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Isolation and production of tandem collagen binding domain from clostridial collagenase ColG and developments in C1Q reagent production for future molecule characterization work

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Isolation and production of tandem collagen binding domain from clostridial collagenase ColG and developments in C1Q reagent production for future molecule characterization work

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Abstract

This thesis covers a two part project: the production methods to create a double collagen binding domain molecule with a growth factor for wound healing applications and the development of a new in-house production method for isolating C1q from bovine blood. The wound healing molecule was created using transformation, sonication, and purification before being tested via electrophoresis SDS page and Western blots to confirm the molecule's presence. The C1q in-house production method utilizes an ultrafiltration flow cell rather than dialysis at a critical point in the process, allowing for researchers to not only be able to use a single small tank but also greatly reducing the amount of buffer needed for filtration.

1. Introduction

Wound healing is a biological process that the human body uses to repair itself when it gets injured. For a wound to heal, it must go through four phases within a certain amount of time and in a specific order (1). These phases work together to allow the body to heal both acute and chronic wounds (1). Acute wounds are breaks in the epithelial integrity of the body and heal normally by the body's cells undergoing a complex healing process (1). Chronic wounds, however, result in portions of the repair process breaking down, resulting in improper issue repair results (1). Therapeutic wound healing drugs are designed to improve wound healing for both types, and for chronic wounds, resolve the improper tissue repair (1).

However, therapeutic wound healing is a complex pathological challenge. A typical therapeutic treatment focuses on reducing the wound burden by using synthetic and biological materials to help the tissue regenerate (1). These materials can speed up the healing process, but they can either be incompatible with the host or they can cause infections, as the biological materials have to come from compatible donors (1). The ideal alternative would be to use materials such as growth factors to activate cellular regeneration, so that the body can heal itself for the most part (1). The issue with this alternative is that it

is difficult to administer the growth factors as they have limited target specificity and short half-lives within the target organism (1).

To reduce both problems, the therapeutic protein that this project will be utilizing has two collagen binding domains and a fibroblast growth factor (1). It also has a protease cleavage site between the collagen binding domain and the fibroblast growth factor as a recombinant protein (1). The collagen binding domains allow for the efficient uptake of the growth factor and for the delivery of growth factors to the wound site (1). Most bacterial collagenases possess multiple collagen binding domains (1). The collagen binding domains allow for the molecule to move material that has been bonded to them (1). In this case, the two collagen binding domains that this project's molecule has shuttles a fibroblast growth factor to a wound site to allow the healing process to begin. The Chemistry and Chemical Engineering departments have been working on the development of this particular molecule, and it has been shown to bind to particular collagen motifs with wounds once to molecule has been exposed to it (1). Dr. Joshua Sakon and his coworkers have built this molecule with two collagen binding domains and has used it successfully to target the collagen the molecule has been exposed to. It also has been able to further "fuse" itself with Fibroblast Growth Factor 2, or bFGF. Like other FGF family members, bFGF possesses cell survival activities as well as broad mitogenic activities that allow it to be involved in tissue repair and cell growth (2). Dr. McKinzie Fruchtl also successfully produced this fusion in *E. coli* (3).

At this point, the Chemical Engineering Department and the Chemistry and Biochemistry Department have a molecule that has two collagen binding domains that can attach to bFGF, but a mechanism that can cleave the bFGF from the collagen binding domains after delivering bFGF to the prospective wound site still requires further investigation. Thus, the project will involve the production of this molecule and the developments currently done to produce C1q (a collagen-based protein complex found in both human and animal blood) in the lab as a reagent for further characterization of the molecule in the future.

2. Methods

2.A. Production of the tandem collagen binding domain molecule with the fibroblast growth factor

To create the tandem collagen binding domain molecule, Drs. Sakon, Beitle, and Elmasheiti designed a plasmid DNA sequence so that it included a tandem collagen binding domain, an MMP protease sequence, and bFGF. This plasmid is called pEMB-1. The DNA sequence was also designed to have six histidine amino acids at the end. This sequence of amino acids was selected to allow for easier purification and identification of the molecule further on in the process. This plasmid contained the 'instructions' via the ribosome in the cell so that the tandem collagen binding domain molecule could be produced by a bacterial strain.

The bacterial strain chosen was *Escherichia coli*, or *E. coli*. *E. coli* was selected because it is well documented, and the molecular biology associated with it is well known. In order to produce the molecule, the *E. coli* needs to take up the final plasmid through a process known as transformation. In transformation, *E. coli* is heated up and exposed to chemicals in a way that temporarily makes its cell wall permeable. The plasmid then diffuses through the cell wall and into the bacterial strain. After the plasmid is transformed into *E. coli*, the bacteria are then quickly cooled on ice, and the cell walls revert, trapping the plasmid in the *E. coli*. The bacteria can then receive the 'instructions' from the plasmid to produce the molecule.

After the *E. coli* is given time to produce the molecule, the *E. coli* cells are 'cracked open' via sonication so that the protein, which is our tandem collagen binding domain molecule, can be released. This protein is then collected, concentrated, and tested through SDS page and Western blot to confirm the presence of the molecule in the sample.

2.A.1. Transformation

One microliter of newly constructed pEMB-1 was transformed to *E. coli* BL21. A stock of *E. coli* was removed from a -80℃ freezer and thawed in ice for 30 minutes. One microliter of pEMB-1 was added to the *E. coli* stock and returned to ice for another 30 minutes. The plasmid was then transformed into *E. coli* by being placed in a water bath at 41℃ for ten seconds, and then was returned to ice for five minutes.

One milliliter of SOC medium was added and put into a shaker at 37℃ and 250 RPM for one hour. One hundred microliters of the medium were then spread onto LB agar ampicillin plate with a concentration of 100 micrograms per milliliter and then placed into an incubator at 37℃ for 17 hours (or overnight). The LB agar ampicillin plate post incubation is shown below in Figure 1.

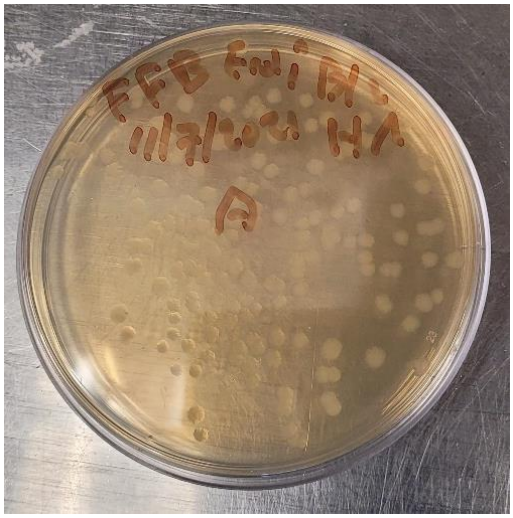

Figure 1. LB agar ampicillin plate with *E. coli* colonies

After incubation, a single colony was pulled from the LB agar ampicillin plate and inoculated in five milliliters of LB media broth along with five microliters of ampicillin at a concentration of 100 micrograms of ampicillin per milliliter. The sample was then incubated overnight at 37℃ and 250 RPM in a shaker.

After incubating overnight, the sample was added to 500 milliliters of LB broth containing 500 microliters of ampicillin. The broth was then placed in the shaker at 37℃ and 250 RPM for two hours.

For induction, 0.119 milliliters of one mM IPTG were added. The broth was placed back in the shaker for four hours.

After four hours, the sample was split into centrifuge containers and centrifuged for one hour at 4500 RPM and 4℃. The cells settled to the bottom of the containers, and the supernatant was poured off and disposed of, as seen in Figure 2.

Figure 2. Centrifuge containers with *E. coli* cells containing pEMB-1 and the target molecule

The containers with the cells at the bottom were then placed into a freezer.

2.A.2. Sonication and FPLC

The centrifuge containers were then pulled from the freezer and five milliliters of ten mM phosphate/ten mM NaCl buffer was added to each bottle. The bottles were then gently swished to resuspend the cells in the bottle. The cells were collected via a pipette in placed in a Falcon tube before being put back on ice.

A sonicator was then used to break apart the cells. The tip of the sonicator was placed one centimeter below the liquid level in the center of the tube so that the tip would not contact the sides. The sonicator

then was set to run for 30 minutes, while pulsing for five seconds then resting five seconds at an amplitude of 40%. While in the lab when the sonicator was running, all personnel were required to wear hearing protection.

After the cells were broken via sonicator, the Falcon tube's contents were then centrifuged for 45 minutes at 4500 RPM and 4°C. The supernatant was poured off and the broken cells were disposed of. For good measure, the liquid was then spun again for four minutes at 14000 RPM via a tabletop centrifuge. The supernatant was then collected again. This supernatant is known as the lysate.

The supernatant was then applied to an FPLC for eight hours. The FPLC, or the IMAC machine, was run with three buffers. Buffer A was 50 mM sodium phosphate/5 mM NaCl and Buffer B was 250 mM imidazole/50 mM sodium phosphate/5 mM NaCl. The FPLC was loaded with a Nickel column, and fractions were collected. The fractions were then placed in a box and frozen overnight.

2.A.3. SDS Page

Based on visual results from the FPLC, fractions 4, 7, 8, 9, 13, and 14 were of particular interest. 15% SDS gels were made and loaded into a Bio-Rad electrophoresis holder. The following Figure 3 describes how the gel wells were labeled:

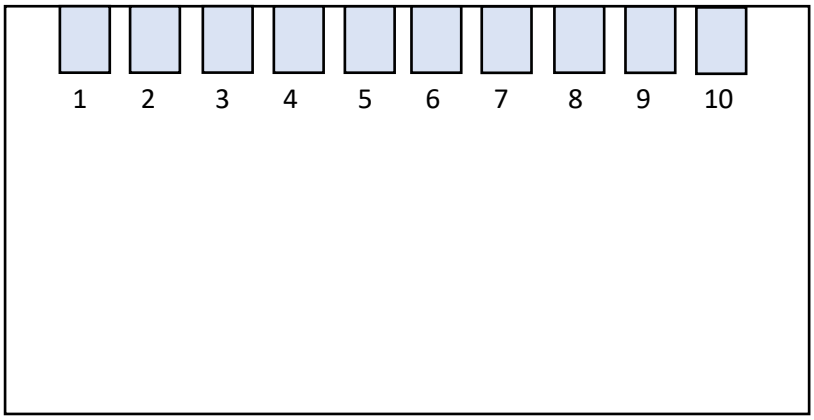

Figure 3. SDS page with numbered wells

The first well was loaded with a protein marker. The second well was loaded with a lysate sample. The third well was loaded with fraction 4, the fourth well was loaded with fraction 7, the fifth well was loaded with fraction 8, the sixth well was loaded with fraction 9, the seventh well was loaded with fraction 13, the eighth well was loaded with fraction 14, the ninth well was loaded with loading dye, and the tenth was loaded with a control sample with a his-tagged protein.

The gel was run for 47 minutes at 180 volts, and then placed in distilled water. The gel was removed from the glass within the water, and then the gel was placed in a strong stain for 30 minutes before being rinsed.

2.A.4. Western blot

The purpose of a Western blot is to check if a sample possesses a His-tag within its structure. For this Western blot, an antibody that interacts with 6-histidine was utilized during the process. The goal is to see a dark band on the Western blot indicating the presence of the his-tag.

To begin, the FPLC samples were taken out of storage. TCA Prep (or Trichloroacetic Acid Protein Precipitation) was added to the samples at a 1:10 ratio to concentrate the protein into a smaller volume. The samples were then centrifuged using a tabletop centrifuge for three minutes at 13000 RPM. Two hundred and fifty microliters of cold acetone were added to each sample and then vortexed via centrifuge for three minutes. The supernatant was disposed of, and the pellet was allowed to air dry for a minute. Then 15 microliters of eight M urea and 15 microliters of loading dye were added to each of the pellets and the pellets were carefully resuspended. The samples were then boiled at 95℃ three to five minutes.

The gel's wells were then loaded with the same scheme as seen in Figure 3. The first well was loaded with ten microliters of protein ladder, the second well was loaded with lysate sample, and wells 3-8 were

loaded with fraction 4, 7, 8, 9, 13, and 14, respectively. Wells 9 and 10 were loaded with loading dye and a control sample with a his-tagged molecule, respectively.

The Western blot gel was then run for 120 minutes at 150 volts and 76 milliamps with the setup as seen below in Figure 4.

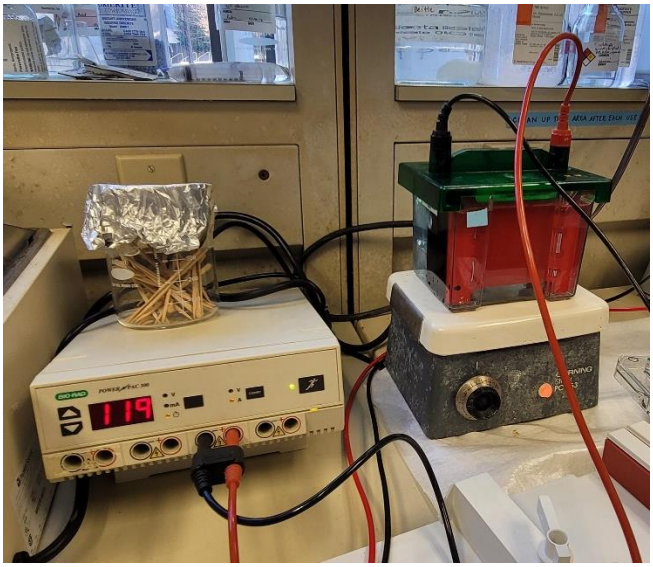

Figure 4. Western blot electrophoresis set up

The membrane on the gel was then carefully removed and placed in the container so that the membrane face that touched the gel was facing upwards. The membrane was then washed three times with TBST (Tris-buffered saline with polysorbate 20). Skim milk was then added to the container and placed on a rocker for 30 minutes.

After the 30 minutes, the membrane was washed three times with TBST. Then six to seven milliliters of TBST, two microliters of his-tag antibody, and 0.2% BSA was added. The 0.2% BSA, or bovine serum albumin, was made of 50 milliliters of TBST and 100 milligrams of BSA. The container was placed back on the rocker overnight.

In the morning, the milk in the container was replaced with TBST and then put on the rocker for three minutes. After three minutes, the TBST was switched out for a smaller amount of TBST and placed back

on the rocker for another three minutes. This was repeated one other time, for a total of three TBST washes after the milk was dumped.

After the last TBST wash, the TBST was removed from the container, and a pipette was used to ensure that all the TBST was removed. Two milliliters of 1-Step NBT/BCIP was pipetted over the membrane at a tilt and allowed to develop for two to three minutes. After the two to three minute time window, distilled water was poured gently over the membrane to stop the membrane from overdeveloping.

2.B. Developments in C1Q reagent production for future molecule characterization

In order to increase our knowledge of the structure that the tandem collagen binding domain molecule takes, x-ray crystallography was attempted. X-ray crystallography is a materials testing technique that aims to capture a three dimensional rendering of a molecular structure from a crystal sample forged out of the target molecule (4). A purified, highly concentrated sample of the molecule is crystallized and exposed to an x-ray beam (4). The synchrotron data collection station processes the resulting diffraction patterns that the x-ray exposed crystal gives off, yielding information about the repeating unit that forms the crystal (4). This repeating unit is the target molecule. The visual readout shows dark spots that can be used to determine the "structure factors," the electron density, and, with the usage of additional methods and the protein sequence, the three-dimensional molecular structure (4).

The first attempts to crystallize the tandem collagen binding domain molecule were unsuccessful, as the images did not render clearly. Dr. Sakon sent a sample of the molecule to a third-party lab, which determined that the main reason for the inability to capture the molecule's image was due to the molecule's extremely dynamic range of motion – in essence, the molecule refused to sit still long enough for the 'photograph'. The molecule is also very small, increasing the challenge of taking a clear render.

To circumvent these two issues, a proposed idea was put on the table: bind the molecule to another well documented molecule to limit the tandem collagen binding domain molecule's motion, with the added

benefit of also increasing the size of the repeating unit for crystallography. Essentially, make a ‘molecular mousetrap.’

The well-documented molecule selected was C1q. C1q is a protein complex that serves a key function as part of the immune system in the blood stream of humans and animals alike (5). Its structure is well documented and, as a bonus, contains collagen binding domains that our molecule can bind to (5).

While purchasing C1q is a viable method of acquiring C1q for the ‘molecular mousetrap,’ investing in the development of an in-house method of acquiring C1q from a raw blood source is of great interest. Raw bovine blood can be obtained for no cost at local slaughterhouses and processing the blood in-house would be significantly cheaper than ordering the same large quantity from a third party. Thus, this part of project was dedicated to development of an in-house production strategy for C1q from a raw bovine blood source.

Raw bovine blood was acquired from B&R Meat Processing in Winslow, Arkansas. Over the course of a few runs, it was determined that by adding in 1.5 milligrams of anti-coagulant EDTA (or Ethylenediaminetetraacetic acid) for each milliliter of blood, the blood did not form as large a clot, allowing more blood for processing. The blood was then centrifuged at 1800xg for one hour and the supernatant was collected for further processing. The pellet left at the bottom formed primarily of fatty proteins and cells was discarded.

To ‘clean up’ the supernatant by removing extraneous proteins, the bovine blood was then loaded into an ultrafiltration flow cell within a walk-in refrigerator. The ultrafiltration flow cell is pictured below in Figure 5.

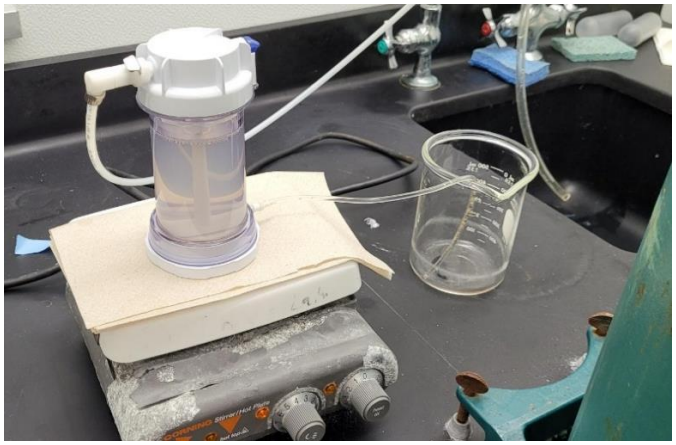

Figure 5. Ultrafiltration flow cell on stirring plate

The selection of the size of the ultrafiltration flow cell, the molecular weight cutoff of the membrane, and the piping of the gas line with a fine pressure control/release valve fell specifically under the purview of this project. The gas line piping can be seen below in Figure 6.

Figure 6. The piping to the gas tank's pressure gauge assembly and ultrafiltration flow cell quick connect

The piping as seen in Figure 6 has a standard gas tank pressure gauge with a fine gas pressure adjuster. This is connected to the plastic assembly branching out to the left of the gauge. The plastic assembly also has a tee built into it with the main pipe feeding into the ultrafiltration flow cell and the downwards pipe

attached to a pressure relief purge. The purge attachment can be set so that it purges the line if the line is above a specific pressure, which can be set by rotating the black plastic fitting. This also allows the line to be quickly purged so that the flow cell can be safely opened and the contents poured out. On the right of Figure 6 is a quick connect fitting that plugs directly into the top of the flow cell, allowing for easy connects and disconnects from the gas tank.

The ultrafiltration cell is capable of processing 200 milliliters in one batch. The bovine blood was diluted with a 85mM NaCl/13.3mM $H_2NaO_4PH_2O$ buffer (with a pH of 6) and carefully pressurized with a nitrogen gas cylinder. The ultrafiltration cell's membrane base had a molecular cutoff of 100 kDa. The molecular weight of C1q is between 410-462 kDa, rendering it too large to pass through the membrane while still allowing smaller unwanted molecules to pass through (6). After processing, the ultrafiltration cell's contents were collected and centrifuged for 1800xg for one hour, and the pellet was collected and washed with the ultrafiltration flow cell buffer. The pellet was then suspended in 0.5 liters of 10 mM EDTA/100 mM NaCl/10 mM benzamide/2.5 mM iodoacetamide/0.2 mM 1,10-phenathroline/25 mM potassium phosphate buffer with a pH of 7.4. Suspending the pellet in this buffer helps preserve the proteins so that they do not degrade and can be temporarily stored.

Further purification via ion-exchange chromatography was carried out via DEAE resin, allowing separation of more proteins based on charge. Ten milliliters of DEAE resin were then equilibrated with 50 milliliters of the pellet suspension buffer. After equilibrating, the resin mix was added to a filter flask and washed twice with more of the buffer and drained via vacuum each time. After the resin beads were fully equilibrated, approximately 240 milliliters of the sample were run through the resin under vacuum and the flowthrough was collected. The next stage in the process is to further 'clean' the samples via dialysis bags in buffer solution, before applying the fractions to further processing.

3. Results

In order to determine whether or not the double collagen binding domain molecule was produced by the *E. coli*, an SDS page and a Western blot were run. The SDS page can be seen below in Figure 7.

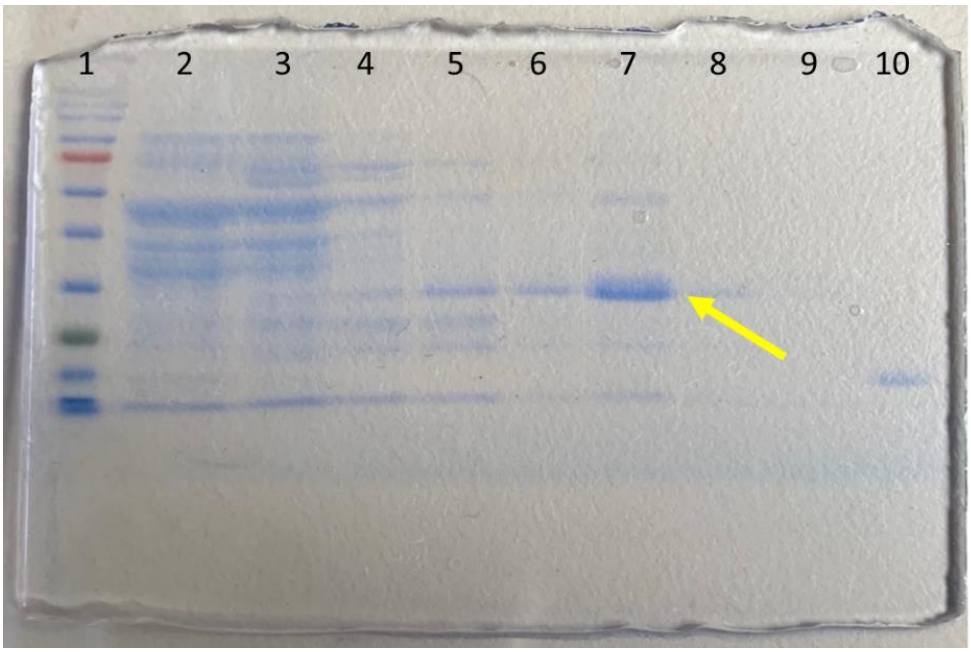

Figure 7. SDS page with numbered wells and an arrow indicating the presence of a double collagen binding domain and a his-tag

For the SDS page shown in Figure 7, the first well was loaded with ten microliters of protein ladder, the second well was loaded with lysate sample, and wells 3-8 were loaded with fraction 4, 7, 8, 9, 13, and 14, respectively. Wells 9 and 10 were loaded with loading dye and a control sample with a his-tagged molecule, respectively.

The SDS page shows a band at the molecular weights of bFGF and the double collagen binding domain, which is approximately 29KDa. On Figure 7, this band is indicated with a yellow arrow. The thickness of the band can be attributed to the fact that fraction 13 was where most of the molecule came through the column.

The Western blot was also run to check for the presence of the his-tag. It can be seen below in Figure 8.

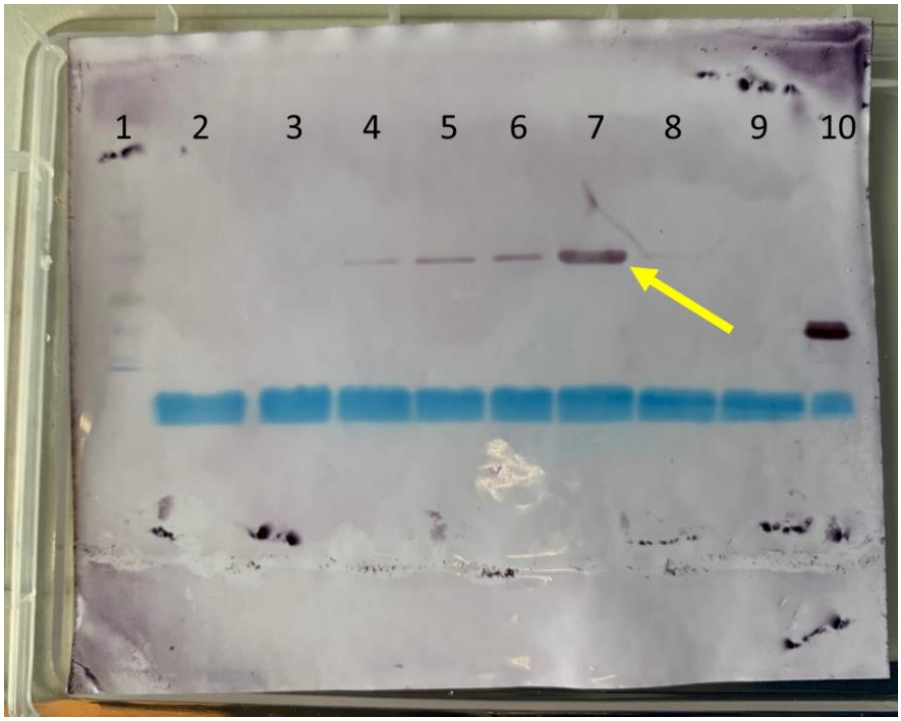

Figure 8. Western blot with numbered wells and an arrow indicating the presence of a his-tag

The Western blot shows a band at the molecular weight of the double collagen binding domain. This indicates that the molecule in question has reacted with the his-tag antibody, showing the existence of a his-tag on the molecule's structure. There is also a band for our control protein in well 10. Between the SDS page in Figure 7 and the Western blot in Figure 8, we have proved the existence of a molecule with our molecule's molecular weight and a his-tag. These two measures combined prove that the molecule was produced.

As for the C1q project, the ultrafiltration cell was able to reliable process 50 milliliters of sample in about 4 hours, allowing the cell to process large quantities of sample by adding in more sample every four hours. The largest result from the C1q reagent production developmental work was the replacement of the previous method of dialysis with the ultrafiltration flow cell. The previous methodology involved making at least 50 liters of dialysis buffer for every five liters of blood and dialyzing in a bathtub (7). The new method with the ultrafiltration flow cell allows the researcher to do the filtration in one tank, the buffer amount needed is significantly smaller, and overall volumes are easy to manage.

4. Discussion

Based on the results of the SDS page and the Western blot, the molecule is being produced effectively, but it is cleaved early by existing proteins in the *E. coli.* In Figure 7, there is a lack of extra bands above the band for the double collagen binding domain in well 7 as seen in the other previous wells. This points to the molecule being produced effectively, but degrading and breaking apart by the time the molecule makes it through chromatography. While this could be due to environmental factors, it is likely that there is an enzyme or a protein in *E. coli* that is cleaving the molecule at the protease site. For future work, either the enzyme or protein causing the premature cleave will need to be removed from the *E. coli* or *E. coli* will need to be replaced altogether.

As for the C1q reagent production method project, the ultrafiltration cell shows great promise in improving the filtration process. Essentially, the first part of the production process is mapped out, leaving the next phase of work to be further purification, concentration of samples, and analysis.

5. Acknowledgements

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