NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOMKAMPUS

Cloning and expression of human recombinant isoform a of glycine-N-acyltransferase

By

Daniel Andries Grundling

1247020

Submitted in partial fulfilment of the requirements for the degree of

MAGISTER OF NATURAL SCIENCE

(M.Sc)

School of Chemistry and Biochemistry

North-West University, Potchefstroom Campus

Potchefstroom, South Africa

Supervisor: Prof. A.A. van Dijk

Potchefstroom

2012

“There is one thing even more vital to science than intelligent methods; and that is, the sincere desire to find out the truth, whatever it may be”

- Charles Pierce

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to the following persons and institutions for their contributions and support towards the completion of this study:

My supervisor Prof A.A. van Dijk for help, guidance, patience and commitment

Retha Potgieter for the ordering of products

Rencia van der Sluis and Lizelle Zandberg for technical guidance

National Research Foundation (NRF)

Funding of NRF FA and DST Biopad BPP007

My parents, my wife, my son and to God.

DECLARATION

I declare that the dissertation for the degree of Master of Natural Science (M.Sc.) at the North-West University: Potchefstroom Campus hereby submitted, has not been submitted by me for a degree at this or another University, that it is my own work in design and execution, and that all material contained herein has been duly acknowledged.

…….. ……..

Daniel Andries Grundling Date

ABSTRACT

Awareness of detoxification, nowadays known as biotransformation, has become an integral part of our daily lives. It is a modern buzz word that is used to promote anything from health food to enhancement of performance in sports. Another lesser known application for detoxification is as a therapy for alleviating symptoms of inborn errors of metabolism. Detoxification is the process where endogenous and xenobiotic metabolites are transformed to less harmful products, in the liver and kidneys, in two phases. Phase 1 detoxification includes oxidation, hydroxylation, dehydrogenation metabolic reduction and hydrolysis. Phase 2 detoxification uses conjugation reactions to increase hydrophillicty of metabolites for excretion in bile and urine. Glycine N-acyltransferse (GLYAT; EC 2.3.1.13) is one of the amino acid conjugation enzymes. There are two variants of human GLYAT. I focused on the full-length mRNA human GLYAT isoform a, with a long term view of using it as a viable therapeutic enzyme for enhanced detoxification of harmful metabolites. I investigated if it is possible to clone and express a biologically active GLYAT. To achieve this goal I used three expression systems: traditional bacterial expression using the pET system; second generation cold shock bacterial expression using the pCOLDTF expression vector to improve solubility of the recombinant protein; and baculovirus expression in insect cells since therein some form of post translation glycosylation of the recombinant protein can occur which might improve solubility and ensure biological activity. The recombinant GLYAT expressed well in all three expression systems but was aggregated and no enzyme activity could be detected. A denature and renature system was also used to collect aggregated recombinant GLYAT and used to try to refold the recombinant protein in appropriate refolding buffers to improve solubility and obtain biological activity. The solubility of the recombinant GLYAT was improved but it remained biologically inactive.

Keywords: detoxification, GLYAT, DNA, RNA, protein expression, enzyme activity, bacterial expression, insect cell expression

OPSOMMING

Die bewusheid van detoksifikasie, hedendaags bekend as biotransformasie, is deel van ons daaglikse lewe. Dit is ‘n moderne sleutelwoord wat gebruik word om gesondheids kos tot sports verbetering te bevorder. ‘n Minder bekende toepassing van detoksifikasie is as terapie vir die verligting van die simptome van ingebore defekte van metabolisme. Detoksifikasie is die proses waar endogeen sowel as uitwendige metaboliete getransformeer word na minder skadelik produkte, in die lewer en niere, in twee fases. Fase 1 detoksifikasie sluit in oksidasie, hidroksilasie, dehidrogenasie, metaboliese reduksie en hidrolise. Fase 2 detoksifikasie gebruik konjugering reaksies om die hidrofilisiteit van die metaboliete te verhoog vir uitskeiding in gal of uriene. Glisien N-asieltransferase (GLYAT; EC 2.3.1.13) is een van hierdie konjugerings ensieme. Daar is twee variante van die menslike GLYAT. Ek het gefokus op die vol lengte mRNA menslike GLYAT isovorm a met die lang termyn visie om die te kan gebruik as ‘n terapeutiese ensiem vir verhoogde detoksifikasie van skadelike metaboliete. Ek het ondersoek ingestel of dit moontlik is om ‘n biologiese aktiewe GLYAT te kan kloon en uit te druk. Om my doel te kan bereik het ek drie ekspressie stelsels gebruik: tradisionele bakteriële ekspressie met die gebruik van die pET stelsel; tweede generasie koue skok bakteriële ekspressie wat gebruik maak van die pCOLFTF ekspressie vektor om die oplosbaarheid van die rekombinante proteïen te verbeter; en bakulovirus ekspressie in insekselle vir post translasie modifikasie glukosilering van die rekombinante proteïen wat kan help met die oplosbaarheid en biologiese aktiwiteit van die proteïen. Die rekombinante GLYAT het goed uitgedruk in al drie die ekspressie sisteme, alhoewel die rekombinante GLYAT onoplosbaar was en geen ensiem aktiwiteit kon waargeneem word nie. Die onoplosbare rekombinante GLYAT was versamel in gebruik in ‘n sisteem waar die rekombinante GLYAT gedenatureer was en gerenatureer was in n reeks van buffers om die oplosbaarheid van die rekombinante GLYAT te verbeter en sodoende ensiem aktiwiteit te verky. Die oplosbaarheid van die rekombinante GLYAT het verbeter maar geen ensiem aktiwiteit is waargeneem nie.

Sleutelterme: detoksifikasie, DNA, RNA, proteïen uitdrukking, bakteriële uitdrukking, ensiem aktiwiteit, inseksel uitdrukking

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

Literature overview

1.1. Introduction

Awareness of detoxification has become an integral part of our daily lives. Everywhere on products, health foods, drinks and diets, detoxification has become a modern day buzzword. Modern man sees detoxification today as a means to a better quality of life, to enhance sports performance and in extreme cases to help drug addicts wean more easily off drugs. It is advertised everywhere, from cereal boxes to commercial companies offering to profile your detoxification ability. A less known application of detoxification is its application as a therapy for alleviating symptoms of inborn errors of metabolism (IEM).

1.1.1. Inborn errors of metabolism

Metabolism (from Greek "metabolismos" meaning “out throw”) is the biochemical modification of chemical compounds in living organisms and cells. This includes the biosynthesis of complex organic molecules (anabolism) and their breakdown (catabolism). Metabolism usually consists of sequences of enzymatic steps, also called metabolic pathways. The total metabolism includes all the biochemical processes of an organism. The cell metabolism includes all chemical processes in a cell. The general pathways are the carbohydrate metabolism, fatty acid metabolism, protein metabolism and nucleic acid metabolism. In these metabolic pathways, many metabolites are formed. Some of these metabolites will continue in the metabolic pathway or will be removed from the pathway via detoxification pathways. The reason for this removal is that some metabolites formed in the pathways can be toxic to the human body (Nelson et al., 2005).

Inborn errors of metabolism (IEM) are a group of disorders where a gene defect has a clinical significant effect on an individual’s metabolic pathway. Metabolites accumulate in the individual and a series of clinical symptoms can then be observed. The defect is passed on in an autosomal recessive or X-linked recessive fashion. This includes diseases like: organic acidemias, fatty acid oxidation defects, primary lactic acidoses, aminoacidopathies, urea cycle defects, disorders of the carbohydrate metabolism, lysosomal storage disorder and peroxisomal disorders (Garrod et al., 1923 and Fernandes et al., 2006).

Detection of some of these diseases is possible by means of neonatal screening. There are over 500 inborn errors of metabolism known (Baric et al., 2001). About 30 inborn errors of metabolism are included in newborn screening programmes. The inclusion of a disease into a newborn screening program occurs when there is some form of treatment that can improve the prognosis for the patient.

1.1.2. Detoxification

Detoxification is the process where endogenous and xenobiotic metabolites are transformed to less harmful products by the liver and kidneys and excreted through bile and urine. These metabolites include metabolites from:

- metabolic pathways (normal metabolism and abnormal metabolism in case of inborn errors of metabolism),
- pharmaceuticals
- food components like preservatives, artificial colourants and flavourants and
- environmental factors such as pollution.

Do deal with these substances the body utilizes a complex detoxification system (Liska, 1998).

In inborn errors of metabolism toxic endogenous metabolites accumulate due to blocked metabolic pathways. In the case of organic acidemias the excess metabolites are then detoxified by amino acid conjugation and carnitine conjugation. Subsequently the body's own levels of glycine and carnitine usually get depleted due to the high excretion of metabolites bound to them. To counteract this depletion, supplementation of glycine and carnitine are given to improve the detoxification ability of the patient and to make the clinical consequences of the organic acidemia more manageable (Fries et al., 1996, Baumgartner., 2003).

Two main detoxification pathways exist (Figure 1). The phase I (functionalization) reactions include oxidation, hydroxylation, dehydrogenation, metabolic reduction and hydrolysis. The cytochrome P450, important in detoxification of pharmaceutical drugs, mono-oxygenase system is a major part of this pathway (Rendic, 2002). Phase II detoxification (conjugation) focuses on the formation of conjugates to increase hydrophillicity. Conjugation with amino acids plays an important role in the detoxification of mitochondrial metabolism.

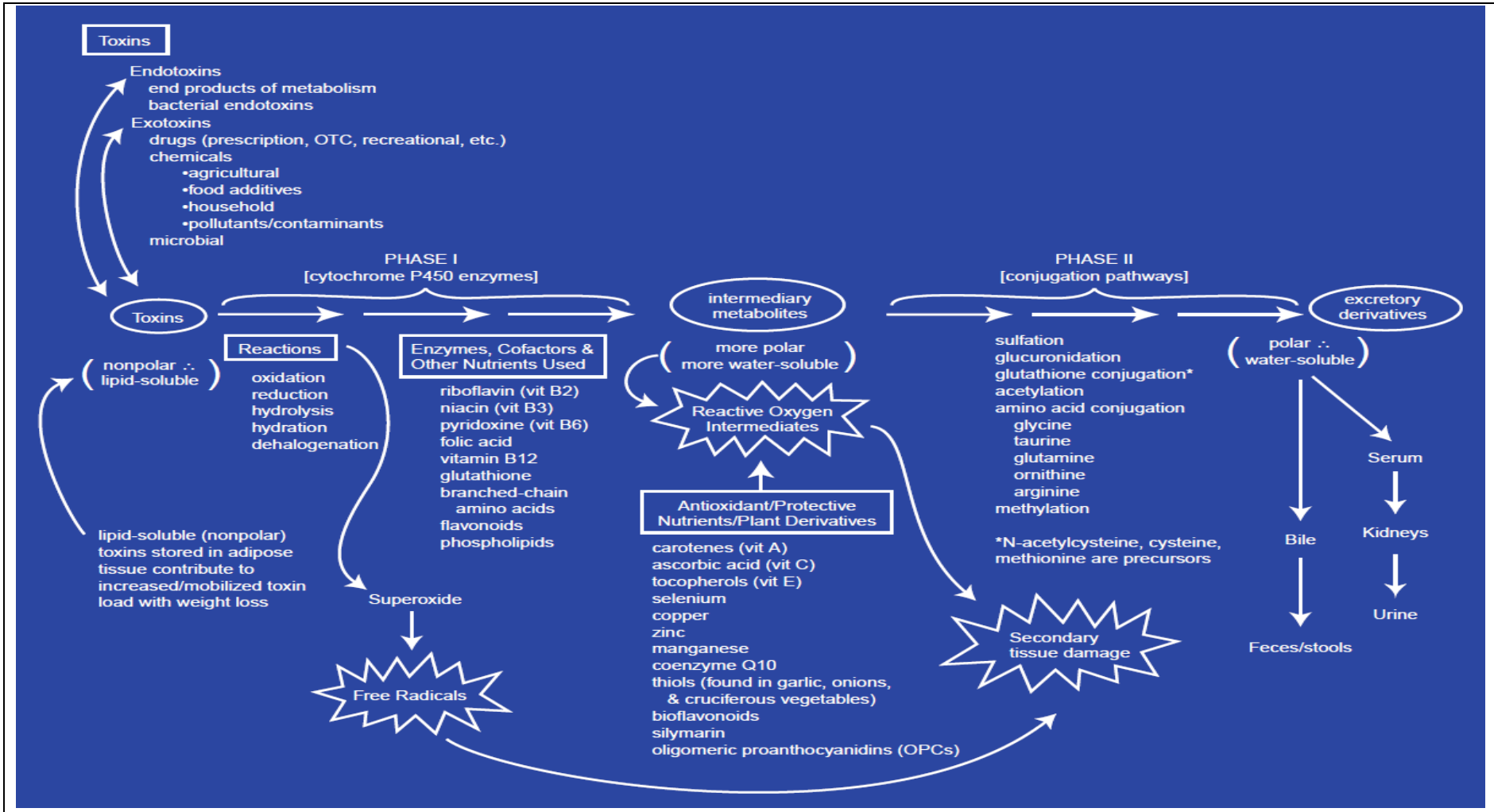


Figure 1: Detoxification pathways with enzymes and co factors used during detoxification in the human liver (used with permission, Liska; 1998)

1.1.3. Phase 1 detoxification

The cytochrome P450 supergene family of enzymes forms the major part of phase 1 detoxification (Liska, 1998., Rendic., 2002), as mentioned above. Phase 1 detoxification is the first step in detoxification of toxins and metabolites. Generally a cytochrome P450 enzyme catalyzes a Phase 1 reaction by using oxygen with NADH as cofactor to add a reactive group, such as a hydroxyl radical. This reactive molecule is then further processed by Phase 2 detoxification. If this does not occur the reactive molecule can damage proteins, RNA and DNA (Liska, 1998).

Protein engineering has been used extensively to investigate and modify the properties of cytochromes P450 enzymes and has lead to major advances in our understanding of their biological roles and catalytic mechanisms. These studies demonstrated the attractive and realistic proposition that protein engineering approaches can be used to modulate cytochromes P450 to catalyze the oxygenation of a molecule of choice. Protein engineering has also been shown to be a key tool for probing the role of active site residues and for dissecting the route of electron transfer through the protein (Miles et al., 2000).

1.1.4. Phase 2 Detoxification

Phase 2 detoxification consists of conjugation reactions. These reactions use cofactors and transfer them to the functional groups introduced by Phase 1 detoxification. This conjugation with the functional groups increases the water solubility of the molecules for better excretion of these molecules through urine and bile. The main pathways in Phase 2 detoxification are: glucuronidation, sulfonation, acetylation, methylation, glutathione conjugation and amino acid conjugation (Caldwell, 1986; Mulder, 1990; Fishman, 1970; Jakoby, 1980; Jakopy, 1981; Paulson et al., 1986; Parkinson , 1995; Liska, 1998).

1.1.4.1. Glucuronidation

UDP-glycuronosyltransferases (UGTs: EC 2.4.1.17) catalyzes the reaction for glucuronidation by transferring a glucuronate group of uridine diphosphoglucuronate to the functional group of the substrate. UGTs are microsomal membrane bound enzymes. The effect of the conjugation is to lend polarity to the substrate making it easier to excrete through bile and urine (Parkinson, 1995).

1.1.4.2. Sulfonation

Sulfotransferase (SULT EC 2.8.2) enzymes catalyze the transfer of a sulfate group from 3-phosphoadenosine 5-phosphosulfate (PAPS) to a hydroxyl group on an acceptor molecule, yielding a sulfonated acceptor and 3-phosphoadenosine 5-phosphate (PAP). This increases the molecule's water solubility to be excreted through bile and urine (Parkinson, 1995).

1.1.4.3. Acetylation

N-acetyltransferase (NAT; EC 2.3.1.5) conjugates acetyl CoA with substrates from Phase 1 detoxification. This is the preferred route of conjugating aromatic amines and hydrazines. N-acetylation occurs in two sequential steps via a ping-pong Bi-Bi mechanism. In the first step, the acetyl group from acetyl-CoA is transferred to a cysteine residue in NAT, with consequent release of coenzyme-A. In the second step, the acetyl group is released from the acetylated NAT to the substrate, subsequently regenerating the enzyme (Parkinson, 1995).

1.1.4.4. Methylation

Methyltransferase (MT; EC 2.1.1.49) catalyzes the transfer of the methyl group to the functional groups of the substrate. MT utilizes S-adenosylmethionine (SAM) as methyl donor. The functional groups are phenols, catechols, aliphatic and aromatic amines and sufhyryl-containing groups are targeted by MT (Parkinson, 1995).

1.1.4.5. Glutathione conjugation

Glutathione S-transferase (GST; EC 2.5.1.18) mediate conjugation by using glutathione (GSH). There are two types of conjugation reaction with glutathione: displacement reactions where glutathione displaces an electron-withdrawing group and addition reactions where glutathione is added to activated double bond structures or strained ring systems. Glutathione conjugates are excreted in bile and converted to cystine and mercapturic acid conjugates in the intestine and kidneys (Parkinson, 1995).

1.1.4.6. Amino acid conjugation

Conjugation of metabolites with amino acids plays an important role in the detoxification of mitochondrial metabolism. Two enzymes are known that use amino acids in detoxification , glycine-N-acyltransferase (EC 2.3.1.13) and glutamine-N-phenylacetyltransferase (EC 2.3.1.14). The GLYAT family of enzymes has been characterized in humans (Webster et al., 1976; Kelley et al., 1994, Zhang et al., 2007, Waluk et al., 2010) and bovine (Nandi et al., 1979; Kelley et al., 1994) of which GLYAT (EC 2.3.1.13) was already referred to above. In drug detoxification, detoxification is usually attributed to the addition of a molecule which makes the target substrate more soluble for excretion in the urine or bile. The primary role of GLYAT has been regarded as one of detoxification of a variety of acyl-CoA esters (Mulder, 1990). It utilizes benzoyl-CoA as well as a wide variety of short and medium, straight- and branched chain, acyl-CoA substrates with glycine as acyl acceptor (Nandi et al., 1979). Glycine conjugation is a very common route for the detoxification of exogenous carboxylic acids, such as salicylate to form salicyluric acid (Levy, 1965) and with benzoate to form benzoylglycine (hippuric acid). It is also the case with endogenously formed carboxylic acids such as propionic acid and isovaleric acid which accumulates in propionic- and isovaleric acidemias respectively, in which case propionylglycine and isovalerylglycine are formed (Bartlett et al., 1974). Conjugation of carboxylic acids with amino acids takes place in the

matrix of the mitochondria of the liver and kidney and effectively results in the joining of the carboxylic acid to the amino nitrogen of the amino acid by a sequential mechanism with the acyl-CoA binding first, followed by glycine to form a ternary complex with the subsequent release of the peptide conjugate (Gatley et al., 1977; Vessey et al., 1995; Schachter et al., 1953). The activity of human GLYAT increases from birth to 18 months, when it reaches adult levels, and remains constant (Mawal et al., 1994).

The amino acid specificity of GLYAT is predominantly for glycine. Some minor affinity for glutamine and asparagine in the case of bovine GLYAT has been reported (Webster et al., 1976; Nandi et al., 1979; Asaoka, 1991). However, acyl-amino acid conjugates with other amino acids, i.e. Ala, Glu, and Ser, have been detected (Hutt et al., 1990; Lehnert, 1981; Lehnert, 1983; Shinka et al., 1985; Rolland et al, 1991).

To date no inborn error of metabolism has been associated with GLYAT itself.

Clearly, the availability of a recombinant GLYAT would significantly benefit studies on the molecular genetics, detailed enzymatic characterization and structural biology of this protein which is important in the detoxification of various endogenous acyl-CoAs. This is still lacking for GLYAT.

1.1.5. Molecular details of GLYAT

Reports on the molecular mass for human GLYAT purified from liver vary, ranging between 24 kDa (Webster et al. 1976), 30 kDa (Mawal et al., 1994) and 30.5 kDa (Kelley et al., 1994). The first GLYAT sequence to be published was that of bovine GLYAT (Vessey et al., 1996). Van der Westhuizen and co-workers identified and sequenced the first putative human GLYAT gene in 1997 (GenBank Accession number AF023466) (Van der Westhuizen et al., 2000). They identified it by matching the N-terminal sequence of bovine GLYAT (Vessey et al., 1996 - EMBL accession number AJ223301) to a 317 bp non-redundant expression sequence tag (Clone ID 124365) of a human fetal liver cDNA clone in the cDNA Clones Bank of Integrated Molecular Analysis of Genomes and their Expression at the Lawrence Livermore National Laboratory (Lennon et al., 1996). The clone consisted of 1083 bp. It contains an open reading frame of 486 bp, encoding for 162 amino acids, and an 86 and 511 bp 5'- and 3'-NTR, respectively. The unmodified translated product of this putative human GLYAT cDNA clone should theoretically have a molecular mass of 18.3 kDa, which is much smaller than native purified human GLYAT (24 - 30.5 kDa) as was previously reported (Webster et al. 1976, Mawal et al., 1994, Kelley et al., 1994). Comparatively, the bovine cDNA sequence contains a much larger open reading frame of 885 bp (295 amino acids) with a 126 and 203 bp 5'- and 3'-NTR, respectively (Vessey et al., 1996). Comparison of the deduced amino acid sequence indicated that the homology between the putative human GLYAT and bovine GLYAT sequences is 77%, while the homology between any of the two GLYAT sequences and the bovine glutamine N-phenylacetyltransferase sequence is 70%. Subsequently,

GLYAT was also identified during different human genome projects. The International Radiation Hybrid Mapping Consortium mapped the GLYAT gene to chromosome 11 (sts-W87532). Two transcript variants, a and b, encoding different isoforms have been found for this gene. Transcript Variant 1 represents the longer transcript and encodes the longer isoform a. Its open reading frame encodes 296 amino acid residues (GenBank NP_964011). Variant 2 differs in the 3' coding region and 3' UTR compared to variant 1. The resulting isoform b is shorter and has a distinct C-terminus compared to isoform a. Its open reading frame encodes 163 amino acid residues (GenBank NP_005829). This sequence is identical to that of the sequence deposited by Van der Westhuizen et al. in 1997 (GenBank AF023466). In recent studies two other members of the GLYAT family of enzymes have been identified GLYATL1 (GenBank DQ084381) and GLYATL2 (Waluk et al., 2010). GLYATL1 is a split gene that consisted out of 7 exons and translates a peptide chain of 302 amino acids in length. The gene was mapped to chromosome 11q12.1 and transcribes in the sense direction of the chromosome (Zhang et al. 2007). GLYATL1 is mostly expressed in the liver and, when it is over expressed, it activates the HSE pathway. The GLYATL2 enzyme is mostly found in the endoplasmic reticulum, expression in salivary gland and trachea is high, but is also detected in spinal cord and skin fibroblasts. GLYATL2 (GenBank NM_145016.3) is expressed on the minus strand, is 34 kDa in size and has a C terminal dilysine motif which is an ER retention or retrieval signal (Waluk et al. 2010). The expression pattern of the GLYATL2 suggests that it might have a barrier function assisting the immune system.

Table 1: Comparison of the members of the human GLYAT family to bovine GLYAT

	HuGLYATa	HuGLYATb	HuGLYATL1	HuGLYATL2	BovGLYAT
Chromosome	11	11	11	11	15
Exons	6	5	7	6	5
Transcript length	2052 bp	1147 bp	2068 bp	1636	984 bp
Protein length	296 aa	163 aa	302 aa	294 aa	294 aa

The Entrez single nucleotide polymorphism database currently lists 137 human GLYAT SNPs. 16 SNPs are in the coding region. 121 SNPs are in the gene's 3'-UTR or in introns. However, nothing is as yet known about the significance of these SNPs, biologically, functionally or otherwise.

1.1.6. Recombinant protein expression systems

To obtain the protein of interest various systems for expression of proteins have been developed for use in the laboratory. This includes bacterial, yeast, insect and mammalian expression systems. Table 2 shows a comparison of these systems and point out their advantages and disadvantages.

Table 2: Comparison of the various expression systems. (Adapted from Genwaybio.com)

Characteristics	E. coli	Yeast	Insect cells	Mammalian cells
Cell Growth	Rapid (30 min)	Rapid (90 min)	Slow (18 -24 H)	Slow (24 H)
Complexity of Growth Medium	Low	Low	High	High
Cost of Growth Medium	Low	Low	High	High
Expression Level	High	Low - High	Low - High	Low - Moderate
Protein Folding	Refolding usually required	Refolding may be required	Proper folding	Proper folding
N-linked Glycosylation	None	High Mannose	Simple, no salic acid	Complex
O-linked Glycosylation	No	Yes	Yes	Yes
Phosphorylation	No	Yes	Yes	Yes
Acetylation	No	Yes	Yes	Yes
Acylation	No	Yes	Yes	Yes
Yield (mg) (per litre culture)	50 - 500	10 - 200	10 - 200	0.1 - 100
Success Rate (%) (soluble or functional)	40 – 60	50 – 70	50 – 70	80 – 95
Project Cost	Low	Low	Moderate	High
Advantage	Simple, robust, lowest cost and highest yield	Simple, low cost	Relatively higher yields, better post translational modification	Natural protein configuration, best post translational modification
Disadvantage	No post translation modification	Longer time for expression, little post translation modification	Longer time for expression, higher cost	Highest cost, little yield

1.1.7. Problem formulation

The Biochemistry Division at the NWU where I was located has a Laboratory for Metabolic Diseases. This laboratory has 25 years experience in the identification and characterization of inherited metabolic diseases from more than 20 000 samples. They have identified 70 different known metabolic diseases and found indications of the presence of as yet undocumented metabolic defects. Recent analysis of urinary organic acids and their conjugates with tandem mass-spectrometry in this laboratory suggests that a benign phenotype might also be related to very effective endogenous detoxification of primary and secondary metabolites formed in patients with metabolic diseases. In our view, this observation correlates with the fact that therapeutic enhancement of endogenous detoxification is one of a few treatments available for metabolic disorders. Increased detoxification is generally achieved by the supplementation of a detoxifying metabolite, mostly either glycine or carnitine. However, to date, little research has been done on the impact of the efficiency of detoxification pathways in patients on the prognosis of their metabolic disorders. The availability of a recombinant GLYAT protein would further our knowledge of the structure and function of this enzyme. An in depth characterization of the enzyme kinetics and substrate specificity can then be performed on the recombinant protein. Through rational protein engineering, substrate specificity might be altered. This in turn can then perhaps lead to a new rational pharmaceutical therapeutic recombinant enzyme to treat a wide range of inborn errors of metabolism. This, in turn, might lead to an improved prognosis for patients, a more normal development and an improved quality of life.

This project was commenced in 2005 and experimental work concluded in 2006. Due to personal changes and career opportunities the writing of this dissertation was concluded in 2012. In the time (2007 – 2012) recombinant bovine GLYAT (Badenhorst et al., 2011), recombinant GLYATL1 (Zhang et al., 2007) and recombinant GLYATL2 has been expressed (Waluk et al., 2010).

1.1.8. Aims

The aim of this project was the cloning and expression of human GLYAT isoform a by:

- Acquiring a synthetic open reading frame (ORF) encoding human GLYAT isoform a cloned into a bacterial expression vector.
- Expression of recombinant GLYAT using bacterial and insect cell expression systems.
- Evaluation of enzyme activity of recombinant human GLYAT isoform a.

Chapter 2

Expression of human GLYAT isoform a in *Escherichia coli* using the pET expression system

2.1. Introduction

Bacterial *Escherichia coli* based expression systems are currently the most commonly used prokaryote expression systems for recombinant proteins. The main advantages of the systems are that they are inexpensive to start up and to maintain, since these bacteria are normally grown on inexpensive carbon sources, biomass accumulation is very rapid and the process is simple to scale-up. The main disadvantage of the system is that the recombinant protein often aggregates as insoluble proteins in the form of inclusion bodies (Baneyx et al., 2004). There are several reasons for inclusion body formation as will be discussed in Chapter 3.

The pET expression system first described by Studier and colleagues in 1991, is nowadays produced commercially by Novagen. It offers a wide variety of expression vectors to produce the recombinant protein of choice (Studier, 1991). The pET expression system contains promoters, multiple cloning sites with different fusion protein segments and protease cleavage sites (Sorenson et al., 2005, Studier, 1991). Host strains must be lysogenized by a DE3 phage fragment that encodes the bacteriophage T7 gene 1 (T7 RNA polymerase) and is controlled by the *lacUV5* promoter inducible by IPTG. This is necessary for expression of the target gene using the pET expression system. The *lacUV5* promoter and the T7/*lac* promoter, encoded by the expression vector, are both repressed by the tetramer LacI. The *lacI* gene is present on the genome of the *E. coli* and the expression plasmid. Expression of the target gene is induced by adding IPTG. IPTG binds to LacI and triggers the release of the protein from the *lac* operator. This then allows T7 RNA polymerase to be transcribed and transcription of the target gene from the T7/*lac* promoter is initiated (Sorensen et al., 2005, Studier, 1991). Figure 2 gives a more visual representation.

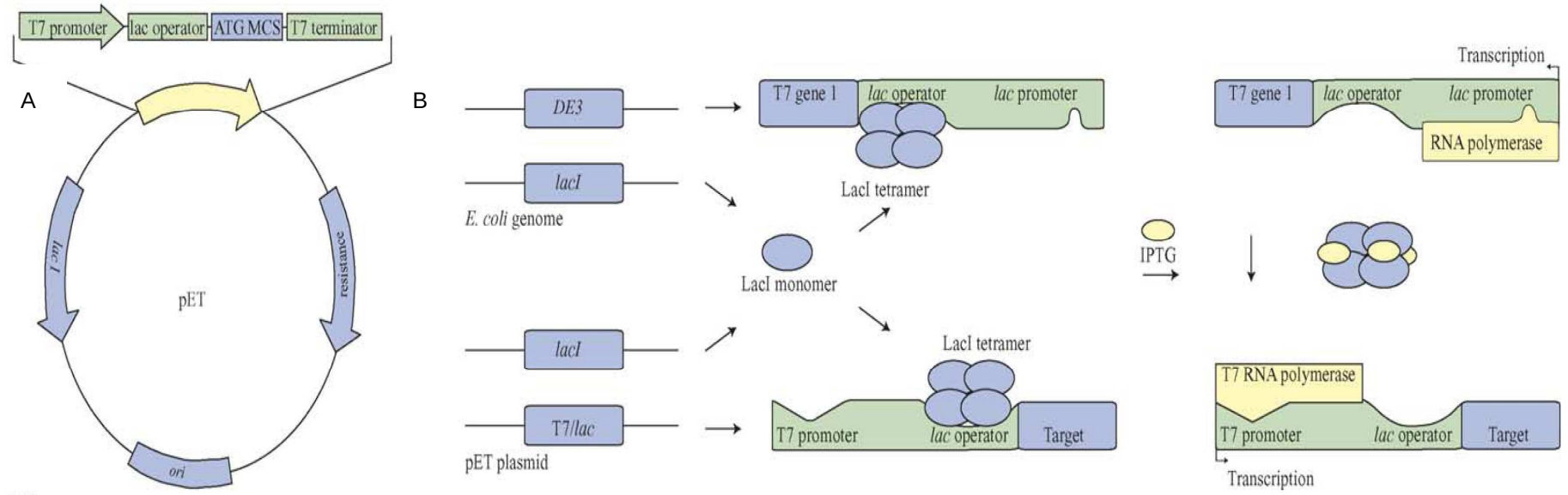


Figure 2: The pET expression system, showing (A) a general pET vector configuration, and (B) the state of the macromolecules before and after induction (used with permission from Sorenson et al., 2005, Studier, 1991).

The following sources were considered to obtain a human gene encoding the complete open reading frame (ORF):

- Human liver
- HepG2 cell cultures
- Having the gene synthesized by a commercial company

Due to the ethical and logistical reasons of isolating the RNA from human livers or isolating the RNA from HepG2 cells, where the expression of the gene was very low, it was decided to commercially synthesize the gene.

A synthetic gene (see 2.2.1) was ordered from Geneart encoding human GLYAT isoform a already cloned into pET32a+ (see Figure 3). This was then used to express a recombinant human GLYAT isoform a using first generation bacterial expression. The aim of this was to produce a soluble and active recombinant protein that can be further used to study the enzyme.

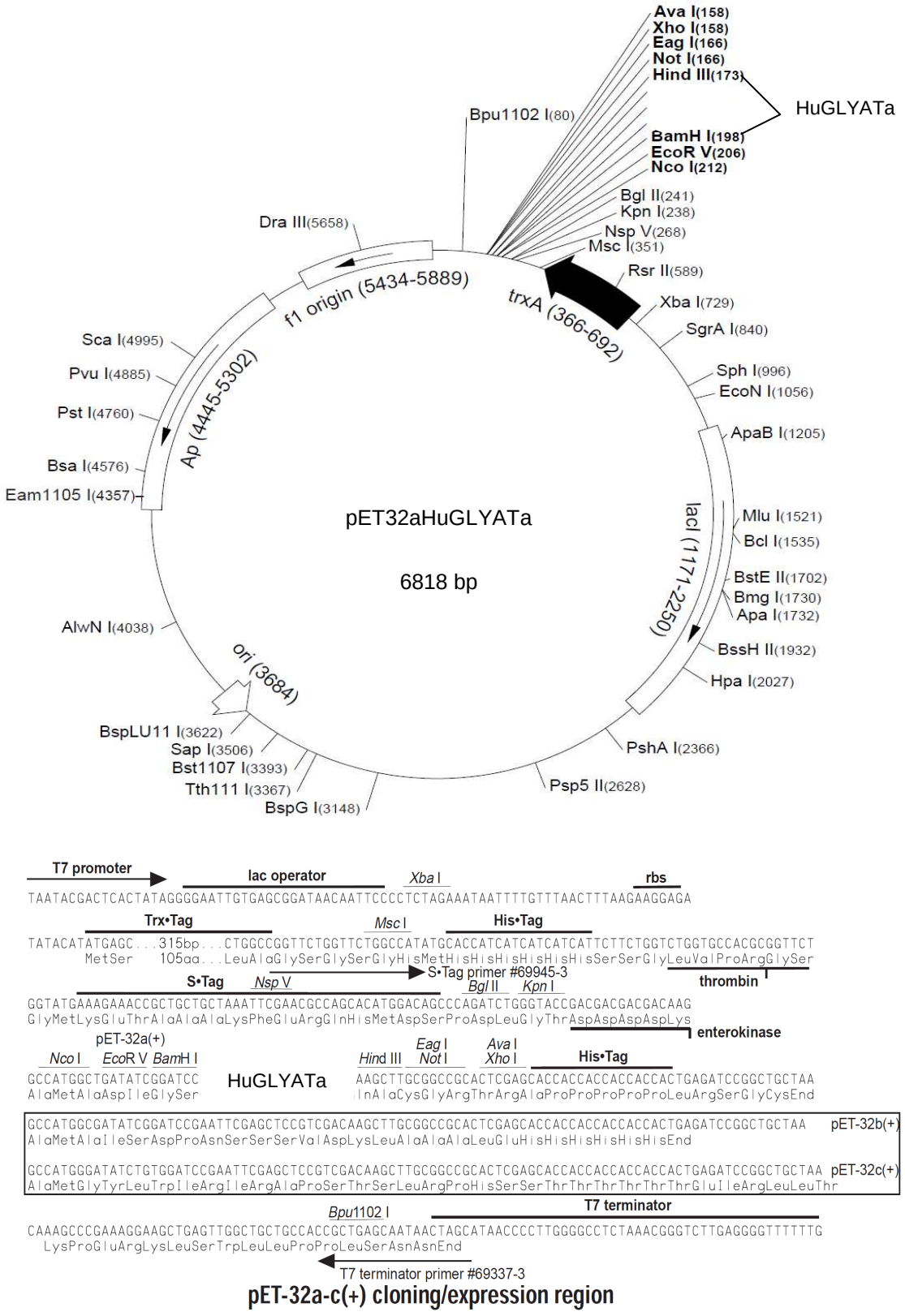


Figure 3: Vector map and sequence map of the region containing the T7 promoter, multiple tags, terminator site and multiple cloning site of pET32a+ showing the position of the insert of the ORF encoding HuGLYATa (Novagen, catalogue no. 69015-3, manual TB122).

2.2. Materials and Methods

2.2.1. Design and synthesis of a synthetic cDNA encoding human GLYAT isoform a and cloned into pET32a+

A commercial company, Geneart, synthesized the gene encoding GLYAT. Synthetic genes can be obtained through various techniques (Engels, JW et al; 1989):

- Ligation of overlapping oligonucleotides by the Khorana procedure (Figure 3, A).
- Sequential ligation of preformed DNA duplexes (Figure 3, B).
- Direct hybridization and cloning of longer oligonucleotides (Figure 3, C)
- Fill-in method of overlapping oligonucleotides (Figure 3, D)
- Fill-in method using autoprimer oligonucleotides (Figure 3, E)

Geneart did not disclose which method was used.

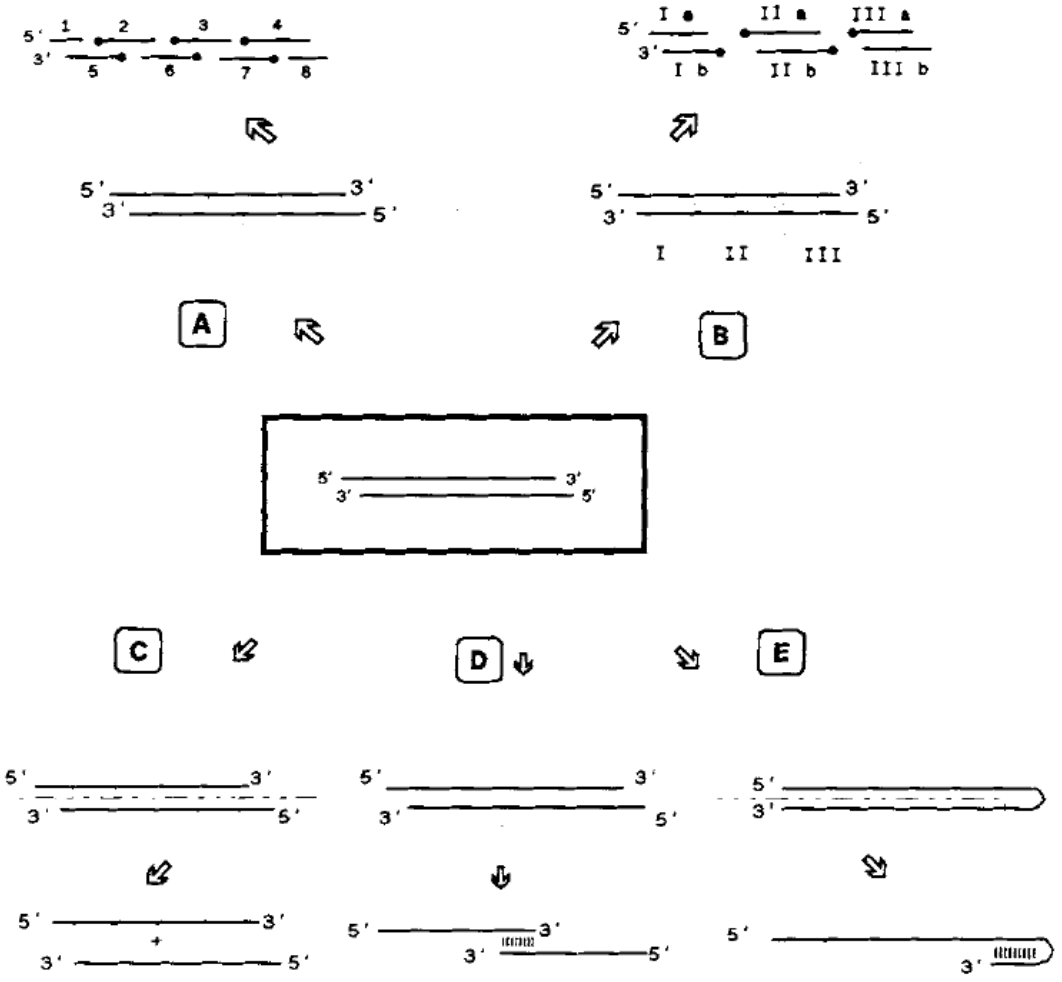


Figure 4: Various gene synthesis techniques (A) the Khorona procedure; (B) sequential ligation; (C) direct hybridisation; (D) method of overlapping oligonucleotides and (E) method using autoprimer oligonucleotides (used with permission Engels, JW et al; 1989)

The cDNA encoding human GLYAT isoform a (HuGLYATa) (Genbank number NM 201648.2) was synthesized and cloned into pET32a+ by Geneart and shipped lyophilized. On arrival the lyophilized pET32a+ HuGLYATa was resuspended in 50 µl 18 Ω MiliQ H_20 and stored at -20 °C.

2.2.2. Bacterial host strains used for the cloning and expression of human GLYAT isoform a

The following host strains were used for the cloning and expression of human GLYAT isoform a.

The SURE® host strain (Stratagene catalogue no. 200238) was used for plasmid production and purification. The SURE® host strain lacks the components for the pathways that catalyze the rearrangement and deletion of nonstandard secondary and tertiary structures in

eukaryotic DNA that impede the cloning of eukaryotic DNA in conventional strains (Stratagene Certificate of Analysis).

The Origami™ host strain (Novagen catalogue no. 70626) was used for the expression of the target gene. Origami™ host strains have mutations in both the thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes to improve the disulfide bond formation in the cytoplasm that may lead to improved formation of an active recombinant protein. Origami™ host strains are ideal to use with pET32 vectors as these vectors contain a thioredoxin fusion tag that further increases the formation of disulfide bonds in the cytoplasm (Novagen, 2007).

2.2.3. Preparation of competent bacterial cells

Cells were made transformation competent by chemical means using the method of Inoue and co-workers (Inoue et al., 1990). To ensure optimal efficiency of the method all glassware used must be very clean and free from any biological residues and detergent. To achieve this, all glassware was washed with chromic acid. The glassware was subsequently rinsed three times with 18Ω H_20 and autoclaved to sterilize the glassware. A scraping of the glycerol stocks of SURE® and Origami™ cells was taken and plated out on LB-agar plates containing 12.5 µg/ml tetracycline and 15 µg/ml kanamycin and incubated overnight (16 hours) at 37 °C. Both SURE® and Origami™ cells have resistance to tetracycline and kanamycin. This eases selection of the cells, as these will be the only bacterial cells growing on the plates. This selection is also useful in cases where the cells may have become contaminated with wild strain bacterial cells. Colonies from the plates were then picked with a sterile toothpick (10 – 12 colonies, measuring 2 – 3 mm in diameter) and inoculated into 250 ml LB medium containing no antibiotics and cultured at 18 °C on a shaking platform (200 – 250 rpm) until the culture reached an optical density of 0.6 at 600 nm. The culture was then placed on ice for 10 minutes. The cultures were then centrifuged at 2500 x g for 10 min at 4 °C. The bacterial pellets were then each resuspended in 80 ml transformation buffer (10 mM PIPES, 15 mM $CaCl_2$, 250 mM KCl), pH adjusted to 6.7 with KOH. After pH adjustment, $MnCl_2$ was added to 55 mM and the buffer was filter sterilized through a 45 µm filter and stored at 4 °C, incubated on ice for 10 minutes and centrifuged again at 2500 g for 10 minutes at 4 °C. The bacterial cell pellet was then gently resuspended in 20 ml transformation buffer while kept on ice. DMSO was added to a final volume 7 % (v/v). The cells were then incubated on ice for 10 minutes. The cell suspensions were divided in 300 µl aliquots in cryogenic vials (Sigma Aldrich catalogue no. CLS430658) and stored in liquid nitrogen.

2.2.4. Transformation of competent bacterial cells with pET32a+HuGLYATa

Transformation of competent bacterial cells was done by means of the heat shock method as recommended in the pET system manual (Novagen, 2007). An aliquot of 100 µl of competent cells was transferred to an Eppendorf tube and 10 ng of plasmid pET32a+HuGLYATa was added to the cells. This mixture was placed on ice for 20 minutes. Cooling down the cells in

the presence of divalent cations like Ca^{2+} makes the cell membrane permeable to the extent that it allows for the uptake of plasmid DNA. The cells were then placed in a water bath 42 °C, for exactly 30 seconds. This heat shock step causes the plasmid DNA to enter the cell. The bacterial cells are then immediately placed on ice for 2 minutes. The cells were then transferred to a 15 ml tube containing 900 µl SOC medium (0.5% Yeast Extract; 2% Tryptone; 10 mM NaCl; 2.5 mM KCl; 10 mM $MgCl_2$; 10 mM $MgSO_4$; 20 mM Glucose) and incubated at 37 °C for 1 hour in a shaking incubator at 200 – 250 rpm. This step helps the cells recover from the transformation procedure. After an hour the cells were plated out on a LB-Agar plate containing 100 µg/ml ampicillin, for selection of cells containing pET32a+HuGLYATa, and incubated overnight in an incubator at 37 °C. Single colonies were then picked up with a sterile toothpick and inoculated in 10 ml LB medium containing 100 µg/ml ampicillin and again grown overnight (16 hours) in a shaking incubator at 37 °C at 250 rpm for plasmid extraction and preparation of glycerol stocks.

2.2.5. Preparation of glycerol stocks

Colonies were picked up from LB-agar plates containing the appropriate antibiotics, with a sterile toothpick and inoculated in 10 ml LB medium containing 100 µg/ml ampicillin and grown overnight (16 hours) in a shaking incubator at 250 rpm at 37 °C. Several 1 ml glycerol stocks were prepared by adding 50 % glycerol to 700 µl of the culture for a final concentration of 15 % glycerol v/v in a 1.5 ml Eppendorf tube. Glycerol stocks were stored at -80 °C (Russell and Sambrook, 2006).

2.2.6. Plasmid extraction of pET32aHuGLYATa

A commercial kit from Invitek (Invisorb® Spin Plasmid Mini Two catalogue no. 0708) was used for plasmid extraction. It uses spin filter technology where the filter selectively binds the plasmid DNA. The kit is based on the alkaline SDS lysis plasmid extraction procedure (Birnboim and Doly, 1979). The authors determined that linear DNA denatures at a pH of about 12.0 – 12.5 but not plasmid DNA. This property can be used to purify plasmid DNA. The method starts of by weakening the cell wall with lysozyme and then completely lyses the cell with SDS and NaOH. Chromosomal DNA is selectively denatured in this high alkaline solution. This high alkaline solution is neutralized with acidic sodium acetate that causes the DNA to renature and aggregate. This also causes the precipitation of protein-SDS complexes. These precipitations are insoluble and ease the extraction of low molecular weight plasmid DNA from the supernatant through ethanol precipitation (Birnboim and Doly, 1979). For small scale plasmid preparations, 5 – 10 ml cultures were grown in a shaking incubator at 250 rpm for 16 hours at 37 °C. An aliquot of 2 ml was transferred to a 2 ml Eppendorf tube and centrifuged for 1 minute at 10 000 g (Eppendorf centrifuge model no 1524) to collect the cells. The cell pellet was then resuspended in 250 µl of Solution A (2 mg/ml lysozyme, 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0) and vortexed to completely resuspend the cell pellet. This is to ensure that the cells to not clump together.

After the cell pellet was completely resuspended 250 µl of Solution B (0.2 N NaOH, 1% sodium dodecyl sulphate (SDS)) was added and the tube was inverted 5 times. This is the cell lysis step which should not be performed for more then 5 minutes. After the lysis step 250 µl of Solution C (3 M sodium acetate pH 4.8) was added and the tube inverted several times. The solution was then centrifuged for 5 minutes at 10000 x g and the clarified supernatant was transferred to a spin filter and placed in a 2 ml receiver tube. The solution was then incubated on the spin filter for 1 minute and then centrifuged for 1 minute at 10000 x g. The filtrate was then discarded and 750 µl wash solution (0.1 M sodium acetate/0.05 M Tris-HCl pH 8, 80 % ethanol v/v) was added and centrifuged again for 1 minute at 10000 x g and the filtrate discarded. The spin filter was then again centrifuged for 3 minutes at 10000 x g to completely remove the ethanol. Ethanol prevents DNA from going into solution. The spin filter is then placed in a 1.5 ml receiver tube and 50 µl of elution solution (10 mM Tris-HCl EDTA pH 8) was added, incubated for 1 minute and centrifuged for 1 minute at 10000 x g. The plasmid preparation was then stored at –20 °C. The quantity and quality of the purified plasmid was determined with a spectrophotometer (NanoDrop ND 1000). The $A_{260/280}$ was used to determine purity and A_{260} was used to determine quantity.

2.2.7. Restriction enzyme analysis of pET32a+HuGLYATa

Restriction enzyme analysis was done to determine if the insert is present in pET32a+HuGLYATa and that the plasmid is the correct size. The restriction enzymes BamHI and HindIII were chosen because the insert was flanked by their respective nucleic acid recognition sites. To linearize the plasmid 0.5 µg of plasmid DNA (from the plasmid extraction) was transferred to an Eppendorf tube and 1X BamHI buffer (Fermentas, catalogue no. ER0051), 10 U BamHI (Fermentas, ER0051) and 18Ω ddH_20 added to a final volume of 20 ul. The reaction mixture was incubated at 37 °C for 4 hours.

To determine if the insert was present, a double digest (according to manufacturers' recommendations) was performed using BamHI and HindIII. Again 0.5 µg of plasmid DNA was transferred to an Eppendorf tube and 1X BamHI buffer, 20 U HindIII (Fermentas, catalogue no. ER0501), 10 U BamHI and 18Ω ddH_20 to a final volume of 20 µl was added to the plasmid DNA. The reaction mixture was incubated at 37 °C for 4 hours. After the 4 hour incubation period, the digested plasmids were visualised on a 1 % agarose gel. The whole 20 µl sample was taken and 3 µl of 2x loading dye (Russell and Sambrook, 2006) added.

2.2.8. Agarose gel electrophoresis

Generally 15 x 10 cm x 0.5 cm 1 % agarose gels were prepared in TAE buffer (50x TAE buffer, 2 M HCl Tris ph 7.5, 50 mM EDTA) as follows: A 50 ml gel mixture was prepared in 1 x TAE buffer in a 100 ml glass bottle and boiled in a microwave oven for 2 minutes or until all the agarose granules were dissolved. The agarose gel was cooled down to 65 °C and 10 µg/ml ethidium bromide (EtBr) was added (Russell and Sambrook, 2006). The gel was then

poured in a caster (15 x 10 cm) with a 10 well comb and alllowed to set for 15 minutes. The gel was then placed in a electrophoresis tank (Mini Subcell, Biorad, catalogue no. 164-0300). The gel was loaded with 5 µl Ladder (O' Gene Ruler, Fermenatas, catalogue no. SM0334) in the first well and 5 µl of each sample with 2 µl of 6 x loading dye (Fermentas, catalogue no. R0611) in the rest of the wells. This was done with all samples unless stated otherwise. The lid of the tank was closed and connected to the power supply unit (Powerpack Basic, Biorad, catalogue no. 164-5050) and run at a 100 V constant for 40 minutes. The gel was visualized by capturing an image under UV light in a gel documentation sytem (Syngene ChemiGenius Bio-Imaging System) and GeneSnap software (Syngene, England).

2.2.9. Expression of the recombinant protein HuGLYATa in Origami™ cells using pET32a+

Frozen glycerol stocks of Origami™ cells containing pET32a+HuGLYATa were used to inoculate 10 ml LB-medium containing 12.5 µg/ml tetracycline, 15 µg/ml kanamycin and 100 µg/ml ampicillin, by scraping a sterile toothpick over the frozen glycerol stocks and transferring the whole toothpick to the medium in a 50 ml Falcon™ tube (BD Bioscience catalogue no. 352070). The culture was then incubated in a shaking incubator at 250 rpm at 37 °C until the culture reached an optical density of 0.6 at $A600_{nm}$. A 1.5 ml volume sample of the culture was taken before induction as a negative control. Protein expression was induced by adding IPTG to a final concentration of 100 µg/ml. Further 1.5 ml samples were taken at hourly intervals to determine protein expression as indicated under Results and Discussion..

2.2.10. Protein extraction of recombinant HuGLYATa expressed in Origami™ cells

For the evaluation of protein expression a 1.5 ml volume of the 10 ml induced cultures was taken and centrifuged at 10000 x g for 5 minutes to collect the cells. The medium was decanted and the last few drops removed from the Eppendorf tube by tapping it gently on a paper towel. BugBuster® protein extraction reagent (Novagen, catalogue no. 70584-3) was used according to the instructions of the manufacturer: 1 x BugBuster® in 1 x PBS buffer, pH 7.4, was added to the pellet by weighing the pellet and adding BugBuster® reagent according to the weight of the pellet. For 1.5 ml of cell culture volume 300 µl of BugBuster® was added to the pellet with 75 units Benzonase nuclease (Novagen, catalogue no. 70746-3) and 500 units rLysozyme (Novagen, catalogue no. 71110-4) which helps reduce viscosity and improve the efficiency of the extraction. The pellet was fully resuspended in the reagent mix and incubated at room temperature for 30 minutes or until the lysate clarified. A 10 µl sample was transferred to a 1.5 ml Eppendorf tube. This was referred to as the total fraction of the protein extraction. After the latter sample was taken the tube was centrifuged for 3 minutes at 10000 x g to pellet the inclusion bodies. A 10 µl sample of the supernatant was taken and transferred to a 1.5 ml Eppendorf tube. This was referred to as the soluble fraction. The supernatant was decanted in to a clean 1.5 ml Eppendorf tube and the inclusion bodies

resuspended in BugBuster® protein extraction reagent and a 10 µl sample was taken. The samples were analysed and visualized by means of 10 % SDS-PAGE.

2.2.11. SDS-PAGE electrophoresis

Proteins were analyzed by SDS-PAGE as described in Current Protocols in Molecular Biology (Gallagher, 1999), which is based on the original method of Laemmli, 1970. A 10 % polyacrylamide separating gel and 3.9% stacking gel was used. A Biorad mini Protean, 76 mm x 70 mm apparatus was used. Protein samples were mixed in a ratio of 1:1 with 2 x SDS sample buffer (62.5 mM Tris-HCl pH 6.8; 25 % glycerol solution; 2 % SDS; 350 mM DTT; 0.001 g bromophenol blue) and boiled for 5 minutes. Samples were electrophoresed at a constant 120 V until the bromophenol blue migrated to the bottom of the gel. A volume of 5 µl of ladder, Biorad Kaleidoscope Prestained Standards (catalogue no. 161-0324) or Fermentas PageRuler™Prestained Protein Ladder (catalogue no. SM0671), was used in the first well and 10 µl of sample was loaded on the rest of the gel. This was used throughout unless otherwise stated. Samples were boiled for 3 minutes beforehand.

The SDS-PAGE gel was stained by using a quick staining method of Fairbanks (Wong et al., 1999). Gels were placed in a microwaveable container and covered with 100 ml Fairbanks A staining solution (0.05% Coomassie, 25% isopropanol, 10% acetic acid). The gel was then heated until the staining solution reached boiling point and cooled at room temperature for 30 minutes on a shaking platform. The staining solution was poured off and the gel rinsed with distilled H_2O. The gel was destained by covering it with 100 ml Fairbanks D destaining solution (10% acetic acid). The destaining solution was heated to boiling point and cooled at room temperature for 30 minutes on a shaking platform. Images of the gel were taken under white light in gel documentation system (Syngene ChemiGenius Bio-Imaging System) using GeneSnap software (Syngene, England).

2.3. Results and Discussion

2.3.1. Design of the synthetic cDNA encoding HuGLYATa

To verify whether the plasmid received, containing the synthesized HuGLYATa from Geneart, was correct, it was transformed into bacterial cells (Materials and Methods 2.2.4). The transformed cells were propagated and plasmid was purified thereafter (Materials and Methods 2.2.6) and the subjected to restriction enzyme analysis.

Design of the synthetic cDNA encoding HuGLYAT was done by using the reference gene in the human genome database (Genbank number NM 201648.2). Only the exons, the parts encoding the protein, were used in the design. This was submitted to Geneart to synthesize. It was also requested that the cDNA be cloned into pET32a+. The plasmid, pET32a+, was used both as a cloning and expression vector. It is renowned for high-level expression of recombinant proteins fused with the Trx•Tag™ thioredoxin protein, which helps with formation

of disulfide bonds, and also provides cleavable His•Tag® and S•Tag™ to facilitate detection and purification of the recombinant protein. In addition, it also contains the strong T7 promoter and the resistance marker for ampicillin for selection of the plasmid (Figure 3). The vector was also chosen for its compatibility with the Origami™ host strain for expression (2.2.2).

BamHI and HindIII were the restriction enzymes chosen for the recognition sites flanking the 5' and 3' sides respectively of the ORF encoding HuGLYATa. These sites were added to the cDNA sequences for synthesis. This helped with easy cleavage of the insert and also for directional cloning of HuGLYATa. The final construct was pET32aHuGLYATa (see Figure 3)

Enzyme digestion of the plasmid was done as described in Materials and Methods 2.2.7. The plasmid was linearized with BamHI to check for the correct size of pET32a+HuGLYATa with an expected length of ± 6818 base pairs. A double digest with BamHI and HindIII was done to see if the insert, HuGLYAT, is present. This should give two bands of 5884 base pairs in length for the plasmid and 918 base pairs in length for the insert HuGLYATa.

Agarose gel electrophoresis (Figure 5) confirmed that the cloning of the synthetic cDNA endcoding human GLYATa into pET32a+ was successful. The band at the position between the size markers of 8000 and 6000 base pairs represents the linearized pET32aHuGLYATa (Fig. 5, lane 3). The very faint band in lane 3 might be due to some plasmids that were not completely digested. The double digestion of pET32a+HuGLYATa (Fig. 5, lane 5) with BamHI and HindIII, resulted in two predominant bands. The large band represents the pET32a+ at 5884 bp which migrated to a position between the size markers of 6000 and 5000 base pairs. The second band represents the insert HuGLYATa (918 bp). The resolution of the ladder is not optimal as it is difficult to accurately determine the size of the bands. By extending the time of the electrophoresis for 10 minutes more, the resolution would have been better.

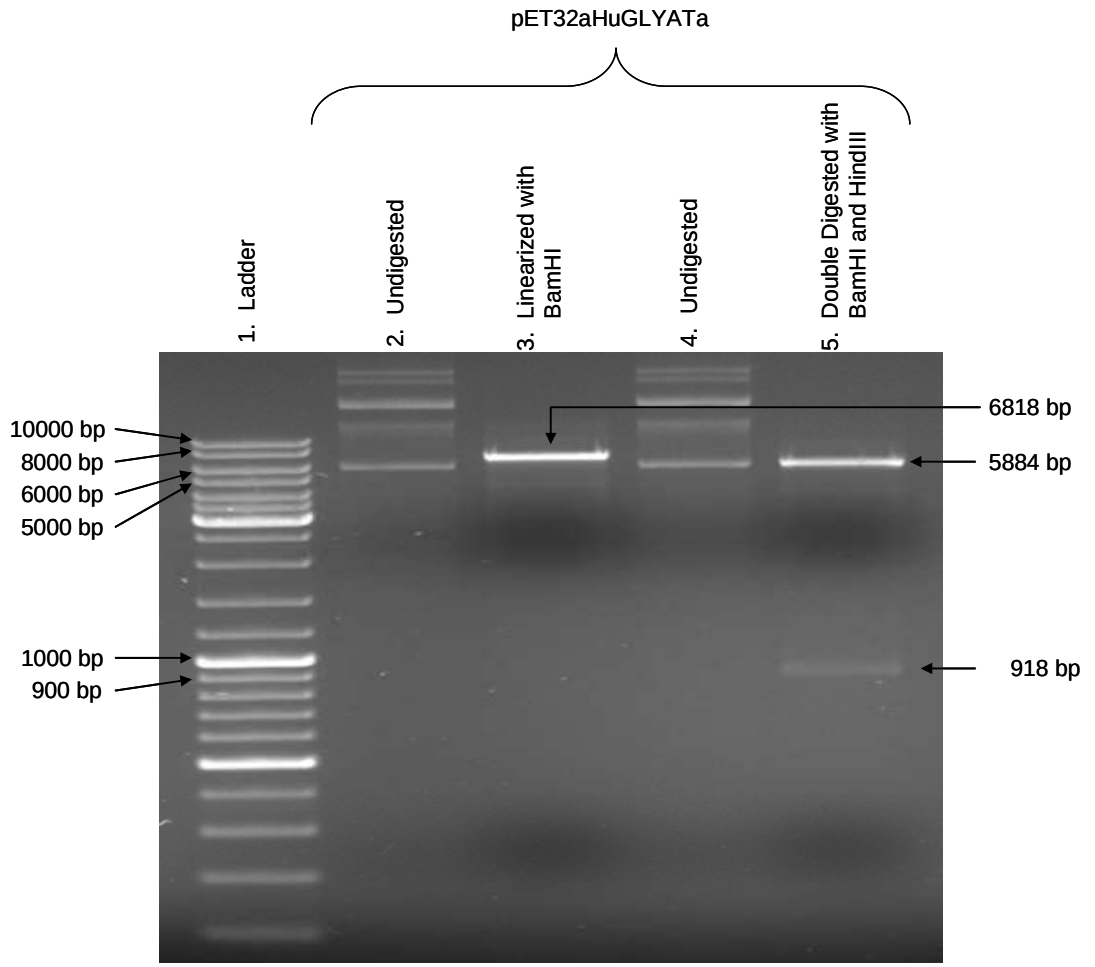


Figure 5: Agaraose gel electrophoretic analysis of the restriction enzyme analysis of HuGLYATa in pET32a+.

Lanes: 1) O' GeneRuler DNA size marker (Fermentas, Catalogue no. SM1173); 2) and 4) undigested pET32aHuGLYATa; 3) pET32aHuGLYATa linearized with BamHI; 5) pET32a+HuGLYATa double digested with BamHI and HindIII

2.3.2. Expression of the recombinant protein HuGLYATa in Origami™ cells using pET32a+

The purified pET32a+HuGLYATa plasmid was transformed into cOrigami™ cells for expression of HuGLYATa. This was done to investigate if there is any expression from the synthetic HuGLYATa and to establish whether the period of IPTG induction affects the level of HuGLYATa expressed.

Expression of HuGLYATa was done as described in MATERIALS AND METHODS 2.2.9. The experimental approach followed was as follows: Five 10 ml cultures were incubated untill an OD A_{600nm} of 0.6 was reached. IPTG induction (2.2.9) of 4 cultures was done at the same time. A 1.5 ml sample was taken as negative control (kept on ice). The rest of the culture

was discarded. Similarly, at hourly time intervals of 1 hour for a period of 4 hours after induction, a 1.5 ml sample was taken and protein extraction was done (see 2.2.10). After the final sample was taken and protein extraction was done, SDS-PAGE (Figure 6) was used to visualize the extracted proteins (see 2.2.11). The total fraction was used and therefore it was not possible to distinguish between soluble and insoluble proteins. This was just to check if expression did take place and to get some indication of the yield of protein after a certain amount of time. A fusion protein ±55 kDa in size was expected for HuGLYATa.

As can be seen from Fig. 6 there was expression of HuGLYATa protein (±55 kDa). The negative control showed that there was no leaky expression which means that the there was tight lac operator suppression control. The increase in yield of HuGLYATa was not as linear as expected. One can see that the is yield increased from 1 hour to 2 hour incubation time. From 3 hours onwards it did not appear if the yield of rHuGLYATa increased. The induction at 3 hours seemed to have decreased in yield. This could be due to mistakes in sample volume extracted from the culture or handling errors during protein extraction. It was concluded then that there would be sufficient recombinant protein produced after 2 hours induction with IPTG for further analysis.

The overall quality of the gel was not satisfactory. The resolution of the gel was poor. This could be due to reagents being too old or that the gel was not well set before electrophoresis. The Biorad Kaleidoscope Prestained Standards (catalogue no. 161-0324) ladder (Fig. 6 lane 1) smeared during electrophoresis. This can be due to overloading of gel or possibly that the ladder was not denatured well enough or a poor batch from the manufacturer. In fact, in our laboratory with this batch of ladder, we never had a good resolution.

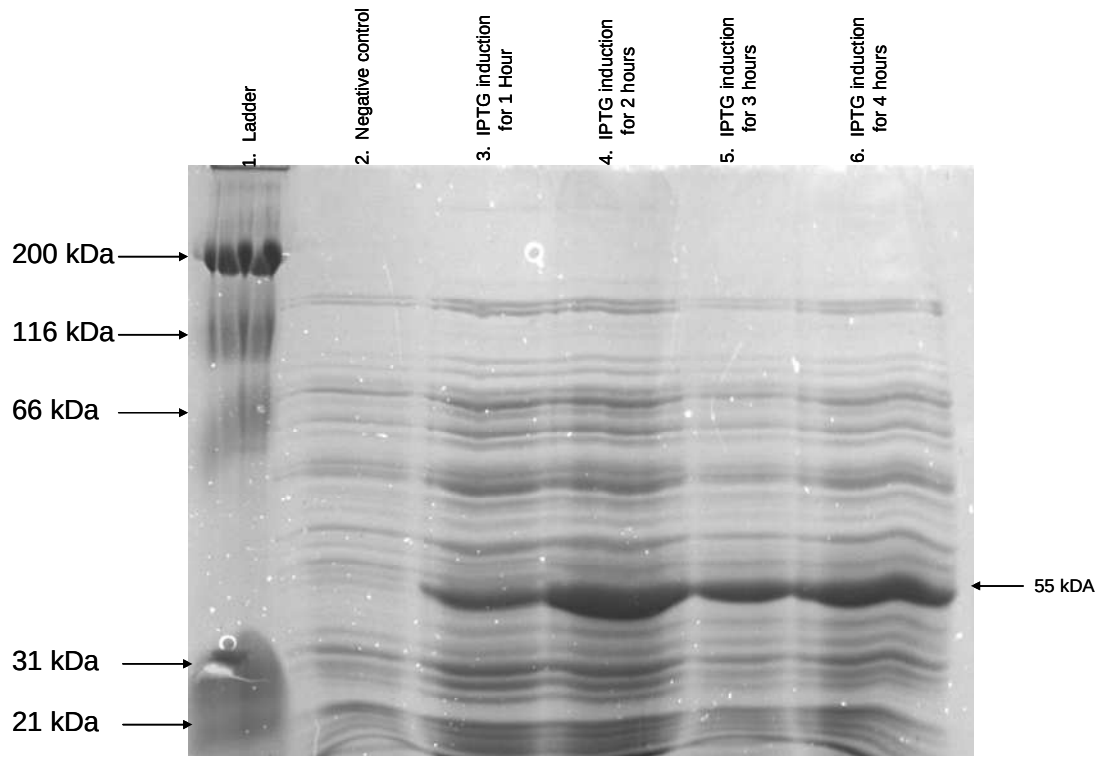


Figure 6: SDS PAGE analysis of recombinant protein expression of HuGLYATa over time.

Lanes: 1) Ladder (Biorad Kaleidoscope Prestained Standards (catalogue no. 161-0324); 2) Negative control before induction; 3) Expression after 1 hour induction with IPTG; 4) Expression after 2 hours induction with IPTG 5) Expression after 3 hours induction with IPTG; 6) Expression after 4 hours induction with IPTG

2.3.3. Investigation as to whether if HuGLYATa is expressed in soluble form

A new 10 ml culture was made and protein extraction was done after 2 hours after induction. Figure 7 showed that the recombinant HuGLYATa (55 kDa in size) was present in the total fraction, which correlated with the findings in the previous experiment. However, HuGLYATa was absent in the soluble fraction and again present in the insoluble inclusion bodies. From the results it was concluded that the recombinant HuGLYATa protein was insoluble. This is not an uncommon problem with bacterial expression of eukaryote proteins. This can be due to misfolding of the recombinant protein during expression causing the protein to aggregate. These aggregated insoluble proteins are usually also biologically inactive.

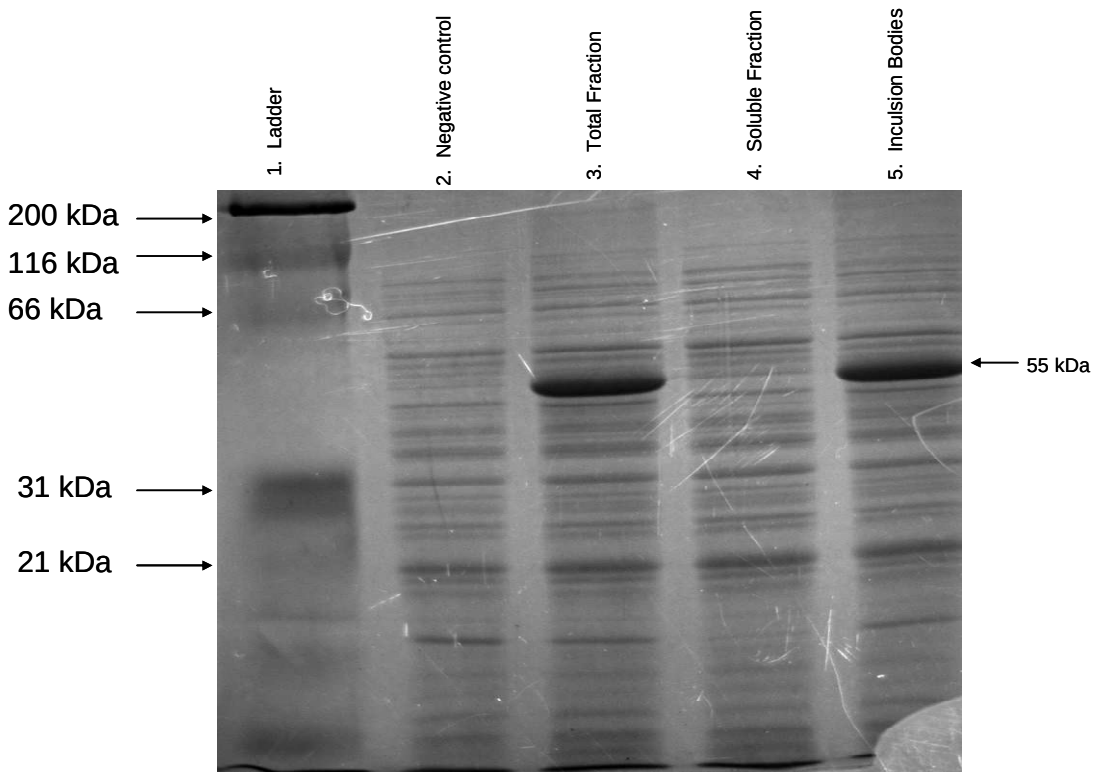


Figure 7: SDS-PAGE analysis of recombinant protein expression of HuGLYATa.

Lanes: 1) Ladder Biorad Kaleidoscope Prestained Standards (catalogue no. 161-0324); 2) Negative control; 3) Total fraction; 4) Soluble fraction; 5) Inclusion bodies.

From this experiment it was clear that this bacterially expressed HuGLYATa is completely insoluble. In Chapter 3 I will deal with purifying the inclusion bodies and investigations to see by means of changing buffer conditions if the protein can be made soluble.

2.4. Summary

E. coli. as an expression system was used for its ease of use. The system is inexpensive to set up and maintain. The system is also easy to scale up if successful expression was achieved. The problem plaguing this system is the misfolding of recombinant proteins that leads to insoluble and biologically inactive recombinant proteins (Baneyx et al., 2004). The gene for HuGLYATa could have been extracted from HepG2 cells or human liver. We decided to have the gene synthetically made by an external company. The gene HuGLYATa arrived already cloned into pET32a+. The pET32aHuGLYATa was then cloned into SURE® host and Origami™ host strain for plasmid production and expression of HuGLYATa respectively. The host strains were plated out on agar plates containing the appropriate antibiotics. This was to ensure that the bacterial cells containing the insert HuGLYATa were the only bacterial colonies present. Colonies were picked up using a sterile toothpick and transferred to a 10 ml LB-Medium containing the appropriate antibiotics and grown overnight in a shaking incubator. The culture was then preserved as glycerol stocks to be used further

in future experiments. Plasmid extractions were done using a commercial kit. Plasmid extractions were digested using BamHI and HindIII to verify that the insert HuGLYATa was present in the bacterial cells. Protein expression was done by inoculating 10 ml LB medium containing the appropriate antibiotics in a shaking incubator until an OD of 0.6 was reached. IPTG induction was used to express the recombinant HuGLYATa. The respective plasmid extractions and protein expression was visualized on an agarose gel and SDS-PAGE respectively.

From the experiments presented in this chapter it was concluded that:

i) The synthetic cDNA encoding fusion HuGLYATa protein was cloned successfully into pET32a+
ii) High level of expression of recombinant protein was obtained
iii) The recombinant HuGLYATa synthesized was insoluble

In this experiment we did not try to manipulate the expression conditions of the protein by changing IPTG concentrations and temperature. I also did not try to see if there is maybe any biological activity in the aggregated protein.

The next step would be to try and denature and renature the insoluble protein (Chapter 3), to use second generation bacterial expression techniques (Chapter 4) and to try and use a eukaryote expression system (Chapter 5).

Chapter 3

Folding and refolding of aggregated recombinant HuGLYATa

3.1. Introduction

In this chapter the problem of aggregation of recombinant HuGLYATa expressed in pET32a+ is addressed.

In *E coli.*, where transcription and translation is tightly coupled, proteins are released every 35 seconds from the ribosome. Small, single domain proteins reach conformation faster than the larger multi domain and recombinant proteins, due to their faster folding kinetics. The larger multi domain and recombinant proteins require the aid of folding modulators (also known as chaperones) in the cell to achieve the right conformation. Folding modulators have different functions which include shielding interactive surfaces from each other and the solvent, catalyzing the rate limiting steps (from an abnormal cis to trans conformation) and formation of the disulfide bonds. Failure of the recombinant protein to correctly interact with the folding modulators in time results in one of two things: partially folded proteins resulting in insoluble aggregates thus resulting in inclusion bodies or degradation of the proteins (Baneyx et al., 2004).

Misfolded proteins occur more frequently in bacterial expression systems that use strong promoters and high inducer concentrations that lead to a product yield of over 50 % of the total cellular protein production, due to folding modulators that are rapidly exhausted. A second cause for protein aggregation when they are used for expression of eukaryotic cells is that bacteria does not support all the post-translation modifications needed by the protein to help with its folding, for example glycosylation and the formation of intra- and intermolecular disulphide bonds. Inclusion bodies are formed when proteins cannot reach their native conformation or when they do not interact with chaperones (Baneyx et al., 2004).

When inclusion bodies are formed there is a variety of ways to optimize the expression system to limit the amount of inclusion bodies that are formed, such as adding folding modulators to the expression of the recombinant protein and reducing inducer concentration might alleviate the aggregation. Alternatively, the inclusion bodies can be isolated for protein refolding by means of commercially available kits (Baneyx et al., 2004).

Most of the kits work on the basis of denaturing the inclusion bodies and refolding proteins by diluting inclusion bodies in an appropriate renaturing buffer. At lower protein concentrations aggregation of the proteins is minimized. The inclusion bodies are solubilzed by an

appropriate denaturant like urea or guanidine hydrochloride, which is also known as chaotropes because they can disrupt the structure of water. The solubilzed protein is then added to a range of different refolding buffers to find the ideal refolding condition for the particular aggregated protein. Most of the buffers contain the widely used glutathione renaturation system, where a mixture of reduced and oxidized glutathione is added to the buffer. This promotes thiolate anion formation to help with disulphide exchange for disulphide bond formation in an alkaline environment (Middelberg, 2002).

As reported in Chapter 2, inclusion bodies were formed during the expression of HuGLYATa. The next logical step was to isolate the inclusion bodies from the bacteria and to chemically refold the recombinant HuGLYATa using a commercial kit.

3.2. Materials and Methods

3.2.1. Preparation of bacterial inclusion bodies containing aggregated recombinant HuGLYATa

A bacterial culture of 10 ml was started from glycerol stocks of Origami™ cells containing pET32aHuGLYATa as described in 2.2.9. The 10 ml culture was then added to LB medium to a final volume of 1 L containing 100 µg/ml ampicilin, 12.5 µg/ml tetracycline and 15 µg/ml kanamycin. The culture was grown to an OD of 0.6 at A600nm. Expression was induced as described in 2.2.9 by adding IPTG to a final concentration of 0.1 M and allowing expression to continue for 24 hours at 250 rpm at 18 °C.

3.2.2. Protein refolding of bacterial inclusion bodies containing aggregated recombinant HuGLYATa

Protein extraction was done as described in 2.2.10. Refolding was done with the Fluka Basic Refolding kit (Fluka, catalogue number 96827). Inclusion bodies were prepared by centrifugation of the 1 L culture at 10 000 x g for 1 hour. The supernatant was discarded and the inclusion bodies were resuspended in 6 M guanidine hydrochloride and allowed to denature for 4 hours at room temperature. The solubilized proteins were then transferred to a whole range of renaturing buffers (see Appendix for full list of renaturing buffers) aliquoted in 1.5 ml Eppendorf tubes. The ratio of solubilized protein to renaturing buffer was 9:1 in a total volume of 1 ml at 4 ºC. The renaturing reaction was performed for 24 hours at room temperature. The samples were visualized using SDS-PAGE (see 2.2.11).

3.2.3. Enzyme assay for GLYAT

The enzyme assay was performed as described by Van der Westhuizen and colleagues, 1999. A crude cytoplasmic extract containing bovine GLYAT was prepared from a bovine liver was used as positive control (a fellow student Mr JHJ Fourie prepared the bovine GLYAT containing extract). The reaction mixture consisted of 25 mM Tris-HCl (pH 8.0), 0.1 mM

benzoyl-CoA, 0.1 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 200 mM Glycine (pH 8.0) and H_2O in a final volume of 500 µl. The crude cytoplasmic extract containing bovine GLYAT and bacterial recombinant HuGLYATa was added to a final concentration of 0.15 µg/ml protein. The reaction mixture was made up in a 1000 µl spectrophotometer cuvet. The reaction was intiated by the addition of 2 µl of the respective enzyme preparations. Glycine dependent CoASH release (DNTB reduction) from bezoyl-CoA was measured at 412 nm at room temperature over a period of 10 minutes using a BioTek spectrophotometer.

3.2.4. The bicinchoninic acid (BCA) protein assay

Protein concentration was determined using the standard BCA protein assay. The BCA assay works on the principle of the reduction of Cu^{2+} to Cu^{1+} in an alkaline medium with the highly sensitive and selective colourimetric detection of Cu^{1+} by bicinchoninic acid. A standard series from 0 µg/µl bovine serum albumin (BSA) (Fluka cat no: A7030) to 10 µg/µl BSA was prepared. The sample supernatant was diluted 10 X (1 part sample to 9 parts ddH_2O). Triplicate dillutions were prepareduced A 200 µl solution of BCA:$CuSO_4.5H_2O$ (50:1) was added to the samples and the standard series. The sample/BCA mixtures were incubated at 37 °C for 30 minutes and then absorbance was read on a microplate fluorescence plate reader at 560nm.

3.3. Results and Discussion

3.3.1. Preparation of bacterial inclusion bodies containing aggregated recombinant HuGLYATa

The results reported in Chapter 2 indicated that expression of HuGLYATa in pET32a+ was achieved but that the recombinant protein was insoluble. To help solve this I turned to denaturing the recombinant protein and renature it in a range of buffers with specific conditions (Appendix).

To do this I followed the procedure of expressing of HuGLYATa as described in 2.2.9. A 10 ml culture of Origami™ containing pET32a+HuGLYATa was started up and grown to an OD600 of 0.6. This culture was then added to LB medium to a final volume of 1 L. This culture was grown to an OD600 of 0.6. At this stage expression was induced by adding IPTG. This was done for 24 hours at 18 °C at 250 rpm. The method described was adjusted to meet the recommendation of the Fluka kit as described in 3.2.1. This is done to obtain as many inclusion bodies as possible and prevent the bacterial culture to overgrow that can lead to protein degradation. The whole 1 L culture was used for protein extraction and was done as described in 2.2.10. SDS-PAGE analysis was done (not shown here) to see if expression was successful. The extracted proteins were then centrifuged for 10 minutes at 10 000 x g to collect all the inclusion bodies.

Expression of HuGLYATa was successful (results not shown). Inclusion bodies collected from this experiment were used for the next step which was protein refolding.

3.3.2. Protein denaturing and refolding of bacterial inclusion bodies containing aggregated recombinant HuGLYATa

Expression of HuGLYATa resulted in inclusion body formation as previously described in Chapter 2. The inclusion bodies from 3.3.1 were collected and used further to denature the inclusion bodies in a suitable solution and then to try and refold that denatured proteins using specific buffers with different sets of conditions.

The inclusion bodies were denaturated using 6 M guandine hydrochloride as per recommendation of the Fluka kit. The denaturing process was performed at 22 °C for four hours. The denatured protein solution was then transferred to a walk in fridge at 4 °C. The renaturing buffers were then added to the denatured protein in ratio of 9:1. This was done in the walk in fridge to slow down the renaturing process and allow the protein to fully interact with the buffer. The pH of the refolding buffers ranged from 6.5 to 8.5 and contained a different concentration of NaCl, PPS and reduced and oxidized glutathione. This promotes disulphide bond formation which can lead to better solubility of protein and promote enzymatic activity. After adding the denatured proteins to the buffers, the renaturing was done for 24 hours at 22 °C. The Eppendorf tubes were centrifuged at 10000 x g for 3 minutes, this was to obtain the soluble fraction to determine if HuGLYATa became soluble after refolding with the renaturing buffers. All soluble fractions were analyzed by SDS-PAGE.

SDS-PAGE analysis (Figure. 8) revealed that in all the buffers recombinant HuGLYATa became soluble if only slightly (not all results shown here). In Figure 8A, lanes 4, 6 and 8 can be seen that the protein slightly increased in solubility pointing to a more favourable buffer for HuGLYATa. In Figure 8B lane 10, using Tris-HCl (pH 8.5) 0.05M, L-GSH reduced 0.005M, L-GSH oxidized 0.001M, the protein became even more soluble as indicated by the more intense band. In figure 8C, lane 3 and 5 showed even beter solubility resulted when renaturation was done in 0.05 M buffer Tris-HCl (pH 8.5) 0.05M, NaCl 0.1M, L-GSH reduced. 0.005M, L-GSH oxidized. 0.001M. The best condition for successful refolding of the protein was 0.05 M buffer Tris-HCl (pH 8.5) 0.05M, NaCl 0.1M, L-GSH reduced. 0.005M, L-GSH oxidized. 0.001M (Fig 8C, lane 3). To determine if HuGLYATa might be enzymatic active in the above mentioned buffer, the protein was used as such in the enzyme assay. The sample was not purified as the sample looked clean enough as was seen on the SDS-PAGE.

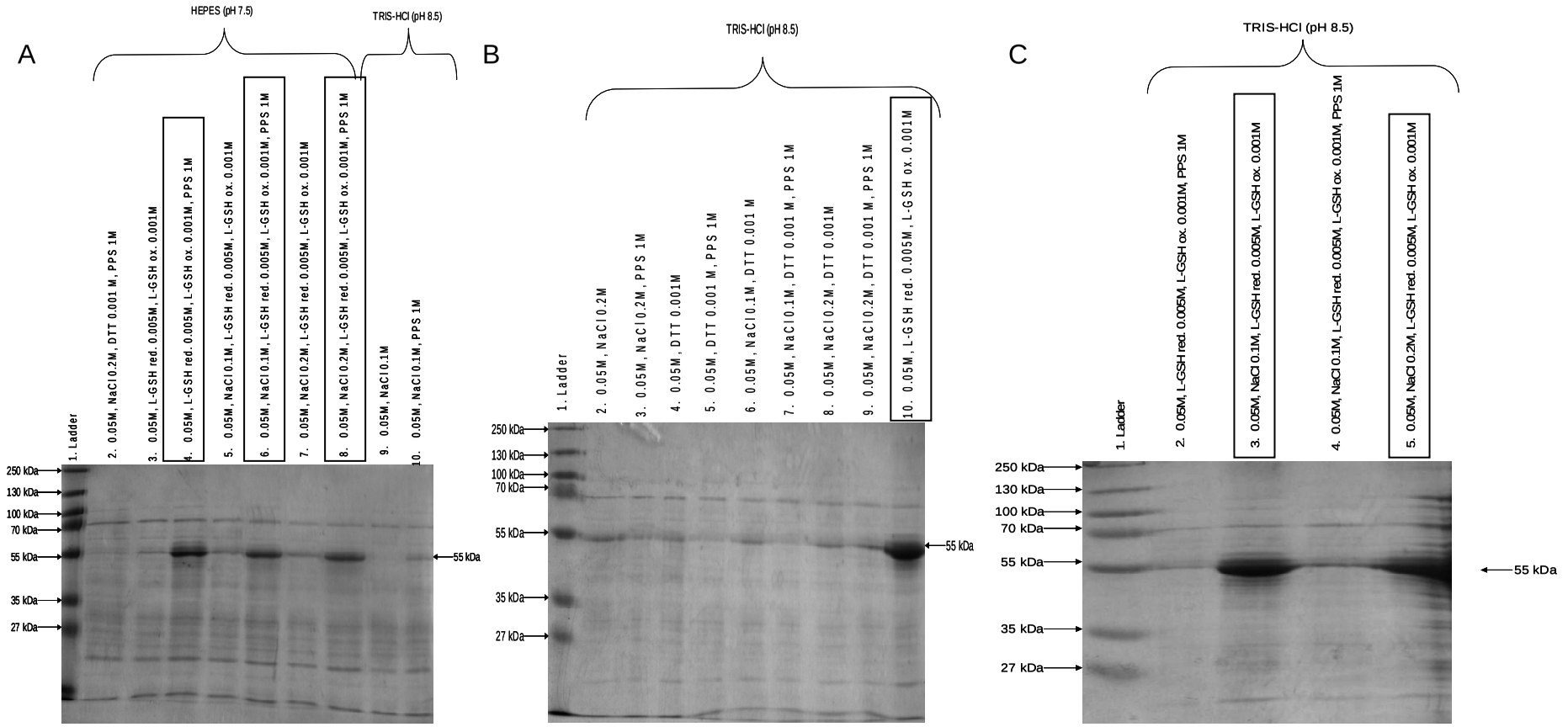


Figure 8: SDS-PAGE analysis for refolding of HuGLYATa.

A, B, C) samples of soluble fractions containing refolded HuGLYATa in different buffers (HEPES (pH 7.5) and TRIS-HCl (pH 8.5) with different components and concentrations of these components as indicated above the lanes.

3.3.3. Enzyme assay for GLYAT

The GLYAT enzyme assay (Van der Westhuizen et al., 2000) was performed as described in 3.2.3 to determine if there was any biological activity present in the enzyme after refolding to make it more soluble. The reagent mixture was prepared for the HuGLYATa, the bovine GLYAT preparation and for a negative control reaction. The amount of HuGLYAT used varied, 2 µl and 20 µl of sample respectively was used. To summarize, six cuvettes was prepared three positive controls where glycine was added and three negative controls where no glycine was added. The results of the enzyme were shown as a graph of absorbance over time (Figure 9).

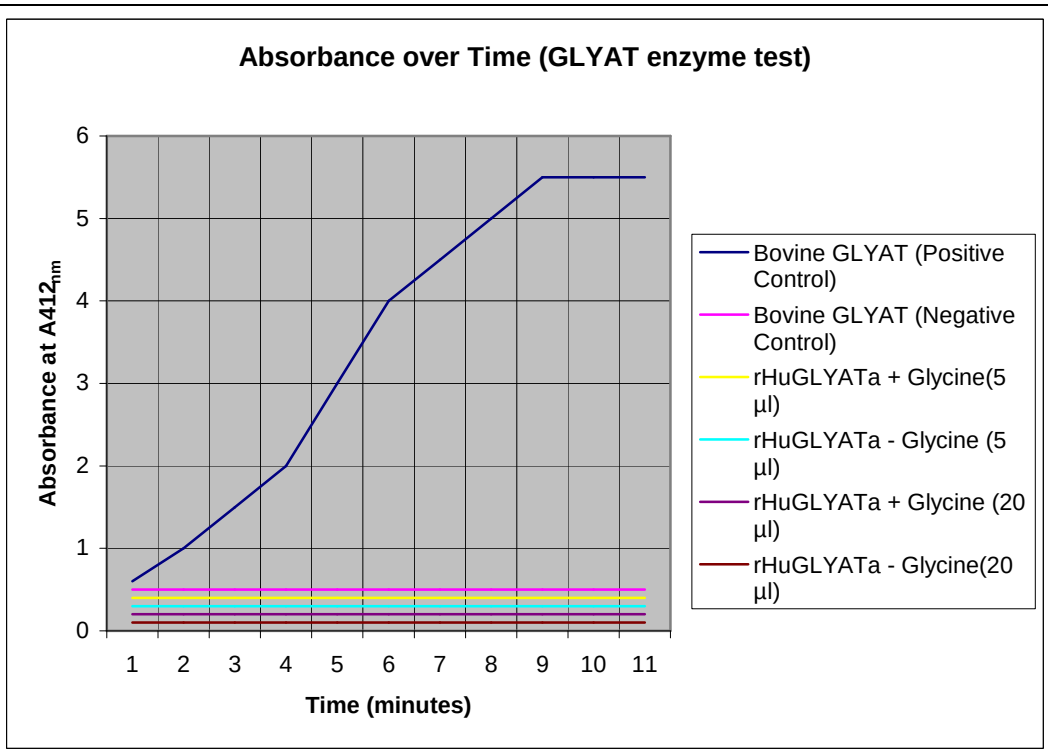


Figure 9: Graph showing absorbance over time for GLYAT enzyme activity assay.

From the results given in Figure 9 the refolded recombinant HuGLYATa is not enzymatically active. The positive control, the crude bovine GLYAT cytoplasmic extract was active as was clear from the logarithmic curve that reflects DNTB reduction. This means that the GLYAT reaction mixture was correctly composed. The negative controls showed a flat line as expected, but so also did the recombinant HuGLYATa. From this was seen that, although the rHuGLYATa was now soluble due to refolding it still had no biological activity.

Therefore, our approach for obtaining a soluble, biologically active human GLYAT enzyme had to change. We then decided to investigate another bacterial expression system in the form the pCold system where cold shock is used to express proteins. This has led to more

soluble and biologically active recombinant enzymes which were insoluble and inactive in the traditional T7 bacterial expression systems (Takara, 2007).

3.4. Summary

In Chapter 2 I encountered the problem that the expressed recombinant HuGLYATa protein was insoluble. I addressed this issue by collecting the aggregated recombinant proteins. This allowed me then to denature the recombinant protein and to renature the recombinant protein in standardize buffers using a commercial kit. This was done to improve solubility of the recombinant protein and possibly get some enzymatic activity. This was the case in this experiment where it was found that the recombinant HuGLYATa became more soluble using 0.05 M buffer Tris-HCl (pH 8.5) 0.05M, NaCl 0.1M, L-GSH reduced. 0.005M, L-GSH oxidized. 0.001M. The soluble recombinant HuGLYATa was used for assaying for enzymatic activity. Unfortunately the enzyme showed no activity as can be seen from the enzyme test (Figure 9).

The next step was to investigate and use a second generation bacterial expression system that uses cold shock for expression of recombinant proteins (Chapter 4).

Chapter 4

Expression of HuGLYATa in *Escherichia coli* using the pCold system

4.1. Introduction

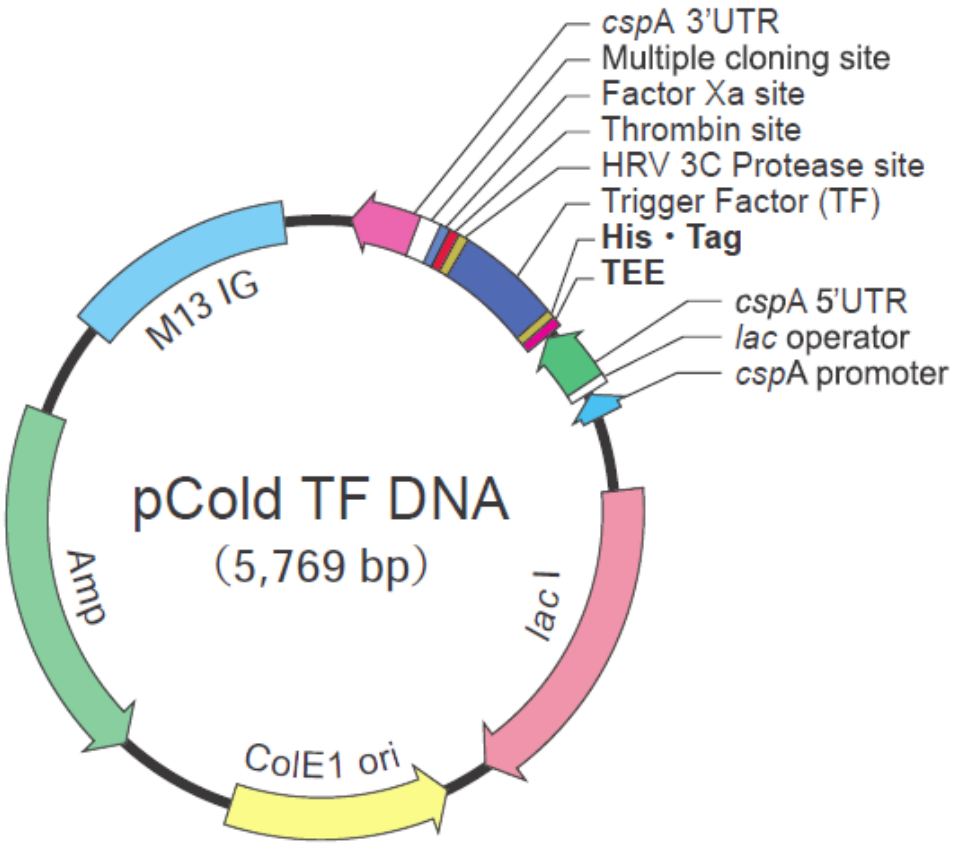
One of the main drawbacks of the first generation T7 based bacterial expression systems, such as the pET system that was used in Chapter 2 and 3, is the inability of the recombinant protein to reach its native conformation. This is usually due to the high-level expression rate of these systems. Under these conditions of over expression, protein aggregates are formed which lead to the formation of inclusion bodies. Strategies that have been developed to combat this problem of aggregation include those where folding modulators are present during expression (Baneyx et al; 2004), the collection of inclusion bodies for denaturing and refolding of the recombinant protein (See Chapter 3) and to express the recombinant protein at lower temperatures (<37 °C).

The strong temperature dependence of hydrophobic interactions favours protein aggregation reactions at higher temperatures, even at 37 °C, which is the temperature used for most of the first generation bacterial expression systems. The reduction in temperature reduces the induction of heat shock proteases and increases the activity and expression of chaperones. This contributes to increased protein stability and correct folding of the protein at lower temperatures. However, decreasing the incubation temperature inhibits the replication, transcription and translation reactions (Sorenson, et al 2004).

The pCold expression system commercialized by Takara was developed to combat the problem of recombinant protein aggregation in the host cell and facilitate protein expression at temperatures lower than 20 °C. The system makes use of cold shock in Escherichia coli for better solubility of the recombinant protein. In this system the recombinant protein is under the control of the cold shock protein A (cspA) promoter and other elements to increase protein production at incubation temperatures as low as 15 °C. The cspA promoter is repressed at 37 °C and highly inducible at 10 °C (Vasina et al, 1998). The low temperature suppresses cellular protein production and suspends cell growth. The result is higher expression of the target protein, increased purity and increased solubility (Takara, 2007).

The aim of the work presented in this chapter was to use this second generation bacterial expression system to investigate if the problem of insolubility and biological inactivity of the recombinant human GLYAT can be resolved. I decided to opt for pColdTF because of the trigger factor (TF) co-expression. It was shown (Nishihara et al., 1999) that with the co-expression of TF, with the recombinant protein, can prevent aggregation. TF associates

itself with nascent protein and facilitates protein folding, allowing the recombinant protein to reach native conformation. The vector map of the expression plasmid pColdTF is shown in Figure 10.



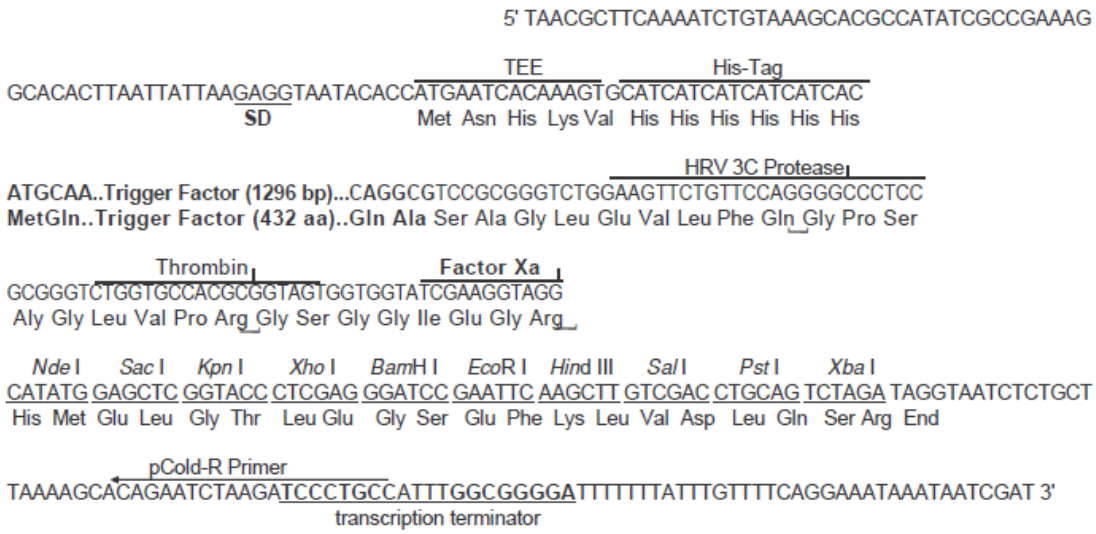


Figure 10: pColdTF Vector map and sequence of the region containing the CspA promoter, histidine tag, trigger factor, terminator site and multiple cloning site (Takara)

4.2. Materials and Methods

4.2.1. Transformation of competent SURE® cells with pColdTF

Transformation of SURE® cells with pColdTF was performed as described in 2.2.4. Glycerol stocks of SURE® cells containing pColdTF were prepared and stored as described in 2.2.5.

4.2.2. Preparation of pColdTF for plasmid extraction and restriction enzyme digestion

Plasmid extraction of pColdTF was performed as described in 2.2.6. Double restriction enzyme digestion was performed as described in 2.2.7. BamHI and HindIII were again used.

4.2.3. Preparation of pET32aHuGLYATa for plasmid extraction and restriction enzyme digestion

Plasmid extraction of pET32aHuGYATa was performed as described in 2.2.6. Double enzyme digestion was performed as described in 2.2.7.

4.2.4. Gel extraction of digested pColdTF and dsDNA fragment containing the open reading frame encoding HuGLYATa

The 500 µl restriction enzyme digestion of pColdTF and ORF encoding HuGLYATa was transferred to a 1 % agarose gel and gel electrophoresed (see 2.2.8). The gel was placed under UV light for about 30 seconds to mark where the linearized plasmid and insert were in the gel. UV light causes mutations in DNA due to the formation of thymidine dimers, therefore the gel was placed only for a very short time under the UV light. The segments were cut out of the gel with a scalpel, placed in 1.5 ml Eppendorf tubes and the gel extraction performed. The linearized plasmid and insert were kept apart for the whole procedure. Gel extraction was performed by using a commercial kit from Qiagen (QIAquick Gel extraction kit, Qiagen, catalogue number 28704). The protocol was followed as recommended by the manufacturer. Gel extraction works on the basis of dissolving the agarose gel in chaotropic salts at 50 °C and capturing the DNA using selective binding silica membranes. The quantity and quality of the extracted linearized plasmid and insert was checked on a spectrophotometer (NanoDrop ND 1000). The A260/280 was used to determine purity and A260 was used to determine quantity.

4.2.5. Ligation of pColdTF with the open reading frame encoding HuGLYATa

Ligation of pColdTF with the DNA encoding open reading frame encoding HuGLYATa was performed as described in Russell and Sambrook, 2006. Ligation was performed using T4 DNA ligase. The T4 DNA ligase catalyzes the formation of a phosphodiester bond between juxtaposed 5'-phosphate and 3'-hydroxyl termini in duplex DNA with blunt or cohesive end termini (Fermentas). The 20 µl reaction mixture consisted out of 100 ng linear vector DNA, 1

x ligation buffer (Fermentas, catalogue number EL0015), 2 units T4 DNA ligase (Fermentas, catalogue number EL0015). Insert DNA was added in a ratio of 3:1 with the linear vector DNA. The ligation was performed for 1 hour at 22 °C. A ligation control was also performed were no insert DNA was added.

4.2.6. Transformation of pColdTF-HuGLYATa into competent SURE® and Origami™ cells

Transformation of pColdTF-HUGLYATa and the ligation control were performed as described in 2.2.4.

4.2.7. Preparation of glycerol stocks of pColdTF-HuGLYATa

Glycerol stocks of SURE® and Origami™ cells containing pColdTF-HuGLYATa were prepared and stored as described in 2.2.5. The SURE® cells were used for amplification of the plasmid for future experiments. The Origami™ cells were used for expression of HuGLYATa using pColdTF.

4.2.8. Expression of the recombinant protein HuGLYATa in Origami™ cells using pColdTF

Expression op pColdTF-HUGLYATa was performed as described in 2.2.9 in 500 ml of LB medium containing the appropriate anitbiotics. The culture was incubated to an OD of 0.6 at A600nm. The shaking platform was then stopped for 30 minutes and the culture cooled to 15 °C. Expression was induced by adding IPTG to a final concentration of 100 µg/ml. The culture was incubated at 250 rpm at 15 °C for 24 hours.

4.2.9. Protein extraction of HuGLYATa expressed in Origami™ cells

Protein extraction of HuGLYATa expressed in Origami™ cells was performed as described in 2.2.10.

4.2.10. Nickel affinity column purification HuGLYATa

Recombinant HuGLYATa containing the 6 X His-tag fused to the N-terminus of the protein was purified by means of nickel affinity column purification. Histidine has a high affinity to divalent metal ions such as nickel. The tagged protein can therefore be purified by binding to immobilized nickel in the column. The Protino Ni-TED 1000 packed columns were used for the purification of the expressed protein from the soluble phase, prepared as described in 2.2.10, (Macherey Nagel catalogue no. NZ74511050) according to the manufacturer's protocol.

4.2.11. The bicinchoninic acid (BCA) protein assay

The test was done as described in 3.2.4

4.2.12. GLYAT enzyme test

The enzyme test was done as described in 3.2.3

4.3. Results and Discussion

4.3.1. Cloning of the dsDNA fragment containing the open reading from enconding HuGLYATa into pColdTF

cSure cells containing pColdTF in 50 ml LB medium culture and extracted as described in 4.2.1. The plasmid pET32aHuGLYATa containing the open reading frame encoding HuGLYATa similarly extracted from a 50 ml culture of cSure cells containing pET32aHuGLYATa. Both plasmids were double digested with BamHI and HindIII as described in Chapter 2, 2.2.5. BamHI and HindIII were chosen as discussed in Chapter 2, 2.3.1. The double digested pColdTF and 918 bp HuGLYATa insert were gel electrophoresed on a 1 % agarose gel. This was done to ensure that digested pColdTF and 918 bp HuGLYATa insert were separated from the DNA fragments created during digestion and to ensure that these fragments do not interfere in the ligation process. The linearized pColdTF and insert HuGLYATa were extracted from the gel as described in 4.2.1. The double digest of the pColdTF and HuGLYATa should have linearized the plasmid and insert respectively creating 5' and 3' overhangs to facilitate directional cloning. This was necessary to ensure the correction orientation of the insert for expression purposes. Ligation of pColdTF and the insert HuGLYATa was done as described in 4.2.1. Two ligation reactions were done, one with plasmid and insert and one with just the plasmid. The latter served as negative control to check if the plasmid ligated on itself which would indicate a incomplete digestion or that the gel extracted linearized plasmid was not pure enough and still contained small fragments of DNA, digested with BamHI and HindIII, from the plasmid. After ligation the two ligation reactions were transformed into cSure cells as described in 2.2.4. The cultures were plated onto a plate containing 100 µg/ml ampicilin. The plates were incubated for 16 hours at 37 °C. As expected there was no growth on the ligation control plate. This indicated that the plasmid did not ligate on itself and both restriction enzyme sites were digested fully. About 200 colonies were detected on the positive plate. This showed that ligation of pColdTF and the insert encoding HuGLYATa was successful. To further verify that this was indeed so, a 50 ml LB medium culture was prepared and inoculated with a colony from the positive plate and incubated 16 hours at 37 °C in a shaking incubator at 250 rpm. From the culture 2 ml was taken for plasmid extraction and glycerol stocks prepared from the rest (as described in 2.2.4 and 2.2.5). The plasmid was called pColdTFHuGLYATa. It was double digested with BamHI and HindIII. The digest were visualized on an agarose gel (Figure 11)

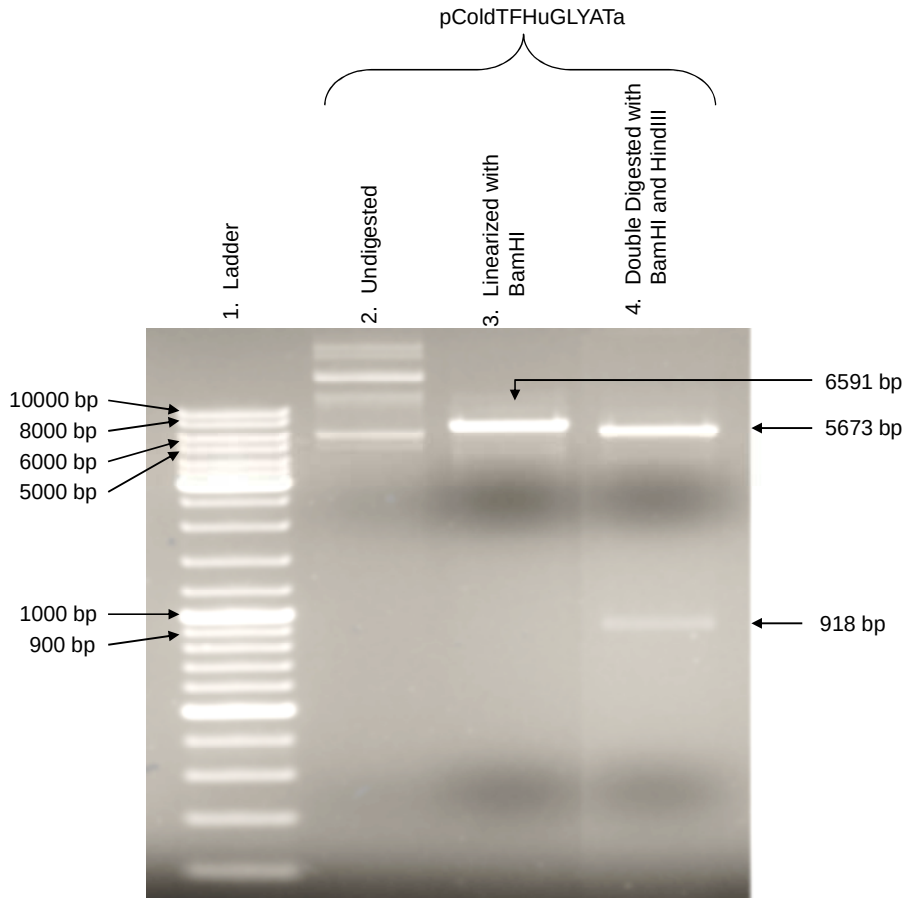


Figure 11: Agaraose gel electrophoretic analysis of the restriction enzyme digest of the ORF of HuGLYATa cloned into pColdTF.

Lanes: 1) O' GeneRuler DNA size marker (Fermentas, Catalogue number SM1173); 2) undigested pColdTFHuGLYATa 3) pColdTFHuGLYATa linearized with BamHI; 4) pColdTFHuGLYATa double digested with BamHI and HindIII

Expected results from the digestion was a linearized plasmid of 6591 bp in size and double digest plasmid 5673 bp in size and insert 918 bp in size. The undigested plasmid showed multiple bands representing the various supercoiled states of the plasmid DNA. The single digestion resulted in one band (Figure 11 lane 3) which was assumed to be the 6591 bp linearized plasmid. Two bands were visible in the double digestion (Figure 11 lane 4) which was assumed to be the 5673 bp plasmid and the 918 bp insert HuGLYATa. This confirmed that ligation of pColdTF and the insert encoding HuGLYATa was indeed successful. Due to the insufficient resolution of the agarose gel fragment size could not be determined more accurately.

4.3.2. Expression of HuGLYATa using the pColdTF vector.

The plasmid pColdTF-HuGLYATa was transformed into cOrigami cells for expression of HuGLYATa (as described in 2.2.4). Expression was performed as described in 2.2.9. The

only change in the procedure was that when the cultured reached an optical density of 0.6 at A600nm, the shaking platform of the incubator was stopped and the culture cooled down for 30 minutes to 15 °C. After the cold shock, protein expression was induced by adding IPTG and incubated at 15 °C for 24 hours shaking at 250 rpm. Protein extraction was done as described in 2.2.10 and visualized by SDS-PAGE. This was to verify the expression of the recombinant protein TF-HuGLYATa fusion protein of 89 kDa in size and to see whether solubility was improved.

The level of expression of the TF-HuGLYATa fusion protein was very high (Figure 12, lanes 2, 3, 4). The gel was severly overloaded resulting in poor resolution. This could have been prevented if the protein extract was diluted and run on another gel. The TF-HuGLYATa fusion protein was soluble as the huge amount of expressed insert was also visible in the soluble fraction (Fig. 12, lane 3). Insoluble HuGLYATa was also present in the protein sample (Fig. 12, lane 4). This was due to over expression of the insert overwhelming the cells, leading to more misfolded proteins as described in Chapter 3, 3.1. A shortcoming on the analysis is that there were no proper controls on the gel, no uninduced sample and no TF only sample.

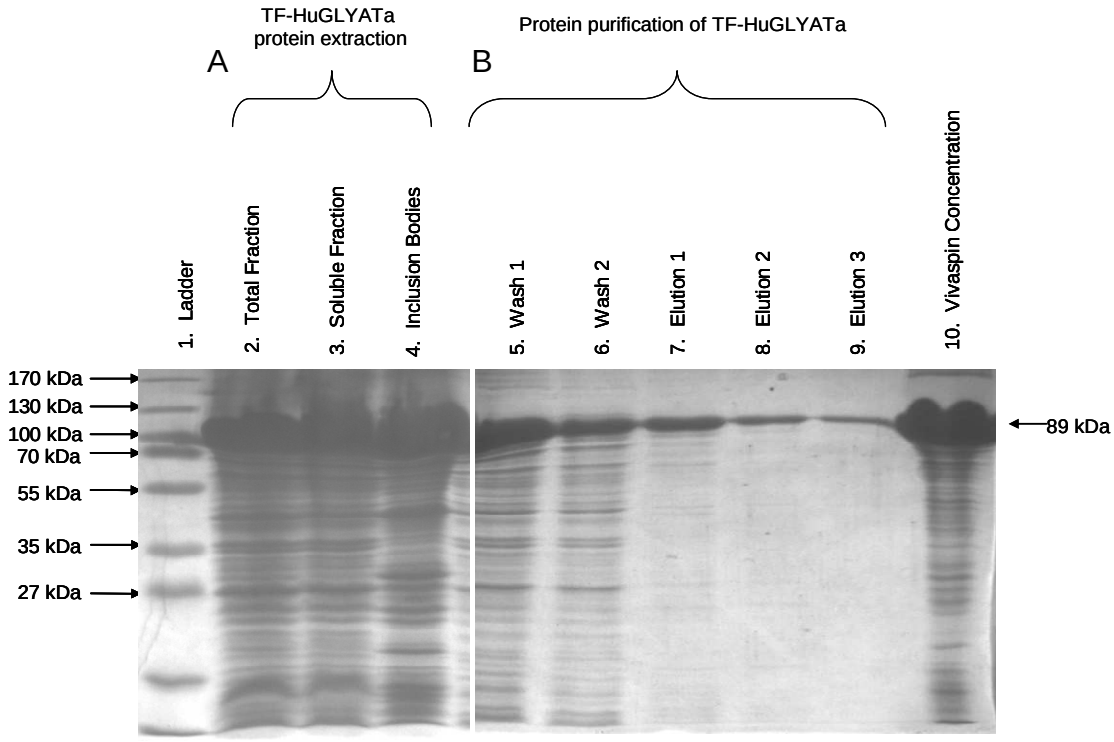


Figure 12: SDS PAGE gel of expression (A) and protein purification (B) of TF-HuGLYATa fusion protein.

Lanes: 1) Fermentas PageRuler™Prestained Protein Ladder (catalogue no. SM0671) A) 2) Total fraction of extraction of TF-HuGLYATa fusion protein 3) Soluble fraction of extraction of TF-HuGLYATa fusion protein 3) 4 inclusion bodies of extraction of TF-HuGLYATa fusion protein B) 5) Wash 1 after binding to Protino columns 6) Wash 2 after binding to Protino columns 7) Elution 1 after binding to Protino columns 8) Elution 2 after binding to Protino columns 9) Elution 3 after binding to Protino columns 10) Protein concentration using Vivaspin

The TF-HuGLYATa fusion protein contains a N-terminal His tag. This allows for the purification of the fusion protein due to the affinity of Histidine for nickel. Protein purification was performed using the Protino Ni-TED packed column. The column contains immobilized nickel that allows for binding of the fusion protein to the matrix in the column. This allows all proteins not containing the His Tag to wash through the column thus leaving the purified fusion protein behind. The columns were then washed out two times to eliminate cellular proteins. The purified fusion protein was then eluted out of the columns. The results were visualized on SDS PAGE. A technical aspect was pointed out to us by Dr. A.C. Potgieter (personal communication) to filter the soluble fraction through two filters. This was to eliminate incomplete fusion proteins from the soluble fraction due to over expression and early termination of translation. These incomplete fusion proteins can potentially “block” the Ni-TED affinity column causing the completed fusion proteins to wash out. The second filter

was held underneath the first column to assure that these completed fusion proteins were captured. Only the second filter was used further to elute the purified TF-HuGLYATa fusion protein. In figure 12B the purification of the recombinant was successful. It was noted that in the washing step of the protocol that some of the recombinant protein was washed out (Figure 12B lanes 5 and 6). The binding capacity of the columns was exceeded and not all the recombinant proteins were captured. The eluted recombinant protein was collected and the recombinant protein was then concentrated, desalted and the buffers changed to 1x NaCl TRIS HCl pH 8.0 by using Vivaspin spin columns as prescribed by the manufacturer. Buffers were changed to wash out imidazole present in the elution buffer of the commercial purification kit. Imidazole interferes with the BCA protein concentration determination and with the GLYAT enzyme test. The results can be seen in Figure 12, lane 10.

4.3.3. Protein concentration determination and GLYAT enzyme activity assay

To determine the concentration of the protein, which was mostly TF-HuGLYATa fusion protein, the BCA test was done as described in 4.2.11. The concentration of the protein was calculated at 5.40 µg/ul (results not shown). The concentrated sample containing TF-HuGLYATa fusion protein was then used to perform the GLYAT enzyme test as described in Chapter 3, 3.2.3. The same result was obtained as in Chapter 3, 3.3.3, in that no enzyme activity was observed (results not shown).

4.4. Summary

In Chapter 2 we used the first generation T7 based bacterial expression system. This led to an expressed recombinant protein but proved to be insoluble. In Chapter 3 we then tried to refold the insoluble recombinant protein by means of denature and renature system. This improved solubility of the recombinant protein but did not help achieve enzymatic activity. To achieve solubility and achieve enzyme activity we turned to the pCold expression system. A trigger factor was co-expressed to facilitate in protein folding. The insert encoding HuGLYATa was excised from the vector by double digestion and gel purified. Extracted pColdTF plasmid was double digested with BamHI and HindIII. The insert HuGLYATa and pColdTF were ligated and expressed. Solubility of the recombinant protein was improved, but the enzyme was still biologically inactive. To solve this problem insect cell expression was chosen next as these systems can perform some post translational modification of the protein, where this was not possible in bacterial expression systems. Baculovirus expression in insect cells was used to try to help solve the problem of biological activity.

Chapter 5

Baculovirus expression of HuGLYATa in insect cells

5.1. Introduction

One of the major drawbacks of bacterial expression systems is their inability to perform post-translational modification, like phosphorylation and glycosylation, of recombinant proteins. These modifications are often essential for biological function and absence thereof can lead to improper folding as seen in Chapter 2 and biological inactivity as seen in Chapters 3 and 4. To overcome these limitations in bacterial expression systems, baculovirus expression is often used. This is a viral system for the production of recombinant proteins in insect cells. Insect cells are eukaryotic cells and can recognize most mammalian protein signal sequences for post-translational modification of the recombinant protein. This allows the recombinant protein to be expressed in its native conformation. However, glycosylation of recombinant proteins in insect cells is not exactly the same as in higher eukaryotic cells. For example *Drosophila* N-linked glycosylation is less complex in that it is not trimmed and sialylated and *Drosophila* proteins have a high mannose content (Invitrogen)(Kost et al, 2005).

Recombinant baculoviruses expressing foreign proteins were initially generated using a highly inefficient homologous recombination process. Insect cells were co-infected with wild-type baculovirus first and then transfected with a plasmid containing the gene to be expressed. This produced a mixture of a very high number of parent and very low number of recombinant progeny viruses. Usually, to indentify the low number of recombinant viruses, a plague assay was performed and recombinant clones were identified microscopically by their distinctive occlusion-negative plaque phenotypes. This was very tedious work because the recombinant viruses were surrounded by parent occlusion-positive virus plaques (O` Reily et al 1992; Luckow et al, 1993; Kost et al, 2005).

To eliminate this tedious plaque procedure to identify recombinant baculoviruses, an *in vivo* bacterial transposition method (Luckow et al., 1993) was developed now known commercially as the Bac-to-Bac® system.

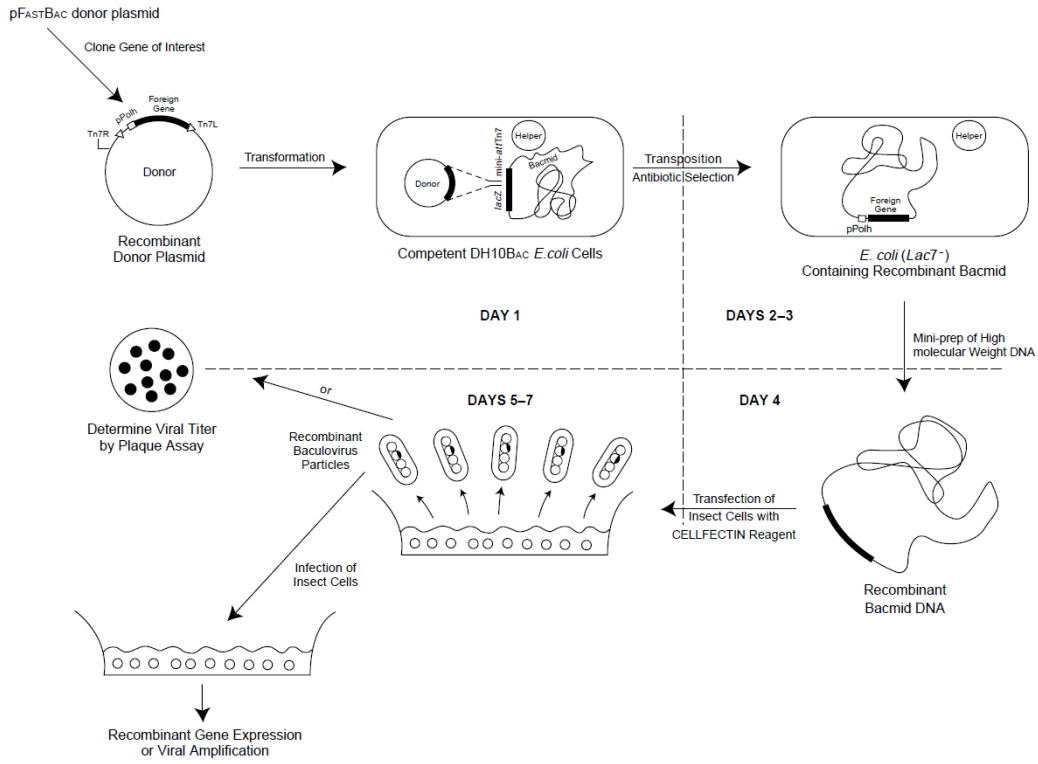


Figure 13: Generation of recombinant bavuloviruses using the Bac-to-Bac® system (Invitrogen)

It is based on the site-specific transposition of an expression cassette into a baculovirus shuttle vector (bacmid) propagated in E. coli. The principle behind this is that the baculovirus DNA genome introduced into susceptible insect cells will be expressed and result into infectious virus particles (Luckow et al, 1993). The advantages of the bacmid being propagated in bacterial cells, is that the bacmid DNA can be altered in the bacterial stage of baculovirus expression before the bacmid is isolated for transfection of the insect cells. Transposition of the gene of interest into the bacmid is achieved by exploiting the properties of the Tn7 transposon. The polyhedrin promoter, in the plasmid vector, of the Autographa californica multiple nuclear polyhedrosis virus (AcMNPV) controls the expression of the gene of interest. The bacmid contains the mini-F replicon, a kanamycin resistance marker and the lacZα gene. On the N-terminus of the lacZα gene is the mini-attTn7 attachment site and does not disrupt the expression of the lacZα gene. This bacmid is the propagated as a large plasmid in Escherichia coli, DH10Bac™ cells. The lacZα gene complements the deletion present in the cells chromosome, expressing the lacZα peptide that colours the colonies cells blue in the presence of X-gal and the inducer IPTG. The gene of interest is then cloned into a donor plasmids' multiple cloning site, which is flanked by the Tn7, and is called the mini-Tn7 site. The mini-Tn7 site confers resistance to gentamicin and contains the polyhedrin promoter, the multiple cloning site and a SV40 poly(A) signal between the Tn7 left and right arm. A helper plasmid in the bacterial cells encodes the transposase, which helps with the transposition of the mini-Tn7 site to the mini-attTn7 site. The helper plasmid confers

resistance to tetracycline. The transposition of the mini-Tn7 site to the mini-attTn7 disrupts the expression of the lacZα gene and this in turn leaves the colonies white in the presence of X-gal and the inducer IPTG. These white colonies are then indicative of a successful transposition of the mini-Tn7 site into the bacmid, resulting in a recombinant bacmid (Luckow VA et al. 1993)(Bac-to-Bac® manual, Invitrogen).

The donor plasmids I used were pFactBacI (Figure 12) and pFastBacHTb (Figure 13).

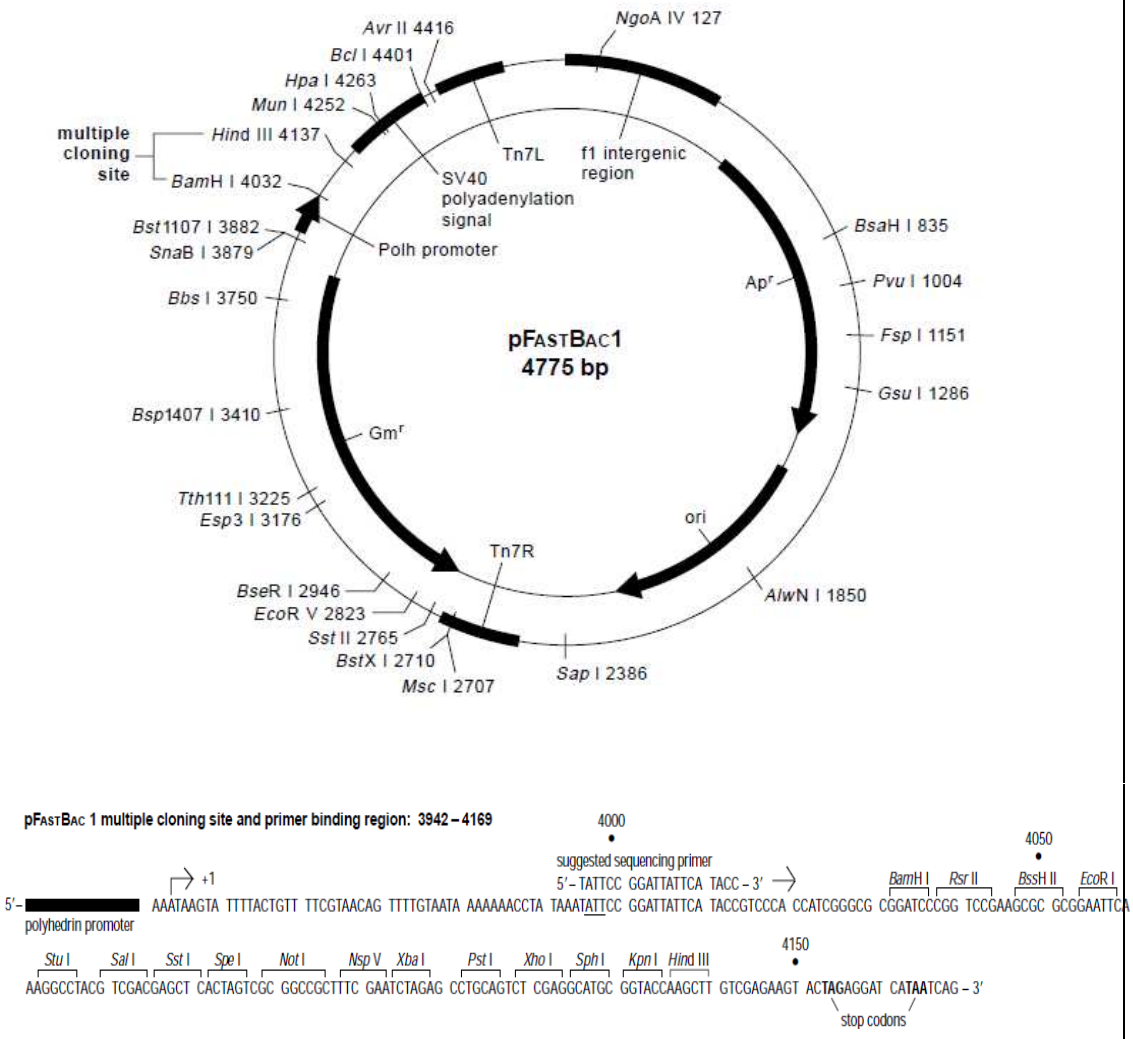


Figure 14: Vector map and multiple cloning site of pFastBacI (Invitrogen).

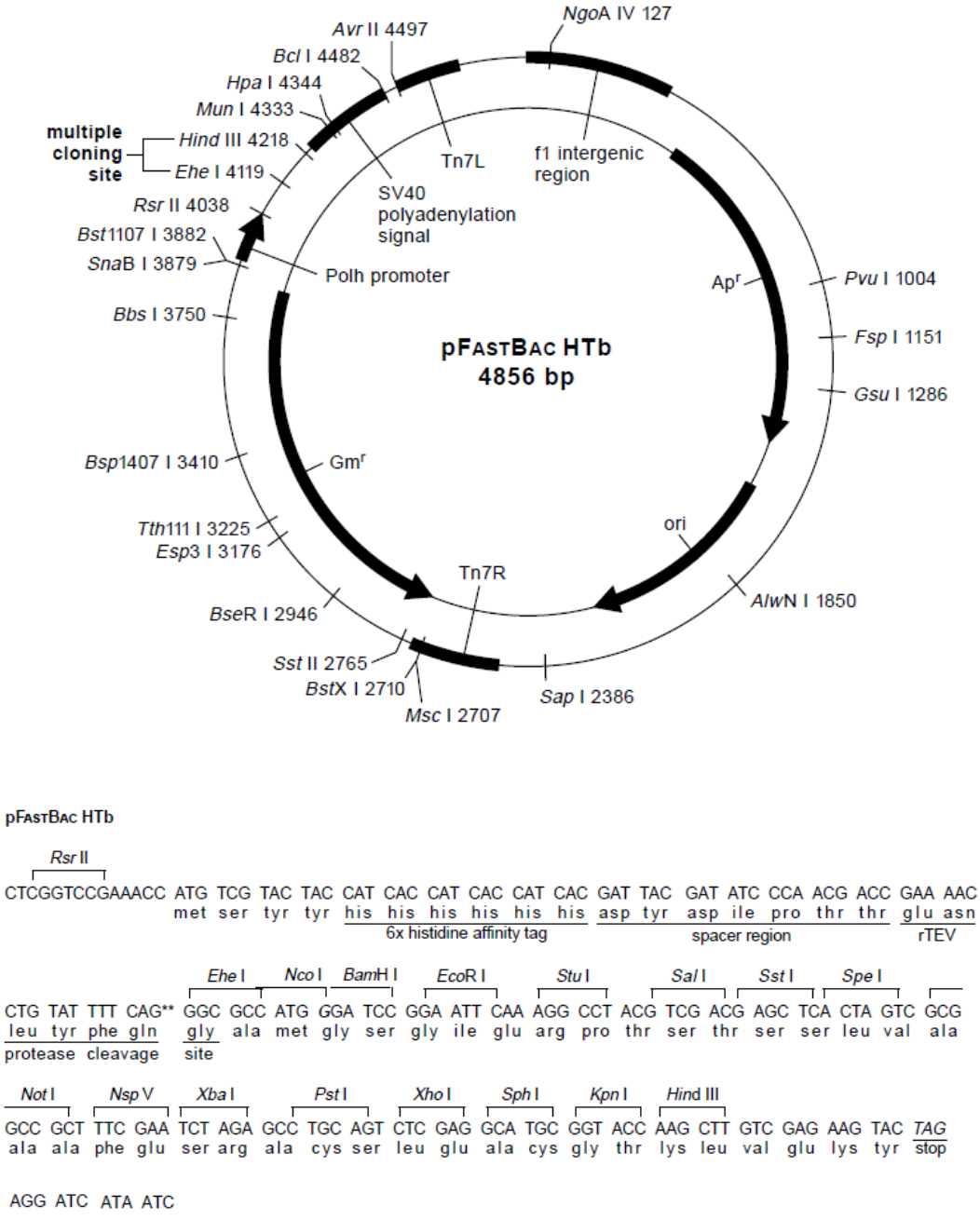


Figure 15: Vector map and multiple cloning site of pFastBacHTb (Invitrogen).

Both of these plasmids contain the polyhedrin promoter for high level expression of the recombinant protein. pFastBacI was chosen to express a GLYAT isoform a directly without a fusion protein as the fusion protein can sometimes be insoluble. Recombinant proteins generated by means of pFastBacHTb have a N-terminal 6x Histidine tag for purification with affinity nickel columns and a TEV cleavage site for removal of the tag. There are three versions of pFastBacHT, this is to ensure that recombinant protein can be cloned with its ORF is in frame with the tag. Therefore, pFastBacHTb was chosen. Furthermore, instead of DH10Bac™ cells, it was decided to use AcBacΔCC cells.

AcBacΔCC cells were produced by the deletion of the genes encoding chitinase and v-cathepsin, in the baculovirus DNA. Chitinase is a secretory protein that burdens the secretory pathway and provides competition for the recombinant secretory protein entering the secretory pathway. V-cathepsin is produced as pro-enzyme and is activated by proteolytic cleavage when cell death occurs or by chaotropic agents like sodium dodecyl-sulfate which results in proteolysis of the recombinant proteins. By deletion of these genes, expression of the recombinant protein is improved (Kaba et al, 2004)

5.2. Material and Methods

5.2.1. Extraction and ligation of pFastBacI and pFastBacHTb with DNA insert HuGLYATa

The plasmids, pFastBacI and pFastBacHTb, were purified from transformed JM109 cells as described in 2.2.6. The purified plasmids were then double digested with BamHI and HindIII as described in 2.2.7 and gel extracted as described in 4.2.4. The insert, encoding HuGLYATa was gel extracted as described in 4.2.4. The insert was double digested with BamHI and HindIII as described in 2.2.6. Ligation of the plasmids with the insert was done as described in 4.2.5.

5.2.2. Transformation of pFastBacIHuGLYATa and pFastBacHTbHuGLYATa into cSure cells

The transformation of pFastBacIHuGLYATa and pFastBacHTbHuGLYATa into cSure cells was done as described in 2.2.4. The cells were stored as glycerol stocks and were prepared from successfully transformed bacterial colonies as described in Chapter 2, 2.2.5.

5.2.3. Transformation of pFastBacIHuGLYATa and pFastBacHTbHuGLYATa into ACC cells and transposition of the HuGLYATa gene into the Bacmid DNA

Transformation of pFastBacIHuGLYATa and pFastBacHTbHuGLYATa into AcBacΔCC cells was done as described in Chapter 2, 2.2.4. The transformed cells were then plated out on a LB-agar plate containing 50 µg/ml kanamycin, 10 µg/ml gentamicin, 10 µg/ml tetracycline, 4 % IPTG and 32 µg/ml X-gal. Plates were placed in an incubator at 37 °C overnight. White colonies were picked up with a sterile toothpick and transferred to a 10 ml LB medium. The cultures were grown overnight at 37 °C in a shaking incubator at 200 rpm.

5.2.4. Extraction of recombinant Bacmid DNA

The method for extraction of the recombinant bacmid DNA was adapted from a method developed in the laboratory of Prof Just Vlak in Wageningen, The Netherlands.

Of the 10 ml overnight cultures, 3 ml of culture was transferred to a 15 ml tube and the cells collected by centrifugation at 9760 x g for 1 minute. The supernatant was decanted and the cells were resuspended in 200 µl GTE buffer which consisted out of 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0 and 0.1 mg/ml Rnase. The resuspended cells were then transferred to a 1.5 ml Eppendorf tube. A volume of 400 µl of 0.2 M NaOH/1 % SDS was added to resuspended cells. A volume of 300 µl chilled KAc solution, (prepared by adding 60 ml 5 M potassium acetate pH 4.8-5.2, 28.5 ml ddH_2O and 11.5 ml glacial acetic acid) was added and the tube inverted several times and placed on ice for 5 minutes. The suspension was then centrifuged in a benchtop centrifuge at 16100 x g for 8 minutes. The clarified supernatant transferred to a new 1.5 ml Eppendorf tube. To precipitate the DNA, 800 µl ice cold isopropanol was added to the supernatant. The precipitated DNA was collected by centrifugation at 16100 x g for 3 minutes. The DNA pellet was washed with 800 µl ice cold 70 % ethanol. The washing step was repeated three times. The ethanol was decanted and the DNA pellet air dried and dissolved in 40 µl TE buffer (pH 8.5). The concentration and purity of the DNA pellet was checked with a NanoDrop spectrophotometer. Analysis of the recombinant Bacmid was done by PCR.

5.2.5. PCR analysis of recombinant Bacmid DNA

PCR analysis of the recombinant Bacmid DNA was done as described in the Invitrogen Bac-to-Bac® system manual. The PCR reaction mixture consisted out of 100 ng Bacmid DNA, 1 x Takara TaqBuffer (Takara, catalogue number R001A) containing 100 mM Tris-HCl pH 8.3, 500 mM KCl and 15 mM $MgCl_2$, 1.6 mM dNTPs, 12.5 pmoles of the forward and reverse primer, Takara Taq polymerase (Takara catalogue number ROO1A) and 18 Ω MilliQ H_2O to a final volume of 50 µl. The reactions were incubated in an Eppendorf Mastercycler® EP at 94 °C for 3 minutes then at 94 °C for 45 seconds, 55 °C for 45 seconds and 72 °C for 5 minutes for 30 cycles and a final elongation cycle at 72 °C for 7 minutes. The samples were then held at 4 °C. Table 3 shows the primers used in this PCR.

Table 3: M13 forward and reverse primers

Primer	Sequence	Length
M13 Forward	5‘ – GTTTTCCCAGTCACGAC – 3’	17 mer
M13 Reverse	5’ – CAGGAAACAGCTATGAC – 3’	17 mer

Primers were synthesized and HPLC purified a commercial company, Metabion.

5.2.6. Transfection of insect cells with recombinant bacmid DNA from pFastBacIHuGLYATa and pFastBacHTbHuGLYATa

Spodoptera frugiperda, Sf9 cells, were used for initial transfection and expression of the recombinant protein. Sf9 cells was started up and incubated in a 75 cm^2 cell culture flask containing 100 ml TC100 growth medium supplemented with 10 % v/v heat inactivated fetal bovine serum. The fetal bovine serum contains growth factors and other various substances to support the metabolism of the Sf9 cells. By heat inactivating the serum substances that can lead to negative expression results is eliminated. TC100 growth medium contains all the necessary salts, amino acids and vitamins to support growth and replication of the cells. The cells are incubated in an incubator at 27 °C for 3 days until cells reached a density of 1 x 10^7. The cells were then washed off the flask floor by using a Pasteur pipette where the point was bend to 45 °. This is done by holding the Pasteur pipette over a Bunsen burner and the bending the glass when it is soft enough to manipulate but not at melting point. Cells were then gently but forcefully washed off the flask floor using the media that they were incubated in. 10 % of the cell culture is then transferred to another 75 cm^2 flask and TC100 growth media supplemented with 10 % v/v fetal bovine serum added to a final volume of 100 ml.

Each well of a 6 well plate (35 mm diameter wells) were seeded with 1.5 x 10^6 Sf9 cells in a total volume of 2 ml growth medium. The growth medium was TC100 (Sigma, cat no T3160). It was supplemented with 10 % heat inactivated fetal bovine serum and antibiotics 0.5 x final concentration (Penicillin and Streptomycin). Cells were incubated for 1 hour at 27 °C to adhere to the well floor. A volume of 10 µl of the recombinant bacmid DNA (1 µg/ml), from pFastBacIHuGLYATa and pFastBacHTbHuGLYATa respectively, was placed at the bottom of a 1.5 ml Eppendorf tube. Fugene 6 transfection reagent (Roche, catalogue number 11815091001) was diluted in TC100 medium to 3 ml from a volume of 75 µl of transfection reagent. The Fugene was carefully dripped in the middle of the medium therefore not to touch the walls of the tube as the Fugene adheres to plastic surfaces. Polypropylene and polystyrene tubes may be used. A 190 µl volume of this mixture was then carefully dripped in the middle onto the bacmid DNA and incubated at room temperature in a laminar flow cabinet for 30 minutes to allow DNA:lipid complexes to form. A 200 µl volume of this was used to transfect the cells by dripping onto the cells from the middle to the edge of the well in a circular motion. After the transfection the cells were left to incubate on a gentle rocking platform for 30 minutes. Insect medium, 1.8 ml TC100, was then added incubated at 27 °C until the cytopathic effects of viral infection were visible from 72 to 96 hours. The medium was then pipetted off the cells and centrifuged at 2440 x g for 2 minutes to pellet cells and cell debris. The remaining supernatant was decanted into another 15 ml Falcon tube. This was the transfection supernatant containing the recombinant AcMNP virus. The supernatant was aliquoted into 1.5 ml Eppendorf tubes and stored at 4 °C away from light to use for further transfections. This was the T1 viral stocks.

5.2.7. Expression of recombinant protein using the virus in the transfection supernatant

For the expression of recombinant proteins 6 well plates were seeded as described in 5.2.6. A volume of 300 µl of the transfection supernatant was diluted to 500 µl with growth medium and the whole volume used to infect the cells. The infected cells were then incubated at room temperature on a gentle rocking platform for one hour. The cells were then covered with growth medium to a final volume of 2 ml in each well. The plates were incubated at 27 °C for 72 hours or until signs of cytopathic effects became visible. The cells were collected by detaching them from the wells by pipetting and the suspension was then transferred to a 2 ml Eppendorf tube. The cells were collected by centrifuging the suspension at 500 g for 2 minutes at 4 °C. The supernatant was decanted into a sterile 1.5 ml Eppendorf tube. This was the P1 viral stocks. The cell pellet was washed with 1 x PBS pH 8.0, two times. The cells were lysed with 200 µl lysis buffer containing 0.5 % NP40 in 1 x PBS pH 8.0 and Complete mini EDTA free protease inhibitors (Roche, cataloque number 11836170001). The soluble fraction was collected by centrifuging the lysate at 10000 x g for 3 mintues. The total, soluble and particulate containing fractions were visualized by SDS-PAGE as described in 2.2.11.

5.3. Results and Discussion

5.3.1. Extraction of plamids pFastBacI and pFastBacHTb and ligation with a DNA insert encoding HuGLYATa

Suitable donor plasmids were chosen to clone the DNA insert encoding HuGLYATa into the bacmid using the Bac-to-Bac® expression system. The plamids pFastBacI and pFastBacHTb were chosen. Plasmid pFastBacI was chosen as the plasmid contains no tags that are expressed with the recombinant HuGLYATa. The reasoning behind this was to produce HuGLYATa alone without any fusion protein tags attached to HuGLYATa. We hoped that the protein alone would reach its native conformation, be soluble and be active. pFastBacHTb was chosen for the His tag expressed with HuGLYATa. A histidine tag was fused to the recombinant protein to allow for easier protein purification. The donor plasmid pFastBacHTb was also chosen because when the insert pFastBavHTBHuGLYATa was inserted into the bacmid that it would be in frame for expression.

Frozen glycerol stocks of JM109 containing pFastBacI and pFastBacHTb were started up using 10 ml LB-medium containing 100 µg/ml ampicillin. The plasmids were extracted using the same technique as described in 2.2.6. The DNA insert encoding HuGLYATa was extracted from previously prepared pET32aHuGLYATa stock. Both the plasmids and DNA insert was double digested with BamHI and HindIII. The plasmids and DNA insert was put on a gel and electrophoresed and the linearized plasmids and DNA insert was gel extracted as described in 4.2.4. The extracted plasmids and DNA insert was then ligated using the

method described in 4.2.5. The plasmids pFastBacIHuGLYATa and pFastBacHTbHuGLYATa were then transformed into cSure cells (2.2.4) and glycerol stocks were made (2.2.5). After transformation into cSure cells, plasmid extraction was again performed to verify successful ligation of DNA insert and plasmid. The extracted plasmids pFastBacIHuGLYATa and pFastBacHTbHuGLYATa were again double digested and resulted into a plasmid pFastBacIHuGLYAT of ±4670 bp in size and insert of ±918 bp and for a plasmid pFastBacHTbHuGLYATa of ±4762 bp in size and insert of ±918 bp.

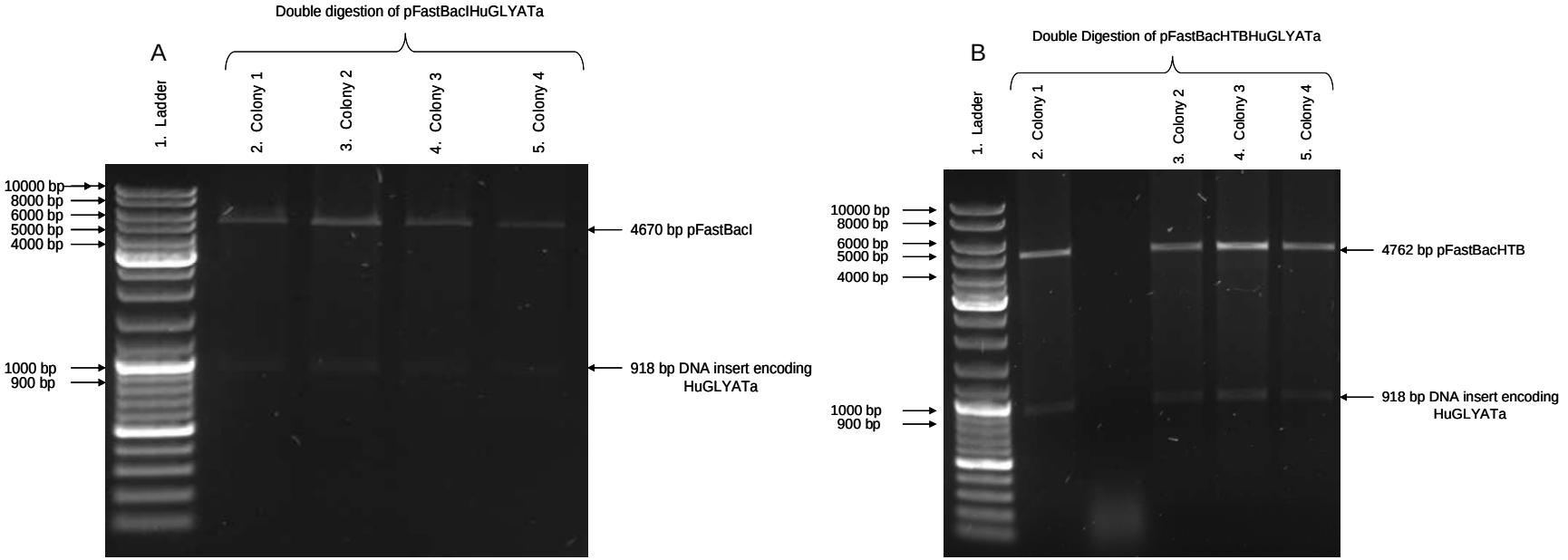


Figure 16: Gel electrophoresis of the double digestion of (A) pFastBacIHuGLYATa and (B) pFastBacHTbHuGLYATa.

(A and B) Lane 1, O' GeneRuler DNA size marker (Fermentas, Catalogue no. SM1173) (A) Lane 2-5 pFastBacIHuGLYATa double digested with BamHI and HindIII, (B) Lane 2-5 single colonies of pFastBacIHTbHuGLYATa double digested with BamHI and HindIII

Successful ligation of pFastBacI and pFastBacHTb with the DNA insert encoding HuGLYATa was observed (Figure 16 A and B). Non-specific ligation is when the insert is inserted into to wrong orientation. This can then lead to some plasmids containing no inserts and effecting expression quality and quantity of HuGLYATa. Non-specific ligation was not observed. This showed that the insert was cloned into the correct orientation with the plasmid. This also showed that the plasmid did not ligate on itself. The next step was to transform the pFastBacIHuGLYATa and pFastBacHTbHuGLYATa into AcBacΔCC cells for transposition into the bacmid.

5.3.2. Transformation of pFastBacIHuGLYATa and pFastBacHTbHuGLYATa into AcBacΔCC

The plasmids, pFastBacIHuGLYATa and pFastBacHTbHuGLYATa, were transformed into competent AcBacΔCC cells for transposition of the DNA insert encoding HuGLYATa into the bacmid. AcBacΔCC cells were chosen because of the deletion of the genes encoding chitinase and v-cathesin (Kaba et al., 2004). This often helps in promoting better production of recombinant protein as discussed in 5.1.

AcBacΔCC cells were made competent by following the procedure in 2.2.3. The plasmids, pFastBacIHuGLYATa and pFastBacHTbHuGLYATa, were transformed into AcBacΔCC cells as decribed in 2.2.4. The transformed cells were then plated out on a LB-agar plate containing 50 µg/ml kanamycin, 10 µg/ml gentamicin, 10 µg/ml tetracycline, 4 % IPTG and 32 µg/ml X-gal. The plates were then incubated at 37 °C overnight. Plates were then examined and the white colonies were picked up with a sterile toothpick and incubated in 10 ml LB medium. The cultures were grown overnight at 37 °C in a shaking incubator at 200 rpm and glycerol stocks were made as described in 2.2.5. White colonies were chosen as these colonies were more likely to contain the correctly transposed DNA insert encoding HuGLYAT from the donor plasmids pFastBacIHuGLYATa and pFastBacHTbHuGLYATa respectively. This is due to the disruption of the expression of lacZα gene and indicative of successful transposition. Blue bacterial colonies were not picked up as this indicates unsuccessful transposition. The next step was to extract the bacmid for PCR analysis to ensure and verify that transposition was indeed successful.

5.3.3. PCR analysis of transposition success of pFastBacIHuGLYATa and pFastBacHTbHuGLYATa into AcBacΔCC

The bacmid was propagated as a large plasmid inside bacterial cells. This allows for rapid identification and verification of recombinant baculovirus, eliminates tedious plaque purification of recombinant virus DNA and rapid production of multiple recombinant viruses for structure studies. To verify that transposition of the desired DNA insert was indeed successful, PCR analysis was performed.

AcBacΔCC cells transformed with pFastBacIHuGLYATa and pFastBacHTbHuGLYATa were started up in 10 ml LB medium containing 50 µg/ml kanamycin, 10 µg/ml gentamicin, 10 µg/ml tetracycline. The cultures were grown overnight at 37 °C in a shaking incubator at 200 rpm. Bacmid extraction was performed as described in 5.2.4. The extracted bacmid was then used in PCR as described in 5.2.5. The amplified PCR product was then gel electropheresed and analysed to determine successful transposition. Expected results for bacmid transposed with pFastBacIHuGLYATa is a amplicon of ±3218 bp in size. Expected results for bacmid transposed with pFastBacHTbHuGLYATa is a amplicon of ±3348 bp in size. Transposition of pFastBacIHuGLYATa and pFastBacHTbHuGLYATa that was not successful into the bacmid will yield a amplicon of ±300 bp. The results are shown in Figure 17.

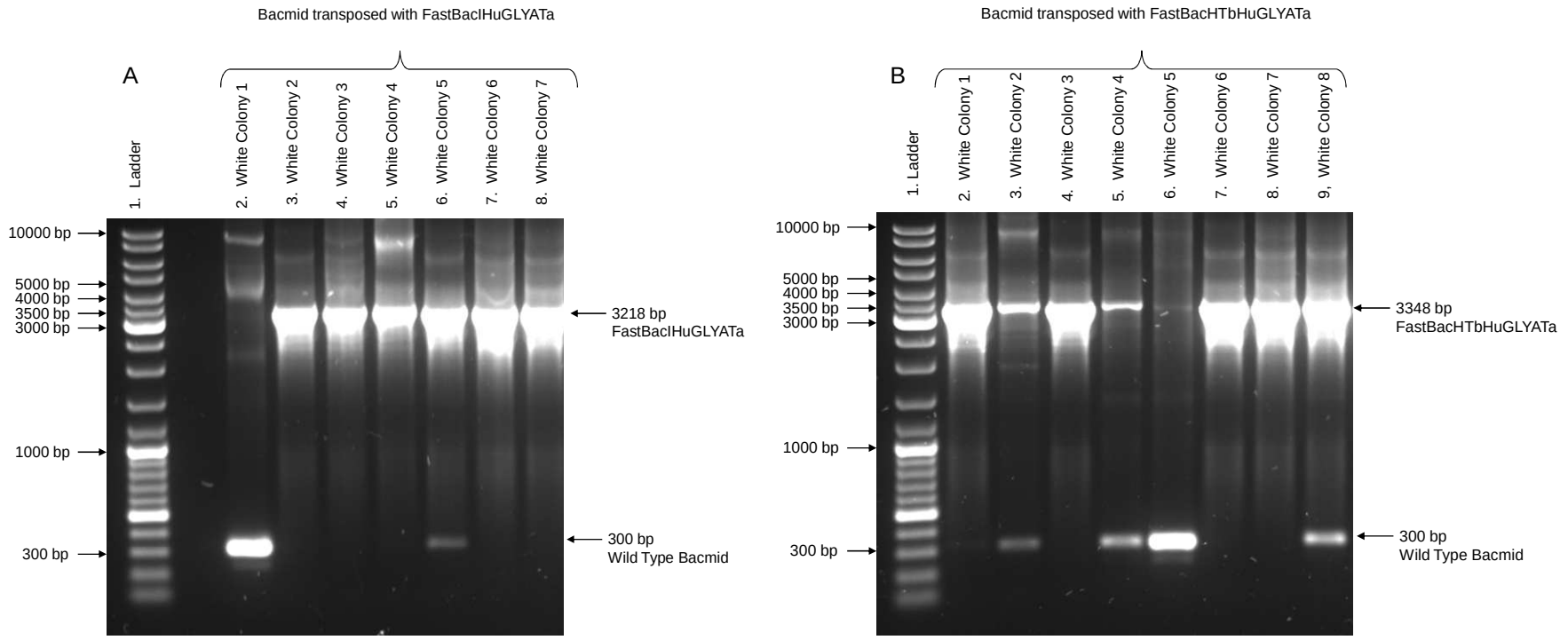


Figure 17: Gel electrophoresis of the PCR analysis of bacmid transposed with (A) pFastBacIHuGLYATa, and (B) pFastBacHTbHuGLYATa**.**

(A and B) Lane 1 O' GeneRuler DNA size marker (Fermentas, Catalogue no. SM1173); (A Lane 2 - 8) DNA of 7 white colonies; (B Lane 2 -8) DNA of 8 white colonies.

As can been seen in Figure 17(A) 7 white colonies were analyzed for successful transposition of FastBacIHuGLYATa. The results showed that 5 of these bacterial colonies were successfully transposed (Figure 17(A) Lanes 3-5, 7 and 8) resulting in an amplicon of ±3218 bp. In lane 2 it showed that transposition was not successful as only the wild type amplicon of ±300 bp was present. In lane 6 transposition was achieved but the wild type bacmid was also still present.

In Figure 17(B) 8 white colonies were analyzed for successful transposition of FastBacHTbHuGLYATa The results showed that 4 of these bacterial colonies were successfully transposed (Figure 17(B) Lanes 2, 4, 7, 8) resulting in an amplicon of ±3348 bp. In lanes 3, 5, 6 and 9 transposition of FasyBcHTbHuGLYATa was achieved but the wild type bacmid ±300 bp was also present.

Therefore, colonies containing both the transposed bacmid and wild type bacmid or wild type bacmid only, were discarded. This is to prevent poor expression levels of the recombinant protein as the wild type often overgrows the recombinant baculovirus. This is also to assure that only the desired recombinant protein is expressed.

The next step was to use the bacmid transposed with FastBacIHuGLYATa and FastBacHTbHuGLYATa, respectively, and transfect *Spodoptera frugiperda* (SF 9 cells) for expression of HuGLYATa.

5.3.4. Expression of FastBacIHuGLYATa and FastBacHTbHuGLYATa

To express the recombinant protein HuGLYATa in Sf9 cells it was necessary to transfect the cells with the bacmid DNA transposed with FastBacIHuGLYATa and FastBacHTbHuGLYATa. The bacmid itself is infectious and forms, in the Sf9 cells, the AcMNP virus containing the insert encoding for HuGLYATa.

The recombinant virus obtained from the initial transfection of the Sf9 cells with the recombinant bacmid, was then used to further transfect Sf9 cells in 6 well plates to express FastBacIHuGLYATa and FastBacHTbHuGLYATa respectively. The respective 6 well plates were then incubated at 27 °C for 72 hours or until signs of cytopathic effects became visible. Each wells' cells were collected and placed in a 2 ml Eppendorf tube. They were centrifuged at 500 x g for 2 minutes. Protein extraction was performed as described in 5.2.7. The results of the protein extraction were visualized on SDS-PAGE. Expected results for FastBacIHuGLYATa were a protein at ±35 kDa and for FastBacHTbHuGLYATa a fusion protein with a His-tag of ±40 kDa.

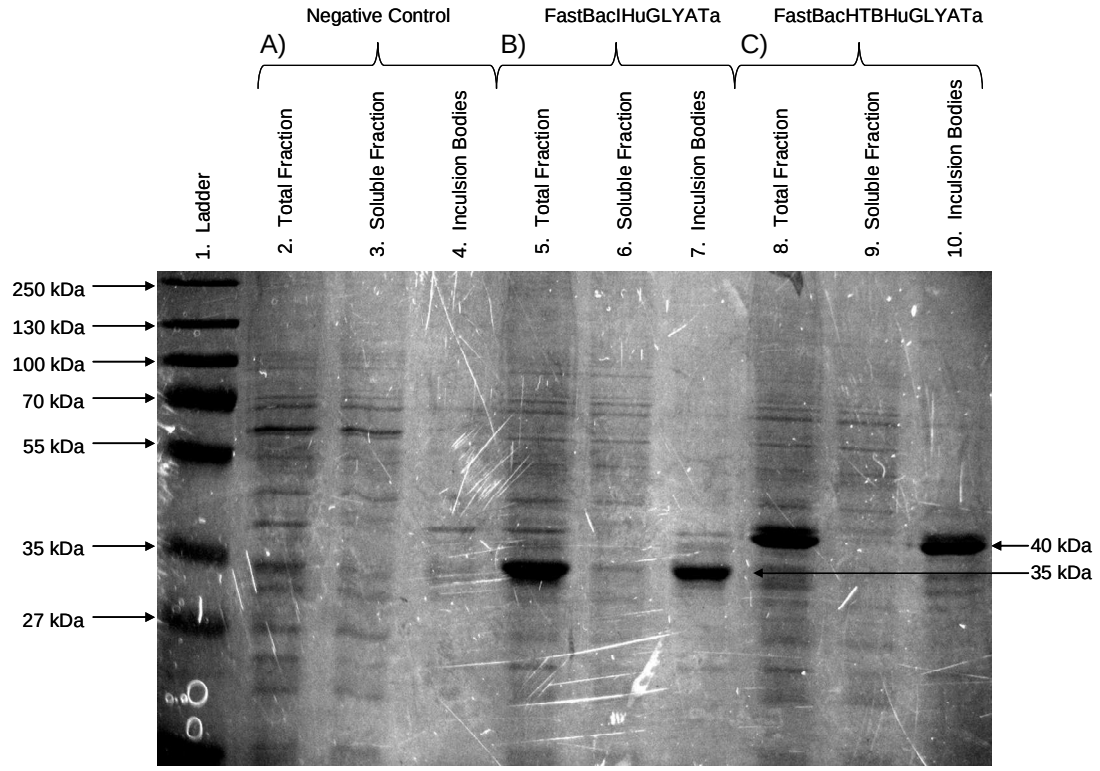


Figure 18: SDS PAGE of the expression of HuGLYATa in Sf9 cells.

Lane 1 Fermentas PageRuler™Prestained Protein Ladder (catalogue no. SM0671); A) Negative Control Lane: 2) Total Fraction 3) Soluble Fraction 4) Inclusion bodies; B) Expression of FastBacIHuGLYATa Lane: 5) Total Fraction 6) Soluble Fraction 7) Inclusion bodies; B) Expression of FastBacHTbHuGLYATa Lane: 8) Total Fraction 9) Soluble Fraction 10) Inclusion bodies.

High levels of expression of FastBacIHuGLYATa at ±35 kDa and FastBacHTbHuGLYATa at ±40 kDa were observed (Figure 18 B and C). The SDS-PAGE smiled as can been seen in the middle of the gel. The negative control (Figure 18 A) showed that the expression achieved was not from the wild type virus. It was also observed that the expressed recombinant HuGLYATa was insoluble (Figure 18 B lane 7 and C lane 10) therefore I did not assayed for enzyme activity.

5.4. Summary

Expressing recombinant proteins using bacterial systems are easy to setup, maintain and are cost effective. The drawback of bacterial systems is their inability to perform post-translational modification. This often leads to recombinant proteins aggregating. These proteins are also biologically inactive. Eukaryotic cell expression is used to try and prevent this aggregation and to express a biologically active protein. Insect cell expression using the Bac-to-Bac® system was used. The DNA insert encoding HuGLYATa was cloned into pFastBacI and pFastBacHTb donor plasmids. This is to express the recombinant protein

alone and with a Histidine tag respectively. AcBacΔCC cells where chosen for production of the recombinant bacmid. They contain deletions of the genes encoding chitinase and v-cathepsin. This helps with improved expression of the recombinant protein.

The DNA insert HuGLYATa where ligated with pFastBacI and pFastBacHTb. The plasmids pFastBacIHuGLYATa and pFastBacHTbHuGLYATa where cloned into cSure cells to prepare sufficient glycerol stocks to use in further experimentation. The plasmids where then cloned into AcBacΔCC cells for transposition of HuGLYATa gene into the bacmid. The cells where then plated out on a LB-agar plate containing 50 µg/ml kanamycin, 10 µg/ml gentamicin, 10 µg/ml tetracycline, 4 % IPTG and 32 µg/ml X-gal. This was to select the correct bacterial colonies containing the correct transposed bacterial colonies. Therefore only white colonies were chosen. The white colonies were then picked up with a sterile toothpick and transferred to 10 ml LB medium containing the necessary antibiotics. The cultures were then grew overnight at 37 °C in a shaking incubato. These culture where then used to extract the recombinant bacmid. PCR analysis was performed on the extracted recombinant bacmid to check transposition success of HuGLYATa into the bacmid. Bacmid containing only HuGLYATa was chosen to transfect insect cells. This was done by using a 6 well plate containing insect cells with a density of 1.5×10^6 cells. The plate where then incubated at 27 °C for 72 hours or until cytopathic effect of viral infection was seen. The cells where then drawn off the cells placed into a 1.5 ml Eppendorf tube and centrifuged. The supernatant was then drawn off. This was the T1 viral stocks. A 6 well plate was again used and the cells again transfected with the T1 viral stock to express the recombinant protein.

The expressed recombinant FastBacIHuGLYATa and FastBacHTbHuGLYATa were well expressed in insect cells but were insoluble and not biologically active.

Chapter 6

Concluding Summary

Detoxification has become a modern day buzzword for enhancing quality of life. Detoxification, now known as biotransformation, is used to promote anything form health food to enhancements of performance in sports. It has a lesser known application as a therapy in alleviating symptoms of inborn errors of metabolism. Inborn errors of metabolism are a group of disorders where a gene defect has a clinical effect on an individuals' metabolic pathway. Metabolites accumulate causing mild to severe clinical symptoms. These metabolites are then expelled by means of detoxification by the liver and kidneys. There are two main pathways of detoxification in the liver. Phase 1 detoxification include: oxidation; hydroxylation; dehydrogenation; metabolic reduction and hydrolysis. Phase 2 detoxification uses conjugation reactions to increase hydrophillicty of metabolites. Here I focused on phase 2 detoxification especially amino acid conjugation as glycine- N-acyltransferase. GLYAT has two variants, we focused on GLYAT isoform a (HuGLYATa). The aim of my master project was the cloning and expression of human GLYAT isoform a. This was achieved by: (1) synthesizing the ORF of human GLYAT isoform a; (2) expressing it in bacterial and insect cell expression systems and (3) evaluating if the recombinant human GLYAT isoform a is enzymatic active. Experimental work was concluded in 2006. Due to personal and work related changes the work was only written up during 2012.

First generation bacterial expression, using *E. coli.* as an expression system, was used for its ease of use. The system is inexpensive to set up, maintain and scale up. HuGLYATa protein was cloned successfully into pET32a+, I achieved high level expression, but HuGLYATa was insoluble and biologically inactive. I addressed the issue of insolubility by collecting the aggregated recombinant proteins. This allowed me then to denature the recombinant protein and to renature the recombinant protein in standardized buffers using a commercial kit. The aggregated HuGLYATa became soluble but remained enzymatically inactive. The next step was then to investigate and use the second generation bacterial expression system that uses cold shock for expression of recombinant proteins. Solubility of the recombinant protein was improved, but the enzyme was still biologically inactive. Insect cell expression was used as these systems can perform some post translational glycosylation modification of the proteins which is, where this was not possible in bacterial expression systems. The expressed recombinant FastBacIHuGLYATa and FastBacHTbHuGLYATa proteins were showed to be insoluble and did not have GLYAT activty. To summarize the results: a good level of expression was obtained with all expression systems. The recombinant HuGLYATa proved to be insoluble using first generation bacterial expression and insect cell expression. Solubility was achieved with second generation bacterial expression using cold shock and

denaturing and renaturing the 67 kDa recombinant HuGLYATa. In all cases the recombinant HuGLYATa did not have any enzymatic inactivity.

By way of reflection and pointing the way forward on how to prepare to achieve a soluble, enzymatic active recombinant HuGLYATa the following come to mind. Changing the conditions under which expression took place for example, lowering the IPTG concentration and temperature in first generation bacterial expression systems. This causes a slowdown in the rate of production of a recombinant protein, allowing the recombinant protein to reach its native conformation (Sorenson et al; 2005). The co-expression of folding modulators in bacterial expression can help with protein folding. Folding modulators can help with refolding the protein on expression for example: folding chaperones like DnaK, GroEL and GroES; holding chaperones like TF and IbpB that holds the recombinant protein when the availability of folding chaperones is low; and disaggregating chaperones for example ClbB to solubilise proteins aggregated under stress (Baynex et al; 2004 and Sorenson et al; 2005)

In the time between my experimental work was completed and writing up of my work, a few developments occurred in my study area. In recent studies two other members of the GLYAT family of enzymes have been identified, GLYATL1 (GenBank DQ084381) and GLYATL2 (Waluk et al., 2010). GLYATL1 is a split gene that consisted out of 7 exons and translates a peptide chain of 302 amino acids in length. The gene was mapped to chromosome 11q12.1 and transcribes in the sense direction of the chromosome (Zhang et al. 2007). GLYATL1 is mostly expressed in the liver and, when it is over expressed, it activates the HSE pathway. The GLYATL2 enzyme is mostly found in the endoplasmic reticulum, expression in salivary gland and trachea is high, but is also detected in spinal cord and skin fibroblasts. The expression pattern of the GLYATL2 suggests that it might have a barrier function assisting the immune system. GLYATL2 (GenBank NM_145016.3) is expressed on the minus strand, is 34 kDa big and has a C-terminal dilysine motif which is an ER retention or retrieval signal (Waluk et al. 2010). In our own laboratory a recombinant bovine GLYAT has been successfully expressed and was found to be biologically active (Badenhorst et al; 2012). In another recent development in our laboratory recombinant human GLYATa was also expressed in a biologically active form (Rencia van der Sluis, personal communication) using some of the constructs developed in my MSc study in combination with chaperones and different purification conditions. Therefore, the research initiated during my MSc study, in finding a recombinant HuGLYATa, has come to fruition.

Appendix

Fluka

Industriestrasse 25
CH-9471 Buchs

E-mail: Flukatec@eurnotes.sial.com
Fax: ++41 81 755 2815

96827 Renaturation Basic Kit for Proteins

Sample: _____ Date: _____
Sample Conzentration (protein): _____ Start Time: _____
Sample Puffer: _____ Temperature: _____
Volume: Total _____ul Sample_____ul Reagent_____ul other possible Additives _____ul

No.	Fluka No.	Reagent Name	Time & Renaturation yield:	Time & Renaturation yield:	Time & Renaturation yield:
1.	72903	MES (pH 6.5) 0.05M, Na-chloride 0.1M			
2.	83047	MES (pH 6.5) 0.05M, Na-chloride 0.1M, PPS 1M			
3.	82953	MES (pH 6.5) 0.05M, Na-chloride 0.2M			
4.	72905	MES (pH 6.5) 0.05M, Na-chloride 0.2M, PPS 1M			
5.	73021	MES (pH 6.5) 0.05M, DTT 0.001M			
6.	71023	MES (pH 6.5) 0.05M, DTT 0.001M, PPS 1M			
7.	93038	MES (pH 6.5) 0.05M, Na-chloride 0.1M, DTT 0.001M			
8.	85312	MES (pH 6.5) 0.05M, Na-chloride 0.1M, DTT 0.001M, PPS 1M			
9.	83809	MES (pH 6.5) 0.05M, Na-chloride 0.2M, DTT 0.001M			
10.	73194	MES (pH 6.5) 0.05M, Na-chloride 0.2M, DTT 0.001M, PPS 1M			
11.	72476	MES (pH 6.5) 0.05M, L-GSH red. 0.005M, L-GSH ox. 0.001M			
12.	77809	MES (pH 6.5) 0.05M, L-GSH red. 0.005M, L-GSH ox. 0.001M , PPS 1M			
13.	72914	MES (pH 6.5) 0.05M, Na-chloride 0.1M, L-GSH red. 0.005M, L-GSH ox. 0.001M			
14.	78569	MES (pH 6.5) 0.05M, Na-chloride 0.1M, L-GSH red. 0.005M, L-GSH ox. 0.001M , PPS 1M			
15.	78582	MES (pH 6.5) 0.05M, Na-chloride 0.2M, L-GSH red. 0.005M, L-GSH ox. 0.001M			
16.	94718	MES (pH 6.5) 0.05M, Na-chloride 0.2M, L-GSH red. 0.005M, L-GSH ox. 0.001M , PPS 1M			
17.	94717	HEPES Na-salt (pH 7.5) 0.05M			
18.	78593	HEPES Na-salt (pH 7.5) 0.05M, PPS 1M			
19.	87062	HEPES Na-salt (pH 7.5) 0.05M, Na-chloride 0.1M			
20.	78579	HEPES Na-salt (pH 7.5) 0.05M, Na-chloride 0.1M, PPS 1M			
21.	87605	HEPES Na-salt (pH 7.5) 0.05M, Na-chloride 0.2M			
22.	72906	HEPES Na-salt (pH 7.5) 0.05M, Na-chloride 0.2M, PPS 1M			
23.	78152	HEPES Na-salt (pH 7.5) 0.05M, DTT 0.001M			
24.	72824	HEPES Na-salt (pH 7.5) 0.05M, DTT 0.001M, PPS 1M			
25.	72588	HEPES Na-salt (pH 7.5) 0.05M, Na-chloride 0.1M, DTT 0.001M			
26.	76983	HEPES Na-salt (pH 7.5) 0.05M, Na-chloride 0.1M, DTT 0.001M, PPS 1M			
27.	73193	HEPES Na-salt (pH 7.5) 0.05M, Na-chloride 0.2M, DTT 0.001M			
28.	80293	HEPES Na-salt (pH 7.5) 0.05M, Na-chloride 0.2M, DTT 0.001M, PPS 1M			
29.	80292	HEPES Na-salt (pH 7.5) 0.05M, L-GSH red. 0.005M, L-GSH ox. 0.001M			
30.	76951	HEPES Na-salt (pH 7.5) 0.05M, L-GSH red. 0.005M, L-GSH ox. 0.001M, PPS 1M			
31.	88503	HEPES Na-salt (pH 7.5) 0.05M, 0.1M Na-chloride, L-GSH red. 0.005M, L-GSH ox. 0.001M			
32.	83068	HEPES Na-salt (pH 7.5) 0.05M, Na-chloride 0.1M, L-GSH red. 0.005M, L-GSH ox. 0.001M, PPS 1M			
33.	83796	HEPES Na-salt (pH 7.5) 0.05M, Na-chloride 0.2M, L-GSH red. 0.005M, L-GSH ox. 0.001M			
34.	80409	HEPES Na-salt (pH 7.5) 0.05M, Na-chloride 0.2M, L-GSH red. 0.005M, L-GSH ox. 0.001M, PPS 1M			
35.	75837	TRIS-HCl (pH 8.5) 0.05M, Na-chloride 0.1M			
36.	91810	TRIS-HCl (pH 8.5) 0.05M, Na-chloride 0.1M, PPS 1M			
37.	82959	TRIS-HCl (pH 8.5) 0.05M, Na-chloride 0.2M			
38.	83943	TRIS-HCl (pH 8.5) 0.05M, Na-chloride 0.2M, PPS 1M			
39.	82305	TRIS-HCl (pH 8.5) 0.05M, DTT 0.001M			
40.	70603	TRIS-HCl (pH 8.5) 0.05M, DTT 0.001M, PPS 1M			
41.	75812	TRIS-HCl (pH 8.5) 0.05M, Na-chloride 0.1M, DTT 0.001M			
42.	81220	TRIS-HCl (pH 8.5) 0.05M, Na-chloride 0.1M, DTT 0.001M, PPS 1M			
43.	72259	TRIS-HCl (pH 8.5) 0.05M, Na-chloride 0.2M, DTT 0.001M			
44.	72991	TRIS-HCl (pH 8.5) 0.05M, Na-chloride 0.2M, DTT 0.001M, PPS 1M			
45.	78217	TRIS-HCl (pH 8.5) 0.05M, L-GSH red. 0.005M, L-GSH ox. 0.001M			
46.	95739	TRIS-HCl (pH 8.5) 0.05M, L-GSH red. 0.005M, L-GSH ox. 0.001M, PPS 1M			
47.	82972	TRIS-HCl (pH 8.5) 0.05M,Na-chloride 0.1M, L-GSH red. 0.005M, L-GSH ox. 0.001M			
48.	72403	TRIS-HCl (pH 8.5) 0.05M, Na-chloride 0.1M, L-GSH red. 0.005M, L-GSH ox. 0.001M, PPS 1M			
49.	85323	TRIS-HCl (pH 8.5) 0.05M, Na-chloride 0.2M, L-GSH red. 0.005M, L-GSH ox. 0.001M			
50.	93039	TRIS-HCl (pH 8.5) 0.05M, Na-chloride 0.2M, L-GSH red. 0.005M, L-GSH ox. 0.001M, PPS 1M			

References

ASAOKA K. (1991). Enzymes that metabolize acyl-CoA in the monkey - their distribution, properties and roles in an alternative pathway for the excretion of nitrogen. Int. J. Biochem. 23, 429 - 434.

BADENHORST C.P., JOOSTE, M., VAN DIJK A.A. (2011). Enzymatic characterisation and elucidation of the catalytic mechanism of a recombinant bovine glycine N-acyltransferase. Drug Metab Dispos. 40(2), 346-352

BARIC, I., FUMIC. F. & HOFFMANN. G.F. (2001) Inborn errors of metabolism at the turn of the millennium. Crout Med J, 42: 379-83

BARTLETT, K., AND GOMPERTZ, D. (1974). The specificity of glycine-acylase and acylglycine excretion in the organic acidemias. Bioch. Med. 10, 15 - 23.

BAUMGARTNER, M. (2003) 3 Methylcrotonyl-CoA carboxylase deficiency. Orphanet encyclopedia. July 2003. http://www.orpha.net/data/patho/GB/uk-MCC.pdf

BAYNEX, F., MUJACIC, M. (2004). Recombinant protein folding and misfolding in Escherichia Coli. Nature Biotechnology 22, 1399 – 1408.

BIRNBOIM H.C., DOLY J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Research Volume 7 Number 6 , 1513 – 1523.

CALDWELL, J. (1986). Conjugation mechanisms of xenobiotic metabolism: mammalian aspects. In Xenobiotic Conjugation Chemistry. Paulson, G.D., Caldwell, J.,Hutson, D.H., and Menn, J.J., eds. American Chemical Society, Washington, DC. pp. 2 - 28.

CHUANG D.T. (1998). Maple syrup urine disease: it has come a long way. J Pediatr, 132(3 Pt 2):S17-23

ENGELS, J.W., UHLMANN E. (1989). Gene Synthesis. Angew. Chem. Int. Ed. Engl. 28, 716-734

FERNANDES, J.; SAUDUBRAY, J.M.; VAN DEN BERGHE, G.; WALTER, J.H. (2006). Inborn Metabolic Diseases : Diagnosis and Treatment (4th ed.). Springer. p. 561 p.

FISHMAN, W.H. (1970). Metabolic conjugation and metabolic hydrolysis. Academic Press, New York.

FRIES M.H, RINALDO P, SCHMIDT-SOMMERFELD E, JURECKI E, AND PACKMAN S. (1996). Isovaleric acidemia: response to a leucine load after three weeks of supplementation with glycine, L-carnitine, and combined glycine-carnitine therapy. J Pediatr. 1996 Sep;129(3):449-52.

GALLAGHER, S.R. 1999. One dimensional SDS gel electrophoresis of proteins. Current Protocols in Molecular Biology. Hobokeng NJ: John Wiley & Sons, Inc.

GARROD, A.E. (1923) Inborn Errors of Metabolism. Second Edition Henry Frowde and Hodder & Stoughton

GATLEY S.J AND SHERRATT H.S.A (1977). The synthesis of hippurate from benzoate and glycine by rat liver mitochondria. Biochem. J. 166, 39 - 47.

GENWAY BIOTECH. 2 APRIL 2011. Comparison of expression systems. http://www.genwaybio.com/technologies/protein-expression Date of access: 8 May 2009.

GREGERSEN N, KØLVRAA S AND MORTENSEN PB. (1986). Acyl-CoA:glycine N-acyltransferase: Organelle localization and affinity toward straight- and branched chained acyl-CoA esters in rat liver. Biochem. Biomed. Metabol. Biol. 36, 98 - 105.

HUTT A.J AND CALDWELL J (1990). Amino Acid Conjugation. In Conjugation reactions in drug metabolism. G.J. Mulder, ed. pp. 273-305. Taylor & Francis, London.

INOUE H, NOJIMA H, OKAYAMA H. (1990). High efficiency transformation of Escherichia coli with plasmids. Gene.Nov 30;96(1):23-8.

JAKOBY, W.B. (1980). Enzymatic basis of Detoxication. Academic Press, New York.

JAKOBY, W.B. (1981). Detoxication and drug metabolism: Conjugation and related systems. Methods of Enzymology,Vol. 77. Williams, R.T., ed. Academic Press, New York.

KABA, S.A., SALCEDO, A.M., WAFULA, P.O., VLAK, J.M. & VAN OERS, M.M. (2004). Development of a chitinase and v-cathepsin negative bacmid for improved integrity of secreted recombinant proteins. J Virol Methods 122, 113–118

KANAZU, T, AND YAMAGUCHI, T. (1997). Comparison of in vitro carnitine and glycine conjugation with branched-chain and cyclic side-chain carboxylic acids in rats. Drug Metab. Disp. 25, 149 - 153.

KELLEY, M, AND VESSEY, D.A. (1994). Characterization of the acyl-CoA: amino acid N-acyltransferases form primate liver mitochondria. J. Biochem. Toxicol. 9, 153- 158.

KOST, T.A., CONDREAY, J.P., JARVIS, D.L. (2005). .Baculovirus as versatile vectors for protein expression in insect and mammalian cells. Natue Biotechonoly Vol 23 No 5:567-575.

LEHNERT W (1981). Excretion of N-isovalerylglutamic acid in isovaleric acidemia. Clin. Chim. ACTA 116, 249 - 252.

LEHNERT W (1983). N-Isovalerylalanine and N-Isovalerylsarcosine: Two new minor metabolites in isovaleric acidemia. Clin. Chim. Acta 134, 207 - 212.

LENNON G, AUFFRAY C. (1996). The I.M.A.G.E Consortium: an integrated molecular analysis of genomes and their expression. Genomics, 33, 151-152

LEVY, G. (1965). Pharmacokinetics of salicylate elimination in man. J. Pharm. Sci. 54, 959 - 967.

LISKA D.J.. (1998). The detoxification enzyme system. Alternative Medicine Review. 3:187-198.

LUCKOW, V.A., LEE, S.C., BARRY, G.F., OLINS, P.O. (1993). Efficient Generation of nfectious Recombinant Baculoviruses by Site-Specific Transposon-Mediated Insertion of Foreign Genes into a Baculovirus Genome Propagated in Escherichia coli. Journal Of Virology, Aug. VOL. 67 p. 4566-4579

MAWAL Y.R, QURESHI I.A. (1994). An immunodetection method for the quantitation of human acyl oA:glycine N-acyltransferase in biological samples. Biochem Mol Biol Int 34:595-601.

MIDDELBERG, A.P.J. (2002). Preparative protein refolding. Trends in Biotechnology Vol.20 No.10:437 – 443.

MILES C.S, OST T.W.B, NOBLE M.A, MUNRO A.W, CHAPMAN S.K. (2000). Protein engineering of cytochromes P-450. Biochimica et Biophysica Acta 1543, 383-40.

MULDER, G.J. (1990). Conjugation Reactions in drug metabolism: An integrated approach. Taylor & Francis, London.

NANDI D.L, LUCAS S.V AND WEBSTER L.T. (1979). 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.

NELSON, DAVID L.; MICHAEL M. COX (2005). Lehninger Principles of Biochemistry. New York: W. H. Freeman and company. pp. 841. ISBN 0-7167-4339-6.

NISHIHARA, K., NISHIHARA K, KANEMORI M, KITAGAWA M, YANAGA H, YURA T: (1999) Chaperone Coexpression Plasmids: Differential and Synergistic Roles of DnaK-DnaJ-GroE and GroEL-GroES in Assisting Folding of an Allergen of Japanese Cedar Pollen, Cryj2, in Eschericia coli. Appl. Environ. Microbiol. 64:1694-1699

NOVAGEN. (2007). pET System Manual 11th edition. http://www.novagen.com Date accessed: 5 April 2007.

O'REILLY, D.R., MILLER, L.K. AND LUCKOW, V.A. (1992) Baculovirus Expression Vectors: A Laboratory Manual, W.H. Freeman and Company, New York.

PAULSON, G.D., CALDWELL, J., HUTSON, D.H., AND MENN, J.J. (1986). Xenobiotic Conjugation Chemistry. American Chemical Society, Washington, DC.

PARKINSON A,. (1995). Casarett and Doull's Toxicology 5th Edtion, 113-186.

RENDIC S. (2002). Summary of information on human CYP enzymes: human P450 metabolism data. Drug Metab Rev, 34, 383-448.

ROLLAND M.O, DIVRY P, ZABOT M.T, GUIBAUD P, GOMEZ S, LACHAUX A AND LORAS I. (1991). Isolated 3-methylcrotonyl-CoA carboxylase deficiency an a 16-month-old child. J. Inher. Metab. Dis. 14, 838 - 839.

RUSSELL D.W., SAMBROOK J. (2006) Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Press, 2006:632–6

SCHACHTER D AND TAGGART J.V. (1953). Benzoyl-Coenzyme A and hippurate synthesis. J. Biol. Chem. 203, 925 - 934.

SHINKA T, INOUE Y, KUHARA T, MATSUMOTO M AND MATSUMOTO I. (1985). Benzoylalanine: Detection and identification of an alanine conjugate with benzoic acid in hyperammonemic patients treated with sodium benzoate. Clin. Chim. Acta 151, 293 - 300.

SORENSON H.P., MORTENSEN K.K. (2005) Soluble expression of recombinant proteins in the cytoplasm of Escherichia coli. Microbial Cell Factories, 4:1

STUDIER J.W. (1991). Use of bacteriophage T7 lysozyme to improve an inducible T7 expression system. J Mol Biol. 219(1):37-44.

TAKARA BIO INC. 2007. Cold shock expression system: pCold DNA. http://www.takara-bio.co.jp Date accessed: 6 January 2009.

VAN DER WESTHUIZEN, F.H., PRETORIUS, P.J., AND ERASMUS, E. (2000) The utilization of alanine, glutamic acid and serine as amino acid substrates for glycine N-acyltransferase. J. Biochem.
Mol. Toxicol. 14, 102–109

VASINA, J.A., PETERSON, M.S,. BANEYX, F. (1998). Scale-up and Optimization of the Low-Temperature Inducible cspA Promoter System. Biotechnol. Prog. 14, 714-721

VESSEY D.A AND HU J (1995). Isolation from bovine liver mitochondria and characterization of three distinct carboxylic acid:CoA ligases with activity toward xenobiotics. J. Biochem. Toxicol. 10, 329 - 337.

VESSEY, D.A., AND LAU, E. (1996). Determination of the sequence of the aralkyl acyl-CoA:amino acid N-acyltransferase from bovine liver mitochondria. J. Biochem. Toxicol. 11, 211 - 215.

WALUK, D.P., SCHULTZ, N. & HUNT, M.C. (2010) Identification of glycine-N-acyltransferase-like 2 (GLYATL2) as a transferase that produces N-acyl glycines in humans. The FASEB journal Vol. 24 2795 - 2803

WEBSTER JR, L.T., SIDDIQUI, U.A., LUCAS, S.V., STRONG, J,M., AND 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. J.Biol. Chem. 251, 3352 - 3358.

ZHANG, H., LANG, Q., LI, J., ZHONG, Z., XIE, F., YE, G., WAN, B., AND YU, L. (2007). Molecular cloning and characterization of a novel human glycine-N-acyltransferase gene GLYATL1, which activates transcriptional activity of HSE pathway. Int. J. Mol. Sci.8, 433–444

“Any opinion, findings and conclusions or recommendations expressed in this material are those of the author(s) and therefore the NRF does not accept an liability in regard thereto”