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UNIVERSITY OF CALIFORNIA SAN DIEGO

Assessment of Gut Microbial Beta Glucuronidase Substrate Specificity in Sex Steroid
Metabolism

A thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

Lai Ying Ivy Tam

Committee in charge:

Professor Varykina Thackray, Chair
Professor Heidi Cook-Andersen, Co-Chair
Professor Susan Golden
Professor Fabian Rivera-Chávez

2024

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University of California San Diego

2024

DEDICATION

This thesis is dedicated to
My mom, dad, Sophia, and Clara
All my friends
And Alejandro
for their unwavering support and encouragement

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LIST OF ABBREVIATIONS

E2	17-beta-estradiol
E2-gluc	Estradiol-glucuronide
FMT	Fecal microbiota transplant
GUS	Beta-glucuronidase
L1	Loop 1
L2	Loop 2
ML	Mini-loop
NAD ⁺	Beta-nicotinamide adenine dinucleotide
NADH	Beta-Nicotinamide adenine dinucleotide, reduced
NL	No loop
NOD	Non-obese diabetic
OD	Optical Density
T	Testosterone
T-gluc	Testosterone-glucuronide
T1D	Type-1 diabetes
UDH	Uronate dehydrogenase
WCE	Whole cell extract
4NG	4-Nitrophenyl β -D-Glucuronide

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I would like to acknowledge my committee chair, Dr. Varykina Thackray, who has mentored me and provided me with extensive support with my research. I am very grateful for the opportunities and guidance that Dr. Thackray has given me, including introducing me to the San Diego chapter of the Association for Women in Science, and I have grown immensely as a scientist under her mentorship.

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ABSTRACT OF THE THESIS

Assessment of Gut Microbial Beta Glucuronidase Substrate Specificity in Sex Steroid
Metabolism

by

Lai Ying Ivy Tam

Master of Science in Biology

University of California San Diego, 2024

Professor Varykina Thackray, Chair
Professor Heidi Cook-Andersen, Co-Chair

Sex differences in the gut microbiome may play an important role in sex-biased physiological processes and diseases. Yet, the mechanisms behind sexual differentiation of the gut microbiome remain unclear. Previous research demonstrated that microbial β -glucuronidase (GUS) enzymes can metabolize glucuronidated estrogen, thus facilitating recirculation of the steroid via the enterohepatic circulation process. Studies have also shown differences in enzyme activity with estriol-glucuronide versus estradiol-glucuronide among gut microbial GUS enzymes, indicating that there is substrate specificity. However, there is a lack of studies exploring bacterial metabolism of testosterone-glucuronide, hindering our understanding of sexual differentiation in gut microbiota. To bridge this gap, I optimized a sex steroid-deconjugation coupled assay to evaluate GUS enzyme substrate specificity with estradiol- and testosterone-glucuronide.

The first part of my study involved assay optimization to evaluate the activity of GUS enzymes in various forms. Results showed that there was GUS enzyme activity from *E. coli*, bead-lysed whole cell extract, and in recombinant form with a 4-nitrophenol substrate. I also optimized the uronate dehydrogenase (UDH) assay and determined that the enzyme was most active at pH 7 and 37°C. Due to wave-length interference from *E. coli*, I found that only recombinant GUS enzyme had significant activity in the coupled assay with 0.5 mM estradiol-glucuronide. In the coupled assay, I demonstrated that the GUS enzyme had substrate specificity for estradiol-glucuronide compared to testosterone-glucuronide. My findings indicate that substrate specificity in gut microbial GUS enzymes may have a direct impact on sexual differentiation of the gut microbiota.

INTRODUCTION

The gut microbiome is associated with many diseases, including autoimmune, metabolic, and reproductive diseases^{1,4,13,24,27}. Changes in the gut microbial composition are observed in individuals with these diseases, often in a sex-biased manner. For instance, type-1 diabetes (T1D) is a microbiome-linked autoimmune disease²⁷. In a mouse model of T1D, nonobese diabetic (NOD) female mice are more susceptible to diabetes compared to male mice, while the sex bias is lost in germ-free mice¹⁵. Additionally, male mice that were castrated to remove the gonads exhibited susceptibility similar to female mice, suggesting that pathogenesis is likely linked to sex steroid levels¹⁴. Changes in the gut microbiota were also associated with susceptibility in the NOD model since fecal microbiota transplant (FMT) from male mice was shown to be protective against T1D in female mice¹⁵. These findings indicate that the gut microbiome is required for the sex bias in the NOD mouse model and that sex steroids are also involved in the pathogenesis of the disease.

Given the complex relationship between the gut microbiome and diseases with sex bias, it is vital to understand the interconnection between gut microbiome and sex steroids as it may provide valuable insights into personalized medicine and treatment for sex-biased health issues. 16S rRNA gene sequencing and metagenomic studies have revealed sex differences in the gut microbial composition between males and females. Host sex is considered one of the top ten contributors to microbial community variation⁷. In addition, males has been reported to have increased abundances in gut bacterial genera including *Prevotella*, *Megamonas*, *Fusobacterium*, and *Megasphaera*, whereas females have increased abundance in genera including *Bifidobacterium*, *Ruminococcus*, and *Akkermansia*²³. These differences in microbial composition

could potentially alter the metabolic functions of the host as gut microbes influence many host biological pathways and functions¹².

Sex differences in the gut microbiome become evident during puberty based on animal and human studies, likely influenced by sex steroid levels. In a dizygotic twins study, significant differences in gut microbial diversity were only observed in pubescent (age 13-17 years) twins of different sexes but not in infant (age 1-12 months) twins of different sexes²⁵. A similar phenomenon was also observed in mice where sex differences in gut microbial composition became noticeable after puberty and were the most significant in adult mice^{5,15,26}. More recently, a study utilizing a genetic hypogonadal mouse model showed that sex differences in microbial communities within the small and large intestine were largely dependent on the hypothalamic-pituitary-gonadal axis²⁰. Altogether, these findings strongly support the idea that activation of the reproductive axis during puberty is required for sex differences in the gut microbiota.

The relationship between sex steroids and the gut microbiome may be bidirectional to maintain homeostasis of sex hormones in the body²². Manipulation of the gut microbiota can result in changes in sex steroid levels. For example, oral gavage of male cecal content led to a significant increase in testosterone levels in female recipient mice¹⁵. While the gut microbiota can affect sex steroid levels, changes in sex hormone levels can also affect the microbial composition of the gut. While the sexes were not compared directly, gonadectomy in male or female mice altered the gut microbial composition^{2,8,10,11,17}. The effect caused by gonadectomy was shown to be reversible by administering testosterone or estrogen to gonadectomized mice^{8,11}, suggesting that sex steroids are sufficient to drive sex differentiation in the gut. Although many studies showed differentiation in the gut microbiome in terms of biological sex and sex steroids,

little is known about the mechanisms involved in sexual differentiation of the gut microbiome and how sex steroids influence gut microbial composition.

Commensal bacteria in the gut microbiome play a critical role in the enterohepatic circulation of sex steroids. Enterohepatic circulation refers to the cycling of sex steroids in the liver, bile duct, and small intestine. It is an important process that controls the balance of excretion of conjugated sex steroids versus reabsorption of deconjugated sex steroids into enterohepatic circulation (Figure 1). Sex steroids are conjugated with glucuronides by liver enzymes and then transported to the gallbladder and small intestine²¹. In the small intestine, glucuronidated sex hormones are deconjugated by a subset of microbes that possess a β -glucuronidase (GUS) enzyme, which results in reabsorption of deconjugated sex steroids into enterohepatic circulation²¹. Possessing functional GUS enzymes could potentially provide these gut microbes a competitive advantage in the gut environment, ultimately driving changes in gut microbial composition.

Previous studies have shown that deconjugation of estrogen is regulated by the GUS enzyme^{6,18,22}. Recent studies have also identified 249 putative GUS enzymes in the human gut microbiome with variable active sites on the enzyme, suggesting that microbial GUS enzymes possess different substrate specificity¹⁹. These active sites differ by the loop regions surrounding the site, affecting enzymatic processing of glucuronidated compounds of different sizes¹⁹. Substrate specificity was observed in 17 out of 35 GUS enzymes when deconjugating estrone-3-glucuronide and estradiol-17-glucuronide to estrone and estradiol, respectively⁶. These studies support the idea of differences in substrate specificity of GUS enzymes for steroid-glucuronide substrates. In addition, it has been shown that testosterone, the main sex hormone present in males, undergoes deconjugation in the gut intestinal tract, specifically in the

small intestine¹³. While the mechanisms regulating microbial metabolism of androgens is less clear, the similarity of the deconjugation process and molecular structure between estrogen and testosterone implies that GUS enzymes are also involved in the hydrolysis of conjugated testosterone.

By exploring the relationship between GUS enzymes and sex steroids, specifically estradiol and testosterone, I aim to investigate if the GUS enzyme has different substrate specificity for the two sex steroids which could provide a potential mechanism for sex differentiation of the gut microbiota. Since deconjugation of sex steroids cannot be directly measured in an enzymatic reaction, I optimized an in vitro sex steroid-deconjugation coupled assay based on a previously published in vitro estrogen-processing coupled assay⁶. The coupled assay involves using the GUS enzyme to deconjugate glucuronidated steroids, and uronate dehydrogenase (UDH) to generate an end-point measurement to indirectly measure GUS activity. I hypothesize that if sex steroids drive sexual differentiation in the gut microbiome, there will be differences in substrate specificity of microbial GUS enzymes with estradiol-glucuronide and testosterone-glucuronide.

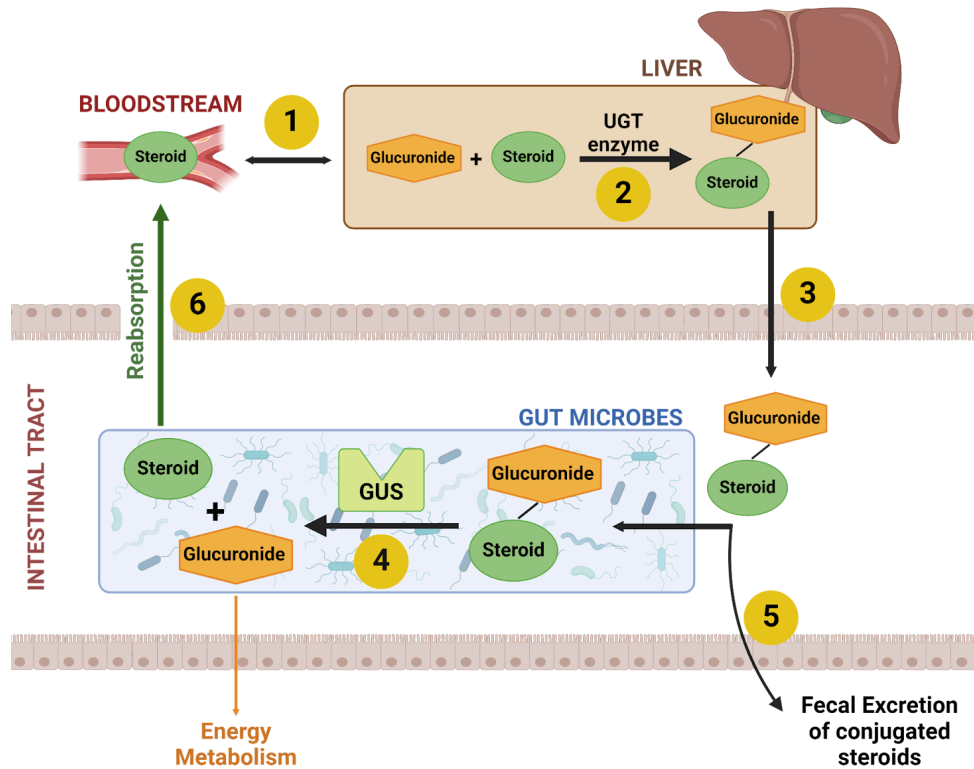


Figure 1. Enterohepatic circulation of sex steroids.

1) Sex steroids in the bloodstream travel to the liver. 2) UDP glucuronosyltransferases (UGT) in the liver conjugate sex steroids with glucuronides. 3) Conjugated steroids are transported into the small intestine via the bile duct, which are then either 4) metabolized and deconjugated by gut microbial β -glucuronidase (GUS) enzymes or 5) excreted from the body through feces. 6) Deconjugated sex steroids are reabsorbed back into the bloodstream and recirculate in the host body. Created with BioRender.com.

MATERIALS AND METHODS

Whole *E. coli* Culture Preparation

An overnight liquid culture of *E. coli* K-12 strain (MG1655) was prepared by inoculating 5 mL of Lysogeny broth (LB) (BioPioneer, #CMLP) with a single bacterial colony from a LB agar plate and incubated at 200 rpm, 37°C for 12-18 hours. A morning *E. coli* culture was prepared by inoculating 30 mL of LB with 300 µL of the overnight liquid culture and then incubated at 200 rpm, 37°C for 2-3 hours until OD600 reached 0.7-0.8.

***E. coli* Whole Cell Extract (WCE) Preparation with Glass Beads**

E. coli pellet was generated by centrifuging 8 mL of an *E. coli* culture grown to OD600 of 0.7-0.8 at 3220 RCF for 20 minutes in an Eppendorf Centrifuge 5810. The supernatant was then removed from the cell pellet using vacuum aspiration and the cell pellet was resuspended in 1 mL of assay buffer (50 mM HEPES-NaCl, pH 7). The *E. coli* Whole Cell Extract (WCE) was prepared by bead lysis method using 0.1 mm glass beads (Fisher Scientific, #50-136-7648) pre-chilled at 4°C prior to use. 600 µL of the resuspended *E. coli* pellet was added to a 2 mL Eppendorf tube containing 3 mg of glass beads which was agitated by a Qiagen Tissuelyzer II for 6 minutes at 30Hz. The homogenate was then centrifuged at 13000 RCF, at 4°C for 10 minutes and the supernatant was collected in a new 2 mL Eppendorf tube and centrifuged at 13000 RCF, at 4°C for another 10 minutes to ensure the WCE was free of glass beads and cell debris. The final supernatant was collected in a new 1.7 mL Eppendorf tube.

Bradford Assay for WCE

The total protein content of the *E. coli* WCE and efficacy of the lysis methods was calculated using a Bradford assay. A set of 1.5 mL Bovine Serum Albumin (BSA) standards were made and diluted for all experiments at concentrations of 1, 2, 5, 10 ug/mL using Lyophilized BSA (Fisher Scientific; #9048-46-8). 10 μ L of *E. coli* WCE, assay buffer blank (50 mM HEPES-NaCl, pH 7), and standards were added to 990 μ L of a 1/5 dilution of Protein Assay Dye Reagent Concentrate (Bio-Rad, #5000006) in a cuvette. Each cuvette was mixed 4-5 times with a pipette and bubbles were popped prior to spectrophotometer measurement. The cuvettes were incubated for 5 minutes at room temperature, and measured at 595 nm using a Bio-Rad SmartSpec Plus UV/VIS Spectrophotometer. Beer–Lambert Law was used to find the concentration of each sample from the slope and y-intercept of the BSA standard, and the absorbance values from the Bradford assays. Concentration of each sample was calculated in ug/ μ L to determine the total protein concentration.

β -glucuronidase 4NG Assay

A 2X 4-Nitrophenyl β -D-Glucuronide (4NG) (Sigma-Aldrich; #10344-94-2) stock solution was made at a concentration of 150 mM in assay buffer (50mM HEPES-NaCl buffer, pH 7). Recombinant *E. coli* β -glucuronidase was purchased as lyophilized powder (Sigma-Aldrich, #G8295) and reconstituted to 5 mg/mL in assay buffer (50mM HEPES-NaCl buffer pH7). Reconstituted β -glucuronidase enzyme was then diluted to 1:25 in assay buffer (50mM HEPES-NaCl buffer, pH 7). 50 μ L of whole *E. coli*, WCE, or diluted recombinant β -glucuronidase were plated with 50 μ L 2X 4NG (75 mM final) on a Corning® 96-well, Clear Flat Bottom, Polystyrene, Non-Treated Microplate (Corning®, #3370). Triplicate wells were

plated for each sample. The microplate was then placed in a TECAN Infinite 200 PRO® plate reader and absorbance was measured at 405 nm every minute over a 60 minute period at 37°C with 5 seconds shaking before each measurement. The change in absorbance relative to time zero was calculated from the equation: $\Delta Absorbance = Absorbance_{time} - Absorbance_{time 0}$.

Uronate Dehydrogenase (UDH) Assay

24kU of recombinant uronate dehydrogenase (UDH) was purchased in 8mL of 3.2 M ammonium sulfate solution (NZYTech, #AE00191). To evaluate optimal UDH enzyme concentration, serial dilutions of 1:25 (2.5U), 1:50 (1.2U), and 1:100 (0.6U) were performed by resuspending UDH in assay buffer (50 mM HEPES-NaCl buffer, pH 7). D-glucuronic acid (Sigma-Aldrich, #G5269) and beta-nicotinamide adenine dinucleotide (NAD⁺) (Thermo Fisher Scientific, #J62337-03) were also resuspended in assay buffer. 20 µL of diluted UDH was plated with a prepared master mix consisting of D-glucuronic acid (20 mM) and NAD⁺ (20 mM), all diluted in assay buffer in 100 µL total volume per well on a Corning® 96-well, Clear Flat Bottom, Polystyrene, Non-Treated Microplate. Triplicate wells were plated for each sample. The microplate was placed in a TECAN Infinite 200 PRO® plate reader and absorbance was measured at 340 nm every minute over a 60 minute period at 37°C with 5 seconds shaking before each measurement. The change in absorbance relative to time zero was calculated from the equation: $\Delta Absorbance = Absorbance_{time} - Absorbance_{time 0}$.

To evaluate optimal pH concentration, a dilution of 1:100 (0.6U) was performed on the UDH enzyme. 20 µL of diluted UDH was plated with a prepared master mix consisting of D-glucuronic acid (20 mM) and NAD⁺ (20 mM), all diluted in assay buffer at various pH (pH 5 - pH 7) in 100 µL total volume per well on a Corning® 96-well, Clear Flat Bottom, Polystyrene,

Non-Treated Microplate. Triplicate wells were plated for each sample. The microplate was placed in a TECAN Infinite 200 PRO® plate reader and absorbance was measured at 340 nm every minute over a 60-minute period at 37°C with 5 seconds shaking before each measurement. The change in absorbance relative to time zero was calculated from the equation:

$$\Delta Absorbance = Absorbance_{time} - Absorbance_{time\ 0}.$$

Sex steroid-deconjugation coupled assay

Estradiol-glucuronide stock solution (5 mM) was prepared by reconstituting β -estradiol 17-(β -D-glucuronide) sodium salt (Sigma-Aldrich, #15087-02-2) in HyPure molecular biology grade water (HyClone, #SH30538.02) while testosterone-glucuronide stock solution (5 mM) was prepared by reconstituting 4-Androsten-17 β -OL-3-ONE Glucosiduronate (Steraloids Inc., #A6980-000) in HyPure molecular biology grade water. UDH was diluted 1:100 (0.6U) in assay buffer (50 mM HEPES-NaCl, pH 7). The assays were conducted in a 200 μ L total volume. 1:25 diluted recombinant *E. coli* β -glucuronidase (51.1U) was plated with reaction master mix consisting of 20 μ L of diluted UDH (0.6U), 2 μ L of NAD⁺ (2 mM final), and 20 μ L of estradiol-glucuronide or testosterone-glucuronide (0.5 mM final), all diluted in assay buffer (50mM HEPES-NaCl buffer, pH 7) on a Corning® 96-well, Clear Flat Bottom, Polystyrene, Non-Treated Microplate (Figure 2). Triplicate wells were plated for each sample. The microplates were placed in a TECAN Infinite 200 PRO® plate reader and absorbance was measured at 340 nm every minute over a 90-minute period at 37°C with 5 seconds shaking before each measurement. The change in absorbance relative to time zero was calculated from the equation: $\Delta Absorbance = Absorbance_{time} - Absorbance_{time\ 0}$.

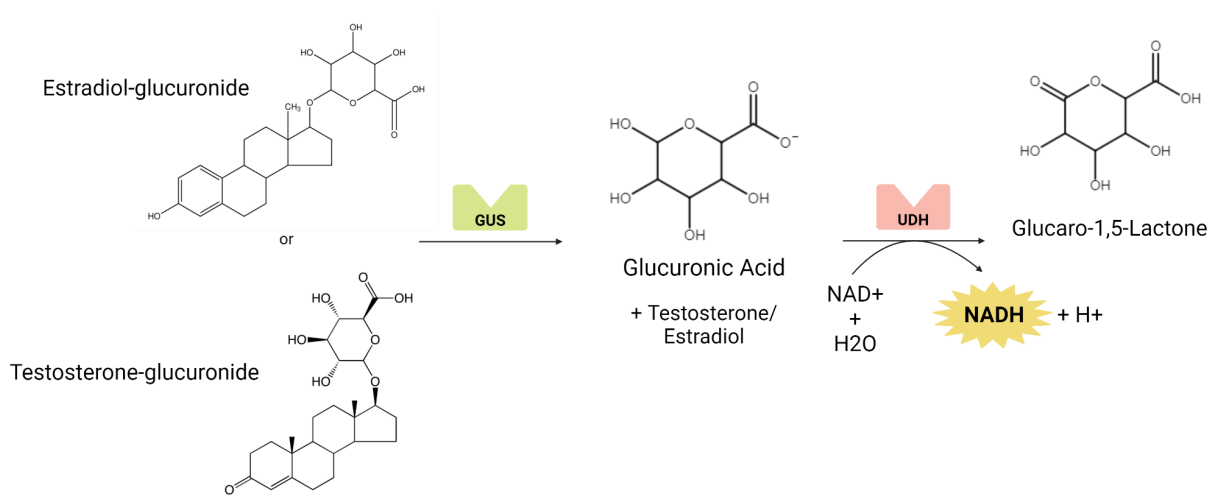


Figure 2. β-glucuronidase (GUS)/Uronate dehydrogenase (UDH) Coupled Assay reaction with estradiol-glucuronide or testosterone-glucuronide. Created with BioRender.com.

Quantification of protein concentration in reconstituted *E. coli* β-glucuronidase

Beer–Lambert Law was used to find the concentration of recombinant *E. coli* β-glucuronidase from the slope and y-intercept of the BSA standard and the absorbance values from the Bradford assays. Concentration of enzymes in each replicate well was calculated in ug/μL and converted to mg/50 μL to determine the concentration in each 50 μL plated well.

Quantification of β-glucuronidase Activity in Coupled Reaction

Beer–Lambert Law was used to calculate the amount of product in mM from the averaged absorbance measurement at each timepoint using the standard curve of β-Nicotinamide adenine dinucleotide, reduced (NADH). The mM products were then converted to μmole. The slope was calculated for the first 20 minute and 60 minute for the reactions with estradiol-glucuronide and testosterone-glucuronide, respectively to determine μmol/min in each reaction. The slope from the β-glucuronidase assay (μmol/min) was divided by the total protein (mg/50 μL) to determine enzyme specific activity (U/mg protein).

Statistical Analysis

The mean and standard error of β -Glucuronidase specific activity for the sex steroid treatments are shown in figure 8. A two-sample t-test ($n=4/\text{group}$) showed statistical significance between the treatment groups, with a p-value of 0.002.

RESULTS

URONATE DEHYDROGENASE (UDH) ASSAY OPTIMIZATION

To employ the sex steroid-deconjugation coupled assay, I first optimized the second reaction which involved UDH performing an oxidation-reduction reaction with glucuronic acid and NAD⁺ to generate an end-point measurement (Figure 2). Figure 4 shows the change in absorbance at 340 nm over 30 minutes from UDH assays with various conditions. While the UDH enzyme at 1:25 and 1:50 dilutions had enzymatic activity, UDH enzyme with 1:100 dilution displayed an optimal curve when reacted with D-glucuronic acid (20 mM) and NAD⁺ (20 mM) at pH 7 and 37°C (Fig 3A). Additionally, pH 7 exhibited an optimal curve in the UDH pH assay compared to pH 5-6 (Fig 3B). As a result, pH 7 and UDH enzyme concentration of 1:100 dilution were employed in the sex steroid-deconjugation coupled assay.

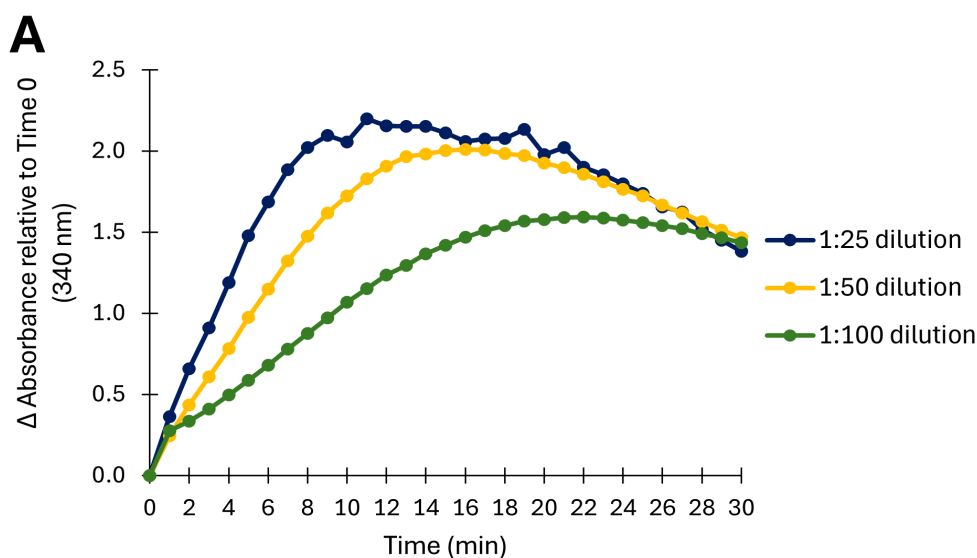


Figure 3. Uronate dehydrogenase (UDH) assay with various conditions.

(A) UDH assay with diluted UDH enzyme. 20 μ L of diluted UDH enzyme, e.g. 1:25 (2.5U), 1:50 (1.2U) and 1:100 (0.6U) was used. Change in absorbance was measured over 30 minutes at 340 nm. (B) UDH assay at various pH. 20 μ L of 1:100 (0.6U) diluted UDH enzyme was used. Change in absorbance was measured over 30 minutes at 340 nm.

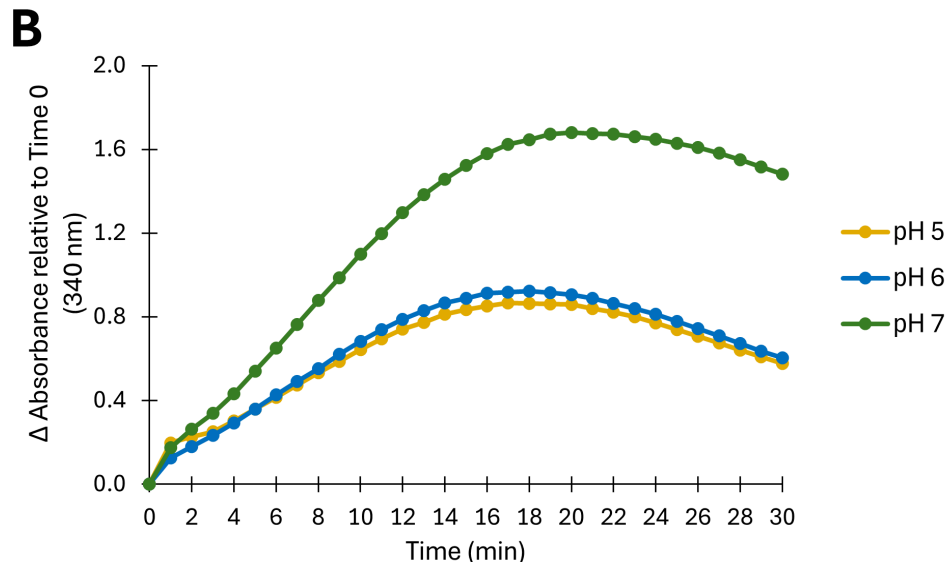


Figure 3. Uronate dehydrogenase (UDH) assay with various conditions, continued

(A) UDH assay with diluted UDH enzyme. 20 uL of diluted UDH enzyme, e.g. 1:25 (2.5U), 1:50 (1.2U) and 1:100 (0.6U) was used. Change in absorbance was measured over 30 minutes at 340 nm. (B) UDH assay at various pH. 20 uL of 1:100 (0.6U) diluted UDH enzyme was used. Change in absorbance was measured over 30 minutes at 340 nm.

β-GLUCURONIDASE (GUS) 4NG ASSAY OPTIMIZATION

Because *E. coli* absorbs at a similar wavelength as NADH, I tested whether recombinant GUS enzyme from *E. coli* and bead-lysed WCE exhibited enzymatic activity in a β-glucuronidase (GUS) 4NG assay with *E. coli* as a positive control (Figure 4-5). GUS enzyme in *E. coli* showed the highest enzyme activity compared to the other two forms of GUS enzymes, with a plateau change in absorbance values of OD 0.3 to 0.4 (Fig 4). In comparison, GUS recombinant enzyme and bead-lysed WCE exhibited enzyme activity with a plateau of absorbance values of OD 0.1 to 0.2 (Figure 4-5). The activity of the WCE obtained from sonication exhibited potential degradation in product after 8 minutes, indicating that this type of WCE was not a viable option for the coupled assay (Figure 5). The control groups, which

contained all components of the coupled reaction except for the GUS enzyme, exhibited minimal to no activity (not shown in graph).

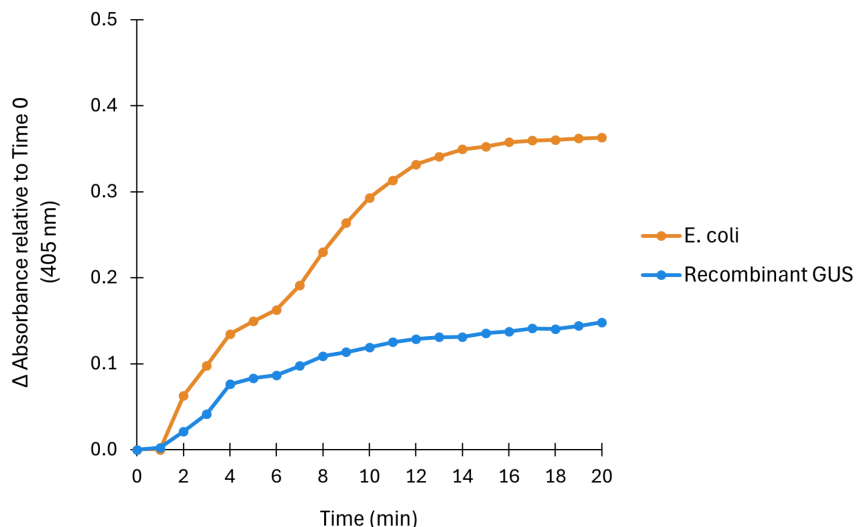


Figure 4. Change in absorbance over time in β -glucuronidase (GUS) 4-nitrophenyl beta-D-glucuronide (4NG) assay with GUS enzymes in *E. coli* (orange) and in recombinant form (blue). 50 μ L of GUS enzymes in *E. coli* or 5 μ L of GUS enzymes in recombinant form were reacted with 4NG substrate (75 mM) at pH 7 and 37°C. Measured over 20 minutes at 405 nm.

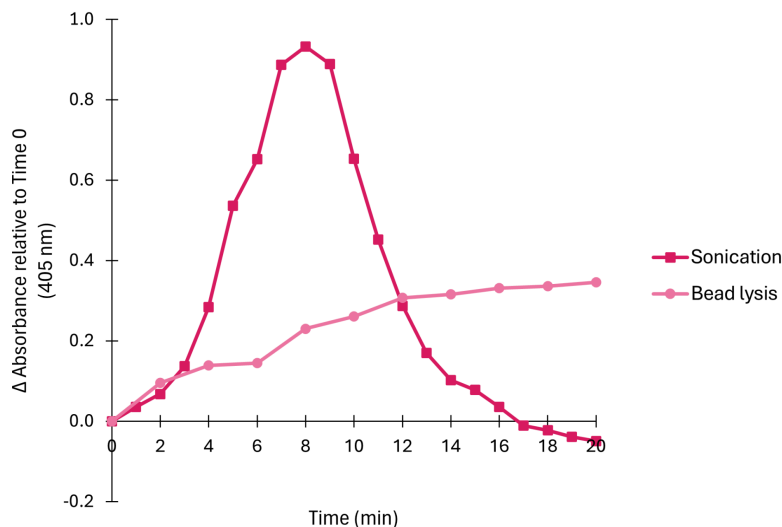


Figure 5. Change in absorbance over time in β -glucuronidase 4-nitrophenyl beta-D-glucuronide (4NG) assay with *E. coli* WCE. *E. coli* was bead-lysed for 6 minutes (circle, light pink) or probe-sonicated for 2 minutes (square, dark pink) to generate WCE. 50 μ L of GUS in WCE was reacted with 4NG substrate (75 mM) at pH 7 and 37°C. Measured over 20 minutes at 405 nm.

SEX STEROID-DECONJUGATION COUPLED ASSAY OPTIMIZATION

Once I had optimized conditions for the GUS and UDH enzyme assays, GUS enzyme in *E. coli*, recombinant form, and bead-lysed WCE were used in the sex steroid-deconjugation coupled assay to determine GUS enzyme activity with steroid-glucuronide substrates. Among the three forms of GUS enzymes, only the GUS enzyme in recombinant form exhibited detectable activity in the coupled reactions with 1 mM of estradiol-glucuronide (E2-gluc), while the GUS enzyme in *E. coli* and the WCE generated from bead lysis showed minimal to no activity based on the change in absorbance values relative to time zero over 60 minutes (Figure 6).

To evaluate whether the assay conditions included excess substrate concentration for the sex steroid-deconjugation coupled assay, recombinant GUS enzyme was diluted to various concentrations and reacted with 0.5 mM E2-gluc (Figure 7). 1:10, 1:25 and 1:50 diluted recombinant GUS enzymes all showed enzyme activity, indicating that 0.5 mM concentration was an excess of substrate in the coupled assay (Figure 7).

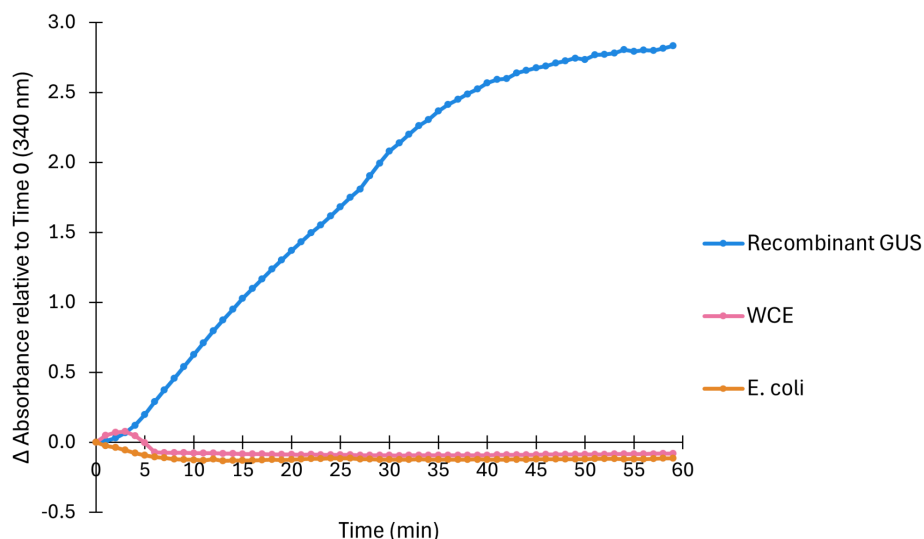


Figure 6. Change in absorbance over time in 1 mM of estradiol-glucuronide coupled reaction with β -glucuronidase (GUS) in recombinant form (blue), *E. Coli* WCE (pink), and *E. coli* (orange). 50 μ L of GUS in various forms were plated in each well. Change in absorbance measured over 60 minutes at 340 nm.

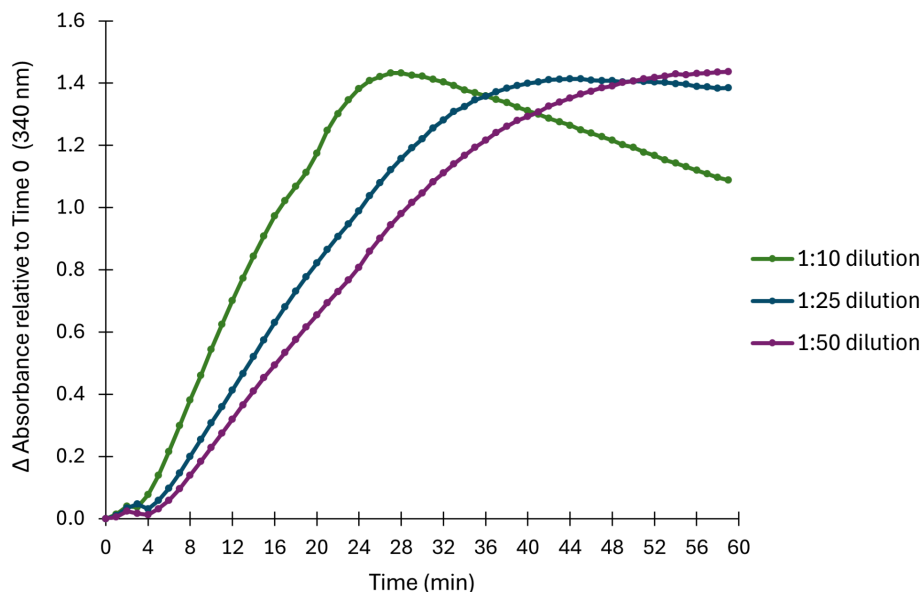


Figure 7. Reaction in substrate excess with diluted recombinant GUS enzyme in coupled reaction with 0.5 mM estradiol-glucuronide. 50 μ L of 1:10 diluted (green), 1:25 diluted (blue), and 1:50 diluted (purple) recombinant GUS in were plated in each well. Change in absorbance measured over 60 minutes at 340 nm.

NADH STANDARD CURVE

Figure 8 shows the standard curve of NADH generated by measuring absorbance of NADH at different concentrations. The path length of the Corning® 96-well, Clear Flat Bottom, Polystyrene, Non-Treated Microplate, was calculated using the Beer's-Lambert Law and absorbance values of NADH between 0.2 mM and 0.5 mM. The average path length generated was 0.591cm and was used to determine concentration of deconjugated sex-steroid products in mM in the sex steroid-deconjugation coupled assay.

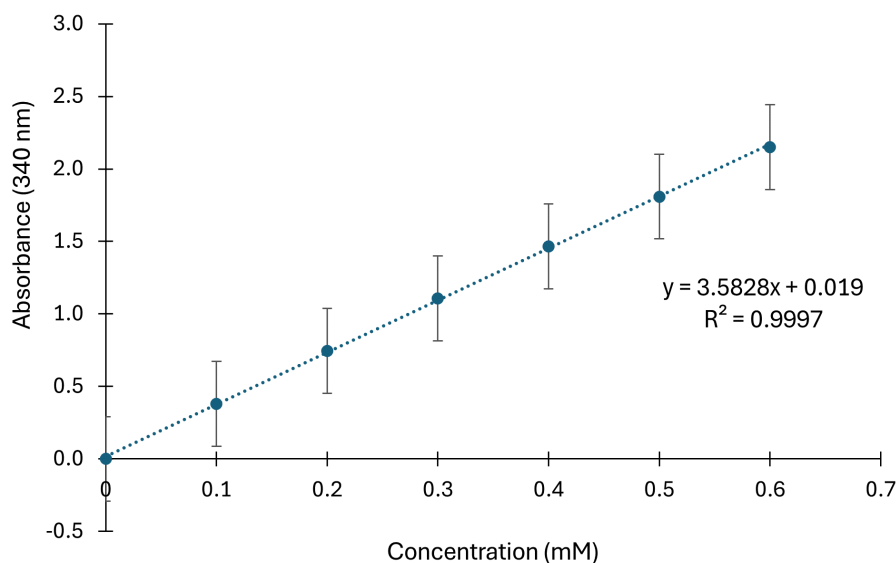


Figure 8. β -Nicotinamide adenine dinucleotide, reduced (NADH) standard curve.

Beer's–Lambert Law was used for 96-well microplate path length calculation. Calculated path length = 0.591cm. Measured at 340 nm.

SEX STEROID-DECONJUGATION COUPLED ASSAY

Once I optimized the conditions for the sex steroid-deconjugation coupled assay, recombinant GUS enzyme was reacted with either 0.5 mM testosterone glucuronide (T-gluc) or estradiol-glucuronide (E2-gluc) in 4 biological replicates performed on different days. The change in enzyme units (ΔU) was calculated using Beer's-Lambert law and the calculated concentration of deconjugated sex-steroid products in mM in the sex steroid-deconjugation coupled assay. The control groups, which contained all components of the coupled reaction except for the GUS enzyme, exhibited minimal to no activity (Figure 9). While E2-gluc results were a bit more variable, the ΔU over time in the coupled reactions with E2-gluc were consistently higher than that with T-gluc (Figure 9). The E2-gluc coupled reaction also plateaued at 0.07 $\mu\text{mol}/\text{min}$ to 0.08 $\mu\text{mol}/\text{min}$ at around 25 minutes whereas T-gluc coupled reaction plateaued at around 0.03 $\mu\text{mol}/\text{min}$ to 0.04 $\mu\text{mol}/\text{min}$ at around 60 minutes (Figure 9). To

calculate specific activity (U/mg) for the GUS enzyme in the coupled reaction, the slopes of ΔU versus time curves were generated (Figure 10). The slopes of E2-gluc coupled reactions were generated from ΔU values between 6-20 minutes (Figure 10A) while the slopes of T-gluc coupled reactions were generated from ΔU values between 6-60 minutes (Figure 10B). Data points before 6 minutes were excluded (Figure 10).

The specific activity (U/mg) for the GUS enzyme with the two substrates was calculated by dividing the slopes from each reaction (Figure 10) with the GUS protein concentration as determined in a Bradford assay. Figure 11 shows the mean and standard deviation of the GUS specific activity for the E2-gluc and T-gluc coupled reactions. A two-sample t-test was performed on the data and a *p*-value of 0.002 was obtained, indicating that there was a statistical significance between the two reactions with different sex steroid-glucuronides (Figure 11).

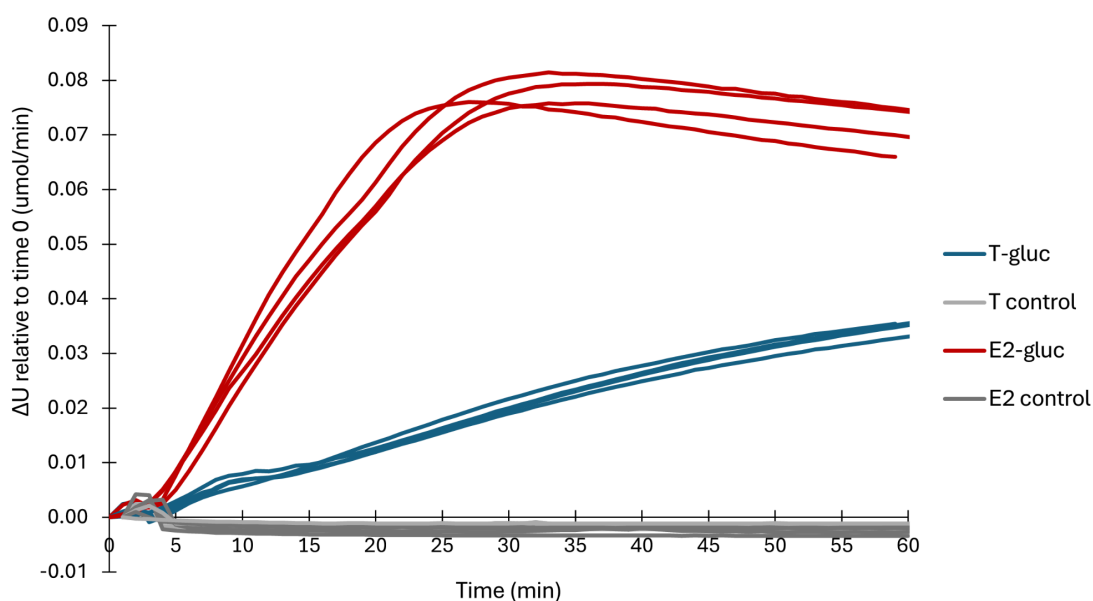


Figure 9. β -glucuronidase/UDH Coupled Assay with 1:25 diluted recombinant GUS enzyme (51.1U). Change in enzyme units (ΔU) was calculated and measured over 60 minutes at 340 nm. T-gluc, Testosterone-glucuronide (blue); T control, Testosterone-glucuronide negative control (light gray); E2-gluc, Estradiol-glucuronide (red); E2 control, Estradiol-glucuronide negative control (dark gray). N=4/group.

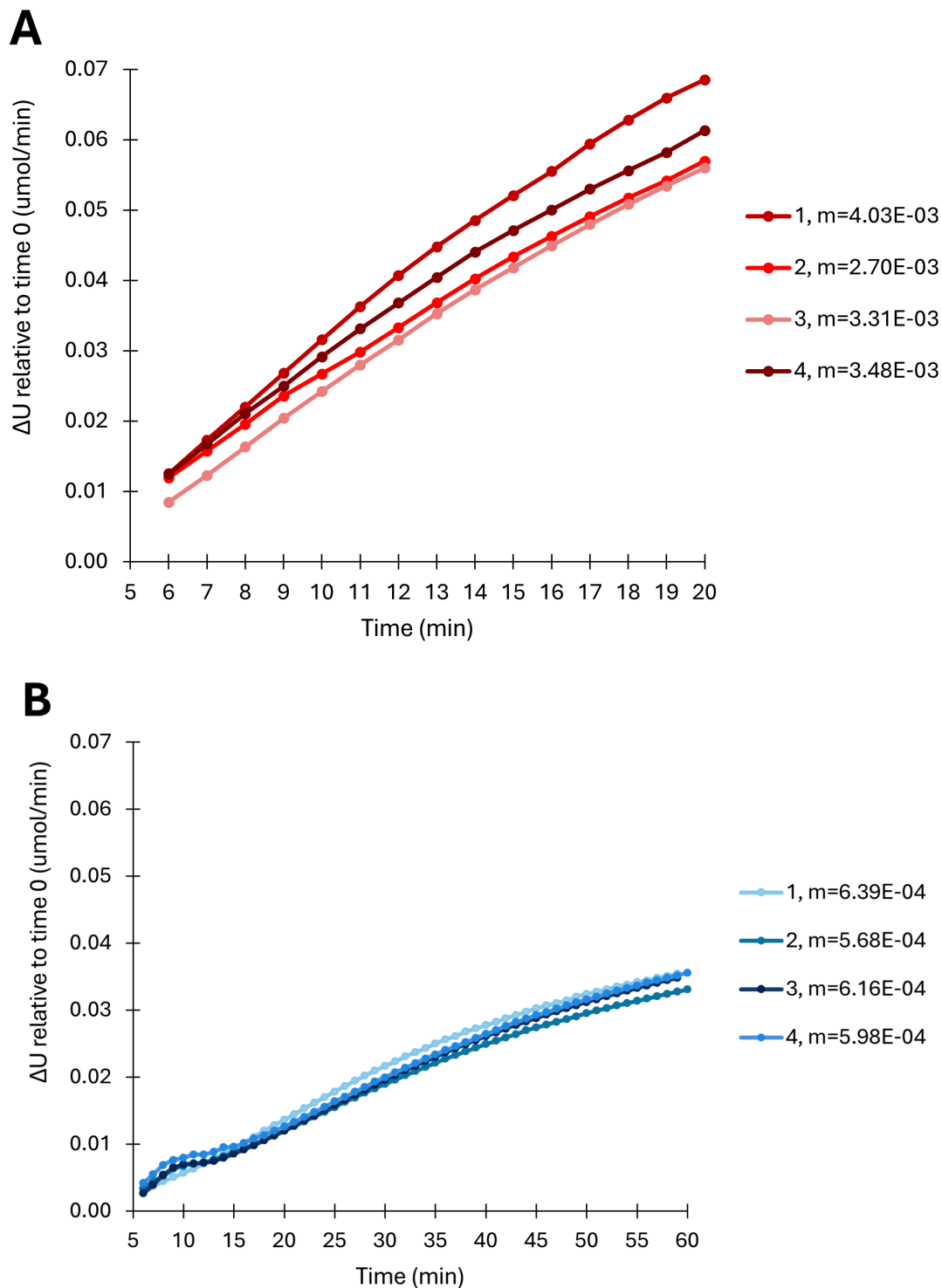


Figure 10. Sex Steroid-deconjugation Coupled Assay with 0.5 mM Sex Steroid-glucuronide. (A) Coupled assay with 0.5 mM estradiol-glucuronide. Slope (m) generated for data points between 6-20 minutes for 4 biological replicates. (B) Coupled Assay with 0.5 mM testosterone-glucuronide. Slope (m) generated for data points between 6-60 minutes for 4 biological replicates.

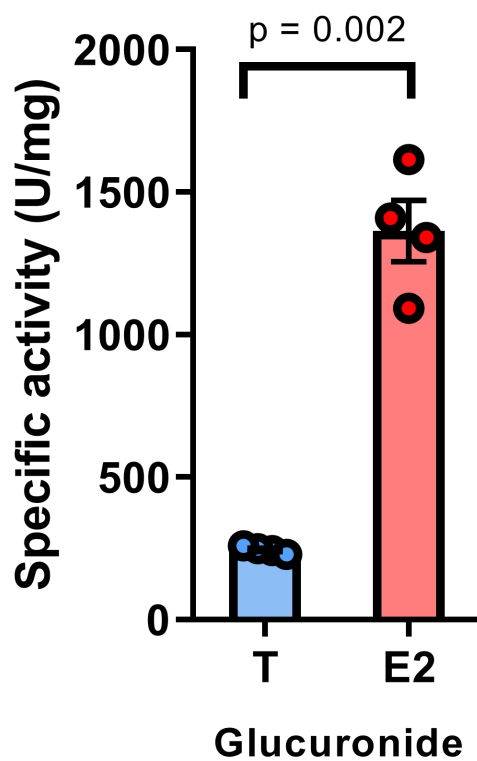


Figure 11. Specific activity of β -glucuronidase (GUS) enzyme in coupled reaction with 0.5 mM of testosterone-glucuronide (T) and estradiol-glucuronide (E2). Bars represent the mean $\pm$ S.E. for each group (N=4/group). *P*-value was determined by a two-sample t-test ($p=0.002$).

DISCUSSION

My study showed that recombinant GUS enzyme purified from *E. coli* (K12 strain) successfully deconjugated estradiol- and testosterone-glucuronide and this enzyme activity could be detected indirectly using a sex steroid-deconjugation coupled assay that I optimized in the lab based on an assay previously described⁶. More specifically, *E. coli* GUS enzyme showed substrate specificity for estradiol-glucuronide over testosterone-glucuronide. These findings indicate that a mechanism for sex differences in the gut microbiome may involve a direct effect of glucuronidated sex steroids on gut microbial composition due to deconjugation by specific gut bacteria that possess a GUS enzyme.

There are a lack of studies on the interaction between glucuronidated androgens and microbial GUS enzymes. My studies showed that *E. coli* (K12 strain) recombinant GUS enzyme can metabolize testosterone-glucuronide, with the caveat that this strain of *E. coli* does not typically occur in the gut. This result implies that gut *E. coli* that possesses a GUS gene that can deconjugate androgen-glucuronides and is in agreement with a previous study that showed urinary androsterone-glucuronide undergoing a hydrolysis process with *E. coli* GUS enzyme⁹. Additionally, a study employing a similar coupled assay to evaluate substrate specificity of GUS enzymes for estrogen-glucuronides showed a ten-fold higher substrate specificity for estrone-glucuronide compared to estradiol-glucuronide with a purified GUS enzyme from a gut *E. coli* strain⁶. Altogether, these results suggest that minor differences in the structure of glucuronidated compounds could lead to large differences in GUS enzyme substrate specificity.

Substrate specificity is most likely related to the varying loop regions flanking the active sites on the GUS enzyme¹⁹. These loop regions have been classified into 6 groups: loop 1 (L1), mini-loop 1 (mL1), loop 2 (L2), mini-loop 2 (mL2), mini-loop 1,2 (mL1,2), and no loop (NL)¹⁹.

E. coli GUS enzymes have been previously identified to have L1 where they are capable of processing smaller glucuronide substrates, such as estrogen-glucuronides, more effectively compared to other types of loop regions¹⁹. Given these findings, future directions could look into how these loop regions interact with testosterone-glucuronide or other glucuronidated steroids to further understand how gut microbial GUS enzymes interact with these important molecules in our body. To gain more understanding of sex steroid metabolism by microbial GUS enzymes, future research should also investigate substrate specificity for other steroid hormones, for instance dihydrotestosterone versus testosterone, or progesterone versus estrogen.

One limitation of my study is that I did not perform the sex steroid-deconjugated coupled assay with GUS enzymes purified from anaerobic gut *E. coli* strains which will be crucial to gain a better understanding of how this microbial enzyme works in the gut environment and in which gut compartments it is active. Depending on the loop regions of the GUS enzymes, the enzyme could reside intracellularly or in the periplasmic space¹⁹. L1 GUS enzymes, such as in *E. coli*, had been shown to be more likely residing intracellularly as they do not contain an N-terminal signal sequence for transportation to the periplasmic space¹⁹. Given that the enzyme is likely intracellular, it is not clear why I did not observe any activity in the sex steroid-deconjugation coupled assay with the WCE obtained with bead lysis, especially since I observed activity in the 4NG assay with the WCE.

In conclusion, the sex steroid-deconjugation coupled assay could serve as a useful tool to investigate gut microbe metabolism in the context of different glucuronidated substrates. The findings from this study provide insight into the relationship between sex steroids and the gut microbiome. Future studies should investigate if glucuronidated sex steroids act as carbon source to promote the growth of bacteria that possess the GUS enzyme. It would also be beneficial to

explore if glucuronidated sex steroids can directly affect the composition of the gut microbial community in vivo and in vitro, to investigate whether possessing functional GUS enzymes could potentially provide gut microbes a competitive advantage in the gut environment, ultimately driving changes in gut microbial composition. It is also worth exploring if inhibition of GUS enzymes in vivo could be used to alter microbial metabolism of steroids as a therapeutic approach to decrease circulating steroid levels. Understanding this complex relationship may improve development of personalized medicine and treatment for sex-biased health issues related to sex steroid levels. Additionally, it may aid in understanding the health implications of hormone replacement therapy..

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