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Protein Isolation Using Peptoid Based Affinity Chromatography

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Protein Isolation Using Peptoid Based Affinity Chromatography

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Abstract

Protein purification is essential for advancements in biotechnology. There are several different methods employed in purifying a particular protein from a complex sample such as a cell lysate. These methods take advantage of differences in the size, charge or binding affinity of the protein. One such method is affinity chromatography which utilizes the binding affinity of a protein toward a certain ligand to purify a protein. This is usually used as a final step to extract the desired protein after the mixture has undergone other purification steps to remove unwanted materials. The goal of this project was to develop a one-step peptoid-based protein purification method.

Poly-N-substituted glycines, or peptoids, were developed in the early 1990s and have been shown to have many biological applications. Peptoid side chains can be manipulated for unique circumstances by utilizing any free amine in synthesis. This is an advantageous quality in the determination of protein ligands.

This study investigated using peptoids as a specific and efficient one-step process for purification methods. It showed there is potential of proteins binding to peptoids by determining protein concentration changes caused by incubation studies. However, these results could not be verified.

Introduction and Background

One type of protein purification technique is column chromatography. It works by having a stationary phase that is a solid adsorbent, and a mobile phase that is a liquid. The liquid is sent through a column and the desired component is absorbed by the solid. This efficient method is generally used to separate all

proteins from cell lysate. One method of the stationary phase utilizes a technique known as immobilized metal ion affinity chromatography (IMAC). IMAC is a process that uses proteins that have an affinity towards metal, usually nickel, copper, cobalt or zinc. These proteins then bind to the solid adsorbent that is composed of microbeads coated in the corresponding metal. Some proteins are not attracted to metal and must be altered. This is done by using recombinant DNA techniques to add a tag that has an affinity towards metal to a particular protein. A disadvantage of using this technique is that IMAC alone cannot provide both efficiency and specificity. Efficiency refers to its ability to extract all of the desired protein, and specificity meaning its capability to extract only the desired protein. It has been suggested that a primary purification step known as aqueous two-phase extraction (ATPE) be used. ATPE is used as a primary step because it is efficient meaning it captures all of the proteins. IMAC must then be used to specifically purify the sample in order to get the desired component.[3]

Poly-N-substituted glycines or peptoids were developed in the early 1990's and have been shown to have many biological applications. Peptoids have a structure similar to that of a peptide (see figure 1); however, peptides have side chains attached to the α-carbon and peptoids have side chains attached to the amide nitrogen. Peptoids can be synthesized using an automated peptide synthesizer, and they are more cost efficient than a peptide because the backbone amines do not have to be protected. The method for synthesizing peptoids is carried out by a submonomer solid-phase protocol shown in Figure 2. This works by having two submonomers that are used to assemble the N-substituted glycines (NSG)

monomers.[4] Peptoids are synthesized to include a unique set of side chains by utilizing any free amine. Peptoids also possess helices that are robust and stable. [2]

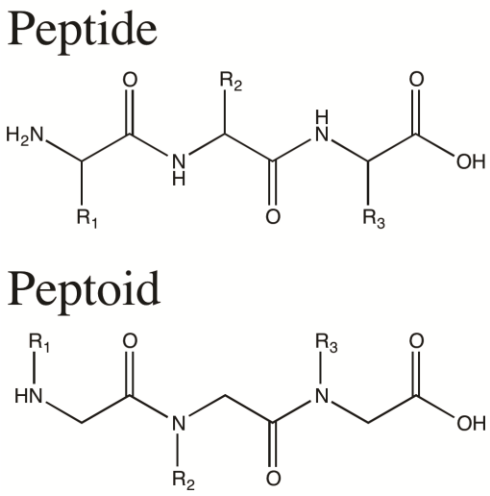

Figure 1: Comparison of peptide and peptoid backbone

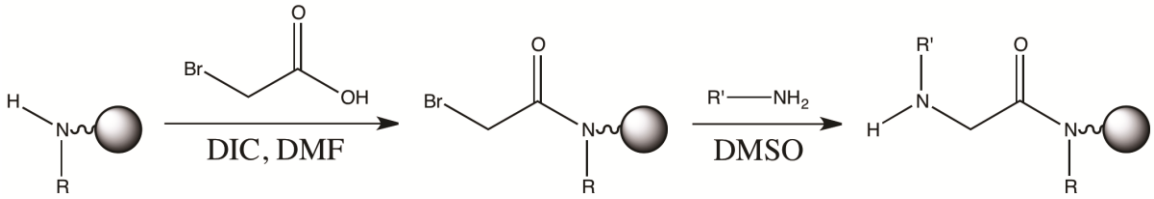

Figure 2: Submonomer solid-phase protocol scheme

It has also been found that properly designed peptoids have the ability to be inexpensive and efficient protein ligands. They have the ability to not only bind efficiently with an unmodified protein but to bind specifically in the presence of other bacterial proteins.[1] These facts about peptoids make them an ideal candidate for affinity based chromatography techniques. The goal of this study was to evaluate the potential of peptoids to bind with protein.

Methods and Materials

Preliminary Binding Experiments

The first test of the potential of peptoids to bind to protein was performed using an unknown peptoid sequence and cell lysate. The peptoid was synthesized previously on amide resin. The experiments were performed with the peptoid attached to the resin. The cell lysate was prepared by sonicating the mixture to break the cell membranes, and the solution was then centrifuged to remove the cell debris. The cell lysate was diluted 4-fold to a final sample volume of 1mL in 3 different buffers: Phosphate Buffer Solution (PBS), 0.02M Na2HPO4 1M Na2SO4(HIC-A) and 0.02M Na2HPO4 (HIC-B). Then 500µL of each protein sample was added to the peptoid resin and incubated for 72 hours at room temperature while being gently shook.

A bicinchoninic acid(BCA) assay was performed to determine the protein concentrations of the samples before and after the incubation period with the peptoid resin. The BCA assay was completed by combing 20 µL of the protein sample , 200 µL of reagent A (mixture containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide), and 5 µL of reagent B(containing 4% cupric sulfate) in a 96-well plate. The samples were then incubated for 30 minutes at 37°C. The assays produce a color change dependent on the amount of protein in the sample. The protein concentrations were then determined by using a nanodrop spectrophotometer at a wavelength of 562nm. A bovine serum albumin (BSA) standard curve was also developed using this method.

Preliminary Validation

A series of validation steps were executed to determine whether the protein was actually binding with the peptoid. First, the protein sample was removed from the sample. Then a high salt concentration solution, 1M NaCl, was added to the peptoid resin in order to break the binding between the peptoid and the protein. Then a SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was done using a 12.5% acrylamide gel at 250 volts, 400 mA for 35 minutes. This was to show the presence of protein in the sample after the high salt wash.

The next step was to liberate the peptoid from the resin. This was done using triflouroacetic acid (TFA) solution. After cleaving the peptoid from the resin another SDS-PAGE was done on the sample using a 12.5% acrylamide gel at 250 volts, 400 mA for 35 minutes.

Peptoid Synthesis

A peptoid that had previously shown protein binding ability was synthesized to further determine the binding potential.[1] The peptoid was synthesized using Zuckerman's sub-monomer protocol on amide resin.[4] Each side chain was prepared with dimethyl formamide (DMF) as a solvent except for 4-(2-aminoethyl) benzene sulfonamide (Nbsa) which utilized dimethyl sulfoxide (DMSO). 7mL of a 1M solution was prepared. The peptoid sequence can be shown in Figure 3. Matrix-assisted laser desorption/ionization (MALDI) was used to verify the successful synthesis of the peptoid. MALDI is a powerful mass spectrometry technique that will show the molecular weight of molecules present in the sample.

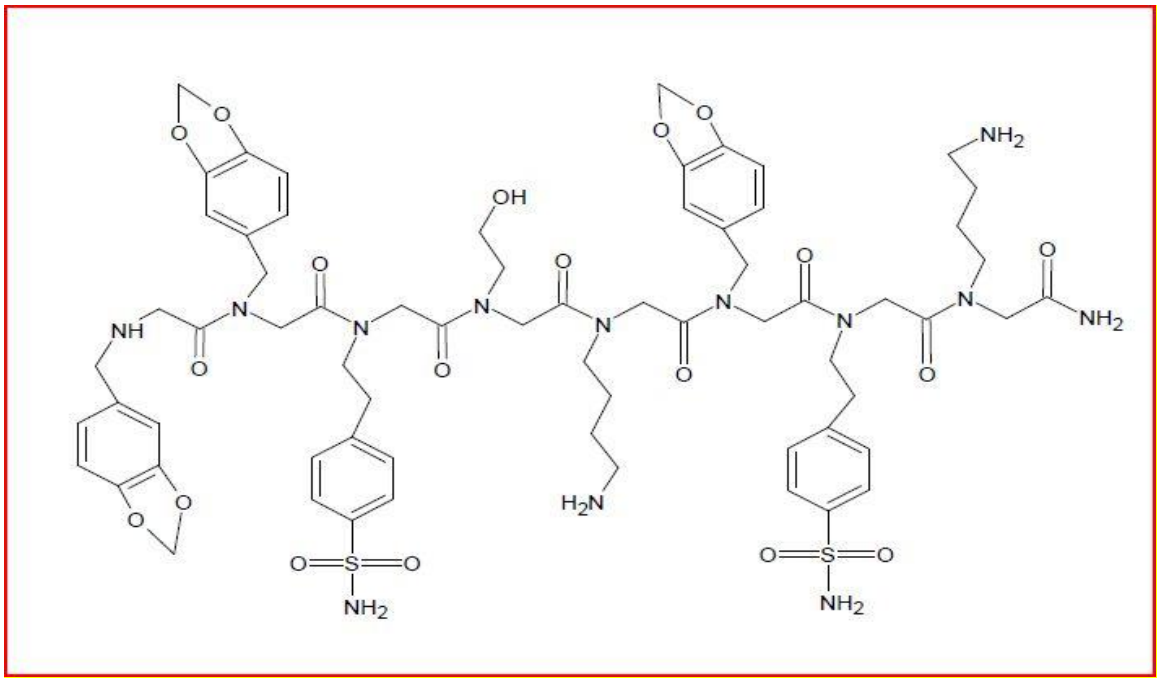

Figure 3: Peptoid sequence utilized with a molecular weight of 1428

Binding Experiments

Table 1 shows a summary of the binding conditions used in the final binding study. The main variations between different samples were the temperature and the protein concentration. Protein 1 had a concentration of 1.72mg/ml and protein 2 had a concentration of 0.663mg/mL. The two temperatures represent room temperature and a standard refrigerator. Each sample was incubated with 112 µL of protein solution. The mass of the peptoid resin was varied as stated.

Table 1: Protein incubation conditions

Sample	Resin Mass	Protein 1	Protein 2	22° C	4° C
1	8.72mg	X			X
2	8.49mg		X		X

3	**8.41mg**	**X**		**X**	
4	**11.13mg**		**X**	**X**	
5	**32.31mg**	**X**		**X**	
6	**40.23mg**		**X**	**X**	

Protein Assay

A BCA protein assay was performed to determine the protein concentration of the samples before and after the incubation period for the novel peptoid with the varied incubation conditions. This assay was carried out as described in the preliminary binding section.

Gel Electrophoresis

SDS-PAGE was performed to verify binding in the new set of samples. First, the resin was treated with a high salt solution (2M NaCl) to release the bound protein from the peptoid resin. Then the gel was performed using a 12.5% acrylamide gel at 250 volts, 400 mA for 35 minutes. This was used to show proteins in the sample.

Results and Discussion

Preliminary binding

The results from the preliminary BCA assay to determine protein concentration can be seen in Figure 4. There is a noticeable difference in the protein concentrations between the original samples and the samples after the incubation

with the peptoid resin. This suggests that the protein in the cell lysate was binding to the peptoid resin with all of the buffer solutions. The largest difference was observed for the HIC-B sample. HIC-A showed a small response indicating that it might not be the most suitable buffer solution for this task. Because of its performance and availability, PBS was used throughout the remainder of the studies.

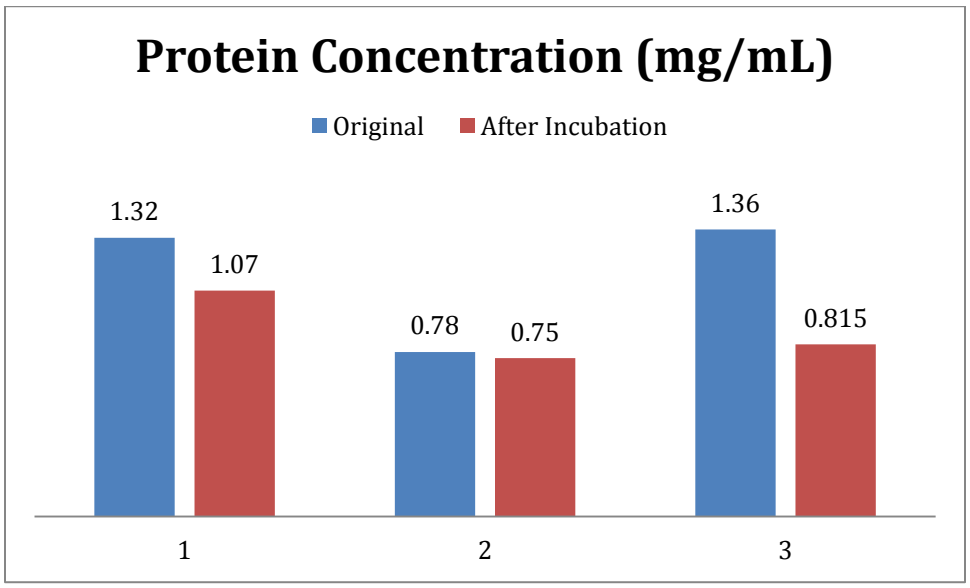

Figure 4: Results from the preliminary binding studies

Preliminary Validation

The initial SDS-PAGE that was completed after the preliminary binding studies is shown below in Figure 5. Figure 5 shows that in the three lanes that the samples were in there was no protein detected. The lane that is labeled total lysate was used as a positive control to show that the gel was working properly and contained the cell lysate before it was diluted and added to the peptoid resin.

Because no protein was detected on the gel, another step had to be taken to break the binding between the protein and the peptoid.

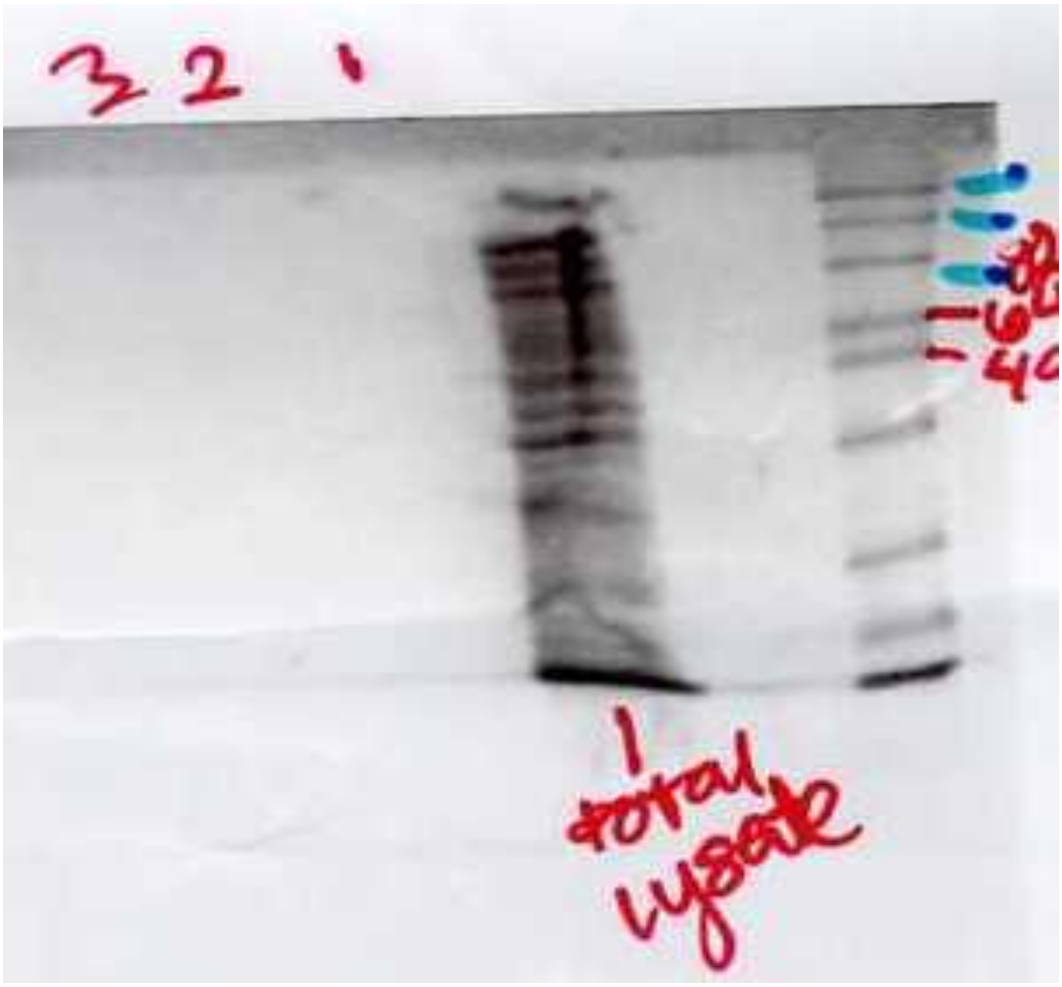

Figure 5: SDS-PAGE for three samples and the total lysate

After the initial SDS-PAGE showed no protein, the peptoid was cleaved from the resin. The cleaved sample then underwent an additional SDS-PAGE. Figure 6 shows the results of that test. It indicates that the lanes experienced blanching. This was probably caused by the pH being too low due to the TFA that was used to cleave the peptoid from the resin. The picture also shows that there may be some protein

at the bottom, but the color may be due to the dye running off the gel or it may be the actual peptoid. Another reason that this gel might not have worked was the protein concentration of the samples may have been too high.

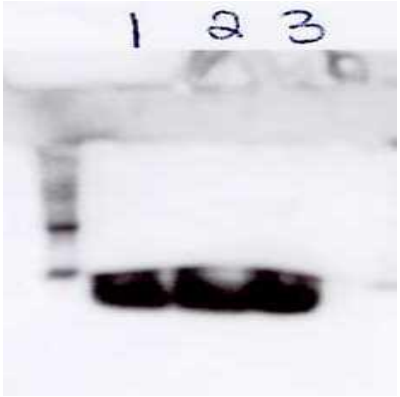

Figure 6: SDS-PAGE of samples after peptoid cleavage

Peptoid Synthesis

The results from the preliminary studies described above led to novel peptoid synthesis for binding determination. After the peptoid was synthesized and cleaved from the resin, MALDI was performed to verify the presence of the peptoid. Figure 7 shows the results from that analysis. The mass spectrometry technique showed that the desired molecular weight of 1428 was not in the sample. This would suggest that there was an error in the peptoid synthesis. This error was not discovered until the after the binding experiments were performed. The results from those experiments were thus from an unknown peptoid source.

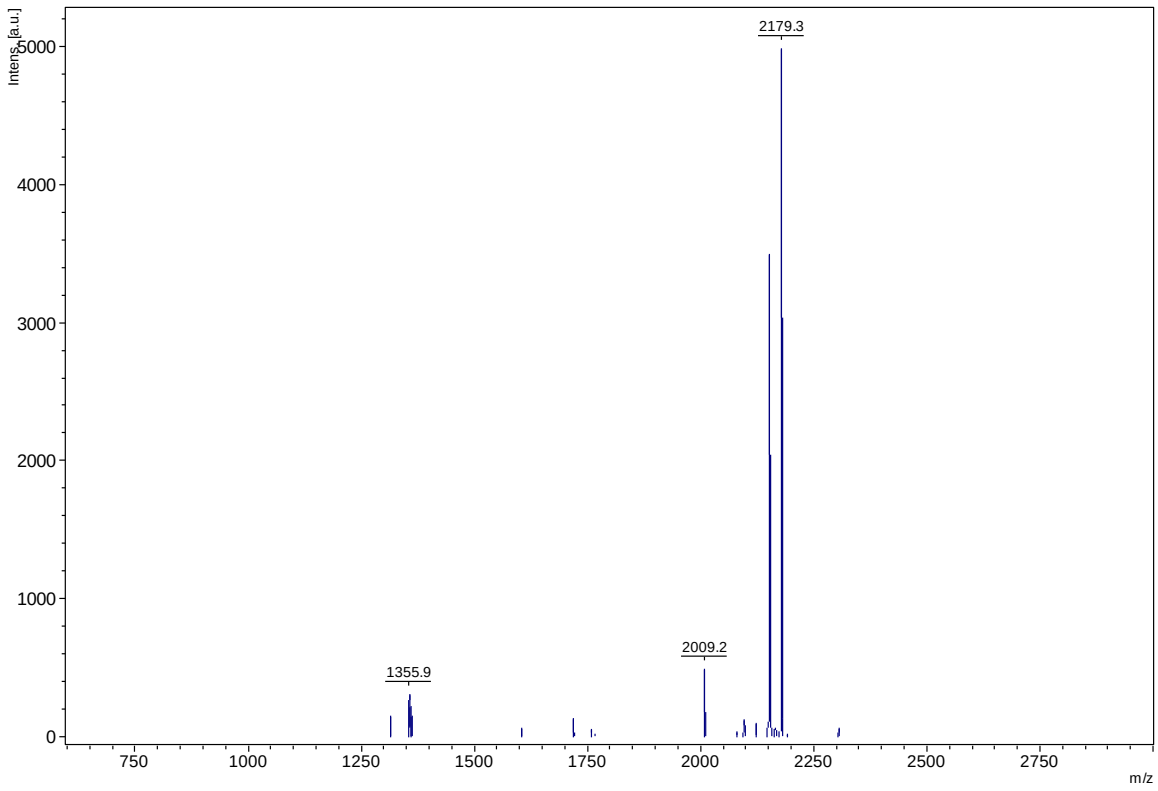

Figure 7: MALDI results showing the molecular weight of the peptoid

Binding Experiments and Protein Assay

Before the MALDI results showing the unsuccessful synthesis of the novel peptoid were obtained, binding experiments were completed. Table 2 shows the results from the BCA of the samples before and after incubation with the synthesized peptoid. The incubation conditions were described in the materials and methods section. Samples 1, 3 and 5 had a starting concentration of 1.72mg/mL. Samples 2, 4 and 6 had a starting concentration of 0.662 mg/mL. This showed that all of the samples had a decrease in concentration with the exception of sample 6. It had too little volume in the sample container to get an accurate protein concentration. Figure 8 shows a visual representation of samples 1 and 3's protein concentration compared to the original protein concentration. Likewise, Figure 9

shows a comparison between samples 2 and 4 and the original protein concentration.

Table 2: Protein concentration after incubation period

Sample	Concentration (mg/mL)
1	1.515
2	0.656
3	1.567
4	0.633
5	1.628
6	----------

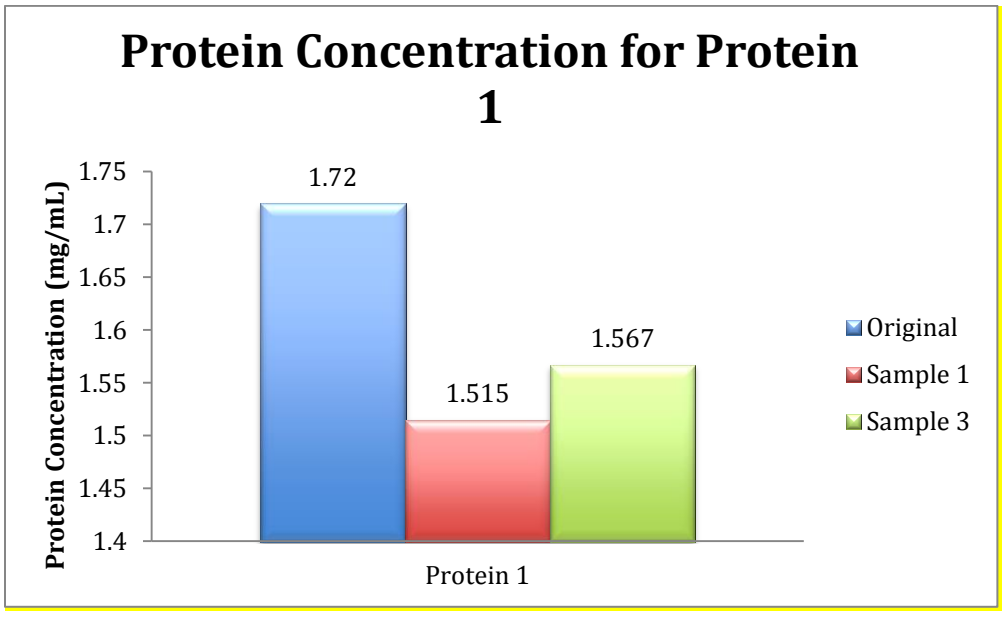

Figure 8: Protein concentration for the 1.72mg/mL protein samples

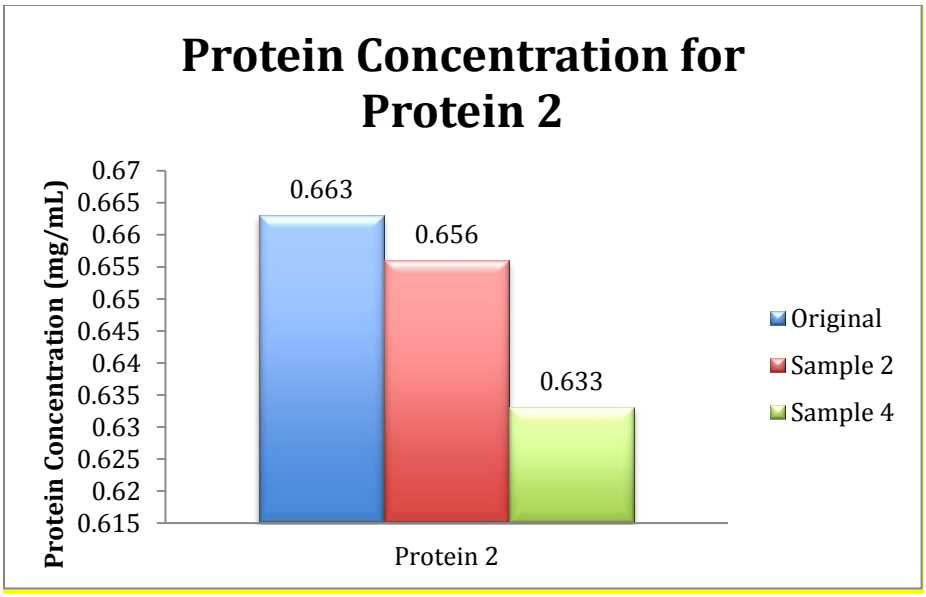

Figure 9: Protein concentration for the 0.663 mg/mL protein samples

Gel Electrophoresis

After the completion of the second set of binding studies and a high salt wash step to disrupt the binding, gel electrophoresis was performed to determine the presence of protein in the sample. The SDS-PAGE showed no protein concentration in sample 2 as shown in Figure 10. This could suggest that the salt concentration was not sufficient means of disrupting the binding between the protein and peptoid. Also, this could mean that the protein was not actually binding to the peptoid.

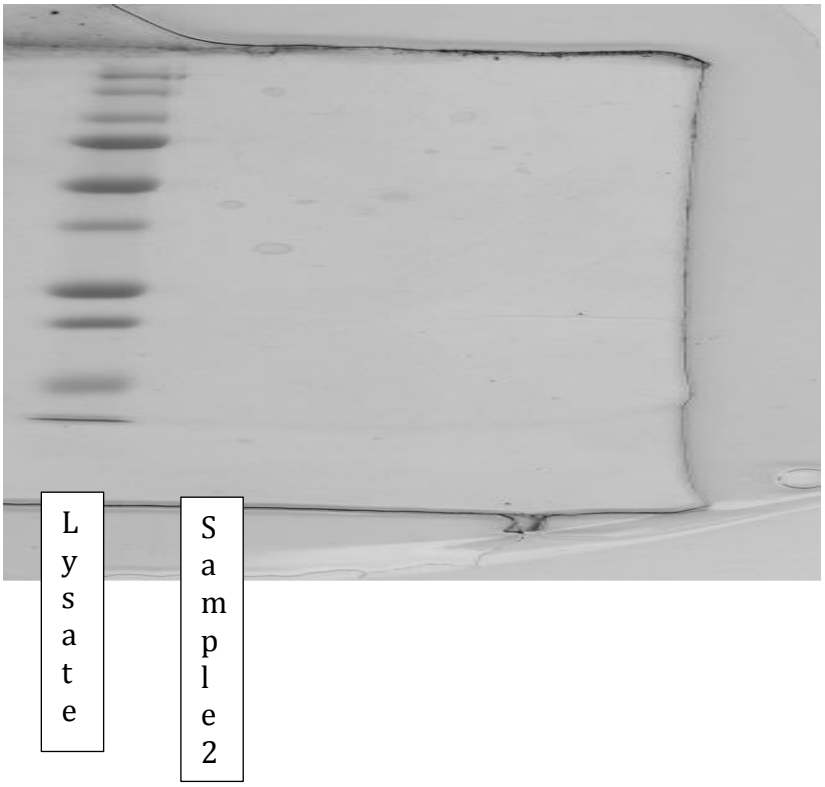

Figure 10: SDS-PAGE showing no protein concentration

Conclusion

This study suggests that there is potential for peptoids to serve as ligands for proteins. This was evident by the changes in protein concentration before and after the protein was incubated with peptoid resin; however, gel electrophoresis was unable to verify the binding potential. Thus, further optimization must be done to verify that the protein is binding to the peptoid.

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References

1. Alluri, P. G., Reddy, M. M., Bachhawat-Sikder, K., Olivos, H. J., & Kodadek, T. (2003). Isolation of Protein Ligands from Large Peptoid Libraries. *J. AM. CHEM. SOC., 125*, 13995-14004.
2. Kirshenbaum, K et al. (1998, April). Sequence-specific polypeptoids: A diverse family of. *Biophysics, Chemistry, 95*, 4303–4308. Retrieved April 14, 2009, from PNAS database: http://www.pnas.org/content/95/8/4303.full.pdf+html
3. Li, Y., & Beitle, R. R. (2002). Protein Purification via Aqueous Two-Phase Extraction (ATPE) and Immobilized Metal Affinity Chromatography. Effectiveness of Salt Addition To Enhance Selectivity and Yield of GFPuv . *Biotechnol. Prog., 18*(5), 1054-1059.
4. Zuckermann, R. N., Kerr, J. M., Kent, S. B. H., & Moos, W. H. (1992). Efficient Method for the Preparation of Peptoids [Oligo(N-Substituted glycines)] by Submonomer Solid-Phase Synthesis . *J. Am. Chem. Soc., 114*, 10646-10647.

This thesis is approved.

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Thesis Committee:

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