# Chapter 2: Cloning and expression of bovine GLYAT in *Escherichia coli*

## 2.1 Introduction

To date no system for the bacterial expression and purification of an enzymatically active recombinant glycine N-acyltransferase (E.C. 2.3.1.13) has been reported in the literature. In previous studies in our laboratory, however, bovine GLYAT has been expressed from pColdIII using its own open reading frame (with no N- or C-terminal fusions) and co-expressed with the GroES-GroEL-TF chaperone system (Section 1.6.2). It was found that the protein was very well expressed, but it was mostly insoluble. The soluble fraction, however, had substantial enzyme activity. (M Snyders, unpublished work). Since no purification tags were fused to the protein, it could not be purified for more detailed investigation. One objective of this study was to confirm this result, and to use the same expression system to express a bovine GLYAT with a histidine tag, which could be purified using nickel affinity chromatography (Section 1.6.3). Another objective was to subject the purified enzyme to further analysis, including the determination of kinetic parameters and comparison to the bovine liver enzyme.

Development of a recombinant expression system is usually of significant value to studying the properties of proteins, especially enzymes. Such a system often allows large amounts of the protein to be produced, which is especially valuable if the protein occurs in small amounts or is found in difficult to obtain tissues. Furthermore, the recombinant enzyme can be manipulated to introduce mutations, truncations fusions, and other modifications. This can significantly aid protein purification and study of its function and is absolutely necessary for engineering new functions (Andersen & Krummen, 2002, Grabowski et al., 1995, Porath et al., 1975, Saeki et al., 2007).

Our long term goal is the development of a recombinant glycine N-acyltransferase with altered substrate specificity, which may be used in the treatment of specific organic acidemias. A recombinant protein expression system is crucial to the development of such a therapeutic enzyme. For example, specific amino acid changes can be introduced, after which the protein is expressed and purified, and its kinetic parameters determined. This allows a systematic approach to be followed in designing a variant of the enzyme with altered properties, whether it be increased activity or perhaps the ability to use a novel substrate. Use of a standardised recombinant expression system would also remove one source of variability (the source and quality of the protein preparation) between researchers, which in turn could help reduce the variation in kinetic parameters reported in the literature (Section 1.4.2).

Ultimately, it would be preferable to develop a human therapeutic enzyme, but to date no successful expression of biologically active human GLYAT (E.C. 2.3.1.13, not to be confused with GLYATL1, which has been expressed in mammalian cells) has been reported. In this chapter the expression, purification and characterisation of recombinant bovine GLYAT, a continuation of the previous work in our laboratory, is described. Since the human and bovine GLYAT enzymes are similar in terms of substrate specificity, the information gained by studying the bovine enzyme can later be applied to human enzyme, when a successful expression system for it is developed (Bartlett & Gompertz, 1974).

A further objective of this study was to investigate the advantage of using the chaperone co-expression (using the pGTf2 plasmid from Takara) strategy. It was shown that expression of bovine GLYAT with chaperone co-expression yields biologically active enzyme, but It has not been determined whether similar results can be obtained without chaperone co-expression.

To summarise, the main aims of this part of the study were firstly to clone bovine GLYAT into a pColdIII vector with a histidine tag and to purify it using nickel affinity chromatography. Secondly, to investigate the effect of chaperone co-expression and, thirdly, to kinetically characterise and compare the recombinant enzyme to one purified from bovine liver.

## 2.2 Materials and methods

The materials and reagents used in this study are listed in Appendix I. The names, suppliers, and catalogue numbers of the reagents used are all included in Appendix I. To simplify the flow of the text, the supplier and catalogue number information is thus not mentioned in the text, but can be found in the appendix, which is organised according to the names used in the text.

The pColdIII vectors used in this study, which contain C-terminal histidine tags with either a long, a short, or no serine-glycine linker, are listed in Table 2.1. These vectors were obtained from Dr AC Potgieter (Deltamune), who modified the original pColdIII vector to contain these different tags.

**Table 2.1: Modified pColdIII expression vectors with C-terminal histidine tags and serine-glycine linkers**

| Vector | Sequence of tag | Molecular weight of tag (Da) | Length of tag (aa) |
|---|---|---|---|
| pColdIII-E | HHHHHH | 840.7 | 6 |
| pColdIII-A | SSGGGGSHHHHHHLQSR | 1814.5 | 17 |
| pColdIII-EH | SSGGGGSGGGGSGGGGSHHHHHHLQSR | 2444.9 | 27 |

Modified pColdIII vectors were obtained from Dr AC Potgieter (Deltamune).

In this study, three strains of *Escherichia coli* were used, namely JM109, Origami, and E. cloni cells. JM109 cells are commonly used for cloning purposes. The recA$^-$ genotype enables cloning of repetitive sequences and other sequences that would normally be restricted. E. cloni cells have inactivated mcr and mrr genes, allowing the direct cloning of methylated DNA from mammalian or plant sources. The recA1 and endA1 mutations enable the isolation of high amounts of high quality plasmid DNA. Origami cells are commonly used for expression studies. Because they contain trxB/gor mutations, a more oxidising than usual environment is found inside these cells. This allows for the formation of disulfide bonds in complex proteins, which is inefficient under normal reducing conditions.

### 2.2.1 Source of the bovine GLYAT coding sequence

The bovine GLYAT coding sequence was originally cloned from bovine liver RNA by means of reverse transcription and PCR amplification as part of a previous study in our laboratory (M Snyders, unpublished results). A pCold-TF plasmid containing this sequence was obtained and used as the template for PCR amplifications of the bovine GLYAT coding sequence.

### 2.2.2 PCR amplification of the bovine GLYAT coding sequence

Using primers NdeF and XhoR from Table 2.2, the coding sequence of bovine GLYAT was PCR amplified. The primers contain NdeI and XhoI restriction endonuclease recognition sites to facilitate directional cloning into expression vectors. The PCR reaction mixtures contained 1X Takara ExTaq buffer, 10 nmol of each dNTP, 25 pmol of each primer, approximately 50 ng of template DNA and 2 units of Takara ExTaq polymerase, in a final volume of 50 µl. Thermal cycling conditions were 94 °C for 1 min, then 30 cycles of 94 °C for 30 seconds, 70 °C for 30 seconds, and 72 °C for 1 minute, followed by a final extension at 72 °C for 10 minutes. Thermal cycling was performed using an Eppendorf thermal cycler.

**Table 2.2: Oligonucleotide primers used in this study**

| Primer name | Oligonucleotide sequence (5' → 3') | $T_m$ (°C) | Length (bp) | Manufacturer |
|---|---|---|---|---|
| NdeFor | GCCGCATATGATGTTCCTGCTGC | 66 | 23 | Inqaba Biotech |
| XhoRev | CTTCTCGAGAGGCTCACAGTTCCACTGG | 70 | 28 | Inqaba Biotech |
| pColdForward | ACGCCATATCGCCGAAAGG | 52 | 19 | Sylvean labs |
| pColdReverse | GGCAGGGATCTTAGATTCTG | 50 | 20 | Sylvean labs |
| M13 Forward | CGCCAGGGTTTTCCCAGTCACGAC | 61 | 24 | Inqaba Biotech |
| M13 Reverse | AGCGGATAACAATTTCACACAGGA | 52 | 24 | Inqaba Biotech |

### 2.2.3 Agarose gel electrophoresis

Agarose gel electrophoresis was used for routine analysis of PCR reactions, restriction digestions and nucleic acid isolations. Standard procedures as set out in the literature were used (Sambrook & Russell, 2001). Unless otherwise stated, 1% agarose gels (6 x 10 x 0.5 cm) were prepared using 1X Tris-Acetate-EDTA (TAE) buffer. Ethidium bromide was added to a final concentration of 0.5 µg/ml to facilitate visualisation of DNA on an ultraviolet light trans-illuminator.

Gels were loaded with samples mixed with one quarter volume of loading dye (50% glycerol coloured with orange G). For purposes of gel purification larger wells were made by taping together the appropriate number of teeth of a comb. DNA molecular size markers (O'GeneRuler SM1173) were always loaded in one lane for estimation of DNA sizes. The gels were then electrophoresed for one hour at 8 V/cm using a Bio-Rad PowerPac Basic system, unless otherwise stated. Directly after electrophoresis gels were photographed using the Syngene ChemiGenius Bio-Imaging system and GeneSnap software.

### 2.2.4 Analysis of DNA concentration and purity

DNA concentration and purity were determined by spectrophotometric analysis using a NanoDrop ND-1000 system. One absorbance unit at 260 nm corresponds to 50 ng/µl of double-stranded DNA. By measuring the absorbance at 260 nm the DNA concentration of the sample can then be calculated. DNA purity is assessed using the A260/A280 ratio. As proteins absorb strongly at 280 nm (owing mostly to tryptophan and tyrosine residues), protein contamination results in a ratio below the ideal value of 1.8 being observed. The device was always blanked with 18.2 Ω water or a buffer appropriate to the sample being analysed.

### 2.2.5 AT cloning of PCR products

In cases where difficulty in cloning was suspected to be the result of inefficient restriction digestion of PCR amplicon ends, TA cloning was used (Sambrook & Russell, 2001). This

system takes advantage of the single dA overhang that Taq polymerases introduce to the 3' end of PCR products. A corresponding 3' dT overhang on the cloning vector hybridises to the dA, and following DNA ligase treatment, a recombinant plasmid containing the amplicon sequence results. If the PCR amplicons contain restriction endonuclease sites, the insert can be cut out of the TA cloning plasmid. Alternatively, sites in the vector multiple cloning site, which flank the cloning site, may be used. If the fragment is cut out of this recombinant vector, it is definitely digested at both ends, which greatly facilitates cloning.

Most TA cloning vectors contain a β-galactosidase coding sequence, interrupted by the break in the plasmid (where the dT overhangs are situated). This property enables a convenient blue-white screening procedure to distinguish vector self ligation from successful recombinant vector formation. If self ligation occurs an intact β-galactosidase coding sequence is formed (negative), but when a gene is inserted between the dT overhangs, the β-galactosidase coding sequence is disrupted (positive). These situations can be distinguished by using the chromogen X-Gal, which is converted to an insoluble blue pigment by the β-galactosidase enzyme. Negative colonies, which express the enzyme, will therefore develop a blue colour. Positive colonies will not have β-galactosidase activity, and will thus remain white. Exceptions, where blue colonies represent positive clones, can occur if the insert is a multiple of three bases long, with no in-frame stop codons, such that the reading frame of the β-galactosidase gene stays open. This can result in residual β-galactosidase activity. Another explanation may be that the colony is not monoclonal, being founded by both a "blue" and a "white" cell.

AT cloning was performed using the pTZ57R/T vector system. Cleaned PCR product and vector were combined in a 3:1 molar ratio. The ligation reactions contained, in 30 µl, 18 pmol of vector and 54 pmol of PCR amplicon. Further, the reaction mixture contained 1X ligation buffer and 5 Weiss units of T4 DNA ligase. Ligation was performed at 4 °C for 24 hours or longer. The reaction could also be scaled down to 15 µl without loss of efficiency.

Of each ligation reaction 3 µl was used to transform electrocompetent *Escherichia coli* cells. Transformation and plating were performed as described in Section 2.2.10, but the agar plates also contained 0.5 mM IPTG for induction of β-galactosidase expression, and 0.002% X-Gal for blue-white screening.

The bovine GLYAT coding sequence was sub-cloned from this vector into the pColdIII expression vectors using the restriction, ligation, and transformation procedures described below. The main expression vector used in this study, pColdIII, is shown in Figure 2.1.

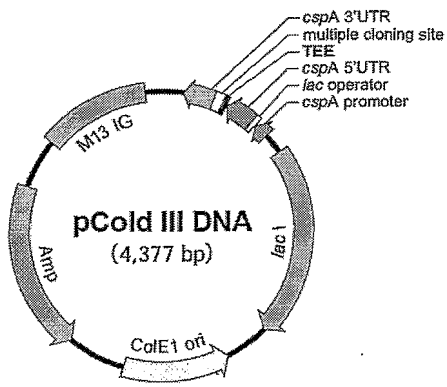


**Figure 2.1 Calculation of the amount of vector and insert DNA to use for ligation.** This figure demonstrates how to work out the amount of DNA, in nanograms, to use in order to have 18 pmol of vector and 54 pmol of insert DNA, a 3:1 ratio.

### 2.2.6 Restriction endonuclease digestions

Restriction enzymes were purchased from Fermentas, and the manufacturer's recommendations were followed for the digestion of plasmids and PCR amplicons. For preparative purposes, 100 µl reactions containing 15 µg plasmid DNA or 5 µg PCR amplicon were set up. The appropriate buffer for the enzyme used (Table 2.3) was added to a 1X final concentration. The reaction mixtures contained 20 units of enzyme per microgram of DNA. Unless otherwise stated, the reactions proceeded at 37 °C from a minimum of six hours to overnight.

After digestion, the DNA was recovered by means of ethanol precipitation (Sambrook & Russell, 2001). Sodium acetate was added to a final concentration of 0.5 M, and absolute ethanol was added to a final concentration of 75%, followed by vortexing and incubation on ice for 10 minutes. The mixtures were then centrifuged at 16 000 g for 20 minutes. The supernatant was decanted and the precipitate washed with 70% ethanol, then dried in a Speed-vac. The DNA was then dissolved in 60 µl of water in the same tube by incubation at 65 °C for 10 minutes. The second buffer and restriction enzyme were then added and the reaction made up to 100 µl with water. This digestion proceeded exactly as the first.

After the second digestion, the desired DNA fragment was purified using agarose gel electrophoresis.

**Table 2.3: Restriction enzymes and buffers used**

| Restriction enzyme | Recognition sequence | Buffer used | Conditions for double digestion |
|---|---|---|---|
| NdeI | CAT ATG | Buffer R | n/a |
| XhoI | CTC GAG | Buffer O | n/a |
| KpnI | GGT ACC | Buffer KpnI | n/a |
| HindIII | AAG CTT | Buffer HindIII | n/a |
| NdeI and XhoI | CAT ATG and CTC GAG | Buffer O | Use a twofold excess of NdeI |

Restriction digestion was often used to screen plasmids for presence of an insert gene. For this purpose double digestions (using two restriction endonucleases simultaneously) were usually performed. Fermentas restriction enzymes work with a system of five buffers that vary, among other things, in concentration of sodium and potassium ions. The buffer is chosen that gives the highest activity and least non-specific recognition. When double digestions are done, a compromise is made. The buffer is chosen that results in the least non-specific recognition for both enzymes and an excess of the disadvantaged enzyme is added. There is a tool on the Fermentas web site (www.fermentas.com) that suggests the optimal conditions for performing double digestions with all the possible combinations of enzymes.

Double digestions for screening were performed in 20 µl reactions containing the appropriate enzymes (10 units, or a twofold excess) and buffer suggested by the double-digest tool. The reactions contained 1 µl of plasmid miniprep (please refer to Section 2.2.11 below), and were incubated at 37 °C for four hours or longer. The reactions were finally analysed by means of agarose gel electrophoresis.

### 2.2.7 Gel purification of desired DNA fragments or products

Using electrophoresis on a 1% agarose gel as described above, a desired DNA fragment can be separated from other fragments of different size (Sambrook & Russell, 2001). After

electrophoresis the correct band is simply excised from the gel and the DNA recovered using spin column technology. The Machery Nagel Nucleospin II kit was used for this purpose. A buffer (NT) is added to the gel, which facilitates the dissolution of agarose at 50 °C. The solution is then passed through a spin column containing a silica membrane. This membrane binds DNA in the presence of the chaotropic salts present in the NT buffer. Enzymes and most contaminants such as dNTPs, salts, primers and agarose do not bind and pass through the column. The membrane is then washed with a buffer (NT3) containing ethanol. DNA remains bound to the column whilst the remaining contaminants (such as ethidium) are washed off. After drying the column by centrifugation for three minutes at 10 000 g the DNA was eluted with 50 µl of water preheated to 80 °C.

### 2.2.8 Ligation reactions

Ligations of digested plasmid vectors and insert genes were performed as described in the literature (Sambrook & Russell, 2001). Reaction mixtures contained, in 30 µl, 18 pmol digested vector, 54 pmol digested insert DNA (a 1:3 vector to insert molar ratio), 5 Weiss units of T4 DNA ligase, and 1X ligation buffer.

The procedure for setting up the ligation reactions was as follows. The plasmid and insert DNA were added to a 250 µl tube. The mixture was then heated to 65 °C for 1 minute, followed by slow cooling of the mixture to 4 °C (using a 30% ramp rate setting with an Eppendorf thermal cycler). This step serves to denature any unwanted self annealing that may have occurred. The slow cooling facilitates effective annealing of the cohesive DNA ends. As only the plasmid and insert are mixed at this point, DNA concentrations are high, which facilitates inter-molecular annealing as opposed to intra-molecular annealing. After this annealing step the buffer and T4 ligase are added, and the reaction made up to 30 µl with water. Ligation reactions were incubated at 4 °C overnight or longer.

### 2.2.9 Preparation of electrocompetent *Escherichia coli* cells

Electroporation is a very fast and efficient means of transforming bacteria with plasmid DNA. Preparation of the electrocompetent cells is also much easier than preparation of

chemically competent cells. Cells are basically grown to mid log phase and then washed several times with water and 10% glycerol to remove salts. The cells are then simply mixed with DNA, placed in a chilled electroporation cuvette and an electric pulse applied (Sambrook & Russell, 2001). The process will be briefly described.

A 50 ml culture of LB medium was inoculated with a single colony from a plate onto which an *Escherichia coli* glycerol stock was streaked. The culture was grown overnight without antibiotics at 37 °C, with vigorous shaking to ensure adequate aeration. In the morning a 200 ml LB culture was inoculated with 2 ml of the overnight culture. The culture was grown at 37 °C with shaking until an optical density at 600 nm of 0.4 to 0.6 was reached. The cells were then harvested by centrifugation (in four 50 ml conical tubes) at 3000 g for 15 minutes. From here all steps were performed at a temperature below 4 °C. The supernatant was discarded and the cell pellets resuspended in an equal volume of ice cold sterile deionised water. The centrifugation was repeated, followed by resuspension in an equal volume of ice cold 10% glycerol. This step was repeated, and after centrifugation the cells from all four tubes were combined in 2 ml of ice cold 10% glycerol. Of this, 60 µl was placed in a pre-chilled electroporation cuvette, and a pulse of 1.8 kV applied for 1 ms to test the cells. If arcing occurred, the glycerol wash step was repeated until this was no longer the case. The cell slurry was then dispensed in 50 µl aliquots into pre-chilled 500 µl tubes. The cells were then snap-frozen in a bath of liquid nitrogen, and transferred to storage at -80 °C.

### 2.2.10 Transformation of electrocompetent *Escherichia coli* cells

Transformation of electrocompetent *Escherichia coli* cells was performed as described in the literature (Sambrook & Russell, 2001). Usually, 10 µl of a 30 µl ligation reaction or 100 pico-grams of super helical plasmid DNA was used for a transformation. A BioRad GenePulser Xcell electroporator and GenePulser cuvettes were used.

Frozen electrocompetent cells (50 µl aliquots) were removed from storage at -80 °C and thawed on ice. The DNA sample was then added and gently mixed. The cell slurry was transferred to a pre-chilled electroporation cuvette, making sure not to form any air

bubbles. A pulse of 1.8 kV was applied for 1 ms. As soon as possible after pulsing, 1 ml of SOC medium was added, and the cells were allowed to recover at 37 °C with gentle shaking for one hour. This allows expression of the antibiotic resistance genes before selection using antibiotics is applied.

Usually, 200 µl of the cell mixtures were spread out on LB agar plates containing 100 µg/ml ampicillin. The plates were left for fifteen minutes to absorb the liquid and then incubated upside-down at 37 °C for 16 hours.

### 2.2.11 Screening of colonies of transformed bacteria

Colonies of transformed cells were screened for presence of the desired insert using either restriction analysis or PCR amplification.

For screening by means of restriction analysis, McCartney bottles containing 5 ml of LB medium (containing 100 µg/ml ampicillin) were inoculated with a colony picked from the plate. For short term preservation, the colonies were streaked onto a master plate prior to inoculation. The cultures were incubated overnight at 37 °C, shaking at 180 rpm.

Plasmid DNA was then isolated from 2 ml of culture as follows. The cells were harvested by centrifugation at 16 000 g for 2 minutes. The supernatant was discarded, and 250 µl of STET buffer (8% sucrose, 5% TritonX-100, 50 mM EDTA, 50 mM Tris) added. The cells were resuspended by vortexing, and boiled at 98 °C for one minute. The boiled lysates were immediately centrifuged for 8 minutes at 16 000 g. The pellet was removed with a toothpick and 5 µl of a 10 mg/ml ribonuclease A solution added. The mixture was incubated at room temperature for ten minutes. DNA was precipitated by addition of 250 µl isopropanol, followed by centrifugation at 16 000 g for 10 minutes. The supernatant was discarded and the DNA washed with 600 µl of 70% ethanol. The DNA was dried in a Speed-vac and dissolved in 20 µl of 1/10 TE buffer by incubation at 65 °C for 10 minutes. Of this plasmid preparation 1 µl was digested using restriction enzymes as described in Section 2.2.6, and the fragments analysed using agarose gel electrophoresis.

### 2.2.12 Long term storage of transformed bacteria

Positive colonies were prepared for long term storage by adding glycerol to a final concentration of 15% to an overnight culture (Sambrook & Russell, 2001). This was done by combining in an Eppendorf tube, 810 µl of culture and 190 µl of 80% glycerol. The stocks were then transferred to -80 °C storage.

### 2.2.13 Midi-preparation of plasmid DNA

Plasmid DNA for sequencing and other manipulations was prepared using the PureYield plasmid midiprep kit from Promega. The instructions of the manufacturer were followed. For standard purification, 50 ml LB cultures containing 100 µg/ml ampicillin were inoculated with the desired clone and incubated at 37 °C overnight with shaking. The cells were harvested by centrifugation at 2000 g for 15 minutes. The cells were then resuspended and lysed using the buffers provided. Proteins and genomic DNA are denatured by the dodecyl sulfate and high pH, while the closed-circular plasmid molecules remain in double-stranded conformation (Sambrook & Russell, 2001). Most proteins and high molecular weight chromosomal DNA were then precipitated by addition of the ammonium acetate-acetic acid buffer, which neutralises the pH and precipitates SDS-protein complexes. After removal of the precipitate, the cleared lysate is passed through a DNA binding column (the principles are the same as for the DNA clean-up columns discussed in Section 2.2.7), and the column washed with the endotoxin removal and column wash buffers. The column is dried by centrifugation at 1500 g for 10 minutes. DNA was eluted with 600 µl of water. The plasmid preparations were analysed with the NanoDrop ND-1000 system.

### 2.2.14 DNA sequence determination

To confirm that a recombinant plasmid contained the gene of interest without any sequence aberrations, Sanger sequencing was used. Samples were sent to the DNA sequencing laboratory of the Central Analytical Facility of the University of Stellenbosch. DNA sequence electrophoretograms were analysed using FinchTV version 1.40

(www.geospiza.com/finchtv) and ClustalX (Larkin et al., 2007) was used to align sequences to reference sequences.

### 2.2.15 Expression of bovine GLYAT from pColdIII and chaperone co-expression

For co-expression of the bovine GLYAT proteins with the Takara chaperones, *Escherichia coli* cells must first be transformed with both the chaperone co-expression plasmid and the GLYAT expression vector. Cells were first transformed with the pGTf2 plasmid for co-expression of the GroEL-GroES-TF chaperone team as described in Section 2.2.10 (selecting with 20 µg/ml chloramphenicol). Cells containing this plasmid are then made competent again, and transformed with the second plasmid, now selecting with both 100 µg/ml ampicillin and 20 µg/ml chloramphenicol. Co-transformed colonies were used to make glycerol stocks which were used to inoculate cultures for expression studies.

The general expression protocol was as follows: the desired strain (Origami cells transformed with the desired expression vector and the pGTf2 plasmid) was used to inoculate 50 ml of LB medium containing 100 µg/ml ampicillin and 20 µg/ml chloramphenicol and the culture was incubated overnight at 37 °C, shaking at 180 rpm. The cells were harvested by centrifugation at 2000 g for 15 minutes, and resuspended in 200 ml of fresh medium containing 50 µg/ml ampicillin and 20 µg/ml chloramphenicol. Tetracycline was added to a final concentration of 100 ng/ml for induction of chaperone expression. The culture was incubated at 37 °C for one hour to allow chaperone expression to take place. The culture was then incubated at 15 °C with gentle shaking for 30 minutes. Induction of GLYAT expression was then induced by addition of IPTG to a final concentration of 0.05 mM, and continuing incubation at 15 °C for 1 to 24 hours.

For expression without chaperone co-expression, the same procedure was followed, except that cells were transformed with only the GLYAT expression plasmid and only ampicillin was used.

### 2.2.16 Cell lysis using the BugBuster protein extraction reagent

Cells were harvested by centrifugation at 2000 g for 10 minutes. BugBuster protein extraction reagent containing rLysozyme and Benzonase nuclease, prepared according to the manufacturer's instructions, was then added (5 ml per gram of wet cell mass). The cells were resuspended by gentle vortexing and incubated at room temperature for 5 minutes to allow for cell lysis. At this point a sample was taken for the total protein fraction. The insoluble material was then removed by centrifugation at 16 000 g for 20 minutes in a 4 °C centrifuge.

### 2.2.17 His tag purification and ultra filtration

The Protino Ni-TED 2000 kit was used for affinity purification of histidine tagged proteins. The cleared cell lysates containing the proteins of interest were passed through Protino Ni-TED 2000 columns that were pre-equilibrated with buffer LEW. The column was then washed four times with 3 ml of buffer LEW. The his-tagged proteins were then eluted with three 4 ml volumes of elution buffer (EB). Imidazole hydrochloride was added to a final concentration of 20 mM to the cell lysates and buffer LEW, to prevent non specific binding to the columns (Verma et al., 2005).

Eluate fractions were pooled and concentrated by means of ultra filtration. Vivaspin 20 ultra filtration devices with 10 kDa molecular weight cut off were used. The pooled eluate was added to the device and then centrifuged for 15 to 30 minutes at 8 000 g. Centrifugation was stopped when the volume was decreased from 12 ml to approximately 1000 µl. To exchange the buffer for storage, 10 ml of 20 mM TrisCl was added after concentration. The volume was then again reduced to approximately 500-1000 µl and the concentrate transferred to storage at 4 °C. All purification steps were performed below 4 °C.

### 2.2.18 Isolation and partial purification of GLYAT from bovine liver

Bovine liver (100 g) was obtained fresh from the Potchefstroom abattoir and homogenised in a blender in 400 ml of 0.13 M KCl, pH 8.0. Centrifugation at 600 g for 10 minutes was

used to remove nuclei and large cell debris. The supernatant was centrifuged for another 10 minutes at 9000 g to pellet the mitochondria. The mitochondrial pellet was resuspended in 50 ml of 0.13 M KCl and subjected to three cycles of freezing to -70 °C, and then thawing. This process disrupts the mitochondrial inner and outer membranes, resulting in release of the contents into the buffer. The solution was then centrifuged for 2 hours at 35 000 g in an ultracentrifuge.

Further enrichment was achieved by ammonium sulfate precipitation. The protein concentration of the supernatant from the previous step was adjusted to 20 mg/ml and ammonium sulfate added to a final concentration of 40% (w/v). After the ammonium sulfate had dissolved, the precipitate was collected by centrifugation at 10 000 g for 15 minutes. The pellet was discarded and more ammonium sulfate added to a final concentration of 60% (w/v). After the ammonium sulfate had dissolved, the precipitate was again collected by centrifugation at 10 000 for 15 minutes. The precipitate was dissolved in 4 ml of a buffer containing 0.1 M KCl and 20 mM TrisCl. The resulting solution was dialysed against 500 ml of 20 mM TrisCl buffer. The solution was divided into 100 µl aliquots and frozen at -70 °C (van der Westhuizen et al., 2000).

### 2.2.19 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SDS-PAGE, as described in the literature, was used for routine analysis of protein expression and purification procedures (Laemmli, 1970, Sambrook & Russell, 2001). In short, samples are boiled with SDS to form complexes with a net negative charge and are then separated according to size by migration through a cross-linked polyacrylamide gel. Finally, the proteins are visualized by staining with Coomassie brilliant blue.

Separating gels generally had a final concentration of 10% acrylamide, unless indicated to be 15%. The composition of the separating gels was 10% acrylamide, 0.27% bisacrylamide, 375 mM TrisCl (pH 8.8) and 0.1% SDS. The composition of the stacking gels was 3.9% acrylamide, 0.1% bisacrylamide, 375 mM Tris-Cl (pH 6.8) and 0.1% SDS. Polymerization was catalysed by addition of 0.008% TEMED and 0.08% ammonium persulfate.

The separating gel was prepared by mixing all the components in an Erlenmeyer flask before addition of the persulfate and TEMED. The gel was then poured into an assembled Bio-Rad Mini Protean gel casting apparatus (70 x 76 mm). The gel was then overlaid with water-saturated isobutanol and left to set for about an hour at room temperature. The butanol was then poured off, and the surface of the gel dried with filter paper. The stacking gel was then prepared and poured on top of the separating gel, followed by insertion of a ten well comb. Again the gel was left to set, after which it was immediately used.

Protein samples were prepared by combining 5 µl of sample with 5 µl of 4X protein loading buffer (please see Appendix I), 9 µl of water and 1 µl of 20X reducing agent. The samples were then mixed and boiled for 5 minutes at 98 °C. Unless otherwise stated, 10 µl of this mixture was loaded onto the gel. For size estimation 5 µl of a protein molecular size marker mixture (Fermentas SM1183) was always loaded in one lane. The loaded gel was then electrophoresed in 1X TGS buffer at a constant current of 30 mA using a Bio-Rad PowerPac Basic system. Electrophoresis was for about 40 minutes, or until the pink dye front reached the bottom of the gel.

The electrophoresed gels were removed from the glass plates, rinsed with water and then submerged in Coomassie gel staining solution with gentle shaking for 60 minutes. The gels were then removed from the staining solution and rinsed with a small volume of methanol-acetic acid gel destain solution before submersion in more destain solution. The destaining gel was gently shaken, with occasional exchange of the destain solution until the gels were no longer blue in colour. The stained gels were placed between two plastic sheets and digitised using an HP digital document scanner.

### 2.2.20 GLYAT enzyme activity assays

Experimental samples were routinely assayed for glycine N-acyltransferase activity using a colorimetric reaction for the detection of coenzyme A (Kolvraa & Gregersen, 1986). Coenzyme A is one of the products of the GLYAT reaction and is amendable to colorimetric analysis using the chromogen DTNB. The free thiol group of the liberated

coenzyme A reacts with DTNB to form a yellow compound that absorbs strongly at 412 nm. By monitoring the increase of absorbance at 412 nm, the progress of a GLYAT reaction can be followed.

Assay mixtures were composed of 25 mM TrisCl, pH 8.0, 100 µM benzoyl-coenzyme A, 0.1 mM DTNB, 200 mM glycine and either 1 or 2 µl of crude bacterial lysate or 1 to 5 µg of protein in a final volume of 100 µl. The concentration of potassium ions was kept at 10 mM as far as possible. Positive controls contained GLYAT purified from bovine liver, and negative controls were set up by omitting glycine from the reaction mixture.

The protein samples were placed in a 96-well plate. The reaction was initiated by addition of the rest of the reaction mixture (prepared in the form of a master mix). This allows for fast and easy mixing of the enzyme and reaction mixture just before measurements begin. The reactions proceeded at 37 °C, with measurements being made every 30 seconds for 10 minutes, unless stated to be longer. A Biotech plate reader and accompanying Gen5 software were used for the analysis.

### 2.2.21 Determination of protein concentration using bicinchoninic acid solution

For determination of the protein content of samples, the bicinchoninic acid method was used (Sambrook & Russell, 2001). This method relies on the colorimetric change that takes place when copper ions ($Cu^{2+}$) are reduced on the surface of proteins to $Cu^{1+}$ ions, which then bind to the bicinchoninic acid, forming a complex that absorbs strongly at 516 nm. The resulting absorbance at 560 nm is proportional to protein concentration, given that concentrations are within the linear range.

Bicinchoninic acid solution and copper sulfate solution (Appendix I) were mixed in a 50:1 v/v ratio. Of this mixture 200 µl was added to 10 µl of protein sample in a 96-well plate. The plate was gently shaken to mix the reactions, which were then incubated at 37 °C for 20 minutes. A standard curve of 2, 4, 6, 8, and 10 µg of protein was always set up for purposes of quantification, using bovine serum albumin (BSA). All reactions were done in triplicate, and the absorbance at 560 nm determined using a Biotech plate reader. The

accompanying Gen5 software was used to plot the standard curve and quantify the protein content of the samples automatically.

### 2.2.22 Calculation of kinetic parameters

For determination of the kinetic parameters, enzyme assays were performed using the Uvicon XS spectrophotometer, as the larger reaction volumes used and the constant light path length of 1 cm make it more accurate than the plate reader which is used for routine assays (Section 2.2.20). For determination of kinetic parameters, various assays were carried out at different substrate concentrations. The initial velocities were used to draw double reciprocal plots. From a double reciprocal (Lineweaver-Burk) plot, the kinetic parameters can be read; a linear regression analysis is performed using the experimentally determined values, and the inverse values of $K_M$ and $V_{max}$ read from the horizontal and vertical intercepts of the plot, respectively. The SigmaPlot 11.0 program was used with the Enzyme Kinetics module (version 1.3) to automatically plot the experimental data and to calculate the kinetic parameters and the respective standard deviations.

The reaction mixtures were 400 µl in volume, and consisted of 25 mM TrisCl, pH 8.0, 0.1 mM DTNB, and varying concentrations of substrate. The amount of enzyme added is described in Section 2.3.8. For the substrate concentrations used, please refer to Section 2.3.8. The assays were carried out at 30 °C (Palmer, 2001), and the change in absorbance (412 nm) over the first three minutes was used to calculate the initial velocities. The change in absorbance per minute was converted to nmol/min by using the extinction coefficient of 13.6 $mM^{-1}cm^{-1}$ for the yellow compound formed. Thus, a rate of 0.1 absorbance units per minute corresponds to 2.941 nmol/minute. All assays were performed in triplicate; the triplicates were experimentally independent of each other, as the whole reaction master mix was prepared fresh for each replicate.

The reactions were set up as follows: The acyl-coenzyme A substrates were made up to 8X the desired concentration, and 50 µl of this solution transferred to the cuvette. This large volume decreases the effect of pipette errors. A master mix would then be set up containing the glycine, buffer, DTNB and enzyme preparation. Of this master mix 350 µl

would then be added rapidly to the cuvette immediately before starting spectrophotometric readings. This automatically mixes the two components without wasting time, as it is important to start spectrophotometric reading as soon as possible.

## 2.3 Results and discussion

### 2.3.1 Cloning bovine GLYAT into modified pColdIII expression vectors

In order to investigate the properties of recombinant bovine GLYAT, it was cloned into a set of three modified pColdIII expression vectors encoding C-terminal histidine tags, to facilitate purification of the recombinant proteins. These tags have spacers between the protein and the histidine tag, serine-glycine linkers of different length, which may enhance the efficiency of purification (Loughran et al., 2006).

In order to clone the coding sequence into the expression vectors, the sequence was PCR amplified using primers containing NdeI and XhoI restriction enzyme sites to facilitate directional cloning (Table 2.2 lists the primers). The PCR amplification was done as described in Section 2.2.2, and the amplicon was visualised on an agarose gel, shown in Figure 2.2. The amplification worked well, with only a little non-specific amplification being visible on the agarose gel. The amplicon was cleaned and digested with the restriction enzymes, as described in Sections 2.2.6 and 2.2.7. Cloning of this digested fragment into the pColdIII expression vectors was unsuccessful. It was suspected that the restriction enzymes did not completely digest the amplicon ends, encumbering the ligation reaction. This problem was solved by first cloning the PCR amplicon into the pTZ57R/T TA cloning vector, and then excising the insert from the recombinant plasmid. This ensures that the insert is digested at both ends, which facilitates sub-cloning into other vectors. The digested insert and pColdIII vectors were visualised on an agarose gel, shown in Figure 2.3. The excised fragment was ligated into the pColdIII vectors. Transformation yielded several colonies of transformants.

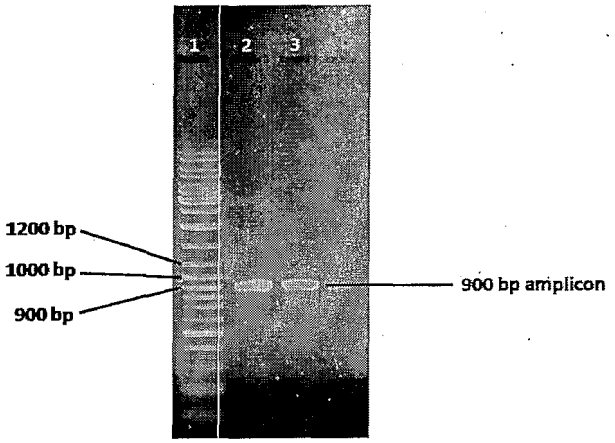


**Figure 2.2 Agarose electrophoretic analysis of the PCR amplification of bovine GLYAT.** Lanes: 1) 5 µl of O'GeneRuler DNA marker; 2) 900 bp amplicon of a PCR with annealing at 68 °C; 3) amplicon of a PCR with annealing at 70 °C.

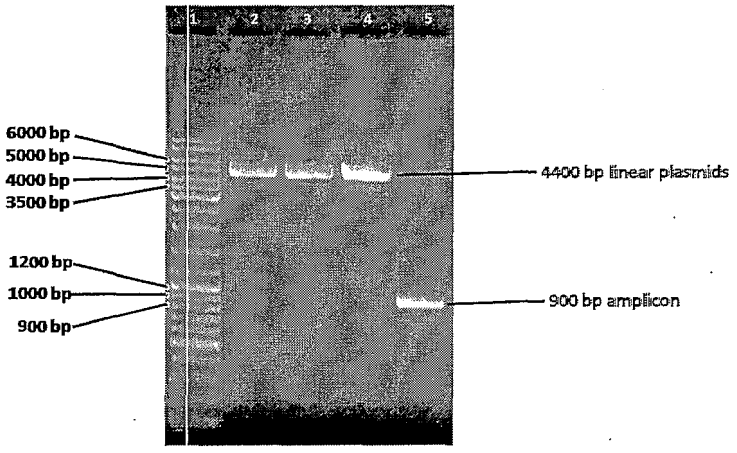


**Figure 2.3 Agarose gel electrophoretic analysis of the pColdIII vectors and bovine GLYAT amplicon after digestion with NdeI and XhoI restriction enzymes.** Lanes: 1) 5 µl of O'GeneRuler DNA marker; 2) digested pColdIII-A; 3) digested pColdIII-E; 4) digested pColdIII-EH; 5) digested bovine GLYAT PCR amplicon.

After ligation and transformation, colonies were screened for desired recombinant plasmids using restriction enzyme digestions, as explained in Section 2.2.11. A colony was considered positive if an excised fragment of approximately 900 bp could be seen on an agarose gel. An example of such a screening is shown in Figure 2.4. Plasmid isolated from positive colonies was sequenced to confirm that the bovine GLYAT sequence had been correctly cloned, without any sequence differences. Sequencing showed that the bovine

GLYAT insert had been successfully cloned into the pColdIII-A, pColdIII-E, and pColdIII-EH expression vectors without sequence aberrations.

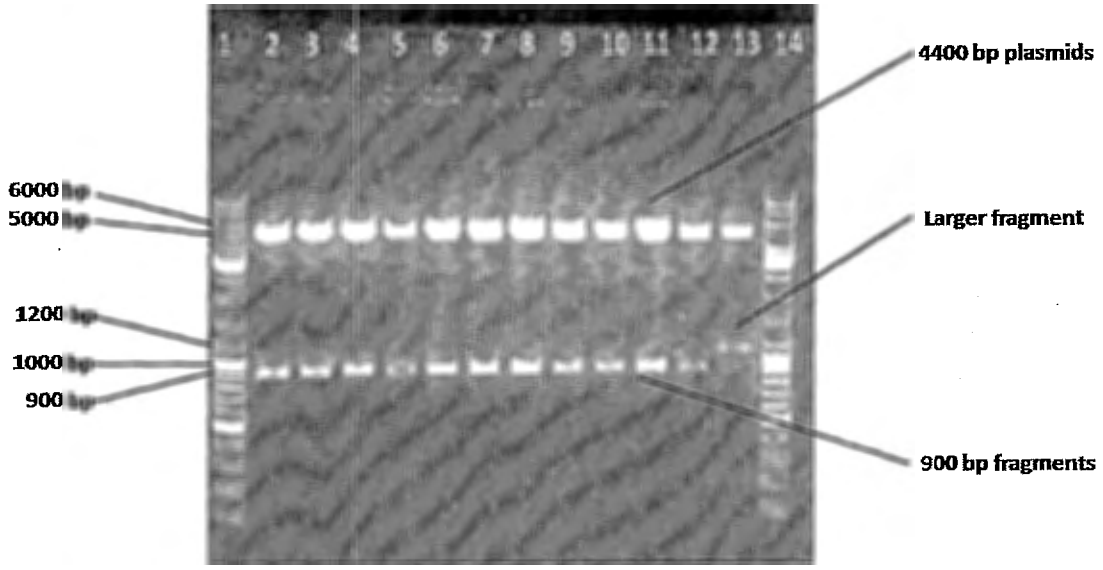


**Figure 2.4 Example of colony screening by means of restriction enzyme digestion using NdeI and XhoI restriction enzymes.** Agarose gel electrophoretic analysis of a restriction enzyme screening for colonies containing the desired recombinant plasmid. Successful recombinant plasmids are identified by excision of the 900 bp bovine GLYAT insert. Lanes: 1) 5 µl of O'GeneRuler DNA marker; 2-12) positive result with a 900 bp fragment excised from the vector; 13) negative result showing an insert larger than 900 bp; 14) 5 µl of O'GeneRuler DNA marker.

## 2.3.2 Bacterial expression of recombinant bovine GLYAT from pColdIII

The first aim of this project was to confirm that bovine GLYAT, without any N- or C-terminal fusions, can be bacterially expressed in a biologically active form, using the pColdIII and GroEL-GroES-TF chaperone co-expression systems discussed in Section 1.8.

### 2.3.2.1 Optimisation of conditions for induction of chaperone expression

Before attempting expression of recombinant bovine GLYAT was attempted, the conditions for chaperone expression were first optimised. It was argued that in order to achieve optimum results, the chaperones should be significantly over-expressed. As demonstrated on the SDS-PAGE gel shown in Figure 2.5 (lanes 4 and 5), significant over expression of

chaperones was not achieved using 10 ng/ml of tetracycline, as suggested by the product manual. By increasing the concentration of tetracycline used for induction to 100 ng/ml, chaperone expression was significantly increased, as visualised on the SDS-PAGE gel shown in Figure 2.5 (lanes 6 and 7). This concentration of tetracycline was not significantly inhibitory to bacterial growth and was used for further work.

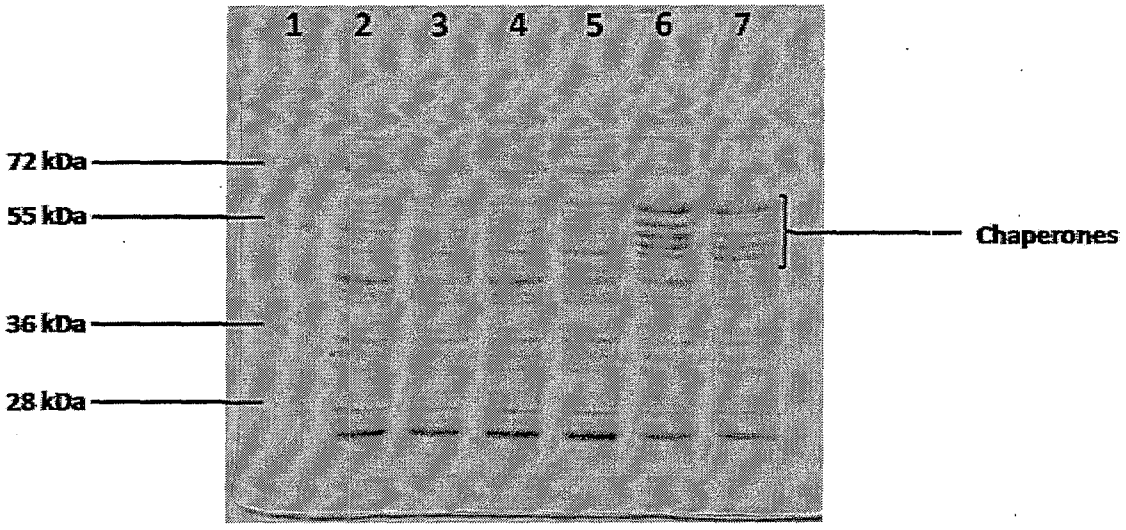


**Figure 2.5 SDS-PAGE analysis of induction of chaperone expression at different tetracycline concentrations.** Lanes: 1) 5 µl of PageRuler protein marker; 2) 0 ng/ml tetracycline, total fraction; 3) 0 ng/ml tetracycline, soluble fraction; 4) 10 ng/ml tetracyclinel, total fraction; 5) 10 ng/ml tetracycline, soluble fraction; 6) 100 ng/ml tetracycline, total fraction; 7) 100 ng/ml tetracycline, soluble fraction.

### 2.3.2.2 Optimisation of the conditions for expression of recombinant bovine GLYAT

A pilot experiment for the expression of recombinant bovine GLYAT was performed by using 1.0 mM IPTG to induce expression for 24 hours. The recombinant protein expressed very well, as shown on the SDS-PAGE gel in Figure 2.6. However, the majority of the expressed bovine GLYAT was insoluble, as visualised on an SDS-PAGE gel (Figure 2.6).

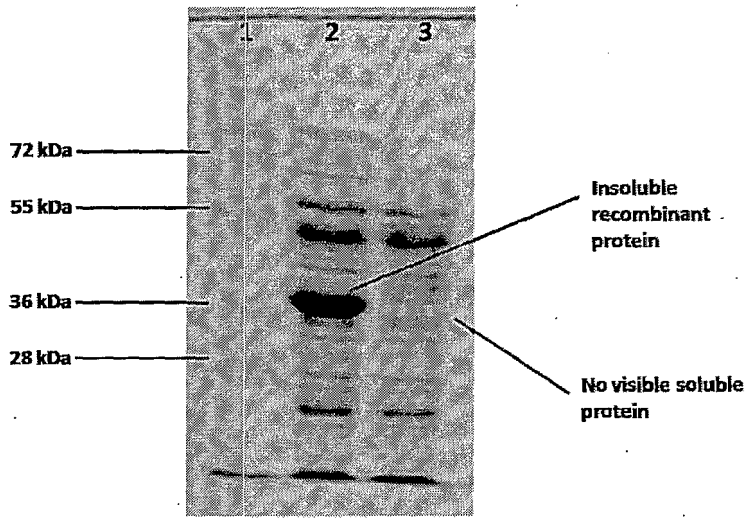


**Figure 2.6 SDS-PAGE analysis of the expression of recombinant bovine GLYAT from the pColdIII vector.** Lanes: 1) 5 µl of PageRuler protein marker; 2) total protein fraction; 3) soluble protein fraction.

It was reasoned that by slowing down the rate of expression, newly formed recombinant GLYAT polypeptides would have more time for proper folding. This was attempted as it could increase folding efficiency, reduce aggregation and increase the yields of soluble protein. The IPTG concentration used for induction, and the time of induction, were both varied in an attempt to increase the yield of soluble recombinant protein.

*The effect of IPTG concentration on the yield of enzymatically active recombinant GLYAT*

To test the effect of IPTG concentration, various IPTG concentrations were used to induce expression of the recombinant bovine GLYAT for 4 hours. The total amount of recombinant protein expressed was the same for concentrations of 0.05 mM, 0.1 mM, 0.2 mM, 0.5 mM and 1.0 mM of IPTG. This is visualised on the SDS-PAGE gel shown in Figure 2.7A. The soluble protein fractions are also visualised on the gel in Figure 2.7B, and it can be seen that there is no obvious difference in the levels of soluble protein expressed at different IPTG concentrations.

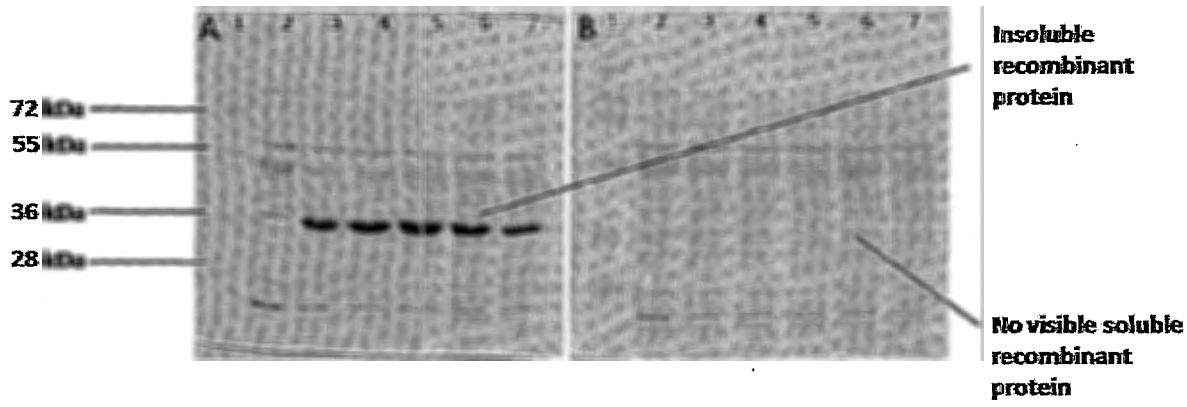


**Figure 2.7 SDS-PAGE analysis of the effect of IPTG concentration on the expression of recombinant bovine GLYAT.** A) total protein fractions. B) soluble protein fractions. Lanes: 1) 5 µl of PageRuler protein marker; 2) un-induced control; 3) 0.05 mM IPTG; 4) 0.1 mM IPTG; 5) 0.2 mM IPTG; 6) 0.5 mM IPTG; 7) 1.0 mM IPTG.

The resolution of SDS-PAGE analysis is not sufficient for judging slight differences in soluble protein expression. For this reason, a more sensitive analysis was performed. Western blotting was not possible, as an antibody for detection of bovine GLYAT was not available. Instead, GLYAT enzyme activity assays were performed, in triplicate, on the soluble fractions of the bacterial lysates, as described in Section 2.2.20. To ensure comparability of the results, the protein content of each lysate was first determined so that a standard amount of 5 µg of protein could be used for each assay. Background activity, resulting from glycine-independent benzoyl-coenzyme A hydrolysis, limits the value of these assays, but comparison to an un-induced control corrected for this. As shown in Figure 2.8, the level of enzyme activity is approximately the same for all IPTG concentrations investigated. However, the difference in levels of enzyme activity between the induced and un-induced samples was significant, as demonstrated in Figure 2.8. From this it can be concluded that the yield of soluble, enzymatically active recombinant GLYAT expressed is independent of the IPTG concentration used for induction. A concentration of 0.05 mM IPTG was thus used for further studies, but this choice was arbitrary.

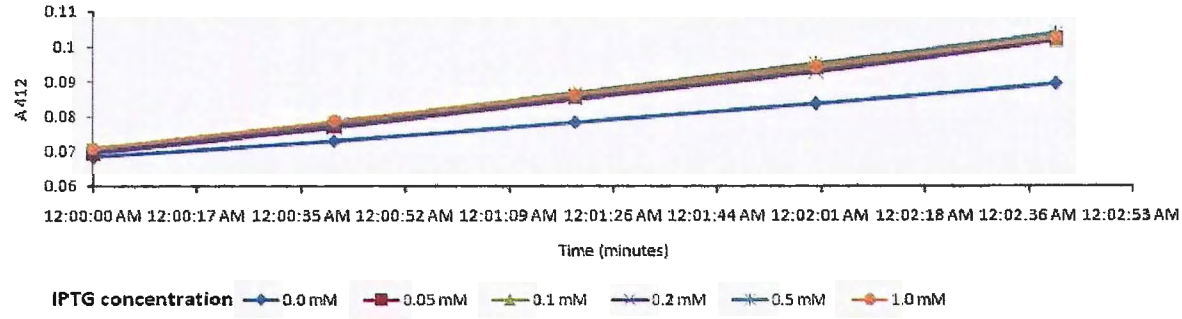


**Figure 2.8 Enzyme assays of recombinant bovine GLYAT expressed using different IPTG concentrations for induction.** The graph shows the change in absorbance at 412 nm with time, using 5 µg of bacterial lysate protein per assay. The legend indicates the concentration of IPTG used.

*The effect of induction time on the yield of enzymatically active recombinant GLYAT*

Time of induction was also varied in an attempt to obtain more soluble protein. Protein aggregation is, like all chemical reactions, concentration dependent. If a protein is largely insoluble and prone to aggregation, this aggregation might be slowed by reducing the concentration of the protein in the cell. It was thus attempted to decrease the amount of recombinant GLYAT expressed by reducing the induction time. Several cultures were thus induced, using 0.05 mM IPTG, for 1, 2, 4, 6 and 20 hours. As shown on the SDS-PAGE gel in Figure 2.9A, the amount of total protein expressed is lowest at 1 hour (lane 3), and increases gradually from 1 to 20 hours (lanes 4 to 7). The amount of soluble recombinant GLYAT visible on an SDS-PAGE gel is however not visibly influenced by the induction time, as shown in Figure 2.9B.

Again, this result was further assessed using the GLYAT enzyme activity assay. The assays were performed in triplicate, and contained 5 µg of soluble protein from each lysate. The result is shown in Figure 2.10. There were very small differences between the levels of enzyme activity for the different induction times. The level of enzyme activity for the 1 hour induction, was, as expected, the highest (Section 2.3.2.2). Strangely, however, the level of GLYAT activity is lowest for the 2 hour induction, and increases from 2 to 20 hours. This is difficult to explain, but it could simply reflect that with the longer induction times, higher proportions of the total cellular protein is represented by recombinant GLYAT (Figure 2.10). This experiment was repeated, and the result confirmed.

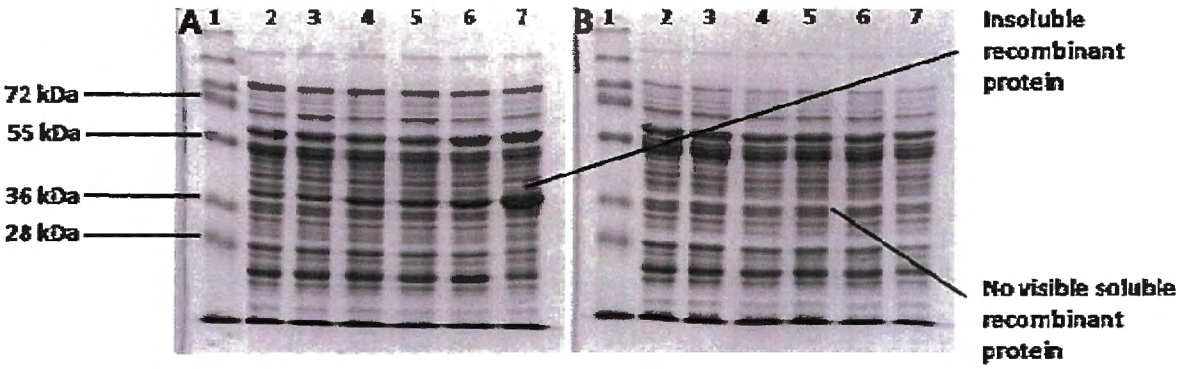


**Figure 2.9 SDS-PAGE analysis of the effect of induction time on recombinant bovine GLYAT expression.** A) total protein fractions; B) soluble protein fractions. Lanes: 1) 5 µl of PageRuler protein marker; 2) un-induced control; 3) induction for 1 hour; 4) induction for 2 hours; 5) induction for 4 hours; 6) induction for 6 hours; 7) induction for 20 hours.

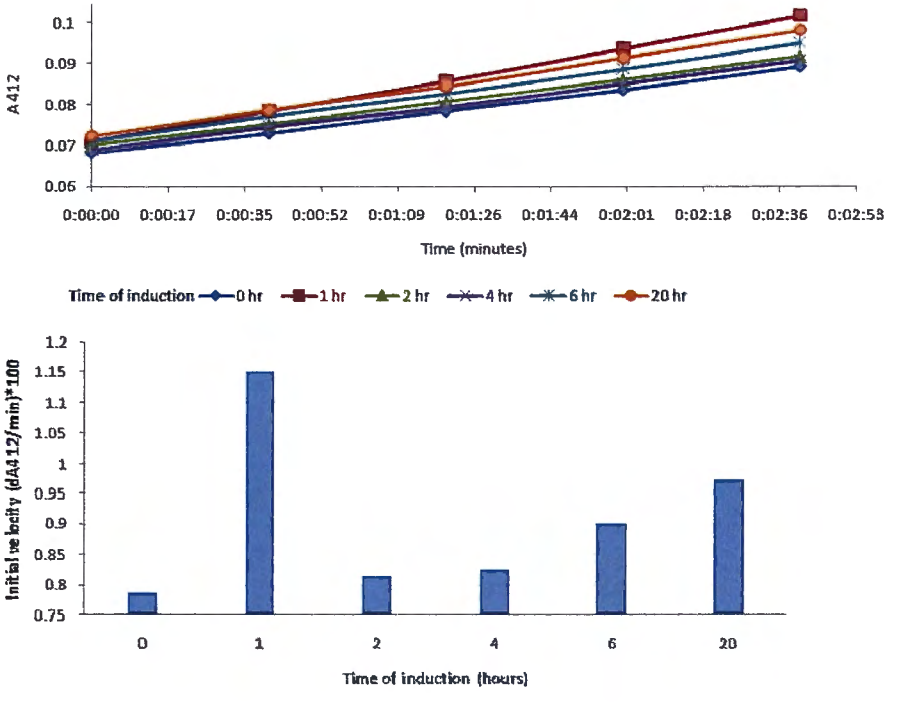


**Figure 2.10 Enzyme assays of recombinant bovine GLYAT expression induced for different lengths of time.** The graph shows the change in absorbance at 412 nm with time, using 5 µg of bacterial lysate protein per assay. The legend indicates the duration of the induction in hours. The bar chart indicates the initial velocity multiplied by 100 with induction time.

In summary, of the conditions tested, induction for 1 hour, using 0.05 mM IPTG for recombinant GLYAT expression and 100 ng/ml of tetracycline for induction of chaperone co-expression resulted in the highest levels of enzyme activity. However, the majority of the recombinant GLYAT expressed was still insoluble.

Since, to this point, experiments were performed using recombinant GLYAT expressed without any purification tags, the assays had to be performed on crude cell lysates. For more detailed analyses, a partially purified enzyme is needed. Expression and purification of a recombinant bovine GLYAT with a C-terminal histidine tag, to facilitate purification, is presented next.

### 2.3.3 Nickel affinity purification of a histidine tagged bovine GLYAT

For kinetic analysis, it is important to have an at least partially purified preparation of the enzyme under investigation (Palmer, 2001). Absolute homogeneity is usually not necessary, unless accurate reaction velocities (per mg of protein) need to be calculated.

To purify the recombinant bovine GLYAT enzyme, the protein had to be expressed with a C-terminal histidine tag to facilitate purification using immobilised nickel affinity chromatography. As discussed in Section 1.6.3, the tagged protein should bind tightly to a resin on which nickel is immobilised, allowing un-tagged proteins to be washed away before the tagged protein is eluted (Porath et al., 1975).

At first, a pilot experiment was carried out. Recombinant bovine GLYAT was expressed from pColdIII-E with a C-terminal histidine tag (this tag has no serine-glycine linker, see Table 2.1) The soluble fraction was passed through a Protino Ni-TED nickel affinity purification column, and purified as described in Section 2.2.17. After purification, SDS-PAGE analysis revealed that the purification was incomplete, as shown in Figure 2.11. Three major protein bands were visible on the gel in the region of the 36 kDa molecular weight marker, and several minor contaminating protein bands of various sizes were also visible. The identities of the proteins responsible for the three major bands are not known. It could not be deduced from size alone which band represented the enzymatically active recombinant bovine GLYAT. An activity assay was performed on this partially purified protein sample, showing significant enzyme activity (results not shown). This means that one of the bands must represent the enzymatically active recombinant bovine GLYAT

enzyme. The purification conditions had to be further investigated in order to achieve a more complete purification.

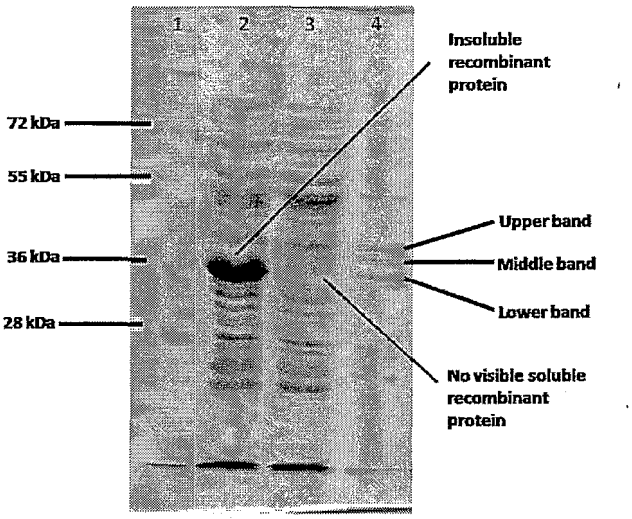


**Figure 2.11 SDS-PAGE analysis of the expression and purification of recombinant bovine GLYAT, without addition of imidazole to column wash buffers.** Lanes: 1) 5 µl of PageRuler protein marker; 2) bovine GLYAT expressed from pColdIII-A, total protein fraction; 3) bovine GLYAT expressed from pColdIII-A, soluble protein fraction; 4) after nickel affinity purification.

It is a common finding that some natural bacterial host proteins can bind non-specifically to the column matrix, resulting in a sub-optimal purification. Since the levels of soluble recombinant bovine GLYAT expressed are low, the final eluate of the purification has to be concentrated significantly. The proteins purified from a 150 ml culture of bacteria was commonly concentrated to a final volume of between 800 µl and 1500 µl. This concentration of the eluate resulted in the co-purifying bands becoming prominent when viewed on an SDS-PAGE gel (Figure 2.11).

One means of reducing this effect is to add a low concentration of imidazole to the cell lysis and wash buffers, which ensures that most of the non-specific contaminants are easily washed off the column. For this purpose 20 mM imidazole was included in the cell lysis reagent and the column wash buffer (LEW). At 40 mM imidazole even hexa-histidine tagged proteins can start eluting, and there are thus limits to how much impurities can be removed using this strategy (Verma et al., 2005). As shown on the SDS-PAGE gel in Figure 2.12, the use of imidazole in the buffers resulted in the majority of the previously co-

purifying proteins being lost. Now only two of the major protein bands were left, the lower two of the original three (Figures 2.11 and 2.12). One of these two bands must represent the enzymatically active bovine GLYAT, but it cannot be deduced from size alone which band. Since the histidine tag is at the C-terminus of the expressed recombinant GLYAT, incomplete translation is probably not accountable for the second protein band, as only completely translated proteins would have the purification tag. Judging by how tightly both these proteins bind, it must be considered that both proteins may have histidine tags.

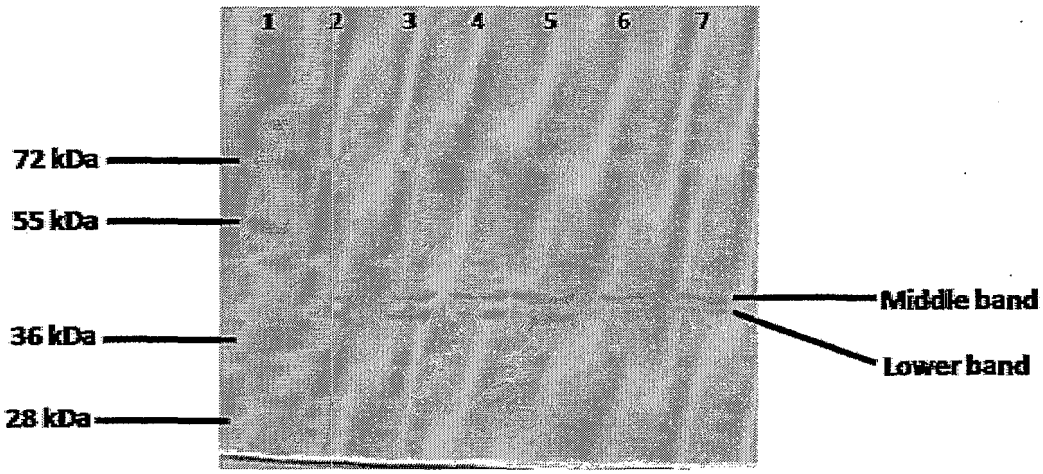


**Figure 2.12 SDS-PAGE analysis of the effect of chaperone co-expression, protease inhibitors and serine-glycine linkers on the yield of purified recombinant bovine GLYAT** Lanes: 1) 5 µl of PageRuler protein marker; 2) bovine GLYAT expressed from pColdIII-A without chaperone co-expression and protease inhibitors; 3) bovine GLYAT expressed from pColdIII-A with chaperone co-expression; 4) bovine GLYAT expressed from pColdIII-A with chaperone co-expression and protease inhibitors; 5) bovine GLYAT expressed from pColdIII-A with the short serine-glycine linker; 6) bovine GLYAT expressed from pColdIII-E without a serine-glycine linker; 7) bovine GLYAT expressed from pColdIII-EH with a long serine-glycine linker.

As mentioned in Section 1.6.3, adding a flexible serine-glycine linker between the protein and histidine tag allows the tag to move freely and access the nickel binding sites on the resin, avoiding steric interference between the matrix and protein (Loughran et al., 2006). To investigate whether this would have an influence on the purification of recombinant bovine GLYAT, the enzyme was expressed with three different C-terminal histidine tags, with either a short, a long, or no serine-glycine linker (Table 2.1). The proteins were purified as described in Section 2.2.17. As shown in Figure 2.12, the different tags have no observable influence on the outcome of the purification.

To confirm that there was no significant difference between the recombinant bovine GLYAT enzymes with the different tags, enzyme assays using 1 µg of purified protein were performed. The assays were performed in triplicate. There was no significant difference between the differently tagged proteins and so the tag with the short linker (Table 2.1) was arbitrarily chosen for further work (enzyme assay not shown).

The possibility of two alternative translation initiation sites to explain the two different proteins was also considered. Figure 2.13 shows the translation of the vector-insert construct, using the reading frame that produces the bovine GLYAT protein. The other reading frames had no significant open reading frames to consider (not shown). It is clear that translation initiation at some position upstream of the intended start site is not a likely explanation for the second protein band, as there is no open reading frame beginning before the intended start site. It is also not likely that translation continues beyond the intended stop codon, as there are multiple stop codons that would prevent this. However, there is a potential internal ribosomal binding site, just upstream of a methionine codon, that would result in translation of a 33.2 kDa protein (Baneyx, 1999). This is smaller than the 36.7 kDa protein obtained using the intended start codon. However, the two bands observed are both larger than 36 kDa, judging from the protein size markers, ruling out this possibility.

There is some evidence suggesting that the lower of the two bands represents the enzymatically active recombinant GLYAT. It was discussed above that the bovine GLYAT sequence was cloned into pColdIII vectors containing C-terminal histidine tags with serine-glycine linkers of different lengths (Table 2.1). The predicted sizes of translation of these three proteins are 37.277 kDa, 36.665 kDa, and 35.692 KDa (long, short, and no linker, respectively). The three proteins, purified by nickel affinity chromatography, were run alongside each other on an SDS-PAGE gel (Figure 2.12, lanes 5, 6 and 7). There is a small size difference between the different proteins. Interestingly, the upper band (thus the middle of the three original bands) is apparently the same size in all three cases. The lower band, however, apparently differs in size for the three proteins. This is expected because of the size of the serine-glycine linkers. This result suggests that this lower band could represent the histidine tagged recombinant bovine GLYAT. It was also noted that the lower

band is in line with the major band of insolubly expressed recombinant protein visible on the SDS-PAGE gel in Figure 2.11 (lanes 2 and 4).

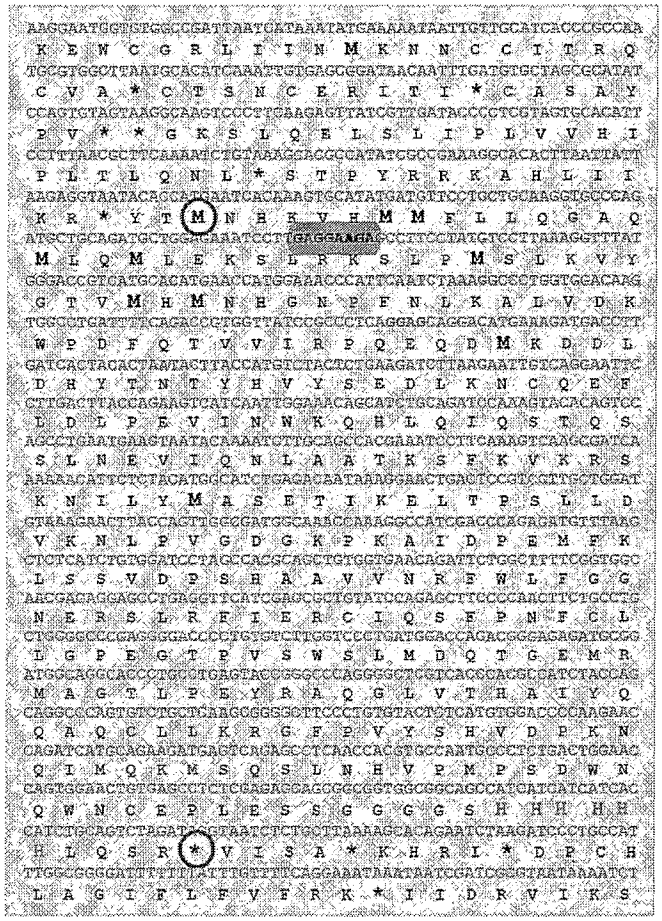

**Figure 2.13 The open reading frame for translation of recombinant bovine GLYAT from the recombinant pColdIII-A-bGLYAT vector.** The normal translation initiation and termination sites are indicated by the circles. A stop codon is depicted by an asterisk, and a start/methionine codon is represented by a bold capital M. The shadowed sequence represents a potential internal ribosomal binding site, with homology to the Shine Dalgarno sequence 5'-UAAGGAGG-3'.

To ensure that proteolytic degradation was not responsible for the smaller band, the purification was performed using protease inhibitors in the cell lysis and wash buffers. This did not have a significant effect on either the appearance of the proteins on an SDS-PAGE gel, or on enzyme activity (Figure 2.12 and Figure 2.14).

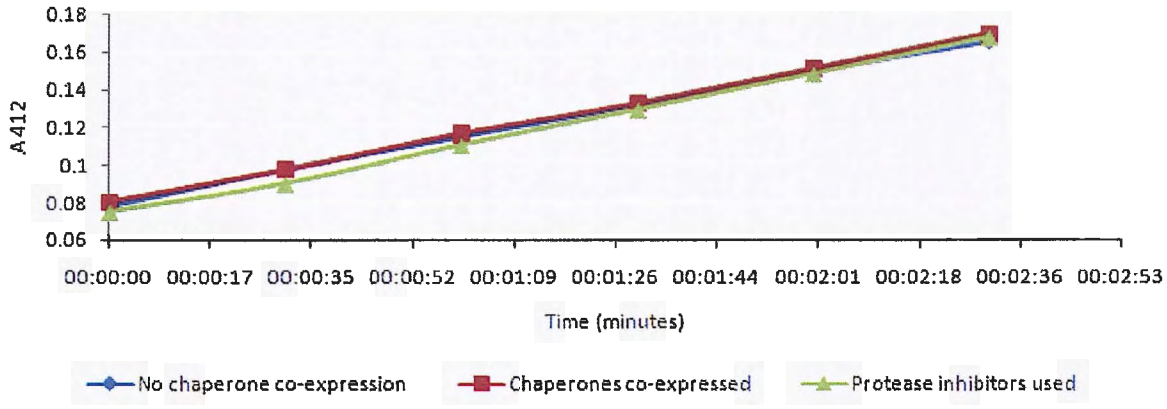


**Figure 2.14 Enzyme assays of recombinant bovine GLYAT expressed with and without chaperone co-expression, and recombinant bovine GLYAT purified in the presence of protease inhibitors.** The graph shows the change in absorbance at 412 nm with time, using 1 µg of protein per assay.

The nature of both major bands could be investigated using mass spectrometry to determine with certainty whether the two bands represent two different forms of recombinant GLYAT or two unrelated proteins. This will be done at a later stage. Another approach to differentiate between the two bands may be to use another purification tag, such as the GST II system, to purify the protein. If the second band represents a protein that binds non-specifically to the nickel affinity column, using a purification system that employs a different biochemical nature may solve the problem.

It will be attempted at a later stage to separate the two proteins using gel filtration and/or chromatofocussing, but for purposes of the present investigation the nickel affinity purification was sufficient.

### 2.3.4 The effect of chaperone co-expression on the yield of active recombinant GLYAT

To assess the contribution of chaperone co-expression to the formation of soluble, enzymatically active recombinant bovine GLYAT, the expression was performed with and without chaperone co-expression. Expression without chaperone co-expression was achieved by using Origami cells transformed with only the pColdIII-A-bGLYAT expression vector, but not the pGTf2 chaperone expression plasmid. Apart from the difference in antibiotic load (chloramphenicol and tetracycline were not added), the conditions were

exactly the same for the two expression experiments. To ensure that the results and conclusion were reliable, this study was performed using enzyme purified by nickel affinity chromatography (Section 2.2.17). As shown on the SDS-PAGE gel in Figure 2.12, the amount of soluble protein obtained is similar, whether chaperones are co-expressed or not. This result was confirmed by doing enzyme activity assays with 1 µg of purified protein from each system. The assays were performed in triplicate, and as shown in Figure 2.14, the activities are comparable. It can thus be concluded that chaperone co-expression does not significantly increase the yield of soluble, active enzyme, and can be left out of the expression protocol.

### 2.3.5 Effect of including hippurate in buffers on the purification of recombinant GLYAT

It is a well established principle that inclusion of the substrate or product of an enzyme in purification buffers tends to stabilise the protein. By binding to the active site of the protein, which is composed of several distinct regions of the primary structure, the inhibitor keeps the tertiary structure intact, preventing denaturation and increasing solubility (Palmer, 2001).

For this reason the purification was also attempted with 0.1 mM hippurate, a product and potent inhibitor of GLYAT, in all the buffers. Since hippurate is 60% inhibitory at 0.1 mM, the final wash step and the elution was without hippurate, to get rid of the inhibitor, which would interfere with subsequent activity assays (Schachter & Taggart, 1954b). SDS-PAGE analysis revealed that the hippurate had no significant effect on the purification efficiency or the amount of recombinant bovine GLYAT obtained (Figure 2.15). Both major protein bands were again purified, independent of the use of hippurate in the buffers.

To confirm this result, GLYAT enzyme activity assays were performed using 1 µg of each protein preparation. As shown in Figure 2.16, the activities of the two enzymes were similar. This suggests that there is no point to adding hippurate to the purification buffers in future experiments.

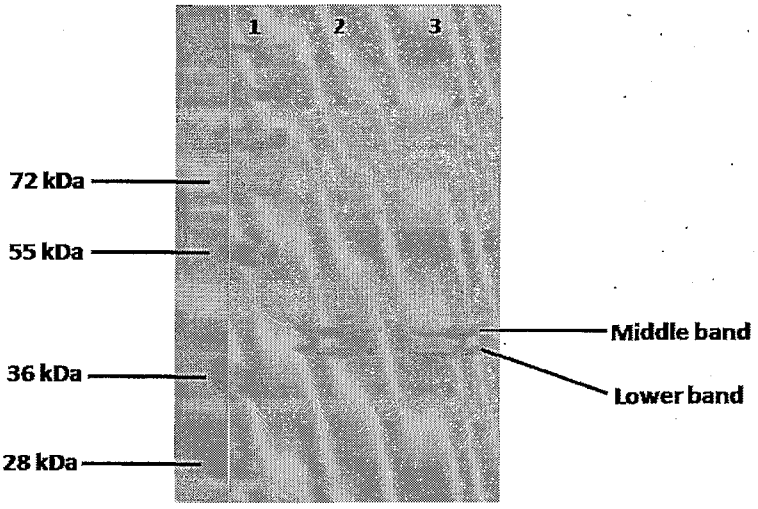


**Figure 2.15 SDS-PAGE analysis of the effect of hippurate on the stability of recombinant bovine GLYAT during purification.** Lanes: 1) 5 µl of PageRuler protein marker; 2) purification in the absence of hippurate; 3) purification using cell lysis and column wash buffers containing 0.1 mM hippurate.

### 2.3.6 Stability of the purified recombinant bovine GLYAT enzyme

It is important that the recombinant enzyme is stable enough to work with, such that its properties do not change in the course of an experiment. Although a detailed investigation of the stability of the purified recombinant bovine enzyme over a period of time was not performed, some general remarks can be made. In 20 mM TrisCl buffer, the partially purified recombinant GLYAT could be stored at 0 °C to 4 °C for weeks, without significant loss of enzyme activity. When frozen overnight at -20 °C in a 20 mM TrisCl buffer, approximately 30% of enzyme activity was lost (Figure 2.16). The enzyme could also be stored at -20 °C in a storage buffer containing 20 mM TrisCl and 50% glycerol for several days without significant loss of enzyme activity.

Thus, the enzyme appears to be more stable after overnight storage in 20 mM TrisCl at 4 °C, or in the glycerol storage buffer at -20 °C, than frozen in 20 mM TrisCl at -20 °C (Figure 2.16). However, since the enzyme was sufficiently stable at 4 °C for the duration of my experiments, detailed storage conditions were not further investigated.

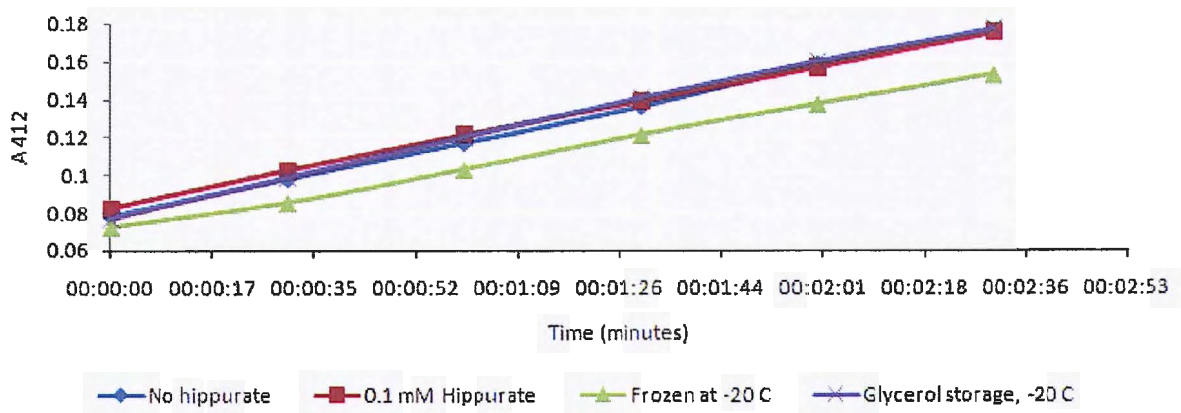


**Figure 2.16 Enzyme assays of recombinant bovine GLYAT purified in the presence and absence of hippurate, and of the enzyme stored under different conditions.** The graph shows the change in absorbance at 412 nm with time. For each assay 1 µg of recombinant protein is used.

### 2.3.7 Partial purification of bovine liver GLYAT for determination of kinetic parameters

For use as a positive control in enzyme assays and for kinetic studies, bovine GLYAT was enriched from bovine liver mitochondria, as described in Section 2.2.18. After isolation of bovine liver mitochondria, the mitochondrial proteins were fractionated using ammonium sulfate precipitation, and the fraction between 40% and 60% ammonium sulfate collected.

The final protein preparation had a specific activity of 0.79 µmol/min/mg of total protein. The enzyme preparation is visualised on the SDS-PAGE gel in Figure 2.17. There are hundreds of protein bands visible on the gel, and it is not known which band represents bovine GLYAT. No antibody was available for Western blotting to visualise the bovine GLYAT enzyme.

The enzyme preparation was not very pure. For the kinetic determinations performed in this study, it was not necessary to have a homogenous preparation of bovine GLYAT. By not using a purified protein, however, the option of calculating accurate reaction velocities and catalytic efficiencies ($V_{max}$ and $K_{cat}$) was made unfeasible. At a later stage, the protein will be purified to homogeneity, both for use in more detailed kinetic studies, and for mass spectroscopic analysis to determine whether the enzyme has any post-translational modifications.

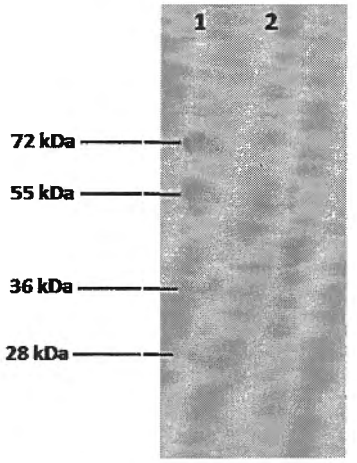


**Figure 2.17 Bovine GLYAT isolated from bovine liver mitochondria.** Lanes: 1) 5 µl of PageRuler protein marker; 2) 2 µl of the bovine liver GLYAT preparation.

### 2.3.8 Kinetic characterisation and comparison of the recombinant bovine GLYAT and GLYAT isolated from bovine liver

With a view of future development of a suite of recombinant therapeutic GLYAT enzymes with a range of substrate specificities, the kinetic parameters of the recombinant bovine GLYAT were determined. The values were compared to those obtained for the native bovine liver enzyme. Because the bovine liver enzyme was not purified to homogeneity, and the recombinant GLYAT could not yet be completely purified, maximal reaction velocities could not be expressed meaningfully in terms of the amount of enzyme used. The apparent $K_M$ values for various substrates could be determined, however, and compared between the bovine liver GLYAT and the recombinant bovine GLYAT.

In a pilot study the amount of protein to be used in each enzyme reaction, for both the bovine liver enzyme and the recombinant enzyme, was determined such that a linear change of 0.24 absorbance units (at 412 nm) in four minutes was observed, using 100 µM benzoyl coenzyme A and 20 mM glycine. This ensures that there is a minute to mix the reactions and start the machine before starting the 3 minute spectrophotometric readings.

This approach allowed comparison of the data for the two enzymes, despite the fact that the amount of protein used is not the same for both enzymes. By setting the initial

velocities equal to each other at the highest substrate concentrations, a reference point was established for the comparison of the data. The apparent $K_M$ values and the relative $V_{max}$ values for the two enzymes could then be compared to each other.

The $K_M$ values for benzoyl-coenzyme A and glycine were determined as described in Section 2.2.22. The apparent $K_M$ values were also determined for benzoyl-, isovaleryl-, propionyl-, octanoyl-, and 3-methylcrotonyl-coenzyme A, with the glycine concentration kept constant at a saturating 200 mM (Nandi et al., 1979). For each substrate, a pilot study with substrate concentrations ranging from 10 to 300 µM was performed to get a rough approximation of the $K_M$ value. An iterative approach was then followed until a good estimate of the $K_M$ value was obtained. Five substrate concentrations above and below this value were then chosen and used for graphical determination of the $K_M$ value. All assays were performed in triplicate. The double reciprocal plots are shown in Figures 2.18a to 2.18e, and the concentrations used in each experiment are indicated in the legends to the figures. The kinetic parameters are summarised in Tables 2.4 and 2.5.

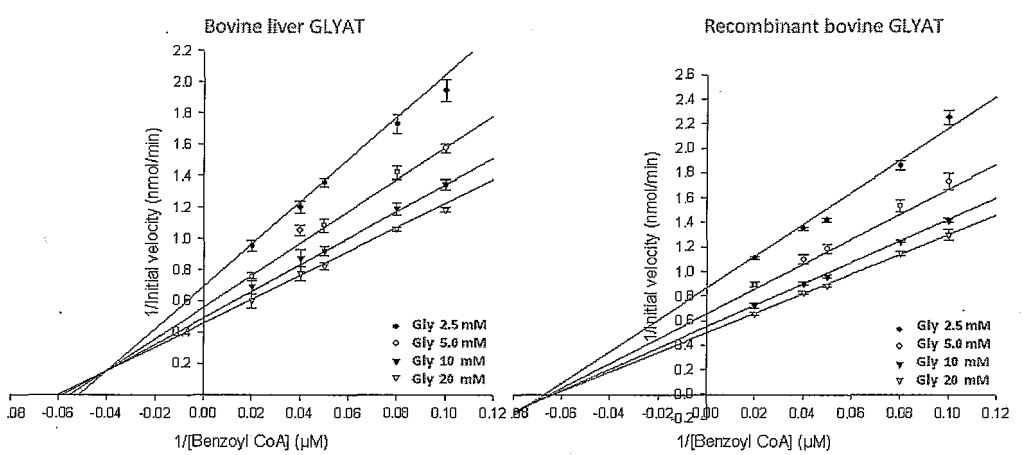


**Figure 2.18 a) Lineweaver-Burk plots for determination of the kinetic parameters for the bovine liver GLYAT and recombinant bovine GLYAT enzymes, using benzoyl-coenzyme A and glycine as substrates.** The plots for the bovine liver GLYAT are on the left, and that for the recombinant GLYAT to the right. Benzoyl-coenzyme A concentrations were 10, 15, 25, 40 and 50 µM. The data points indicate average values ± standard deviation, with n = 3. Of the bovine liver GLYAT and the recombinant bovine GLYAT, 420 ng and 30 ng, respectively, were used per assay.

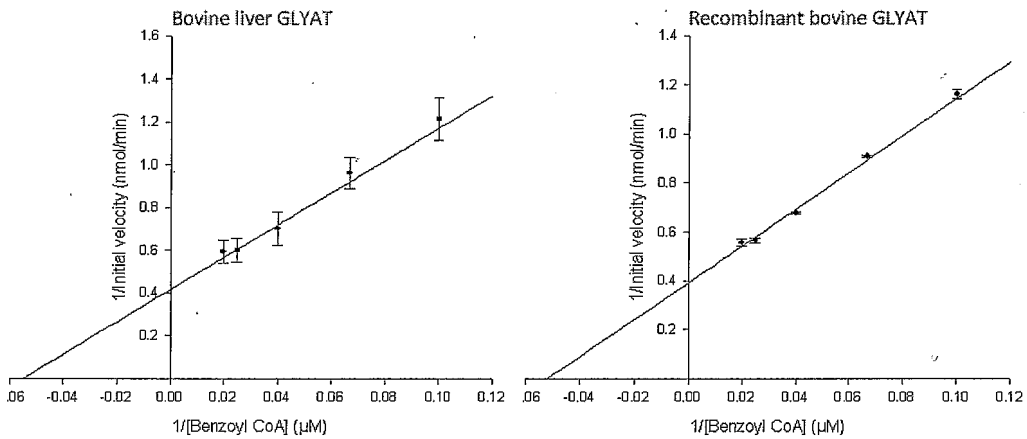


**Figure 2.18 b) Lineweaver-Burk plots to determine the Km values of benzoyl-coenzyme A for the bovine liver GLYAT and recombinant GLYAT enzymes.** The plot on the left is for the bovine liver GLYAT, and the plot on the right for the recombinant GLYAT. Benzoyl-coenzyme A concentrations were 10, 15, 25, 40 and 50 µM. The data points indicate average values ± standard deviation, with n = 3. Of the bovine liver GLYAT and the recombinant bovine GLYAT, 420 ng and 30 ng, respectively, were used per assay.

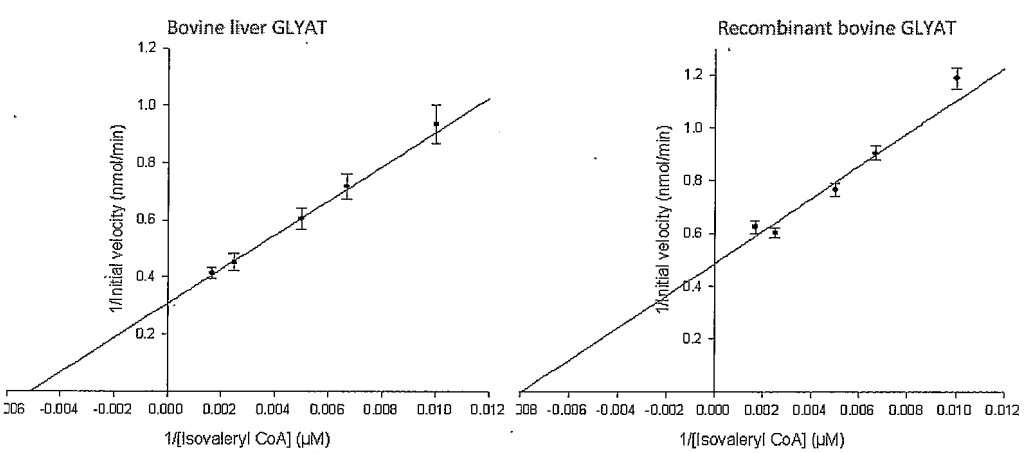


**Figure 2.18 c) Lineweaver-Burk plots to determine the Km values of isovaleryl-coenzyme A for the bovine liver GLYAT and recombinant GLYAT enzymes.** The plot on the left is for the bovine liver GLYAT, and the plot on the right for the recombinant GLYAT. Isovaleryl-coenzyme A concentrations were 100, 150, 200, 400 and 600 µM. The data points indicate average values ± standard deviation, with n = 3. Of the bovine liver GLYAT and the recombinant bovine GLYAT, 420 ng and 30 ng, respectively, were used per assay.

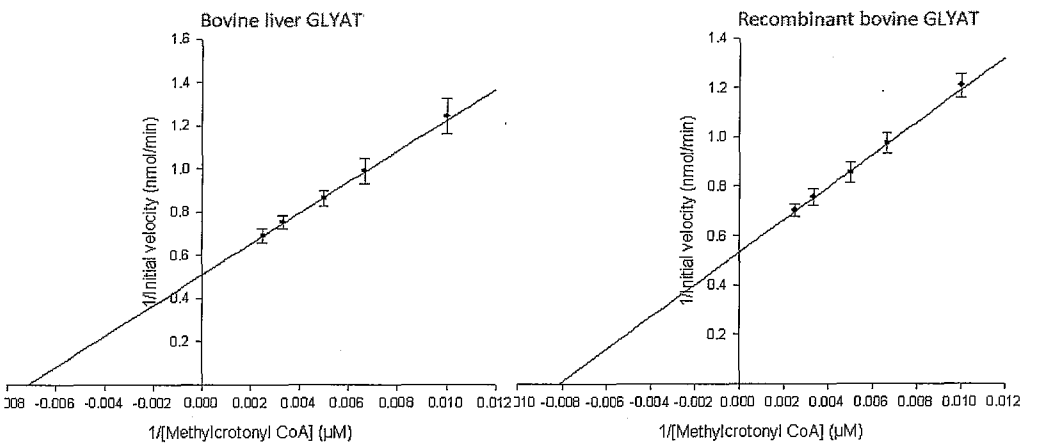


**Figure 2.18 d) Lineweaver-Burk plots to determine the Km values of 3-methylcrotonyl-coenzyme A for the bovine liver GLYAT and recombinant GLYAT enzymes.** The plot on the left is for the bovine liver GLYAT, and the plot on the right for the recombinant GLYAT. Methylcrotonyl-coenzyme A concentrations used were 100, 150, 200, 300 and 400 µM. The data points indicate average values ± standard deviation, with n = 3. Of the bovine liver GLYAT and the recombinant bovine GLYAT, 420 ng and 30 ng, respectively, were used per assay.

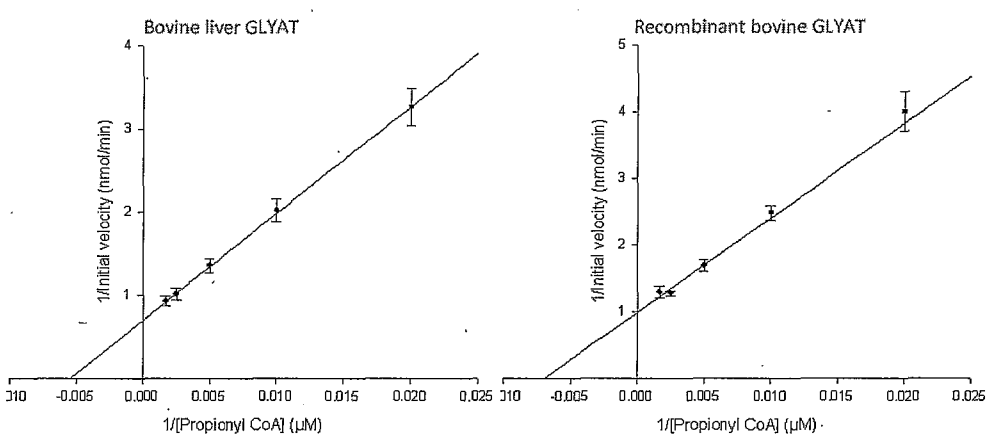


**Figure 2.18 e) Lineweaver-Burk plots to determine the Km values of propionyl-coenzyme A for the bovine liver GLYAT and recombinant GLYAT enzymes.** The plot on the left is for the bovine liver GLYAT, and the plot on the right for the recombinant GLYAT. Propionyl-coenzyme A concentrations used were 50, 100, 200, 400 and 600 µM. The data points indicate average values ± standard deviation, with n = 3. Of the bovine liver GLYAT and the recombinant bovine GLYAT, 420 ng and 30 ng, respectively, were used per assay.

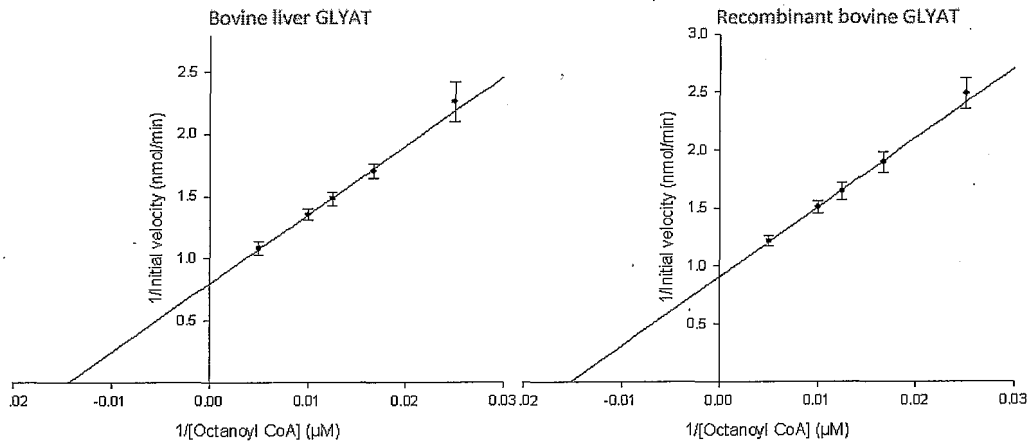


**Figure 2.18 f) Lineweaver-Burk plots to determine the Km values of octanoyl-coenzyme A for the bovine liver GLYAT and recombinant GLYAT enzymes.** The plot on the left is for the bovine liver enzyme, and the plot on the right for the recombinant enzyme. Octanoyl-coenzyme A concentrations used were 40, 60, 80, 100 and 200 µM. The data points indicate average values ± standard deviation, with n = 3. Of the bovine liver GLYAT and the recombinant bovine GLYAT, 420 ng and 30 ng, respectively, were used per assay.

**Table 2.4: Kinetic parameters for the bovine liver GLYAT enzyme and the recombinant bovine GLYAT enzyme using benzoyl-coenzyme A and glycine**

| GLYAT enzyme | $K_M$ benzoyl-coenzyme A (µM) | $K_M$ glycine (mM) | Relative $V_{max}$ (nmol/min) |
|---|---|---|---|
| | | | |
| GLYAT from bovine liver (420 ng protein) | 15.91 ± 2.51 | 1.60 ± 0.51 | 2.34 ± 0.15 |
| Recombinant bovine GLYAT (30 ng protein) | 16.26 ± 1.23 | 2.33 ± 0.28 | 2.23 ± 0.07 |

**Table 2.5: $K_M$ values for benzoyl-coenzyme A and various amino acids for the human and bovine GLYAT enzymes**

| | $K_M$ for acyl-coenzyme A (µM) | | Relative $V_{max}$ (nmol/min) | |
|---|---|---|---|---|
| Peptide conjugate | Bovine liver | Recombinant | Bovine liver (420 ng protein) | Recombinant (30 ng protein) |
| Benzoylglycine | 18.17 ± 5.44 | 19.25 ± 1.60 | 2.41 ± 0.28 | 2.56 ± 0.09 |
| Isovalerylglycine | 195.29 ± 31.68 | 126.66 ± 19.69 | 3.26 ± 0.2129 | 2.06 ± 0.11 |
| Propionylglycine | 184.29 ± 30.13 | 143.14 ± 22.63 | 1.44 ± 0.09 | 1.01 ± 0.06 |
| 3-methylcrotonylglycine | 139.98 ± 24.19 | 123.14 ± 19.15 | 1.96 ± 0.13 | 1.88 ± 0.10 |
| Octanoylglycine | 69.60 ± 9.50 | 66.09 ± 8.43 | 1.26 ± 0.07 | 1.10 ± 0.06 |

For all the substrates investigated, the $K_M$ values and relative $V_{max}$ values correspond reasonably between the bovine liver and recombinant GLYAT enzymes (Table 2.4 and Table 2.5). The experiments were performed in independent triplicates, with a separate master mix made up for each replicate, suggesting that the parameters were reliable. It was discussed in Section 1.4.3 that there is great variation in the values of kinetic parameters reported in the literature, which limits the value of a comparison of the experimentally determined parameters to the literature (Bartlett & Gompertz, 1974, Gregersen et al., 1986, Gron et al., 1978a, Kelley & Vessey, 1986, Kelley & Vessey, 1993, Kelley & Vessey, 1994, Kolvraa & Gregersen, 1986, Mawal & Qureshi, 1994, Nandi et al., 1979, Schachter & Taggart, 1954a, van der Westhuizen et al., 2000). The $K_M$ value for benzoyl-coenzyme A reported in the literature varies between 9 µM and 160 µM (Table 1.2). The values of approximately 16 µM for the bovine liver and recombinant GLYAT enzymes obtained here thus fall well within the range reported in the literature (Table 2.4). The $K_M$ values reported for glycine (using benzoyl-coenzyme A as acyl donor) range from 2 mM to 15 mM (Table 1.2). Again, the values determined in this study are comparable, being approximately 2 mM for both enzymes (Table 2.4). The $K_M$ value reported for the bovine liver enzyme for both propionyl-coenzyme A and isovaleryl-coenzyme A is 180 µM, which is of the same order as that determined in this study (Table 2.4). These values correspond well, considering the variation in values reported for benzoyl-coenzyme A. The $K_M$ value reported for 3-methylcrotonyl-coenzyme A is 14 µM (Table 1.2). The value determined in this study was significantly higher (Table 2.4). This difference is difficult to explain, considering the rather good correspondence between the experimental values and literature values for the other substrates. No $K_M$ value for octanoyl-coenzyme A is reported in the literature, and so the values determined here could not be compared to anything.

These results suggest that the kinetic properties of the bovine liver GLYAT and the recombinant bovine GLYAT are similar. Therefore, further studies on the substrate specificity of bovine GLYAT, and its relationship to the amino acid sequence of the enzyme, can take advantage of this recombinant bovine GLYAT expression system.

## 2.4 Summary

In a previous study in our laboratory, it was found that bovine GLYAT, expressed at 15 °C from pColdIII in the presence of over-expressed GroES-GroEL-TF chaperone proteins, was partially soluble and had significant enzyme activity. This part of the study had three main objectives. Firstly, the expression system had to be verified to yield soluble and enzymatically active recombinant bovine GLYAT, and expression conditions optimised. Secondly, a recombinant bovine GLYAT with a C-terminal histidine tag had to be constructed to facilitate protein purification using nickel affinity chromatography. Thirdly, the kinetic properties of the purified recombinant bovine GLYAT had to be determined for several substrates, and compared to that of the enzyme purified from bovine liver mitochondria.

The expression of bovine GLYAT, without any N- or C-terminal fusions, from the pColdIII vector, with co-expression of the GroEL-GroES-TF chaperone team, resulted in a recombinant protein that was mostly insoluble (Figure 2.11). A very small amount of soluble, enzymatically active recombinant bovine GLYAT was produced, however. This confirms the observation made in an earlier study in our laboratory that recombinant bovine GLYAT, expressed using the system described, is slightly soluble and enzymatically active (M Snyders, unpublished work). It was determined that the co-expression of the chaperone proteins had no observable benefit in terms of the amount of soluble protein produced, or the enzymatic activity of this soluble protein (Figure 2.12 and Figure 2.13). The IPTG concentration used for induction, and the duration of induction were both changed from that recommended by the pCold Manual. It was found that expression with induction for 1 hour with 0.05 mM IPTG yielded more soluble, enzymatically active recombinant bovine GLYAT than the standard induction for 24 hours with 1 mM IPTG (Figure 2.10). However, most of the recombinant protein expressed was still insoluble. This problem may be solved by using a more advanced eukaryotic expression system, such as a mammalian expression system or a Drosophila expression system. This approach will be investigated in a later study.

The purification of the recombinant bovine GLYAT described in Section 2.3.3 was not optimal, as the recombinant bovine GLYAT was not purified to homogeneity (Figure 2.11). Several minor proteins with a range of sizes co-purify with the recombinant GLYAT. Furthermore, three prominent proteins, with sizes in the range of the 36 kDa marker were purified. It was found that adding 20 mM imidazole in the cell lysis and column wash buffers prevented the co-purification of the minor proteins, and one of the three major proteins (Figure 11.12). There are now two major proteins that are purified by the nickel affinity chromatography, and an argument could be made that the smaller protein represents the histidine tagged recombinant bovine GLYAT. It will be attempted at a later stage to use another purification system, such as GST-II tagging, which may solve the problem if the second protein co-purifies by binding to the nickel affinity columns. Alternatively, gel filtration or chromatofocussing could be used to separate the two proteins. It can then be determined whether they represent different forms of recombinant bovine GLYAT, or if one is just a non-specific co-purifying protein.

The kinetic parameters were determined for both the GLYAT extracted from bovine liver and recombinant bovine GLYAT, using several acyl-coenzyme A substrates and glycine. The values obtained for the two enzymes were comparable and corresponded well to the values reported in the literature. This suggests that the bacterially expressed recombinant bovine GLYAT can be used to study the substrate specificity of the GLYAT enzyme.

The conclusion of the work described in this chapter is that the recombinant bovine GLYAT enzyme, expressed and purified as described, has biochemical characteristics similar to the GLYAT enzyme purified from bovine liver. This suggests that it may be used in future investigations of various properties of the enzyme. In terms of our long term goal of developing a recombinant therapeutic GLYAT enzyme for the treatment of organic acidemias, this was an important first step. The system will enable us to start investigating the relationship between protein sequence and enzyme substrate specificity. This would facilitate a systematic approach to development of a GLYAT enzyme with altered substrate specificity or increased activity. When a system for the expression and purification of biologically active recombinant human GLYAT is developed, the knowledge gained by studying the recombinant bovine enzyme could be used to aid rational design of a

therapeutic human GLYAT. It is our long term goal to use such a novel recombinant GLYAT in the treatment of organic acidemias.

Based on the work presented in this chapter, a peer reviewed scientific paper for publication and a patent application are currently being prepared. The patent will cover the use of a recombinant GLYAT enzyme for therapeutic purposes.