



Evaluating the effect of substrate competition on the detoxification rate of glycine conjugation

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DECLARATION

I hereby declare that this thesis is the result of my own independent work and has not been submitted for any degree of other qualification at any other university. Where I have used to work of others, this has been appropriately acknowledged and referenced.

This thesis was conducted in accordance with the North-West University guidelines for academic integrity and ethical research conduct.

Name: Christo Botha

Signature:

A handwritten signature in black ink, appearing to be 'C. Botha', with a horizontal line extending from the end.

Date: 30/05/2025

² *It is the glory of God to conceal a thing: but the honour of kings is to search out a matter. (Proverbs 25:2)*

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²³*And whatsoever ye do, do it heartily, as to the Lord, and not unto men;* ²⁴*Knowing that of the Lord ye shall receive the reward of the inheritance: for ye serve the Lord Christ" (Col. 3:23-24)*

ABSTRACT

Detoxification is a critical metabolic process that enables cells to manage and eliminate potentially harmful compounds. Among the various pathways involved, glycine conjugation plays a supporting yet significant role in handling excess acyl-CoA metabolites, particularly those arising under metabolic stress. This pathway involves the mitochondrial enzyme glycine N-acyltransferase (GLYAT), which facilitates the conjugation of glycine with acyl-CoA substrates, aiding in their conversion into more water-soluble forms that can be excreted from the body.

The aim of this study was to investigate how competition between substrates affects the efficiency of this conjugation process. To do this, human GLYAT was expressed recombinantly in *Escherichia coli*, and its enzymatic activity was validated using a colorimetric assay. Throughout the expression process, multiple protocol parameters, such as inducer type, temperature, and lysis conditions were optimized to improve solubility and yield of the active protein.

In order to analyze product formation in detail, a ^1H -NMR-based method was developed and validated. This technique allowed for accurate quantification of glycine conjugates, while also addressing common analytical challenges such as spectral interference and variability between replicates. Using this approach, substrate competition experiments were performed with benzoyl-CoA, phenylacetyl-CoA, and isovaleryl-CoA. The results showed distinct patterns of competition and inhibition, indicating that substrate availability can strongly influence GLYAT activity.

These findings not only provide a robust framework for studying enzyme kinetics in the context of detoxification but also offer insight into the biochemical dynamics relevant to disorders like isovaleric acidemia, where glycine conjugation may play a compensatory therapeutic role.

Key terms

Glycine conjugation, GLYAT, detoxification, recombinant expression, ^1H -NMR, acyl-CoA substrates, isovaleric acidemia, substrate competition

LIST OF ABBREVIATIONS

°C	Degrees Celsius
CoA	Coenzyme A
CV%	Coefficient of Variation
DMPA	Dimethylphenylalanine
g	Gram
GLYAT	Glycine N-acyltransferase
GLYATL1	Glycine N-acyltransferase-like 1
HA	Hippuric Acid
His-tag	Polyhistidine tag (typically 6x His)
HRP	Horseradish Peroxidase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IVA	Isovaleric Acidemia
IVD	Isovaleryl-CoA Dehydrogenase
kU	Kilo Units (1,000 enzyme activity units)
L	Liter
mL	Millilitre
mM	Millimolar
m/v	Mass per volume
µg	Microgram
µL	Microliter
µM	Micromolar
NMR	Nuclear Magnetic Resonance
OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
RPM	Revolutions Per Minute
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TSP	Trimethylsilyl Propionate
UFC	Ultra Filtration Cartridge
v/v	Volume per volume
w/v	Weight per volume
×g	Relative Centrifugal Force (times gravity)

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CHAPTER 1 INTRODUCTION

1.1 Introduction

Metabolic detoxification is a vital cellular process responsible for converting potentially harmful compounds into more water-soluble and excretable products. The glycine conjugation pathway is one of many detoxification pathways and plays a crucial but underappreciated role, especially in neutralizing carboxylic acid-containing metabolites that are not easily handled by other detoxification pathways (Grant, 1991, Knights and Miners, 2011). This process involves the conjugation of acyl-CoA intermediates with glycine, catalyzed by the mitochondrial enzyme glycine N-acyltransferase (GLYAT, EC:2.3.1.71)(Vetting et al., 2005).

One of the unique characteristics of glycine conjugation is its flexibility in handling a wide range of endogenous and exogenous acyl-CoA substrates, including those that accumulate under pathological conditions. In this way, glycine conjugation can act as a metabolic “safety valve”, providing an alternate route for detoxification when primary pathways such as β -oxidation are impaired or overwhelmed (Badenhorst et al., 2013a). This auxiliary role becomes especially important when toxic intermediates build up due to inborn errors of metabolism or environmental overload (Heindel et al., 2017). Under such circumstances, the glycine conjugation pathway may help reduce the burden on the liver and mitochondria by enabling the excretion of these compounds in conjugated, less harmful forms.

One example where glycine conjugation could serve as a therapeutic alternative is isovaleric acidemia (IVA), a rare inborn error of metabolism. IVA is caused by a deficiency in isovaleryl-CoA dehydrogenase (IVD), resulting in the accumulation of isovaleryl-CoA, a potentially toxic metabolite derived from leucine catabolism (Vockley and Ensenauer, 2006b). Studies have suggested that GLYAT may conjugate isovaleryl-CoA with glycine to form isovalerylglycine, a less toxic compound excreted in urine (Tanaka et al., 1966, Kühn et al., 2023). However, this hypothesis remains insufficiently tested.

A key challenge in studying this pathway is the limited information on substrate competition, especially when multiple acyl-CoA substrates are present (Schulke and Sass, 2021, van der Sluis et al., 2017a). This is significant, as humans are exposed to a wide array of xenobiotic and endogenous substrates throughout their lifetime(Walker et al., 2019). Understanding whether certain substrates inhibit or outcompete others during glycine conjugation is essential for predicting detoxification efficiency and possible therapeutic applications.

To address this knowledge gap, the aim of this study was to evaluate the effect of substrate competition on the detoxification rate of glycine conjugation. This was achieved through the recombinant expression of human GLYAT, verification of enzymatic activity using Ellman's assay (Ellman, 1959a), and the development of a ^1H -NMR-based method for quantifying glycine conjugation products.

By providing a reproducible method for assessing substrate competition in glycine conjugation, this study contributes toward a deeper understanding of detoxification mechanisms, particularly in the context of inborn errors of metabolism such as IVA.

1.2 Dissertation outline

1.2.1 Chapter 2: Literature review.

This chapter aims to explore the role of detoxification in metabolic health, the pathology of isovaleric acidemia, and the functional biochemistry of GLYAT and its paralogues. This is done to provide background context and give motivational reasoning behind the study.

1.2.2 Chapter 3: Recombinant expression of GLYAT.

Chapter 3 revolves around the method optimization of expressing recombinantly active GLYAT enzymes. In this section the reader can see the logical flow of trouble shooting and the improvement in expression results. This chapter ends with an Ellman's assay where the GLYAT enzyme activity is measured using preferred substrates.

1.2.3 Chapter4: Developing a ^1H -NMR method to evaluate substrate competition in glycine conjugation.

Chapter 4 outlines the development and validation of a quantitative NMR method to analyze glycine conjugation, focusing on establishing detection limits and assessing reproducibility. This encompasses the main objective of the study and investigates the influence of substrate competition using benzoyl-CoA, phenylacetyl-CoA, and isovaleryl-CoA as competing substrates in the glycine conjugation reaction.

1.2.4 Chapter 5: Conclusion and future prospects.

Chapter 5 discusses the implications of the findings, limitations of the study, and suggestions for future research.

CHAPTER 2 LITERATURE REVIEW.

2.1 Introduction

Metabolic detoxification plays a fundamental role in maintaining cellular homeostasis and protecting the body from toxic metabolites and xenobiotics. This intricate process involves multiple phases of biotransformation, where harmful compounds are converted into less toxic, more excretable forms (Brodie et al., 1955b). Among these, the glycine conjugation pathway is particularly significant due to its role in neutralizing acyl-CoA metabolites (Badenhorst et al., 2013a). Dysfunction in detoxification pathways can lead to multifactorial diseases, worsened by the accumulation of toxic intermediates, highlighting the clinical importance of understanding these processes (Heindel et al., 2017).

One such condition where impaired detoxification has profound effects is isovaleric acidemia (IVA), an inborn error of metabolism characterized by the inability to properly break down the amino acid leucine (Vockley and Ensenauer, 2006b, Tanaka et al., 1966). This results in the buildup of isovaleric acid, leading to severe metabolic crises. Recent studies suggest that the glycine conjugation pathway could provide a therapeutic avenue for managing IVA by facilitating the detoxification of isovaleric acid through the formation of isovalerylglycine (Rinaldo et al., 1991).

In this review I explore the cause of Isovaleric acidemia and its relationship to the glycine conjugation pathway. I then discuss the glycine *N*-acyltransferase enzyme (GLYAT), which is responsible for catalysis in the glycine conjugation pathway. Section 2.2 is a short summary on detoxification as a defence mechanism followed by a discussion on Isovaleric acidemia in section 2.3. Section 2.4 will focus on the glycine conjugation pathway with a closer look at the GLYAT enzyme. Lastly, I will highlight a problem statement for my study derived from the topics that are mentioned.

2.2 Biotransformation and detoxification: A metabolic defense strategy

The concept of detoxification in the human body dates back to early medicine, but its underlying mechanisms were not fully understood. It was not until the 19th century, with major advancements in fields like physiology and organic chemistry, that scientists were able to identify toxins and metabolic byproducts from the urine of patients. This supported the first idea of the body's ability to absorb, transform and lastly excrete metabolic compounds as seen fit (Fortney et al., 2018).

Key discoveries later in the 20th century would lead to a rapid advancement in our understanding of metabolic detoxification. For instance, Bernard B. Brodie and colleagues were one of the first to show how drugs were able to undergo enzymatic transformation in the body. This research laid the groundwork for understanding how enzymes metabolize and detoxify xenobiotic substances (Brodie et al., 1955a).

Advancements in the field of metabolism and detoxification has become a necessity as it is estimated that humans are exposed to roughly two million xenobiotic compounds in their lifetime (Patterson et al., 2010). Not only is the “detoxification system” of our body under immense pressure from all these xenobiotic compounds, but diseases that alter the human metabolism and its capability of detoxification are also becoming more prevalent (Heindel et al., 2017).

As our understanding of the human metabolome and its detoxification methods increases, it becomes increasingly evident that biotransformation is a fundamental process in human metabolism. Depending on which stage of metabolism is being examined, biotransformation can be divided into various steps; nevertheless, the enzymes that mediate the reactions are a key component in all of them (Beiras, 2018).

The human metabolism can also be viewed as extremely delicate, and when subjected to massive changes, such as xenobiotic overload or systematic flaws, it can crumble and have serious consequences. This is why detoxification is so crucial to metabolism. It is the primary line of defence for clearing the body of metabolites that accumulate as a result of improper functioning.

2.2.1 Detoxification and diseases.

Because our bodies' metabolisms are so diverse and fragile, when subjected to a wide range of drastically varied settings, this "perfect balance" of homeostasis is not always achieved. Defective detoxification can be caused by several factors, including genetic variance, xenobiotic chemical excess in the environment, and dietary inadequacies (Aronica et al., 2022). These factors can affect the detoxification process in multiple ways, and what begins as a minor error in the system can have far-reaching implications, leading to a variety of chronic diseases such as cancer, cardiovascular disease, diabetes and even in extreme cases has been shown to lead to neurodegenerative conditions like Alzheimer's disease (Norman et al., 2013)

The association between external influences and changes in detoxification systems has been studied extensively and one phenomenon that arises more frequently than not is changes to

detoxification enzymes themselves. This can either be through genetic mutation or up or down regulation of a gene.

For instance, the absence of GSTT1 (glutathione S-transferase theta 1) expression has been associated with lung cancers (Hosgood et al., 2007). Another study showed that single-nucleotide polymorphism (SNP) in the gene *CYP1A1* (P-oxidase cytochrome A 1) were much more frequent in patients with glaucoma than healthy individuals (Costa et al., 2014).

These enzymatic alterations do not always occur as a direct result of external factors but can also be caused by mutations that are genetically inherited. Inherited metabolic diseases are more commonly referred to as inborn errors of metabolism (IEMs) and typically have an early onset (Mansoor et al., 2024). There are already more than a thousand separate IEMs, and each one has a unique set of symptoms that stem from various malfunctioning aspects of the human metabolism, particularly the detoxification pathways (Ferreira et al., 2021). One IEM that will be focused on, is isovaleric acidemia.

2.3 Isovaleric acidaemia

Isovaleric acidemia (IVA) is a rare autosomal inherited disorder which is caused by a deficiency in the isovaleryl-CoA dehydrogenase (IVD) enzyme (Vockley and Ensenauer, 2006a). The enzyme plays a key role in the catabolic pathway of leucine, which is an essential amino acid, by converting isovaleryl-CoA into methylcrotonyl-CoA. Without proper function of the enzyme, isovaleryl-CoA accumulates in the body and leads to toxic derivative build-up (Fig.2.3.1) (Vockley and Ensenauer, 2006b).

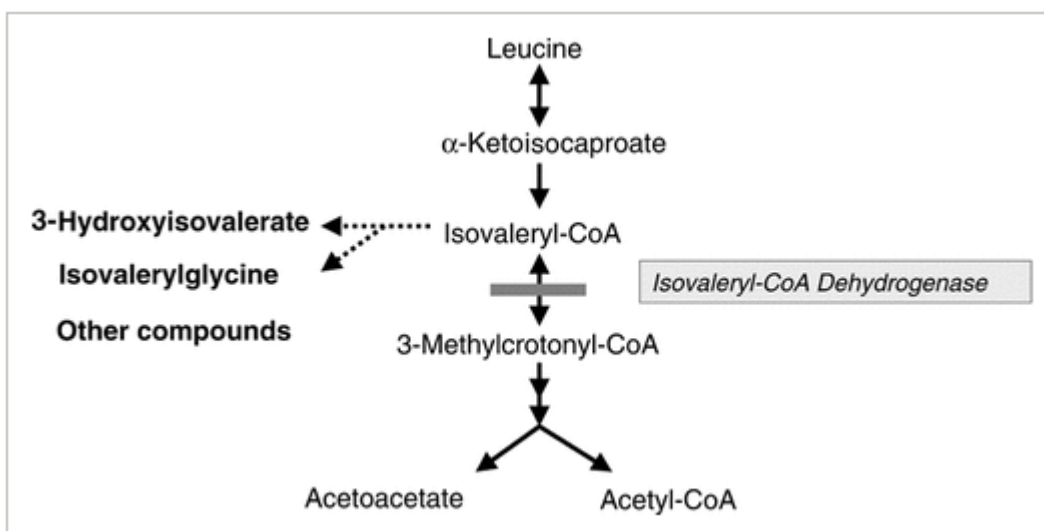


Figure 2.3.1 Catabolic pathway of leucine.

Isovaleryl-CoA dehydrogenase catabolizes isovaleryl-CoA to 3-methylcrotonyl-CoA. (Vockley and Ensenauer, 2006a)

2.3.1 Clinical manifestations and diagnosis

It appears from the reviewed literature that IVA typically manifests in two different ways. Within the first two weeks of the patient's existence, the neonatal form emerges. Symptoms of the disorder include vomiting, poor eating, lethargy, convulsions, and the distinctive "sweaty feet" smell. Sometimes the disease can result in life-threatening complications, such as a coma, if treatment is not received (Nyhan et al., 2011).

The second form is referred to as a chronic form, which can manifest at any age and can involve recurrent episodes of metabolic crises that are triggered by factors such as stress, infection, or high-protein intake. Symptoms commonly found in intermittent IVA include metabolic acidosis, hypoglycemia, vomiting, and lethargy (Nyhan et al., 2011).

IVA is most frequently found through neonatal screening, which identifies high C5-acylcarnitine levels. This is due to the fact that C5-acylcarnitine is a crucial indicator of isovaleric acid accumulation (Frazier et al., 2006). Urine organic acid analysis is then used for confirmatory testing to check for increased amounts of 3-hydroxyisovaleric acid and isovalerylglycine. IVD gene mutations can also be confirmed by genetic testing (Carr, 2009).

2.3.2 Treatment and management

Patients diagnosed with IVA are often placed on a modified diet to reduce the risk of a metabolic crisis. The diet revolves around the idea of lower leucine and protein intake. This limits the substrate feed for the leucine catabolic pathway and reduces the production of isovaleryl-CoA build-up which IVA patients cannot catabolize (Chatvuttinun et al., 2021).

Supplementing with glycine and carnitine is another popular therapeutic approach. By giving isovaleryl-CoA conjugates to bind to, this technique tackles the second half of the route. As previously indicated, isovaleryl-CoA can produce secondary, non-toxic metabolites such as isovalerylglycine and isovalerylcarnitine, which are more easily eliminated and lessen the initial accumulation of isovaleryl-CoA (Chinen et al., 2017). In a study that isolated and identified *N*-isovalerylglycine of IVA patients, no significant increase in other short chain fatty acids, such as *N*-butyric and *N*-caproic acids, were seen (Tanaka and Isselbacher, 1967b). It was suggested that isovaleryl-CoA cannot be utilized by primary pathways like β -oxidation, meaning that there is a heavy reliance on the glycine conjugation pathway for alleviating isovaleryl-CoA build-up in the form of isovalerylglycine. Historically, the assumption that glycine conjugation plays a major role in isovaleryl-CoA detoxification was based primarily on indirect evidence, such as the presence of isovalerylglycine in patient urine. Prior to recent development, only three studies had directly investigated whether GLYAT enzymes could conjugate isovaleryl-CoA to glycine (Kühn et al., 2023).

However, the recent study by (Kühn et al., 2023) provided direct experimental evidence that GLYAT and its paralogue GLYATL1 can indeed catalyze the formation of isovalerylglycine through *in-silico* and *in-vitro* validation. This finding supports the long-standing hypothesis and emphasizes the need for more detailed investigation into the enzymatic properties, substrate preferences, and physiological regulation of GLYAT family enzymes, especially in the context of inborn errors of metabolism such as isovaleric acidemia.

2.4 Glycine conjugation

The glycine conjugation pathway falls under the Phase II detoxification of metabolism. This component of the metabolism occurs primarily in the liver and is utilised to neutralise metabolites from Phase I detoxification or other toxins that accumulate in the body (Grant, 1991). Phase II detoxification accomplishes this by making toxic metabolites more water-soluble, allowing them to be easily excreted, via conjugation reactions. The glycine conjugation pathway is one of these important processes.

Currently it is known that the glycine conjugation pathway consists of a two-step enzymatic reaction, but for this review, the second step of the pathway involving glycine *N*-acyltransferase (GLYAT) will be looked at more closely. This pathway largely includes the conjugation of carboxylic acids with mainly glycine, which increases their water solubility and urinary excretion (Figure 2.4.1). The enzyme glycine *N*-acyltransferase (GLYAT)(EC:2.3.1.13) catalyses the process by transferring activated acyl-CoA intermediates to glycine, resulting in the formation of glycine conjugates such as hippuric acid from benzoic acid (Knights et al., 2007b). This conjugation reduces the toxicity of potentially harmful metabolites, preventing their accumulation and subsequent cellular damage.

The pathway primarily targets carboxylic acids, both endogenous and exogenous, for detoxification. Key substrates that are often utilized include aromatic acids like benzoic acid, which is widely used in the food industry as a preservative leading to over accumulation in the body (Shu et al., 2016). Another common substrate is salicylic acid, which is commonly found in medication such as aspirin (Visagie et al., 2024).

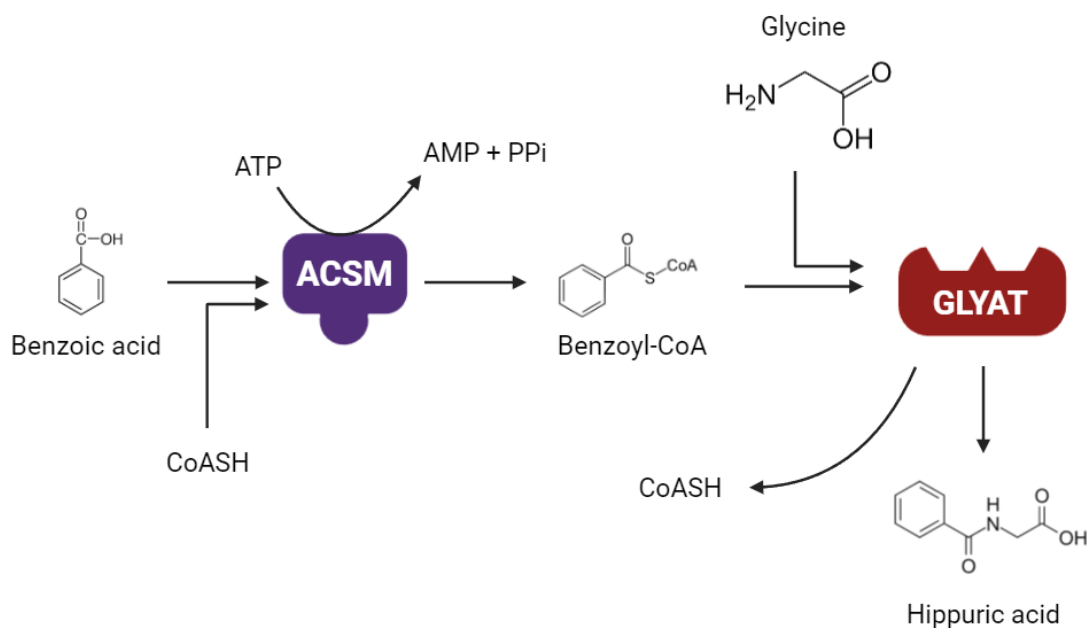


Figure 2.4.1 Glycine conjugation pathway.

Precursor substrate (Benzoic acid) being activated in tandem with CoASH molecule in the presence of ATP (Step 1). Intermediate metabolite (benzoyl-CoA) is then conjugated to an amino acid acceptor (Glycine) to form Hippuric acid and releasing CoASH. The image was generated using Biorender.

2.4.1 Structure and function of GLYAT

Glycine N-acyltransferase (GLYAT) is a mitochondrial enzyme that belongs to the Gcn5-related N-acyltransferase (GNAT) superfamily, meaning that it primarily facilitates acetylation as mentioned above (Vetting et al., 2005). The enzyme's active site is tailored to accommodate both acyl-CoA substrates and glycine, causing transfer of acyl groups to the amino acid. Residues are a key component to any enzyme active site and one study investigated murine GLYAT enzymes and concluded that lysine 19 may be important for enzyme function as this is where acetylation primarily takes place (Waluk et al., 2012).

Studies pertaining to the molecular structure of GLYAT are very few, but some recent studies have provided insight into the enzyme's catalytic mechanism. Ebrecht *et al.* (2021) has recently recombinantly expressed and purified GLYAT from *Bos taurus* (bGLYAT) and solved the molecular structure using molecular replacement (MR) and single-wavelength anomalous diffraction phasing. They concluded that while certain regions of the enzyme were as per prediction conservative to the GNAT superfamily, like a mixed open central beta-sheet with helical

crossovers allowing for catalytic activity, there were also other regions with unknown functions that were most likely playing a role in post-translational modifications. Another recent study carried out computational predictions of the human GLYAT and GLYATL1, but this was focused on substrate preference and interaction rather than structural function analysis (Kühn et al., 2023). This shows that there is so much that still needs to be understood about the GLYAT structure as it can vary between each paralogue in the GLYAT family.

2.4.2 Genetic variation and its impact on GLYAT function.

GLYAT is encoded by the *GLYAT* gene which is located on chromosome 11q12.3 (HGNC, 2025; NCBI Gene, 2025). The gene is comprised of six exons and multiple transcripts variants have been shown to occur through alternative splicing, which ultimately leads to different isoforms of GLYAT.

The GLYAT gene is considered highly conserved, as evidenced by analysis of genetic variation in the open reading frame (ORF) among Caucasian individuals (van der Sluis et al., 2015). This conservation underscores the critical physiological role of the glycine conjugation pathway. However, certain single nucleotide polymorphisms (SNPs) resulting in amino acid substitutions have been identified and shown to influence the enzyme's kinetic properties, including substrate affinity and catalytic efficiency (Rohwer et al., 2021, Schulke and Sass, 2021). These recent studies have expanded on the topic of exploring the prevalence and impact of GLYAT polymorphisms in different populations and their potential role in metabolic disorders.

Functional analyses of genetic variations have raised the question of the impact this could have on metabolism as GLYAT facilitates the excretion of various endogenous and xenobiotic compounds. Understanding the genetic makeup of GLYAT and its variations is crucial, as these differences can influence responses to environmental toxins and susceptibility to certain diseases that were mentioned before. Further research into GLYAT polymorphisms and their functional consequence will further our understanding of metabolic diversity.

2.4.3 GLYAT paralogues and substrate specificity

Genetic variation causing polymorphisms to occur does not solely make up the diversity pool of the GLYAT family of enzymes. Since the early studies focusing on GLYAT it has been hypothesized that there are closely related N-acyltransferases, that even though they are similar on a genetic and structural level, showcases very different binding affinities to certain substrates (Matsuo et al., 2012). An example of this is a study that focuses on purifying N-acyltransferase

from bovine liver mitochondria. When the enzymes were subjected to physiological test, difference in substrate specificity was observed (Nandi et al., 1979b). Where GLYAT is known to primarily conjugate benzoyl-CoA to glycine, GLYATL1 (E.C:2.3.1.68) is shown to preferably conjugate glutamine to phenyl-acetyl-CoA (Nandi et al., 1979b). While this was an interesting phenomenon at first, the reason for the different paralogues of GLYAT was still a mystery.

A study that helped with answering this question was Webster *et al.* (1976) that were one of the first to show that benzoyl-CoA is a primarily utilized substrate in the amino acid conjugation of GLYAT. They continued to show that when enzymes of different localized extracts of mitochondria were used in kinetic analysis, different preferred substrates would appear in the analyses. They concluded in part that substrate specificity can be attributed to enzyme localization.

Up until now different GLYAT proteins have been isolated and biochemically purified from different organs like the kidney and liver of human, bovine and rhesus monkey species' (Mawal and Qureshi, 1994a, Schachter and Taggart, 1953). Figure 2.4.2 shows a diagram depicting some of the different paralogues of GLYAT that have been identified so far and their presumed role in detoxification. Both GLYAT and GLYATL1 utilize medium chain acyl-CoA that are produced from Acyl-CoA synthetase medium-chain family member enzymes (ACSM2A/B) in the mitochondrial matrix and GLYATL3 (E.C: 2.3.1.13) which uses long-chain acyl-CoA's.

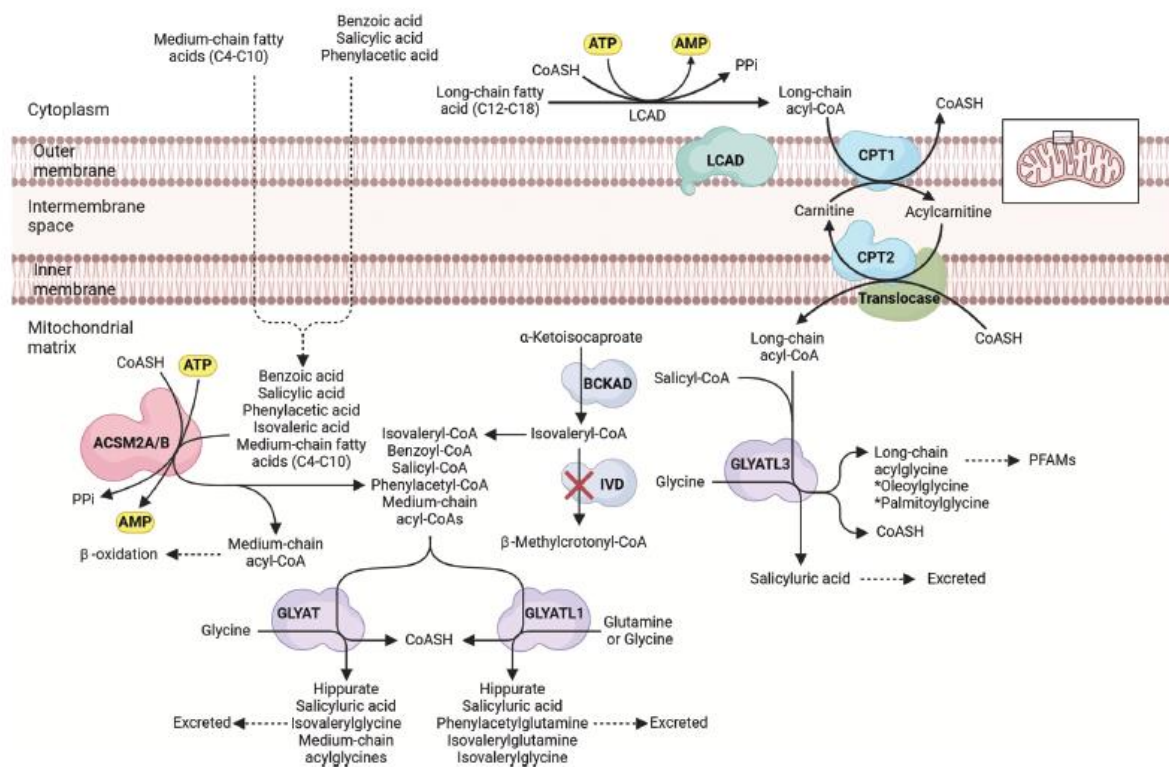


Figure 2.4.2 Different paralogues of GLYAT and their assumed role in detoxification.

GLYAT and GLYATL1 conjugate different medium-chain acyl-CoA's to Glycine and L-Glutamine respectively. GLYATL3 predicted to conjugate long-chain acyl-CoA to glycine.

Currently only two out of the three paralogues depicted above have been characterized in terms of their genetic make-up, structural moiety as well as substrate specificity, but the reason as to their substrate preference is still not fully understood. GLYAT is the most studied between all paralogues as it's kinetics parameters has been established whereas the other's have not been studied (Van der Sluis et al., 2017b).

(Kühn et al., 2023) aimed to understand the difference between GLYAT and GLYATL1 better and simulated computational models of each enzyme (Figure 2.4.3), which could explain the substrate preference differences between the paralogues. This is one of the few studies that furthered our understanding of the GLYAT family and their substrate preference variability.

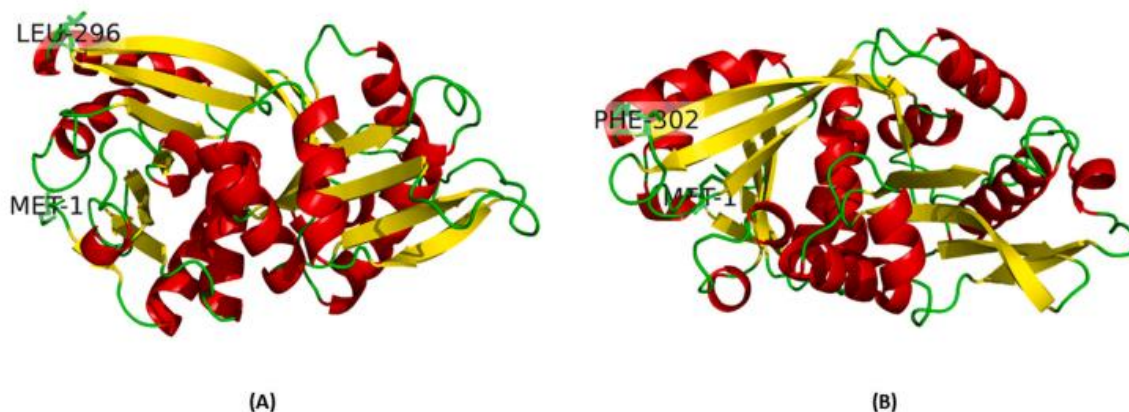


Figure 2.4.3 Secondary structure prediction of GLYAT (A) and paralogue GLYATL1 (B) (Kühn et al., 2023)

2.4.4 Influence of Co-factors on GLYAT activity.

Variations in these paralogues influence substrate specificity and catalytic efficiency, contributing to the enzyme family's versatility across different metabolic contexts. However, the complexity of GLYAT regulation goes beyond genetic factors alone. Co-factors, including substrate availability, CoA derivatives, and feedback mechanisms can potentially modulate GLYAT activity too. These co-factors usually have a big impact on enzyme function and exploring both genetic and cofactor-mediated regulation influence on GLYAT can result in a much more comprehensive understanding of its role in metabolic detoxification.

Even though this is a very important part of understanding any enzyme, there is a scarcity in studies focusing on this aspect. A study done by (Badenhorst et al., 2014) pointed out certain substrates that can have an effect on the glycine conjugation pathway. They noted that glycine conjugation, compared to glucuronidation, is not as effective in increasing water solubility of aromatic acids. They continued to explain that over accumulation of free benzoates, which is caused by dietary preservatives, can cause a depletion in glycine levels which show negative influence on brain neurochemistry.

Another study interestingly showed that GLYAT downregulation facilitates tumor growth and metastasis in breast cancer cells by possibly playing a role in the PI3K/AKT/Snail pathway that helps with migration signalling (Tian et al., 2021). Lastly, a very early study which aimed to identify N-acyltransferases, revealed that certain cations can serve as both inhibitors and catalysts,

depending on the N-acyltransferase (Nandi et al., 1979a). Even though the findings in these studies are very interesting phenomena, they have not really been investigated on a deeper level.

2.5 Problem statement

In recent years, there has been growing interest in the role of glycine conjugation as part of the body's detoxification system, especially in relation to disorders like isovaleric acidemia (IVA). In IVA, the accumulation of isovaleryl-CoA poses a serious metabolic challenge, and glycine conjugation offers a potential route for its safe elimination. However, while the pathway has been studied in terms of its general function and structure, there is still a limited understanding of how different substrates interact within this system.

One particular area that has not been explored in enough detail is the effect of substrate competition and inhibition on GLYAT, the key enzyme involved in glycine conjugation. In metabolic conditions like IVA, the build-up of isovaleryl-CoA could potentially interfere with the conjugation of other substrates, either by outcompeting them or through inhibition. This raises important questions about whether such interactions affect the efficiency of detoxification, especially under conditions where multiple substrates are present.

2.5.1 Research Aim

The aim of this study is to investigate how substrate competition influences the activity of glycine N-acyltransferase (GLYAT), with a particular focus on the role of isovaleryl-CoA as a potentially competitive or inhibitory substrate in the glycine conjugation pathway.

2.5.2 Research objectives

- To recombinantly express human GLYAT and verify its enzymatic activity.
- To develop and validate a reproducible H^1 -NMR-based method for detecting and quantifying glycine conjugation products.
- To evaluate the influence of various acyl-CoA substrates (including benzoyl-CoA, phenylacetyl-CoA, and isovaleryl-CoA) on GLYAT activity under controlled conditions.
- To determine whether isovaleryl-CoA acts as a competitive inhibitor in glycine conjugation when other substrates are present.

CHAPTER 3 RECOMBINANT EXPRESSION OF AN ENZYMATICALLY ACTIVE GLYCINE N-ACYLTRANSFERASE (GLYAT).

3.1 Introduction

The aim of this study was to evaluate the effect of substrate competition on the detoxification rate of glycine conjugation. To do this, enzymatically active recombinant GLYAT needed to be expressed in bacteria (Figure 3.1.1). Although recombinant human GLYAT has been successfully expressed previously (Kühn et al., 2023, Grundling, 2012, Schutte, 2020) several steps needed to be optimized to improve expression (i.e. removing arabinose, optimizing the lysozyme and protease inhibitor concentrations as well as reducing the temperature at which expression was induced).

Previous studies also purified recombinant GLYAT and determined its enzyme kinetic parameters using a colorimetric assay (Schutte, 2020, Van der Sluis et al., 2013). The drawback of this assay is that the specific product that is being formed cannot be identified or quantified and therefore this assay is not suitable to evaluate product competition. To overcome this dilemma as well as bypass the laborious purification step of nickel affinity chromatography, a new method will be developed using NMR (Chapter 4).

After sequence confirmation of GLYAT inserted in the pHis17 plasmid, the successful expression of GLYAT was evaluated using SDS-PAGE and western blot analysis. The enzyme activity of GLYAT was confirmed using an Ellman's assay and aliquots were stored from one batch for further analyses to reduce any batch effects. The final method is summarized in section 3.2.2, while the trouble shooting and rationale behind the optimization steps are discussed in section 3.3.

It is important to note that although this study focused on the expression and characterization of GLYAT, GLYATL1 was also expressed during the optimization steps to determine if the steps would increase the expression of GLYATL1. GLYATL1 will be characterized in a future study.

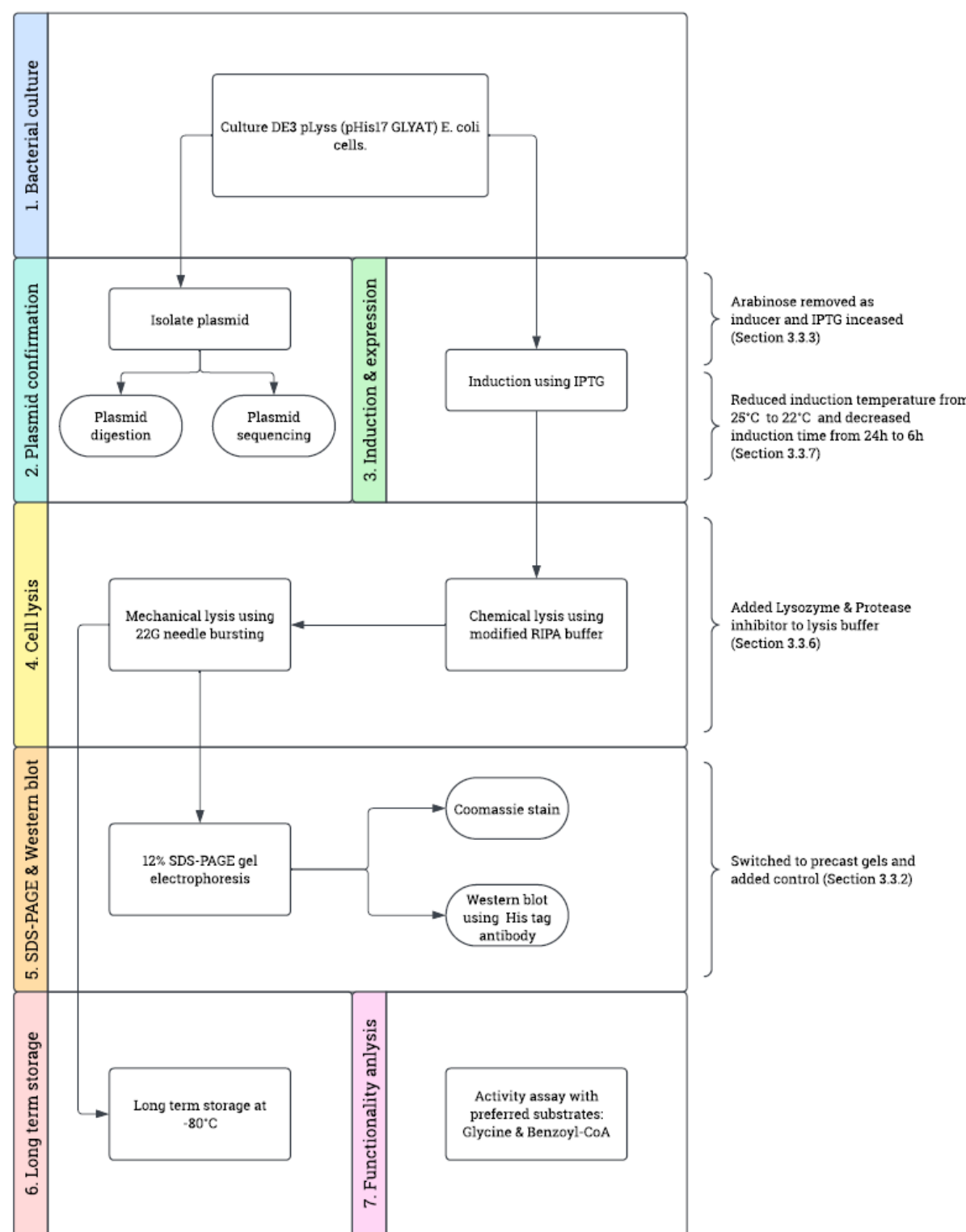


Figure 3.1.1 A Flowchart representing the experimental flow of Chapter 3

Sections are in different colour codes. Brackets also show trouble shooting steps along with section referral.

3.2 Materials and Methods

3.2.1 Materials

All materials utilized in this chapter can be found in Appendix AA. This will include the reagents, tools and machinery.

3.2.2 Methods

Plasmid Verification and Storage

Plasmids encoding human *GLYAT* and *GLYATL1* were isolated from *E. coli* C41(DE3)pLysS cells using a commercial plasmid miniprep kit (e.g., GeneJET Plasmid Miniprep Kit, Thermo Scientific), following the manufacturer's protocol. DNA concentration and purity were determined using a Nanodrop™ spectrophotometer (Thermo Scientific), which reliably measures absorbance at 260 nm for nucleic acid quantification and provides A260/A280 ratios to assess sample purity.

To confirm the integrity of the plasmids and the presence of the insert, restriction enzyme digestion was performed. Approximately 3 µg of purified plasmid DNA was incubated with the restriction enzyme *NdeI*, which cleaves within the insert of both the *GLYAT* and *GLYATL1* constructs. This digestion linearizes the plasmid, allowing for size confirmation of the construct. Reactions were prepared according to the enzyme manufacturer's instructions (New England Biolabs), containing DNA, 10 µl 1X reaction buffer, and 2 µL of *NdeI* in a 100 µL total volume (filled with Nuclease-free water). Digestion was performed at 37 °C for 40 minutes, followed by enzyme inactivation at 65 °C for 20 minutes.

Digested products were analysed via agarose gel electrophoresis. A 1% agarose gel was prepared using 1X TAE buffer and stained with ethidium bromide. Samples were mixed with loading dye and loaded alongside a DNA ladder. Electrophoresis was run at 100 V for 45 minutes, and bands were visualized using a transilluminator.

To further confirm plasmid identity, the isolated plasmids were submitted for Sanger sequencing at the Central Analytical Facilities (CAF) in Stellenbosch. Sequencing was performed from the T7 promoter to the T7 terminator region using standard primers provided by CAF: the T7 promoter primer (5'-TAATACGACTCACTATAGGG-3') and the T7 terminator primer (5'-GCTAGTTATTGCTCAGCGG-3'). The retrieved chromatogram and sequence data were aligned and compared to the expected reference sequence (NCBI Reference Sequence: NM_001389712.2) using the Benchling sequence analysis platform.

Expression of recombinant GLYAT and GLYATL1

Glycerol stocks of C41(DE3)pLysS *E. coli* cells which contained either the pHis17_GLYAT (containing the S₁₅₆ SNP) or pET23a(+)_GLYATL1 (NCBI Reference Sequence: NM_001389712.2) plasmid were incubated in 20 mL of sterile media (2% m/v bacto-tryptone, 1.25% m/v yeast extract, 0.625% NaCl, 0.5% Na₂HPO₄, 0.1% KH₂PO₄, 0.2% m/v glucose, 100 µg/mL carbenicillin, and 35 µg/mL chloramphenicol) (Sigma Merck) at 37 °C and 250 RPM (New Brunswick Innova 40 shaking incubator), overnight. The following day, the starter culture was inoculated in 100 mL of fresh media at a ratio of 1:100 and grown at 37 °C and 250 RPM until the OD₆₀₀ was between 0.5 and 0.6. The expression of recombinant GLYAT or GLYATL1 was then induced by supplementing the media with 0.05 mM IPTG, 5 g/L arabinose, and 0.2% m/v glycine or 0.2% m/v glutamine (Sigma Merck) (for GLYAT or GLYATL1, respectively), then lowering the temperature to 25 °C and leaving the cells for 24 h at 250 RPM (New Brunswick Innova 40 shaking incubator).

Thereafter, the cells were pelleted at 4,500 x g and 4 °C for 10 min, the clarified supernatant was discarded, and the cells were resuspended in 10 mL of a modified RIPA buffer (0.29% m/v NaCl, 3.6% m/v NaH₂PO₄, 1% v/v Triton X-100)(Sigma Merck) which was supplemented to 1 U/10 mL of Pierce Universal Nuclease (Thermo Fisher Scientific). Each suspension was left on ice for 15 min before being passed through at 22-G needle 10 times. The lysate was centrifuged at 14500 x g and 4 °C for 30 min whereafter 9 mL of the clarified supernatant was removed for purification.

The results described in this chapter will refer to a "total fraction," which is the supernatant before the final centrifugation step, and a "soluble fraction," which is the clarified supernatant after centrifugation. This distinction is made because expressed enzymes are generally soluble and easier to isolate compared to their insoluble inclusion body counterparts (Sørensen and Mortensen, 2005). The soluble fraction should, therefore, theoretically contain the soluble enzyme, while cell debris and insoluble proteins are removed during this centrifugation step. Experiments will also contain an "empty cell" lane. This refers to *E. coli* cells that do not contain a recombinant plasmid and therefore should not be able to express any protein when induced. This ensures that bands of interest are the target protein and not general cellular protein.

SDS-PAGE and Western Blot Analysis of Recombinant GLYAT and GLYATL1

Recombinant GLYAT and GLYATL1 proteins were analyzed by SDS-PAGE and western blotting. Initial SDS-PAGE gels were cast manually following standard protocols (Cold Spring Harb Protoc, 2015), using a 12% resolving gel with a 4% stacking gel. For improved reproducibility, commercially pre-cast Mini-PROTEAN TGX 12% gels (Bio-Rad) were used in subsequent experiments.

Protein samples were mixed with 5× Laemmli buffer containing β -mercaptoethanol, heated at 95 °C for 5 minutes, and loaded onto the gel. Electrophoresis was carried out in 1× Tris-Glycine-SDS running buffer at 150 V for 45–60 minutes.

Proteins were transferred onto PVDF membranes using the Trans-Blot Turbo transfer system (Bio-Rad) under the manufacturer's recommended conditions (25 V, 1.3 A, 30 minutes). Membranes were equilibrated in Towbin transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) prior to transfer.

Following transfer, membranes were blocked with 5% bovine serum albumin (BSA) in TBS-T for 2 hours at room temperature. After blocking, membranes were incubated overnight at 4 °C with a diluted 6×-His Tag Monoclonal Antibody (HIS.H8), HRP-conjugated. Washing steps were performed using TBS-T (10× TBS, 0.1% Tween-20) between each step.

Immunoreactive bands were visualized using AEC substrate prepared according to Rodig (2019), whereby AEC was dissolved in sodium acetate buffer (pH 5.2) and combined with hydrogen peroxide. The solution was filtered and applied to the membrane until bands were clearly visible.

Quantitative Enzyme Activity Assay Using DTNB and Spectrophotometry

The enzymatic activity of recombinant GLYAT was assessed using a colorimetric Ellman's assay, which quantifies the release of free coenzyme A (CoA-SH) through its reaction with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB). This reaction forms a yellow-coloured 2-nitro-5-thiobenzoate (TNB) anion, which absorbs at 410 nm and can be monitored spectrophotometrically to estimate enzyme activity (Ellman, 1959b, Riddles et al., 1979)

The assay was conducted in a 96-well format using a BioTek spectrophotometric plate reader. Each 100 μ L reaction contained final concentrations of 0.1 M Tris-HCl buffer (pH 8.0), 200 mM glycine, 100 μ M acyl-CoA (specifically benzoyl-CoA), and 100 μ M DTNB. A 10 μ L volume of the

soluble protein fraction was added, delivering approximately 5 µg of total soluble protein (including recombinant GLYAT) lysate per reaction. The protein concentration of the soluble lysate was determined prior to the assay using the Qubit™ Protein Assay Kit (Broad Range, Thermo Fisher Scientific) according to the manufacturer's instructions. The remaining volume was adjusted with molecular-grade water.

All components, except for acyl-CoA and the enzyme, were pre-mixed and pipetted into a sterile, single-use, flat-bottom 96-well plate, which was kept on ice during setup. The plate was then heated to 33 °C before rapid and careful addition of acyl-CoA and protein. Absorbance was measured at 410 nm at 20-second intervals for a total duration of 10 minutes.

3.3 Results and discussion

3.3.1 Verification and sequencing of the pHis17_GLYAT and pET23a_GLYATL1 vectors

To ensure the integrity of the constructs used for recombinant protein expression, both the pHis17_GLYAT (Figure 3.3.1.) and pET23a (+)_GLYATL1 (Figure 3.3.2) plasmids were verified before any expression experiments were conducted. These plasmids encode for human GLYAT and its paralogue GLYATL1, respectively, and were previously cloned into expression-compatible vectors with appropriate selection markers and fusion tags. While only GLYAT was taken forward for downstream characterization and activity analysis, GLYATL1 was included at this early stage to evaluate whether any expression optimizations would also be compatible with this related enzyme.

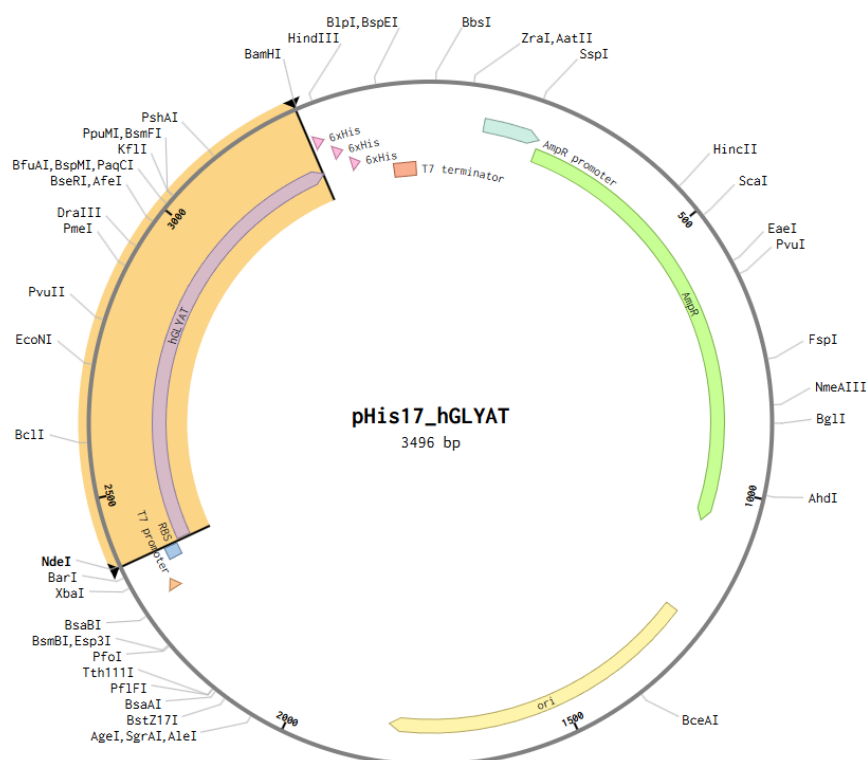


Figure 3.3.1 Plasmid map of the pHis17_GLYAT construct used for recombinant expression.

The GLYAT insert is highlighted on the map, flanked by the T7 promoter and terminator sequences. A pink arrow indicates the location of the N-terminal 6x His tag, which facilitates both detection (via western blot) and purification (via Ni-affinity chromatography). Restriction enzyme sites (NdeI) used for plasmid verification are also indicated on the map.

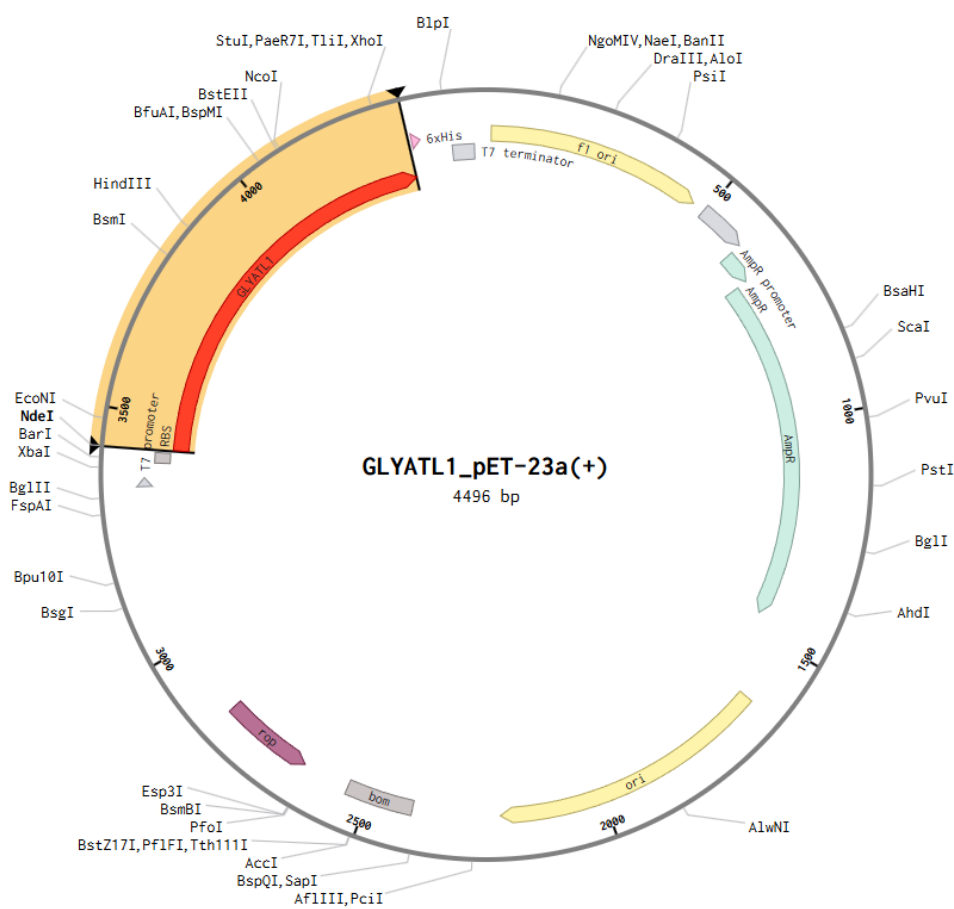


Figure 3.3.2 Plasmid map of the pET23a (+)_GLYATL1 construct used for recombinant expression.

The GLYATL1 gene insert is highlighted, with the T7 promoter and terminator sequences flanking the insert. A pink arrow marks the location of the C-terminal 6x His tag, used for downstream detection and purification. The NdeI restriction site used for plasmid linearization is also indicated.

Restriction enzyme digestion was performed to confirm the correct size of the recombinant plasmids prior to sequencing and expression. This verification step helps ensure that the plasmid contains the full insert without major rearrangements or degradation. For both GLYAT and GLYATL1 constructs, the restriction enzyme NdeI was selected, as its recognition site is located within the coding region of the respective gene inserts. Digesting the plasmids with NdeI should therefore produce a linearized plasmid if the insert is present and intact, while undigested plasmids typically display a more diffuse banding pattern due to supercoiling. The resulting digestion products were visualized by agarose gel electrophoresis (Figure 3.3.3).

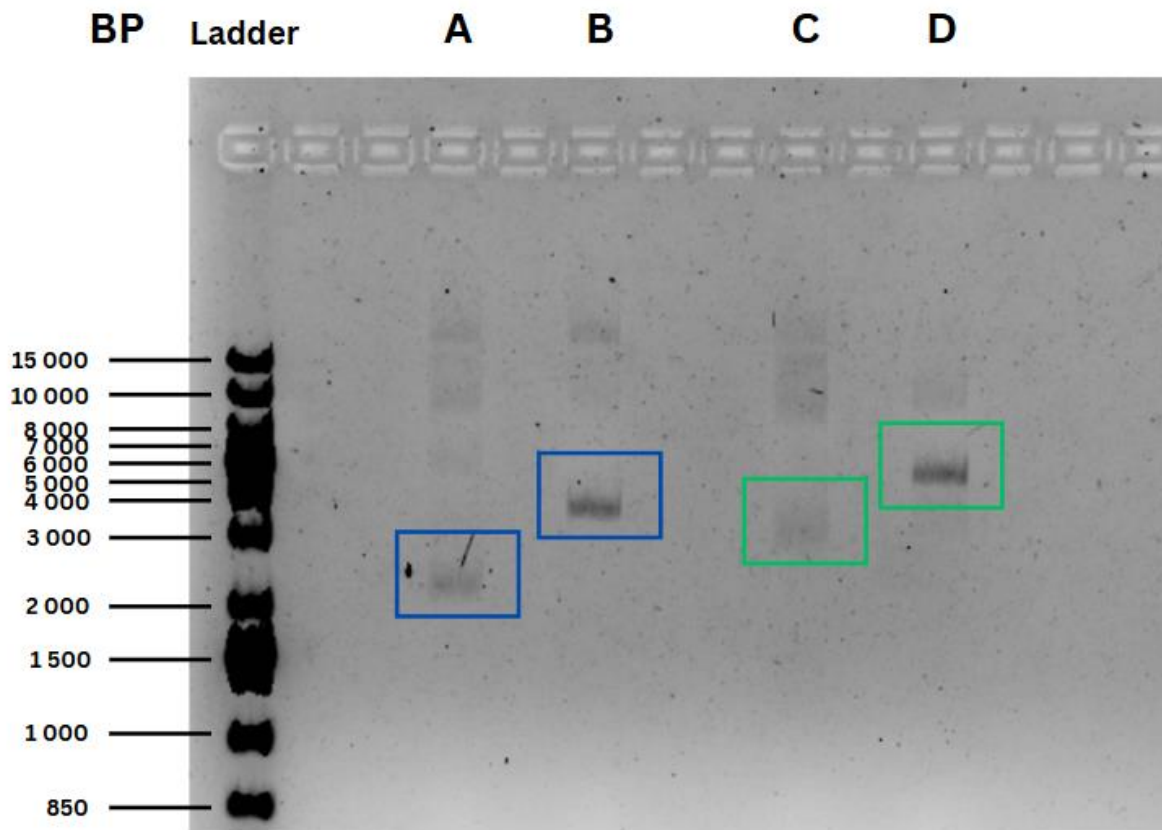


Figure 3.3.3 Agarose gel (1%) of plasmid DNA extracted from *E. coli* DE3 pLysS glycerol stocks and subjected to restriction digestion using NdeI.

Samples were run in 1 x TAE buffer with a DNA ladder (left lane) for size estimation.

Lane A: Undigested pHis17_GLYAT plasmid showing a diffuse, lower-migrating band typical of supercoiled plasmid DNA (~3.4 kb). **Lane B:** pHis17_GLYAT plasmid digested with NdeI, producing a single, clearer band at ~3.4 kb, consistent with linearized plasmid. **Lane C:** Undigested pET23a(+)_GLYATL1 plasmid showing a supercoiled band near ~4.4 kb. **Lane D:** pET23a(+)_GLYATL1 plasmid digested with NdeI, producing a distinct linearized band at ~4.4 kb. Blue boxes highlight GLYAT plasmids; green boxes indicate GLYATL1.

Upon digestion with NdeI, both GLYAT and GLYATL1 plasmids produced distinct bands that migrated more slowly than their undigested counterparts. This shift in mobility is consistent with the transition from a compact supercoiled conformation to a relaxed linear form. Supercoiled plasmids (Lanes A and C) typically migrate faster through an agarose matrix due to their condensed structure, whereas linearized plasmids (Lanes B and D) are less compact and thus

migrate more slowly, appearing higher in the gel. This is a well-documented characteristic of plasmid DNA (Lee et al., 2012). Importantly, the linearized GLYAT plasmid migrated near the expected ~3.4 kbp region, while the GLYATL1 plasmid aligned near ~4.4 kbp, confirming the presence and approximate size of the full-length constructs.

The diffuse or fuzzy appearance of the bands across all samples could be attributed to several common technical issues. Overheating during electrophoresis may cause partial DNA denaturation of the gel bands, resulting in smearing or fuzzy migration patterns (Green & Sambrook, 2020). Additionally, minor degradation of plasmid DNA, potentially due to residual nuclease activity or repeated freeze-thaw cycles, can produce fragmented products that blur band definition (Wilfinger et al., 1997). Uneven gel polymerization or excess salt in the sample buffer can further contribute to streaking and band diffusion.

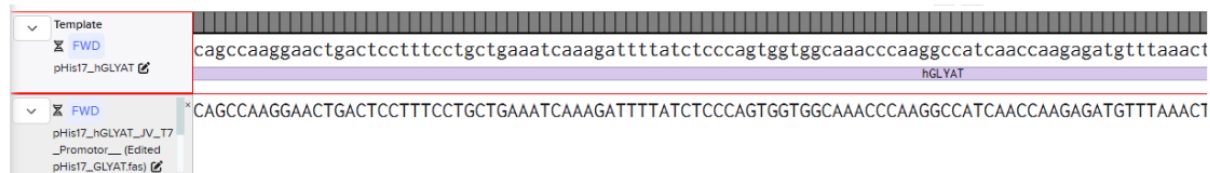
Following confirmation of plasmid size by restriction digestion, both the pHis17_GLYAT and pET23a(+)_GLYATL1 plasmids were subjected to Sanger sequencing at the Central Analytical Facilities™ (CAF), Stellenbosch. Sequencing was performed using a forward primer positioned near the T7 promoter, enabling coverage from the promoter region through to the T7 terminator. Although sequencing was conducted in only the forward direction, this approach provides comprehensive coverage of the insert, sufficient for confirming sequence identity. Future studies may benefit from sequencing both strands to further validate base calls across complementary strands; however, given the complementary nature of DNA, a high-quality forward read can reliably confirm the integrity of the inserts.

The returned sequences (see Appendix AB) were aligned against their respective reference sequences using Benchling's alignment tool. The GLYAT insert demonstrated greater than 95% sequence identity with the human GLYAT reference sequence (NCBI Reference Sequence: NM_201648.3), while the GLYATL1 insert showed comparable identity with the human GLYATL1 reference sequence (NCBI Reference Sequence: NM_001389712.2). These findings are summarized in Figures 3.3.4 and 3.3.5, which display sequence alignments.

A



B



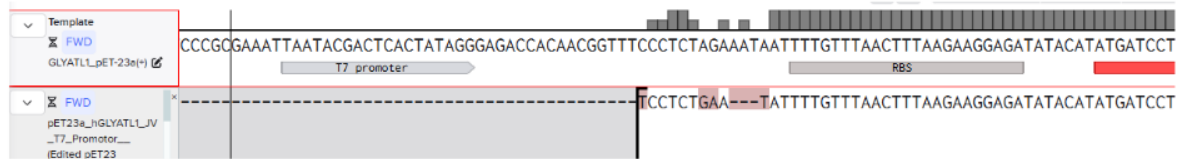
C



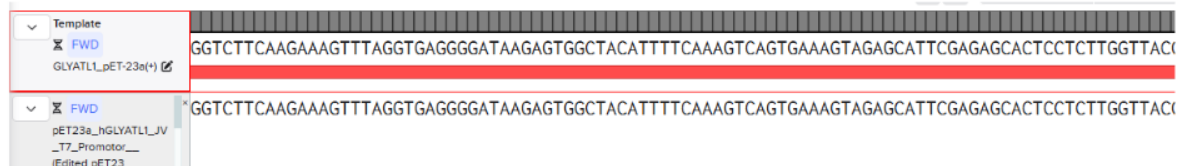
Figure 3.3.4 Sequence alignment of the pHis17_GLYAT insert with the human GLYAT reference sequence (NCBI Reference Sequence: NM_001389712.2).

The figure is divided into three panels showing distinct regions of the insert: (A) the beginning, (B) the middle, and (C) the end. The top grey bar represents the reference sequence, while the bottom bar shows the sequencing reads obtained from Sanger sequencing. Correct base pair matches are indicated in grey, and mismatches or low-confidence base calls are highlighted in red. Note the perfect alignment in the middle region (B) and the minor discrepancies at the sequence termini (A and C), consistent with typical Sanger sequencing artifacts due to signal decay.

A



B



C

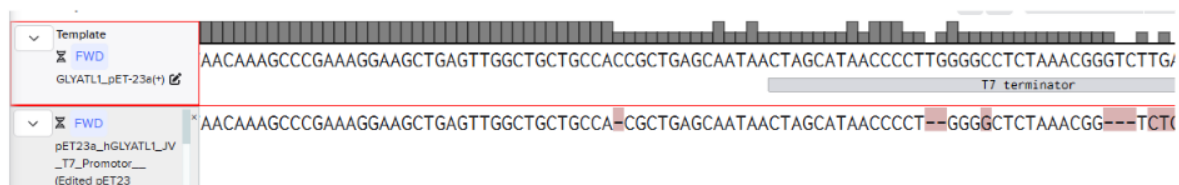


Figure 3.3.5 Sequence alignment of the pET23a(+)_GLYATL1 insert with the human GLYATL1 reference sequence (NCBI RefSeq: NM_203406.3).

Panels (A), (B), and (C) correspond to the beginning, middle, and end of the insert, respectively. The top grey bar denotes the reference sequence and the bottom bar the Sanger sequencing reads. Base pair matches are shown in grey; mismatches or low-confidence calls are marked in red. The middle panel (B) exhibits complete concordance, while minor mismatches appear near the ends (A and C), typical of end-read sequencing noise.

In both constructs, the middle panels (B) exhibit perfect concordance with their reference sequences, indicating high-quality sequence data throughout the central coding regions. The beginning (A) and end (C) panels reveal a small number of mismatches and low-confidence base calls, highlighted in red. This pattern is consistent with common Sanger sequencing artifacts arising from signal decay and reduced quality near primer binding sites and terminator regions, a phenomenon known as end-read noise or sequencing signal drop-off (Smith et al., 1986).

Overall, the sequencing data confirms that both GLYAT and GLYATL1 plasmids contain full-length, mutation-free inserts, thereby validating their suitability for subsequent expression and functional analyses.

3.3.2 Optimization of the expression protocol.

In the past several studies have successfully expressed both recombinant GLYAT and GLYATL1 (Van der Sluis et al., 2013, Lourens et al., 2022). However, the protocol in these studies varied slightly when compared to the most recent study (Kühn et al., 2023)(Table 3.3.1).

Table 3.3.1 Method comparison of the different expression protocols used for GLYAT and GLYATL1 expression.

Study	Van der Sluis <i>et al.</i> , 2013 <i>Characterisation of the influence of genetic variations on the enzyme activity of a recombinant human glycine N-acyltransferase.</i>	Kühn <i>et al.</i> , 2023 <i>The glycine N-acyltransferases, GLYAT and GLYATL1, contribute to the detoxification of isovaleryl-CoA - an in-silico and in vitro validation.</i>	Pieter Lourens 2022 <i>The establishment of a model system in which variants of the medium-chain ligase (ACSM) and glycine N-acyltransferase (GLYAT) can be co-expressed and analysed.</i>
Cell line	OrigamiB(DE3) cells containing the pGro7 chaperone expression plasmid.	C41(DE3)pLysS <i>E. coli</i> cells	C41(DE3)pLysS <i>E. coli</i> cells
Construct	pET32a(+)_hGLYAT	pHis17_GLYAT pET23a(+)_GLYATL1	pHis17_GLYAT pET23a(+)_GLYATL1
Initial growth media	10 ml cultures containing: 2% bacto-tryptone, 1.25% yeast extract, 0.625% NaCl, 0.5% Na ₂ HPO ₄ ·12H ₂ O, 0.1% KH ₂ PO ₄ , 0.2% glucose. 50 µg/ml ampicillin, 20 µg/ml chloramphenicol. 2 mg/ml L-arabinose	20 mL culture containing: 2% m/v bacto-tryptone, 1.25% m/v yeast extract, 0.625% NaCl, 0.5% Na ₂ HPO ₄ , 0.1% KH ₂ PO ₄ , 0.2% m/v glucose. 100 µg/mL carbenicillin, 35 µg/mL chloramphenicol	25 mL culture containing: 1% tryptone (w/v), 0.5% yeast extract (w/v), 1% NaCl (w/v) 34 ng/µl chloramphenicol, 100 ng/µl carbenicillin, 0.2% Glucose(w/v), 0.5% Glycine (w/v), 5 mM pantothenic acid

	0.5% Glycine (w/v), Incubate overnight at 37 °C	Overnight at 37 °C and 250 RPM	Overnight at 37°C and 200 rpm.
Gene expression conditions	Harvest cells at 4000 ×g; 4 °C for 5 min. Resuspended in 100 ml of the same antibiotic containing medium and 0,5% Glycine. Incubated at 37 °C until the OD (600nm) reaches 0.4. The cultures were then transferred to 28 °C for 24 h. Note! No addition of IPTG.	100 ml media containing: 1 mL of the overnight culture. 2% m/v bacto-tryptone, 1.25% m/v yeast extract, 0.625% NaCl, 0.5% Na ₂ HPO ₄ , 0.1% KH ₂ PO ₄ , 100 µg/mL carbenicillin, 35 µg/mL chloramphenicol When the OD (600nm) is between 0.5 and 0.6, induce by adding the following: 0.05 mM IPTG, 5 g/L arabinose 0.2% m/v glycine (GLYAT) 0.2% m/v glutamine (GLYATL1) Incubate at 25 °C for 24h at 250 RPM.	100 mL media containing: 1 mL of the overnight culture. 1% tryptone (w/v), 0.5% yeast extract (w/v), 1% NaCl (w/v) 34 ng/µL chloramphenicol 100 ng/µL carbenicillin 5 mM glycine When the OD (600nm) is between 0.8 and 1.0, induce by adding the following: 1mM IPTG Incubate overnight at 25 °C and 200 RPM.
Lysis buffer	10 ml of buffer, pH 8.0 containing: 50 mM NaH ₂ PO ₄ , 300 mM NaCl,	10 ml of buffer containing: 0.29% m/v NaCl (50 mM) 3.6% m/v NaH ₂ PO ₄	5 ml of buffer containing: 50 mM NaH ₂ PO ₄ 300 mM NaCl (pH 8.0)

	10% (v/v) glycerol, 1% (v/v) Triton X-100, 10 μ l of Lysonase Bioprocessing Reagent Protease inhibitors added.	(300 mM) 1% v/v Triton X-100 1 U/10 mL of Pierce Universal Nuclease No Protease inhibitor.	10% Glycerol (v/v) 1% Triton X-100 (v/v) 1 μ L of Pierce® Universal Nuclease (5 kU). No Protease inhibitor.
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Several steps were therefore optimized through adaptation from previous protocols (i.e. the removal of arabinose and increasing the concentration of IPTG, adding and optimizing concentrations of protease inhibitor and lysozyme in the lysis buffer, also reducing the temperature and time during the induction phase).

Firstly, Dr Kuhn lowered IPTG concentrations but added arabinose as an induction reagent. This was done because too much IPTG can be regarded as toxic to the cell, so it was lowered and supplemented with arabinose. This is a mistake as vector expression prefers a single inducer and co-induction can cause one inducer to repress the other (Živič et al., 2025). Also, this particular plasmid used in the expression system does not rely on a PBAD promoter which works well with arabinose but rather a T7 promoter system in which IPTG is known to induce. This was the first optimization step applied to the expression protocol (Section 3.3.3)

Secondly, two of the three protocols did not contain any protease inhibitor or lysozyme in the lysis buffer. Protein aggregation prevention during expression can be challenging as the process of lysis can also play a major role in this. Small characteristics like buffer conditions or hydrophobic patch exposure can also cause downstream aggregation.

According to Strandberg & Enfors (1991), protein aggregation results in inclusion bodies forming in the cell which are clusters of protein, especially overexpressed protein. Palmer & Wingfield (2004) discussed different ways to combat these inclusion bodies, and a common method is the addition of lysozyme. Lysozyme, which is used to facilitate better cell lysis, allows for the isolation of inclusion bodies from the rest of the cell debris by sheering cell structural proteins (Singh et al., 2015). This helps target inclusion bodies more specifically in downstream lysis.

Protease inhibitor is also an important reagent in cell lysis as this can help with the prevention of degradation of proteins of interest and act as a “safeguard” for the proteins intact after lysis (Leimgruber et al., 2002). These two compounds were later added to the lysis protocol (Section 3.3.4)

3.3.3 Removal of arabinose and increase in IPTG concentration from 0.05 mM to 1 mM.

Arabinose was completely removed from the induction protocol and IPTG concentration increased from 0.05 mM to 1mM. Additionally, glycine levels were increased from 0.2% to 0.5% (m/v) to match the conditions used in previously successful expression protocols (Table 3.3.1), where glycine likely supports substrate availability during expression of glycine-conjugating enzymes. (Figure 3.3.6)

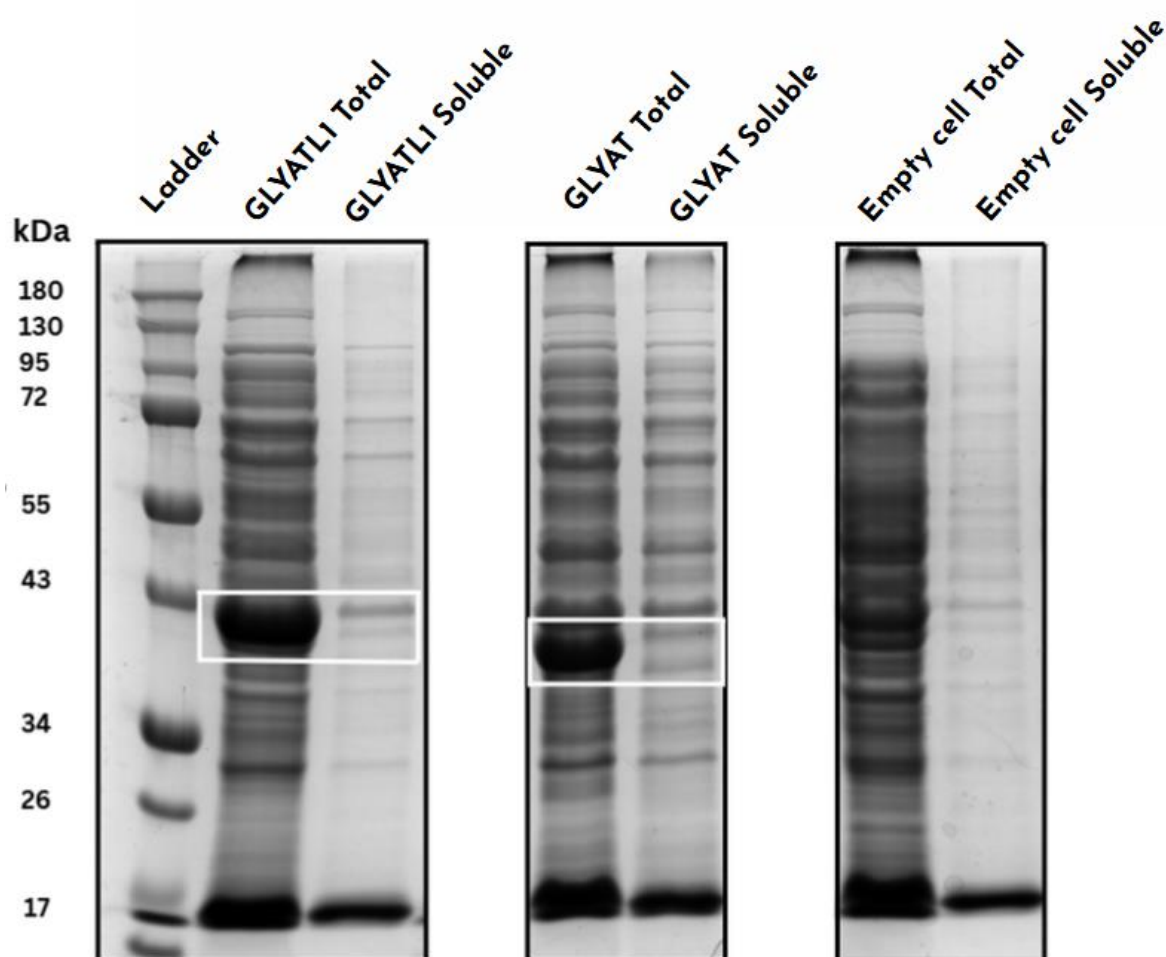


Figure 3.3.6 Coomassie stain of SDS-PAGE gel (12% Tris Glycine extended) loaded with soluble and total fractions of expressed GLYAT cell lysate.

Protein ladder (BIO-RAD™, #1610374) serving as a marker. White boxes indicate predicted location of GLYAT (~34 kDa) and GLYATL1 (~35 kDa).

In the white boxes specifically, dark bands can be observed in the total fraction of the samples, but these prominent bands were not present in the soluble fraction. The experiment was repeated several times but ultimately the same outcome was presented.

It was theorized that the protein was present in the soluble fraction but at a very low concentration after centrifugation and that it was not distinguishable from the background protein. The next step in the experimental workflow was a western blot in which anti-bodies could specifically bind to the His tag of the expressed GLYAT enzymes. If successful, clear bands would form if the protein of interest was present (Figure 3.3.7).

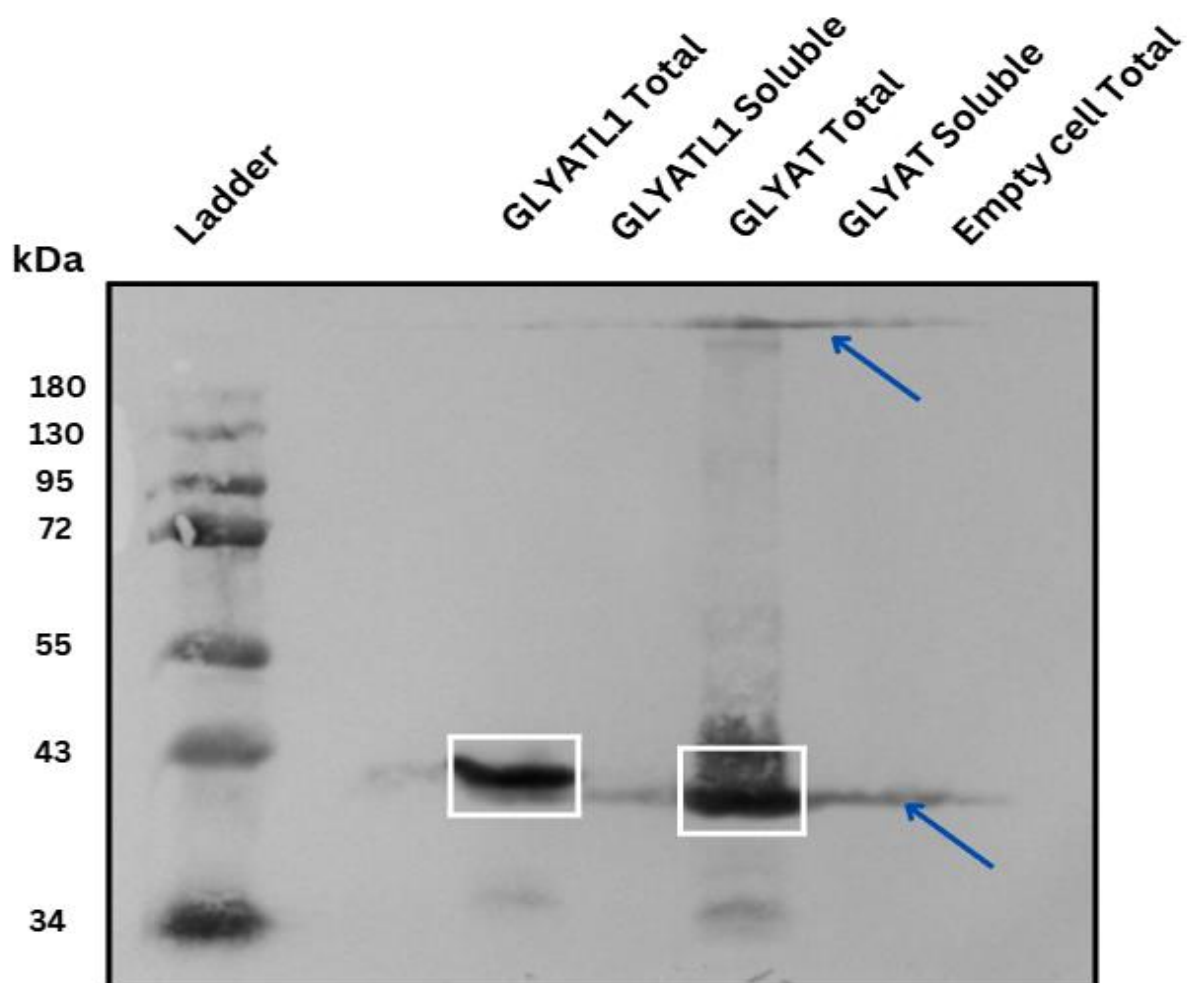


Figure 3.3.7 Western blot blocked with 5% BSA and incubated with 6x – His tag Monoclonal Antibody (HIS.H8), HRP activated.

Blot includes both soluble and total fraction lysate of samples and control. White boxes indicate predicted location of GLYAT (~34 kDa) and GLYATL1 (~35 kDa). Blue arrows show “lines” forming on blot potentially caused by non-specific binding or insufficient blocking.

Figure 3.3.7 shows two clear bands forming in the predicted area of the total fraction of the samples, but similar bands in the soluble fraction are not present. Small bands can be seen in soluble fractions, but these were similar to the bands at the top of the gel. This is indicated with blue arrows and was most likely due to non-specific binding. This experiment clearly showed that the protein is in fact present in the total fraction but for some reason does not solubilize as it should.

3.3.4 Addition of lysozyme and protease inhibitor to lysis buffer.

Efficient cell lysis and protein preservation are critical when working with recombinant proteins prone to aggregation or degradation. In earlier optimization steps, significant protein aggregation was suspected, potentially resulting in the formation of inclusion bodies. To improve lysis efficiency and protect the expressed protein, lysozyme and protease inhibitors were added to the lysis buffer. Lysozyme facilitates cell wall degradation by hydrolysing the β -1,4-glycosidic linkages in the peptidoglycan layer of *E. coli*, which can aid in the mechanical disruption of inclusion bodies and promote more complete cell lysis (Singh and Panda, 2005, Palmer and Wingfield, 2004). Protease inhibitors help prevent post-lysis degradation of the target protein, which can be especially important when expression stress leads to protease release (Jutras et al., 2016). The combination of these additives improves recovery and stability of overexpressed proteins, particularly those that are partially insoluble or prone to proteolysis.

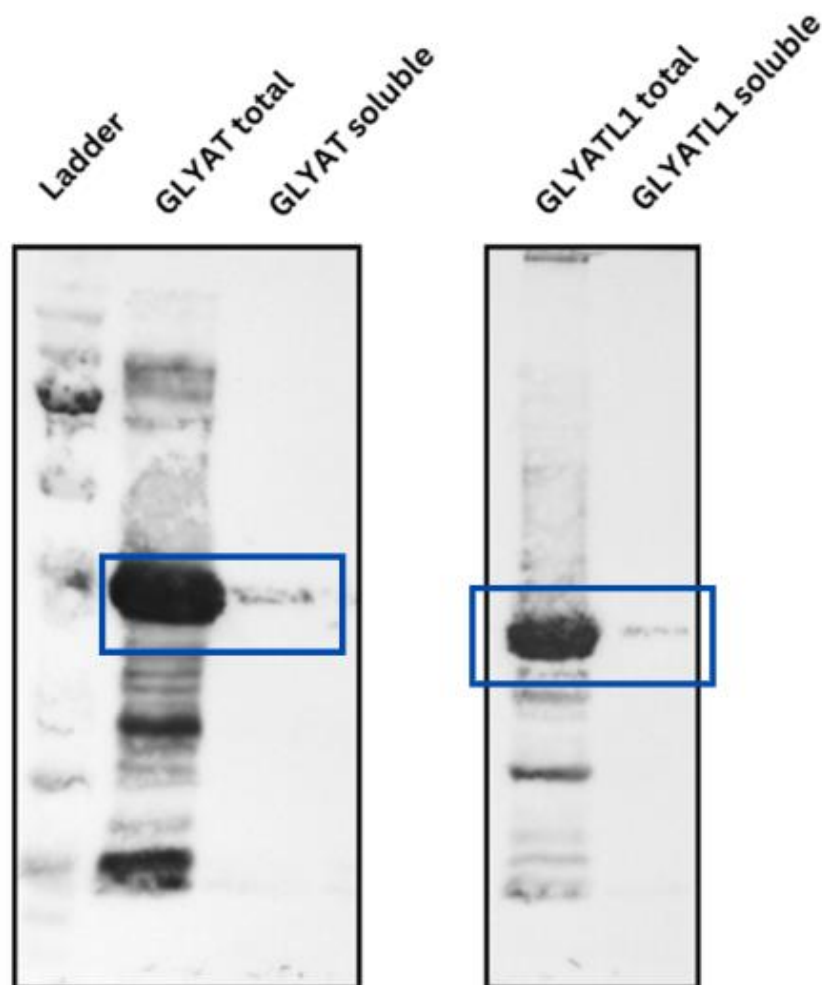


Figure 3.3.8 Western blot blocked with 5% BSA and incubated with 6x – His tag Monoclonal Antibody (HIS.H8), HRP activated.

Lanes contain both total and soluble fractions of lysate after the addition of lysozyme and protease inhibitor. Bands can be seen as indicated in blue boxes.

In Figure 3.3.8 thick bands can be seen in the total fraction lysate as indicated in the blue boxes. Faint bands can also be seen in the soluble fraction at the same location with no other band formation occurring in the lanes. While these bands are present, they are not prominent enough to be declared a definitive band. Improvements are observed but changes still need to be made to improve the concentration of the enzymes in the soluble fraction.

3.3.5 Temperature and induction time adaptations to the expression protocol.

While both GLYAT and GLYATL1 were included in initial expression experiments, the subsequent figures in this section focus solely on GLYAT. Faint bands corresponding to GLYATL1 were observed in some early SDS-PAGE analyses; however, these signals were not consistently reproducible across repeated experiments. As a result, and given time constraints, later optimization steps, including adjustments to induction temperature and incubation time, were applied only to GLYAT. The decision to focus further analysis on GLYAT reflects its more reliable expression profile under the tested conditions.

While the lysis buffer improvements focused on downstream optimization, upstream causes also needed to be investigated. Francis and Page (2010) suggests a few different strategies to optimise protein expression in *E. coli*. One of the methods is decreasing the time that induction occurs. This is because overexpression can become destructive to the cell and limiting expression can yield better protein solubility (Bolognesi and Lehner, 2018). In this study the expression time was therefore reduced from 24 hours to only 6 hours.

Another strategy to optimise protein expression is the adjustment of temperature, especially lowering the temperature during induction. Huang et al., (2021) did a study to compare different induction temperatures and showed significant yield increase of protein at lower temperatures. In this study, induction temperatures were reduced from 25 °C to 22 °C (Figure 3.3.9).

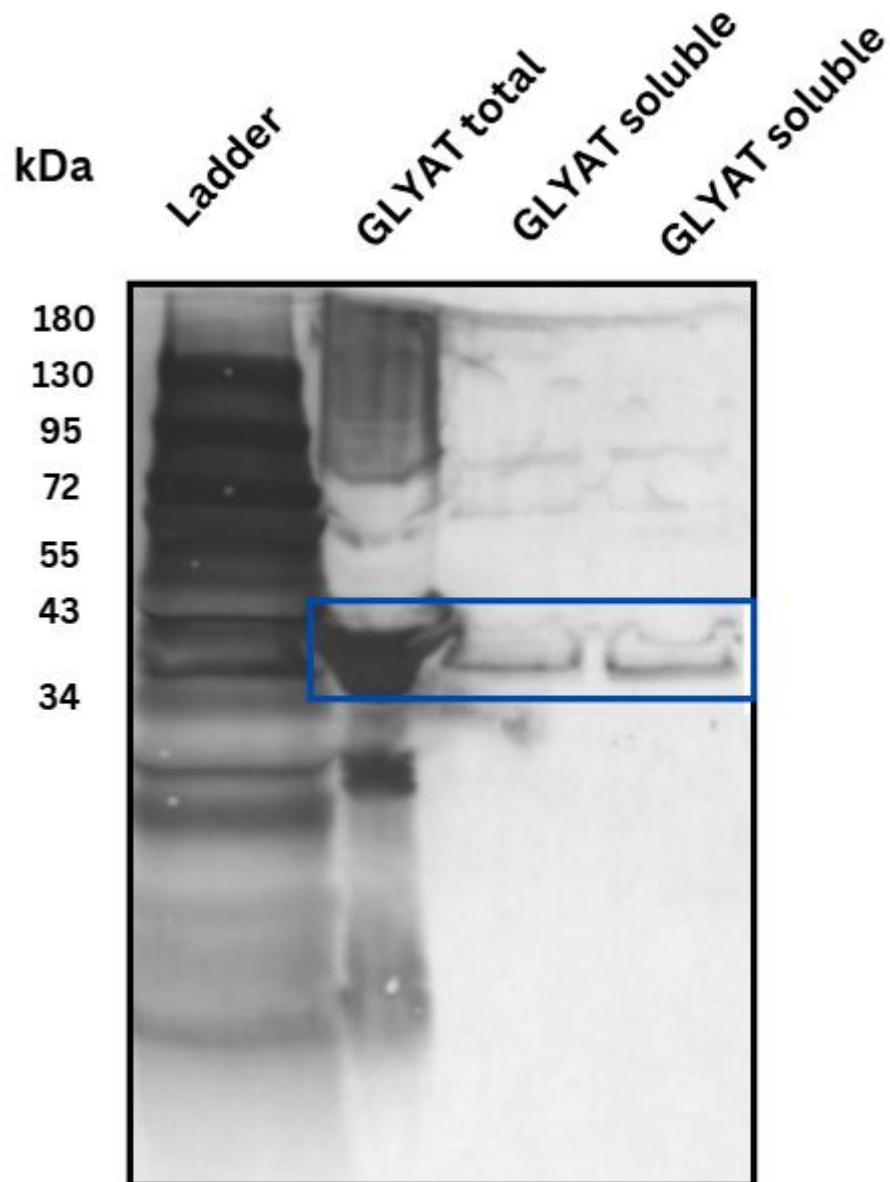


Figure 3.3.9 Western blot of 12% TGX SDS-PAGE gel, blocked with 5% BSA, incubated with 6x – His tag Monoclonal Antibody (HIS.H8), HRP activated.

The blot shows both total and soluble fraction lysate after temperature and induction time reductions during induction. Band formation can be seen in the predicted location as indicated in the blue boxes.

In Figure 3.3.9 shows successful band formation in the soluble fraction that are also less fuzzy and prominent when compared to the western blot before the expression adaptations. This showed that the proteins were more soluble and possible confirmation changes were being reduced. The western blot technique will need adjustment for neater and clearer results. Because of the limited time of the study, progression in the overall study was more important as this trouble shooting

phase was unforeseen. The last step was to culture and express GLYAT enzymes which were aliquoted and flash frozen and finally stored at -80 °C for further use.

3.3.6 Enzyme activity analysis using preferred substrates (glycine & benzoyl-CoA).

After expression and lysis, confirming that the recombinant GLYAT enzyme was catalytically active was essential before proceeding to the next phase of the study, substrate competition analysis using NMR spectroscopy. Despite suboptimal expression levels, it was necessary to establish that the enzyme retained its activity under experimental conditions, as inactive or degraded enzyme would invalidate downstream kinetic and competition assays.

The enzyme activity assay employed 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), also known as Ellman's reagent. This compound reacts with free coenzyme A (CoA) molecules released during the enzymatic reaction to produce a yellow-coloured product, TNB^{2-} , which absorbs at 410 nm. As GLYAT catalyses the conjugation of benzoyl-CoA with glycine, each reaction liberates a CoA molecule. The rate of colour formation, therefore, directly correlates with enzyme activity and can be quantitatively monitored via spectrophotometry.

A previous study analysed GLYAT reaction kinetics and plotted the initial reaction rate (v) against substrate concentration, producing a sigmoidal curve, indicative of substrate activation, when benzoyl-CoA concentrations were held constant (van der Sluis et al., 2017). Ideally, the current reaction system should demonstrate a similar kinetic profile under consistent conditions.

The experiment included two control conditions: one lacking benzoyl-CoA and another lacking glycine. These were necessary to confirm that any observed activity was due to the intended enzymatic reaction, rather than spontaneous hydrolysis or background interference. Reactions were carried out at 33 °C for 10 minutes, with absorbance readings taken every 20 seconds using a BioTek plate reader. Each condition was run in separate wells on a 96-well reaction plate (Figure 3.3.10).

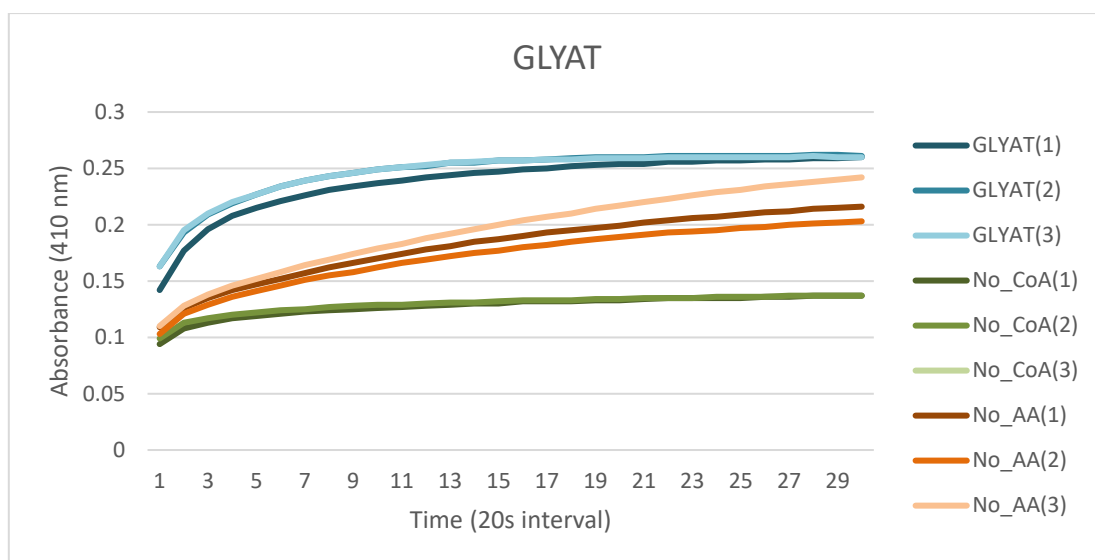


Figure 3.3.10 Enzyme activity of recombinant GLYAT measured by DTNB assay.

Absorbance at 410 nm (y-axis) was recorded every 20 seconds for 10 minutes (x-axis) at 33 °C. Blue curves represent triplicate reactions containing both glycine and benzoyl-CoA, orange curves exclude glycine, and green curves exclude benzoyl-CoA. The increase in absorbance in the complete reaction confirms enzymatic activity. Minor activity in glycine-free controls likely results from residual glycine carried over from expression. No activity was observed without benzoyl-CoA.

Figure 3.3.10 shows the reaction curves over time. Samples containing both benzoyl-CoA and glycine (blue traces) produced a clear increase in absorbance, consistent with active CoA release. No measurable activity was observed in samples lacking benzoyl-CoA (green), validating the specificity of the assay. Interestingly, samples lacking glycine (orange) still demonstrated a measurable degree of activity. This unexpected result was attributed to residual glycine carried over from the expression step, where glycine was supplemented in the growth media to support protein expression. Although no glycine was intentionally added during the assay setup, trace amounts likely remained in the lysate supernatant and were sufficient to support limited product formation.

Despite these complexities, the assay successfully confirmed that the GLYAT enzyme was catalytically active. This verification was critical to justify progression to NMR-based substrate competition experiments, which required a functional enzyme capable of interacting with multiple acyl-CoA substrates under controlled conditions.

3.4 Conclusion

This chapter detailed the successful cloning, expression, and functional validation of recombinant GLYAT, along with initial attempts at expressing GLYATL1. While GLYATL1 expression produced faint signals in early experiments, these were not reproducible, and subsequent optimization efforts focused exclusively on GLYAT. Through iterative changes, including the removal of co-inducers, adjustment of IPTG concentrations, modifications to induction temperature and duration, and the incorporation of lysozyme and protease inhibitors during lysis, GLYAT expression was improved to the extent that an active enzyme preparation was obtained.

Although expression yields were not fully optimized, the incremental protocol refinements developed in this phase serve as a strong foundation for future optimization efforts. Each step provided insight into conditions affecting solubility and activity, which can be further explored and fine-tuned in follow-up experiments.

The DTNB-based activity assay confirmed that GLYAT was enzymatically active under the tested conditions. However, this assay lacks substrate specificity, as it detects total CoA release regardless of which substrate is used. As a result, while enzyme functionality was confirmed, the assay cannot distinguish between different acyl glycine products formed. To overcome this limitation, the next stage of the study employed nuclear magnetic resonance (NMR) spectroscopy, which enables direct detection of specific conjugation products and allows for a more detailed investigation of substrate competition within the glycine conjugation pathway.

CHAPTER 4 DEVELOPING A NUCLEAR MAGNETIC RESONANCE (H^1 -NMR) SPECTROSCOPY METHOD TO EVALUATE SUBSTRATE COMPETITION IN THE GLYAT REACTION

4.1 Introduction

The aim of this study was to evaluate to what degree substrate competition affects the detoxification rate of glycine conjugation. In Chapter 3, recombinant GLYAT was expressed, and the Ellman's test was used to confirm that the expressed protein was enzymatically active (Šinko et al., 2007). While this assay is useful for determining enzyme kinetics of glycine conjugation when using only one acyl-CoA substrate, it cannot be used to evaluate substrate competition as the individual glycine conjugates cannot be quantified

To overcome this limitation, an NMR method was developed to quantify the individual glycine conjugates formed during the glycine conjugation reaction. Nuclear Magnetic Resonance (NMR) spectroscopy is a powerful analytical technique that examines atomic nuclei interactions in magnetic fields, allowing for molecular structure determination and metabolite quantification (Palmer, 2004). Previous studies have demonstrated the use of NMR spectroscopy to investigate the interactions between recombinantly expressed enzymes and their substrates, providing insights into enzyme kinetics and substrate competition. For example, H^1 NMR has been used to monitor the catalytic activity of recombinant rat skeletal muscle cytosolic sialidase, enabling real-time observation of substrate hydrolysis and product formation (Albouz-Abo et al., 2005). Additionally, saturation transfer difference (STD) NMR has been applied to study recombinant GH10 xylanases from *Geobacillus stearothermophilus*, identifying key substrate-enzyme interactions and competitive binding effects (Balazs et al., 2013). Furthermore, ^{13}C NMR spectroscopy has been employed to characterize substrate binding and reaction kinetics in recombinant *Coprinus cinereus* peroxidase (CIP), demonstrating its utility in evaluating enzyme specificity and competition (VEITCH et al., 1994).

Although these studies highlight the effectiveness of NMR in studying recombinant enzymes and their substrate interactions, research into GLYAT substrate competition has been limited. Early studies did explore the ability of GLYAT to accept multiple acyl-CoA substrates and noted evidence of substrate competition (Webster et al., 1976, van der Westhuizen et al., 2000). However, these investigations relied on indirect enzymatic or metabolic assays and lacked the

resolution to quantify the different glycine conjugates formed. As a result, the precise extent of substrate competition and product distribution in GLYAT-mediated conjugation remains incompletely characterized. Analysing the degree of substrate competition of an enzyme is crucial to understanding the role an enzyme plays in xenobiotic detoxification. Since multiple substrates can be utilized for GLYAT mediated glycine conjugation (Tanaka and Isselbacher, 1967a, Moldave and Meister, 1957a), understanding the substrate preference and competition dynamics allows for a closer look at the efficiency of metabolizing toxic compounds. This can be useful in understanding conditions wherein the detoxification of alternative metabolites occur (i.e. detoxification of Isovaleryl-CoA in Isovaleric acidemia).

For this study, glycine conjugation reactions were conducted using recombinant GLYAT with its preferred substrates, glycine and benzoyl-CoA. Reaction product (hippuric acid) was analysed via ^1H NMR to quantify the substrate consumption and hippuric acid formation. After the establishment of baseline values, additional reactions were analysed that incorporated phenylacetyl-CoA and Isovaleryl-CoA. These substrates have been implicated in GLYAT-mediated glycine conjugation, but their competitive interactions and inhibitory effects remain uncharacterized (Gegersen et al., 1986, Moldave and Meister, 1957b).

4.2 Materials and Methods

4.2.1 Materials

All materials utilized in this chapter can be found in Appendix B. This includes the reagents, tools and machinery.

Buffer preparation

A standard NMR phosphate buffer (also referred to as the urine buffer, following Dona et al., 2014) was prepared to ensure consistent spectral conditions and metabolite stability during ^1H -NMR analysis. The buffer was formulated by dissolving 20.4 g of potassium phosphate monobasic (KH_2PO_4) in 80 mL of deuterium oxide (D_2O) to yield a 1.5 M solution. This high-concentration phosphate buffer provides a stable chemical environment and minimizes variability in chemical shifts across samples, helping to “lock” the pH and ionic strength during NMR analysis (Malatji et al., 2017).

To facilitate absolute quantification and chemical shift referencing, 100 mg of trimethylsilyl-2,2,3,3-tetradeuteriopropionic acid (TSP) was included as an internal standard (Malatji et al.,

2017). To prevent microbial contamination during storage and analysis, 13 mg of sodium azide was dissolved in 10 mL of D₂O and added to the buffer. The buffer was sonicated for thorough mixing, then adjusted to pH 7.4 using potassium hydroxide pellets. The solution was finally made up to 100 mL with D₂O and stored at 4 °C until use.

After ultrafiltration, 54 µL of filtered sample was mixed with 6 µL of this buffer to obtain a 90:10 sample-to-buffer ratio prior to NMR acquisition.

4.2.2 Methods

Enzyme reactions

Reagents and protein aliquots were removed from storage and thawed on ice. Enzyme reactions were prepared in a total volume of 100 µL at room temperature in sterile Eppendorf tubes. Each standard reaction contained the following final concentrations: 100 mM Tris-HCl buffer (pH 8), 100 mM glycine, 300 µM acyl-CoA substrate, and approximately 5 µg of total soluble protein (containing recombinant GLYAT) lysate (added as a 10 µL aliquot of the soluble lysate). The protein concentration of the lysate was determined using the Qubit™ Protein Assay (broad range), and the enzyme quantity was estimated based on the total protein content of the lysate. Molecular-grade water was added to adjust the total reaction volume.

The acyl-CoA substrates tested in this study included benzoyl-CoA, phenylacetyl-CoA, and isovaleryl-CoA, each tested either individually or in combination, depending on the specific experimental condition. For negative control reactions, specific reagents (e.g., glycine or acyl-CoA) were omitted and replaced with molecular-grade water to maintain a consistent reaction volume. The reaction mixture is quickly placed within an incubator (37 °C) for 10 minutes.

Sample preparation for NMR analyses

Following the enzyme reaction, samples were prepared for NMR analysis through ultrafiltration to remove macromolecules that may interfere with spectral clarity. Ultra Centrifugal Filters (UFC5100BK, Millipore®) were pre-washed to eliminate residual chemicals, such as glycerol-based preservatives, commonly present in regenerated cellulose membranes (Cong et al., 2023). If not adequately removed, these residues can leach into the filtrate and compromise metabolite detection.

To minimize this risk, filters were pre-washed by adding 400 μL of Milli-Q ultrapure water followed by centrifugation at $21,500 \times g$ for 5 minutes. After each spin, filters were externally rinsed with distilled water and gently shaken to dry. This process was repeated five times to ensure thorough decontamination.

After the enzyme reaction was complete, the entire 100 μL reaction mixture was transferred into the washed centrifugal filter and centrifuged at $21,500 \times g$ for 30 minutes. This ultrafiltration step removes proteins and other macromolecules that can cause broad background signals in ^1H -NMR spectra (Brown et al., 2022). These broad signals can overlap with metabolite peaks, leading to reduced resolution and inaccurate quantification.

Following filtration, 54 μL of the filtrate was mixed with 6 μL of a standard urine phosphate buffer (prepared as described in Section 4.2.1) to obtain a 90:10 sample-to-buffer ratio. The mixture was then transferred to an NMR tube for analysis.

^1H NMR analysis

^1H -NMR spectra were acquired at 500 MHz using a Bruker Avance III HD NMR spectrometer equipped with a 5 mm triple resonance inverse (TXI) probe head (^1H , ^{15}N , ^{13}C) and x, y, z gradient coils. Each spectrum consisted of 128 transients collected over 23,000 data points with a spectral width of 6000 Hz (12.0 ppm). The sample temperature was maintained at 300 K throughout the analysis to ensure stable conditions (Mason et al., 2018).

To suppress the H_2O resonance at 4.70 ppm, the NOESY-presat pulse sequence was employed, applying single-frequency irradiation during a relaxation delay of 4 seconds with a 90° excitation pulse of 10 μs . Acquisition time and receiver gain were set to 2.7 seconds and 64, respectively. A total of 4 dummy scans were performed, followed by 128 scans for each sample, resulting in a total acquisition time of approximately 15 minutes and 45 seconds per sample. These acquisition parameters were selected based on established standard operating procedures (SOPs)(Beckonert et al., 2007, Dona et al., 2014).

NMR spectra were pre-processed using Bruker TopSpin, including spectral alignment, chemical shift referencing, phase correction, and baseline correction to ensure consistency for subsequent quantification. Bruker AMIX was used for peak selection, identification with an in-house compound library, and quantification.

4.3 Results and discussion

Ensuring the reliability and reproducibility of scientific results requires rigorous method standardization and quality control. In analytical techniques such as ^1H NMR spectroscopy, even minor variations in sample preparation, instrument calibration, or data acquisition can lead to inconsistencies that compromise the validity of results (Dumas et al., 2006). To mitigate these issues, researchers must undergo thorough training to familiarize themselves with the intricacies of the technique and establish standardized protocols that minimize experimental variability.

One of the primary aspects of standardization in this study was optimizing the substrate and enzyme concentrations to balance reagent usage efficiency with obtaining sufficiently strong NMR signals for accurate analysis. The initial substrate concentration range (2.5 μM , 5 μM , 10 μM , 20 μM and 40 μM) was selected based on concentrations reports in prior studies investigating GLYAT enzyme kinetics and activity (Rohwer et al., 2021). These concentrations allowed exploration within a physiologically relevant range while considering detection limits of NMR. It is important to note that the substrate concentration can influence GLYAT activity, with higher concentrations potentially leading to substrate inhibition, as reported in van der Sluis et al. (2017a) and Knights and Vessey (2010). Therefore, the range tested also aimed to identify concentrations that minimize inhibitory effects while maintaining detectable product formation for quantification.

Beyond reagent concentration optimization, reproducibility was assessed by performing repeated experiments under identical conditions to quantify variability through coefficient of variance (CV%). Replicate reactions (triplicate) were conducted, and spectra were analysed to ensure peak positions and intensities on the spectra remained consistent across all runs.

Instrument calibration and data acquisition were also standardized to reduce potential sources of error. This included regular calibration using a reference compound (Trimethylsilyl propanoic acid (TSP), shimming to optimize magnetic field homogeneity, and maintain consistent acquisition parameters such as spectral width and relaxation (Mason et al., 2018, Beckonert et al., 2007).

Additionally, sample preparation was standardized by ensuring uniform mixing, consistent reaction times, and controlled temperature conditions (10 minutes at 37 °C). The wash step was also optimized by aligning the reaction tubes in the centrifuge so that the filter membranes were oriented perpendicular to the axis of rotation. This helped ensure even centrifugal force across the membrane surface, improving flow-through consistency and minimizing sample retention.

Through these standardization steps, this study established a robust methodological framework that ensures reliable and reproducible results in the investigation of GLYAT-mediated glycine conjugation.

4.3.1 Reagent concentration range establishment.

The first experiment aimed to establish the lower limit of detection (LOD) for key compounds in the glycine conjugation reaction, specifically hippuric acid, benzoyl-CoA, and glycine. Determining the LOD is essential when working with limited sample quantities and metabolites present at extremely low concentrations. For example, the enzyme reaction in Chapter 3 was performed in a 100 μL volume, where the substrate of interest (benzoyl-CoA) does not exceed 100 μM . Establishing the LOD helps optimize experimental conditions by ensuring that reagent use is efficient while still allowing reliable detection of target compounds against the sample background.

To simulate real sample conditions, the test samples contained expressed protein aliquots (soluble fraction of the lysate) from Chapter 3, providing a comparable matrix composition and background. This aliquot is expected to contain both the enzyme of interest and cellular metabolites. While this results in a less 'clean' sample, it serves as a practical representation of the real sample matrix, ensuring that the background noise observed in the spectra is comparable to that of actual experimental conditions. This approach helps establish a more realistic baseline for determining the limit of detection (LOD), accounting for the complexity of biological samples."

The samples were then spiked with pure hippuric acid (the enzyme reaction product) at concentrations of 2.5 μM , 5 μM , 10 μM , 20 μM , and 40 μM respectively in duplicates (Figure 4.3.1).

Several studies have supported the use of micromolar concentrations for ^1H NMR spectroscopy at 500 MHz, especially when working with limited sample volumes as in this case. For instance, a study utilized spectral fitting with a limit of approximately 50 μM , noting that the actual detection limit based on spectral noise could be as low as 10 μM (de Graaf et al., 2011). While lower concentrations are possible, it is recommended that peaks are well above baseline noise and not overlap with other resonances (Hafer et al., 2020).

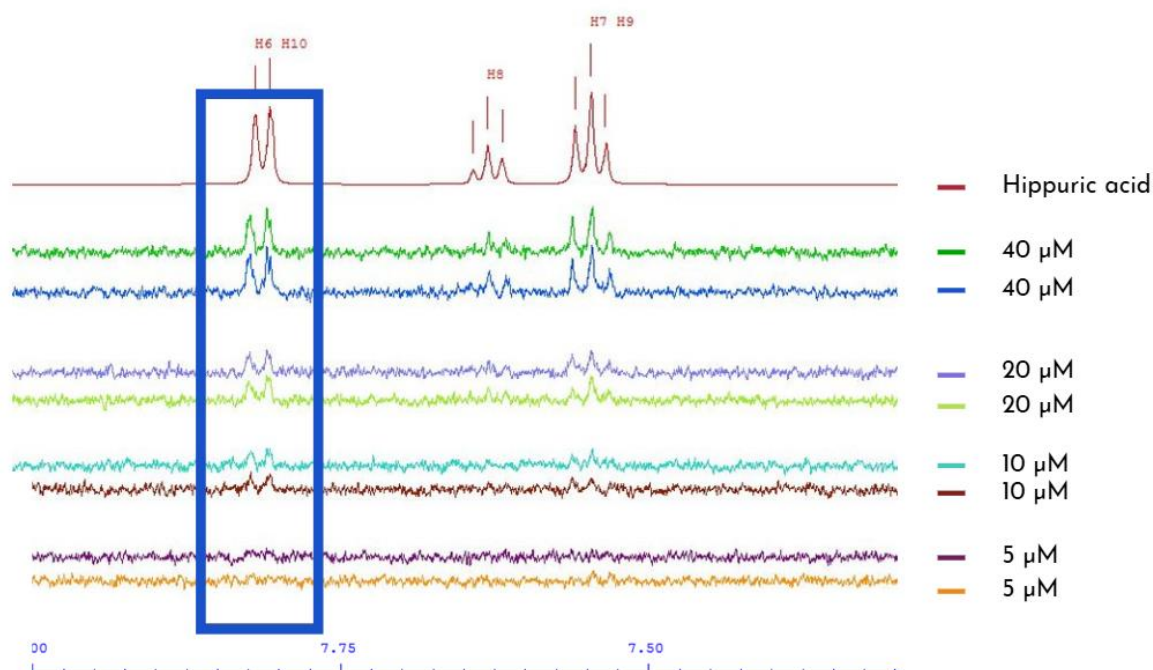


Figure 4.3.1 ^1H NMR spectra of samples spiked with different concentrations of hippuric acid, with TSP as an internal standard.

The blue box highlights the expected region of the hippuric acid peaks. Sample concentrations are arranged from lowest to highest, from bottom to top. The top spectrum represents pure hippuric acid, serving as a reference from an in-house database.

Figure 4.3.1 presents a series of ^1H NMR spectra corresponding to samples spiked with varying concentrations of hippuric acid, along with a reference spectrum of pure hippuric acid. As the concentration of hippuric acid increases, the intensity of the peaks within the highlighted region (blue box) becomes more pronounced. In NMR spectroscopy, peak intensity is directly proportional to the number of nuclei contributing to the signal, meaning that larger peaks indicate a higher concentration of the corresponding compound. This relationship allows for the quantification of hippuric acid in the samples based on peak integration (Pauli et al., 2012).

In the ^1H NMR spectrum of pure hippuric acid, three distinct clusters of peaks are observed, corresponding to different proton environments within the molecule. These arise from the aromatic ring protons, the methylene group adjacent to the nitrogen (N-CH_2), and the amide proton (Wei et al., 2011). Despite the presence of multiple peak clusters, an accurate quantification of hippuric acid can be achieved by integrating a single, well-resolved peak cluster (in the blue box) (ENFSI,

2017; USP, 2020). Selecting a single peak simplifies the analysis and minimizes potential integration errors, provided that the chosen peak is free from overlap and baseline distortions.

The peaks observed in the 40 μM reaction are more distinct compared to those at lower concentrations of hippuric acid. Based on this trend, concentrations below 50 μM are expected to produce peaks that may not be easily distinguishable from background noise and, therefore, cannot be confidently quantified.

In the previous enzyme reactions (Chapter 3), a substrate concentration of 100 μM was used, which consistently yielded quantifiable product formation. While this concentration exceeds typical physiological levels, where acyl-CoA concentrations in liver mitochondria are generally reported to be in the 1–10 μM range (Mawal and Qureshi, 1994b), the 100 μM concentration was retained in this study to ensure detectable signals in the NMR spectra, particularly given the method's sensitivity limitations (Rohwer et al., 2021; Kühn et al., 2023).

Although this concentration may not fully reflect *in vivo* conditions, it allows for robust comparative analysis of substrate competition under standardized and reproducible conditions.

In the enzymatic reaction, benzoyl-CoA reacts with glycine to form benzoylglycine (hippuric acid) and coenzyme A. The GLYAT mediated reaction proceeds in a 1:1 stoichiometry between benzoyl-CoA and hippuric acid. Therefore, it was concluded that the concentration of hippuric acid can directly reflect the initial concentration of benzoyl-CoA (Nandi et al., 1979c).

4.3.2 Reproducibility assessment and variability analysis.

Reproducibility is a critical aspect of method development, ensuring that quantified values remain consistent across repeated experiments. A key statistical measure for assessing variability is the coefficient of variation (CV%), which expresses the standard deviation as a percentage of the mean. A low CV% indicates high precision, demonstrating that the method yields reliable and reproducible results (Brenes, 2024).

In the context of NMR analysis of enzyme reactions, maintaining consistent peak positions and intensities across replicates is essential for accurate quantification. High variability in these values may indicate inconsistencies in sample preparation, instrument sensitivity, or reaction conditions, potentially impacting data interpretation. Therefore, evaluating CV% provides a quantitative measure of the method's robustness and helps identify sources of experimental variation.

Following the establishment of the desired concentration range, samples will now contain a complete enzyme reaction. Each sample will include active recombinantly expressed GLYAT enzyme along with its preferred substrates: 100 mM glycine and 100 μ M benzoyl-CoA. The enzyme reaction will be carried out at 37 °C for 10 minutes, after which the reaction matrix will be filtered and analysed via NMR.

To ensure rigorous assessment, the experiment included two control groups, similar to those used in the enzyme activity assay (Table 4.3.1). The Benzoyl-CoA control group contained all reagents except benzoyl-CoA, while the Glycine control group contained all reagents except glycine. These controls were designed to systematically exclude each essential substrate from the reaction to determine whether the formation of the product (hippuric acid) was dependent on the presence of that specific component. This approach helps confirm that any observed product formation is truly enzyme-catalysed and substrate-dependent. However, as the assay uses a bacterial lysate rather than purified GLYAT, the potential contribution of other endogenous *E. coli* enzymes cannot be fully ruled out. Nonetheless, the inclusion of substrate-specific controls allows for the assessment of whether product formation depends on the presence of key substrates, reducing the likelihood that observed signals arise from non-specific or non-enzymatic reactions.

Table 4.3.1 Experimental design of standard reaction test

Sample Group	Contains GLYAT (Y/N)	Contains Glycine (Y/N)	Contains Benzoyl-CoA (Y/N)	Number of Samples (n)
Benzoyl-CoA Control	Y	Y	N	3
Glycine Control	Y	N	Y	3
Standard Reaction	Y	Y	Y	3

Note: Y and N indicate the presence or absence of a given reagent.

After NMR analysis, the targeted reagents were quantified (Table 4.3.3.2).

Table 4.3.2 Raw data for metabolite concentrations (μM)

Sample Group	Glycine (μM)	Hippuric Acid (μM)	Benzoyl-CoA (μM)
Normal	887.53, 705.51, 892.38	153.79, 150.21, 172.83	0, 0, 0
No B-CoA	697.30, 813.05, 912.74	0, 0, 0	0, 0, 0
No Glycine	0, 0, 0	134.98, 133.02, 49.23,	60.53, 62.10, 70.51

Table 4.3.3 Coefficient of variation (CV%)

Sample Group	Glycine (%)	Hippuric Acid (%)	Benzoyl-CoA (%)
Normal	12.86	7.65	0.00
No B-CoA	13.35	0.00	0.00
No Glycine	0.00	46.29	8.34

From this data, several important trends can be observed. Overall, the CV% for most reagents and sample groups is relatively low, with most values falling below 15%, indicating good reproducibility. However, a notable exception is hippuric acid in the "no glycine" control group, which shows a CV% of 46.29%. This high variability is likely due to one of the triplicates having a concentration approximately one-third of the others, significantly increasing the variation.

Additionally, not all metabolites are detected in every sample group, which aligns with the expected reaction mechanism. For example, in the "no benzoyl-CoA" group, benzoyl-CoA is

absent, as none was added, and hippuric acid is also undetected since benzoyl-CoA is essential for its formation. Similarly, in the "no glycine" group, glycine is absent (as expected), yet concentrations of hippuric acid are detected, highlighting a key anomaly of the reaction that required further investigation.

Interestingly, in the "normal" group, where all reagents were present, no benzoyl-CoA was detected. This initially appeared to suggest complete substrate conversion.

However, further analysis revealed that the average concentration of hippuric acid was 158.94 μM , which exceeds the initial 100 μM of benzoyl-CoA added to the reaction. Given that hippuric acid is produced in a 1:1 ratio with benzoyl-CoA, this indicates likely over-quantification of the product. This discrepancy may be attributed to low signal-to-noise ratios in the NMR spectra, which can lead to integration errors and artificially inflated concentration values. Additional factors such as peak overlap, baseline artifacts, or matrix effects may also have contributed to the inaccurate quantification. These findings highlighted the importance of using signal quality thresholds and caution in interpreting peak integration results in low-concentration NMR studies. The use of an isotopically labelled internal standard could help correct for integration error and matrix effects by providing a reliable reference for quantification, improving accuracy and consistency in future experiments. For instance a study by Akira and Hashimoto (2001) used ^{13}C -labelled benzoic acid to monitor hippuric acid formation via NMR.

Contrastingly glycine concentration averaged only 828.47 μM , which was significantly lower than the expected $\sim 100\text{ mM}$. This discrepancy represented one of the major inconsistencies in the experiment and requires further investigation. Several factors may have contributed to this underestimation, including incomplete filtration resulting in glycine loss during sample preparation, poor signal resolution or integration inaccuracies due to the proximity of glycine peaks to neighbouring signals, or interference from overlapping metabolites in the sample matrix. Identifying the source of this deviation was essential to ensure the accuracy and reliability of the method.

4.3.3 Assessment of Glycine loss and background interference.

Based on these findings, two initial hypotheses were formed to explain the unexpected results. Firstly, the detection of hippuric acid in a reaction mixture that had not been spiked with glycine was unexpected, as glycine is a necessary substrate for the formation of benzoylglycine (hippuric acid). It was therefore postulated that residual glycine may have been present in the protein aliquot derived from the cell lysate, as the enzyme had not undergone purification. This

endogenous glycine could have served as a substrate, enabling the formation of hippuric acid despite the absence of externally added glycine.

Secondly, in reactions where glycine had been intentionally added, its final measured concentration was significantly lower than predicted, indicating a possible loss of glycine during sample preparation. One potential cause was that glycine did not fully pass through the filter.

To test these hypotheses, a comparative experiment was conducted, analysing glycine concentrations in a control sample (without protein aliquot) versus a test sample containing the cellular lysate background. In addition, the internal standard Dimethyl phenylalanine (DMPA) was introduced prior to filtration, allowing assessment of metabolite loss by comparing its known pre-filtration concentration to the post-filtration measurement.

Dimethylphenylalanine (DMPA) was selected as an internal standard to assess metabolite loss during filtration due to its favourable molecular properties. Structurally, DMPA contains two equivalent methyl groups attached to the α -carbon, producing a sharp, isolated singlet in the ^1H -NMR spectrum, which facilitates accurate quantification (National Centre for Biotechnology Information, n.d.). Its moderate size, nonpolar aromatic side chain, and lack of involvement in cellular metabolic pathways make it unlikely to bind non-specifically to proteins or enzymes. These features, combined with its solubility and stability under the experimental conditions, made DMPA a suitable candidate for detecting potential metabolite retention or loss during the filtration process.

Six samples in total were prepared, consisting of two experimental groups. The first group ("Unspiked Controls") included three samples that did not receive any additional glycine, while the second group ("Glycine-Spiked") comprised three samples spiked with 100 mM glycine. In all samples, the internal standard Dimethylphenylalanine (DMPA, 0.5 mM) was added prior to filtration. This setup enabled assessment of potential glycine loss by comparing glycine concentrations between spiked and unspiked groups, and evaluation of overall metabolite retention by measuring DMPA levels post-filtration (Table 4.3.4).

Table 4.3.4 Experimental design for glycine retention and filtration loss test.

Sample Group	Protein (Y/N)	Glycine 100 mM (Y/N)	DMPA 0.5 mM (Y/N)	Number of Samples (n)
Unspiked Controls	Y	N	Y	3
Glycine-Spiked	Y	Y	Y	3

Note: Y and N indicate the presence or absence of a given component.

Table 4.3.5 Raw data for metabolite concentrations (μM)

Sample Group	Glycine (μM)	DMPA (μM)
Unspiked Controls	71.39, 52.51, 66.86	0.575, 0.473, 0.500
Glycine-Spiked	131.86, 130.26, 106.79	0.527, 0.586, 0.449

Table 4.3.6 Coefficient of variation (CV%)

Sample Group	Glycine (%)	DMPA (%)
Unspiked Controls	14.96	10.30
Glycine-Spiked	11.27	13.25

From the results, we can see that the internal standard DMPA, which was added at a known concentration (50 μ M) prior to filtration, exhibited a coefficient of variation (CV%) between 10% and 13% across both control and sample groups. Since DMPA is a small, non-reactive compound not involved in cellular metabolism, this variation is interpreted as the baseline technical variability associated with filtration and sample handling.

Glycine, which was similarly spiked before filtration, showed a comparable CV range, suggesting that any observed decrease in glycine concentration is within the expected margin of technical loss. This implies that glycine was not selectively retained, bound, or otherwise blocked during the process. Instead, its variation reflects general metabolite retention variability, comparable to that observed for DMPA. It was also evident that glycine was present in samples that had not been spiked with external glycine. This confirms the initial hypothesis that residual glycine, likely originating from the cellular lysate, was already present in the protein aliquot. As a result, hippuric acid formation in samples containing only benzoyl-CoA can be explained by the availability of this endogenous glycine.

A follow-up experiment was designed to further investigate the presence of residual glycine in protein aliquots and to confirm its role in unintended hippuric acid formation. This experiment was carried out at a larger scale and included multiple sample groups. Two glycine “control” groups (triplicates) without protein were included to assess technical variability introduced by pipetting and filtration. One group contained 100 mM glycine, and the other 5 mM. The rationale for using both concentrations was to determine whether the previously observed glycine detection anomalies were related to high substrate saturation (as in the 100 mM group), or whether similar effects could still be observed at physiologically relevant concentrations (5 mM), which might point toward systematic matrix interactions or consistent glycine loss independent of concentration. These were followed by two additional groups in which the same glycine concentrations were combined with the protein aliquot, previously shown to contain residual glycine. Another group included only the protein aliquot, to quantify the endogenous glycine content. Lastly, a key test group was prepared by adding benzoyl-CoA to the protein aliquot without any spiked glycine, to assess whether hippuric acid could form under these conditions (Table 4.3.7).

Table 4.3.7 Experimental design for residual glycine and hippuric acid formation test.

Sample Group	Protein (Y/N)	Glycine (Y/N)	Benzoyl-CoA (Y/N)	Number of Samples (n)
Pure Glycine (100 mM)	N	Y	N	3
Pure Glycine (5 mM)	N	Y	N	3
Protein + Glycine (100 mM)	Y	Y	N	3
Protein + Glycine (5 mM)	Y	Y	N	3
Protein only	Y	N	N	3
Full reaction without Glycine	Y	N	Y	3

Note: Y and N indicate the presence or absence of a given component.

From this experiment, the “pure glycine” control groups (without added protein) displayed glycine levels that matched the expected concentrations. In contrast, all groups containing the protein aliquot exhibited interference in the spectral region used for glycine quantification (Figure 4.3.2). Although glycine was almost certainly present, its signal was confounded by overlapping peaks from glycerol, making accurate quantification unreliable. As illustrated in Figure 4.3.2, the glycine peak is partially obscured by neighbouring glycerol signals, highlighting the difficulty in resolving these metabolites due to spectral overlap. This interference likely explains the inconsistent or undetectable glycine levels in these samples. Notably, hippuric acid formation was observed only in the test group containing benzoyl-CoA but no added glycine, suggesting that trace glycine in the protein aliquot was sufficient to support the reaction.

The source of glycerol contamination is likely from the protein aliquot itself, as glycerol is included in enzyme storage buffers to stabilize proteins at -80°C . Additionally, residual glycerol from the

cellulose filters used during sample preparation cannot be ruled out. Regardless of its origin, glycerol presents a significant challenge for NMR-based metabolite quantification. Resolving this interference will be essential for accurately assessing substrate competition and reaction stoichiometry in future experiments.

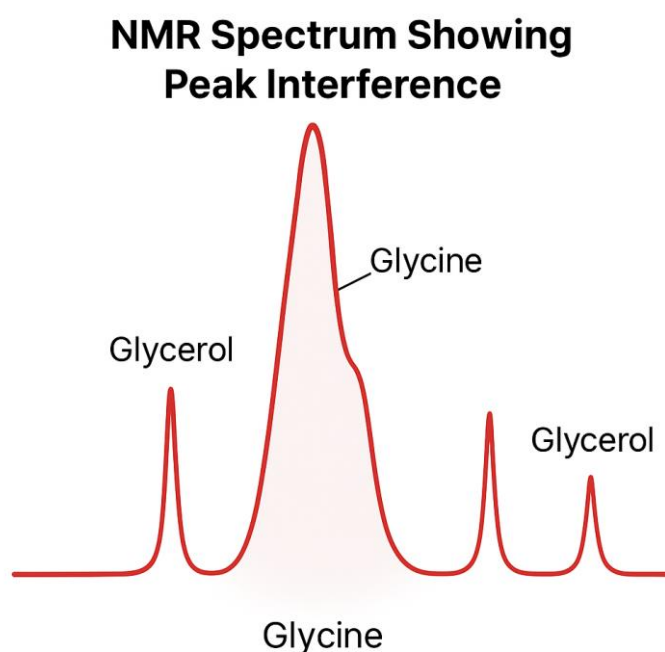


Figure 4.3.2 NMR spectrum showing peak interference between glycine and glycerol.

Overlapping signals in the glycine region hinder accurate quantification due to spectral crowding from glycerol.

4.3.4 Mitigation of Glycerol-derived spectral overlap.

Peak overlap in NMR spectra is a common analytical challenge, particularly in complex biological samples. One widely used strategy to resolve overlapping peaks is adjusting the sample's pH. Shifting the pH can influence the chemical environment of nuclei near protonation sites, thereby altering their resonance frequencies and potentially separating otherwise clustered signals (Putko et al., 2024). This technique is especially effective for metabolites with functional groups sensitive to protonation and deprotonation.

To explore this approach, a follow-up experiment was conducted in which the pH of the internal standard buffer (containing TSP) was systematically varied across samples, ranging from pH 3 to 8. The goal was to determine whether a pH-induced shift could separate the overlapping glycine and glycerol peaks. Despite the theoretical promise, altering the pH in this case did not sufficiently resolve the spectrum overlap between glycine and glycerol (results not shown). As such, alternative strategies were required to overcome this interference and enable accurate metabolite quantification.

Given the persistent peak interference, attention turned to the possibility that the source of the problem was not spectral overlap alone, but actual glycerol contamination introduced during sample preparation.

Glycerol contamination in NMR samples is a well-documented issue and is often attributed to components introduced during sample preparation. One notable source is the regenerated cellulose membrane found in centrifugal filters, which are commonly used to remove macromolecules from crude lysates while allowing smaller metabolites to pass through (Amicon™, 41104916). These membranes are preserved in glycerol during manufacturing, and insufficient washing can result in residual glycerol leaching into the filtrate. Standard protocols recommend multiple washing steps to minimize glycerol carryover (Mason et al., 2018).

To address this issue, the filter orientation during centrifugation was modified to improve the efficiency of the washing process. Instead of the random positioning, filters were oriented perpendicular to the axis of the centrifuge rotor. This adjustment allowed for more uniform solvent distribution across the entire membrane surface, rather than concentrating flow through one side. Preliminary results indicated that this modification led to improved glycerol removal, as evidenced by reduced peak interference in the NMR spectra (Figure 4.3.3). While not entirely eliminating the contamination, this optimized washing approach represents a meaningful step toward more reliable metabolite quantification.

To assess whether residual glycerol in filtered lysates would interfere with glycine quantification, an experiment was done to distinguish endogenous glycerol signals from those of exogenously added glycine. This approach aimed to determine whether glycine peaks could still be clearly resolved and quantified despite the presence of background glycerol contamination. Two sample groups were prepared: one containing only the protein lysate and buffer (unspiked controls), and another where 100 mM glycine was added prior to filtration (glycine-spiked). Both groups were treated under the same conditions to maintain consistency. The experimental design is summarized in Table 4.3.8.

Table 4.3.8 Experimental design for glycine-spiked filtration test and glycerol interference analysis

Sample ID	Protein present (Y/N)	Glycine 100 mM (Y/N)	Purpose
Sample 1	Y	N	Unspiked control
Sample 2	Y	N	Unspiked control
Sample 3	Y	N	Unspiked control
Sample 4	Y	Y	Glycine spiked
Sample 5	Y	Y	Glycine spiked

Note: Y and N indicate the presence or absence of each component. Unspiked controls were used to observe baseline glycerol signals from the lysate. Glycine-spiked samples were used to assess peak separation and potential interference. Only two replicates were included for this group due to one sample failing quality control, likely caused by an issue during sample preparation or loading. The remaining replicates were used for analysis.

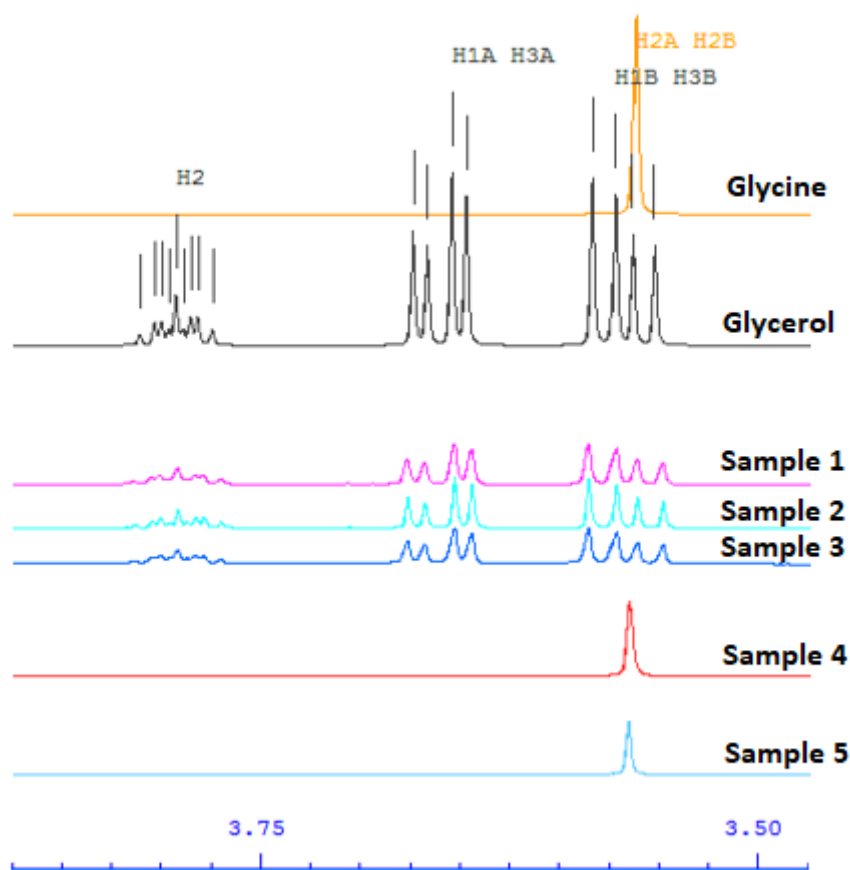


Figure 4.3.3 Representative NMR spectra showing glycine and glycerol peak positions across experimental samples.

The top traces correspond to pure glycine (yellow) and pure glycerol (grey) from the internal database. Sample 1–3 spectra represent unspiked controls with only the protein aliquot present. Sample 4 and 5 spectra include 100 mM glycine. The blue box highlights the region of interest containing overlapping glycerol and glycine peaks. Larger glycine signals in samples 4 and 5 demonstrate reduced interference under glycine-excess conditions.

Figure 4.3.3 displays representative NMR spectra from the glycine retention and filtration loss experiment. The top two traces show spectra for pure glycine and pure glycerol, respectively, taken from the in-house reference database. These serve as spectral references for peak identification. Glycine peaks (yellow trace) and glycerol peaks (grey trace) are clearly distinguishable.

Samples 1 to 3 represent unspiked control samples containing the protein aliquot, which includes the cell lysate background but no added glycine. These samples reflect the baseline level of glycerol-related interference inherent to the protein preparation. Samples 4 and 5 were prepared

identically but were additionally spiked with 100 mM glycine to evaluate whether glycine signals could be clearly distinguished from glycerol in a high-glycine background. As shown in the blue-highlighted region, samples 4 and 5 exhibit significantly larger glycine peaks relative to glycerol. In fact, no visible glycerol peaks were observed in these samples, which is due to the strong intensity of the glycine signal out scaling the smaller glycerol peaks, effectively masking them in the spectrum.

Additionally, glycerol contamination was reduced compared to earlier experiments, likely due to the optimized alignment of the filter tubes (perpendicular to the centrifuge axis), which helped minimize glycerol carryover from the filter membranes. Nevertheless, this setup demonstrated successful glycine signal detection in the presence of background glycerol, it is important to note that this outcome was not consistently reproducible across all experiments. In subsequent tests, significant glycerol interference re-emerged, likely due to variability in the glycerol content of the protein aliquots themselves. Since these aliquots originate from crude lysates and were manually pipetted, the amount of residual glycerol introduced into each sample may have fluctuated depending on how much lysate or filter residue was carried over. This variability underscores the need for tighter control during sample handling and further refinement of the preparation protocol to achieve reliable and interference-free glycine quantification.

To further address this variability and improve quantification accuracy, an additional strategy was implemented to mathematically account for overlapping peaks. Glycerol produces multiple distinct resonance clusters in the NMR spectrum due to its various proton environments, whereas glycine typically presents as a single sharp singlet. Leveraging this difference, a method was adopted in which a non-overlapping glycerol peak cluster was quantified independently, serving as a reference for glycerol content. This value was then used to estimate glycerol's contribution to the overlapping region that also contained the glycine signal. By subtracting the estimated glycerol portion, the remaining signal could be attributed to glycine with greater confidence. This subtraction-based interference correction method is a form of peak deconvolution or overlap correction, commonly used to address signal overlap in complex metabolic mixtures. Similar techniques have been employed in metabolic profiling studies involving complex mixtures, where signal overlap is a recurring challenge (Veselkov et al., 2009, Emwas et al., 2019b) This approach provided a practical and reproducible workaround for peak interference in cases where complete removal of glycerol was not achieved.

While the initial aim of this study was to investigate the variance in substrate competition and inhibition, particularly with regard to acyl-CoA's role in glycine conjugation via GLYAT, it became evident that quantifying glycine usage at low concentrations was not feasible with the methods at

hand. The challenge of distinguishing the small amounts of glycine used, especially when present in excess (~100 mM), led to a shift in focus away from glycine quantification. Given that glycine was in excess and the limiting reagent was the acyl-CoA (~100 μ M), the more critical and insightful analysis is centred on the acyl-CoA products formed. This shift allowed for a clearer investigation into the enzyme's activity and its interactions with acyl-CoA, providing a more direct and measurable pathway for understanding the enzymatic reaction. Thus, the latter part of this study focused on the acyl-CoA substrate and its resulting products, offering a more precise avenue for assessing the enzyme's functionality and substrate specificity.

4.3.5 Analysis of acyl-CoA substrate competition and product formation.

4.3.5.1 Benzoyl-CoA conjugation and Hippuric acid formation

To investigate substrate competition and inhibition, a series of stepwise experiments was conducted in which different acyl-CoA substrates were incrementally introduced into the glycine conjugation reaction. The aim was to observe how the presence of additional substrates would influence product formation, shedding light on substrate preference and potential inhibitory effects. Understanding these dynamics is particularly relevant for patients with metabolic disorders such as isovaleric acidemia (IVA), where substrate accumulation can impact detoxification efficiency. By modelling how different acyl-CoA's compete for GLYAT, these experiments can help clarify how patients metabolize CoA substrates under physiological and pathological conditions. This approach also provides a framework for future studies involving GLYAT variants or disease-associated mutations, where altered enzyme behaviour could further affect substrate usage and detoxification capacity. The starting condition involved only benzoyl-CoA, a well-characterized and preferred substrate of GLYAT. In the subsequent experiment, phenylacetyl-CoA was introduced alongside benzoyl-CoA. This substrate was chosen due to its known preference by GLYATL1, the GLYAT variant mentioned in this study.

Finally, isovaleryl-CoA was added as a third substrate. This acyl-CoA is clinically relevant, as its accumulation is a hallmark of isovaleric acidemia, a disorder that glycine conjugation may help to mitigate. This progressive experimental design was intended to reveal how substrate presence and competition affect CoA utilization and conjugate formation, offering insight into GLYAT's role in both normal metabolism and therapeutic detoxification.

To initiate the substrate competition experiments, the first reaction focused on benzoyl-CoA as the main CoA donor used in glycine conjugation catalysed by GLYAT. The experiment was structured in a stepwise manner to isolate the effects of each substrate. A consistent protein aliquot containing the expressed enzyme was used in all samples. Four groups were prepared:

enzyme only (serving as background control), enzyme with 100 mM glycine, enzyme with 100 μ M benzoyl-CoA, and enzyme with both 100 mM glycine and 100 μ M benzoyl-CoA. This layout enables direct comparison between conditions to assess whether hippuric acid formation is dependent on both substrates being present. The experimental setup is summarized in Table 4.3.9, and the corresponding NMR spectra are shown below to illustrate the observed product formation (Figure 4.3.4).

Table 4.3.9 Experimental design for residual glycine and hippuric acid formation test.

Sample Group	Contains GLYAT (Y/N)	Contains Glycine (Y/N)	Contains Benzoyl-CoA (Y/N)	Number of Samples (n)
Protein aliquot control	Y	N	N	3
Glycine Control	Y	Y	N	2
Benzoyl-CoA Control	Y	N	Y	2
Standard Reaction	Y	Y	Y	2

Note: Y and N indicate the presence or absence of a given component. Samples were excluded due to insufficient filtrate volume, which prevented proper NMR tube loading. This resulted in one group having three replicates while the others had only two.

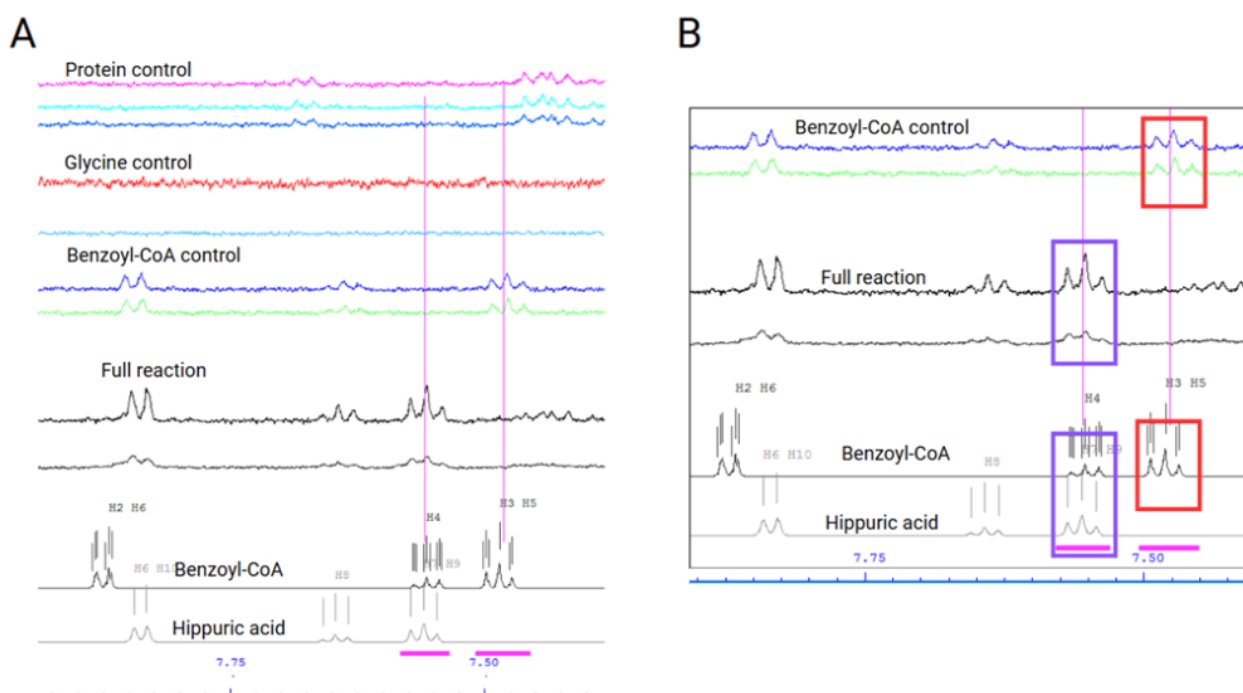


Figure 4.3.4 NMR spectra of glycine conjugation with benzoyl-CoA.

^1H -NMR spectra from each experimental group (see Table 4.3.9) are shown: Benzoyl-CoA Control, Glycine Control, and Standard Reaction (100 mM glycine, 100 μM benzoyl-CoA, and GLYAT enzyme). Peaks corresponding to benzoic acid and hippuric acid were identified using reference spectra. Hippuric acid formation is assumed to be observed only in the full Reaction group, indicating enzymatic activity.

Figure 4.3.4 presents representative ^1H -NMR spectra from the glycine conjugation reaction with benzoyl-CoA, showcasing the spectral profiles of each experimental condition. Panel A offers an overview of all experimental groups, while Panel B zooms in on the relevant spectral regions for benzoyl-CoA and hippuric acid. Reference spectra for benzoic acid and hippuric acid were used to annotate key peaks, highlighted in red (benzoyl-CoA) and purple (hippuric acid), respectively.

Notably, no significant peaks corresponding to either benzoyl-CoA or hippuric acid are observed in the enzyme-only and glycine-only controls. This absence supports the conclusion that the background signal from the enzyme-containing lysate does not significantly interfere with detection of the metabolites of interest, affirming the specificity of the observed peaks in the experimental groups.

In the full reaction condition (enzyme + glycine + benzoyl-CoA), characteristic hippuric acid peaks are clearly visible, indicating successful enzymatic conjugation catalysed by GLYAT. Quantification of the hippuric acid peaks yielded concentrations of 420.99 μM and 459.79 μM in

the two replicate samples (Table 4.3.10), confirming product formation at a robust level under these conditions. This variability could stem from differences in the biological activity or concentration of the enzyme present in the protein aliquots. As the recombinant enzyme is derived from a soluble lysate fraction rather than being purified, variations in expression level or folding efficiency between aliquots could influence reaction efficiency. Additionally, minor fluctuations in NMR sensitivity or shimming quality may contribute to peak intensity differences, though this is partially mitigated by the consistent benzoyl-CoA peak intensities observed in the control group, where the CoA ester is expected to remain unreacted.

Table 4.3.10 Quantified hippuric acid concentrations from full reaction samples (μM)

Sample Group	Hippuric Acid (μM)
Full Reaction 2	459.79
Full Reaction 2	420.99

An important limitation of the analysis is the partial spectral overlap between benzoyl-CoA and hippuric acid peaks. While reference spectra suggest clear separation between the two compounds in isolation, this resolution diminishes within the complex matrix of the reaction mixture. As such, the peaks identified in the full reaction group as hippuric acid (purple boxes) partially overlap with benzoyl-CoA signals. Despite this, their absence in the benzoyl-CoA-only control supports the hypothesis that these peaks predominantly represent newly formed hippuric acid. However, this spectral ambiguity may have contributed to an overestimation of hippuric acid concentrations, as some signal intensity from residual benzoyl-CoA may have been integrated into the product region. This could explain why quantified hippuric acid levels exceeded the initial 100 μM substrate concentration. Additional factors such as baseline drift, peak broadening, or small variations in NMR integration settings may also have compounded this effect. Nonetheless, the observed peaks in the full reaction remain consistent with enzymatic activity, and this quantification caveat should be considered in interpreting the results.

4.3.5.2 Phenylacetyl-CoA as competing substrate and spectral interference analysis

To expand the substrate competition analysis, the next experiment introduced phenylacetyl-CoA to assess its behaviour in the GLYAT-catalysed glycine conjugation pathway. The primary objectives were twofold: (1) to determine whether phenylacetylglycine formation could be observed and distinguished via H^1 -NMR, and (2) to evaluate whether the presence of phenylacetyl-CoA or its conjugated product interferes with the product formation, detection or resolution of hippuric acid peaks.

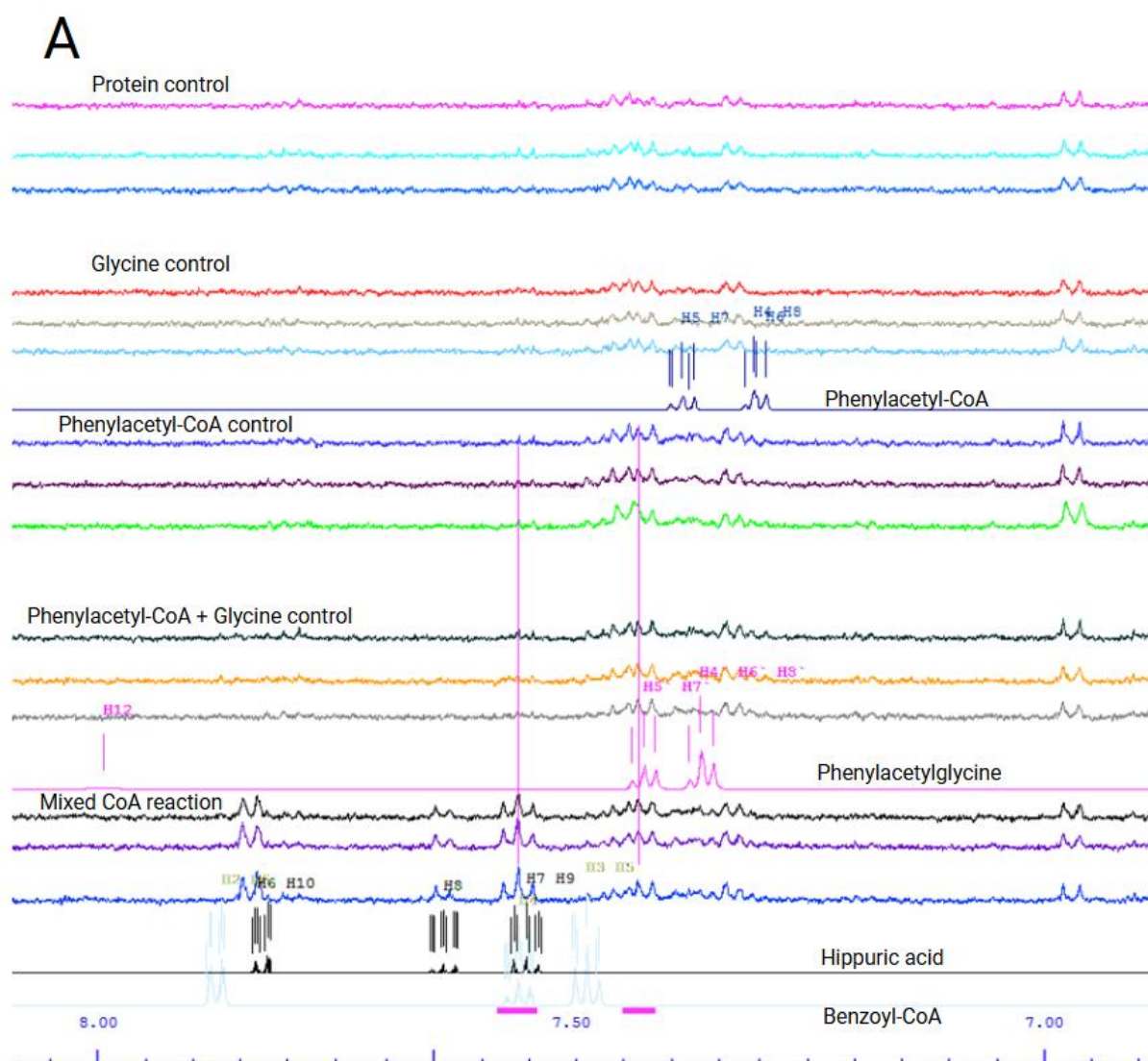
The experiment was structured in a stepwise manner using five experimental groups, each prepared in triplicate to ensure reproducibility. A consistent protein aliquot containing the expressed GLYAT enzyme was used across all conditions. The first group included only the protein aliquot, serving as background noise control. The second group added 100 mM glycine to the protein aliquot, ensuring glycine itself did not produce interfering signals. The third group introduced 100 μ M phenylacetyl-CoA to the protein aliquot, allowing assessment of phenylacetyl-CoA and phenylacetic acid peak behaviour within the lysate matrix. The fourth group combined phenylacetyl-CoA (100 μ M) and glycine (100 mM) with the protein aliquot to evaluate the formation of phenylacetylglycine. Finally, the fifth group included both phenylacetyl-CoA and benzoyl-CoA (each at 100 μ M), alongside 100 mM glycine, to investigate potential substrate competition effects during conjugation. A summary of the sample layout is provided in Table 4.3.11.

Table 4.3.11 Experimental design for assessment of phenylacetyl-CoA reactivity and potential substrate competition with benzoyl-CoA in glycine conjugation reactions.

Sample Group	GLYAT Present (Y/N)	Glycine (100 mM) (Y/N)	Phenylacetyl-CoA (100 μ M) (Y/N)	Benzoyl-CoA (100 μ M) (Y/N)	Number of Samples (n) (Y/N)
Protein aliquot control	Y	N	N	N	3
Glycine Control	Y	Y	N	N	3

Phenylacetyl-CoA Control	Y	N	Y	N	3
Phenylacetyl-CoA + Glycine Reaction	Y	Y	Y	N	3
Mixed CoA Reaction	Y	Y	Y	Y	3

Note: Y and N indicate the presence or absence of a given component.



B

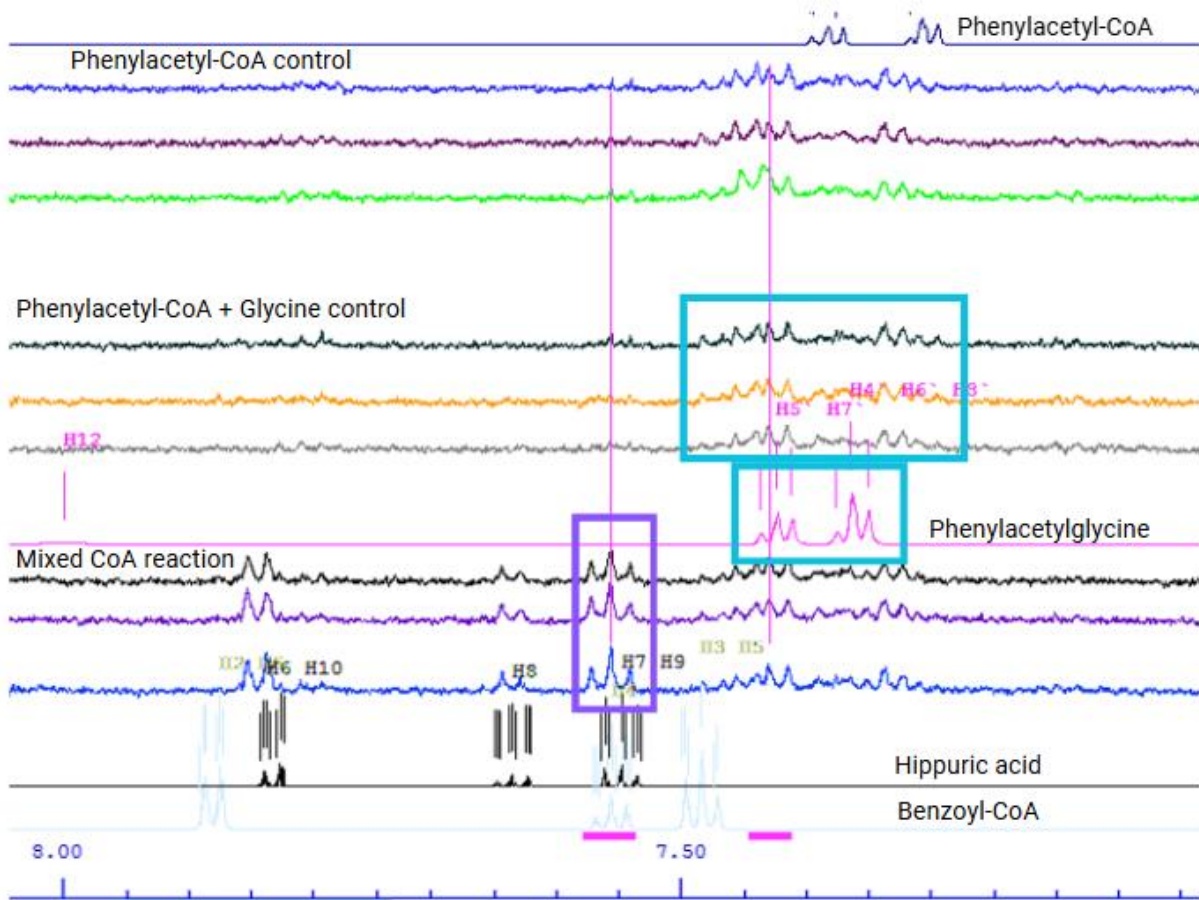


Figure 4.3.5 ^1H -NMR spectra of glycine conjugation reactions involving phenylacetyl-CoA and substrate competition with benzoyl-CoA.

(A) Overview of representative spectra from all experimental groups, arranged top to bottom in the same order as Table 4.3.11. **(B)** Zoomed-in view of selected spectral regions from the phenylacetyl-containing groups. Purple box highlights hippuric acid peaks observed in the final sample group. Aqua blue boxes indicate the expected phenylacetylglycine region based on a pure standard and observed peaks in the phenylacetyl-CoA + glycine group.

From the data presented in Figure 4.3.5, the hippuric acid peaks remain relatively distinct, even in the presence of phenylacetyl-CoA, suggesting that benzoyl-CoA continues to be effectively processed by GLYAT under competitive conditions. Quantification of hippuric acid peaks revealed that in the benzoyl-CoA-only group, concentrations reached 420.99 μM and 459.79 μM (Table 4.3.12), while the group with added phenylacetyl-CoA yielded lower hippuric acid levels of 150.64 μM , 229.57 μM , and 225.68 μM . This reduction indicates partial substrate competition or

inhibition, yet the sustained product formation highlights that benzoyl-CoA remains a highly preferred substrate. These quantitative results support and strengthen the qualitative observation that phenylacetyl-CoA does not fully suppress hippuric acid formation.

Table 4.3.12 Quantified Hippuric Acid Concentrations (μM) in Substrate Competition Assays

Experimental Group	Hippuric Acid (μM)
Benzoyl-CoA only	420.99, 459.79
Benzoyl-CoA + Phenylacetyl-CoA	150.64, 229.57, 225.68

In contrast, interpreting phenylacetyl product formation proved significantly more challenging. Reference spectra of both phenylacetyl-CoA and phenylacetyl-glycine from an in-house library were included to aid peak identification. Although peaks do appear near the expected phenylacetyl-glycine region in the spectra of phenylacetyl-CoA-containing samples, similar signals are also present in the protein-only and glycine control groups. This strongly suggests that the observed peaks arise from background metabolites in the lysate rather than phenylacetyl-glycine specifically. Notably, phenylacetyl-CoA is not the preferred substrate of GLYAT. Previous work, such as the study by (Kühn et al., 2023), has demonstrated that GLYATL1 displays significantly higher catalytic efficiency toward phenylacetyl-CoA than GLYAT. This may explain the lack of clear phenylacetyl-glycine formation in the current experiment, which utilized GLYAT. Future studies comparing phenylacetyl-glycine production between GLYAT and GLYATL1 could provide clearer insights into isoform-specific substrate preferences and detoxification roles.

However, upon closer inspection, a small, distinct peak appears within the expected cluster only in the phenylacetyl-CoA-positive samples (highlighted further in Figure 4.3.6.).

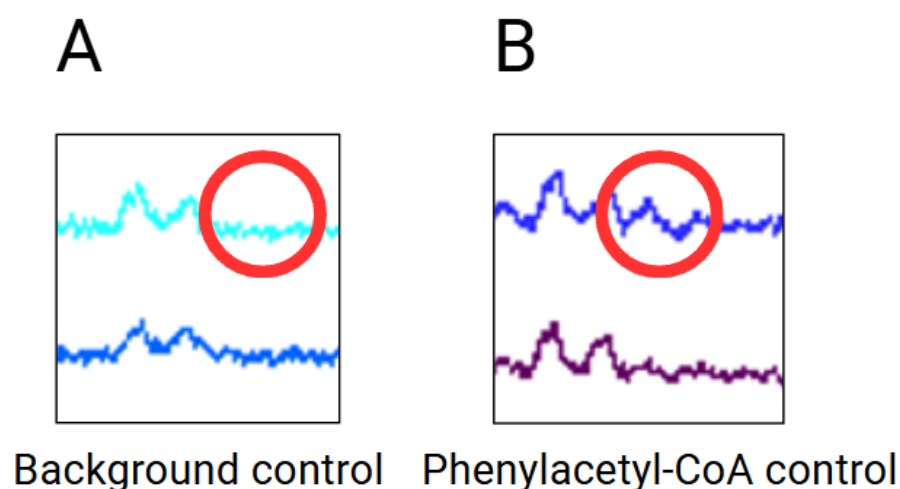


Figure 4.3.6 Shows peak cluster difference between samples.

Samples only containing protein aliquot (A) and sample spike with 100 μ M phenylacetyl-CoA at location of phenylacetic acid on the spectra. Peak difference can be observed as indicated by red circles.

This subtle spectral feature may indicate the presence of phenylacetylglycine, though likely at low concentrations insufficient to rise clearly above the complex lysate background. These findings suggest that while phenylacetyl-CoA may undergo some degree of glycine conjugation, its product formation under current conditions is minimal or below NMR detection limits.

Consequently, future competition and inhibition analysis may need to rely primarily on quantifying hippuric acid levels as the readout for substrate competition, rather than attempting direct detection of alternative CoA-derived products.

The limited visibility of phenylacetylglycine signals, despite the presence of its precursor substrates, highlights a key limitation of the current setup. The low concentrations of CoA derivatives may fall below the NMR detection threshold, particularly in the context of background interference from the lysate. This observation supports the increase in acyl-CoA concentrations in future experiments to improve signal clarity and enhance the reliability of product identification in complex sample matrices.

4.3.5.3 Addition of Isovaleryl-CoA to assess substrate competition.

To further investigate substrate competition in the GLYAT-catalysed glycine conjugation pathway, isovaleryl-CoA was introduced as an additional CoA donor. This substrate is of particular interest due to its physiological relevance in metabolic disorders such as isovaleric acidemia. The aim of this experiment was to determine whether isovaleryl-CoA can be reliably detected in the current assay system, whether isovalerylglycine is formed under the tested conditions, and how the presence of multiple acyl-CoA substrates may influence product formation.

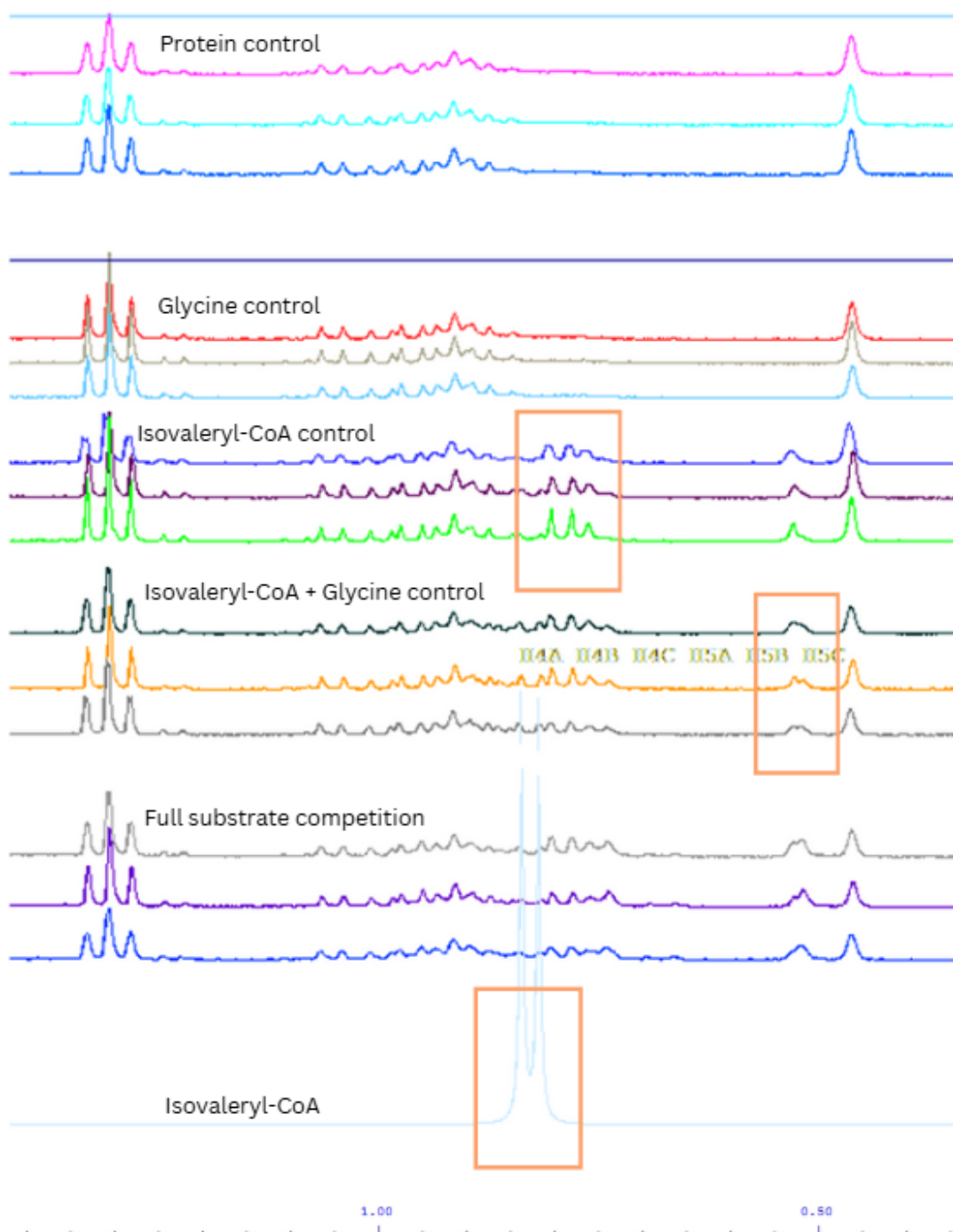
Five experimental groups were prepared, each in triplicate. The first group consisted of only the protein aliquot to serve as background control, followed by a glycine-only control group to assess any background peaks derived from glycine addition. The third group included the protein aliquot with 100 μ M isovaleryl-CoA to evaluate peak formation of the substrate in the absence of glycine. The fourth group contained both isovaleryl-CoA and glycine to monitor potential isovalerylglycine formation. Finally, the fifth group included all three acyl-CoA substrates: benzoyl-CoA, phenylacetyl-CoA, and isovaleryl-CoA, alongside glycine and the enzyme-containing lysate, to assess possible competition among substrates under the same reaction conditions. The full experimental layout is summarized in Table 4.3.13. Notably, a group containing both benzoyl-CoA and isovaleryl-CoA, without other competing substrates, was not included in this initial experimental setup. However, to address this limitation, a dedicated group featuring benzoyl-CoA and isovaleryl-CoA was included in a subsequent experiment (see Section 4.3.6), allowing for a more targeted analysis of substrate competition between these two physiologically relevant acyl-CoA's.

Table 4.3.13 Experimental design for isovaleryl-CoA inclusion and substrate competition test.

Sample Group	GLYAT Present (Y/N)	Glycine (100 mM) (Y/N)	Isovaleryl-CoA (100 μM) (Y/N)	Phenylacetyl-CoA (100 μM) (Y/N)	Benzoyl-CoA (100 μM) (Y/N)	Number of Samples (n) (Y/N)
Protein aliquot control	Y	N	N	N	N	3
Glycine Control	Y	Y	N	N	N	3
Isovaleryl-CoA Control	Y	N	Y	N	N	3
Isovaleryl-CoA + Glycine Reaction	Y	Y	Y	N	N	3
Full Substrate Competition Reaction	Y	Y	Y	Y	Y	3

Note: Y and N indicate the presence or absence of a given component.

A



B

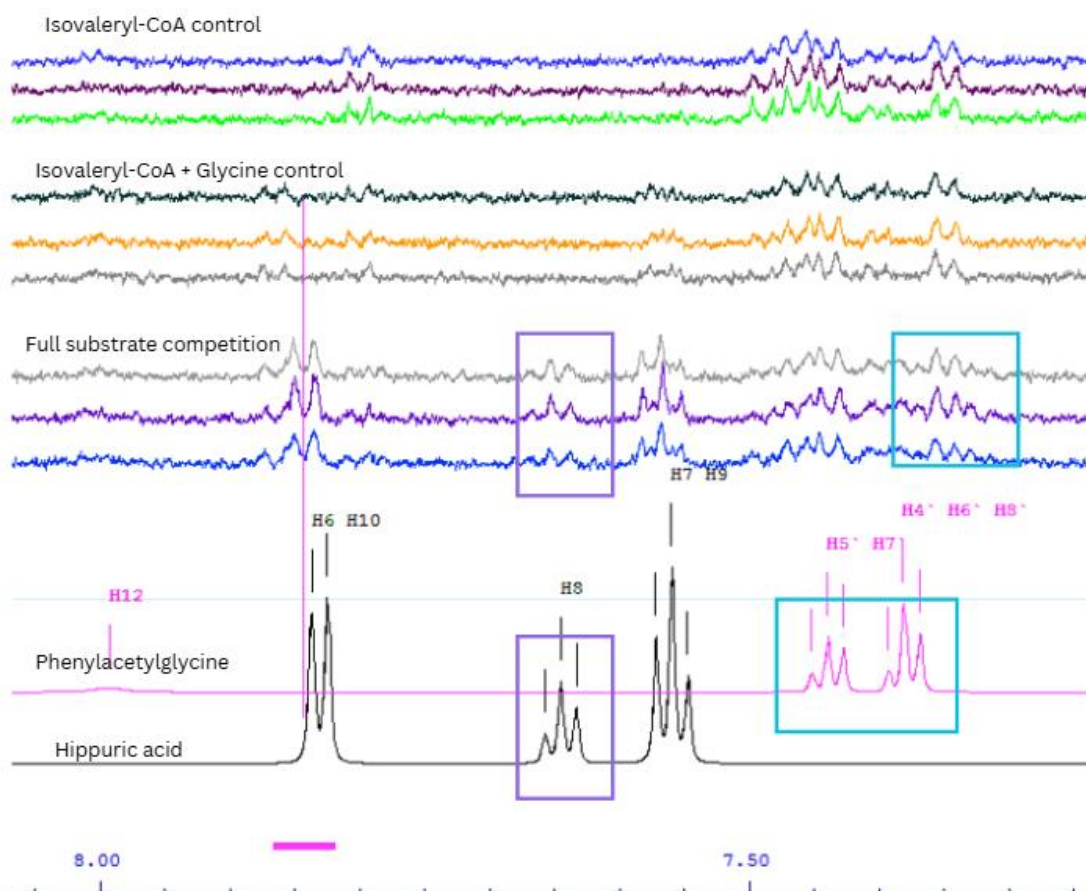


Figure 4.3.7 Representative ^1H -NMR spectra of GLYAT reactions with isovaleryl-CoA.

(A) Overview of all experimental groups arranged according to Table 4.3.6.3. Orange boxes indicate peaks near the isovaleric acid reference region. (B) Zoomed-in regions highlighting hippuric acid (purple boxes) and phenylacetyl-related peaks (aqua blue boxes).

Figure 4.3.7 consists of two panels. Image A displays all sample groups arranged on the NMR spectrum according to Table 4.3.13. At the bottom, a pure isovaleric acid compound from the in-house reference library is shown, with its corresponding peak cluster highlighted in an orange box. While no clear peak formation matching the reference compound can be seen in the experimental samples, additional minor peaks appear only in the groups spiked with isovaleryl-CoA. These are also indicated with orange boxes. Although their presence is intriguing, the peaks are neither prominent nor aligned with the reference signal, so it would be premature to identify them as isovaleric acid or isovalerylglycine.

It is worth noting that in NMR spectroscopy, metabolite signals may shift or be altered in complex biological matrices due to interactions, derivatization, or partial metabolism, resulting in chemical shifts that differ from pure standards (Emwas, 2015). This may explain the emergence of non-matching peaks in the isovaleryl-CoA samples.

As discussed previously, the lack of distinguishable peaks for phenylacetyl- and isovaleryl-derived products suggested that future reactions may benefit from increased acyl-CoA concentrations to enhance signal visibility and improve product identification confidence.

Panel B revisits the regions associated with hippuric acid and phenylacetyl-glycine. Phenylacetyl-related peaks (aqua blue boxes) continued to appear across all samples, including controls, complicating definitive identification. A subtle, unique peak previously observed in phenylacetyl-CoA samples (Figure 4.3.5) reappeared here, possibly indicating the compound's presence, though its signal remained buried beneath the background.

Hippuric acid peaks (purple boxes) are again observed exclusively in the final group consistent with the presence of benzoyl-CoA and enzymatic activity. However, the quantified concentrations 195.01 μM , 201.12 μM , and 163.25 μM were lower than the reaction group from the reproducibility experiment (Section 4.3.5.2) which lacked isovaleryl-CoA, suggesting that the additional substrate may have contributed to substrate competition (Table 4.3.14). The coefficient of variation (CV%) for this group was approximately 10.5%, indicating that the observed reduction in hippuric acid was unlikely to result from sample variability alone.

Two key insights emerged from these experiments: (1) hippuric acid formation remained the most reliable indicator of enzymatic activity, consistent with literature describing benzoyl-CoA as GLYAT's preferred substrate; and (2) due to ambiguous signals, product formation from phenylacetyl-CoA and isovaleryl-CoA cannot yet be reliably tracked using NMR in this sample matrix. Consequently, evaluation of substrate competition would primarily rely on monitoring hippuric acid formation, which may offer translational insights, such as advising patients with metabolic disorders on dietary strategies to minimize competitive substrate load.

Table 4.3.14 Quantified hippuric acid concentrations (μM) in the presence of all three CoA substrates (benzoyl-CoA, phenylacetyl-CoA, isovaleryl-CoA).

Sample Group	Hippuric Acid (μM)
Full Substrate Competition	195.01, 201.12, 163.25

To address the limitations encountered thus far, the final experiment will involve simultaneous addition of all three acyl-CoA substrates at an elevated concentration ($300\ \mu\text{M}$). The aim is to improve signal clarity for each product, particularly hippuric acid, to enable more accurate assessment of competition and potential inhibition.

4.3.6 Quantification of Hippuric Acid Under Substrate Competition Conditions

This section describes the final experiment in which all three acyl-CoA substrates (benzoyl-CoA, phenylacetyl-CoA, and isovaleryl-CoA) were added simultaneously at increased concentrations ($300\ \mu\text{M}$) to assess their combined effect on hippuric acid formation. In contrast to previous experiments, quantitative NMR analysis was performed to evaluate potential substrate competition or inhibition.

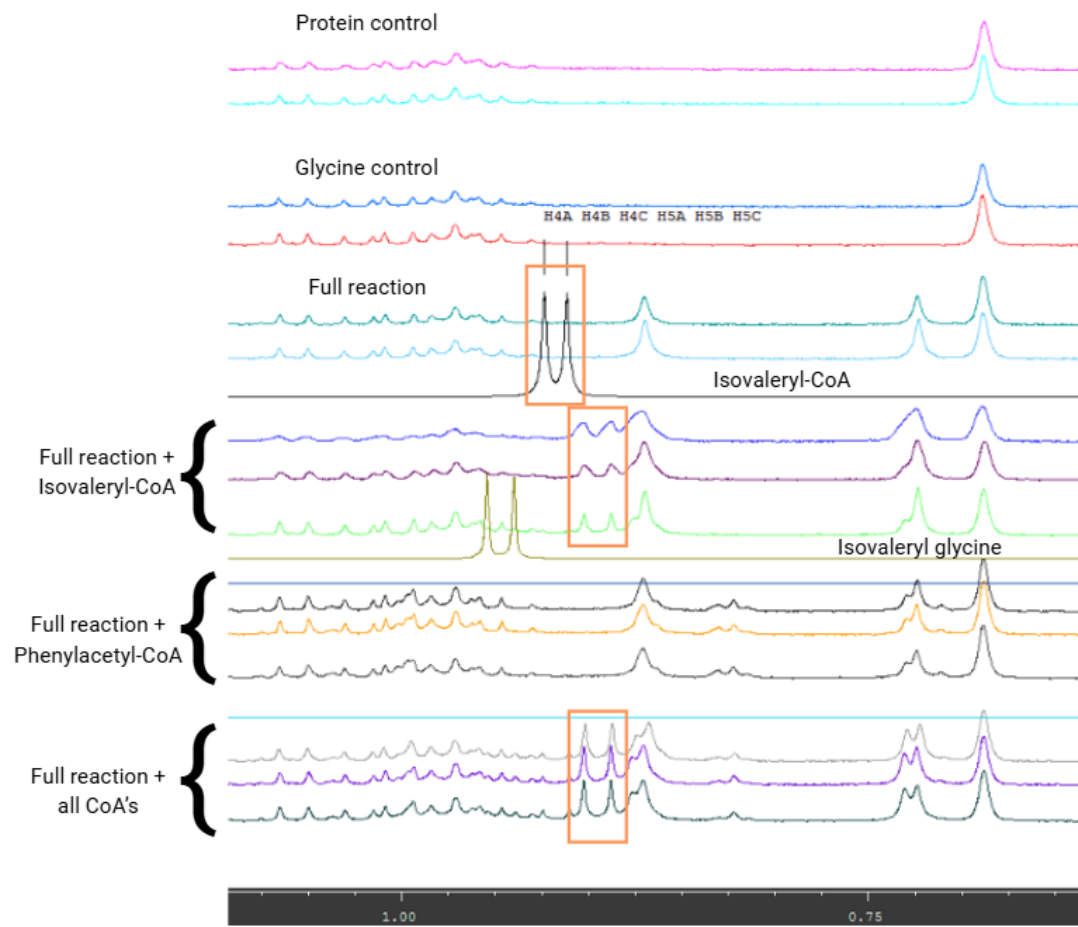
In this experiment, various combinations of CoA donors were tested in the presence of $100\ \text{mM}$ glycine and the same protein aliquot used previously. Control groups included samples with only the protein aliquot, with or without glycine, as well as a standard reaction group containing only benzoyl-CoA as the CoA donor. Experimental groups tested the effect of adding isovaleryl-CoA, phenylacetyl-CoA, or both in combination with benzoyl-CoA, to evaluate changes in hippuric acid formation. The experimental design is summarized in Table 4.3.15.

4.3.15 Experimental design for quantitative NMR analysis of acyl-CoA substrate competition

Sample Group	GLYAT Present (Y/N)	Glycine (100 mM) (Y/N)	Benzoyl-CoA (300 μ M) (Y/N)	Phenylacetyl-CoA (300 μ M) (Y/N)	Isovaleryl-CoA (300 μ M) (Y/N)	Number of Samples (n) (Y/N)
Protein aliquot control	Y	N	N	N	N	2
Glycine Control	Y	Y	N	N	N	2
Standard Reaction	Y	Y	Y	N	N	2
Reaction + Isovaleryl-CoA	Y	Y	Y	N	Y	3
Reaction + Phenylacetyl-CoA	Y	Y	Y	Y	N	3
Reaction + All CoA's (Full Mix)	Y	Y	Y	Y	Y	3

Note: Y and N indicate the presence or absence of a given component.

A



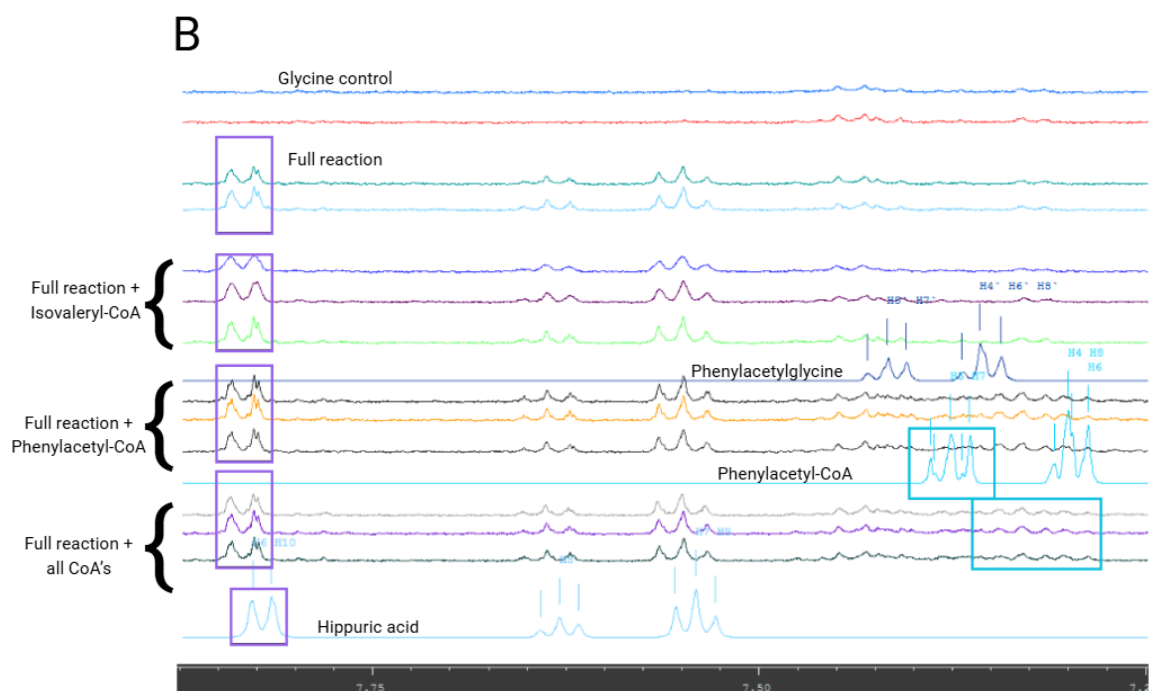


Figure 4.3.8 NMR spectra of final acyl-CoA competition experiment with increased acyl-CoA concentrations (300µM).

A) Spectral region showing isovaleryl-associated peaks across all sample groups (Table 4.3.15). Orange boxes highlight new peaks in the "Reaction + Isovaleryl-CoA" and "Reaction + All acyl-CoAs" groups, potentially representing isovaleryl derivatives. **B)** Spectral region showing hippuric acid (purple boxes) and phenylacetyl-associated peaks (aqua blue boxes). Hippuric acid peaks are distinct in all benzoyl-CoA samples. Phenylacetyl signals appear only in relevant groups but remain unclear.

Table 4.3.16 Quantification summary of hippuric acid peaks across experimental groups.

Sample Group	n	Raw Values (μM)	Mean (μM)	CV (%)
Protein aliquot control	2	2.70, 32.71	17.71	119.82
Glycine Control	2	49.60, 28.15	38.87	39.02
Standard Reaction	2	375.52, 524.36	449.94	23.39
Reaction + Isovaleryl-CoA	3	618.77, 613.15, 595.40	609.10	2.00
Reaction + Phenylacetyl-CoA	3	597.06, 563.90, 585.29	582.08	2.89
Reaction + All CoAs (Full Mix)	3	401.09, 558.70, 621.97	527.25	21.57

Several observations can be drawn from Figure 4.3.8. alongside the quantified hippuric acid peak values in Table 4.3.16. Firstly, hippuric acid peaks are now clearly distinguishable from the background noise, especially when compared to earlier experiments. This highlights that

increasing the concentration of CoA substrates positively impacted the qualitative detection of products, particularly for hippuric acid.

Looking at the quantified values, a few patterns emerge. The background controls (protein only and protein + glycine) still yield quantifiable values (17.71 μM and 38.87 μM , respectively), which are unexpectedly high given that no benzoyl-CoA was added. This likely reflects over-quantification due to background interference in a crowded matrix, a recurring issue in previous experiments.

Despite the low CV% in some of the competition groups suggesting some technical consistency, the absolute values are still biologically implausible and emphasize the importance of careful interpretation when working with complex lysates.

Interestingly, the "standard reaction" group shows a mean hippuric acid concentration of 449.94 μM , well above the ~ 300 μM substrate that was added. This may again point to over-quantification, perhaps exacerbated at higher peak intensities due to overlap or baseline distortion. It's important to note that NMR, while robust for structural elucidation and relative quantification, is not as sensitive or selective as other analytical platforms like LC-MS, which may explain some of the observed inflation.

While ratio-based analysis of substrate competition is conceptually preferable to relying on absolute quantification, since it can normalize for variability in sample preparation and signal intensity, the current data set exhibits considerable variability that undermines the reliability of ratio interpretations. Specifically, the calculated ratios in the final substrate competition group paradoxically return to near 100%, likely due to the inconsistent quantification values. Consequently, ratio analysis was not performed on this data set. Future studies with improved sample consistency and sensitivity could benefit greatly from ratio-based approaches to better elucidate substrate competition dynamics.

Additionally, the use of an isotopically labelled internal standard could also provide a valuable normalization factor, potentially reducing quantification variability. Incorporating such standards into NMR assays has been demonstrated in prior metabolomics research (e.g., (Emwas et al., 2019a)) to improve accuracy by compensating for variations in sample concentration, matrix effects, and instrument response. Thus, implementing labelled internal standards in future work may enhance the robustness and interpretability of quantitative data.

4.4 Conclusion

One of the main objectives was to explore whether inhibition or substrate competition could be inferred from changes in hippuric acid production. In theory, if isovaleryl-CoA or phenylacetyl-CoA compete with benzoyl-CoA, a reduction in hippuric acid production would be expected. While there is a decreasing trend in hippuric acid levels in the presence of these additional acyl-CoA's, the differences are not pronounced enough relative to the high baseline and variability (CVs around 20%) to confidently confirm inhibition. This limits the interpretability of the data in terms of enzyme kinetics and substrate preference.

From a methodological standpoint, this experiment supports the potential of NMR-based quantification in enzyme studies, provided sample preparation is improved. A cleaner system (e.g., partially purified enzyme rather than lysate) would likely reduce background interference and metabolite transformation, improving both qualitative and quantitative reliability. This is especially important for resolving low abundance or structurally similar compounds.

Regarding metabolite identity, the increased acyl-CoA concentrations did help clarify the isovaleryl-related peaks. These peaks are now more distinctly separated from the noise, though they still do not match the reference standard precisely. This supports the possibility of derivatization or metabolic transformation in the lysate. Literature supports this phenomenon: metabolites often undergo minor modifications (e.g., conjugation, oxidation) in biological matrices, which shift their NMR profiles slightly as mentioned before.

On the other hand, phenylacetyl peaks remain elusive. Even at higher concentrations, they remain indistinct and are largely obscured by background signals. One possible explanation is that phenylacetyl-CoA or its derivatives are diverted through other metabolic pathways more rapidly in the lysate, or that their products overlap with existing lysate components. A cleaner system or orthogonal validation using another analytical technique could help resolve this ambiguity.

In summary, this final experiment shows encouraging progress in peak clarity and quantification with increased substrate concentrations, especially for hippuric acid. However, high background, possible over-quantification, and unclear phenylacetyl profiles suggest that further refinement, particularly in sample preparation, will be necessary for drawing robust conclusions about enzyme kinetics and substrate competition using NMR.

CHAPTER 5 CONCLUSION AND FUTURE PROSPECTS.

5.1 Introduction

Historically, the glycine conjugation pathway was considered a relatively minor route within Phase II detoxification and was often overlooked compared to more extensively studied pathways such as glucuronidation and sulfation (Knights et al., 2007a). However, emerging insights into its role in conjugating and eliminating toxic acyl-CoA species, particularly those arising from inborn errors of metabolism, have led to a reassessment of its metabolic significance (Badenhorst et al., 2014, Temellini et al., 1993). More recent studies have further highlighted the pathway's physiological relevance. For instance, research in mouse models of non-ketonic hyperglycinaemia suggests that glycine conjugation helps regulate glycine levels and mitigate the buildup of toxic intermediates (Leung et al., 2020). A key enzyme in this process is glycine N-acyltransferase (GLYAT), which catalyses the conjugation of glycine with acyl-CoA's to form less toxic, excretable metabolites such as hippuric acid (Badenhorst et al., 2013a).

In disorders such as isovaleric acidemia, where isovaleryl-CoA accumulates due to a defect in leucine catabolism, glycine conjugation provides an important alternative pathway for detoxification (Vockley et al., 2019, Tanaka et al., 1966, Yudkoff et al., 1978). Nevertheless, isovaleryl-CoA, an intermediate of leucine catabolism, is not a preferred substrate for GLYAT (Schutte, 2020), raising the question of how substrate specificity or competition affects its detoxifying capacity under metabolic stress.

This study was designed to explore GLYAT's substrate specificity and potential competitive dynamics between different acyl-CoA species using a combination of biochemical and analytical approaches. Recombinant GLYAT was expressed and used in ^1H NMR spectroscopy to monitor product formation in multi-substrate reactions. This approach aimed to improve understanding of GLYAT's functional behaviour under conditions that mimic physiological substrate mixtures.

5.2 Methodological advancements

Recombinant expression and assay development

A key methodological focus of this study was the recombinant expression of human glycine N-acyltransferase (GLYAT). An initial attempt was also made to express its paralogue GLYATL1, which was of theoretical interest due to its distinct substrate preferences and potential relevance to substrate competition studies. While GLYATL1 was successfully expressed, its expression was considerably weaker and less consistent compared to GLYAT. As a result, the methodological

and experimental focus of the project shifted primarily to GLYAT, for which robust expression and downstream activity assays could be reliably developed.

Expression was carried out using *E. coli* C41 (DE3) cells transformed with a pHis17 expression vector, which includes a His-tag to facilitate purification. The induction strategy was optimized compared to previous protocols by replacing dual inducers (IPTG and arabinose) with IPTG alone. This adjustment, along with a reduction in induction time and temperature, helped mitigate issues related to overexpression, which had previously resulted in insoluble protein aggregates. Further refinements included the addition of lysozyme and protease inhibitors during lysis, which improved the solubility of the expressed protein.

Both GLYAT and GLYATL1 were successfully expressed, although GLYATL1 appeared at much lower levels and exhibited poorer solubility. Despite the presence of His-tags, affinity purification via nickel chromatography proved unsuccessful for GLYATL1 and inconsistent for GLYAT. Given the high cost of column-based purification and the need for sufficient enzyme for downstream assays, the soluble fraction of the GLYAT-expressing lysate, obtained after centrifugation, was used as the enzyme source for downstream activity assays. Validation of successful expression was achieved using SDS-PAGE and western blotting, although band clarity remained suboptimal. Nevertheless, activity was confirmed using Ellman's assay, demonstrating that the GLYAT enzyme retained functional activity consistent with predicted catalytic behaviour.

NMR method development for substrate analysis

A major methodological contribution of this study was the development of a novel NMR-based assay to analyse substrate utilization by GLYAT. Traditional enzymatic activity assays, such as the Ellman's assay, detect the release of CoA and are well-suited for confirming catalytic activity. However, they lack the specificity required to distinguish between multiple acyl-CoA substrates in competition scenarios. Given the focus of this study on substrate competition and inhibition among different CoA species, a more discriminating analytical method was required.

Although mass spectrometry offers excellent sensitivity and is widely regarded as a gold standard for metabolite quantification, its sample preparation steps, such as protein precipitation or potential derivatization, can introduce artifacts or alter the reaction environment (Dettmer et al., 2007, Zhang et al., 2012). In contrast, NMR spectroscopy allowed direct, non-invasive monitoring of intact reaction mixtures, better simulating physiological conditions and preserving the enzymatic context. For these reasons, NMR was explored as a method more suited to this project's goals.

The core objective of the method was to quantify specific product formation, primarily hippuric acid, as a proxy for substrate preference and, eventually, inhibition. The experimental setup used the soluble fraction of *E. coli* lysate containing GLYAT. While the use of crude lysate (specifically, the soluble fraction) introduces complexity into the spectra, it also provided a pragmatic approach in the absence of a fully purified enzyme. Method development involved optimizing several parameters, beginning with the minimum concentration of substrates required for reliable quantification. Significant interference from background glycerol and overlapping signals posed challenges, especially given the reliance on integration of relatively small product peaks.

To address this, a subtraction-based correction step was developed, wherein the glycerol peak contribution was estimated from an isolated cluster and computationally removed from the overlapping region. This analytical refinement improved quantification without needing to alter the biological sample, preserving the integrity of the enzyme reaction. While the future use of purified enzyme could reduce some sources of spectral complexity, it would not eliminate all background signals, as purified enzymes are typically stored in glycerol-containing buffers. Therefore, any potential benefit would depend on further optimization of storage protocols and filtration steps.

Despite technical hurdles, the method proved more successful than anticipated. Product peaks corresponding to hippuric acid could be consistently identified and integrated. This allowed qualitative reasoning about relative substrate preference, for instance, if hippuric acid made up 80% of the total product, it suggested 20% inhibition or redirection of catalytic activity by an alternate substrate like isovaleryl-CoA. Though a full substrate competition study was not achieved within the project timeframe, the method lays a solid foundation for this type of quantitative analysis once refined.

Current limitations include poor sensitivity at low concentrations, signal overlap, and variability in absolute quantification. Nonetheless, this NMR-based assay demonstrates promising potential for monitoring substrate usage by GLYAT in a physiologically relevant, non-invasive format. With further improvements, particularly through enzyme purification and signal enhancement, this approach could support deeper mechanistic investigations into substrate selectivity and inhibition within the glycine conjugation pathway.

5.3 Evaluation of methodological outcomes

Ellman's assay and enzyme validation

Initial validation of the recombinantly expressed GLYAT enzyme was performed using Ellman's assay. The enzyme exhibited an increase in absorbance over time in the presence of benzoyl-

CoA and glycine, consistent with enzymatic activity. The shape of the progress curve was qualitatively similar to typical enzymatic reactions, supporting the conclusion that the expressed GLYAT retained functional catalytic capacity. Notably, when benzoyl-CoA was absent, no measurable activity was detected, and omission of glycine resulted in significantly reduced reaction rates. This reduction is likely due to glycine that was present in the lysate as a result of its supplementation during protein expression, which may have supported minimal catalysis. Nevertheless, these findings confirmed the enzyme's activity and suitability for subsequent NMR-based analysis.

NMR-based substrate monitoring and product formation

NMR spectroscopy was employed to detect and quantify the formation of glycine-conjugated products in reactions containing benzoyl-CoA, isovaleryl-CoA, and phenylacetyl-CoA. The hippuric acid (HA) peaks were the most prominent and reliably detectable, consistent with benzoyl-CoA being the preferred substrate of the GLYAT enzyme used. In contrast, phenylacetylglycine and isovalerylglycine peaks were comparatively subtle and closer to baseline noise, suggesting that either these products formed in lower amounts due to substrate competition, or that the NMR method's sensitivity at this concentration range (~300 μ M substrate, with lower product formation) was insufficient for robust quantification.

In some cases, minor chemical shift changes or new peaks were observed within the expected regions for product formation, particularly in the phenylacetyl cluster, indicating conversion to product. However, these peaks were not significantly above baseline and lacked sufficient signal-to-noise ratio for confident quantitative interpretation. This emphasizes the need for enhanced sensitivity, such as through cleaner sample preparation, higher substrate spike concentrations, or longer reaction times.

Competitive/Inhibitory dynamics

A final set of experiments aimed to quantify hippuric acid formation across different competitive substrate conditions. Interestingly, the HA concentrations increased in the presence of competing substrates, reaching even higher levels than the standard benzoyl-CoA-only reaction. While this contradicts expectations based on competition, it is likely attributed to noise limitations and the inability of the NMR method to accurately resolve small concentration differences at low micromolar levels. It is also possible that excess glycine allowed all available benzoyl-CoA to be converted over time, masking any real-time competition effects. Delaying filtration or reducing glycine concentration in future time-course experiments may resolve these limitations.

Nevertheless, the relative prominence of HA peaks confirms benzoyl-CoA as the preferred substrate under the conditions tested.

Insights into enzyme behaviour

Overall, the study demonstrated that GLYAT preferentially conjugates benzoyl-CoA with glycine, and that competitive substrates can be conjugated to a lesser extent. The enzyme displays activity in a multi-substrate environment, but precise quantification of non-dominant products remains difficult. The presence of distinct product peaks in reactions with isovaleryl-CoA confirms its role as a viable, albeit less favoured, substrate, supporting earlier literature on the glycine conjugation of branched-chain acyl-CoA's.

Comparison to literature and future directions

The findings align with previous studies, suggesting that GLYAT can exhibit different substrate use. While this study used a single GLYAT variant, future work could explore paralogue-specific differences (e.g., GLYATL1 vs. GLYAT) to further dissect substrate specificity. The observation of isovalerylglycine formation in reactions with isovaleryl-CoA and glycine supports clinical findings in metabolic detoxification literature. GLYAT being a potential crucial enzyme in alleviating Isovaleric acidemia.

5.4 Theoretical and clinical implications

Although the primary goal of this study was developing a novel method, the experimental results contribute to a growing body of evidence that positions GLYAT as an adaptive detoxification enzyme capable of handling multiple acyl-CoA substrates. Previous research has established that glycine conjugation plays a role in detoxifying xenobiotics and endogenously generated acyl-CoA's (Badenhorst et al., 2013b). This study supports and extends that view by demonstrating that GLYAT can catalyse the conversion of several structurally diverse acyl-CoA substrates, not just benzoyl-CoA, to their glycine-conjugated forms, albeit with varying efficiency.

The detection of isovalerylglycine formation in the presence of isovaleryl-CoA is particularly noteworthy in the context of isovaleric acidemia, a metabolic disorder characterized by the accumulation of isovaleryl-CoA. Previous studies have shown that patients with this disorder benefit from glycine supplementation, which facilitates the formation of isovalerylglycine, enhancing excretion and reducing toxicity (Tanaka et al., 1966). The results in this study suggest that GLYAT could play a significant role in this detoxification route. While not tested under physiological conditions, the enzymatic conversion of isovaleryl-CoA to a more excretable form

implies that GLYAT could help buffer toxic metabolite buildup in affected individuals, opening future avenues for enzyme-based or glycine-enhancement therapies.

Moreover, the pronounced formation of hippuric acid compared to other products highlights substrate preference, where the enzyme selectively processes some substrates over others. While this study observed, what is assumed to be prioritization of benzoyl-CoA, literature suggests that substrate specificity may vary based on GLYAT paralogues and genetic variants (Rohwer et al., 2021). For example, GLYATL1 has been implicated in the conjugation of phenylacetyl-CoA (Guan et al., 2020), suggesting substrate-specific roles among family members. This paralogue diversity could have important implications in personalized medicine, particularly in the context of metabolic disorders or pharmacogenomics.

5.5 Study limitations

While this study presents a novel approach to investigating substrate competition and inhibition in GLYAT-mediated glycine conjugation using NMR, several limitations should be acknowledged.

One of the study's limitations is that GLYAT activity was measured using a crude soluble lysate rather than purified enzyme. This choice was partly practical, helping to avoid time-consuming and costly purification steps. More importantly, it was also intentional: using the lysate created a complex mixture that better reflected a biological sample like urine, where various metabolites are present. This allowed the NMR method to be tested in a more realistic setting. That said, using unpurified lysate came with challenges. Background reactions may have occurred due to small molecules left over from the expression system, including glycine. In the Ellman's assay, for example, some enzymatic activity was detected even without added glycine. The lysate also contained other components, such as glycerol, which contributed to overlapping signals in the NMR spectra. These factors complicated the analysis but also demonstrated that the method could still work in a less-than-ideal matrix, which is often the case in real biological samples.

Second, while NMR spectroscopy offers high-resolution structural insights and the ability to simultaneously monitor multiple metabolites, its sensitivity becomes a limitation when quantifying products at low micromolar concentrations. Initial limit tests confirmed that low concentrations could be qualitatively observed when signal intensity was scaled, but precise quantification of subtle differences, especially in the presence of competition, proved challenging. As a result, confidence in the quantification of less abundant conjugates such as phenylacetylglycine or isovalerylglycine was reduced, and small changes in product formation may have gone undetected.

Third, the substrate panel was limited to benzoyl-CoA, isovaleryl-CoA, and phenylacetyl-CoA. These were deliberately selected to represent both the canonical substrate (benzoyl-CoA), a clinically relevant toxic metabolite (isovaleryl-CoA), and a substrate reportedly favoured by a GLYAT paralogue (phenylacetyl-CoA via GLYATL1). While broader screening may have further elaborated the enzyme's capacity, the inclusion of more CoA derivatives was considered unnecessary unless emerging research identifies specific metabolites with strong inhibitory or competitive potential.

Fourth, the kinetic setup of the reaction, particularly the use of a fixed 10-minute incubation period and an excess of glycine may have limited the resolution needed to observe fast acting competition effects. Reducing the reaction time or using lower glycine concentrations could improve temporal and kinetic specificity in future studies. However, such refinements should follow the establishment of a robust baseline method, which this study aimed to provide.

Finally, the study presents a largely one-dimensional view of substrate interaction, with limited insight into the mechanistic basis of competition or enzyme preference. It remains unclear whether structural properties, binding affinities, or upstream cellular regulation are driving substrate selectivity. This conceptual limitation highlights the need for integrative approaches, such as combining this method with other omics (e.g., proteomics, metabolomics, or transcriptomics), to better understand the factors influencing substrate triage in physiological contexts. This will be further elaborated in the future prospects section.

5.6 Future prospects

This study provides a novel method foundation for analysing substrate competition and inhibition in glycine conjugation via NMR. While the potential applications are broad, from mechanistic biochemistry to therapeutic exploration, future work should first focus on method refinement to ensure robustness and reproducibility before broader ambitions are pursued.

In-Vitro refinements and kinetic modelling

Rather than expanding the substrate range immediately, future research should aim to optimize the current system by minimizing experimental noise, eliminating interfering background components, and improving enzyme purity. Real-time analysis of competition could be explored by running parallel reactions with varying time points, potentially combining NMR with activity-based assays. However, such an approach would require a high-throughput and highly sensitive analytical framework. The current setup, though innovative, should be streamlined and simplified before deeper kinetic modelling or complex inhibition studies are attempted.

Translational considerations and systems-level biology

This in vitro system is best viewed as a controlled tool to understand specific enzyme-substrate interactions, especially in the context of substrate overlap and interference. Its utility lies in isolating variables that are often too complex to parse in vivo. Nevertheless, it opens interesting avenues for downstream investigations, for instance, linking known genetic variations in GLYAT or its paralogues to altered substrate preference or detoxification efficiency. This could form part of a broader strategy integrating in-silico predictions with in-vitro validation to understand enzyme behaviour in more personalized or pathological contexts.

Therapeutic and diagnostic horizons (Long-Term)

While clinical translation remains distant, this kind of analysis could eventually support diagnostics by identifying disruptions in glycine conjugation capacity, either through reduced formation of hippuric acid or altered product profiles. In the longer term, refined multi-dimensional enzyme studies may reveal underappreciated regulatory points or therapeutic targets, such as modifiable enzyme variants or cofactor dependencies. However, realizing these applications will depend on first establishing a more robust and sensitive analytical model.

Overall, the priority should be realism over optimism: refining this novel method is the most immediate and impactful next step.

5.7 Closing thought

This study contributes a fresh methodological approach to investigating substrate interactions within the glycine conjugation pathway, an area that has historically received limited attention compared to other metabolic detoxification mechanisms. By combining recombinant enzyme expression with NMR-based product analysis, it highlights both the feasibility and the challenges of probing substrate competition in vitro.

More broadly, it underscores that glycine conjugation is not merely a secondary detoxification route but a potentially adaptive mechanism capable of handling a variety of metabolic intermediates under stress or overload conditions. In doing so, this work opens new avenues for thinking about enzyme flexibility, substrate triage, and the metabolic interplay between endogenous and xenobiotic compounds.

While further optimization is needed before the method can be routinely applied, this study serves as an interesting steppingstone. It invites a more nuanced look at detoxification pathways, encouraging both method development and conceptual re-evaluation of how we approach enzyme behaviour in complex biochemical networks.

BIBLIOGRAPHY

- AKIRA, K. & HASHIMOTO, T. 2001. Hippuric acid test using ¹³C-labelling and NMR spectroscopy. *Clin Chem Lab Med*, 39, 215-7.
- ALBOUZ-ABO, S., TURTON, R., WILSON, J. C. & VON ITZSTEIN, M. 2005. An investigation of the activity of recombinant rat skeletal muscle cytosolic sialidase. *FEBS Letters*, 579, 1034-1038.
- ARONICA, L., ORDOVAS, J. M., VOLKOV, A., LAMB, J. J., STONE, P. M., MINICH, D., LEARY, M., CLASS, M., METTI, D., LARSON, I. A., CONTRACTOR, N., ECK, B. & BLAND, J. S. 2022. Genetic Biomarkers of Metabolic Detoxification for Personalized Lifestyle Medicine. *Nutrients*, 14.
- BADENHORST, C. P. S., ERASMUS, E., VAN DER SLUIS, R., NORTJE, C. & VAN DIJK, A. A. 2014. A new perspective on the importance of glycine conjugation in the metabolism of aromatic acids. *Drug metabolism reviews*, 46, 343-361.
- BADENHORST, C. P. S., RENCIA, V. D. S., ELARDUS, E. & AND VAN DIJK, A. A. 2013a. Glycine conjugation: importance in metabolism, the role of glycine N-acyltransferase, and factors that influence interindividual variation. *Expert Opinion on Drug Metabolism & Toxicology*, 9, 1139-1153.
- BALAZS, Y. S., LISITSIN, E., CARMIEL, O., SHOHAM, G., SHOHAM, Y. & SCHMIDT, A. 2013. Identifying critical unrecognized sugar–protein interactions in GH10 xylanases from *Geobacillus stearothermophilus* using STD NMR. *The FEBS Journal*, 280, 4652-4665.
- BECKONERT, O., KEUN, H. C., EBBELS, T. M. D., BUNDY, J., HOLMES, E., LINDON, J. C. & NICHOLSON, J. K. 2007. Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. *Nature Protocols*, 2, 2692-2703.
- BEIRAS, R. 2018. Chapter 12 - Biotransformation. *In: BEIRAS, R. (ed.) Marine Pollution*. Elsevier.
- BOLOGNESI, B. & LEHNER, B. 2018. Reaching the limit. *eLife*, 7, e39804.
- BRENES, A. J. 2024. Calculating and Reporting Coefficients of Variation for DIA-Based Proteomics. *Journal of Proteome Research*, 23, 5274-5278.
- BRODIE, B. B., AXELROD, J., COOPER, J. R., GAUDETTE, L., LA DU, B. N., MITOMA, C. & UDENFRIEND, S. 1955a. Detoxication of drugs and other foreign compounds by liver microsomes. *Science*, 121, 603-4.

- CARR, L. 2009. Chapter 16 - Metabolic and Degenerative Disorders of Childhood. *In: WARNER, T. T. & HAMMANS, S. R. (eds.) Practical Guide to Neurogenetics*. Philadelphia: W.B. Saunders.
- CHATVUTTINUN, S., WATTANASIRICHAIGOON, D., CHAVASIT, V., DUMRONGWONGSIRI, O., TIM-AROON, T., SUTHUTVORAVUT, U. & CHONGVIRIYAPHAN, N. 2021. Clinical utility of low branched-chain amino acid modular diets in patients with isovaleric aciduria and maple syrup urine disease. *Malaysian Journal of Nutrition*, 27.
- CHINEN, Y., NAKAMURA, S., TAMASHIRO, K., SAKAMOTO, O., TASHIRO, K., INOKUCHI, T. & NAKANISHI, K. 2017. Isovaleric acidemia: Therapeutic response to supplementation with glycine, l-carnitine, or both in combination and a 10-year follow-up case study. *Molecular Genetics and Metabolism Reports*, 11, 2-5.
- COSTA, N. B., SILVA, C. T., FRARE, A. B., SILVA, R. E. & MOURA, K. K. 2014. Association between CYP1A1m1 gene polymorphism and primary open-angle glaucoma. *Genet Mol Res*, 13, 10382-9.
- DE GRAAF, R. A., CHOWDHURY, G. M. & BEHAR, K. L. 2011. Quantification of high-resolution (1)H NMR spectra from rat brain extracts. *Anal Chem*, 83, 216-24.
- DETTMER, K., ARONOV, P. A. & HAMMOCK, B. D. 2007. Mass spectrometry-based metabolomics. *Mass Spectrometry Reviews*, 26, 51-78.
- DONA, A. C., JIMÉNEZ, B., SCHÄFER, H., HUMPFER, E., SPRAUL, M., LEWIS, M. R., PEARCE, J. T. M., HOLMES, E., LINDON, J. C. & NICHOLSON, J. K. 2014. Precision High-Throughput Proton NMR Spectroscopy of Human Urine, Serum, and Plasma for Large-Scale Metabolic Phenotyping. *Analytical Chemistry*, 86, 9887-9894.
- DUMAS, M.-E., MAIBAUM, E. C., TEAGUE, C., UESHIMA, H., ZHOU, B., LINDON, J. C., NICHOLSON, J. K., STAMLER, J., ELLIOTT, P., CHAN, Q. & HOLMES, E. 2006. Assessment of Analytical Reproducibility of 1H NMR Spectroscopy Based Metabonomics for Large-Scale Epidemiological Research: the INTERMAP Study. *Analytical Chemistry*, 78, 2199-2208.
- ELLMAN, G. L. 1959a. Tissue sulfhydryl groups. *Arch Biochem Biophys*, 82, 70-7.
- EMWAS, A.-H., ROY, R., MCKAY, R., TENORI, L., SACCENTI, E., GOWDA, N., RAFTERY, D., ALAHMARI, F., JAREMKO, L., JAREMKO, M. & WISHART, D. 2019a. NMR Spectroscopy for Metabolomics Research. *Metabolites*, 9.

- EMWAS, A.-H. M. 2015. The strengths and weaknesses of NMR spectroscopy and mass spectrometry with particular focus on metabolomics research. *Metabonomics: Methods and protocols*, 161-193.
- EMWAS, A. H., ROY, R., MCKAY, R. T., TENORI, L., SACCENTI, E., GOWDA, G. A. N., RAFTERY, D., ALAHMARI, F., JAREMKO, L., JAREMKO, M. & WISHART, D. S. 2019b. NMR Spectroscopy for Metabolomics Research. *Metabolites*, 9.
- FERREIRA, C. R., RAHMAN, S., KELLER, M., ZSCHOCKE, J. & GROUP, I. A. 2021. An international classification of inherited metabolic disorders (ICIMD). *Journal of Inherited Metabolic Disease*, 44, 164-177.
- FORTNEY, L., PODEIN, R. & HERNKE, M. 2018. Chapter 106 - Detoxification. *In: RAKEL, D. (ed.) Integrative Medicine (Fourth Edition)*. Elsevier.
- FRANCIS, D. M. & PAGE, R. 2010. Strategies to optimize protein expression in E. coli. *Curr Protoc Protein Sci*, Chapter 5, 5.24.1-5.24.29.
- FRAZIER, D. M., MILLINGTON, D. S., MCCANDLESS, S. E., KOEBERL, D. D., WEAVIL, S. D., CHAING, S. H. & MUENZER, J. 2006. The tandem mass spectrometry newborn screening experience in North Carolina: 1997–2005. *Journal of Inherited Metabolic Disease*, 29, 76-85.
- GRANT, D. M. 1991. Detoxification pathways in the liver. *J Inherit Metab Dis*, 14, 421-30.
- GREGERSEN, N., KØLVRAA, S. & MORTENSEN, P. B. 1986. Acyl-CoA: Glycine N-acyltransferase: In vitro studies on the glycine conjugation of straight- and branched-chained acyl-CoA esters in human liver. *Biochemical Medicine and Metabolic Biology*, 35, 210-218.
- GRUNDLING, D. A. 2012. *Cloning and expression of human recombinant isoform a of glycine–N–acyltransferase*. North-West University.
- GUAN, R., HONG, W., HUANG, J., PENG, T., ZHAO, Z., LIN, Y., YU, M. & JIAN, Z. 2020. The expression and prognostic value of GLYATL1 and its potential role in hepatocellular carcinoma. *Journal of gastrointestinal oncology*, 11, 1305.
- HAFFER, E., HOLZGRABE, U., KRAUS, K., ADAMS, K., HOOK, J. M. & DIEHL, B. 2020. Qualitative and quantitative ¹H NMR spectroscopy for determination of divalent metal cation concentration in model salt solutions, food supplements, and

- pharmaceutical products by using EDTA as chelating agent. *Magnetic Resonance in Chemistry*, 58, 653-665.
- HEINDEL, J. J., BLUMBERG, B., CAVE, M., MACHTINGER, R., MANTOVANI, A., MENDEZ, M. A., NADAL, A., PALANZA, P., PANZICA, G., SARGIS, R., VANDENBERG, L. N. & VOM SAAL, F. 2017. Metabolism disrupting chemicals and metabolic disorders. *Reprod Toxicol*, 68, 3-33.
- HOSGOOD, H. D., 3RD, BERNDT, S. I. & LAN, Q. 2007. GST genotypes and lung cancer susceptibility in Asian populations with indoor air pollution exposures: a meta-analysis. *Mutat Res*, 636, 134-43.
- HUANG, C.-J., PENG, H.-L., PATEL, A. K., SINGHANIA, R. R., DONG, C.-D. & CHENG, C.-Y. 2021. Effects of Lower Temperature on Expression and Biochemical Characteristics of HCV NS3 Antigen Recombinant Protein. *Catalysts*, 11, 1297.
- JUTRAS, P. V., MARUSIC, C., LONOCE, C., DEFLERS, C., GOULET, M. C., BENVENUTO, E., MICHAUD, D. & DONINI, M. 2016. An Accessory Protease Inhibitor to Increase the Yield and Quality of a Tumour-Targeting mAb in *Nicotiana benthamiana* Leaves. *PLoS One*, 11, e0167086.
- KNIGHTS, K. & VESSEY, D. A. 2010. Enzymology of Amino Acid Conjugation Reactions.
- KNIGHTS, K. M. & MINERS, J. O. 2011. Amino Acid Conjugation: A Novel Route of Xenobiotic Carboxylic Acid Metabolism in Man. *Encyclopedia of Drug Metabolism and Interactions*.
- KNIGHTS, K. M., SYKES, M. J. & MINERS, J. O. 2007a. Amino acid conjugation: contribution to the metabolism and toxicity of xenobiotic carboxylic acids. *Expert Opin Drug Metab Toxicol*, 3, 159-68.
- KÜHN, S., WILLIAMS, M. E., DERCKSEN, M., SASS, J. O. & VAN DER SLUIS, R. 2023. The glycine N-acyltransferases, GLYAT and GLYATL1, contribute to the detoxification of isovaleryl-CoA - an in-silico and in vitro validation. *Computational and Structural Biotechnology Journal*, 21, 1236-1248.
- LEE, P. Y., COSTUMBRADO, J., HSU, C. Y. & KIM, Y. H. 2012. Agarose gel electrophoresis for the separation of DNA fragments. *J Vis Exp*.
- LEIMGRUBER, R. M., MALONE, J. P., RADABAUGH, M. R., LAPORTE, M. L., VIOLAND, B. N. & MONAHAN, J. B. 2002. Development of improved cell lysis, solubilization and imaging approaches for proteomic analyses. *PROTEOMICS*, 2, 135-144.
- LEUNG, K. Y., DE CASTRO, S. C. P., SANTOS, C., SAVERY, D., PRUNTY, H., GOLD-DIAZ, D., BENNETT, S., HEALES, S., COPP,

- A. J. & GREENE, N. D. E. 2020. Regulation of glycine metabolism by the glycine cleavage system and conjugation pathway in mouse models of non-ketotic hyperglycinemia. *J Inherit Metab Dis*, 43, 1186-1198.
- LOURENS, P., VAN DER SLUIS, R. & LINDEQUE, J. 2022. The establishment of a model system in which variants of the medium-chain ligase (ACSM) and glycine N-acyltransferase (GLYAT) can be co-expressed and analysed.
- MANSOOR, S., QAMAR, R. & AZAM, M. 2024. Inborn errors of metabolism: Historical perspectives to contemporary management. *Clinica Chimica Acta*, 562, 119883.
- MASON, S., TERBURGH, K. & LOUW, R. 2018. Miniaturized 1H-NMR method for analyzing limited-quantity samples applied to a mouse model of Leigh disease. *Metabolomics*, 14, 74.
- MATSUO, M., TERA, K., KAMEDA, N., MATSUMOTO, A., KUROKAWA, Y., FUNASE, Y., NISHIKAWA, K., SUGAYA, N., HIRUTA, N. & KISHIMOTO, T. 2012. Designation of enzyme activity of glycine-N-acyltransferase family genes and depression of glycine-N-acyltransferase in human hepatocellular carcinoma. *Biochemical and Biophysical Research Communications*, 420, 901-906.
- MAWAL, Y. R. & QURESHI, I. A. 1994a. Purification to homogeneity of mitochondrial acyl coA: glycine n-acyltransferase from human liver. *Biochemical and biophysical research communications*, 205, 1373-1379.
- MOLDAVE, K. & MEISTER, A. 1957a. Synthesis of phenylacetylglutamine by human tissue. *Journal of Biological Chemistry*, 229, 463-476.
- NANDI, D. L., LUCAS, S. V. & WEBSTER, L. T., JR. 1979a. Benzoyl-coenzyme A:glycine N-acyltransferase and phenylacetyl-coenzyme A:glycine N-acyltransferase from bovine liver mitochondria. Purification and characterization. *J.Biol.Chem.*, 254, 7230-7237.
- NORMAN, R. E., CARPENTER, D. O., SCOTT, J., BRUNE, M. N. & SLY, P. D. 2013. Environmental exposures: an underrecognized contribution to noncommunicable diseases. *Reviews on environmental health*, 28, 59-65.
- PALMER, I. & WINGFIELD, P. T. 2004. Preparation and extraction of insoluble (inclusion-body) proteins from Escherichia coli. *Curr Protoc Protein Sci*, Chapter 6, 6.3.1-6.3.18.

- PATTERSON, A. D., GONZALEZ, F. J. & IDLE, J. R. 2010. Xenobiotic metabolism: a view through the metabolometer. *Chem Res Toxicol*, 23, 851-60.
- PAULI, G. F., GÖDECKE, T., JAKI, B. U. & LANKIN, D. C. 2012. Quantitative ¹H NMR. Development and Potential of an Analytical Method: An Update. *Journal of Natural Products*, 75, 834-851.
- RIDDLES, P. W., BLAKELEY, R. L. & ZERNER, B. 1979. Ellman's reagent: 5,5'-dithiobis(2-nitrobenzoic acid)—a reexamination. *Analytical Biochemistry*, 94, 75-81.
- RINALDO, P., WELCH, R. D., PREVIS, S. F., SCHMIDT-SOMMERFELD, E., GARGUS, J. J., O'SHEA, J. J. & ZINN, A. B. 1991. Ethylmalonic/adipic aciduria: effects of oral medium-chain triglycerides, carnitine, and glycine on urinary excretion of organic acids, acylcarnitines, and acylglycines. *Pediatr Res*, 30, 216-21.
- ROHWER, J. M., SCHUTTE, C. & VAN DER SLUIS, R. 2021. Functional Characterisation of Three Glycine N-Acyltransferase Variants and the Effect on Glycine Conjugation to Benzoyl-CoA. *International Journal of Molecular Sciences*, 22, 3129.
- SCHACHTER, D. & TAGGART, J. V. 1953. Benzoyl coenzyme A and hippurate synthesis. *Journal of Biological Chemistry*, 203, 925-934.
- SCHULKE, D. & SASS, J. 2021. Frequent sequence variants of human glycine N-acyltransferase (GLYAT) and inborn errors of metabolism. *Biochimie*, 183.
- SCHUTTE, C. 2020. *Characterization of the enzyme activity and substrate specificity of GLYAT haplotypes*. North-West University (South Africa).
- SHU, Y., YU, B., HE, J., YU, J., ZHENG, P., YUAN, Z., CHEN, D. & MAO, X. 2016. Excess of dietary benzoic acid supplementation leads to growth retardation, hematological abnormality and organ injury of piglets. *Livestock Science*, 190, 94-103.
- SINGH, A., UPADHYAY, V., UPADHYAY, A. K., SINGH, S. M. & PANDA, A. K. 2015. Protein recovery from inclusion bodies of *Escherichia coli* using mild solubilization process. *Microbial Cell Factories*, 14, 41.
- SINGH, S. M. & PANDA, A. K. 2005. Solubilization and refolding of bacterial inclusion body proteins. *Journal of Bioscience and Bioengineering*, 99, 303-310.

- TANAKA, K., BUDD, M., EFRON, M. & ISSELBACHER, K. 1966. Isovaleric acidemia: a new genetic defect of leucine metabolism. *Proceedings of the National Academy of Sciences*, 56, 236-242.
- TANAKA, K. & ISSELBACHER, K. J. 1967a. The Isolation and Identification of N-Isovaleryl-glycine from Urine of Patients with Isovaleric Acidemia. *Journal of Biological Chemistry*, 242, 2966-2972.
- TEMELLINI, A., MOGAVERO, S., GIULIANOTTI, P., PIETRABISSA, A., MOSCA, F. & PACIFICI, G. 1993. Conjugation of benzoic acid with glycine in human liver and kidney: a study on the interindividual variability. *Xenobiotica*, 23, 1427-1433.
- TIAN, X., WU, L., JIANG, M., ZHANG, Z., WU, R., MIAO, J., LIU, C. & GAO, S. 2021. Downregulation of GLYAT Facilitates Tumor Growth and Metastasis and Poor Clinical Outcomes Through the PI3K/AKT/Snail Pathway in Human Breast Cancer. *Front Oncol*, 11, 641399.
- VAN DER SLUIS, R., BADENHORST, C. P., ERASMUS, E., VAN DYK, E., VAN DER WESTHUIZEN, F. H. & VAN DIJK, A. A. 2015. Conservation of the coding regions of the glycine N-acyltransferase gene further suggests that glycine conjugation is an essential detoxification pathway. *Gene*, 571, 126-34.
- VAN DER SLUIS, R., BADENHORST, C. P., VAN DER WESTHUIZEN, F. H. & VAN DIJK, A. A. 2013. Characterisation of the influence of genetic variations on the enzyme activity of a recombinant human glycine N-acyltransferase. *Gene*, 515, 447-453.
- VAN DER SLUIS, R., UNGERER, V., NORTJE, C., A. VAN DIJK, A. & ERASMUS, E. 2017a. New insights into the catalytic mechanism of human glycine N-acyltransferase. *Journal of Biochemical and Molecular Toxicology*, 31, e21963.
- VAN DER WESTHUIZEN, F. H., PRETORIUS, P. J. & ERASMUS, E. 2000. The utilization of alanine, glutamic acid, and serine as amino acid substrates for glycine N-acyltransferase. *J Biochem Mol Toxicol*, 14, 102-9.
- VEITCH, N. C., TAMS, J. W., VIND, J., DALBØGE, H. & WELINDER, K. G. 1994. NMR studies of recombinant Coprinus peroxidase and three site-directed mutants. *European Journal of Biochemistry*, 222, 909-918.
- VESELKOV, K. A., LINDON, J. C., EBBELS, T. M., CROCKFORD, D., VOLYNKIN, V. V., HOLMES, E., DAVIES, D. B. & NICHOLSON, J. K. 2009. Recursive segment-wise peak alignment of biological (1)h NMR spectra for improved metabolic biomarker recovery. *Anal Chem*, 81, 56-66.

- VETTING, M. W., LP, S. D. C., YU, M., HEGDE, S. S., MAGNET, S., RODERICK, S. L. & BLANCHARD, J. S. 2005. Structure and functions of the GNAT superfamily of acetyltransferases. *Arch Biochem Biophys*, 433, 212-26.
- VISAGIE, J. L., ARUWAJOYE, G. S. & VAN DER SLUIS, R. 2024. Pharmacokinetics of aspirin: evaluating shortcomings in the literature. *Expert Opinion on Drug Metabolism & Toxicology*, 20, 727-740.
- VOCKLEY, J. & ENSENAUER, R. 2006a. Isovaleric acidemia: New aspects of genetic and phenotypic heterogeneity. *American Journal of Medical Genetics Part C: Seminars in Medical Genetics*, 142C, 95-103.
- VOCKLEY, J., ZSCHOCKE, J., KNERR, I., VOCKLEY, C. W. & MICHAEL GIBSON, K. 2019. Branched Chain Organic Acidurias. In: VALLE, D. L., ANTONARAKIS, S., BALLABIO, A., BEAUDET, A. L. & MITCHELL, G. A. (eds.) *The Online Metabolic and Molecular Bases of Inherited Disease*. New York, NY: McGraw-Hill Education.
- WALKER, D. I., VALVI, D., ROTHMAN, N., LAN, Q., MILLER, G. W. & JONES, D. P. 2019. The metabolome: A key measure for exposome research in epidemiology. *Curr Epidemiol Rep*, 6, 93-103.
- WALUK, D. P., SUCHARSKI, F., SIPOS, L., SILBERRING, J. & HUNT, M. C. 2012. Reversible Lysine Acetylation Regulates Activity of Human Glycine α -N-Acyltransferase-like 2 (hGLYATL2): IMPLICATIONS FOR PRODUCTION OF GLYCINE-CONJUGATED SIGNALING MOLECULES *. *Journal of Biological Chemistry*, 287, 16158-16167.
- WEBSTER, L. T., SIDDIQUI, U. A., LUCAS, S. V., STRONG, J. M. & MIEYAL, J. J. 1976. Identification of separate acyl- CoA:glycine and acyl-CoA:L-glutamine N-acyltransferase activities in mitochondrial fractions from liver of rhesus monkey and man. *Journal of Biological Chemistry*, 251, 3352-3358.
- WEI, S., ZHANG, J., LIU, L., YE, T., GOWDA, G. A., TAYYARI, F. & RAFTERY, D. 2011. Ratio analysis nuclear magnetic resonance spectroscopy for selective metabolite identification in complex samples. *Anal Chem*, 83, 7616-23.
- WILFINGER, W. W., MACKEY, K. & CHOMCZYNSKI, P. 1997. Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. *Biotechniques*, 22, 474-6, 478-81.

- YUDKOFF, M., COHN, R. M., PUSCHAK, R., ROTHMAN, R. & SEGAL, S. 1978. Glycine therapy in isovaleric acidemia. *The Journal of Pediatrics*, 92, 813-817.**
- ZHANG, A., SUN, H., WANG, P., HAN, Y. & WANG, X. 2012. Modern analytical techniques in metabolomics analysis. *Analyst*, 137, 293-300.**
- ŽIVIČ, Z., LIPOGLAVŠEK, L., LAH, J. & HADŽI, S. 2025. A single vector system for tunable and homogeneous dual gene expression in *Escherichia coli*. *Scientific Reports*, 15, 99.**

ANNEXURES:

Appendix AA: Materials Used in Chapter 3

Product	Supplier
GeneJET Plasmid Miniprep Kit	Thermo Scientific
Nanodrop™ Spectrophotometer	Thermo Scientific
NdeI Restriction Enzyme	New England Biolabs
10× NEB Buffer	New England Biolabs
Nuclease-free Water	Thermo Scientific
Agarose	Sigma-Aldrich
Ethidium Bromide	Thermo Scientific
DNA Ladder	Thermo Scientific
Gel Loading Dye	Thermo Scientific
Transilluminator	Thermo Scientific
Lysozyme	Sigma-Aldrich
Protease Inhibitor Cocktail	Sigma-Aldrich
IPTG	Sigma-Aldrich
RIPA Lysis Buffer Components:	

• NaCl • NaH₂PO₄ • Triton X-100	Merck Sigma-Aldrich Sigma-Aldrich
Pierce Universal Nuclease	Thermo Scientific
SDS-PAGE Reagents	Bio-Rad
Bio-Rad Mini-PROTEAN TGX Gels	Bio-Rad
Laemmli Sample Buffer	Bio-Rad
Tris-Glycine-SDS Running Buffer	Bio-Rad
PVDF Membrane	Bio-Rad
Towbin Transfer Buffer Components: Tris, Glycine, Methanol	Sigma-Aldrich
Bovine Serum Albumin (BSA)	Sigma-Aldrich
Anti-6xHis HRP Antibody	Thermo Scientific
Ellman's Reagent (DTNB)	Sigma-Aldrich
Microplate Reader	Thermo Scientific

Appendix AB: GLYAT and GLYATL1 Sanger Sequencing Results

The following is the forward Sanger sequencing result for the GLYAT insert using the T7 promoter primer. The sequence is presented in the 5' → 3' direction.

CAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGATGTTACCATTGC
AAGGTGCCCAGATGCTGCAGATGCTGGAGAAATCCTTGAGGAAGAGCCTCCCAGCATCCTTAAAGGTTTA
TGGAAGTGTCTTTACATAAACCATTGGAAATCCATTCAATCTGAAGGCTGTGGTGGACAAGTGGCCTGA
TTTTAATACAGTGGTTGTCTGCCCTCAGGAGCAGGATATGACAGATGACCTTGATCACTATACCAATACTT
ACCAAATCTACTCCAAAGATCCCCAAAAGTGTGAGGAATTCCTTGAGTACCAGAACTCATCAACTGGAAA
CAGCATTTACAGATTCAAAGTTCACAGCCTAGCCTGAATGAGGCTATACAAAATCTTGCAGCCATTAAGTC
CTTCAAAGTCAAACAAACACAACGCATTCTCTATATGGCAGCTGAAACAGCCAAGGAAGTACTCCTTTCC
TGCTGAAATCAAAGATTTTATCTCCAGTGGTGGCAAACCCAAGGCCATCAACCAAGAGATGTTTAACT
CTCATCCATGGATGTTACCCATGCTCACTTGGTGAATAAATTCTGGCATTTTTGGTGGTAATGAGAGGAGCC
AGAGATTCAATTGAGCGCTGCATTGAGACCTTTCCACCTGCTGTCTCCTGGGGCCTGAGGGGACCCCTGTG
TGCTGGGATCTAATGGACCAGACTGGAGAGATGAGAATGGCAGGCACCTTGCCGGAATACCGGCTCCATG
GCCTTGTGACGTATGTCATCTATTCCCACGCCAGAAATTGGGCAAACCTGGGTTTCTGTCTATTCTCAT
GTAGACTACAGCAATGAAGCTATGCAAAAATGAGTTACACACTGCAACATGTTCCCATTTCCAGAAGCTG
GAACCAGTGGAAGTGTGTACCTCTGGGATCCCATCATCATCATCATTAAGCTTCACCACCACCACC
ACCACTAAGAATTCCATCATCATCATCATCATTA.

The following is the forward Sanger sequencing result for the GLYATL1 insert using the T7 promoter primer. The sequence is presented in the 5' → 3' direction.

TCCTCTGAATATTTTGTTTAACTTTAAGAAGGAGATATACATATGATCCTACTGAATAACTCCCATAGC
TGCTGGCCCTATACAAATCCTTGCCAGGAGCATCCCTGAGTCCCTGAAGGTGTATGGCTCTGTGTATCAC
ATCAATCAGGGAACCCCTTCAACATGGAGGTGCTGGTGGATTCCCTGGCCTGAATATCAGATGGTTATTAT
CCGGCCTCAAAGCAGGAGATGACTGATGACATGGATTACATACAAAACGTATATCGTATGTTCTCCAAA
GAGCCTCAAAAATCAGAAGAAGTTTTGAAAAATTGTGAGATCGTAAACTGGAAACAGAGACTCCAAATCC
AAGGTCTTCAAGAAAGTTTAGGTGAGGGGATAAGAGTGGCTACATTTTCAAAGTCAGTGAAGTAGAGCA
TTCGAGAGCACTCCTCTTGTTACGGAAGATATTCTGAAGCTCAATGCCTCCAGTAAAAGCAAGCTTGGA
AGCTGGGCTGAGACAGGCCACCCAGATGATGAATTTGAAAGTGAAACTCCCAACTTTAAGTATGCCAGC
TGGATGTCTCTTATTCTGGGCTGGTAAATGACAACTGGAAGCGAGGGAAGAATGAGAGGAGCCTGCATTA
CATCAAGCGCTGCATAGAAGACCTGCCAGCAGCCTGTATGCTCGGCCAGAGGGAGTCCCGGTCTCATGGG
TAACCATGGACCTTCTTGTGAAGTAGGAATGGCCTACAGCATGGAAAAATACCGAAGGACAGGCAACAT
GGCAGAGTGATGGTGGGATACATGAAATATCTGCGTCAGAAGAATATTCATTTTACATCTCTGTGTTG
GAAGAAAATGAAGACTCCCGCAGATTTGTGGGGCAGTTTGGTTTCTTTGAGGCCTCCTGTGAGTGGCACC

AATGGACTTGCTACCCACAGAATCTAGTTCCATTTCTCGAGCACCACCACCACCACCTGAGATCCGGCT
GCTAACAAAGCCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACGCTGAGCAATAA.

Appendix B: Materials Used in Chapter 4

Product	Supplier
Tris-HCl Buffer	Sigma-Aldrich
Glycine	Sigma-Aldrich
Benzoyl-CoA	Sigma-Aldrich
Phenylacetyl-CoA	Sigma-Aldrich
Isovaleryl-CoA	Sigma-Aldrich
Molecular-grade Water	Thermo Scientific
Milli-Q Ultrapure Water	Merck Millipore
Deuterium Oxide (D ₂ O)	Sigma-Aldrich
Potassium Phosphate Monobasic (KH ₂ PO ₄)	Sigma-Aldrich
Trimethylsilylpropionic acid-d ₄ (TSP)	Sigma-Aldrich
Sodium Azide	Sigma-Aldrich
Potassium Hydroxide Pellets	Sigma-Aldrich
UFC5100BK Ultrafiltration Filters	Millipore®
Eppendorf Tubes (1.5 mL, sterile)	Eppendorf
Qubit™ Protein Assay Kit	Thermo Scientific

Bruker Avance III HD 500 MHz NMR Spectrometer	Bruker
Bruker TopSpin and AMIX Software	Bruker
NMR Tubes (5 mm)	Sigma-Aldrich