Abstract:
The increase of global terrorism, and the potential increase of the use of biological warfare, it is necessary to develop new, faster, and simpler methods for detecting pathogens in the field. These methods need to be rapid, efficient, specific, selective, and portable. These are all of the qualifications that help to define a biosensor. A biosensor is a device capable of measuring concentrations of biologically important chemicals, or a device that uses a biological material to detect an analyte. In this instance, the biosensor is incorporated in a device called Surface Plasmon Resonance detector device that utilizes a thin, gold-coated glass slide as a sensor surface. Interacting with the gold surface via a thiol terminus is a capture agent that consists of an organic dendritic tether. Attached to the free end of the tether, the dendrimer has an amide-bonded adipic hydrazide terminus. The bioactive end of the molecule, which is covalently attached to the dendrimer, consists of a glycosylated protein or antibody where the sugar has been functionalized to an aldehyde. Polyclonal antibodies are naturally glycosylated, but monoclonal and recombinant antibodies do not always have sugars attached. The goal then is to develop a method that attaches a sugar to the protein (antibody) of choice enzymatically so that the antibody is specifically orientated and the active site is available and unaffected by the glycosylation process. There is a human enzyme called O-glycosyl transferase that attaches an N-acetyl glucosamine to serine and threonine residues within other proteins. This enzyme only glycosylates the serine and threonine within a specific recognition sequence. The purpose of this analysis is to engineer a promiscuous O-glycosyl transferase that will add the sugar to serine residues that are located anywhere within the protein. Specifically, the POGT will glycosylate a serine residue that is located in a linker sequence of a recombinant antibody. The POGT will be engineered and tested using standard and novel biochemical techniques.
GLYCOSYLATION OF ANTHRAX PROTECTIVE ANTIGEN:
ENGINEERING OF A PROMISCUOUS O-GLYCOSYLTRANSFERASE

by

Deborah Anne Hyman

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Biochemistry

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ABSTRACT

With the increase of global terrorism, and the potential increase of the use of biological warfare, it is necessary to develop new, faster, and simpler methods for detecting pathogens in the field. These methods need to be rapid, efficient, specific, selective, and portable. These are all of the qualifications that help to define a biosensor. A biosensor is a device capable of measuring concentrations of biologically important chemicals, or a device that uses a biological material to detect an analyte. In this instance, the biosensor is incorporated in a device called Surface Plasmon Resonance detector device that utilizes a thin, gold-coated glass slide as a sensor surface. Interacting with the gold surface via a thiol terminus is a capture agent that consists of an organic dendritic tether. Attached to the free end of the tether, the dendrimer has an amide-bonded adipic hydrazide terminus. The bioactive end of the molecule, which is covalently attached to the dendrimer, consists of a glycosylated protein or antibody where the sugar has been functionalized to an aldehyde. Polyclonal antibodies are naturally glycosylated, but monoclonal and recombinant antibodies do not always have sugars attached. The goal then is to develop a method that attaches a sugar to the protein (antibody) of choice enzymatically so that the antibody is specifically orientated and the active site is available and unaffected by the glycosylation process. There is a human enzyme called O-glycosyl transferase that attaches an N-acetyl glucosamine to serine and threonine residues within other proteins. This enzyme only glycosylates the serine and threonine within a specific recognition sequence. The purpose of this analysis is to engineer a promiscuous O-glycosyl transferase that will add the sugar to serine residues that are located anywhere within the protein. Specifically, the POGT will glycosylate a serine residue that is located in a linker sequence of a recombinant antibody. The POGT will be engineered and tested using standard and novel biochemical techniques.
CHAPTER 1

INTRODUCTION

Within the past five years, the world has seen a dramatic increase in global terrorism. Of particular concern is the emergence of bioterrorism and the threat of biological warfare. Because of this, it has become necessary to develop techniques that are capable of detecting biological agents and pathogens. These techniques must be rapid, efficient, specific, selective, and portable. It is also necessary that they do not require sterile culture or Enzyme-Linked Immunosorbant Assay (ELISA), they can be used in a variety of non-laboratory settings (1), and can be used by people with varying skill levels and knowledge. The creation of such a method would be a valuable tool to first responders to an incident, such as police, fire department, etc., especially if the system were simple enough that even non-scientific personnel could use it effectively.

The techniques that meet these criteria involve the use of biosensors. Over the last thirty years, the demand and value of biosensors has increased along with the quality of the technology. A biosensor is a device capable of measuring concentrations of biologically important chemicals, or a device that uses a biological material to detect an analyte. It consists of two fundamental parts: a biological component that selectively interacts with a particular analyte, and a physical component, the transducer, that can convert the interaction to a quantifiable signal.
(2). The device that will ultimately be used as a detector in this project utilizes a thin, gold-coated glass slide that is the sensor surface.

These methods are dependent on the differences that occur between a normal bioactive surface consisting of just the capture agent, and a surface where the capture agent has snared the analyte of interest. There are two main ways this can be accomplished. The first system involves the attachment of the capture agent (antibody or protein) directly to the gold surface. This causes several potential problems such as random orientation of the protein, and the protein may or may not stay attached to the gold surface. By attaching the protein or antibody to a tether that is more strongly attached to the gold, these problems are eliminated. In this project, the capture agent is attached to an organic dendritic tether with a thiol terminus interacting with the gold surface, and at the other end, the dendrimer has an amide-bonded adipic hydrazide terminus. The biomolecule is a protein or antibody that has been glycosylated (either naturally, or in the laboratory), in which the sugar has been functionalized to an aldehyde. The aldehyde can then be covalently attached to the dendritic tether. The result is a flexible, stable unit where the protein is in a specific orientation so that its active site is not compromised by the chemistry involved in creating this system, and the active site is available to capture analyte. This system can be applied to a number of antibody/antigen relationships such as anthrax, cholera, brucellosis, etc. or any system where there is a specific and selective interaction between capture agent and analyte.
The organic dendrimer is adsorbed to the gold surface forming a Self-Assembled Monolayer (SAM) (3). This reaction occurs spontaneously and utilizes the strong interactions that occur between the thiol end of the dendrimer and the gold molecules. This forms a stable yet flexible tether to which the capture agent can then be attached. The dendrimer was synthesized by Scott Tarter of MPA Technologies and is shown in Figure 1.

At the free end of the dendrimer is an adipic hydrazide which is capable of reacting with an aldehyde via a spontaneous dehydration reaction. The aldehyde is obtained by oxidizing a sugar moiety with periodate. The sugar is attached either naturally, chemically, or enzymatically to an antibody. One possible antibody to use in this system is a single chain recombinant antibody with a peptide linker consisting of the sequence (glyglyglyser)₃₋₅. Although the initial testing of the system utilized Anthrax Protective Antigen (PA), a monoclonal anti-anthrax antibody called mAb 14B7 provided by Steve Leppla can be used for this purpose. It is the precursor for the recombinant antibody that will ultimately be the capture agent in the sensor system. The entire reaction scheme is shown in Figure 1.

This system is convenient for polyclonal antibodies that are naturally glycosylated, but will not work with monoclonal or recombinant antibodies that do not already have sugars attached to them (4). A method needs to be developed that would glycosylate an antibody in a specific manner. This is to ensure that when the antibody is attached to the dendrimer, the antibody will be in a specific orientation so that its active site is available, and will not be altered in the process.
FIGURE 1. Provided by Brenda Spangler (Sensopath Technologies). Overall reaction scheme for creation of biosensor demonstrating attachment of glycosylated PA to dendritic SAM. The first step depicts the dendrimer alone, the second step shows the glycosylation and oxidation of PA, and in the last step, the completed biosensor. Ultimately, the PA will be replaced with an antibody for the PA antigen.

There are several methods that have been utilized to attach a sugar to a peptide. One such method involves the formation of N-linked glyco-Asparagine motif through diimide-mediated coupling of glycosylamines with an Asp side chain, but this method is often plagued by the ready anomerization of the glycosylamine (5). Many of the current methods require several involved steps and have many reagents that would need to be removed from the solution in order to purify the protein system. The chemicals used would cause the proteins to unfold and lose their natural function.
Other methods will glycosylate proteins indiscriminately using 2-
aminomethoxymethyl thioglycosides and reductive amination methods, or using
activated acyl azides for the formation of amides from proteinaceous amines and
carboxylate ester terminus spacer arm carbohydrates (6). These methods and others
like them result in high levels of functionalization, but the major drawbacks to these
techniques are a lack of residue selectivity, and the techniques may alter the overall
charge of the protein or destroy the sugars involved. Most methods that have been
developed so far are capable of either glycosylating only a short peptide chain, or
adding a glycan chain to an already attached sugar residue (6). Up until now, a
successful method has not been developed that will glycosylate a protein at a specific
amino acid residue that will not affect the structure or function of the protein.
Therefore, in order to effectively glycosylate a protein, an enzymatic method must be
devised and utilized.

The goal of my research was to develop an enzymatic method of attaching an
N-acetylglucosamine sugar to Anthrax Protective Antigen (PA). This was
accomplished by engineering a promiscuous form of a human glycosyltransferase
enzyme.

The enzyme employed is called O-linked N-acetylglucosamine transferase, or
OGT. OGT uses UDP-N-acetylglucosamine (UDPAG) as a substrate to attach the N-
acetylglucosamine molecule to the oxygen of serine, threonine, and possibly tyrosine
residue via an O-linkage. Figure 2 shows the general reaction mechanism of OGT.
FIGURE 2. General reaction scheme showing POGT facilitating the attachment of N-acetylglucosamine to a serine residue within a target sequence and subsequent oxidation of the sugar to aldehydes.

OGT was initially isolated by Lubas et al. from rat liver as a heterotrimer of 350 kDa containing two 110 kDa subunits and one 78 kDa subunit. The larger subunit has been cloned from humans (7). The enzyme has nine tandem tetratricopeptide repeats (TPR's) at the N-terminus. The TPR is a 34 amino acid repeat containing the eight following loosely conserved residues: WLGYAFAP (7, 8). It is believed that these repeats are responsible for substrate recognition and specificity, but are not required for catalytic activity (7). The native enzyme recognizes and glycosylates the specific amino acid sequence YSDSPSTST
(TyrSerAspSerProSerThrSerThr) within a protein, forming O-linkages between the serines in the target sequence, and the enzyme’s substrate sugar which is UDP-N-acetylglucosamine (UDPAG).

We hypothesized that removal of the first six tetratricopeptide repeats of OGT responsible for binding the specific target site on the protein should reduce the substrate specificity of the enzyme and make it more promiscuous. Not only should it be able to glycosylate a test sequence, but it should glycosylate a (GGGS)_4 linker sequence located within the anthrax single chain recombinant antibody provided by George Georgiou (University of Texas, Austin). It should also theoretically be able to glycosylate anthrax PA, which is not naturally glycosylated, or any protein that contains an available serine or threonine oxygen.

As an additional test of this concept, glycosylation was attempted of a generation 2 PAMAM dendrimer containing multiple terminal hydroxyl groups, provided by Joel Morgan in Professor Mary Cloninger’s lab at Montana State University. This test utilized the same methods of glycosylation, detection, and oxidation that were used to glycosylate a protein. This portion of the work has not been successfully analyzed at the current time.

The goals of this research were to develop a promiscuous OGT enzyme (POGT) using a variety of recombinant DNA and molecular biology techniques, modify and perform a glycosylation assay to attach N-acetylglucosamine to PA with both OGT and POGT, develop a simple technique to functionalize the attached sugar
to an aldehyde, and use a chemical method to detect the presence of aldehydes in solution. The work presented here describes the successful completion of these goals. It also leaves the door open for future researchers in a variety of disciplines to continue this work in several different directions.
CHAPTER 2
EXPERIMENTAL

pET32b Plasmid Construct

Native OGT DNA received from John Hanover of NIH (7) consisted of the Lv4F human OGT clone (9) that was subcloned into the plasmid vector pET 32b (Novagen) using the EcoRV and NotI restriction sites to make clone A. This was the clone that was used for expression of native OGT in the following experiments.

Transformations of Native OGT DNA into Nova Blue, BL21 and BL21 (DE3) E.Coli Cells

The OGT DNA construct in pET32b from above was transformed by mixing 1 µL plasmid DNA, 20 µL of Nova Blue E.Coli cells, and 80 µL of ice cold water in a sterile 1.5 mL microcentrifuge tube. This was added to a chilled electroporation cuvette and the cuvette was placed into an Eppendorf electroporator. The solution was zapped with 1500 volts for 2 nanoseconds. After this, 500 µL of room temperature SOC was added and everything was shaken at 180 rpm for four h at 37°C. Fifty microliters of cells were spread on 50 µg/mL LB carbanecillin agar plates made from a 25-mg/mL carb stock solution. For transformation into E. Coli BL21 cells, instead of 1 µL of DNA, 2 µL were used.
Production of Usable Quantities of Cells

A colony was picked from either the E.Coli BL21 cells or the E.Coli BL21(λDE3) cell plate with a sterile pipette tip and then deposited into a 15 mL sterile tube that contained 3 mL of LBCarb50 broth (LB broth containing 50μg/mL Carbenicillin). The cells were incubated at 37°C for 6 h, and then used to inoculate a larger quantity of LBCarb50 broth, usually 300 to 500 mL. These were then allowed to grow another 8 to 14 h in an incubator shaker at 37°C.

Glycerol Stocks

For these, 1 mL of cells was mixed with 150 μL of 80% sterile glycerol, or 0.5 mL of cells was mixed with 75 μL of 80% sterile glycerol. The cells were then frozen at -20°C or -70°C.

Colony Pick PCR

A colony pick PCR was done to determine the presence of the OGT gene insert in Nova Blue colonies on the original transformed plate. In order to do this, 20 μL of sterile water was pipetted into a PCR tube, and the colony of choice was circled on the LBCarb50 plate and picked with a small pipet tip. The tip was swished in the water, and then the tube was boiled for 5 minutes at 100°C in a thermal cycler. The tube was removed from the thermal cycler and centrifuged for five minutes to collect the sediment.
In another PCR tube, 12.5 μL of 2x master mix (Promega, Madison, WI) containing Taq DNA Polymerase (50 U/μL in reaction buffer pH 8.5); 400μM each of dATP, dGTP, dCTP, and dTTP; and 3mM MgCl₂, 1.5 μL of 10 μM Forward T7 promoter primer (and later pET 32 b and S-tag forward primer, both from Novagen), 1.5 μL of 10 μM Reverse T7 terminator primer, and 10 μL of the boiled DNA were added. Subsequent colony picks used 1.5 μL of 10 μM pET32b (Novagen) forward primer or S-tag primer because they were closer in sequence to the insert. The sample was placed into a thermal cycler using the program 444Taq which consists of: 94°C for 3 m, 94°C for 30 s, 40°C for 30 s, 72°C for 1 m, 40 cycles of this, then 72°C for 5 m, and held at 15°C overnight if necessary.

**Agarose Gel Electrophoresis (AGE)**

Agarose Gel Electrophoresis was done to verify the presence of the OGT insert. A 1.3% gel was made that consisted of 0.4 g agarose in 30 mL of 1x TAE (40mM Tris acetate, 1mM EDTA, pH 8.0) buffer and 3 μL of 10 mg/mL ethidium bromide. Typically 12 μL of the PCR sample was mixed with 3 μL of Promega 6x AGE loading buffer (premixed, contains orange G, bromophenol blue, and zylene cyanol) and then 12-15 μL of sample was loaded onto the agarose gel. Labeled PCR markers were used as size standards.
DNA Isolation

Cultures of 1 to 10 mls of cells containing the construct were grown in LBCarb50 broth (LB broth with 50 μg/ml carbanecillin). The DNA was isolated using either Wizard Kit (Promega) or Qiagen alkaline lysis miniprep kits and following the respective protocols.

Sequencing of Vector (pET 32b) and Insert (OGT gene)

DNA was extracted from E. Coli BL21 cells, and an ethanol precipitation was done (32). For the Qiagen extraction, 25 μL of 7.5 M ammonium acetate, then 187.5 μL of 95% ethanol was added. The quantities were doubled for the Wizard kit. The mixture was centrifuged for 20 minutes at 10,000 rpm in a microcentrifuge. The supernatant was aspirated carefully so the pellet was not disturbed. The pellet was washed with 125 μL of 70% ethanol, and centrifuged for 20 minutes. The supernatant was aspirated, the pellet was air-dried, and enough nuclease free water was added to give a final DNA concentration of 30-50 ng/μL.

Big Dye™ Sequencing Reaction

Big Dye™ sequencing mix, 4μL (obtained from Mary Bateson in Prof. D. Ward's lab at MSU), 2 μL of 5x sequencing buffer, 10 μL of DNA template (from ethanol precipitation), and 3.2 μL of S-tag primer (from Novagen) was added to a PCR tube. To prevent evaporation, 40 μL of mineral oil was added to the top. The programs
used were designated 444seq or OGT1: 96°C for 30 s, 50°C for 15 s, 60°C for 4 m, repeat this 25 times, and then hold at 4°C. The program OGT1 differs only in that step 2 is 45°C instead of 50°C for 15 s. To completely sequence the gene, eight primers and the S-tag primer were designed based on both their location in the gene for the OGT Lv4F clone (GeneBank accession number U77413) and their absence of secondary structures as determined by a program obtained from www.cybergene.se/primer.html (Figure 3). They were synthesized by Integrated DNA Technologies. The annealing temperature was changed from 50°C (444seq) to 45°C (OGT1), based on the optimal annealing temperatures of the new primers.

Clean-up of Big Dye™ Extension Products

After the PCR was completed, the tubes were spun briefly. Then, the bottom (aqueous) layer was pipetted onto parafilm and the oil was separated from the aqueous layer (mineral oil is absorbed by the parafilm) by rolling the droplet away from the oil until the drop was clean. The drop was picked up and placed into a sterile 1.5-mL centrifuge tube. Then, 80 μL of 75% isopropanol was added, vortexed briefly, and left to sit at room temperature for 15 m, to precipitate DNA extension products. The tube was centrifuged for 20 m at 10,000 rpms. The supernatant was aspirated, and then the pellet was washed with 250 μL of 75% isopropanol and vortexed. It was then centrifuged for 5 m at 10,000 rpms. The supernatant was again aspirated, and the sample was dried in a 90° water bath for one minute. The samples were then sequenced by either Mary Bateson or Peggy Bunger (Montana State University).
Expression of Native OGT

A colony was picked from the E. Coli BL21(λDE3) transformation plate, inoculated in 3 mL of LBCarb50 broth and incubated in a 37°C incubator shaker for 8 h. This culture was added to 250 mL of LBCarb 50 broth and allowed to grow for another 6 hours. The cells were induced with 100 mM IPTG (isopropylthio-β-D-galactoside) to achieve a final concentration of 1mM IPTG (1:100 dilution), for 8 hours.

Time Course Induction Assay

In order to determine the optimum time of expression, the same procedure as above was used, except 25μL aliquots of cells were taken out at time intervals of t=0, 15 m, 30 m, 1 h, 2 h, 6 h, 8 h, 10 h, 24 h, 36 h, 72 h. The aliquots were spun down, the supernatant was discarded, and the cells were resuspended in 50 μL of SDS-PAGE sample buffer.

SDS-PAGE Gel

In 10 mL total volume of 6x sample buffer, 5 mL of 1M Tris-HCl pH 6.8, 1.2 g SDS, 3.1 g DTT (dithiothreitol or Cleland’s reagent), 60 mg bromophenol blue, and 5 mL of glycerol. In 1x sample buffer there were 5 mL of 50 mM Tris, 1 mL 6x buffer. The 2x SDS buffer consisted of 10 mL of 100 mM Tris HCl, 4% w/v SDS, 0.2% bromophenol blue, 20% v/v glycerol, and 200 mM DTT. For 500 mL of 5x Tris-glycine running buffer: 25 mM Tris or 2x dilution of 50 mM Tris, 1 M glycine, and 0.1% SDS, pH8.5.
This was diluted 1:5 to run as a 1x gel buffer solution with final concentrations of 5 mM Tris, 200 mM glycine, and 0.02% SDS. Gels were run at 120 volts for 45 minutes in a BioRad Ready Gel electrophoresis Cell.

**Zinc Stain**

The Zinc Stain kit was obtained from BioRad. Solution A was zinc sulfate, solution B was imidazole. Both were provided as 10X concentrates and diluted 1:10 with deionized water. The gel was placed in solution A for 10 minutes and shaken at a low speed. The gel was removed and placed into solution B for one minute until the color developed, and then the gel was rinsed in water for 3 minutes. The gels were then viewed on a black background.

**Protein Purification and Isolation**

In order to purify the protein, 250 mL of IPTG induced E.Coli BL21(λDE3) cells were spun down in a centrifuge for 15 minutes at 15,000x g and 4°C. The supernatant was discarded, and the remaining cells were frozen at -70°C for 24-48 hours. At a later time cells were resuspended in 15 mL of TEAN buffer (50 mM Tris pH 7.4, 1 mM EDTA, 3 mM sodium azide, 200 mM NaCl). Lysozyme (1 mL of 20 mg/mL solution) was added and the solution was allowed to sit at room temperature for 30 m. The cells were sonicated, on ice, on a Branson sonicator for five minutes on duty cycle 50%, power output 7. The cells were centrifuged at 4°C for 20 minutes at 13,000 rpm. The
supernatant, which should contain the OGT protein, was poured off and both supernatant and pellet were subjected to SDS-PAGE to determine the location of the protein.

**S-Tag Rapid Assay**

The pET 32b plasmid contained the sequence for an S-tag. The S-tag encodes a 15 amino acid peptide that binds with high affinity to the 104 amino acid s-protein derived from pancreatic ribonuclease A. The OGT gene was inserted into the plasmid near the sequence so that the 15 amino acid S-tag peptide was expressed fused to the OGT protein. A rapid assay was used to determine the concentration of the S-tagged protein based on the interaction of the 15 amino acid S-tag peptide fused to OGT with ribonuclease S-protein. Neither protein alone has enzymatic activity, but when mixed together, they form an active ribonuclease enzyme. The presence of the S-tag sequence is shown by the assay of ribonuclease activity. The components, which consisted of S-tag standard or sample lysate, 10x S-tag assay buffer, and S-tag grade S-protein, were assembled in a sterile 1.5 mL microcentrifuge tube according to the instructions provided with the kit.

All of the reagents were added, with the S-protein added last. The tubes were incubated at 37°C for exactly 5 minutes. The reactions were stopped by adding 100 µL of 25% TCA at 0°C. The tubes were vortexed and placed on ice for 5 minutes, then centrifuged at maximum speed for 10 minutes. The contents of the tubes were read at 280 nm. Tube 1 contained the blank, so it was used to zero the spectrophotometer. The concentration of the unknown sample was calculated using the equations $A_i = ε_i b_i c_i$, and
$A_2 = \varepsilon_2 b_2 c_2$, where $b_1 = b_2 = 1 \text{ cm}$, and $\varepsilon_1 = \varepsilon_2$. Thus, $A_1 / c_1 = A_2 / c_2$, where $A_1$ is the absorbance of the standard tube (#2), $C_1$ is the concentration of the standard (0.05 pmol/μL), $A_2$ is the absorbance of the unknown, and $C_2$ is the concentration of the unknown. The concentration of the unknown was found by solving the above equation for $C_2$ and substituting the known numbers ($C_2 = A_2 C_1 / A_1$). The number generated is in pmol/μL. This number is multiplied by the dilution factor, in this case 200, then converted by calculations to μg/mL. The final concentration was used for further testing.

**S-Tag Dot Blot**

An S-Tag dot blot test was done to determine the presence of expressed protein based on the fact that the plasmid used contains an S-tag marker that is expressed along with the protein of interest. Novagen protocol for the S-tag dot blot test of tagged proteins was followed. Briefly, the lysate was diluted from the concentration determined in the rapid assay to concentrations ranging from 2 μg/mL to 200 μg/mL. The final concentrations were 2 μg/mL, 10, 50, 100, and 200 μg/mL. The lysate was diluted with a solution of 10 mM Tris-HCl, 25 mM EDTA, pH8. The following solutions were made: 10x Tris-buffered saline (TBS), (100 mM of Tris-HCl pH 8.0 and 1.5 M NaCl), TBST + 1% gelatin (1/10 TBS, 1/10 10% gelatin, 1/200 20% Tween20, and 80% of the total volume of water). TBST (Tris-buffered saline and 0.1% Tween 20) without gelatin was also made. A 1:5000 dilution of S-Protein Alkaline Phosphatase Conjugate (AP) was made in TBST. The developer was 60 μL 83 mg/ml NBT (Nitroblue Tetrazolium) and 60 μL 42 mg/mL BCIP (5-Bromo-4-Chloro-3-indoylphosphate) per 15 mL of 1x AP
buffer. The 1x AP buffer was diluted from 20x AP buffer included with the kit. All of
the solutions were provided with the kit except for TBS and water. For the dot blot, a 1
μL dot of each concentration of lysate, BSA (negative control), and S-tag standard was
placed on a piece of nitrocellulose (Imobilon-P) and allowed to air dry for one hour at
37°C. The membrane was dipped in methanol for 15 seconds and allowed to air dry for
several minutes. The membrane was incubated in TBST + 1% gelatin at room
temperature for 15 minutes to block excess protein binding sites. The membrane was
incubated with the 1:5000 dilution of S-protein AP conjugate at room temperature for 15
minutes. The membrane was washed four times with 25 mL of TBST at room
temperature. Color was developed with the developing solution made above and
incubated at room temperature until the color developed. A positive result was a purple
dot. The blots were rinsed with deionized water and allowed to air dry.

**S-Tag Thrombin Purification**

Novagen provided the S-tag purification kit including instructions. The S-
protein agarose is a 50% slurry in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM
EDTA, and 0.02% sodium azide. The other reagents were made in the lab. The binding/
wash buffer was a 10x solution consisting of 200 mM Tris-HCl, pH 7.5, 1.5 mM NaCl,
and 1% TritonX-100. The storage buffer final concentration was 1x bind/ wash buffer
and 0.02% sodium azide (20 mM Tris-HCl, pH 7.5, 0.15 mM NaCl, and 0.1% TritonX-
100). The elution buffer was 1x bind/ wash buffer plus 3 M MgCl₂.
The S-protein agarose was suspended by inversion and 2 mL of slurry, which is equivalent to 1 mL of resin, and was added to 1 mL of undiluted OGT lysate. They were mixed and incubated at room temperature with shaking for 30 minutes, then centrifuged at 5000 xg for ten minutes. The supernatant was carefully decanted and discarded. The resin was resuspended in 5 mL of 1x bind/wash buffer and mixed gently. Centrifuging, pouring and resuspending were repeated two more times. Purification of one sample of lysate stopped here and this was used as “OGT on resin” for the glycosylation assay.

Another sample was processed as above, then eluted with MgCl₂ in the following manner: the resin was resuspended in 1 mL 1x bind/wash buffer and 3 M MgCl₂. The solution was incubated at room temperature for ten minutes. It was mixed gently every few minutes in order to keep the resin suspended in solution. The slurry was transferred to a 0.45 spin filter placed in a collection tube (Novagen) and centrifuged for 5 minutes at 500 x g to remove agarose beads. Elution buffer was added to the resin in the upper chamber and centrifuged for five minutes at 500 xg. The flow through that is collected contains purified and unbound OGT protein. The unbound OGT was only used to determine concentration.

OGT Glycosylation Assay

The glycosylation reaction was performed in a total volume of 100 μL. The procedure was the same for both OGT and POGT. Anthrax Protective Antigen (PA, MW 83,000) was glycosylated as follows: For the reaction, 2.5 μg of anthrax PA, 20 μL of OGT on the S-tag resin, 5 μL of 0.01 mM UDPAG (uridine 5’-diphospho-N-
acetylglucosamine), and 72.5 μL of buffer (40 mM tris, pH 7.4, 12.5 mM MgCl₂) were placed in a 1.5 mL microcentrifuge tube. The final concentrations of each reagent utilized were: approximately 10 μg of OGT, 2.5 μg of PA, and 0.033 μg (5.1 μM) of UDPAG. The mixture was incubated at 37°C for 2 hours with shaking at 200 rpm. The reaction was centrifuged at maximum speed for 5 minutes and the supernatant was removed and saved. The remaining resin was washed with 125 μl of water and spun again. The supernatant was removed and added to the first supernatant. The rinse was repeated a total of 3 times. The collected supernatant was then filtered using a 0.22 μm filter. The resulting eluant was then ready to test for glycosylation using the glycoprotein carbohydrate test. If shown to be positive for carbohydrate, the protein was used for attachment to a tether on an SPR chip.

**Oxidation to Aldehyde and Glycoprotein Carbohydrate Test**

The oxidation and carbohydrate tests are based on the PIERCE protocols, but with some modifications. The glycoprotein detection reagent, AHTT (4-amino,3-hydrazino-1,2,4, triazol-5-thiol), (0.5 g) was dissolved in 10 mL of 1M NaOH to make a 0.5% solution. In a 96 well polystyrene assay plate, 50 μL of sample and standards were pipetted into the wells. 25 μL of 10mM NaIO₄ were added to each well to oxidize the carbohydrate. The solution was mixed for 30 seconds and let sit at room temperature for ten minutes. Freshly made 0.5% AHTT solution (150 μL) was added, and the mixture was incubated at room temperature for 1 hour to allow the color to develop. The plate was read at 640 nm on a Dynatech plate reader. A purple color was indicative of the
presence of aldehyde derived from carbohydrate oxidation. Standards included 2.5 mg/mL lysozyme (negative control), 2.5 and 0.25 mg/mL ovalbumin (positive control), 2.5 mg/mL BSA (negative control), and 0.25 mg/mL fetuin (positive control). Water was used as the blank. Alternatively, the test was performed in a test tube, a PCR tube, or on a glass slide. Standards and sample (0.2 mL each) were placed in test tubes. Sodium periodate (0.1 mL of 10 mM) was added. The mixture was vortexed and incubated at room temperature for 10 minutes. AHTT solution was added, and the resulting solution was incubated at room temperature (20-25 minutes). For the glass slide and the PCR tube, 10 µL of test solution, 10 µL of 10mM periodate, and 30 µL of AHTT were mixed on a clean glass slide and incubated as previous.

**Bgl II Double Cut**

Bgl II enzyme and buffer were obtained from New England Biolabs (NEB). The standard procedure was to mix 4 µL of 10x buffer (NEB 3), 2 µL of Bgl II enzyme, 20 µL of purified native OGT DNA, and 14 µL of water in a PCR tube. The reaction was incubated at 37°C for 1 hour. The samples were then run on 1.2-% agarose gels to separate the plasmid containing POGT from the 441 base pair double cut fragment that had been excised from the beginning of the OGT gene. The remainder of the POGT DNA that remained on the gel was excised and then further processed.

Subsequently, the method was modified and agarose gels were no longer run on the PCR products. Instead, a 30,000-mwco spin column was used to separate the Bgl II enzyme, which is very large, and the cut fragment from the plasmid. The Bgl II remains
on the filter, while the excised piece and the plasmid are found in the eluant. This seems to be because the spin columns are made to be used with proteins and the plasmid and excised piece are linear DNA, which would flow right through the pores. The column was spun for twelve minutes at maximum speed, rinsed with 100 µL of water, spun again, then the plasmid, in the eluant, was removed and further processed.

**Ligation of Bgl II Cut**

In a PCR tube, 17 µL of DNA from the Bgl II cut, 2 µL of ligation buffer, and 1 µL of T4 DNA ligase were mixed. The program used on the thermal cycler was called NBT4. This was run for three hours at 16°C, then 10 minutes at 65°C to inactivate the enzyme. The ligation mixture was then used to transform Nova Blue cells.

**NCO I Cut**

In a PCR tube, 4 µL of 10X Promega Buffer D (60 mM Tris-HCl pH 7.9, 1.5 M NaCl, 60 mM MgCl₂, and 10 mM DTT), 2 µL of NCO I, 1 µL of 50x BSA, and 33 µL of DNA were mixed. The same program on the thermal cycler was used for NCO that was used for the Bgl. This was done to compare a single DNA cut to a double cut resulting in excision of a fragment on an agarose gel.

**Transformation of Construct into E.Coli Nova Blue, E.Coli BL21, and E.Coli BL 21 (λDE3) cells**

A 1 µL aliquot of the ligation mix (or previously isolated POGT DNA) was added to 20 µL of competent cells (Novagen). Then 80 µL of ice cold water was added. The
solution was placed in an ice cold electroporation cuvette. This was put into an
electroporator and shocked at 1500 volts. Next, 250 µL of room temperature SOC was
added and the cells were allowed to recover with gentle shaking at 37°C for 3 ½ hours.
The cells were then plated on LBCarb50 plates, and allowed to grow overnight. Single
colonies were picked and inoculated in LBCarb50 broth as previously described.

**PCR of Native OGT and POGT**

To compare the size of the OGT and POGT constructs, PCR of each using T7
promoter primer (forward), and T7 terminator primer (reverse) were used (32). The
program used was 444Taq, described previously. The DNA used was a colony picked
and mixed into sterile water, boiled and centrifuged as previously described (32). The
supernatants were electrophoresed on a 1.2% agarose gel to compare their sizes to each
other and standards.

**Expression of POGT**

A colony was picked from the E.Coli BL21(λDE3) transformation plate and
inoculated in 25 mL of LBCarb50 broth and incubated overnight. Broth (200 mL) was
inoculated with 500 µL of this culture, which was grown for 15 h at 37°C with shaking.
Protein expression was induced with 2 mL of 100 mM IPTG for a final IPTG
concentration of 1 mM. Cells were allowed to express for 7 h. The remaining 25 mL of
cells were induced with IPTG and a time course assay was done. Samples of 500 µL
were taken at t=0, 30 min, 1 hour, 2,4,8,12,24,36, and 48 hours. These were then
electrophoresed on an SDS-PAGE gel to detect how much protein appeared in the
\(-100,000\) Da band. All procedures that were used to process, quantify, and purify native
OGT were repeated for POGT.
FIGURE 3: Sequence of Lv4F clone of OGT (GeneBank accession number U77413). Sequence begins at base number 1774 of the full DNA sequence. Restriction sites in the OGT DNA are Not I, |EcoRV, and Primers used for sequencing are shown in red. The other Bgl II cut site is located on the pET 32b plasmid.
CHAPTER 3
RESULTS

Finding an OGT Colony that Contained Insert

Native human OGT DNA from the NIH lab of John Hanover was transformed into Nova Blue E.coli cells (Novagen). A single colony was picked and another LBCarb plate was streaked. This colony was shown via PCR to have no detectable insert. Therefore, sixteen additional colonies were picked from the original Nova Blue plate and treated by PCR using T7, pET32b, and S-tag forward promoter primers. The S-tag primer was the closest in sequence to the insert and was the optimal primer to use. Of the sixteen colonies tested for the presence of insert, only one, designated number nine was shown to have insert when run on a 1.2% agarose gel. An LBcarb50 plate was streaked with colony nine, and from this, four subsequent colonies were subcloned on separate plates 1-4 and then subjected to PCR to detect insert. Of the four colonies only 3 contained the OGT DNA insert. For the rest of the project, colonies were used from plate 1 that was subcloned from the original colony shown to contain insert. This was re-streaked about every 3 weeks on a fresh LBcarb50 plate.

Sequencing of Native OGT

The initial sequencing of OGT used the S-tag forward promoter primer (Novagen) for the front end of the gene. Eight other primers were constructed based on their location in the OGT gene (from the Lv4F clone sequence, GenBank accession number
Sequencing was poor due to secondary structures such as palindromes and hairpin loops that formed within the all of the primers except 8F.

A second set of primers was designed that did not contain any hairpin loops or palindromes [www.cybergene.se/primer.html]. These were used for subsequent sequencing (Figure 4). The sequence data was retrieved and the information was entered into the BLAST [www.ncbi.nlm.nih.gov] search database. The results were then compared to the known sequence.

**FIGURE 4:** DNA sequences within the Lv4F OGT clone that were used as primers for successful sequencing of the entire gene

Sequencing of the OGT gene was successful with the new primers and the new corresponding annealing temperature. The sequence matched the sequence of the Lv4F clone that was retrieved from the NCBI web site (Figure 3).

**Expression of OGT**

OGT DNA was transformed into the expression host E.coli BL21 (λDE3) and plated on LBCarb50 agar plates. A colony was picked and inoculated in LBCarb50 broth. The cultures grew for 8 h at 37°C in an incubator shaker. Protein expression was then induced with IPTG and the cells were allowed to express for eight hours. The SDS-PAGE gel showed a protein at a molecular weight of greater than 112,000 that
corresponds with the size of the OGT protein (Figure 5). The time course assay showed that the optimal time of expression is four to six hours after induction (data not shown). However, the SDS-PAGE indicates that expression occurs in the absence of IPTG, which means that the cells grew too long before induction. It was later discovered, when two distinct lines showed up on an agarose gel of the isolated and purified DNA, that there were two plasmids that were present in the transforming DNA, so they needed to be separated, identified, and the correct plasmid transformed into E.coli BL21 (λDE3) cells. The plasmids were separated by running the DNA on an agarose gel, and excising both plasmids from the gel. They were identified by sequencing the front end of the gene. A usable sequence was obtained from the correct plasmid, labeled plasmid 2, while the incorrect one contained a random nucleotide sequence.

**Restriction Enzyme Bgl II Cut**

The Bgl II cuts were performed on purified OGT 1C DNA. Initially, enzyme and buffer were procured from Promega. The first ten attempts were run at 37°C for 1.5 hours and 65°C for 15 minutes to deactivate the enzyme. The next 22 attempts ran at 37°C for 4 hours followed by heating to 65°C for 15 minutes. For all of these attempts, samples were run on a 1.2% agarose gel to compare uncut DNA, supposedly double Bgl II cut DNA, and single NCO cut DNA and to separate the ~410 base pair fragment cut from the plasmid. The samples were then extracted from the gel and used for ligation. None of the Bgl II cuts seemed to be successful even though there appeared to be size differences between the cut and the uncut DNA on the gel. This actually led to the
discovery of two different plasmids contained in the original DNA preparations. For the Bgl II cuts, varying amounts of reagents were used to try to obtain a positive result.

![SDS-PAGE gel of OGT (lane 1), POGT (lane 2), and molecular weight standards. Lane 3 is the cell lysate from OGT t=0 (no IPTG), and lane 4 the cell lysate from POGT t=0. The top line of the standards represents 112,000 Daltons. OGT and POGT weigh ~100,000 Daltons.](image)

**FIGURE 5**: SDS-PAGE gel of OGT (lane 1), POGT (lane 2), and molecular weight standards. Lane 3 is the cell lysate from OGT t=0 (no IPTG), and lane 4 the cell lysate from POGT t=0. The top line of the standards represents 112,000 Daltons. OGT and POGT weigh ~100,000 Daltons.

**Ligation of Bgl II Cut**

Both Promega and New England Biolabs T4 DNA ligase were used. The NEB enzyme proved to be more successful. The first seven ligations were performed at 22°C for 3 hours, then 70°C for ten minutes to deactivate the enzyme. Several ligations were performed at 15°C overnight, and most were done at 37°C for 3 hours and 65°C for ten minutes. Most of the ligation reactions were run on a 1.2% agarose thin gel to compare to uncut, single cut, and Bgl cut DNA. This was also done to get an estimate of
concentration. The successful protocol for the ligation used 2 \( \mu \text{L} \) buffer, 1 \( \mu \text{L} \) of T4 DNA ligase, and 17 \( \mu \text{L} \) of DNA and was run using the NBT4 program.

The ligation mixes were then transformed into E.coli BL21, BL21 (\( \lambda \)DE3), or Nova Blue cells. If colonies grew, then the ligation was determined to be successful. Most of these were unsuccessful.

**Successful Bgl II Cut, Ligation, and Transformation**

Several protocols for digestion by Bgl II restriction enzyme were tried. The following proved to be effective: the reaction mixture was incubated for 1 hour at 37°C. Agarose gel electrophoresis and protocols for extracting DNA from the gels may have been responsible for interfering with the sticky ends of the cut plasmid, making ligation impossible. Therefore, in lieu of a gel, a 30,000 molecular weight cut off spin column (mwco) was used after digesting the DNA in order to separate the 410 base pair fragment that was excised from the remaining plasmid DNA from the larger plasmid fragment. This new treatment proved effective to prepare DNA for subsequent ligation reactions.

The Bgl cut DNA that was not run on a gel, but spun in the 30,000 mwco spin column was initially into E.coli BL21 cells; there were no colonies present. NEB advised transforming constructs into E.coli Nova Blue cells rather than E.coli BL21 cells because Nova Blue cells are EndA\(^-\) and RecA\(^-\), which should make it easier for the construct to replicate and make transformation more successful. The ligated DNA was able to transform the E.coli Nova Blue cells indicating the creation of cut and re-ligated OGT.
DNA. The DNA was later extracted from the Nova Blue cells and successfully transformed into E.coli BL21 and E.coli BL21(λDE3) cells.

**PCR to Verify Cut**

A PCR was performed on both native OGT DNA and POGT DNA to verify Bgl cut and ligation. This was done using T7 forward and reverse primers based on the T7 phage promoter and terminator genes respectively and following the same procedure used to verify the presence of insert initially. The resulting samples were run on a 1.2% agarose gel and a 400 base pair size difference was noted between the samples (Figure 6).

![Image of T7 PCR results](image_url)

**FIGURE 6:** Agarose gel of T7 PCR results. Lane 1 is Lambda Hind III size markers (Promega), Lane 2 is native (uncut) OGT DNA, Lane 3 is POGT DNA (after Bgl II cut), and the ~400 base pair fragment. POGT DNA is smaller than native OGT by ~400 base pairs.
Sequencing of POGT

The DNA was transformed into E.coli BL21 cells in order to maximize sequencing efficiency. Only the beginning of the 3' terminus of POGT DNA was sequenced because the only difference between it and the native OGT DNA was the removal of a 400 base pair fragment from the front end of the gene. The S-tag primer was used.

When sequencing DNA from native OGT, the usable sequence begins around 300-350 base pairs from the beginning of the Lv4F clone sequence. The Bgl II cut site occurs at the sequence AGATCTCT, which is located 712 base pairs from the beginning of the gene. When POGT was sequenced, the sequence began around 735 base pairs. This indicates that there was a ~ 400 base pair segment removed from the DNA via the Bgl cut and ligation (Figure 7, compare to Figure 2).

```
tccggaaaca gtgggggtag gaaaactcgg cctcaagttg cgccctctag gtagcacttg
61  aaaacatgac aagggcccgt agttgtttg ataagagaac tccagcatag agccttatag
121  caacgtacct ccacagtaag tccagctgta aggtggcgct ttggtgac agacatgaac
181  atggtgttcc gcattgcgtt ttgggtggcg cagcgcagca ccgagcagcc
cattagtttc ggacgaacag agccatatct gcagcttgac ttaggtggcg tcagagaagg
241  ccacagctcc cccacatcctt cttttcctcctttcctt catcattttt cctccaagc
301  ccacagctcc cccacatcctt cttttcctcctttcctt catcattttt cctccaagc
361  tttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
Expression of POGT

DNA was transformed into E.coli BL21(λDE3) cells and allowed to grow for 17 hours. After induction with IPTG, cells were allowed to express for 8 hours. The time to maximal expression for POGT was approximately 8 hours but all the sample times run showed expressed protein, indicating that the cells grew too long before induction. The SDS-PAGE time course assay was done two times with the same result for both runs.

S-tag Dot Blots and Rapid Assays

The dot blot and assay were performed on the lysate derived from the BL21(DE3) expressing cells. A positive result on the S-tag dot blot was represented by a purple dot. For both OGT and presumed POGT, there were dots at higher concentrations of lysate, but not the lower concentrations. The rapid assay results were as follows:

Table 1: S-tag rapid assay results for OGT and POGT

<table>
<thead>
<tr>
<th>Tube contents</th>
<th>Absorbance of standard</th>
<th>Absorbance of &quot;unknown&quot;</th>
<th>Concentration of lysate</th>
</tr>
</thead>
<tbody>
<tr>
<td>OGT</td>
<td>0.492</td>
<td>1.107</td>
<td>2250 µg/ml</td>
</tr>
<tr>
<td>POGT</td>
<td>0.264</td>
<td>1.215</td>
<td>4600 µg/ml</td>
</tr>
</tbody>
</table>

This indicates that there is a high concentration of expressed protein in the lysate.

This assay gives an idea of the amount of lysate to add to the S-tag resin in order to purify the protein and use the resin efficiently.
OGT Glycosylation Protocols

There were three versions of this protocol. The first was a duplication of Hanover's assay (7), then the next two versions had major modifications. The first protocol is suspect, because in order to utilize the necessary concentrations of components, it is not possible to use a 40μL total solution, as Hanover's assay did (7). His assay used two synthetic peptides; one was the control peptide with the sequence YSDSPSTST, and the other peptide was the experimental with the sequence (GGGS)$_4$. After the assay, a formic acid column was used with a cation exchange resin. This method was not used due to its complicated nature. A simpler method was devised to purify products based on size rather than charge.

The successful protocol, diothiothreitol reducing agent used in Lubas' protocol was eliminated from all reagent solutions because it interfered with the subsequent glycosylation assay that used periodate. Anthrax Protective Antigen (PA) was used as a substrate for OGT and POGT. This was a convenient protein because the biosensor was designed with anthrax in mind, and also PA is not naturally glycosylated, which is shown in tube one of the periodate test (Figure 8).

Oxidation of Sugar to Aldehyde and AHTT Test

This test was performed on water, unglycosylated PA, PA that was glycosylated by OGT, and PA that was glycosylated by POGT. It appears that with the OGT, there is
some form of glycosylation occurring, as evidenced by the purple color of the solution (Figure 8), but proteins glycosylated by POGT are more positive, indicating a greater concentration of aldehydes in the solution. The color intensity is proportional to the amount of carbohydrate present.

Table 2: Results of glycosylation and AHTT test

<table>
<thead>
<tr>
<th>Sample</th>
<th>Glycosylated by OGT</th>
<th>Glycosylated by POGT</th>
<th>Assay Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (control)</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PA</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PA</td>
<td>-</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>

A + indicates a positive result (or protein was present) which is the darkness of the purple solution upon addition of AHTT. A – indicates a negative result.

FIGURE 8: Results of AHTT test for glycosylation of PA. Tube 1 is unglycosylated PA, Tube 2 is PA glycosylated by native OGT, and Tube 3 is glycosylated by POGT. PA glycosylated by POGT is a darker purple than PA glycosylated by native OGT
Detection of Glycosylated PA via SPR

The glycosylated and oxidized and NaIO₄ reduced PA was flowed across an SPR slide containing the dendritic tether. Figure 9 shows the results of this procedure PA did become attached to the tether indicated by a steady rise in refractive index and that a significant quantity remained attached to the tether indicated by a refractive index above baseline after a rinse with PBS (phosphate buffered saline). We therefore assume that glycosylation and oxidation were successful, otherwise attachment would not occur. For the SPR detection, PA was glycosylated using POGT, separated from the enzyme and concentrated, then treated with NaIO₄ to oxidize any sugars coupled to the protein.

The treated PA was introduced to an SPR slide previously prepared with a self-assembled monolayer composed of a dendritic dithiol tether terminated with a hydrazide. Figure 9 shows coupling of PA to tether, indicated by a steady increase in refractive index from a time of zero seconds until the PBS rinse at a time of 70,000 s.

Coupling was accomplished by recycled flow overnight. Figure 9 shows a portion of the coupling period. After coupling, PBS was introduced at the indicated point on the plot. While some of the PA was removed from the surface, indicated by the drop in refractive index, the refractive index stabilized above the previous baseline indicating stable coupling of PA to the tether. This was corroborated by an influx of anti PA following presumed coupling of PA to SAM on SPR slide. Polyclonal antibody (Ligocyte) was introduced. Binding to PA on the SAM is indicated by the rise in
refractive index, confirming presence of PA on the SAM. The SPR experiment was
designed and performed by Brenda Spangler and Scott Tarter.

FIGURE 9: SPR sensogram showing addition of glycosylated oxidized PA and
subsequent addition of Polyclonal Anti-PA. The sensogram shows that after ~22 hours
PA still remained on the gold slide, and was able to capture Polyclonal Anti-PA.
CHAPTER 4

DISCUSSION

Identification of DNA

One of the initial difficulties faced was being able to find a Nova Blue colony that contained insert after the original transformation. The DNA that was sent by Hanover (7) showed three plasmids when run on an agarose gel. We initially thought that two of the plasmids contained different OGT clones (7), and the third was the native DNA. Once a colony was found that contained the full OGT insert, as demonstrated by a positive result on an agarose gel after treatment of the isolated DNA with T7 promoter primer PCR, it was used throughout the project.

At several points, a second plasmid began showing up on agarose gels from plasmid preparations. There was no way to know which plasmid was the correct one that contained native OGT, just from the gel. The two plasmids were extracted from the gel and sequenced. One of the plasmids contained native OGT DNA sequence, and the other plasmid appeared to be empty pET 32b DNA sequence with no insert. Expulsion of the insert by the plasmid is a common problem, the empty plasmid needed to be separated from the one containing insert. This occurred with POGT DNA as well.

We confirmed the presences of OGT and POGT by DNA sequencing. The DNA sequences that were produced matched that of GenBank (9,10). The sequencing was a difficult process until the correct primers were created, and the correct annealing temperatures were determined. For POGT, only the 3' of the gene was sequenced
(approximately 400-500 base pairs) to confirm that the 400 base pair fragment was missing from the 3' end of the gene. This indicates the creation of POGT DNA when the sequencing results showed the gene starting ~400 base pairs into the native OGT DNA sequence (Figure 7).

**Engineering of POGT**

In order to do a successful excision of the native OGT DNA, many factors had to be taken into account. Since we needed to make a double cut, the amount of Bgl II needed to be doubled. When there were two plasmids, it was difficult to know exactly what was going on. It is possible that the empty plasmid was just being cut once and that was showing up on the gel giving a false positive. The subsequent ligations and transformations were good indicators that the Bgl II cut was unsuccessful.

Once the plasmids were separated, there were other issues to deal with. After the Bgl II cut, agarose gels were run to compare uncut DNA, single cut DNA, and double Bgl II cut DNA. The bands on the gel that were thought to be double Bgl II cut DNA were excised from the gel and purified. This was an easy and convenient method to get pure concentrated DNA and remove the cut out fragment. After consulting NEB, it was determined that running an agarose gel on the cut DNA was interfering with the sticky ends, making re-ligation of the ends impossible. The solution to this problem was to use a 30,000 mwco spin column to remove the fragment and other molecules that might interfere. The liquid remaining on the top contained the cut piece of DNA (still ~2400 base pairs in length). This was what was eventually used for the successful ligation.
The Bgl II cut was shown to be successful in three ways. First, sequencing OGT and POGT DNA showed an approximately 400 base pair difference in size representing removal of the front end of the gene. Second, the PCR based on the T7 promoter primer and subsequent gel of the PCR products show a ~400 base pair size difference, indicating excision of the correct size fragment. Third, the Bgl II double digest was successful, DNA could be relegated and successfully transformed into E.coli Nova Blue, BL21, or BL21(DE3) cells.

**Glycosylation of PA**

The glycosylation protocol was revised several times. The peptide substrates proved extremely difficult to work with especially the (GGGS)$_4$, because it was difficult to quantitate. The protein that was glycosylated by Hanover (7) was casein kinase II. This is a natural substrate for OGT. We determined that casein kinase II is already glycosylated, and the OGT adds more sugars to it. It therefore was not a good substrate to work with because it was difficult to tell the difference between “unglycosylated” and glycosylated.

Lysozyme was used as a negative control throughout, as was water. All of the samples were treated differently depending on which protein was in them. The protocol that we wanted to develop, and eventually did develop, treated every sample the exact same way.

The decision to use PA was an easy one. It is not naturally glycosylated, it is large (83,000), and it fits in with the ultimate goal of the project. The biosensor is
versatile enough that the capture agent could be either antibody or antigen. Either way, unpurified serum could be run across the SPR slide and the analyte captured. The sensogram shows PA as the capture agent and Anti-PA from serum (provided by Steve Leppla) as the analyte. Eventually, Brenda Spangler and Sensopath Technologies will use the antibodies as capture agents, and PA or another antigen as analyte. It is shown in Figure 9 that PA has successfully captured the Anti-PA from the serum.

**Future Work**

There are many directions that can be taken with this work. OGT is an intriguing protein that has not been well studied. It needs to be crystallized so that its secondary and tertiary structures can be elucidated. So far, there is no structural information about OGT beyond the primary structure and DNA sequence. Other properties of the protein need to be studied, as well as the function within the human body. Very little is known up to this point where OGT fits into the metabolic scheme.

The ability to glycosylate any protein is important for the sake of advances in medicine. By glycosylating dendrimers, studies can be done on protein carbohydrate interactions. O-linked glycosylation of proteins as a post-translational modification is less well known than N-linked glycosylation. O-linked glycosylation in general and OGT activity specifically has been implicated in several disease and metabolic disorders such as embryonic development, insulin resistance, and Alzheimer’s disease. By knowing more about this enzyme, some of the medical conditions associated with O-linked glycosylation can be further studied and perhaps cured. In learning more about the
process of glycosylation and glycosylated proteins, more will be known about the cellular processes of signal transduction and post-translational modifications.

Biosensors are being utilized in an ever increasing number of applications. Not only can they be used to diagnose diseases and infections, but can be used to detect meat and bone meal in animal feed samples. This is becoming more important because of the recent discovery of Bovine Spongiform Encephalopathy in Canada. Studies are being done to show the accuracy and reliability of using a biosensor to detect banned ruminant proteins in animal feeds.

One application that we were beginning to work on using this methodology was the glycosylation of a generation 2 PAMAM dendrimer (Figure 10) in collaboration with Mary Cloninger at Montana State University. The dendrimer was prepared by Joel Morgan. If this monodisperse macromolecule can be successfully glycosylated using the enzymatic method presented here, it would have widespread implications in the world of carbohydrate chemistry.
FIGURE 10: Generation 2 dendrimer is shown in blue. Isothiocyanate is added to convert terminal amines to hydroxyls. The dendrimer is glycosylated in a similar manner to PA.


33. www.cybergene.se/primer.html