile in absorbance units.[10]

GafD	Area under curve (pixels)	Calculated concentration (mg/mL)
GafD with imidazole	20660	0.344
GafD desalted	19950	0.332

Table 1: Estimated area under the curve and calculated concentration of GafD via concentration curve (Fig. 5).

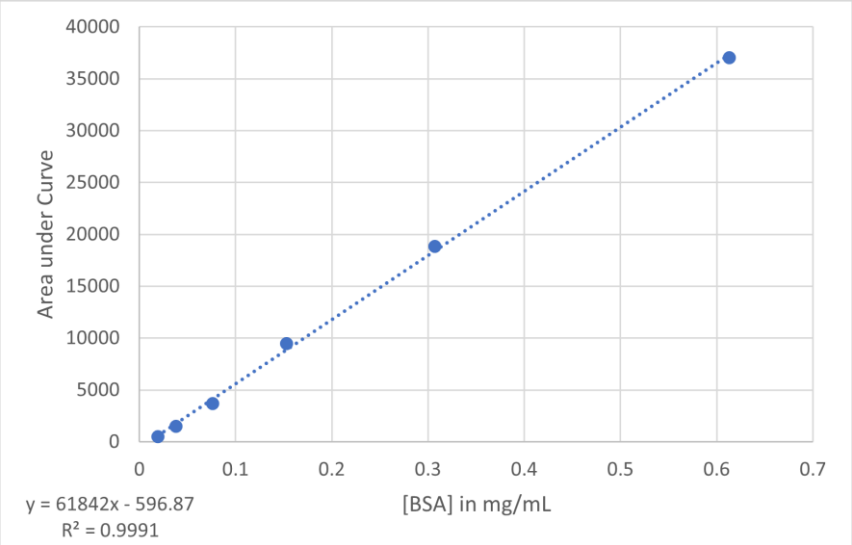

Figure 5: Gel based quantification of GafD concentration utilizing a BSA calibration curve (r^2=0.9991) with calculated protein concentration based on one technical replicate.

GafD/GlcNAc Binding Curve

The indirect ELISA proved to be successful in characterizing the binding curve; however, the maximum binding has yet to be reached, creating a greater need for higher and more accurate protein concentrations (Fig. 6). Optimization of the assay was done by determining the laminin incubation period and the ideal antibody concentration, as well as developing a direct ELISA functional control. Based on preliminary results, the GafD produced is functional and has affinity towards GlcNAc.

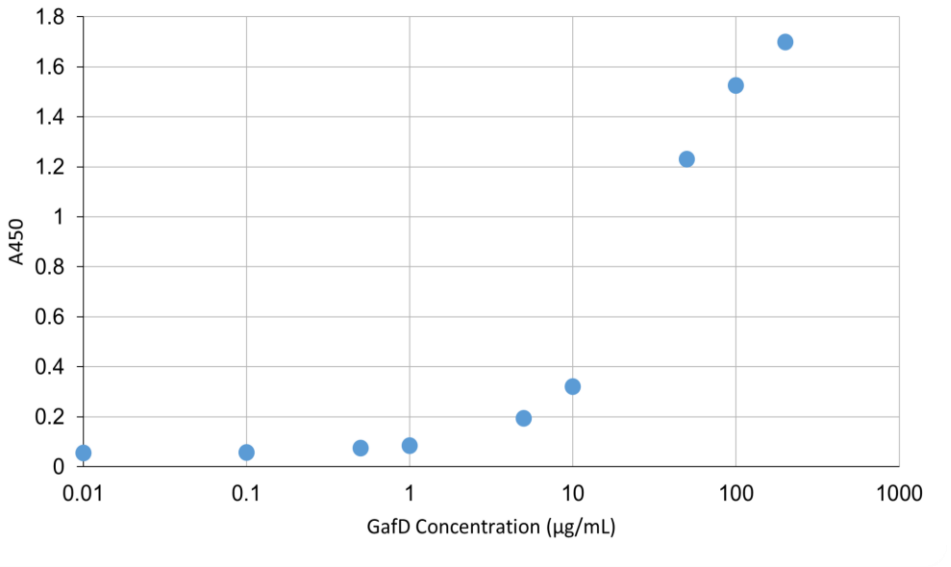

Figure 6: GafD/GlcNAc binding curve characterized by ELISA.

Surface Plasmon Resonance

The methods for SPR are currently being optimized to troubleshoot a seeming lack of specific binding. Although the functionality of the coupling reaction of the ligand of interest to the chip was confirmed, based on the results of the trace, the GafD did not specifically bind to the biotinylated GlcNAc (Fig. 7). Prior to SPR, the function of the GafD sample to specifically bind to GlcNAc was confirmed via a GlcNAc gravity column. However, when GafD from the same expression was applied to both channels of the SPR, channel 2 containing biotin-streptavidin coupled GlcNAc and channel 1 acting as a control, both provided the same response (Fig. 7). The same GafD also failed to show specific binding in an ELISA. This meant that no specific binding was seen between the GafD with confirmed function and immobilized GlcNAc. This could be due to several factors including the steric interactions of the ligand presentation, the concentration of the ligand, the age of the protein sample, and/or batch-to-batch issues. It is currently unclear what the specific cause is, however, going forward, we aim to remedy this by increasing blocking to decrease non-specific binding, varying lectin concentration to allow for more surface area for the lectin-ligand complex to form, or by developing a procedure to immobilize a GlcNAc containing glycoprotein such as laminin or BSA-GlcNAc on a carboxyl sensor so a larger signal change could occur. Protein storage will also be optimized by decreasing the amount of time between the purification and testing and by further improving storage buffer conditions.

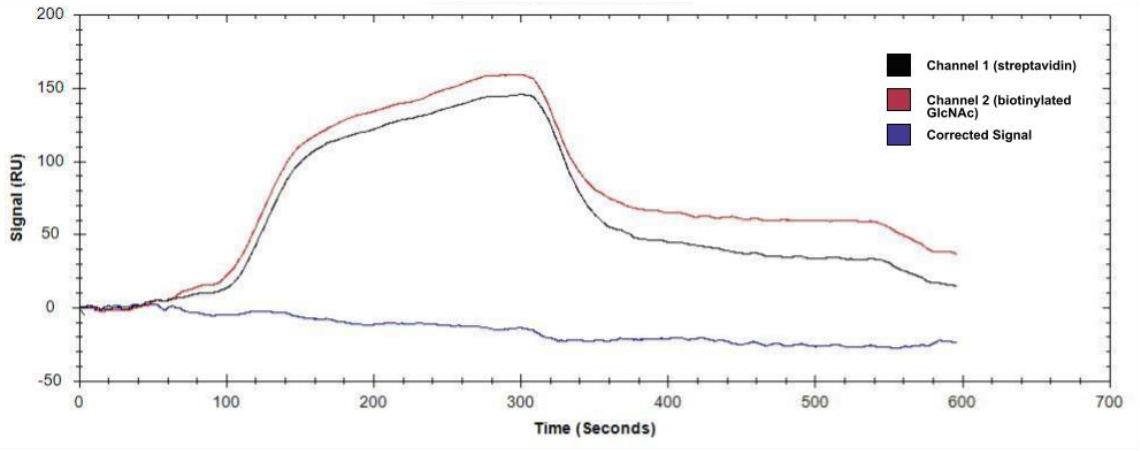

Figure 7: An overlay of the SPR response signals (in response units) of channel 1 (black), channel 2 (red), and the corrected signal between the two (blue). Channel 1 acts as a control, while channel 2 contains the ligand of interest, and the blue trace measures the difference of the signals.

Conclusion

The expression and purification methodologies of GafD have been optimized. At this point, a 1.0 L liquid culture will reliably yield 2 mL of 0.3 mg/mL pure GafD, using Co^{2+}-affinity FPLC. We have determined one main cause of protein loss is in syringe filtration when the sample is prepared for FPLC and have developed a method to minimize this issue. Although the issues surrounding readings with the Nanodrop Lite have not yet been resolved, the gel-based quantification method developed to circumvent this issue has proven successful at simultaneously quantifying the protein concentration and visualizing protein purity.

Going forward, the overall yield of GafD needs to be increased to reach maximum binding to a GlcNAc ligand in a binding assay so that a full characterization of the lectin/carbohydrate interaction can occur. Optimization of the SPR procedure will also continue in order to quickly characterize the binding between GlcNAc and GafD. Once inhibitors are ready to be tested, conditions for a competitive ELISA-like assay and SPR assay will be developed.

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