LARGE-SCALE ENZYMATIC SYNTHESIS OF UDP-LINKED SUGARS

by

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ABSTRACT

ALLENA DIVINE OPOKU. Large-Scale Enzymatic Synthesis of UDP-Linked Sugars. (Under the direction of DR. JERRY TROUTMAN)

Glycans or carbohydrates covalently linked to proteins or lipids play important roles in the interactions of bacteria and their hosts. One of the main sugars that are used to form these glycan structures is Uridine 5'-diposho-N-acetylglucosamine (UDP-GlcNAc) which is primarily used as a donor for N-acetylglucosamine (GlcNAc). GlcNAc is important since it is a key component of bacterial cell wall peptidoglycan, as well as other bacterial surface structures. In addition, UDP-GlcNAc can be modified to form other sugar moieties to be used in other bacterial glycan biosynthetic pathways with the use of sugar-modifying enzymes. This study aims to optimize the production of UDP-GlcNAc in a 'one-pot synthesis' format to collect on a larger scale to then exploit the versatility to form other UDP-linked sugars that are not commercially available. These enzymatic reactions are tracked by high performance liquid chromatography (HPLC) to visualize the formation of UDP-GlcNAc. This system can then be used to analyze the shift in retention times when modifying UDP-GlcNAc to other UDP-linked sugars. This study also aims to compare the structures of different sugar-modifying enzymes that can impact their substrate promiscuity and selectivity.

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of the way even when I felt that I would never make it through. Your compassion, empathy, realness and excitement has been one of the main reasons that I have made it this far. Thank you for being my mentor, I would not have it any other way.

DEDICATION

For my family, my fiancé and my cats for somehow managing to stick around for all my shenanigans. To the ones that could not be here with me on earth, thank you for guiding me in spirit.

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2CN	2-nitrileaniline
4-keto-sugar	2-acetamido-2,6-dideoxy-α-d-4-ketohexulose
A. thaliana	*Arabidopsis thaliana*
Ara	arabinose
ATP	adenosine 5′-triphosphate
B. fragilis	*Bacteroides fragilis*
B. infantis	*Bifidobacterium infantis*
BP	bactoprenyl phosphate
BPP	bactoprenyl diphosphate
C. jejuni	*Campylobacter jejuni*
CDC	Centers for Disease Control and Prevention
CE	capillary electrophoresis
CEalign	combinatorial extension alignment
cef	cell envelope fraction
CPSA	capsular polysaccharide A
diNAcBac	N,N-diacetylbacillosamine
E. coli	*Escherichia coli*
ECA	enterobacterial common antigen
Gal	galactose
GalK	a phosphotransferase
GalNAc	N-acetylgalactosamine

GHMP	galactokinase, homoserine kinase, mevalonate kinase and phosphomevalonate kinase
Glc	glucose
GlcA	D-glucuronic acid
GlcAK	a phosphotransferase
GlcN	glucosamine
GlcNAc	N-acetylglucosamine
GlcNAz	N-azidoacetylglucosamine
GlcNTFA	N-trifluoroacetylglucosamine
GlmU	nucleotidyltransferase and acytltransferase
GT	glycosyltransferases
GTP	guanosine-5'-triphosphate
HPLC	high performance liquid chromatography
IPTG	isopropyl β-D-thiogalactopyranoside
kDa	kilodaltons
LB	lysogeny broth
LOD	limit of detection
LPS	lipopolysaccharides
Man	mannose
ManNAcA	N-acetylmannosaminuronic acid
NAD	nicotinamide adenine dinucleotide
NahK	a phosphotransferase
Ni-NTA	nickel nitrilotriacetic acid

P. multocida	*Pasteurella multocida*
PglD	an acetyltransferase
PglE	an aminotransferase
PglF	a hydro-lase
PGT	phosphoglycosyltransferase
PpA	phosphatases
RMSD	root mean square deviation
SDS-PAGE	sodium dodecyl-sulfate polyacrylamide gel electrophoresis
TLC	thin layer chromatography
UDP	uridine diphosphate
UMP	uridine monophosphate
USP	nucleotidyltransferase
UTP	uridine triphosphate
V. vulnificus	*Vibrio vulnificus*
WbpP	an epimerase
WcfR	an aminotransferase
WecA	an initiating phosphoglycosyltransferase
WecC	a dehydrogenase
Xyl	xylose

CHAPTER 1: INTRODUCTION

1.1: Bacteria Effect on Human Health

Bacteria inhabit almost every part of the human body, whether it's living on the skin, in the oral cavity, or in the large and small intestines.[1] These organisms play a large role in our overall health as many factors of infections, diseases and autoimmune diseases can be based on the absence or the presence of a particular microbes.[2] When this occurs in the case of an infection, antibiotics are used to treat them. Antibiotics can be split into two categories, broad-spectrum and narrow spectrum. Broad-spectrum antibiotics kill most bacterial species while narrow spectrum targets only a certain subgroup of bacteria. Broad-spectrum antibiotics are given to patients the most since there are minimal options of narrow spectrum antibiotics, but can create a disturbance in the human gut microbiome.[3]

The use of antibiotics can cause a microbial fallout called dysbiosis where there is an imbalance in the ratio of Gram-negative and Gram-positive bacteria leaning towards the elimination of Gram-positive bacteria.[4] Due to Gram-positive bacteria only having one membrane, they are more susceptible to antibiotics compared to the double membranes of Gram-negative bacteria. The use of narrow spectrum antibiotics seems like a great option to combat this but dysbiosis can still occur, especially if its targeting bacteria that is gram-positive. As mentioned by Dr. Santanu Datta, "A recent review connecting the antibiotic type and dysbiosis reported that antibiotics like azithromycin that mainly target Gram-positive bacteria cause significantly more dysbiosis than broad-spectrum antibiotics like penicillin." This purges the body of beneficial bacteria that needs to be present. Broad-spectrum antibiotics then seem like a fair trade off from using narrow spectrum antibiotics to minimize the likelihood of dysbiosis

occurring. Broad-spectrum antibiotics that are used against pathogens will also slowly become ineffective due to the bacteria evolving their mechanisms to become resistant.[4]

According to the Centers for Disease Control and Prevention (CDC), approximately 35,000 people die due to antibiotic resistant pathogens per year in the US alone.[5] Some non-traditional mechanisms that cause antibiotic resistance or loss of effectiveness are related to their complex surface polysaccharides which have been largely unexplored. One way to combat this is to create glycoconjugate vaccines that can improve the immune response to bacterial polysaccharides. Being able to understand, replicate and exploit these surface polysaccharides would be beneficial to form glycoconjugate vaccines that can target specific pathogens.[6]

1.2: Bacterial Glycans

In the Troutman lab, there is a focus on complex carbohydrate structures that are produced in both Gram-negative and Gram-positive bacteria known as glycans which are often found on the outer membrane. Glycans are carbohydrates that are covalently attached to proteins or lipids that are linked together in a specific arrangement. These sugars can be simple monosaccharides or more complex oligosaccharides and polysaccharides.[7] Glycans display a wide variety of structures that are different from one species to another. The variation of glycan structure can occur even within the same species. In nature, these glycans have a wide variety of functions such as cell recognition, signaling, and immune response.[8, 9] Some of these glycans can consist of a singular repeating unit while others can be composed of four or more different sugars in different linkages that are then repeated to form a polymer. This makes it difficult to study

these glycan structures from bacteria due to having a larger monosaccharide variety and linkages compared to eukaryotes in humans.[10]

Regardless of the purpose of the glycan, most complex glycans of multiple sugar types are built on a lipid-embedded polyisoprenoid anchor called bactoprenyl phosphate (BP) which is composed of 55 carbons.[11, 12] This serves as a carrier for the production of numerous bacterial glycans including peptidoglycan, O-antigen, exopolysaccharides and more.[11-13] The formation of glycans starts with BP undergoing a phosphoglycosyl transfer reaction with a phosphoglycosyltransferase (PGT) that starts this process where a sugar-phosphate group is attached to BP to initiate a particular glycan pathway.[13] Next, subsequent glycosyltransfers occur with glycosyltransferases (GT) to catalyze the stepwise elongation by sequential addition of sugars (Figure 1).[14]

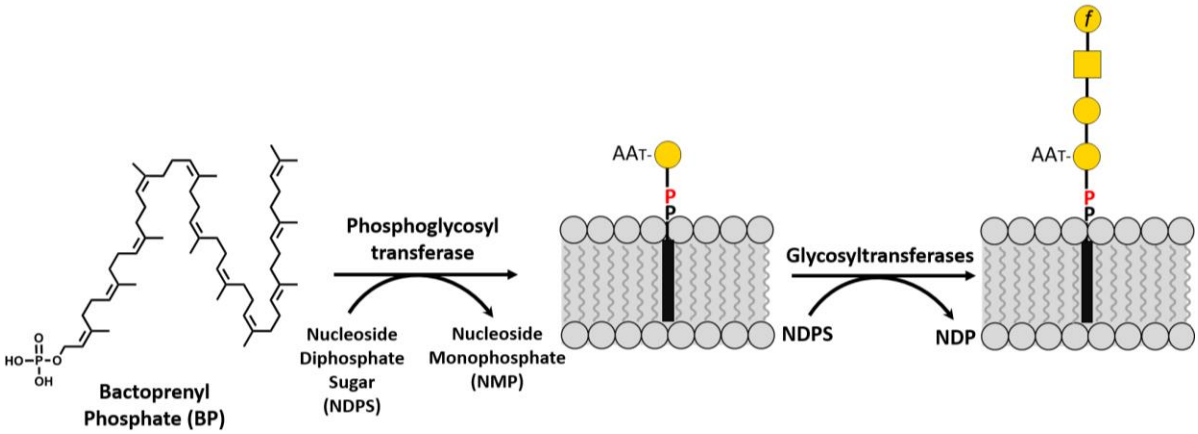

Figure 1: Example of Common BP Pathway Forming Glycans using capsular polysaccharide A (CPSA) found in B. Fragilis

To get an understanding of how important BP is in the production of glycans, Figure 2 shows the different bacterial glycan synthesis pathways from a small subset of bacteria. These specific pathways have been studied *in vitro* in the Troutman Lab where the underlined enzymes are either PGT or GT and the sugar known or hypothesized to be transferred is shown above that

enzyme. The others shown are sugar-modifying enzymes that alter the structure of the sugar before their addition to the glycan with a PGT or GT. This flowthrough of different glycan structures shows the ideal synthesis of these glycans where we can add these enzymes sequentially like bacteria rather than one at a time. Some of the final glycans that are being produced can vary depending on the bacteria.

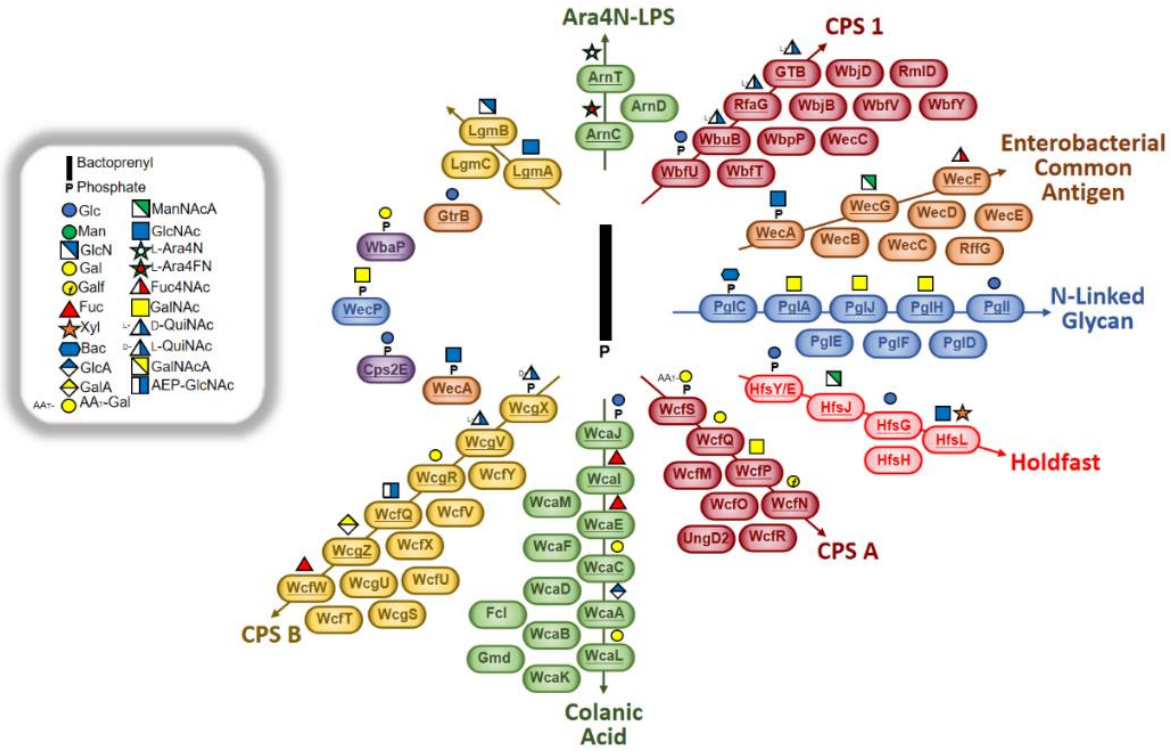

Figure 2: Glycan-Building Toolkit of The Troutman Lab created by Colleen R. Eade

1.3: Fluorescent BP and cef Side Affects

The formation of glycans consists of an initiation step with PGT then subsequent GTs with BP being a major participant in the synthesis of glycans. Most of the research done to form these glycan structures are in multiple steps where the UDP-Linked sugar is formed, which is purified, and then a PGT enzyme is used to transfer the UDP-Linked sugar to BP.[11-13, 15] To ensure the formation of the BPP-sugar, this can be tracked with a fluorescent BP whose peak will shift with the addition of a sugar. The structure of BP is important since it starts with this

molecule as a backbone to build glycans on, but it is a large molecule that is very difficult to synthesize and difficult to detect with current analytical methods. To get around this setback, the Troutman lab has shown in previous work that using a 2-nitrileaniline-tagged BP (2CN-BP) (Figure 3) was useful and dependable for its use to track glycan assembly.[11, 13] This fluorescent tag attached to the BP will allow the process of adding sugars or modifying sugars to be tracked using high performance liquid chromatography (HPLC).

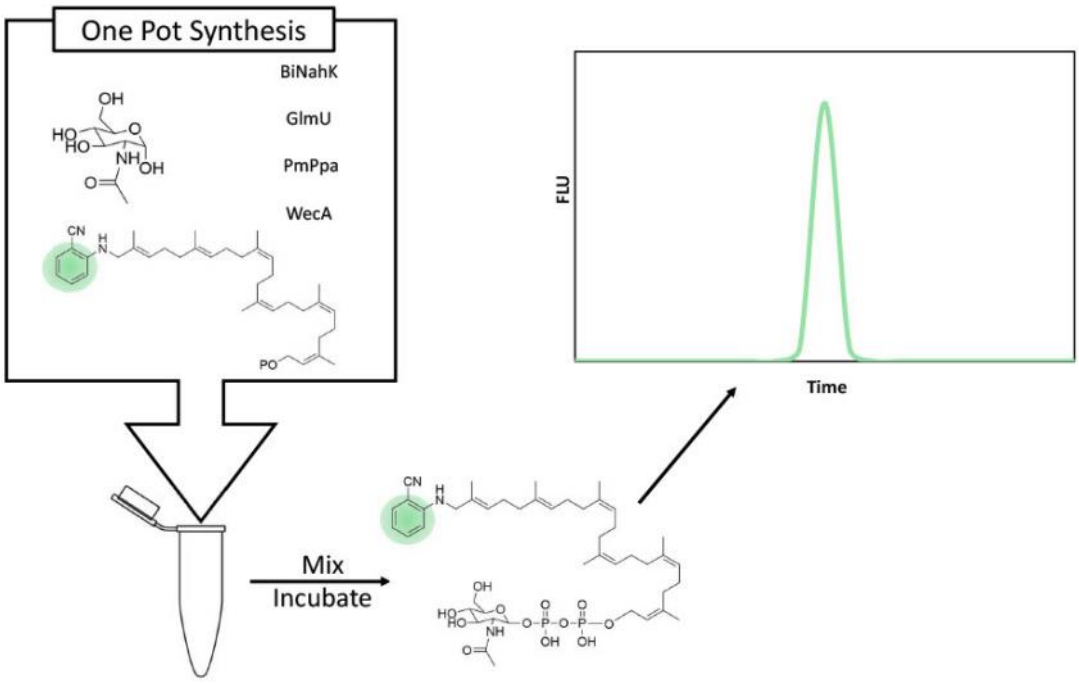

Figure 3: Scheme of the formation of BPP-GlcNAc in a One pot Synthesis method with an example chromatograph displaying the fluorescent peak of the BPP-GlcNAc

When attaching the sugar to BP, the enzymes used to complete this process are typically insoluble proteins compared to most of the enzymes used to alter sugar structures which are usually soluble proteins.[16] These proteins are soluble in water and are easier to isolate and obtain like PglE and PglD which are used to help synthesize Uridine 5'-diposho-N,N-diacetylbacillosamine (UDP-diNAcBac).[17] Usually, when using an insoluble protein, the entire cell envelope fraction (cef) will be used rather than attempting to isolate the individual

enzyme.[13, 18, 19] Most researchers use the cef due to the requirement of membrane components for appropriate function.[13, 19]

When using cef in chemoenzymatic reactions, there are other transmembrane proteins that are present that may cause secondary products to be formed in conjunction with the expected product. This has been seen before in the Troutman Lab with the enzyme WecA.[13] When running a preliminary reaction that contained only WecA and the 2CN-BP, an unknown product was produced without a sugar donor being present. But, when the sugar donor was added, there was full turnover to the expected product. The unknown product peak was a background transferase involved in the modification of the lipid A portion of LPS in native E.coli.[11] Due to the multiple transmembrane domains in the cef, the 2CN-BP was being utilized for a different purpose within the cef until the sugar donor for a particular pathway was presented (Figure 4).

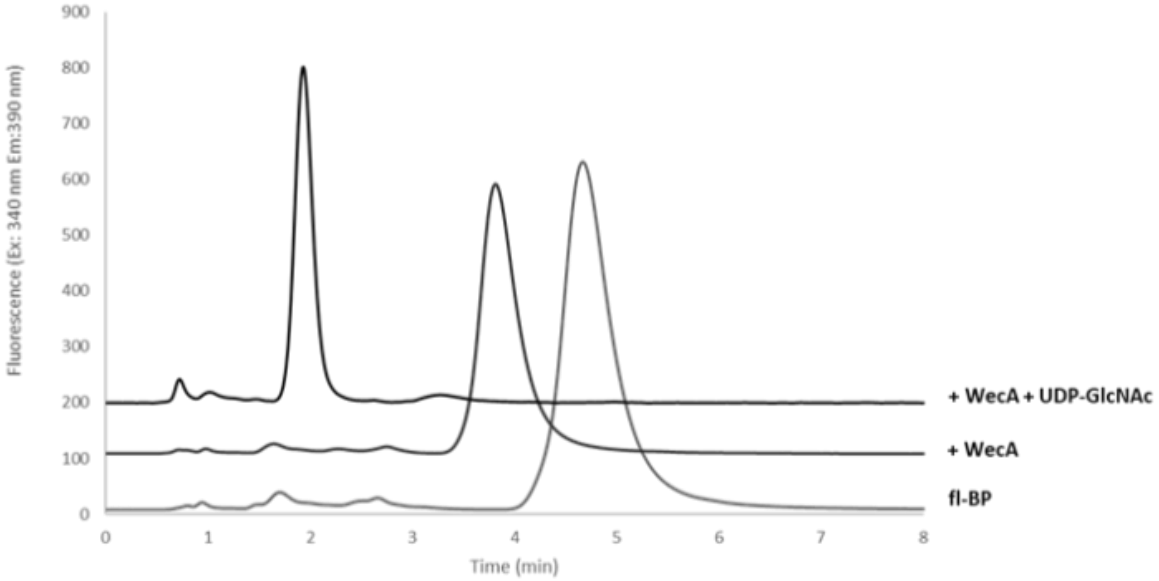

Figure 4: Formation of BPP-GlcNAc with step addition of phosphoglycosyltransferare to fluorescent BP. Background reaction occurring with the reaction containing fluorescent BP and the WecA enzyme.

1.4: Chemo-enzymatic vs Chemical Synthesis Techniques for Glycans

There are many different methods for synthesizing sugar nucleotides. The main two that are used are chemical synthesis and chemoenzymatic synthesis. Chemical synthesis is where a target product is formed from readily available starting materials. Chemoenzymatic synthesis uses isolated enzymes or whole cells in conjugation with a chemical synthesis to create the desired product. Even though chemical synthesis has been used and refined to produce compounds, it can be problematic when working with sugar nucleotides. The need for using multiple protection/deprotection steps, low solubility of nucleotides in organic solvents and vigorous reaction conditions make chemical synthesis a difficult task to achieve.[20] These are also one of the main reasons that most chemical synthesis of sugar structures do not include the nucleotide link. For example, to chemically synthesize CPSA, it will take 17 steps to form all the individual sugar and assemble them together to make one repeating unit of CPSA with a low overall yield.[21] This does not take into account the time needed to form each sugar and the time taken to assemble them into the appropriate glycan structure.

In comparison, chemoenzymatic synthesis can yield desired sugar nucleotides using multiple enzymes in a one pot reaction.[22] This method tends to obtain a higher percent yield, less hands-on time with the reaction, and less purification steps. As shown in Scheme 1 with N-acetylglucosamine (GlcNAc) as an example, a kinase catalyzes the transfer of a phosphate group from adenosine 5′-triphosphate (ATP) to a specific molecule, forming sugar-1-phosphate. Following this, a sugar nucleotide pyrophosphatase reaction occurs where the uridyl group is transferred from uridine-5′-triphosphate (UTP) to sugar-1-phosphate to obtain the sugar nucleoside diphosphate product. An inorganic pyrophosphatase is then added to degrade the

pyrophosphate that is formed in the reaction, pushing the reaction forward to form the desired product (Figure 5).[22] The salvage pathway to form UDP-Linked sugars have become the dominant approach in recent years compared to chemical synthesis.

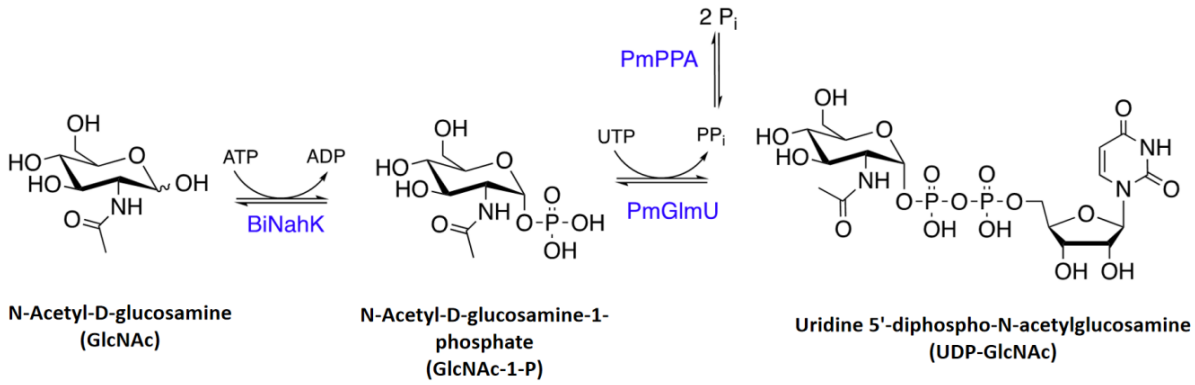

Figure 5: Scheme of Chemoenzymatic Synthesis of UDP-GlcNAc via a One-Step Reaction

1.5: Versatility and Capabilities of UDP-GlcNAc

Even with the structural complexity of glycoconjugates and oligosaccharides where there are what seems almost infinite building blocks for bacteria, there are only nine common building blocks for eukaryotic glycoproteins and glycolipids, one of them that is also found in bacteria is N-acetyl-glucosamine (GlcNAc).[23, 24] When observing the Glycan Toolbox of the Troutman lab in Figure 2, GlcNAc can be found in 2-3 different bacterial glycan structures either as a product from a PGT transferase reaction or a glycosyltransferase reaction from Uridine 5'-diposho-N-acetylglucosamine (UDP-GlcNAc). In addition, UDP-GlcNAc can be modified to form other sugar moieties to be used in other bacterial glycan pathways (Figure 6). Most of the sugar nucleotides that UDP-GlcNAc can be modified to are not commercially available or the cost per milligram is exceptional. For example, the lowest price to source UDP-GlcNAc from was $3.52 per mg (Millipore) compared to Uridine 5'-diphospho-N-acetylgalactosamine (UDP-GalNAc), which would cost $62.00 per mg (Sigma-Aldrich). The only difference between these two sugar

nucleotides is the positions of one hydroxyl group. There are also cases where some of these sugar nucleotides are not commercially available, or it costs thousands of dollars to synthesize rare sugar nucleotides.

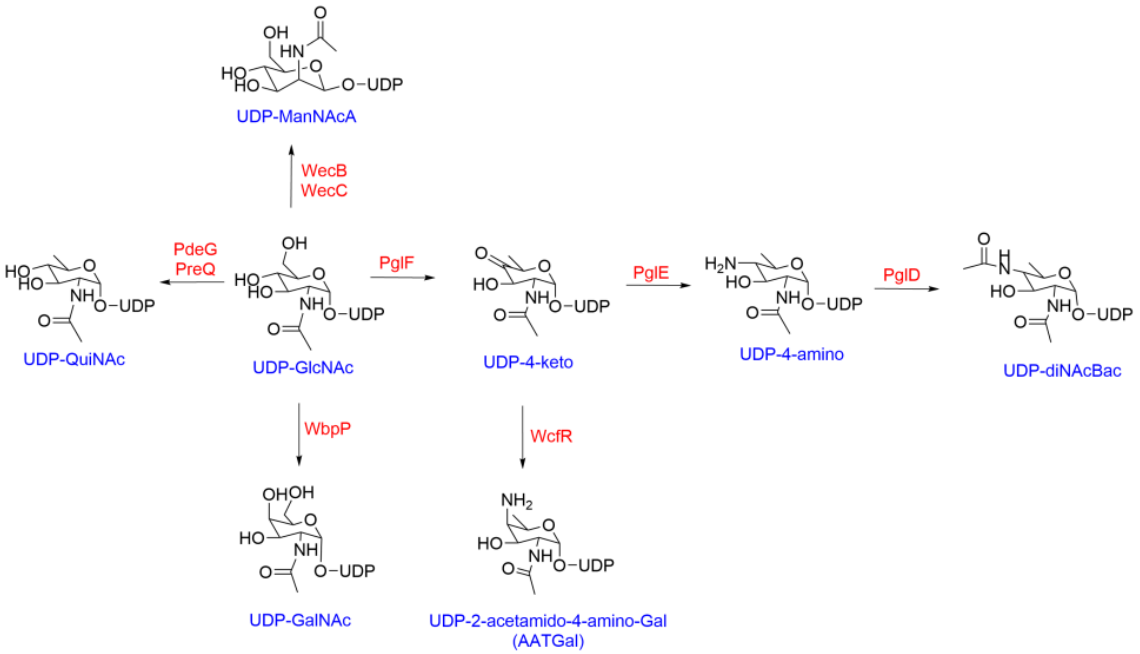

Figure 6: Flow graph displaying UDP-GlcNAc's ability to transform from one sugar nucleotide to another.

The chemoenzymatic synthesis of UDP-GlcNAc can be split into two different methods. In the first, one of the reactions is set up where GlcNAc is synthesized with the kinase, purify the sugar-1-phosphate, then use the uridyltransferase and inorganic pyrophosphatase to get UDP-GlcNAc and purify again.[25] The second method starts with GlcNAc and uses a one-pot synthesis where all the enzymes are added at once. Once the product is formed, we then purify the final product.[26] The first method is useful to explore the activity of each enzyme to ensure it isn't active when adding other cofactors to make rare sugar nucleotides since some steps of making the sugars may require the same cofactor. The second method for large scale, streamlined chemoenzymatic synthesis as you can produce grams of UDP-GlcNAc with this method without

having to sacrifice time for purification steps. For this project, the second method will be utilized to synthesize UDP-GlcNAc.

If the formation of UDP-GlcNAc can be optimized, other enzymes from different bacteria can be used to create new sugar nucleotides. One of the possible UDP-GlcNAc derivatives that can be formed is part of the N-Linked glycan found on the surface of the pathogenic *Campylobacter jejuni* with the sugar modifying enzymes Pgl F, E, and D. Alternatively a similar sugar can be formed from enzymes in the *B. fragilis* CPSA biosynthesis system using the enzymes PglF from *C. jejuni* and WcfR from *B. fragilis*. This type of system could be used for other glycan formation pathways including those associated with Enterobacterial Common Antigen (ECA) found in *E. coli*, CPS1 in *Vibrio vulnificus*, and Holdfast in *Caulobacter crescentus*.

1.6: Different Types and Functions of Sugar Modifying Enzymes

UDP-GlcNAc to form other sugar nucleotides requires sugar-modifying enzymes and a pyrophosphatase. Sugar modifying enzymes are a diverse set of proteins that have the ability to alter sugar nucleotides with the addition, removal, or modification of functional groups that are attached to a sugar nucleotide.[27] Some of the common types of sugar modifying enzymes that can be utilized in chemoenzymatic reactions that this thesis will investigate are sugar isomerases, lyases, transferases, oxidoreductases and hydrolases (Table 1).

Sugar isomerases catalyze an intermolecular rearrangement where bonds are broken and formed to convert a molecule from one isomer to another. A subgroup that fits in this category

are sugar epimerases which involve the conversion of a sugar epimer to its counterpart. Having the ability to alternate between different sugar epimers increases the carbohydrate diversity. One example of a sugar epimerase that is used in the Troutman lab is the conversion of UDP-GlcNAc to UDP-GalNAc with WbpP from *V. vulnificus* where the stereochemistry of the hydroxyl group at carbon 4-position is inverted (Figure 6).

Sugar lyase enzymes catalyze the elimination of various chemical bonds, forming a new double bond or ring structure using means other than hydrolysis and oxidation.[28] A subclass of this category are hydro-lyases where there is a breakage of a carbon-oxygen bond.[29] These types of enzymes help add diversity to the sugar nucleotides to be utilized in other parts of glycan synthesis. An example of a hydro-lase is PglF in the conversion from UDP-GlcNAc to UDP-2-acetamido-2,6-dideoxy-α-d-4-ketohexulose (UDP-4-keto-sugar) where the hydroxyl group on carbon 4 is turns into a keto group (Figure 6).

Sugar transferases catalyze a transfer of a particular functional group from a substance to the sugar.[30] The functional group that is attached can vary but the main ones seen are amino-, methyl, and phosphate groups.[30] One of the subcategories for sugar transferases are sugar phosphotransferases. Sugar phosphotransferases catalyze the transfer of a phosphate group from ATP/GTP to the enzyme's substrate where an alcohol group is an acceptor.[31] Phosphorylation is a crucial step in chemoenzymatic routes to produce complex sugar nucleotides since this is usually the beginning of the synthesis process. Another subcategory in sugar transferases are nucleotidyltransferases which transfer a phosphorous-containing groups that also contains a nucleoside.[32] Nucleotidyltransferases are also essential since this is used as a precursor for

glycosyltransfer in building glycans. An example of both subcategories can be found in the synthesis of UDP-GlcNAc with NahK being used as a sugar phosphotransferase and GlmU being used as a nucleotidyltransferase (Scheme 1).

Sugar oxidoreductases catalyze an oxidation/reduction reaction where there is a transfer of hydrogen and oxygen atoms from one substance to the sugar molecule.[33] A sub-class of this enzyme are sugar dehydrogenases which oxidize a substrate by transferring hydride to an electron acceptor, like NAD, which forms a ketone or an aldehyde.[34] This oxidation/reduction enzyme helps with redox balancing and contributes to bacteria's ability to make these complex sugar structures. An example displaying this is WecC from *E. coli* which aids in the formation of UDP-ManNAcA (Figure 6).

Sugar hydrolases catalyze the formation of two products that come from a substrate by hydrolysis.[35] A subgroup of this is phosphatases where this enzyme uses water to cleave phosphate groups that contain phosphoanhydrides.[36] This enzyme ensures that these phosphate groups are broken down to be used in other systems in bacteria and to have the reaction become more favorable to form the sugar nucleotide.[37] An example of this would be the use of PpA in the synthesis of UDP-GlcNAc. Even though PpA technically is not used as a sugar modifier, it is needed to form these rare sugar nucleotides by making the reaction favorable.

Table 1: Classification of Enzymes by Types of Reactions

Class	Type of Reaction	Example
Oxidoreductases	Oxidation/reduction reactions that transfers hydrogen oxygen atoms from one substance to another	Dehydrogenases
Transferases	Transfer of functional group(s) from one substance to another	Phosphotransferases, Nucleotidyltransferases
Hydrolases	Two products being formed from a substrate due to hydrolysis	Phosphatases
Lyases	The cleavage of C-C, C-O, C-N and other bonds due to elimination that leave double bond or can add groups to double bonds	Decarboxylase
Isomerases	Structural or geometric changes within a molecule	Epimerases
Ligases	Joining two molecules together with hydrolysis of a diphosphate bond with triphosphate	Synthetase
Translocases	Assists in moving another molecule or ion	Transporter

1.7: Synthesis of UDP-GlcNAc Enzymes Comparison

For this thesis, the main sugar modifying enzymes that will be discussed are Nahk and GalK from *Bifidobacterium infantis*, USP and GlcAK from *Arabidopsis thaliana*, and GlmU from *Pasteurella multocida*. BiNahK is a sugar phosphotransferase that plays an essential role in the beginning synthesis of UDP-GlcNAc. The enzyme catalyzes the transfer of a phosphate group from ATP onto GlcNAc by using the alcohol group on C1 as an acceptor.[38] BiGalK is also a sugar phosphotransferase from the same species as NahK that utilizes ATP to transfer the phosphate group onto carbon 1, but it is the beginning synthesis of UDP-GalNAc using GalNAc as a substrate.[39] AtGlcAk is another sugar phosphotransferase that also utilizes ATP that uses the same previous methods but uses D-glucuronic acid (GlcA) as a substrate to help synthesize UDP-GlcA.[40] There has been data showing that BiNahk can phosphorylate GlcNAc, GalNAc and GlcNAc moieties like N-azidoacetyl glucosamine (GlcNAz) while BiGalK and AtGlcAK have only been shown to phosphorylate with either one monosaccharide or very slim selection of other monosaccharides.[26, 39-41] Structural analysis and sequence analysis can be used to get an

understanding of why BiNahK has the ability to be very versatile with substrates while BiGalk and AtGlcAK have a strict substrate specificity.

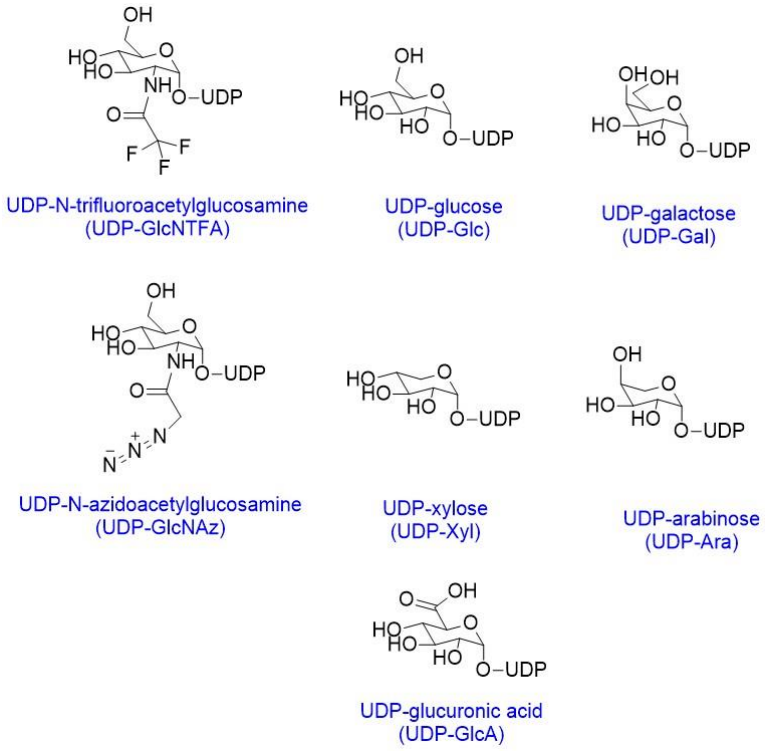

Figure 7: UDP-linked sugars that BiNahK, AtGlcAK, PmGlmU and AtUSP can assist in synthesizing.

PmGlmU is a bifunctional enzyme that is involved in the synthesis of UDP-GlcNAc. PmGlmU has 2 domains, an N-terminal acetyltransferase that catalyzes a transfer of an acetyl group to carbon 2 on glucosamine-1-phosphate (GlcN-1-P) and C-terminal uridyltransferase that catalyzes transfer of UMP from UTP to form UDP-GlcNAc.[42] This enzyme is also able to synthesize UDP-GlcNAc analogues like UDP-GlcNTFA and UDP-GlcNAz.[41] There will be some discussion of the acetyl transferase for structural analysis of PmGlmU but focus for structural comparison will be on the C-terminal domain that performs a uridyltransferase reaction. AtUSP is used in salvage pathways as a nucleotidyltransferase with multiple monosaccharide 1-phosphates to their respective UDP-sugar.[43] This broad specificity has been shown with glucose-1-phosphate (Glc-1-P), galactose-1-phosphate (Gal-1-P), xylose-1-

phosphate (Xyl-1-P), arabinose-1-phosphate (Ara-1-P) and glucuronic acid -1-phosphate (GlcA-1-P).[43] While both are nucleotidyltransferases, there is a vast difference between the two on their substrate specificity. Structural analysis and sequence analysis can help determine why AtUSP has a broad substrate acceptance compared to PmGlmU.

CHAPTER 2: MATERIALS AND METHODS

2.1: Expression and Purification of Enzymes

2.1.1: Soluble Protein Overexpression of NahK, GlmU and PpA

E. Coli strain C41 transformed with plasmids encoding either NahK, GlmU or PpA (commercially synthesized) were cultured in 5mL of lysogeny broth (LB) with carbenicillin (100 ug/mL) which was grown at 37 °C under shaking at 220 rpm overnight. The starter culture was diluted 1:100 into fresh 1L of LB with carbenicillin then incubated at 37°C with shaking at 220 rpm. When OD600 reached between 0.4 - 0.6, the temperature of the shaker was decreased to 16 °C for 30 minutes then 0.5 mM Isopropyl β-D-thiogalactopyranoside (IPTG) was added to the culture then incubated with shaking at 16 °C overnight. Cells were harvested by centrifugation at 5,000 x g for 15 minutes at 4 °C. The cell pellet was resuspended in lysis buffer (50 mM Tris-HCl, 20 mM Imidazole, 200 mM NaCl) and sonicated for 12 minutes. To remove any unlysed cells and membrane fractions, cell lysate underwent centrifugation at 150,000 x g for 60 minutes at 4 °C and the supernatant was collected. Purification was performed by loading the supernatant onto 2 mL of equilibrated nickel nitrilotriacetic acid (NI-NTA) resin and washed 3 times with 4 times the column volume with wash buffer (50 mM Tris-HCl, 50 mM Imidazole, 200 mM NaCl). The purified proteins were eluted with elution buffer (50 mM Tris-HCl, 500 mM Imidazole, 300 mM NaCl) 6 times in increments of 1mL. Elutions that contained the target protein were dialyzed with Tris-HCl buffer and stored at -70 °C. Elutions that contained GlmU were desalted with a HiTrap® Desalting column rather than dialysis to remove imidazole and other components then stored at -70 °C.

2.1.2: SDS-PAGE and Western Blot of Enzymes

After protein purification, each protein was analyzed using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 14% polyacrylamide gels where one was stained with Coomassie and the other transferred to a nitrocellulose membrane. The nitrocellulose membrane was stained with Ponceau for visualization then used for Western Blot analysis. The nitrocellulose membrane was washed with deionized water and blocked overnight in a 5% milk solution containing phosphate buffered saline pH 7.4 with 0.3% Tween-20 (PBST). The nitrocellulose membrane was washed for 15 minutes with PBST then incubated in primary antibody (1:10,00 dilution anti-His rabbit antibody) for 4 hours at 4 °C. The nitrocellulose membrane was washed again for 15 minutes with PBST then incubated in secondary antibody (1:10,00 dilution anti-rabbit goat antibody conjugated to alkaline phosphatase) for 2 hours at 4 °C. Membrane was washed for 45 minutes with PBST and proteins of interest were detected with NBT/BCIP.

2.2: Chemoenzymatic Synthesis of UDP Linked Sugars

2.2.1: Synthesis of UDP-GlcNAc

A small-scale 50 µL reaction was prepared containing 20 mM of GlcNAc, 20 mM UTP, 20 mM ATP, 10 mM $MgCl_2$, and 20 mM Tris-HCl (pH 8.0) then was adjusted with 1 M NaOH to pH 8.0. Enzymes were added at a final concentration of 0.18 mg/mL of NahK, 0.31 mg/mL of GlmU and 0.75 mg/mL of PpA. The reaction was incubated at 37 °C in a shaker at 80 rpm overnight. HPLC was used to visualize the formation of UDP-GlcNAc.

A large-scale containing 20 mM of GlcNAc, 20 mM UTP, 20 mM ATP, 10 mM $MgCl_2$, and 20 mM Tris-HCl (pH 8.0) was adjusted with 1M NaOH to pH 8.0. Enzymes were added at a final concentration of 0.20 mg/mL of NahK, 0.34 mg/mL of GlmU and 0.83 mg/mL of PpA was added. The reaction was incubated at 37 °C in a shaker at 80 rpm for 24-48 hours then analyzed by HPLC and thin layer chromatography (TLC) (Ethyl Acetate: Methanol: Water = 3:2:1) with p-Anisaldehyde staining was used to visualize the formation of UDP-GlcNAc.

2.2.2: Synthesis of UDP-GalNAc

The reaction for a small-scale 50 µL reaction system uses the same method stated for the synthesis of small-scale UDP-GlcNAc reaction with the addition of 0.27 mg/mL of WbpP. HPLC was used to visualize the formation of UDP-GalNAc and consumption of UDP-GlcNAc. The reaction for a large-scale 15 mL reaction system uses the same method stated for the synthesis of large-scale UDP-GlcNAc reaction with the addition of 0.31 mg/mL of WbpP. HPLC was used to visualize the formation of UDP-GalNAc and consumption of UDP-GlcNAc.

2.3: General Methods for Reaction Analysis

2.3.1: HPLC Analysis

Analysis of all reactions to form a sugar nucleotide were done with High Performance Liquid Chromatography (HPLC) using an Agilent 1100 series HPLC with an Zorbax NH_2 column (4.6 ×150 mm, 5 µm) with an isocratic mobile phase consisting of 97% 100 mM ammonium acetate pH 4.5 and 3% Acetonitrile at 1 mL/min for 15 minutes. Absorbance at 260 nm was used for detection of sugar nucleotides.

2.3.2: CE Analysis

Capillary Electrophoresis (CE) was performed on UDP-GlcNAc reactions using a P/ACE MDQ Plus system (SCIEX) equipped with a photodiode array detector monitoring absorbance at 260 nm. The running buffer was composed of 20 mM phosphate/ 20 mM borate buffer pH 3.00 using a bare silica capillary (75 μm × 40 cm) with an effective length of 30 cm (40 cm total). The capillary was conditioned before each run by being washed with 0.1 M NaOH for 2 min, water for 5 min, and running buffer for 5 min. In general, the sample and standard were prepared with a dilution using water by a ratio of 1:10. Sample was introduced by voltage injection for 5.0 s at 5.0 kV, and the separation was performed at 10 kV (reverse polarity).

2.4: Structure Analysis for Sugar Modifying Enzymes

Each enzyme's structure was analyzed based on results obtained from an UniProtKB/Swiss-Prot (National Center Biotechnology Information) conserved domain search with the AlphaFold predicted structure. Active sites were then aligned in the Pymol molecular visualization software to better study the 3D structure model. Clustal Omega was used to generate alignments between each enzymes sequence for comparison.

CHAPTER 3: RESULTS AND DISCUSSION

3.1: Protein Expression and Precipitation Issues

The three sugar modifying enzymes that will be utilized to synthesize UDP-GlcNAc are BiNahK, PmGlmU and PmPpA. The three enzymes were expressed and isolated as soluble protein with the use of Ni-NTA purification. BiNahK, PmGlmU and PmPpA were all expressed successfully with Coomassie displaying prominent bands for each enzyme. To ensure that our proteins of interest were present, an anti-His Western blot was used to detect the hexahistidine tagged proteins. In this case, Western blot confirmed that the proteins have been isolated (Figure 8).

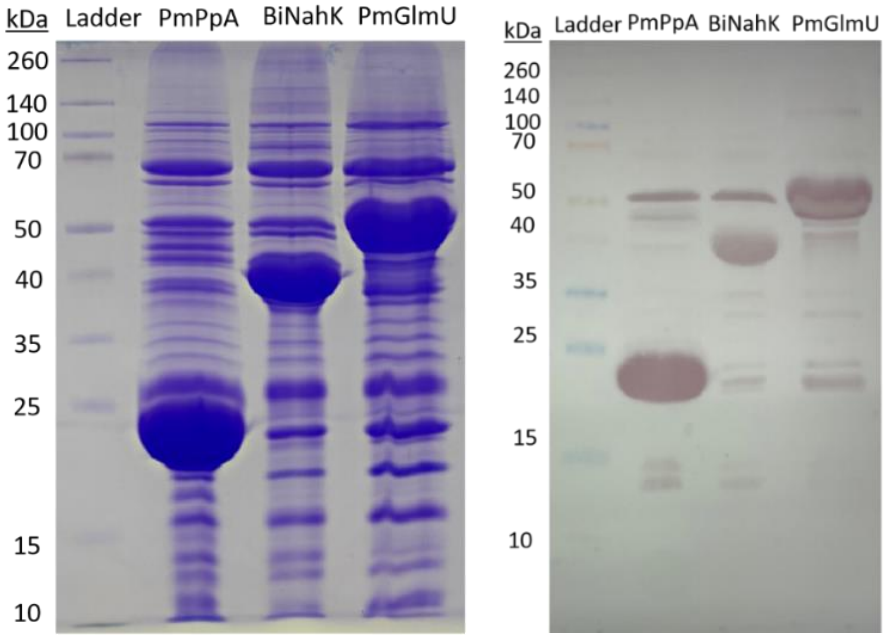

Figure 8: LEFT: SDS-PAGE gel analysis of NahK, GlmU and PpA RIGHT: Western Blot analysis of NahK, GlmU and PpA

After isolating each enzyme from Ni-NTA, the standard protocol involves using dialysis to remove excess salts including imidazole which is required to elute the protein off of the column.[44] When using this technique, the protein solution is placed in a dialysis membrane that has a specific molecular weight cutoff which is then submerged in a large volume of buffer. Each

enzyme underwent dialysis and did not show any complications except PmGlmU. With PmGlmU, there was a noticeable amount of precipitation that formed in the dialysis membrane that was not present for PmPpA and BiNahK. It was possible that PmGlmU was too concentrated and that induced misfolding and aggregation leading to protein precipitation. To combat this, a different type of buffer exchange method was needed to avoid precipitation.

As an alternative we used a HiTrap® desalting column to remove small molecule components from the protein preparation.[45] PmGlmU was loaded onto the column and was pushed through the column at a rate of 5 mL/min with dialysis buffer. As the protein solution and buffer pass through the column, the small molecules are separated from PmGlmU. Flow through was collected in four 0.5 mL fractions and analyzed by SDS-PAGE stained with Coomassie (Figure 9).

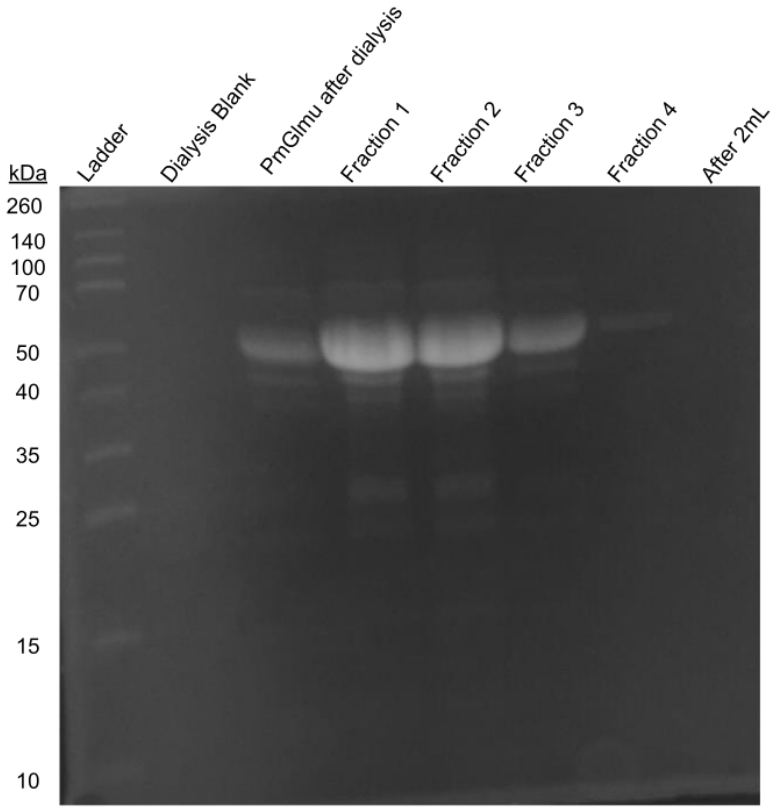

Figure 9: Coomassie Stain of different versions of PmGlmU

When analyzing the Coomassie gel, there was a faint signal for PmGlmU after dialysis, but the solution contained precipitates. For the desalting column fractions, there were noticeable signals for fraction 1, fraction 2 and fraction 3. Fraction 4 displayed a faint signal with no signal for the last (optional) fraction collected after the mandatory 2 mL fraction collection (Figure 9). We observed no precipitation with the desalting column and later compared the activity of protein prepared by desalting and dialysis.

3.2: Chemoenzymatic Reactions

3.2.1: Chemoenzymatic Synthesis of UDP-GlcNAc

The chemoenzymatic synthesis of UDP-GlcNAc has been performed by other research labs in the past.[22, 26, 46] The Troutman lab recently wanted to follow suit in creating this UDP-linked sugar but found some challenges that hindered us. A previous member in the group attempted to chemoenzymatically synthesize UDP-GlcNAc but the amount formed was very low in concentration, only just above the limit of detection (LOD) by HPLC (Data Not Shown). With this information provided, there was a need to use an analytical technique that was higher sensitivity, could utilize smaller sample volumes and produce better separation.

We chose to use Capillary Electrophoresis (CE) for a more sensitive detection of UDP-GlcNAc product which separates analytes based on their charge-to-size ratio by applying an electric field across a capillary tube filled with an electrolyte solution.[47] In normal polarity CE, the anode (positive electrode) is located at the inlet and the cathode (negative electrode) is at the outlet. This creates an electroosmotic flow (EOF) which moves towards the cathode that is negatively charged, carrying analytes towards the detector.[48] Smaller and more highly charged

molecules migrate faster than larger less charged molecules. To further enhance detection and separation, reverse polarity CE was utilized instead of normal polarity. Reverse polarity switches the location of detection from the cathode, that is now the injection, and the anode, becoming the outlet with the detection now located at the anode.[49] When this occurs, the EOF can be suppressed or reversed which in this case was suppressed. With the EOF suppressed, cations migrate towards the cathode, neutral ions stay static with no movement to either side and anions migrate toward the anode which means only the anions are being detected.[49] With the reagents used, the main analytes that would be detected would be ATP, UTP and UDP-GlcNAc due to their negative charges on the phosphate groups so they will be able to migrate to the detector.

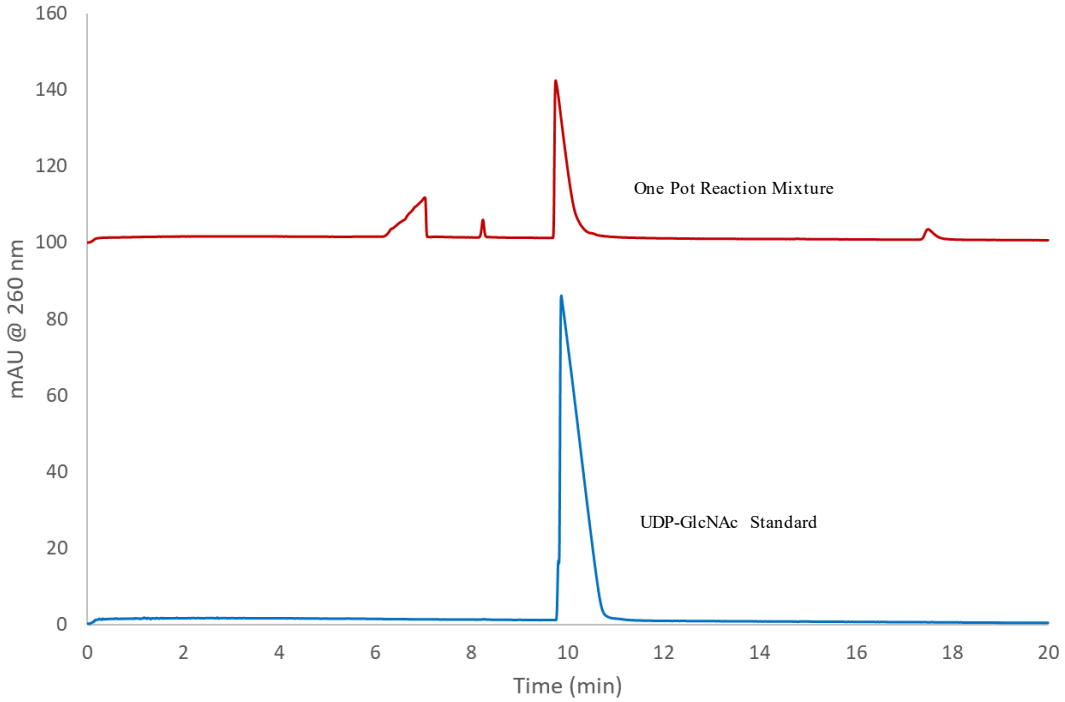

Figure 10: CE Chromatogram showing the migration time for UDP-GlcNAc One-Pot reaction compared to the UDP-GlcNAc Standard

When analyzing the data from CE, there is formation of UDP-GlcNAc from the one-pot reaction. The one-pot reaction mixture and the standard were diluted 10x before being analyzed by CE, meaning the concentration of this UDP-GlcNAc is higher than what is being displayed in the chromatogram, so this has the possibility of being detected on HPLC (Figure 10). With this

evidence, there was a need to demonstrate that this reaction was reproducible and explain why this reaction is displaying better yield conversion than previous attempts.

These conditions for each reaction contained the same concentration of each enzyme, buffer, GlcNAc, adjusted to pH 8 and used the desalted purified PmGlmU. One of the reactions was incubated for 24 hours while others were incubated for 12 hours, this was done to see if the reaction will continue to produce UDP-GlcNAc even after a 12-hour incubation. After each reaction, HPLC was used with a standard of UDP-GlcNAc to determine if the one-pot reactions formed the desired product. Each analysis produced a chromatogram that had similar retention times to the UDP-GlcNAc standard with the third reaction that was incubated 24 hours double in the amount of product. The retention times were consistent across all runs, indicating successful and reproducible product formation of UDP-GlcNAc using the one-pot synthesis method (Figure 11).

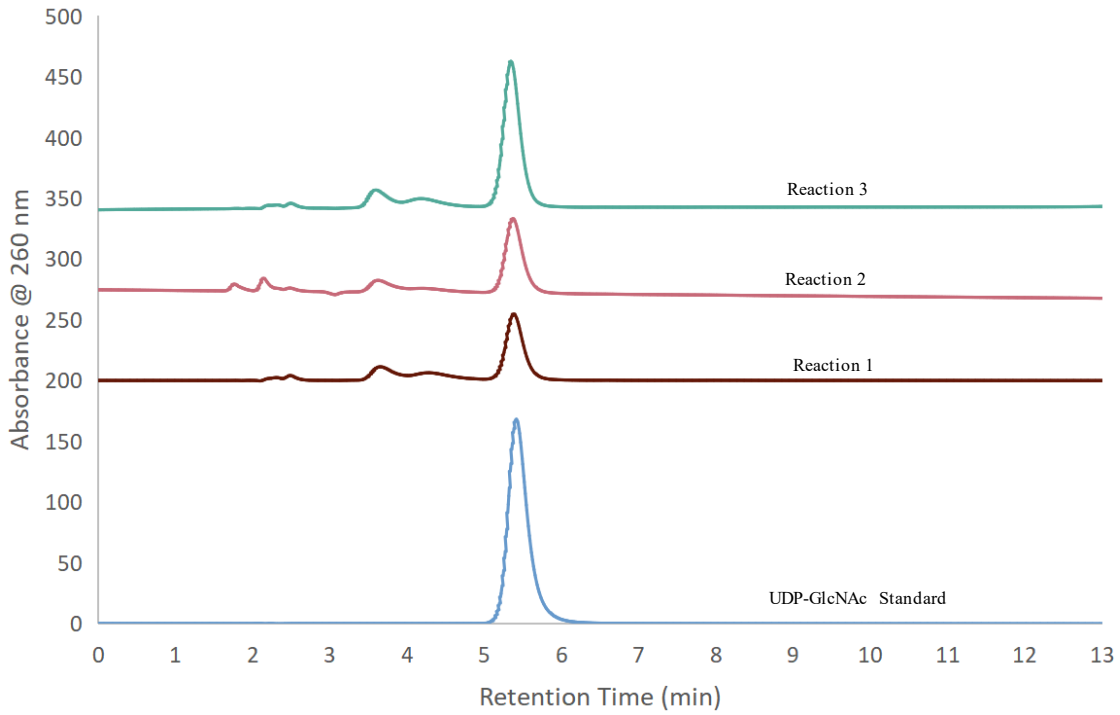

Figure 11: Chromatograms of different one-pot synthesis of UDP-GlcNAc against the standard UDP-GlcNAc. Reaction 1 and Reaction 2 were run for 12 hours, Reaction 3 was run for 24 hours.

One of the possible reasons there was little to no turnover to form UDP-GlcNAc was the pH of the reaction mixture regardless of the buffer used and the correlating pH. However, when utilizing the one-pot synthesis method with the use of Tris-HCl buffer at a pH of 8, the reaction did not produce UDP-GlcNAc. Because the other components of the reactions were at a concentration close to that of the buffer it was possible that these components impacted the overall pH. To determine if the pH was the problem, the one-pot reaction mixture's pH was tested after adding all the components except the three enzymes. The pH of the reaction was between 3-4 even with the addition of the buffer meaning its necessary to adjust the pH after. To acknowledge that the pH also played a big role, a one-pot reaction without pH adjustment was analyzed by HPLC to track formation of UDP-GlcNAc (Figure 12). The one-pot reaction without pH adjustment showed little to no formation of UDP-GlcNAc.

As mentioned previously, there were issues with the nucleotidyltransferase protein, PmGlmU, precipitating when attempting to remove imidazole from the preparation. The protocol when this occurred in dialysis requires that the precipitate is removed, and the supernatant continues to undergo dialysis. Even though the concentration of the protein would be lower, it should still be functional. After following the protocol for HiTrap® desalting mentioned previously, a comparison was made with two different versions of PmGlmU utilizing HPLC (Figure 12). The two versions consist of PmGlmU after dialysis and PmGlmU after the desalting method. There was only the formation of UDP-GlcNAc with the desalted version of PmGlmU. With these findings, it is reasonable to conclude that the method of removing small molecules and salts from PmGlmU and pH have been the main factors that impacted the formation of UDP-GlcNAc using the one-pot method (Figure 12).

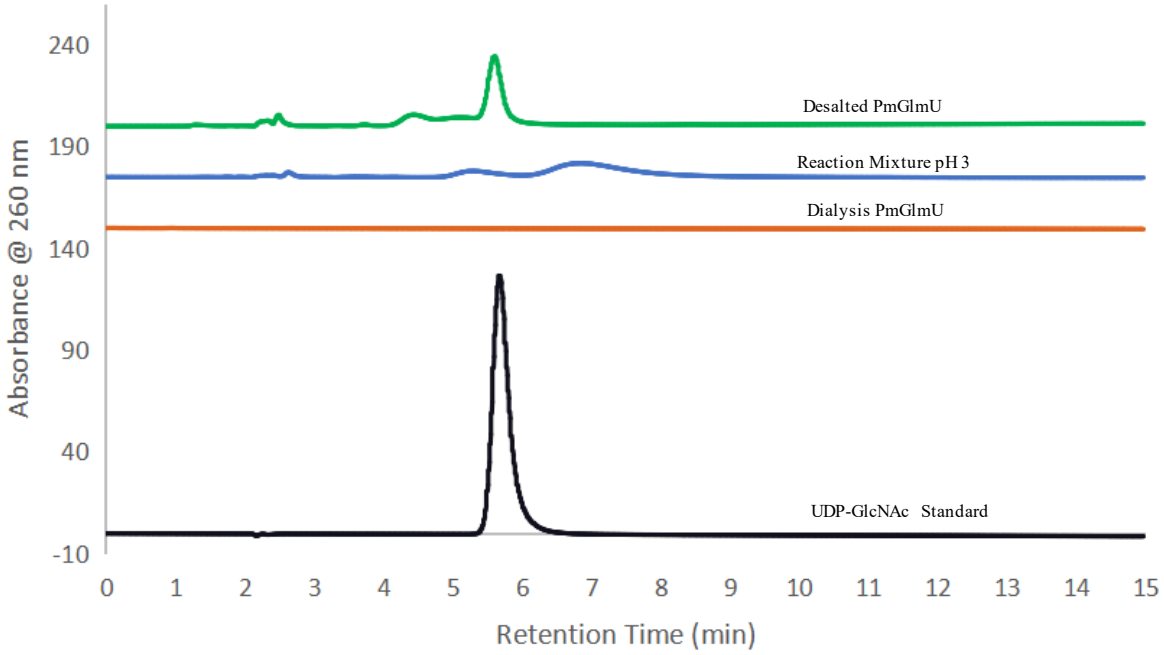

Figure 12: Chromatograms showing the formation of UDP-GlcNAc from different one-pot reactions containing different components.

With the success producing UDP-GlcNAc in a small-scale reaction, the next step was to scale up the process to a larger reaction volume. As mentioned previously, UDP-GlcNAc is an essential substrate that is used for bacterial cell ways and cell signaling. Being able to synthesize UDP-GlcNAc on a larger scale, we are able to provide ample supply of the sugar nucleotide for ongoing research to make other rare sugar nucleotides. Additionally, producing larger quantities of UDP-GlcNAc that can be done in the research lab rather than depending on a manufacturer makes this method cost effective. Lastly, to utilize UDP-GlcNAc in an industrial setting, larger amounts of the substrate will be required and needs to be efficient and low effort like the one-pot synthesis method. The large-scale reaction consisted of increasing the quantities of all the reagents, buffer, and enzymes while maintaining the same conditions for the small-scale reaction but in a larger vessel. After 24 hours of incubation, the large-scale reaction was analyzed first with TLC to determine if there was any formation of UDP-GlcNAc (Figure 13).

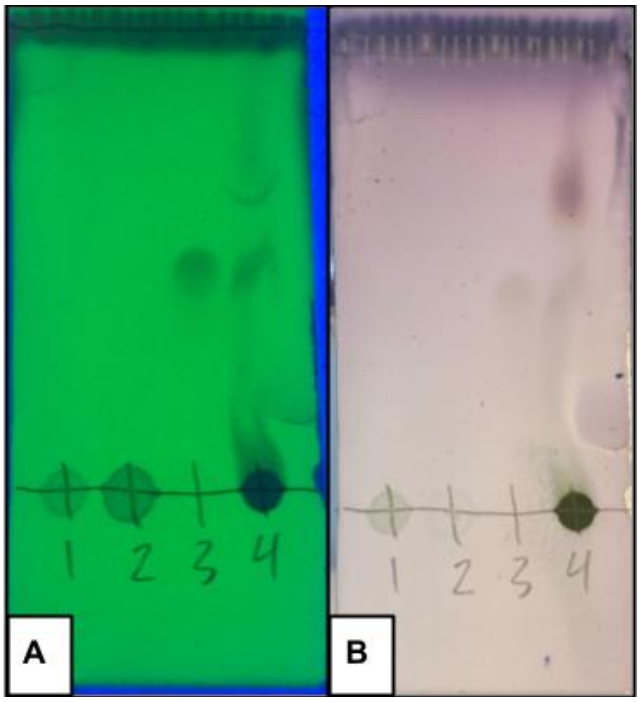

Figure 13: TLC analysis of large-scale enzymatic synthesis of UDP-GlcNAc. A) UV light analysis; B) p-anisaldehyde sugar stain analysis Lane 1: ATP; Lane 2: UTP; Lane 3: UDP-GlcNAc Standard; Lane 4: UDP-GlcNAc One-Pot Reaction

Once that was confirmed, the reaction was analyzed using HPLC to confirm the formation of UDP-GlcNAc. The HPLC chromatograms from the large-scale reaction displayed the presence of UDP-GlcNAc at the expected retention time when compared to the standard of UDP-GlcNAc, meaning that scaling up the reaction does not impede the efficiency of the reaction (Figure 14). With the larger-scale reaction, there are opportunities for optimization of reaction conditions to improve the yield shown. With the use of a large-scale reaction, the environment for the reaction is more stable compared to the smaller scale where minor variations could negatively impact the turnover to UDP-GlcNAc. Once the reaction is tuned to produce UDP-GlcNAc at an efficient rate, then purification can be utilized to be used in other research projects that uses this sugar nucleotide.

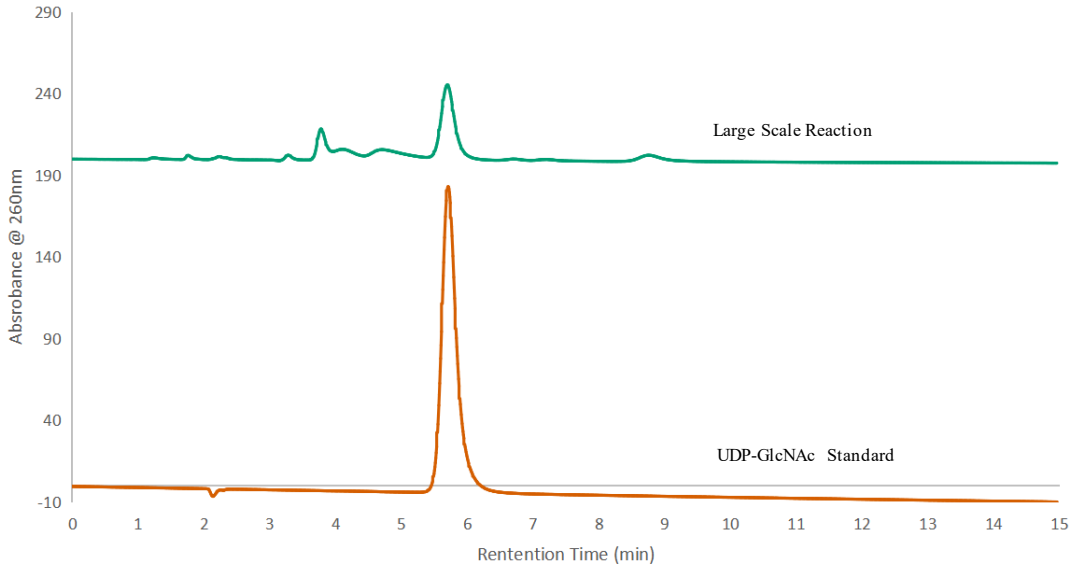

Figure 14: Chromatograms displaying the formation of UDP-GlcNAc in one-pot synthesis on a large scale compared the standard for UDP-GlcNAc.

3.2.2: Chemoenzymatic Synthesis of UDP-GalNAc

After successfully preparing UDP-GlcNAc from a one-pot reaction, the next step was to replicate this approach to form UDP-GalNAc. The reasoning for this approach is due to the drastic price difference between UDP-GlcNAc and UDP-GalNAc with the only structural difference being the position of one hydroxyl group. There has been evidence showing a way to synthesize UDP-GalNAc with GalNAc.[26] Since the one-pot synthesis was successful for UDP-GlcNAc, being able to use the same method with UDP-GalNAc without having to change the substrate GlcNAc to GalNAc would be cost-effective for the Troutman Lab.

The ability to use the same reaction components and conditions for UDP-GlcNAc to synthesize UDP-GalNAc is due to the epimerase enzyme VvWbpP.[50] With the one-pot synthesis of UDP-GlcNAc, using VvWbpP will then take that final product and invert the hydroxyl on carbon-4 to form UDP-GalNAc. With this information, the one-pot synthesis for UDP-GalNAc was attempted on a small scale to determine if this reaction will have turnover to the final

product. The reaction was set up using the same procedure as the one-pot UDP-GlcNAc synthesis with the addition of VvWbpP incubated over the course of 12 hours. There has been research done explaining that with PaWbpP, NAD(H) is tightly bound in the protein and does not require additional cofactors for the reaction to occur.[51]

After incubation, the reaction mixture was analyzed using HPLC for detection of UDP-GalNAc based on the retention of a commercial standard. The chromatogram displayed a mixture of UDP-GlcNAc and UDP-GalNac (Figure 15). This was to be expected based on previous experiments completed in the Troutman lab utilizing VvWbpP to convert UDP-GlcNAc to UDP-GalNAc where the reaction yield was a ratio of 7:3 (UDPGlcNAc/UDP-GalNAc).[50] This partial turnover was also mentioned for *Pseudomonas aeruginosa* WbpP, which has about 70% identity to the VvWbpP.[51] This is due to the equilibrium between UDP-GlcNAc and UDP-GalNAc where the formation of UDP-GlcNAc is preferred over UDP-GalNAc.

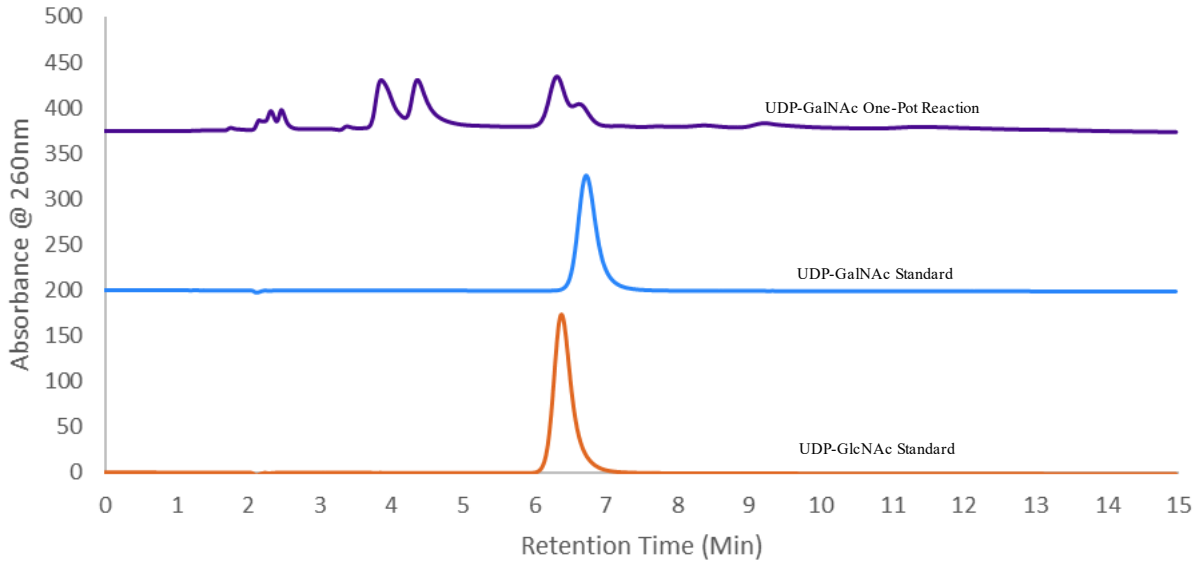

Figure 15: Chromatograms displaying the formation of UDP-GalNAc in one-pot synthesis on a small scale compared the standards for UDP-GlcNAc and UDP-GalNAc

Following the success of forming UDP-GalNAc with the small-scale reaction, I next scaled up the reaction. This reaction followed the same protocol used for the large-scale one-pot synthesis of UDP-GlcNAc with the addition of VvWbpP with an incubation of 24 hours to help produce as much of UDP-GalNAc as possible. After 24 hours, the reaction was analyzed with TLC to visualize the formation of UDP-GalNAc. After using TLC, the reaction mixture was analyzed on HPLC. The HPLC chromatograms for this reaction mirrored that of the small-scale reaction displaying the presence of UDP-GlcNAc and UDP-GalNAc shown in a partial turnover (Figure 16). The consistency of the results from the small-scale reaction to the large-scale shows that the one-pot reaction is reliable as the formation of UDP-GalNAc is present but also the base reaction of UDP-GlcNAc is repeatable to be used with other sugar enzyme modifiers.

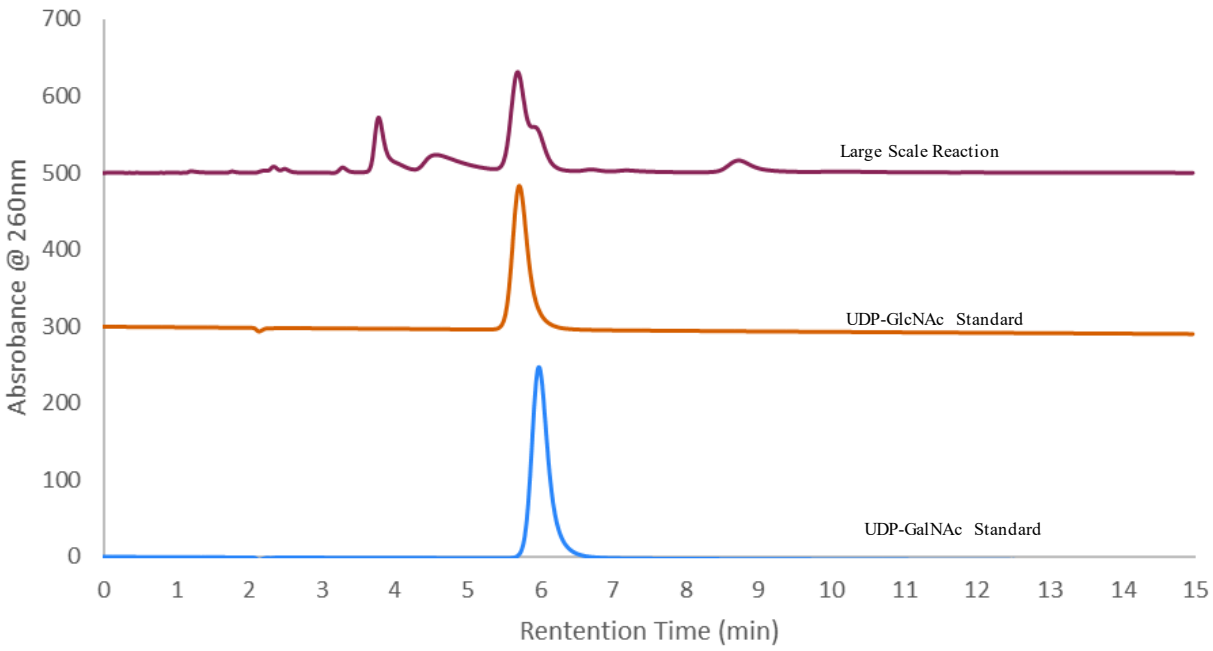

Figure 16: Chromatograms displaying the formation of UDP-GalNAc in one-pot synthesis on a large scale compared the standards for UDP-GlcNAc and UDP-GalNAc

3.3: Structural Comparison of Sugar Modifying Enzymes

3.3.1: Kinases: BiNahK, BiGalK, and AtGlcAK

The structures of BiNahk, BiGalK and ATGlcAK are of great interest due to their ability to transfer a phosphate group from ATP to a particular sugar. Since these three enzymes have similar functions, we should be able to analyze their predicted structures for comparison. This comparison should help visually identify what structural differences can cause the specificity of BiGalk and ATGlcAK then the promiscuity of BiNahk which the Troutman Lab utilizes.

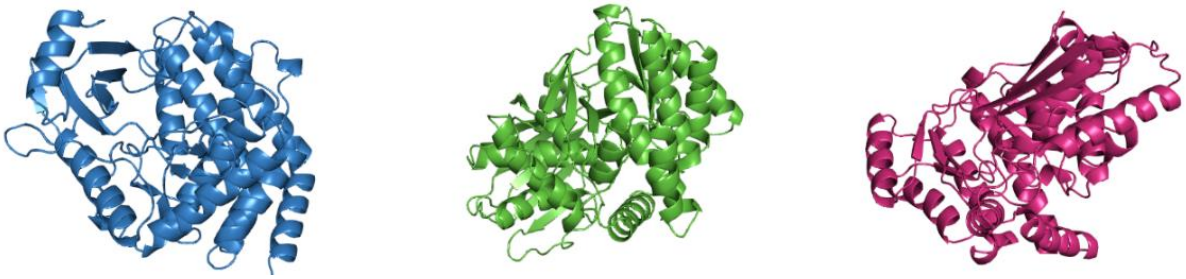

Figure 17: Predicted structures of BiNahk (left, blue), BiGalK (center, green) and AtGlcAK (right, pink)

When discussing BiNahK, this enzyme is normally utilized to synthesize UDP-GlcNAc. ATP with the addition of GlcNAc and the cofactor Mg^{+2} produces GlcNAc-1-P, and H^{+}. As for BiGalK, it is used to synthesize UDP-Gal with the same catalytic activity as BiNahK but using Gal as a substrate to produce Gal-1-P. Then for AtGlcAK, it is used to synthesize UDP-GlcA that also uses the same catalytic activity as BiNahK but uses GlcA as the substrate to make GlcA-1-P. Visually looking at each enzyme, BiNahK appears densely packed with α-helices tightly arranged to one side. This can also be seen with BiGalK as well but with the secondary structures being closer to each other compared to BiNahK. AtGlcAk appears to be more elongated when compared to BiNahK and BiGalK which are more compact. This leads to the idea of comparing

the sequence between them to determine how similar these enzymes are to each other (Figure 17).

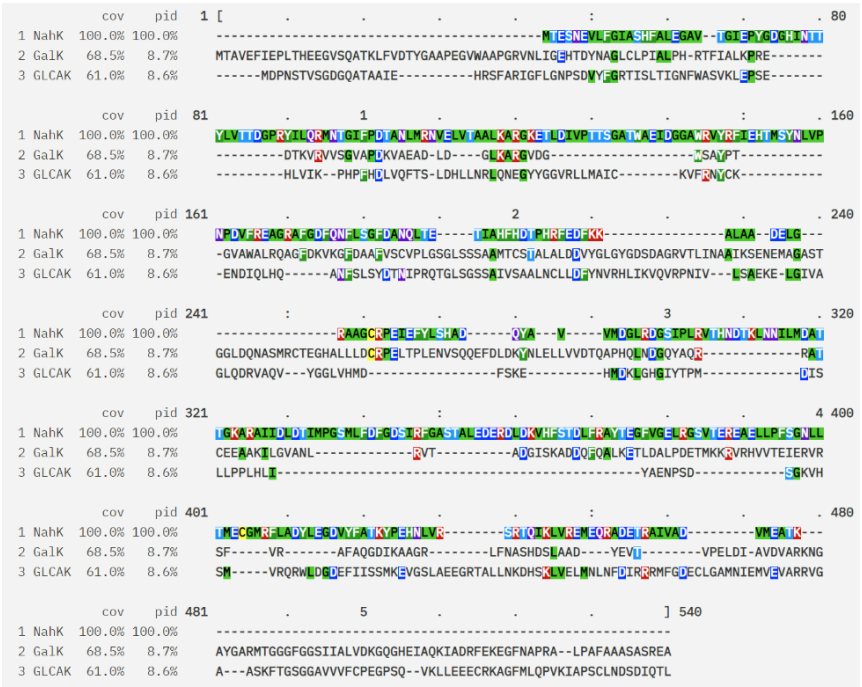

Figure 18: Sequence alignment similarity comparison between BiNahK, BiGalK and AtGlcAK

Based on the sequence alignment data, there is very low sequence similarity when comparing BiGalK to BiNahK and AtGlcAK to BiNahK (Figure 18). Due to AtGlcAK deriving from a plant, the sequence similarity between the two will be low, which was expected. The low sequence similarity between BiNahk and BiGalK could be due to both enzymes having the same function but utilizes a different substrate. Even with the use of different substrates, one could have an expectation that there would still be a decent amount of similarity since these two enzymes come from the same bacteria. To visualize the three enzymes structure similarities, Pymol's "CEalign" function was used rather than the "align" function.

The align function uses sequence alignment and a structural superimposition to see how well two or more proteins compare. This function can only be utilized when proteins have at

least 30% sequence similarity. Due to our BiGalK and AtGlcAK not having a strong similarity to BiNahK, the align function cannot be used. CEalign utilizes the Combinatorial Extension (CE) algorithm which is based on the alignment pathway determined by aligned fragment pairs (AFPs) from two proteins.[52] AFPs are then extended in a combinatorial approach to determine the best overall alignment that maximizes the structural similarity between the two proteins.[52] This alignment method allows for comparisons of each protein's overall structure and compares structural motifs or domains that are conserved between them.

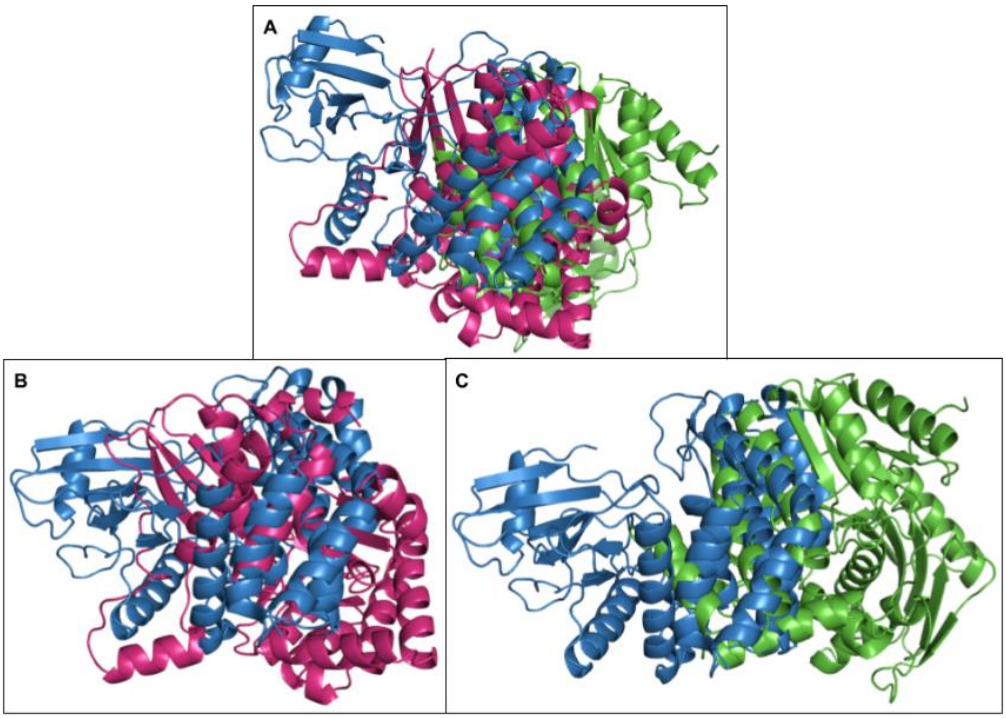

Figure 19: Superimposed images of the three proteins. A) Superimposed image of BiNahK (blue), BiGalK (green) and AtGlcAK (pink). B) Superimposed image of BiNahK and AtGlcAK. C) Superimposed image of BiNahK and BiGalK

Once CEalign was used, these proteins were superimposed to analyze any correlating structures between the three proteins (Figure 19). The RMSD between BiNahK and GlcAK lowered from 20.763 to 5.654 while the RMSD between BiNahK and BiGalK lowered from 21.354 to 6.267. While these values are higher than what most will consider for enzymes to be similar, there is still a dramatic change in value showing they are related. Some of the α-helices

for all three proteins are structurally similar. BiNahK can use a wider range of substrates including GlcNAc, GlcNAz, GalNAc, and mannose (Man) which could be due to the domain where the binding area is located more exposed compared to AtGlcAK and BiGalK. This wider domain area has been shown to phosphorylate larger structures like kanamycin A which is an antibiotic (Figure 20).[53] There is little similarity between these enzymes compared to what was expected using CEalign.

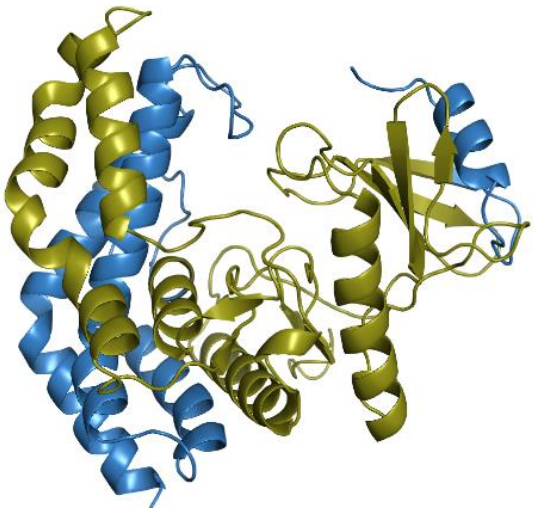

Figure 20: Highlighted domain on BiNahK. The olive color represents the domain for the aminoglycoside phosphotransferase.

Due to the low similarity of BiNahK between BiGalK and AtGlcAK, there was interest in seeing if the similarity between BiGalK and AtGlcAK was higher. When using the regular align function, the similarity is higher than when they were compared to BiNahK (Figure 21D). The RMSD between the two enzymes was enhanced from 10.481 to 4.776 once the CEalign function was used. Discussing how these enzymes are more similar to each other than BiNahK, there has to be an analysis of their domains. When visually looking at the structure for BiGalK, there are two domains, GHMP kinase N-terminal and GHMP kinase C-terminal (Figure 21A).[54]

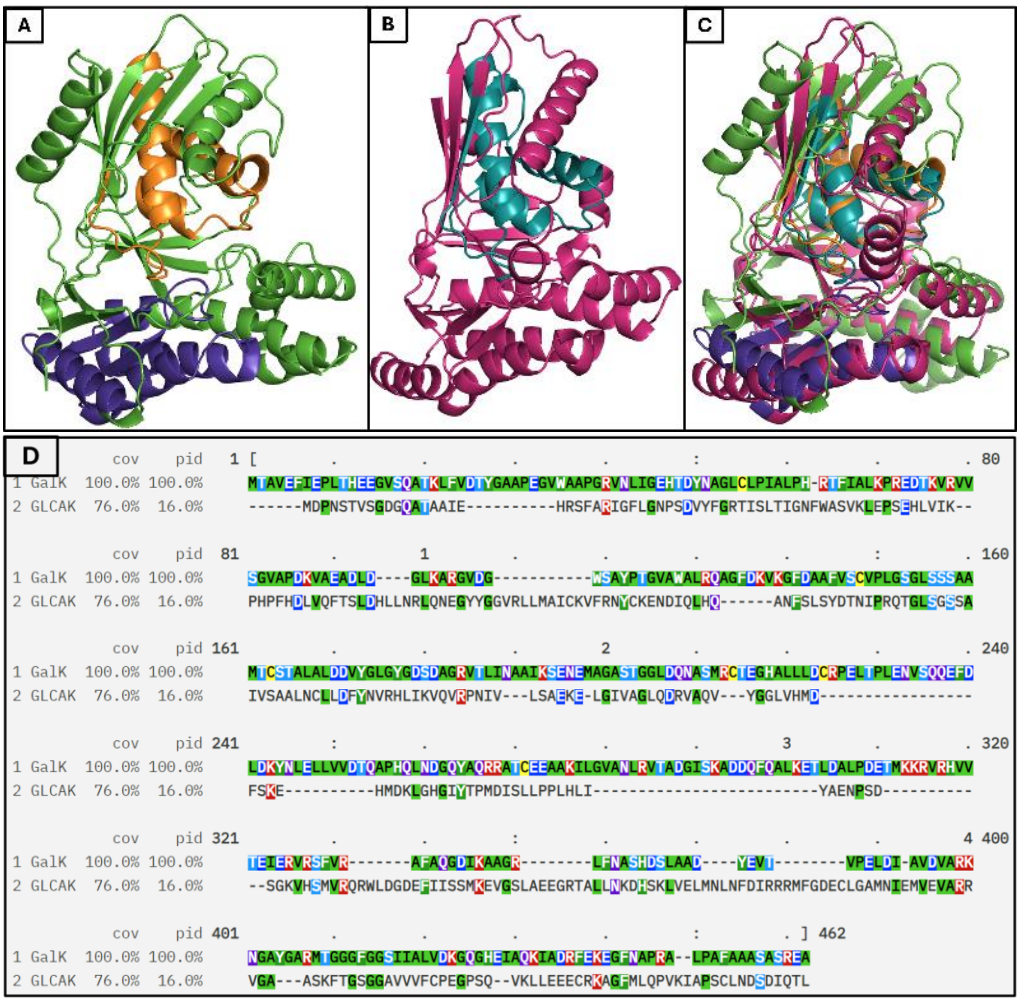

Figure 21: A) Structure of BiGalK with highlighted domains. Orange- N Terminal, Purple- C Terminal. B) Structure of AtGlcAK with highlighted domain. Teal- N Terminal. C) Overlay of images A and B. D) Sequence alignment similarity comparison between BiGalK and AtGalK

The GHMP kinase family represents a group of ATP dependent enzymes that are involved in phosphorylating specific substrates.[55] Galactokinases and glucuronokinases are some of the main families in the GHMP kinase family so there have been studies showing these domains with a central β-sheet surrounded by α-helices (Figure 21A-B).[56, 57] When comparing the two enzymes, both display the GHMP kinase N-terminal domain with similar layout of the α-helices and β-sheets. It has not been mentioned that AtGlcAK has the characteristic GHMP kinase C-terminus like BiGalK but when analyzing the structures, there are some overlaps with similar secondary structures that mimic the C-terminus (Figure 21C). Experimentally, AtGlcAK has

been shown to only utilize GlcA while BiGalK has the ability to accept GalA and Gal. The structure for AtGlcAK appears to be more compact compared to BiGalK which could lead to AtGlcAK being specific and only using one substrate.

3.3.2: Nucleotidyltransferases: PmGlmU and AtUSP

Another set of enzymes that are of interest are PmGlmU and AtUSP with their ability to transfer uridyl group from UTP to a sugar-1-phosphate. In for the synthesis of UDP-GlcNAc, PmGlmU is the enzyme used as a nucleotidyltransferase and can use other motifs of GlcNAc-1-P. This has not been shown with AtUSP to utilize GlcNAc-1-P as a substrate to synthesize UDP-GlcNAc, but has shown that it can form smaller UDP-linked sugars.[26] This comparison should help visually identify what structural differences are responsible for the versatility of AtUSP and the promiscuity of PmGlmU.

When analyzing PmGlmU, this bifunctional enzyme is comprised of three parts: N-terminal acetyltransferase domain, a linker that holds the N and C-terminal together and the C-terminal uridyltransferase domain (Figure 22). This enzyme is utilized to synthesize UDP-GlcNAc. Acetyl CoA with the addition of GlcN-1-P in PmGlmU produces GlcNAc-1-P, CoA and H^+. The uridyltransferase activity requires UTP, Mg^{+2} and H^+ with the addition of GlcNAc-1-P to produce UDP-GlcNAc and diphosphate. The acetyltransferase region consists of left-handed β-sheets in a corkscrew structure where the binding sites are facing the outer portion of the tunnel towards the end of the structure while the linker is an α-helix that's attached to both terminals (Figure 22B).

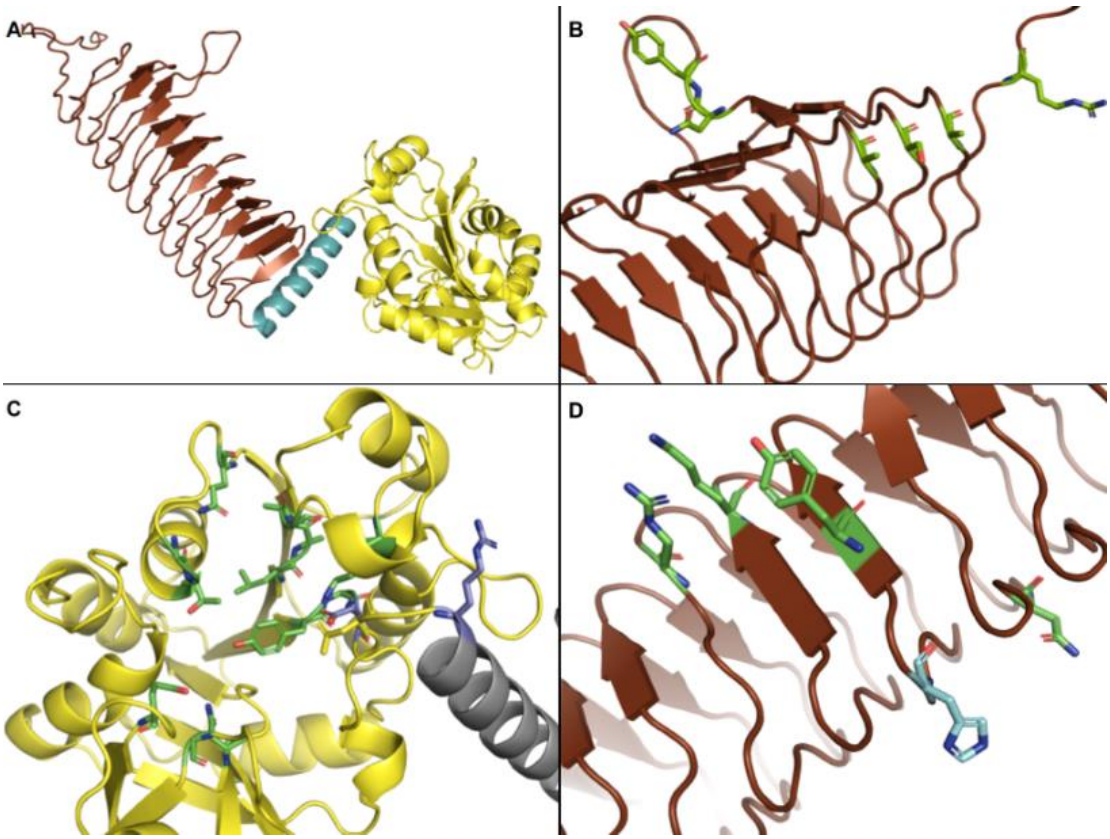

Figure 22: A) Predicted structure of PmGlmU depicting the 3 segments found in the structure. Brown - N terminal acetyltransferase, Mint – Linker, and Yellow – C terminal uridyltransferase. B)Acetyl-CoA Binding sites in lime green. C) UDP-GlcNAc binding sites in gr green and Mg^{+2} binding sites in lavender. D) UDP-GlcNAc binding sites in green, active site labeled light blue.

The uridyltransferase region contains a mixture of α-helices and β-sheets where the binding sites are facing the center along with two Mg^{+2} ion binding sites towards the linker region. There have been studies with GlmU from other bacteria demonstrating that the two-metal-ion mechanism can play a large role for uridyltransfer in GlmU.[58] One of the Mg^{+2} ions help stabilize the reactive groups of GlcNAC-1-P and UTP to help facilitate their interaction. The second Mg^{+2} ion interacts with UTP to create a strained conformation to stabilize the transition state.

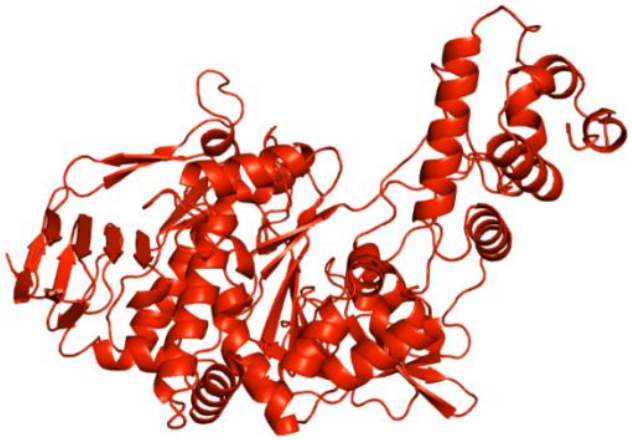

Figure 23: Predicted structure of AtUSP generated through Pymol

Compared to PmGlmu, little work has been done in analyzing the active and binding sites for AtUSP (Figure 23) but there have been many UDP-sugar formation reactions to test its substrate specificity. For the catalytic activity of the uridyltransferase, UTP, Mg^{+2} ions and H^{+} with the addition of a monosaccharide-1-P produces a UDP-monosaccharide and diphosphate. Sequence alignment of PmGlmU and AtUSP shows an underwhelming amount of similarity between the two proteins like the results for the kinases (Figure 24).

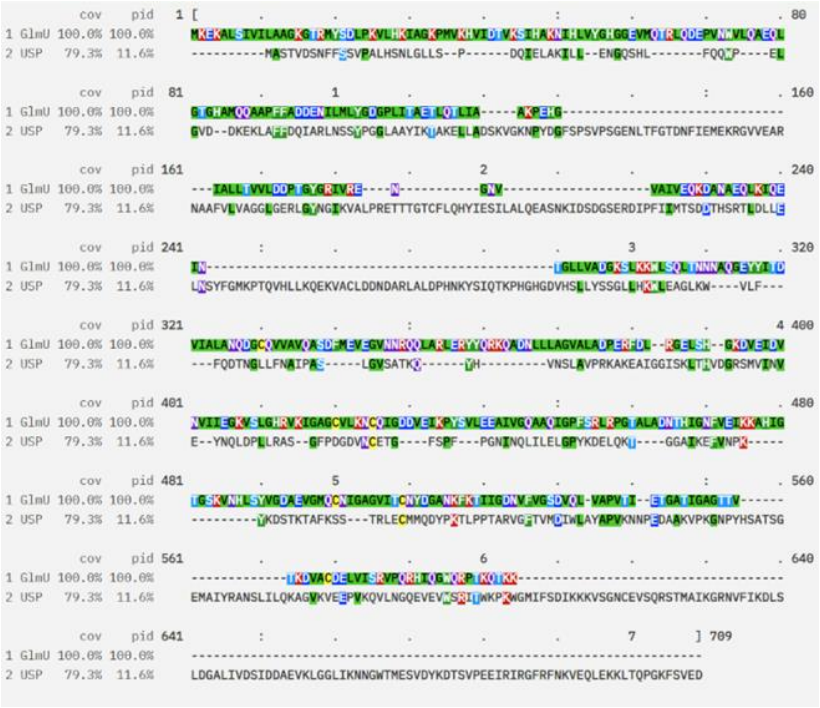

Figure 24: Sequence alignment similarity comparison between PmGlmu and AtGlcAK, PID = 11.6%

With the sequence identity between PmGlmU and AtUSP being very low, CEalign was implemented to have a better representation of the two protein structures superimposed on each other (Figure 25). Before utilizing CEalign, the RMSD was reported with the regular align function as 17.620. After utilizing CEalign, the RMSD was reported as 3.511 which is a drastic change showing there are more structural similarities than what is being shown when looking at just the sequence. Once this function was used, it became possible to see where some of the similarities and differences are.

Starting with similarities, PmGlmU and AtUSP showed significant overlap, with similar α-helices and β-sheet structures. The core structure, consisting of a central ß-sheet flanked by α-helices, is retained, which is required for binding UTP and sugar-1-phosphate substrates and is a common structural motif among uridylyltransferases.[59-61] This alignment shows that these enzymes likely use a similar mechanism. In comparison to PmGlmU, AtUSP displays extra α-helices and extended loop segments making AtUSP larger than PmGlmU. Exploring the binding pocket for each enzyme, the PmGlmU binding pocket is deeper than AtUSP. This correlates to the different UDP-linked sugars each enzyme is able to produce. For PmGlmU, this enzyme is able to accommodate larger and modified sugars like GlcNAc-1-P, GlcNTFA-1-P and GlcNAz-1-P whereas AtUSP is best suited for smaller, more common sugars like Gal-1-P, GlcA-1-P and GalA-1-P.[26]

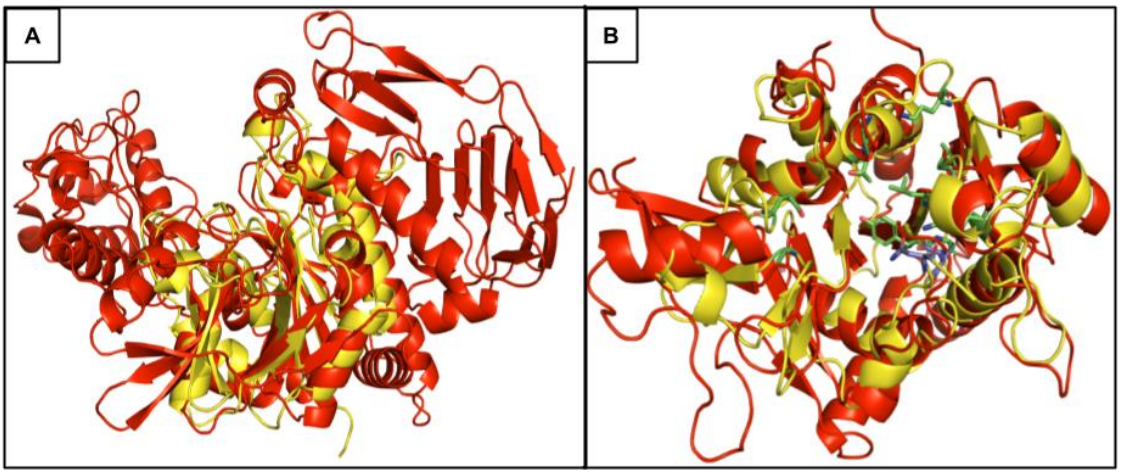

Figure 25: A) Superimposed image of AtUSP and PmGlmU using CEalign from Pymol. Yellow- PmGlmU, Red- AtUSP B) Same image of superimposed image but with the binding groups of PmGlmU visible. UDP-GlcNAc binding sites in green and Mg^{+2} binding sites in lavender

CHAPTER 4: CONCLUSIONS AND FUTURE WORK

4.1: Synthesis of UDP-linked Sugars

The Troutman lab has recently wanted to follow suit in the use of One-Pot synthesis to chemoenzymatically create UDP-linked sugars. The ability to make these UDP-linked sugars in-house would significantly lower the cost of the in vitro synthesis of complex carbohydrates. There have been many attempts to utilize this method without success. The proposed work displayed sought to optimize this method in the Troutman group to understand what could be impeding the formation of UDP-linked sugar production.

The main sugar that I focused on synthesizing was UDP-GlcNAc. UDP-GlcNAc was of great interest due to its ability to be modified to form other UDP-linked sugars that may not be available commercially. The crucial issues that halted the formation of UDP-GlcNAc were the pH of the reaction and the precipitation of PmGlmU. The reaction mixture contained a buffer that should have maintained the pH through the reaction, but the addition of many reagents caused the pH to lower significantly regardless of the buffer's pH. As for PmGlmU, the protein would precipitate after attempting to remove salts making the protein non-functional. Once the pH was adjusted after all reagents and the use of HiTrap® desalting for PmGlmU, there was detectable levels of UDP-GlcNAc formed in the reaction.

Once there was confirmation of UDP-GlcNAc being formed, the next step that was taken was utilizing this in a large-scale formation. This portion was more streamlined since the main issue of attempting to synthesize UDP-GlcNAc was resolved with the production of UDP-GlcNAc occurring in the large-scale version. The next step was to attempt the synthesis of UDP-

GalNAc using the same method and reagents utilized in synthesizing UDP-GlcNAc. The addition of VvWbpP was able to give partial conversion from UDP-GlcNAc to UDP-GalNAc. Partial conversion has been seen in previous experiments in the Troutman lab utilizing this enzyme. This is due to UDP-GlcNAc being a more favorable product than UDP-GalNAc, this would be evident with other epimerases, so this is not just specific to VvWbpP. The same partial turnover was shown for the larger scale of the reaction.

Future work that can follow from this is synthesizing rarer UDP-linked sugars from the base reaction mixture of UDP-GlcNAc. There are rare sugar nucleotides that cannot be commercially found like UDP-diNAcBac and UDP-ManNAcA that can be derived from UDP-GlcNAc. These UDP-linked sugars have been synthesized before but had purification steps in between and occurred in different reaction vessels.[18, 50] The process of being able to do this in one reaction vessel without having to constantly monitor the formation would be preferable.

Another area that this can be taken to is to use this in forming BPP-sugars. Instead of introducing the already synthesized UDP-linked sugar to fluorescent BP, there can be an attempt to append the one pot synthesis with fluorescent BP to track the formation of BPP-sugar. This would be a continuation of the work that has been shown in the Troutman lab by adding everything in one-pot to track the formation of BPP-sugars based on PGTs.[13] The main things to keep in mind when attempting these other reactions is the concentration of GlcNAc as once it is too concentrated, this can cause the reaction mixture to exhibit high viscosity which impacts the conversion rate.[26] Another important point to keep in mind is the different PGTs used and what cofactors that may be needed might not be compatible with the base one-pot reaction of UDP-

GlcNAc. With the information from this thesis, there is a wide range of opportunities to use this flexible method of synthesizing UDP-linked sugars and BPP-sugars.

4.2: Sugar Modifying Enzymes

Understanding sugar modifying enzymes and how their structures can influence their ability to accept or reject specific substrates are important aspects to keep in mind when attempting to chemoenzymatically synthesize UDP-linked sugars. There are certain sugar modifying enzymes that could have the same role and possibly utilize the same substrate but display improved performance. The proposed work displayed sought to analyze the structures of sugar-modifying enzymes that perform similar tasks to understand what attributes in these enzymes make different substrates preferred.

BiNahK, BiGalK and AtGlcAK were sugar phosphotransferases that were compared to get a better understanding of the substrate promiscuity of BiNahK and substrate specificity of BiGalK and AtGlcAK. It was shown that the sequence similarity between each enzyme was very low. To get a better structural comparison, CEalign was utilized to get the maximum comparison between each structure. This method led us to some structure similarity but still relatively lower than what was expected. Due to the low similarity between each enzyme, the next step was to compare BiGalk and AtGlcAK to determine if their structure similarity would stay as low or would improve. Data showed that BiGalK and AtGlcAK were more similar to each other than to BiNahK which was most likely due to both enzymes being part of the GHMP kinase family. This family has conserved N and C terminal domains that aid in the transfer of a phosphate group onto the monosaccharide which was visualized in the structure comparison.

PmGlmU and AtUSP were the sugar nucleotidyltransferases that were also compared to understand why PmGlmU can accommodate larger and modified substrates compared to AtUSP. These two enzymes showed similar results to the kinases as the sequence comparison was very low and had to utilize CEalign to get an accurate visual comparison. The alignment between the two drastically improved and were more like each other than the kinases. Both enzymes displayed a core structure that contained central ß-sheet flanked by α-helices in the binding region. The main differences that most likely contributes to their substrate acceptance was the size of the binding areas, the shallower AtUSP accepts smaller sugar-1-phospates compared to the deeper, flexible binding region of PmGlmU that can use larger substrates. This work showed the ability to use a different alignment tool to get a more accurate visual comparison rather than only depending on sequence comparison.

Future work that can follow with this information could investigate the similarity between the same enzyme produced from different bacteria to determine which performs better in forming different sugar-1-phosphates. For example, there was a study done to analyze how well NahK from two different bacteria utilized different modifications of GlcNAc and GalNAc.[62] The data showed that they displayed different amounts of conversion even when in the same pH and concentration of Mg^{+2}. There were also differences in conversion based on which substrate was being introduced. Being able to carry out this type of comparison visually will help to see what differences can impede or improve product turnover with different substrates in other one-pot reactions.

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