Continuation/Research Progression Projects Form (7)

Required for projects that are a continuation/progression in the same field of study as a previous project.
This form must be accompanied by the previous year's abstract and Research Plan/Project Summary.

Student's Name(s) Farihah Chowdhury

To be completed by Student Researcher: List all components of the current project that make it new and different from previous research. The information must be on the form; use an additional form for previous year and earlier projects.

Components	Current Research Project	Previous Research Project: Year: 2018-2019
1. Title	Potential Pitfalls in Protein Structure Determination via Protein Crystallography	The Structural Resolution of Bovine Thyroglobulin
2. Change in goal/ purpose/objective	To identify common errors throughout the crystallogenesis and structural analysis of proteins. The two proteins used were insulin and thyroglobulin.	To determine the structure of thyroglobulin.
3. Changes in methodology	Using ATLAS and gelatin to try and crystallize thyroglobulin, and multiple screening kits and chemicals to find ligands for insulin.	Using Hampton Research and PEG screening kits and lysozyme as a nucleation inducing reagent to crystallize thyroglobulin.
4. Variable studied	Pitfalls during the processes of crystallogenesis and structural refinement of thyroglobulin and insulin.	Small angle scattering and bead model for thyroglobulin.
5. Additional changes	Gelatin, not lysozyme, was used and an entirely new screening kit that was newly developed by a collaborating scientist to work on thyroglobulin. Additionally, insulin was included in this project, while last year's project only focused on thyroglobulin.	

Attached are:

☑ Abstract and Research Plan/Project Summary, Year 2018-2019

I hereby certify that the above information is correct and that the current year Abstract & Certification and project display board properly reflect work done only in the current year.

Farihah Chowdhury — Student's Printed Name(s)

Signature

01-20-20 — Date of Signature (mm/dd/yy)

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Structural Resolution of Bovine Thyroglobulin

Farihah Chowdhury & Mariam Sheikh

2018-2019 Research Plan

1. RATIONALE

Thyroglobulin is a protein located in the thyroid and controls hormone production. These hormones work to modulate behavior, central nervous system function, seasonal changes in physiology, and energy metabolism in vertebrates (Holzer et al., 2016). In addition, it is a dimeric glycoprotein with a molecular mass of 660 kDa. Specifically, bovine thyroglobulin is heavily decorated with alpha gal and can be used to diagnose the allergy (Apostolovic et al., 2017). For these reasons, the structure of bovine thyroglobulin is crucial to find and can lead to new information about the relationship between alpha gal and the IgE antibodies.

Alpha gal, an oligosaccharide, is a major blood group substance in mammals such as cattle and pigs. Studies strongly suggest that bites from the Lone Star Tick Amblyomma americanum infect the human host with the carbohydrate alpha-gal (Commins & Platts-Mills, 2013). After some time, when beef or another red meat is consumed, an immune response is initiated by the IgE antibodies, that results in an immediate allergic reaction characterized by symptoms of anaphylaxis (Sim et al., 2017). Currently, the structure of bovine thyroglobulin is unresolved. The aim of this research is to find the structure of the protein using various techniques, such as protein crystallography, small angle X-ray scattering, and Size Exclusion Chromatography.

Small angle X-ray scattering is a technique that can be used to determine how the shape and size of proteins in solution averaged over all conformations. This information, then can be used to generate a structure of proteins, with the solution scattering data also being able to account for structural flexibility (Castellanos et al., 2016). There are numerous articles contradicting the formation of thyroglobulin crystals. Efforts to crystallize thyroglobulin have revealed that it is most likely a flexible protein (Mezghani et al., 1997). This method seems to have been ineffective for other researchers, including previous research through our institution. However, further attempts will be made using different methods and buffer solutions to crystallize thyroglobulin in this project, since information from crystallization coupled with small angle X-ray scattering can produce more information about a protein and its interactions (Kovalchuk et al., 2016)

As a result of the fluctuating radii of gyration with different concentrations of NaCl and thyroglobulin with SAXS, it was concluded that size exclusion chromatography should be used as well. Size exclusion Chromatography (SEC) separates different compounds according to their size and when performed with aqueous solvents, it is known as gel filtration. This procedure desalted the protein and aided to elute proteins to identify and isolate different species (Nagy & Vekey, 2008). Since thyroglobulin is a dimeric protein, the use of SEC to separate the different particles, analyzing the distinct oligomeric states of thyroglobulin with SAXS was made possible (Gentile, Salvatore, & Salvatore, 1995).

RESEARCH QUESTIONS:

Is lysozyme a nucleation inducing reagent of thyroglobulin?

Will Small Angle X-Ray Scattering and Size Exclusion Chromatography aid in the process of resolving the structure of thyroglobulin?

HYPOTHESIS:

It was hypothesized that lysozyme is a nucleation inducing reagent of thyroglobulin and that SAXS and SEC will aid in the process of resolving the structure of thyroglobulin.

2. METHODS

2.1. Thyroglobulin and Lysozyme Crystallization Screening

Various screening kits and buffer solutions will be screened with different ratios of thyroglobulin and lysozyme mixed. PEG Rx 1 and PEG Rx 2 # 1-48 screening kits, Ammonium sulfate at a molarity of 1.5, and Phosphate buffer with a pH of 7.2 will each be screened with a 2:1 and 1:1 ratio of thyroglobulin to lysozyme. 1:1 ratios will be 40 mg/ml of thyroglobulin dissolved in Phosphate buffer pH 7.2 with 40 mg/ml of lysozyme dissolved in Sodium Acetate Trihydrate at 0.02 M. The 2:1 ratios will be 30 mg/ml of thyroglobulin dissolved in Phosphate buffer with a ph of 7.2 and 20 mg/ml of lysozyme will be dissolved in Sodium Acetate Trihydrate at 0.02 M.

1:1 ratios will be made to screen crystallization using the HR 121 and 122 screening kit. Thyroglobulin will be dissolved in dH2O at 40 mg/ml and combined with Lysozyme which will be dissolved in sodium acetate trihydrate at 40 mg/ml. This will be repeated replacing dH2O with phosphate buffer for solution HR 121 #40 as this is the primary solution that contained crystals in a previous experiment.

All of the plates for screening will be made using the hanging drop method. 350µl of the buffer being used will be put into each well, and then a 5µl drop of the thyroglobulin solution with or without lysozyme will be put on a cover slide and placed on top of the well. These plates will then be stored in a temperature controlled refrigerator to form crystals.

2.2. Future analysis of crystals

Specific crystals, ones of good and defined size and shape, will be fished and placed into pucks in liquid nitrogen. The crystals will then be placed into the AMX and FMX beamlines to create raster files. The purpose of the AMX beamline is to determine the structure of macromolecules using x-ray diffraction and the purpose of using the FMX beamline is to determine the structure and function of macromolecules with micro-diffraction while limiting radiation damage (BNL NSLS II). The raster files will then be converted in CCP4i. The protein structure will then be analyzed on COOT.

2.3. Small Angle Scattering

First, optimal salt and protein concentration will be identified. To identify optimal protein concentration, thyroglobulin concentrations of 0.12, 0.25, 0.5, 1.0, 2.0, 3.0, and 5.0 mg/ml was analyzed. For optimal salt concentration, NaCl concentrations of 175, 200, 350, and 500 mM will be analyzed. Then, 60 microliters of each solution will be put into PCR tubes and centrifuged. After, the tubes will be inserted into the LiX beamline. The LiX beamline, or the Life Science X-Ray Scattering beamline, is used to analyze the composition of biomolecular solutions using small angle x-ray scattering. After the issues with crystallization in the last project, small angle scattering was presented as a method to determine more information about the structure of the protein, and it has been used for this purpose in other studies as well. The first tube for each trial consisted of the buffer solution for a control. Python code in Jupyter notebooks will then be used to generate the SAXS figures, SAXS-Buffer figures, and Guinier plots for each solution.

2.4 Size Exclusion Chromatography

Size Exclusion Chromatography will then be used to separate different compounds by size. Thyroglobulin is a dimeric protein, therefore the use of SEC to separate the different molecules will make analyzing the distinct oligomeric states of thyroglobulin with SAXS possible.

The Superdex 200 Increase columns will be used in order to separate monomer, oligomer, and aggregated forms of thyroglobulin, and perform SEC. The Superdex columns are high performance gel filtration columns used to check for the homogeneity of a solution (GE, 2013).

First, solutions of thyroglobulin and Phosphate buffer with a pH of 7.2 will be created, with concentrations of 5 mg/ml and 10 mg/ml. 100µl of each solution will be created for both trials, and filtered down with the VWR centrifugal filter by centrifuging the tube at 7200 rpm at 4°C for 3 minutes. Then 60µl of each solution will be put into PCR tubes and centrifuged again at the same parameters previously used. It is essential to decrease the chance of any bubbles appearing in the solution since they can detrimentally affect the SEC system.

To prepare the SEC system, Phosphate buffer will be used to purge the line. Then, the Superdex column will be attached to the system, and the buffer will run through the column for 10 minutes in order to equilibrate the system. Then, the samples will be placed inside the system, where there is an autoinjector that will be injecting the samples into the tubes and running them through the column.

To analyze the data from SEC, python code in Jupyter notebooks will be used to generate the plot of the scattering of the solution, depicting a peak that can help to determine the size and shape of the protein. This data then will then be converted into .dat files, which will later be uploaded into PRIMUS to determine more information about thyroglobulin.

3. SAFETY RISKS:

The synchrotron presents a radiation risk when the beamlines are running. However, the facility is well controlled by Brookhaven National Lab, including numerous alarms and safety precautions put into place. All minors working in the synchrotron also wear TLDs which track the amount of radiation exposure, helping to ensure the safety of everyone. Lastly, many training courses were taken to be fully prepared to operate the beamline and work in the labs.

4. BIBLIOGRAPHY:

1. Apostolovic, D., Krstic, M., Mihailovic, J., Starkhammar, M., Velickovic, T. C., Hamsten, C., & van Hage, M. (2017). Peptidomics of an in vitro digested α-Gal carrying protein revealed IgE-reactive peptides. Scientific reports, 7(1), 5201.
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12. Yuqian Luo, Yuko Ishido, Naoki Hiroi, Norihisa Ishii, and Koichi Suzuki, “The Emerging Roles of Thyroglobulin,” Advances in Endocrinology, vol. 2014, Article ID

189194, 7 pages, 2014. https://doi.org/10.1155/2014/189194.

Addendum:

Title: ***Advancements in the*** Structural Resolution of Thyroglobulin

Part A

1. **RATIONALE**

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Part B

RESEARCH QUESTIONS:

1. ***How effective is lysozyme as a nucleation inducing reagent of thyroglobulin?***
2. ***What is the bead model of bovine thyroglobulin?***

HYPOTHESIS:

It is hypothesized that lysozyme will be a highly effective nucleation inducing reagent of thyroglobulin and that the bead model of bovine thyroglobulin will be a complex globular structure containing alpha and beta helices, factoring in flexibility.

EXPECTED OUTCOMES:

It is expected that lysozyme will be an effective nucleation reagent and thyroglobulin will be a globular protein. It is also expected that the size exclusion chromatography data will yield that thyroglobulin is a flexible structure.

Part C

3. **METHODS**

Thyroglobulin and Lysozyme Crystallization Screening

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the amount of radiation exposure, helping to ensure the safety of everyone. Lastly, many training courses were taken to be fully prepared to operate the beamline and work in the labs.

Chemicals such as ammonium sulfate and crystal screening buffers from the Hampton Research 121/122 and PEG Rx1/Rx2 will be utilized in the attempt to crystallize thyroglobulin. These chemicals will be handled in a laboratory setting and students will wear protective equipment including gloves, goggles, and lab coats.

4. **BIBLIOGRAPHY:**

1. Apostolovic, D., Krstic, M., Mihailovic, J., Starkhammar, M., Velickovic, T. C., Hamsten, C., & van Hage, M. (2017). Peptidomics of an in vitro digested α-Gal carrying protein revealed IgE-reactive peptides. *Scientific reports*, *7*(1), 5201.

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OFFICIAL ABSTRACT and CERTIFICATION

Advancements in the Structural Resolution of Bovine Thyroglobulin

Farihah Chowdhury and Mariam Sheikh
William Floyd High School, Mastic Beach, NY

Bovine thyroglobulin shares an epitope of alpha-gal, which can cause an allergic reaction when IgE antibodies against α- gal are present in the human body, therefore resolving its molecular structure may provide insight into the mechanism by which alpha-gal initiates an immune response (Sim et. al, 2017). The structure of bovine thyroglobulin is currently unresolved, therefore, attempts were made to crystallize the protein and to create a bead model using Small Angle X-Ray Scattering (SAXS). Crystallogenesis was attempted, tested at macromolecular crystallography beamlines using numerous buffers and lysozyme as a nucleation inducing reagent, but was unsuccessful. Small angle X-ray scattering was used in an effort to obtain a low-resolution structure and radius of gyration. SEC was conducted on the LiX beamline, and the data indicated that bovine thyroglobulin's radius of gyration is about 78. The analysis of this data also allowed for the creation of a bead model, providing more insight into the structure of thyroglobulin. When the bead model and scattering profiles from our data were compared to the tentative structure and profile depicted by the amino acid sequence of bovine thyroglobulin, there were many similarities that could be drawn, further validating the results from this research.

Category
Pick one only—
mark an "X" in box
at right

Animal Sciences	☐
Behavioral & Social Sciences	☐
Biochemistry	■
Biomedical & Health Sciences	☐
Biomedical Engineering	☐
Cellular & Molecular Biology	☐
Chemistry	☐
Computational Biology & Bioinformatics	☐
Earth & Environmental Sciences	☐
Embedded Systems	☐
Energy: Chemical	☐
Energy: Physical	☐
Engineering Mechanics	☐
Environmental Engineering	☐
Materials Science	☐
Mathematics	☐
Microbiology	☐
Physics & Astronomy	☐
Plant Sciences	☐
Robotics & Intelligent Machines	☐
Systems Software	☐
Translational Medical Sciences	☐

1. As a part of this research project, the student directly handled, manipulated, or interacted with (check ALL that apply):

   ☐ human participants ☐ potentially hazardous biological agents

   ☐ vertebrate animals ☐ microorganisms ☐ rDNA ☐ tissue

2. I/we worked or used equipment in a regulated research institution or industrial setting: ■ Yes ☐ No

3. This project is a continuation of previous research. ☐ Yes ■ No

4. My display board includes non-published photographs/visual depictions of humans (other than myself): ☐ Yes ■ No

5. This abstract describes only procedures performed by me/us, reflects my/our own independent research, and represents one year's work only ■ Yes ☐ No

6. I/we hereby certify that the abstract and responses to the above statements are correct and properly reflect my/our own work. ■ Yes ☐ No

This stamp or embossed seal attests that this project is in compliance with all federal and state laws and regulations and that all appropriate reviews and approvals have been obtained including the final clearance by the Scientific Review Committee.