Expression of recombinant human metallothionein 2A as internal standard for mass spectrometric analysis of metallothioneins

By

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Uitdrukking van rekombinante mens metallotionien 2A as intêrne standaard vir massa spektrometriese analise van metallotioniene

Deur

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Abstract

The induction of metallothionein (MT) expression in mitochondrial disorders has been well studied on the transcription level by means of RNA measurements in an attempt to understand and confirm the function of this protein in the deficient cells and organs (Olivier, 2004:42; Pretorius, 2006:44; Reinecke, 2004:89). However, MT expression induction still needs to be verified on protein (translation) level in order to confirm previous findings and to gain better perspective on the significance of MT expression induction. Therefore, it is necessary to use a technique that is capable of quantifying MT accurately in biological material. Due to the lack of sensitivity and selectivity of many commonly used techniques (Dabrio et al., 2002:125), it is necessary to develop a mass spectrometric based quantification technique to detect and quantify MT-2A selectively and accurately.

For quantification of human MT-2A in biological material using a mass spectrometry-based method, a MT (MT-2A) standard similar to the native form but with a slightly different mass was required. Due to the lack of pure human MT standards and high cost of pure rabbit MT standards, it was decided to create a recombinant human MT-2A with different mass due to additional N-terminal amino acids. In addition, native human MT-2A is also required to develop and optimize an MS quantification technique in a future study. Therefore, pure (98 %) rabbit MT standard, which is highly similar to human MT-2A, was purchased to serve as a positive control for MS detection in this study and which can also be used to develop and optimize an MS quantification technique in a future study.

An expression vector for human MT-2A was constructed with the use of recombinant DNA techniques. The correct construct was identified and characterized with PCR and verified by sequencing. This newly created expression vector was transformed into four *E.coli* BL21(DE3) strains to express a modified human recombinant MT-2A ($MT\text{-}2A^{\Delta}$) using induction with IPTG. This protein comprised of a full length human MT-2A sequence, but excluding the N-terminal Met and including an N-terminal His-tag. $MT\text{-}2A^{\Delta}$ expression in the selected strains was extensively optimized and monitored with SDS-PAGE. *E.coli* BL21(DE3) CodonPlus-RIL cells proved to be the

strain that expressed MT-2A$^{\Delta}$ at the highest relative levels. Expressed MT-2A$^{\Delta}$ was isolated and purified using a three step purification procedure which included heat treatment, metal chelating chromatography and RP-HPLC. Relative pure (70 %) MT-2A$^{\Delta}$ was successfully obtained as confirmed with SDS-PAGE and mass spectrometry. Removal of the His-tag from MT-2A$^{\Delta}$ with thrombin protease cleavage was, however, unsuccessful. In addition, it was observed that this protein was, compared to native commercially obtained MT-2A, unstable and after extensive purification still had a lower than required purity. It was concluded from this studies' results that, although it was successfully produced, this recombinant MT-2A protein would not be suitable as an internal standard for MS analysis of human MT-2A. On the other hand, rabbit MT-2E (as alternative) holds great promise as internal standard since it is stable and pure.

Opsomming

Die indusering van metallotionien (MT) uitdrukking in mitochondriale defekte is noukeurig bestudeer op die transkripsie vlak deur die meting van *RNS* met die doel om die funksie van die proteïen in die defektiewe selle en organe te verstaan en te bevestig (Olivier, 2004:42; Pretorius, 2006:44; Reinecke, 2004:89). Indusering van metallotionienuitdrukking moet egter nog geverifieer word op die proteïen (tanslasie) vlak om vorige bevindinge te bevestig en om beter perspektief te verkry oor die belangrikheid van MT indusering. Daarom is dit noodsaaklik om 'n gepaste tegniek te gebruik wat in staat is om MT akkuraat in biologiese materiaal te kwantifiseer. Weens die gebrek aan sensitiwiteit en selektiwiteit by verskeie algemeen-gebruike tegnieke (Dabrio et al., 2002:125), is dit nodig om 'n massa spektrometries-gebasseerde kwantifiseringstegniek te ontwikkel om MT-2A selektief en akkuraat te kwantifiseer.

'n MT (MT-2A) standaard wat byna dieselfde struktuur as die natuurlike vorm het, maar met 'n geringe massaverskil, is nodig vir die kwantifisering van menslike MT-2A in biologiese materiaal deur massaspektrometrie. Weens die gebrek aan suiwer mens MT-standaarde en hoë koste van suiwer haas MT-standaarde, is daar besluit om rekombinante menslike MT-2A te produseer wat 'n effense massaverskil het weens addisionele N-terminale aminosure. Natuurlike mens MT-2A is ook nodig om in die toekoms 'n MS-kwantifiseringstegniek te ontwikkel en te optimaliseer. Daarom was suiwer (98 %) haas MT standaard ook aangekoop, wat baie dieselfde is as mens MT-2A, om te gebruik as positiewe kontrole vir MS deteksie in hierdie studie maar verder ook gebruik kan word om 'n MS kwantifiseringstegniek te optimaliseer in 'n toekomstige studie.

'n Uitdrukkingsvektor vir menslike MT-2A was gekonstrueer deur die gebruik van rekombinante DNS-tegnieke. Die korrekte vorm is geïdentifiseer met PKR en geverifieer deur DNS-volgordebepaling. Hierdie nuwe uitdrukkingsvektor is in vier *E.coli* BL21(DE3) sellyne getransformeer om 'n gemodifieerde menslike rekombinante MT-2A ($MT\text{-}2A^{\Delta}$) uit te druk deur gebruik te maak van IPTG-indusering. Hierdie proteïen bestaan uit 'n vollengte mens MT-2A volgorde, maar sonder die N-

terminale metionien en sluit 'n N-terminale "His-tag" in. MT-2A$^{\Delta}$ uitdrukking in die selektiewe sellyne is ekstensief geoptimaliseer en gemonitor deur SDS-PAGE. *E.coli* BL21(DE3) CodonPlus-RIL selle het MT-2A$^{\Delta}$ by die hoogste relatiewe vlakke uitgedruk. Uitgedrukte MT-2A$^{\Delta}$ is geïsoleer en gesuiwer deur die gebruik van 'n drie-stap suiweringsprosedure wat hittebehandeling, metaalaffiniteitschromatografie en omgekeerde fase HPLC insluit. Relatiewe suiwer (70 %) MT-2A$^{\Delta}$ is suksesvol verkry soos bevestig deur SDS-PAGE en massaspektrometrie. Daarteenoor is die "His-tag" nie suksesvol van die MT-2A$^{\Delta}$ met trombien protease vertering verwyder nie. Daar is ook opgemerk dat die proteïen, in vergelyking met kommersieel gekoopte natuurlike MT-2A, onstabiel was en selfs na ekstensiewe suiwering was die suiwerheid van die proteïen laer as wat nodig was. Alhoewel die proteïen suksesvol geproduseer is, is uit die resultate die gevolgtrekking gemaak dat die rekombinante MT-2A proteïen nie gepas is om as interne standaard te dien vir MS analise van mens MT-2A nie. Daarteenoor het haas MT-2E (as alternatief) belowende resultate gegee om as interne standaard te dien omdat dit suiwer en stabiel is.

1Co 13:2 And though I have *the gift of* prophecy, and understand all mysteries, and all knowledge; and though I have all faith, so that I could remove mountains, and have not charity, I am nothing.

1Co 13:8 Charity never faileth: but whether *there be* prophecies, they shall fail; whether *there be* tongues, they shall cease; whether *there be* knowledge, it shall vanish away.

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List of Abbreviations and Symbols

Symbols

°C ..... Degrees Celsius

µg ..... Microgram

µl ..... Micro-litre

µm ..... Micrometer

µM ..... Micro-molar

α ..... Alpha

& ..... And

~ ..... Approximately / about

β ..... Beta

% ..... Percentage

> ..... Larger than

2YT ..... Twice as much yeast extract and tryptone as LB media

..... Number

+ ..... positive charge

A

A ..... Absorbance at the specific wavelength

AAS ..... Atomic absorption spectrometry

ACN ..... Acetonitrile

Ag ..... Silver

amu ..... Atomic mass unit

ATP ..... Adenosine triphosphate

Au ..... Gold

B

BCA ..... Bicinchoninic acid

Bi ..... Bismuth

bp ..... Base pairs

BSA ..... Bovine serum albumin

C

$CaCl_2$ ..... Calcium chloride

Cam ..... Chloramphenicol

CBB ..... Coomassie Brilliant Blue

Cd ..... Cadmium

$CdCl_2$ ..... Cadmium chloride

cDNA ..... Complementary DNA

CE ..... Capillary electrophoresis

cELISA ..... Competitive ELISA

CNS ..... Central nervous system

Co ..... Cobalt

C-terminal ..... Carboxyl terminal

Cu ..... Copper

$CuSO_4$ ..... Copper (II) sulphate

Cys ..... Cysteine

D

Da ..... Dalton

DMSO ..... Dimethyl sulphoxide

DNA ..... Deoxyribonucleic acid

DNS ..... deoksiribonukleïensure

DTT ..... Dithiothreitol

E

E.coli ..... *Escherichia coli*

EDTA ..... Ethylenediaminetetraacetic acid dipotassium

ELISA ..... Enzyme-linked immunosorbent assay

ESI ..... Electro-spray ionisation

EtBr ..... Ethidium bromide

EtOH ..... Ethanol

F

FKBP ..... FK506 binding protein

FT ..... Flow through

G

g ..... G-force (9.80665 m/s^2)

g ..... Gram

GIF ..... Growth inhibitory factor

GSH ..... Glutathione

GSSG ..... Oxidized glutathione

GST ..... Glutathione-S-transferase

H

h ..... Hour

H_2O ..... Water

H_2O_2 ..... Hydrogen peroxide

HCl ..... Hydrochloride

HF ..... Heat fraction

Hg ..... Mercury

His ..... Histidine

HMW ..... High molecular weight

HPLC ..... High performance liquid chromatography

I

ICP-MS ..... Inductively-coupled plasma MS

IPTG ..... Isopropyl-β-D-thiogalactopyranoside

K

Kan ..... Kanamycin

KCl ..... Potassium chloride

kDa ..... Kilo-Dalton

kHz ..... Kilo-Hertz

kV ..... Kilo volt

L

l ..... Litre

LB ..... Luria-Bertani

LC ..... Liquid chromatography

LMW ..... Low molecular weight

M

M ..... Molar

mA ..... Milli amperes

MCS ..... Multiple cloning site

mg ..... Milligram

$MgCl_2$ ..... Magnesiumchloride

min ..... Minutes

ml ..... Millilitre

mM ..... Milli-molar

$MnCl_2$ ..... Manganese chloride

mRNA ..... Messenger RNA

MS ..... Mass spectrometry

MT ..... Metallothionein

MT-2A$^{\Delta}$ ..... Recombinant modified human MT-2A

MT-2A* ..... Recombinant MT-2A without His-tag

m/v ..... mass to volume ratio

m/z ..... relative mass to charge ratio

N

NaCl ..... Sodium chloride

NADH ..... Nicotinamide adenine dinucleotide

Ni ..... Nickel

$NiSO_4$ ..... Nickel (II)-sulfate

ng ..... Nanogram

nm ..... Nanometer

nM ..... Nanomolar

$NO^{\bullet}$ ..... Nitric oxide

N-terminal ..... Amino terminal

O

O_2 ..... Oxygen

O_2^{-} ..... Superoxide

OD ..... Optical density

$OH^{\bullet}$ ..... Hydroxide

OXPHOS ..... Oxidative phosphorylation

P

PCA ..... Perchloric acid

PCR ..... Polymerase chain reaction

PIPES ..... 2-[4-(2-sulfoethyl)piperazin-1-yl]ethanesulfonic acid

PKR ..... Polimerase ketting reaksie

Prom ..... Promoter

R

RAPS ..... Rapid plasmid isolation

RIA ..... Radioimmunoassay

RNA ..... Ribonucleic acid

RNS ..... Reactive nitrogen species

RNS ..... Ribonukleïensure

ROS ..... Reactive oxygen species

RP-HPLC ..... Reverse Phase HPLC

rpm ..... Revolutions per minute

RT-PCR ..... Reverse transcription PCR

S

SDS-PAGE ..... Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEC ..... Size exclusion chromatography

T

TB ..... Transformation buffer

TCA ..... Trichloroacetic acid

TEMED ..... N, N, N', N' –tetramethylethylenediamine

Term ..... Terminator

TF ..... Total fraction

TFA ..... Trifluoroacetic acid

Tris-HCl ..... Tris-hydrochloride

U

U ..... Unit

UV ..... Ultraviolet

V

v/v ..... Volume to volume ratio

X

Xaa ..... Amino acid other than Cysteine

Z

Zn ..... Zinc

$ZnSO_4$ ..... Zinc sulphate

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Chapter 1

Introduction

Mitochondria are essential for life and especially for most eukaryotic cells due to its crucial role in energy metabolism. The final pathway of mitochondrial energy metabolism, oxidative phosphorylation, consists of five enzyme complexes (I to V) which are located in the inner membrane of the mitochondrion (Ballard & Whitlock, 2004:730; Chinnery & Schon, 2003:1189; Leonard & Schapira, 2000:299; Zeviani & Di Donato, 2004:2153). Mitochondrial disorders occur in about 1 per 3 000 births and is characterized by low energy output as a result of deficient OXPHOS (Cohen & Gold, 2001:625; Naviaux, 2004:354). The biochemical and clinical manifestations of these disorders are heterogeneous and vary from single to multi-system dysfunction (Chinnery & Schon, 2003:1190; Leonard & Schapira, 2000:301; Naviaux, 2004:352). For this reason, much research has been done on the mitochondrial deficiencies with special attention to the production of and protection responses (including transcriptional) against reactive oxygen species (ROS), which may be the most significant consequence in these disorders.

Gene expression analyses of mitochondrial disorders show the over- and under expression of many genes. The over-expression of certain genes is believed to compensate losses and protect against damage caused by ROS, low energy output and apoptosis. A similar study was done by van der Westhuizen et al. (2003:15) on complex I (also known as NADH-ubiquinone oxidoreductase), the most frequent deficient complex in the mitochondria (Smeitink et al., 1998:1574). Metallothionein (MT) expression was found to be significantly increased in complex I deficient fibroblast cell lines. This was confirmed in HeLa cells treated with rotenone which also results in complex I deficiency (Reinecke, 2004). As mentioned before, a key consequence of OXPHOS deficiencies is the production of ROS (Kirkinezos & Moraes, 2001:452; Scheffler, 2000:21), which are believed to be responsible for the induction of metallothionein gene expression (Suzuki et al., 2005:51).

ROS is known to damage DNA, proteins and lipids and cause a vicious destructive cycle (Scheffler, 2000:22). Since MT is induced by ROS, it is believed that MT plays an important role under these circumstances and it is hypothesized that it protects against oxidative damage. This hypothesis led to a series of *in vitro* research projects to study MT's expression during oxidative stress related disorders (Semete, 2004), complex I deficiency (Olivier, 2004) and MT's functional properties in complex I deficiency (Reinecke, 2004) which ultimately led to an *in vivo* study in mice (Pretorius, 2006). MT's expression was measured by the quantification of RNA levels in the cells or tissues. This is a common approach but literature has shown that there is not always a correlation between the RNA- and protein levels. It is therefore important to measure MT levels in addition to RNA levels in these studies. The difficulty to specifically measure MT isoforms as stated by Olivier (2004:74), has been a weakness in the evaluation of the expression and role of MT in these previous studies.

Many methods are used in an attempt to specifically detect and measure MT, but many have failed to do so or have limitations (as discussed in Section 2.2). As technology and our knowledge of it develop, more possibilities are created. ESI-MS (electrospray ionisation mass spectrometry) can be used to identify and measure large biomolecules such as proteins. ESI-MS has been successfully applied in the identification and characterization of MT, but its ability to quantify is unexplored as can be seen from the absence of reports in the literature. The absence of pure MT standards as reference material, the structural variants that exist and the poor immunogenicity of MT are probably the main reasons for the lack of good quantification methods. This dissertation will describe a study that was undertaken to develop recombinant human MT-2A with the aim to use it in future studies to develop a mass spectrometric-based technique for the quantification of MT-2A in biological samples.

Chapter 2

Literature Overview

2.1 METALLOTHIONEIN

2.1.1 What is Metallothionein?

Metallothionein (MT) is a small intracellular, non-enzymatic protein (61-68 amino acids in length) (Haq et al., 2003:211) and is part of the low molecular weight class of proteins with a mass of 6000 - 7000 Da depending on the type and number of metal ions bound (Coyle et al., 2002:627). Metallothionein has high cysteine content (30 %) and lacks aromatic acids (Alhama et al., 2006:56; Coyle et al., 2002:627; Ghoshal & Jacob, 2001:358; Stillman, 1995:462). This high sulphur content gives the protein a high affinity for metal binding, which in return makes the protein heat resistant (Beattie, 1998:261; Rosenberg, 2003:877; Vasak, 2005:16). Metallothionein can bind 7 - 12 metal ions per protein-molecule, depending on the valence of the metals (Coyle et al., 2002:628; Merrifield et al., 2002:158). The primary structure of this protein also contributes to its ability to bind metals. The ability of MT to bind metals contribute to its biological function, which is zinc and copper homeostasis, detoxification of toxic heavy metals and the protection against oxidative damage.

2.1.2 Structure

The tertiary structure of MT depends on whether metals are bound or the protein is oxidized. Metallothionein wraps its metal-binding clusters around the metals to form S_{Cys}-M-S_{Cys} bonds (maximum exposure of metals to thiol groups). This wrapping of the thiol groups around metals cause that MT consist of two definite globular domains (Figure 2.1), the α (alpha) domain containing the C-terminal and the β (beta) domain that contains the N-terminal (Rigby Duncan & Stillman, 2006:2102; Stillman, 1995:463; Vasak, 2005:14; Vasak & Hasler, 2000:178; Winge & Miklossy, 1982:3475). The first 30 amino acids, contributing to the β-domain, consist of nine cysteine amino acids which bind three divalent metals. The remaining 31 or so amino

acids which make the α-domain consist of eleven cysteine amino acids, which bind four divalent metals (Coyle et al., 2002:628; Dabrio et al., 2001:329; Guo et al., 2005:1789; Merrifield et al., 2002:158; Stillman, 1995:465; Vasak & Hasler, 2000:178; Winge & Miklossy, 1982:3475).

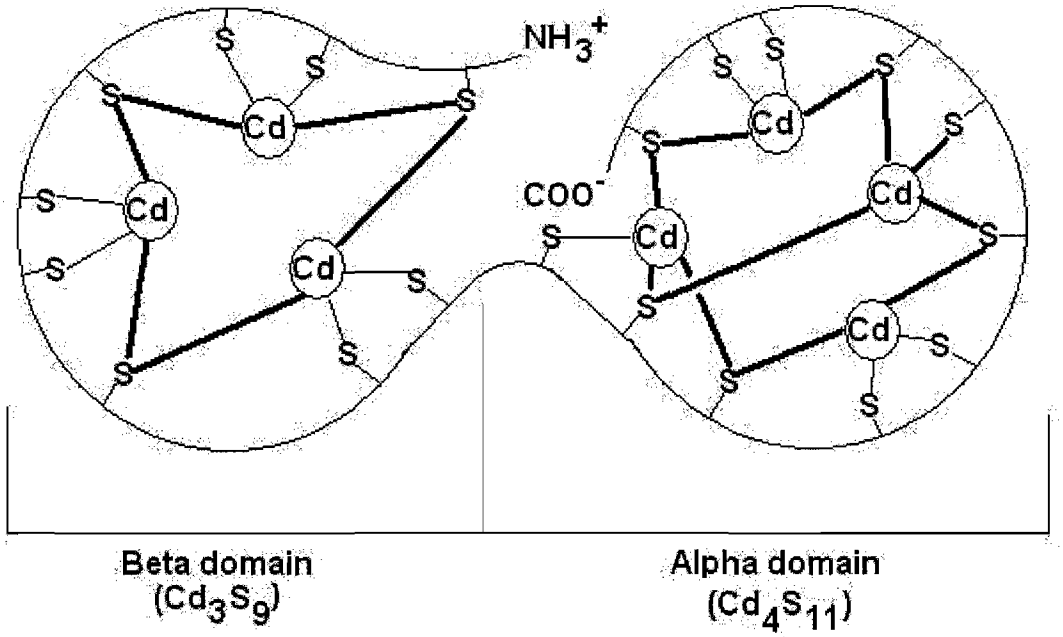

Figure 2.1: Schematic illustration of the tertiary structure of metallothionein. Metallothionein has a dumb-bell-like shape when bound to metals with two distinct domains. These domains are highly similar, depending on the metals bound. The peptide folds around the metals, which are tetrahedrally bound to the thiolate clusters (Rigby & Stillman, 2004:1276; Rigby Duncan & Stillman, 2006:2102; Romero-Isart & Vasak, 2002:389; Wang et al., 2007:3). MT is a random, disordered structure when no metals are bound (Romero-Isart & Vasak, 2002:389; Simpkins et al., 1998:19; Stillman, 1995:466). Divalent metals have four Cys bindings each and monovalent metals have only three Cys bindings each (Merrifield et al., 2002:158). This figure was modified from Winge & Miklossy (1982:3475).

The cysteines in this protein form characteristic Cys-Xaa-Cys, Cys-Xaa-Xaa-Cys and Cys-Cys sequences, where Xaa is any non-cysteine amino acid (Coyle et al., 2002:628; Ghoshal & Jacob, 2001:358; Stillman, 1995:465; Vasak & Hasler, 2000:178). The cysteine residues are externally orientated and therefore exposed to the environment, to rapidly scavenge metals and oxygen radicals. This consequently protects the protein from oxidative and proteolytic damage (Davis & Cousins, 2000:1085; Ma, 2005:31; Stürzenbaum et al., 1998:441). It also prevents the

formation of disulphide bindings under physiological conditions and preserve the cysteine clusters for metal binding (Rigby & Stillman, 2004:1276). MT, however, is easily oxidized under non-physiological conditions where high levels of oxygen are available, resulting in the formation of disulphide bridges between the cysteines. This causes the tertiary structure of the protein to curl and tighten (Rigby & Stillman, 2004:1276; Stillman, 1995:472).

The N-terminal of mammalian MT is usually acetylated, but non-acetylated isoforms had been identified. Whether this acetylation has biological importance other than MT degradation remains unclear (Ghoshal & Jacob, 2001:359; Orga & Suzuki, 1999:22; Sanz-Nebot et al., 2003:389).

Binding of metals makes MT heat resistant and resistant to acid precipitation (Rosenberg, 2003:877). When the pH of the protein solution is lowered, the bound metals begin to dissociate from the protein. Most of the heavy metals dissociate when the pH is lowered below 3 except for copper which dissociates at a pH lower than 2. It was established that metal dissociation happens in a specific pattern, leaving certain species of metal bound to MT. This metal dissociation patterns of MT was the first indicator of the tertiary structure of metallothionein and its consistence of two domains. At neutral pH it was observed that the M_7-MT forms (where M is a metal) are present but when the pH is gradually lowered a dominant form of M_4-MT appears. Further lowering of the pH results in a predominantly apoMT. When a gradual lowering of pH is done, the M_3-MT species is not detected, which can be interpreted that the metals bound to the β-domain dissociate first (Guo et al., 2005:1789).

2.1.3 Isoforms

The metallothionein family consists of four major isoforms MT-1, MT-2, MT-3 and MT-4, with most organisms expressing at least the first two isoforms (MT-1 and MT-2) (McSheehy & Mester, 2003:312; Simes et al., 2003:312; Vasak & Hasler, 2000:177; Vasak, 2005:14). There is little difference between these isoforms (Figure 2.2), with only two amino acids difference between some subisoforms of MT-1 (McSheehy &

Grey areas in text contain additional trivial information.

Mester, 2003:312). In humans the other three metallothionein isoforms (MT-2, MT-3 and MT-4) have no known functional subisoforms. Many MT isoforms are due to genetic polymorphism (Chassaigne & Łobiński, 1999:109; Vallee, 1995:28). Humans have 17 MT genes clustered within the q13 domain of chromosome 16. However, only ten of the 17 MT genes on chromosome 16 is functional and encode MT-1 (A, B, E, F, G, H and X), MT-2 (also known as MT-2A), MT-3 and MT-4 (Coyle et al., 2002:628; Haq et al., 2003:212; Hunziker & Kägi, 1985:381; Schmidt et al., 1985:7735; Stennard et al., 1994:364; Vallee, 1995:28).

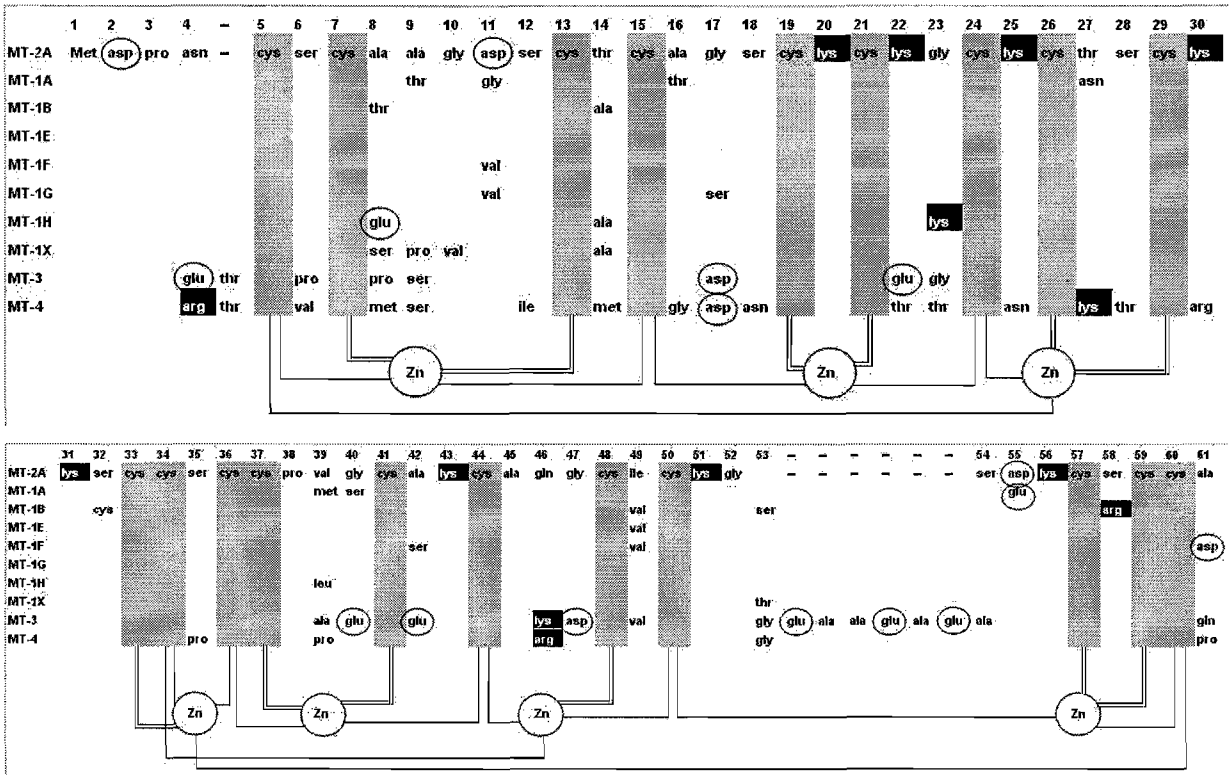

Figure 2.2: Homology of the human MT gene family. Metallothioneins have a high degree homology in their primary structure especially of the cysteines in the structure (Palmiter et al., 1992:6337). There is little difference in amino acid composition with all cysteines on similar positions in the sequence (grey areas). This homology is not only found in the human isoforms, but can be seen when interspecies comparison of the primary structure is done and indicates that this protein should have an important function (Haq et al., 2003:211; Ghoshal & Jacob, 2001:359). The amino acids in the circles and black squares contain negative and positive charge, respectively at neutral pH. This figure was modified from Coyle et al. (2002:629) and Sanz-Nebot et al. (2003:386).

MT-1 and MT-2 are expressed in almost all major organs especially the liver, pancreas, intestine, kidneys and the brain (Coyle et al., 2002:628; Haq et al.,

2003:212; Sato & Kondoh, 2002:10; Vasak, 2005:14). The MT-2 isoform accounts for more than 80 % of all human MT expression (Coyle et al., 2002:628; Davis & Cousins, 2000:1086; Studer et al., 1997:65; Tan et al., 2005:130). MT-3 has seven additional amino acids to its structure at positions 5 and 55-60 when compared to the other expressed metallothionein isoforms (Palmiter et al., 1992:6336; Vallee, 1995:30) and is mainly expressed in the brain (especially the hippocampus and glutaminergic neurons) and skin. MT-3 is the only MT isoform with a specific function reflected by its original name of "neuronal growth inhibitory factor" (Coyle et al., 2002:628; Haq et al., 2003:212; Palmiter et al., 1992:6335; Vallee, 1995:30; Vasak, 2005:14). Pretorius (2006) also found MT-3 mRNA being expressed in the liver of mice, albeit in very small relative quantities. MT-4, the most recently discovered isoform, is expressed in the brain, skin (Davis & Cousins, 2000:1085) and epithelial cells of the tongue (Haq et al., 2003:212; Sato & Kondoh, 2002:11).

2.1.4 Function

Metallothionein has many diverse functions due to their metal-thiolate clusters (Vasak, 2005:15). These can be grouped into primary and secondary functions where primary functions are the work MT do on molecular level.

Metallothionein has three primary functions:

1. zinc and copper homeostasis;
2. detoxification of toxic, heavy metals such as cadmium, mercury, silver;
3. protection against oxidative damage caused by reactive oxygen species (ROS) and reactive nitrogen species (RNS) by acting as a free radical scavenger.

The name, "metallothionein", is derived from its main ability, which is to bind metals via thiol groups (Cys) on the protein. Metallothionein has a strong affinity towards metals, especially toxic, heavy metals. It plays an important role in metal regulation of the body. It is responsible for zinc and copper uptake (when in overdose), distribution (intra- and intercellular), releasing (when needed by enzymes) and to a lesser extent storage (Davis & Cousins, 2000:1087; Ghoshal & Jacob, 2001:359; Rigby Duncan & Stillman, 2006:2102; Vallee, 1995:24; Vasak & Hasler, 2000:179). This ability to

regulate metals means that MT also regulates some enzymes dependent on these metals and therefore plays a role in metabolism and DNA transcription regulation. About 300 enzymes in all six classes are dependent of metals such as zinc (Coyle et al., 2002:637; Vasak, 2005:16) and during time of need, MT releases the metals to the enzymes (Vasak & Hasler, 2000:180). MT also donates or accepts zinc from zinc-finger transcription factors and plays a role in some transcriptional responses (Vasak, 2005:16).

The metals bound to MT under physiological conditions (zinc and copper) are replaced during conditions of heavy metal overdose, because the affinity of MT for these metals is much stronger (Romero-Isart & Vasak, 2002:392; Sato & Kondoh, 2002:10; Stillman, 1995:473). Because of metallothionein's higher affinity toward toxic metals, many investigators believe that metallothionein's biological function is the detoxification of heavy metals (Kang, 1999:263; Stillman, 1995:466; Vallee, 1995:25) as can be seen in aquatic animals during heavy metal pollution. MT thus protect against heavy metal toxicity (Alhama et al., 2006:57; Vasak, 2005:15) such as hepatotoxicity, nephrotoxicity, hematotoxicity, immunotoxicity and bone damage (Dabrio et al., 2002:124).

The metal affinity of MT is as follows: Hg(II) > Ag(I) = Cu(I) > Cd(II) > Zn(II) (Romero-Isart & Vasak, 2002:392; Sato & Kondoh, 2002:10; Stillman, 1995:473).

Many cell-free *in vitro* studies on MT have led to the discovery that MT act as a free radical scavenger for many reactive oxygen species such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), nitric oxide ($NO^\bullet$) and hydroxyl ($OH^\bullet$) radicals (Choi, 2003:239; Ghoshal & Jacob, 2001:360; Hussain et al., 1996:146; Kang, 1999:264). Since these initial discoveries, many more *in vitro* studies with cell cultures and *in vivo* studies were done that confirmed the protective role of MT against oxidative stress (Davis & Cousins, 2000:1086; Kang, 1999:264; Reinecke, 2004:114; Vasak, 2005:15). This ability of MT has been studied thoroughly by many research groups (Hussain et al., 1996:146; Suzuki et al., 2005:536). MT is an extra-ordinarily effective free radical scavenger which can scavenge $OH^\bullet$ 300 times more effective than glutathione (Hussain et al., 1996:150; Kang, 1999:264; Sato & Kondoh, 2002:10) one of best known anti-oxidants of the body. A connection of MT/thionein and

GSH/GSSG has been established. Just as MT exchange metals with enzymes and transcription factors, the same reduction and oxidation exchange (redox control) can occur between glutathione and MT. MT can be oxidized when many oxidized glutathione species exist, to form thionein (apoMT) en reduced glutathione (Coyle et al., 2002:637).

Mitochondria have a central role in the generation of physiological amounts of ROS and RNS in a controlled environment. With mitochondrial disorders, this control is lost which leads to high levels of ROS and RNS, which in return affect other biomolecules and organelles in the cell (especially the mitochondrion). As mentioned in Chapter 1, the expression of MT is up-regulated in mitochondrial complex I disorder (van der Westhuizen et al., 2003:17) and most certainly plays a key role in protecting the cell from the increasing levels of ROS and RNS. The increased expression of MT was confirmed by Semete (2004) in oxidative stress related disorders and by Olivier (2004) in complex I deficiency. The protective properties of MT in complex I deficiency was studied by Reinecke (2004) in HeLa cells and later by Pretorius (2006) in rotenone induced complex I deficient mice.

Most of MT's functions have been uncovered by traditional animal, cell culture and *in vitro* models. However, knockout and knock-in mice models have recently been used to study all functional aspects of MT (Coyle et al., 2002:631; Davis & Cousins, 2000:1086; Sato & Kondoh, 2002:18; Suzuki et al., 2005:536), with "knockout" meaning gene interruption which leads to loss of function and "knock-in" meaning gene amplification which leads to gain of function (Vasak & Hasler, 2000:4). Some scientific groups, working with these mice models have noted that these models did not add much new knowledge about the function of MT (Vasak & Hasler, 2000:179). The new discoveries via these models only led to more debate about MT's necessity when executing these important biological functions, such as in zinc metabolism/ homeostasis. Because MT-null mice are born without any obvious pathological deficiency, it is unlikely that MT is essential for zinc homeostasis during normal situations (Davis & Cousins, 2000:1086; Sato & Kondoh, 2002:11). Only during stress conditions such as high levels of cadmium are the difference exploited with MT-null mice not coping as well as the wild-type mice (Park et al., 2001:97).

The same conclusion can be made about MT's necessity in protecting against oxidative damage during normal situations. Only during oxidative stress conditions can a difference be seen between wild type and MT-null mice. However, there is still a debate on MT's necessity in protecting against oxidative damage, which can be ascribed to the reactivity and extremely short half-life of free radicals *in vivo*, especially hydroxyl radicals. MT must therefore be close to the production site of free radicals to scavenge sufficiently (Kang, 1999:269). MT is a cytoplasmic protein, but has been detected in the nucleus and inner-membrane space of liver mitochondria (Coyle et al., 2002:637; Pérez & Cederbaum, 2003:451; Sato & Kondoh, 2002:16; Ye et al., 2001:2321). In the cytoplasm MT protects against oxidative damage done by *tert*-butylhydroperoxide. In the nucleus, MT protects against oxidative damage of N-methyl-N'-nitro-N-nitrosoguanidine, ultraviolet rays and hydrogen peroxide (Sato & Kondoh, 2002:16).

The three biological functions discussed earlier are considered as the primary molecular functions of metallothionein. These molecular (primary) abilities result in secondary functions of MT which can be explained as the functions of MT seen on the macro-level. This class of secondary functions is diverse and extensive due to the combination of the three primary abilities. Some of the best studied secondary functions are:

1. protection against stress-related conditions and neural and mitochondrial related diseases (Davis & Cousins, 2000:1086);
2. cell growth, proliferation and differentiation (Studer et al., 1997:66; Tan et al., 2005:130; Vasak, 2005:16);
3. anti-apoptotic effects (Kang, 1999:269; Vasak & Hasler, 2000:180);
4. reduction of neural inflammatory response during CNS injury to promote recovery (Vasak, 2005:16);
5. protection against xenobiotics (Coyle et al., 2002:632);
6. neural growth inhibition is a function of MT-3.

MT-3 is structurally and functionally different from the other metallothionein isoforms and is also induced by different factors. As mentioned, this isoform is commonly found in the central nervous system (CNS) and is also known as "growth inhibitory

factor" (GIF). MT-3 is the only isoform with an established, distinct biological function (Coyle et al., 2002:637; Vallee, 1995:30; Vasak, 2005:16).

Surprisingly, no distinct physiological function has been ascribed to the other MT isoforms (even after ~50 years of research) (Coyle et al., 2002:640; Haq et al., 2003:211; Dabrio et al., 2002:125; Vasak & Hasler, 2000:179). The many protective functions of MT depend mainly on its expression induction under stress conditions and in the presence of toxic agents (Dabrio et al., 2002:124). There is therefore a direct relationship between induction and function, which means that both need to be studied together.

2.1.5 MT induction

Metallothionein expression is recognized to be induced by an increasing variety of chemical and physical stressors or factors, many of which act indirectly (Table 2.1).

Table 2.1: Some of the best known inducers of MT expression. The table is compiled of the data collected from the following published articles: Andrews (2000:100); Coyle et al. (2002:631); Davis & Cousins (2000:1087); Ghoshal & Jacob (2001:365-369); Haq et al. (2003:217); Jacob et al. (1999 :302); Suzuki et al. (2005:534); Temara et al. (1997:31).

Inducers	Examples
Metal ions	Zn (physiological inducer), Cd, Hg, Au, Bi, Cu, (Ni & Co can also induce MT expression but do not bind to the protein)
ROS and RNS	H_2O_2, $OH^{\cdot}$, NO (nitric oxide), High O_2 tension, free radical producing agents (diethyl maleate, paraquat, menadione), *tert*-butyl hydroquinone
Hormones and second messengers	Glucocorticoids, progesterone, estrogen, catecholamines, glucagon
Inflammatory agents and cytokines	Lipopolysaccharide, interleukin-1, interleukin-6, interferon-γ, tumour necrosis factor α
Tumour promoters	Phorbol esters
Cytotoxic agents	Ethanol, diethyl maleate, paraquat, doxorubicin, cisplatin, bleomycin
Stress-producing conditions	Starvation, infection, inflammation, ultraviolet radiation

MT-3, however, is not always induced by the same factors which stimulate MT-1 and MT-2 expression (Vallee, 1995:30).

Most of the inducing agents listed in Table 2.1 are not physiological inducers, which mean that during these studies high (non-physiological) concentrations of these agents were used. This list of non-physiological inducing agents and factors is ever growing but the physiological inducers are fixed. These do not need to be in high, toxic levels to induce MT expression (Coyle et al. 2002:631).

The direct and indirect regulation of the promoter of the MT genes has been well studied and reviewed (Coyle et al., 2002:631; Davis & Cousins, 2000:1087) but will not be discussed in this dissertation as this is not the main focus of this study.

2.1.6 Recent interest regarding MT

There are a few properties of metallothionein that makes it an interesting protein to study. Firstly, metallothionein's wide occurrence in nature (eukaryotic and prokaryotic animals and plants) and secondly its ability to bind metals and scavenge reactive oxygen and nitrogen species (Stillman, 1995:463). Scientists have studied MT's role in diseases such as Alzheimer's, Menke's disease and previously mentioned mitochondrial disorders and as biomarker in nature.

- **MT as a biomarker:** Because MT expression is induced by heavy metals in all organisms containing the gene, MT can be used as a biomarker for heavy metal pollution in aquatic animals (Alhama et al., 2006:57; Dabrio et al., 2002:131; Erk et al., 2002:1211; Infante et al., 2006:186; Temara et al., 1997:29; Vasak & Hasler, 2000:178). The European Commission and other international scientific organizations proposed MT to be included in environmental monitoring programs (Dabrio et al., 2002:131). Thus the accurate quantification of MT is important to fulfil its role as biomarker (Alhama et al., 2006:57; Dabrio et al., 2002:131). This also led to the development of a lot of sensitive and quick quantifying techniques to measure MT routinely.

- **MT in diseases:** A decrease in MT expression is believed to contribute to some diseases such as Alzheimer's-, Menke's- and Wilson's diseases. When the brains of Alzheimer's disease patients were studied, it was found to have better neural survival due to less neural growth inhibition. The MT-3 (GIF) levels were compared with normal brains and were found to be decreased in the brains of the Alzheimer's disease patients (Vallee, 1995:30; Vasak, 2005:16; Vasak & Hasler, 2000:180; Yu et al., 2001:40). MT-1 and MT-2 was found to be increased in the Alzheimer's patients brains (Vasak, 2005:16) but decreased according to Yu et al. (2001:40). Since MT-3 has lower metal-binding affinity but higher metal-binding capacity necessary for zinc metabolism in the brain (especially buffering zinc fluctuation), a decrease in MT-3 can cause serious zinc fluctuations. The increase of other metallothionein isoforms fail to compensate and correct the disturbance of metal homeostasis in the brain due to their lower metal-binding capacity (Palumaa et al., 2005:210). A decrease in MT expression is one of the possible causes of Menke's and Wilson's disease which involve the incorrect metabolism of copper (Stillman, 1995:468).

- **Functional studies:** Metallothionein's function and necessity in organisms are the main focus of many laboratories, especially since MT does not have a distinct and established physiological function. It can only be described as a race between scientists to "discover" or prove a distinct function.

- **MT therapy:** MT therapy (especially MT-2A) may hold promise in the treatment of a series of diseases. Published data from Køhlera et al. (2003:134) suggests that MT-2A therapy in various pathological conditions can achieve beneficial effects and might prevent disease progression. Certain companies (China Grand [Shanghai] Imp & Exp Co., Ltd.) already commercialized MT products such as MT capsules which can be taken orally (Anon, 2006).

MT plays an essential role in cell survival and can serve as an important biomarker. A quick and accurate quantification method for MT is essential to utilise MT as biomarker, for studying its role in disease and therapy or when functional studies are done.

2.2 METALLOTHIONEIN QUANTIFICATION METHODS

The elucidation of the specific roles that MT play in organisms is supported by the identification, quantification and structural characterization of MT (Dabrio et al., 2002:125; Łobiński et al., 1998:46). Traditional functional studies of MT were done by monitoring the transcriptional response of specific MT isoforms (Dabrio et al., 2002:125). This approach, however, is not sufficient as many organs in different organisms have been identified to contain trace amounts of MT-3 mRNA, but most of them do not contain any detectable protein levels (Haq et al., 2003:219; Kaplan et al., 1995:137). Therefore, functional studies now include the translational response by measuring protein levels.

Since MT lack aromatic amino acids, it has low detection sensitivity with common laboratory methods (Meloni et al., 2005:77; Sayers et al., 1999:859). Besides the general detection problem, MT also consists of many isoforms which is physically and chemically very similar, making isoform specific detection very difficult. For this reason, a good separation technique is necessary as well as a good detection system (Łobiński et al., 1998:46; Prange & Schaumlöffel, 2002:442; Sanz-Nebot et al., 2003:380). Many methods have been used in the past and present in an attempt to specifically detect and quantify MT. These techniques can be divided into two sections namely separation and detection techniques. In the following sections the principles, advantages and disadvantages of these techniques will be discussed.

2.2.1 Separation Techniques

2.2.1.1 Electrophoresis

- **Northern Blotting:** This is a very common technique which can be performed in most laboratories. Northern blotting uses mRNA, which is very unstable, to study gene expression (Dabrio et al., 2002:129; Pérez & Cederbaum, 2003:445). MT expression is often investigated by this method, but due to the RNA's low stability, many experimental problems are often experienced (Kaplan et al., 1995:140).

This method gives poor, unspecific results most likely due to homology of MT isoforms.

- **SDS-PAGE**: SDS-PAGE is used for the electrophoretic separation of proteins and peptides. The expression of MT can be studied by quantifying the amount of MT protein, which is much more accurate than mRNA detection (Dabrio et al., 2002:130). Previous studies did not always show a correlation between the mRNA quantity and protein levels (Vasak, 2005:16; Vasak & Hasler, 2000:179; Vasconcelos et al., 1996:671). SDS-PAGE can thus be utilized to indicate the amount of MT and in exceptional situations can distinguish between MT-1 and MT-2 (and some times MT-3). The subisoforms of MT-1, however, cannot be distinguished from one another. Most of the time MT is associated with band broadening on the gel and a reduced electrophoretic mobility (Meloni et al., 2005:77). Proteins are commonly visualized in the gel by Coomassie® Brilliant Blue staining, which binds to hydrophobic areas. Since MT does not have aromatic residues, it is not efficiently stained by Coomassie Brilliant Blue (Alhama et al., 2006:56; Mizzen et al., 1996:80). An alternative staining method is the use of silver staining, which reacts with peptide bonds.

2.2.1.2 Capillary electrophoresis

As the technique's name imply, this technique entails electrophoresis in a capillary column. Capillary electrophoresis (CE) is different from chromatography as it uses an electrical field over the column (not pressure) to elute the samples. The samples are, for this reason, identified according to their migration times (and not retention times). Capillary electrophoresis is, along with RP-HPLC (Section 2.2.1.3), reportedly the best separation technique for metallothionein with the capability to separate positive, neutral and negative ions in a single run. It is also much cheaper than HPLC (Álvarez-Llamas et al., 2001:118; Beattie, 1998:256; McSheehy & Mester, 2003:312; Prange & Schaumlöffel, 2002:442; Schaumlöffel et al., 2002:159; Virtanen & Bordin, 1998:236).

Other advantages of this method are the little sample preparation needed, rapid analysis and separation at low pH (which is necessary to keep the metallothionein protein in its metal-free form) (Knudsen et al., 1998:167; Pröfrock et al., 2003:1414;

Richards & Beattie, 1995:30). Thionein (or apoMT) can be better resolved when no metals are bound (Beattie, 1998:257; Knudsen et al., 1998:170; Richards & Beattie, 1995:30), as different metals can cause different migration times and detection. Virtanen et al. (1996:399), however, disagree with this statement. Although this is a very effective technique for MT isoform separation, some researchers state that it does not always have enough resolution for the MT-1 subisoforms (Nischwitz et al., 2003:147). The basic design of CE limits its use with detectors such as mass analysers and requires complicated coupling interfaces (Álvarez-Llamas et al., 2001:107; Łobiński et al., 1998:46; Prange & Schaumlöffel, 2002:442; Rosenberg, 2003:849).

2.2.1.3 Chromatographic techniques

- **Size exclusion (SEC):** With this technique molecules are chromatographically separated via their molecular size and to a lesser extent their shape. In general this separation method does not give satisfactory resolution to discriminate between proteins which have a small amino acid difference and cannot separate MT-1 from MT-2 (Nischwitz et al., 2003:149). This technique is better applied as a clean-up step to separate the MT fraction (light-weight proteins) from other proteins in a biological sample (Álvarez-Llamas et al., 2001:108; Beattie, 1998:261; Łobiński et al., 1998:46; Nischwitz et al., 2003:147; Prange & Schaumlöffel, 2002:448; Rosenberg, 2003:848).

- **Anion Exchange:** This type of chromatography relies on the attraction of opposite charged particles. MT-1 and MT-2 proteins, which have (natural) negative charges under neutral, hydrophilic conditions, can be separated via anion exchange chromatography (McSheehy & Mester, 2003:312; Prange & Schaumlöffel, 2002:449; Richards & Beattie, 1995:30). This separation technique, however, cannot effectively discriminate between the subisoforms (Łobiński et al., 1998:46; Prange & Schaumlöffel, 2002:449). Another disadvantage of this method is its use of ionic buffers such as Tris-HCl, which make it less favourable to use online with mass spectrometry (Prange & Schaumlöffel, 2002:451).

- **Reverse Phase (RP):** As the name imply, it is chromatography between a non-polar stationary phase and relatively polar mobile phase. With this technique hydrophobic compounds are better retained than hydrophilic compounds (Chassaigne & Łobiński, 1999:111). RP-HPLC, along with CE, is the best known techniques for separation of metallothionein isoforms and subisoforms (Beattie, 1998:266; Chassaigne & Łobiński, 1999:111; Hunziker & Kägi, 1985:379; Poleć et al., 2002:205; Prange & Schaumlöffel, 2002:451). RP-HPLC gives good resolution between isoforms and subisoforms that have the same net charge and only differ in hydrophobic character. Another advantage is that reverse phase columns do not bind metals, which means that no metal contamination of metal-free MT occur and no metal loss of metal-bound MT. The following requirements, however, limits its use: it uses expensive organic solvents, the columns are relatively expensive and analysis takes relatively long, compared to other chromatographic methods (Chassaigne & Łobiński, 1999:111; Łobiński et al., 1998:46; Nischwitz et al., 2003:147; Prange & Schaumlöffel, 2002:451).

2.2.2 Detection or Quantification Techniques

Not only is a good separation method needed for MT quantification, but also a selective and sensitive detection system to help discriminate between the MT isoforms in biological material. This is where the combination of techniques is very useful and powerful (Łobiński et al., 1998:46; Prange & Schaumlöffel, 2002:442). Most of the separation techniques can be coupled to any the following detection methods.

The detection techniques most commonly used to measure MT can be separated into two groups: direct detection and indirect detection. The indirect techniques quantify the metals bound to MT and then estimate the MT concentration (Dabrio et al., 2002:125). The direct methods measure the protein directly. The RNA analysis techniques are also classified as direct detection.

The indirect detection methods, which measure the metal content rather than the protein itself, depend on the saturation of metallothionein with metals for detection. This is why the indirect methods are also called *saturation* techniques.

2.2.2.1 Saturation techniques

- **Atomic absorption spectrometry (AAS):** As mentioned, this technique is based on metal detection. Metals are quantified to estimate the MT content (Del Ramo et al., 1995:122). Thus, the metal saturation step when preparing MT for analysis is critical. This also means that analysis must be done at neutral or basic pH since metals dissociate at lower pH. This approach is in fact speculative since not all MT proteins in a mixture are always saturated with metals and some contain metals of higher affinity (Del Ramo et al., 1995:123). An advantage is that the metal content from raw (untreated) MT can be studied along with affinity studies, but quantification is not recommended (Sanz-Nebot et al., 2003:381).

- **Spectrophotometric detection:** This is a very common detection technique fitted on most liquid chromatographic (LC) systems. Compounds are detected via UV or fluorescence depending on the compound and pre-analysis preparation (such as fluorescent probe derivatization) (Alhama et al., 2006:53). Standard protein spectrophotometry using UV absorption at 280 nm cannot be used to detect MT as MT lacks aromatic amino acids. Peptide bonds (detection at 200 nm) or metals bound to the protein can be used to detect MT (Beattie, 1998:262; Chassaigne & Łobiński, 1999:111; Richards & Beattie, 1995:30; Simes et al., 2003:313; Stürzenbaum et al., 1998:438). Unfortunately, the use of any wavelength under 250 nm has the disadvantage that many substances absorb light at these wavelengths. Fluorescent detection can be used when MT is derivatized with a fluorescent probe, especially those that bind the thiol-groups of the protein (Alhama et al., 2006:53). This derivatization in association with LC separation can be a powerful detection method. Unfortunately, one cannot possibly be sure which MT isoforms were detected since selectivity occurs only via retention times (Chassaigne & Łobiński, 1999:115; Dabrio et al., 2002:128). There is only a primary selection of the separation method and not a secondary selection of the detection system. Thus, while other compounds with cysteine groups can also be

derivatized, it is easy to see why sometimes speculative, contradicting and confusing data can be seen with such detection (Alhama et al., 2006:57; Álvarez-Llamas et al., 2001:116; Sanz-Nebot et al., 2003:380).

- **Inductively-coupled plasma mass spectrometry (ICP-MS):** Similar to AAS, it is an element-specific detector but uses the element mass. This is probably the most used detection system for MT and has generally replaced AAS as preferred detection method (Prange & Schaumlöffel, 2002:442). It has high sensitivity and the lowest detection limit of all the detection methods described here. But, as with AAS, samples need to be pure from other interfering metallo-compounds. Though ICP-MS has the best detection sensitivity and excellent selectivity, it is only useful to a limited extent. It only provides element specific information and not molecular or structural information which is especially needed when unknown and un-purified tissue protein mixtures are analyzed (Álvarez-Llamas et al., 2001:118; McSheehy & Mester, 2003:311; Rosenberg, 2003:843).

2.2.2.2 PCR techniques

The introduction of PCR techniques such as RT-PCR (reverse transcription PCR) into the field of MT gene expression research simplified mRNA measurements and has replaced techniques such as Northern blotting. The relatively unstable mRNA is converted to the more stable cDNA form and this way the mRNA levels can be quantified "indirectly" (Dabrio et al., 2002:129; Kaplan et al., 1995:137). Some commonly used PCR methods for metallothionein RNA quantification is: semi quantitative RT-PCR (comparing target mRNA's integrated optical density to corresponding human phosphoglyceraldehyde dehydrogenase), competitive RT-PCR (using known concentrations of cRNA mimic as internal standard) and Real-time PCR (Dabrio et al., 2002:130; Kaplan et al., 1995:138; Reinecke, 2004:137). These techniques, especially real time PCR, are very sensitive and selective but do not give any data on the protein levels.

2.2.2.3 Immunological techniques

Immunological techniques consist of an antibody reaction with a target molecule which can be visualized by radioactivity, chemiluminescence, fluorescence or enzymatic reactions. These techniques usually have high sensitivity and selectivity

and can be used on proteins as well as nucleic acids. Commonly used immunological techniques are enzyme-linked immunosorbent assay (ELISA), cELISA (competitive ELISA), radio-immuno assay (RIA) and immunoblotting. Immunological techniques, however, has low selectivity for MT (Olivier, 2004:69) and can only distinguish between the isoforms MT-1 and MT-2 (not MT-3 or between subisoforms). This low selectivity is due to the following: (1) the small size of the protein, which does not give efficient antibody binding and activation when producing antibodies (Dabrio et al., 2002:130; Łobiński et al., 1998:46); (2) the homology of the isoforms and; (3) the differences in structure of apo-MT, oxidized MT and metal-bound MT where various metals bound can also give structural differences (Valles Mota et al., 2000:195). Apostolova et al. (1998:331), however, developed an antibody not influenced by metals bound or released from the MT proteins. Except for the poor selectivity, immunological techniques are still sensitive and capable to detect MT in biological samples which contain low levels of the protein (Dabrio et al., 2002:129).

2.2.2.4 Electrochemical techniques

The most used electrochemical technique to detect and quantify metallothionein is the Brdicka method (Erk et al., 2002:1212). Measurement is based on the amount of thiol-(SH) groups in the mixture (Dabrio et al., 2002:125). The total MT content in a mixture can thus be determined with relative high sensitivity (but low selectivity). Other redox systems in the mixture can interfere with the results and for this reason a high purity sample is required. (Dabrio et al., 2002:129; Łobiński et al., 1998:46; Valles Mota et al., 2000:194).

2.2.2.5 Mass spectrometry

The highly sensitive mass spectrometric (MS) detector detects molecules by their mass-to-charge ratio and is chosen more often than other detectors because of its high selectivity. Electrospray ionization (ESI) produces intact multiple charged molecular ions which means that proteins and other large biomolecules can be detected by mass spectrometry (Łobiński et al., 1998:46; Prange & Schaumlöffel, 2002:451; Rosenberg, 2003:844; Vestling, 2003:122). The basic amino acids (lysine, arginine and histidine) as well as the N-terminal play an important role in protonation (Wilson & Walker, 2003:553). ESI-MS is also attractive because of its universal fit to chromatographic systems and because it can handle most LC flow rates (Rosenberg,

2003:845; Vestling, 2003:122). Despite the increasing doubt in the mass detector for accurate protein quantification (Garbis et al., 2005:9), it remains the best direct, specific and universal detection method which is used in proteomic studies.

Mass spectrometry is an essential technique for MT characterization and quantification (Dabrio et al., 2002:128). Unfortunately, most published studies focus on MT identification and characterization in a mixture and less on quantification (Prange & Schaumlöffel, 2002:452; Sanz-Nebot et al., 2003:381). Although this technique is a direct approach, quantification still is a difficult task (Dabrio et al., 2002:128). Except for the technique-associated difficulties for measuring proteins there is also the nature of MT to consider, such as the different forms of MT (apo-MT, oxidized MT and metal-bound MT) and the absence of pure commercial standards to optimize this technique (Sanz-Nebot et al., 2003:392). These difficulties can, however, be overcome by acquiring a MT standard to optimize preparation (to attain uniformity) and analytic conditions, which is the rationale behind this study.

2.3 PROBLEM STATEMENT AND OBJECTIVE

Mitochondrial disorders are accompanied not only by low energy output, but by increasing oxidative stress. Oxidative stress is commonly known to cause damage to various molecules, organelles, cells and ultimately organs. This damage can manifest as various diseases such as cancer, neurological diseases, organ failure, just to name a few. The chemical imbalance caused by these mitochondrial disorders is known to cause secondary effects other than damage. Many epigenetic- and cell signal transduction pathways are affected which stimulate or inhibit the expression of specific genes. Many of these expressed genes serve as defense mechanisms against the imbalances like ROS.

Van der Westhuizen et al., (2003:17) found one protein's expression to be markedly increased during mitochondrial complex I deficiency during a microarray-based investigation of gene regulation. This protein is metallothionein, which is believed to act as an antioxidant in situations of high oxidative stress. The induction of metallothioneins in mitochondrial disorders was studied further in *in vitro* and *in vivo* studies by means of RNA level measurements (Olivier, 2004:42; Pretorius, 2006:44). This was done in relation to oxidative stress measurements and damage or phenotypic observation (Reinecke, 2004:89). Reinecke et al. (2004:86) showed that the levels of induction of MT mRNA expression differ from protein levels.

From these and other studies it is clear that MT should be measured on protein level to investigate the expression and role of MT more accurately. A method to quantify MT quickly and accurately in biological material need to be established, which can be used to measure and compare the MT levels in biological samples of various types.

Based on current literature (Section 2.2), the methods used for MT analysis and available analytical capacity at this institution, it was concluded at that a mass spectrometric-based technique would be possible and holds the most promise for future research. One other consideration was that quantification of human MT-2A

would be a priority as this isoform is the key ubiquitous active isoform involved in humans. For detection and quantification with mass spectrometry, however, a pure standard of chemical and physical similarity to metallothionein (MT-2A) is necessary for technique optimization. The lack of such pure MT standards is emphasized in many publications (Section 3.1). Before the technique could be established and optimized, pure (95 %) MT standards are required.

Therefore, the objective of this study was to obtain or develop two pure MT standards: one similar to MT-2A but different in mass to serve as internal standard for quantification and the other (MT-2A) to use for method optimization (in future studies).

The strategy outlined in Section 2.4 was followed for these aims to have been reached (Figure 2.3).

2.4 STRATEGY AND EXPERIMENTAL DESIGN

The strategy to obtain the objective of this study and develop useful MT standards for MT-2A quantification is summarized in Figure 2.3. As mentioned before, this strategy is aimed at developing a standard to be used in mass spectrometric quantification of MT-2A in biological samples for future research. The rationale behind each step of the strategy and methodology will be discussed in more detail in the following chapters, but briefly include the following steps:

Firstly, the human MT-2A gene had to be inserted into an expression vector of choice by cutting it from pIRESneo-MT2 and ligate it to pET28B. The ligation products were transformed into *E.coli* cells to confirm ligation and ensure sufficient stock of the new constructs. The new vector constructs was screened with restriction analysis and PCR to confirm the correct insertion of MT-2A into the vector. The new construct was also sequenced to confirm successful ligation.

Secondly, the new construct, pET28B-MT2, was transformed into expression strains and the recombinant modified MT-2A (containing an N-terminal His-tag) was expressed. Expression conditions were optimized to ensure optimal expression. After expression, the cells were harvested and broken to collect the lysate (containing the recombinant protein).

Thirdly, the recombinant MT-2A fraction was extracted from the collected samples by heat treatment and further isolated by means of metal affinity chromatography. The purity of the isolated recombinant modified MT-2A was further increased by separating it from other interfering proteins with RP-HPLC. Thrombin was used to remove the His-tag from the protein which was again purified with RP-HPLC. The final product was analyzed with mass spectrometry to confirm the successful removal of the tag and purity before its use as *internal standard* for mass spectrometry quantification of MT in biological material.

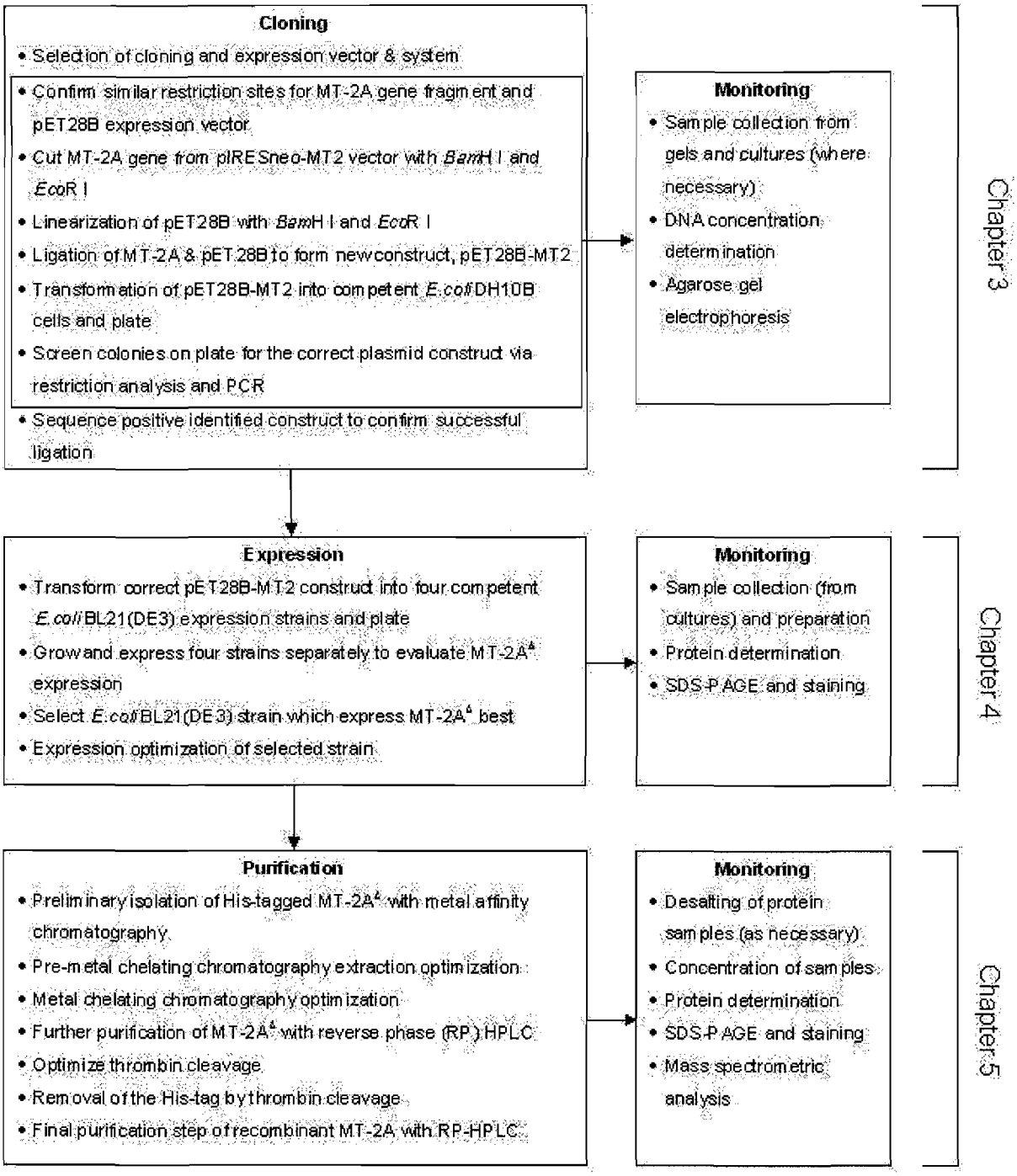

Figure 2.3: Strategy for the developing of MT standards for mass spectrometric analysis of MT-2A. The process of developing an internal standard is separated into three chapters. Chapter 3 describes the cloning procedure followed by Chapter 4 describing the expression of recombinant MT-2A. Chapter 5 focuses mainly on the purification of the expressed recombinant standard.

Chapter 3

Cloning of human MT-2A

3.1 INTRODUCTION

3.1.1 General considerations

In Section 2.3 it was stated that the objective of this study was *to obtain two pure standards: one to serve as internal standard for quantification and the other to use for method optimization*. An internal standard of known concentration and similar structure and mass to the target molecule (human MT-2A in this case) is required for accurate quantification of similar target molecules in biological samples using mass spectrometry. A second standard (the target molecule itself, MT-2A) is also required to optimize the quantification technique before its use on biological material. Two important prerequisites that were put forward for these standards were that they had to be relatively pure (>95 %) and, in the case of the internal standard, enough had to be available for regular use in analysis.

Commercially obtained MT standards (isolated from animal tissue) were considered for these purposes. It was reasoned that commercial MT-2A can be used to prepare concentration ranges to optimize the method. Furthermore, any other commercial MT of similar structure but with different mass not resembling any human MT isoform, such as rabbit MT-1A, can be used as the internal quantification standard, assuming that the response factors for MS detection are similar between the internal standard MT and target MT (MT-2A).

Unfortunately, most commercial MT standards are not pure and consist of more than one isoform, which cannot be used for technique optimization or quantification. The absence of pure commercial metallothionein standards, especially human MT-2A, is a common limitation for the development and optimization of techniques for MT identification and quantification (Dabrio et al., 2002:88; Łobiński et al., 1998:46; Sanz-Nebot et al., 2003:796; Virtanen et al., 1996:398). Many research groups settled with the less pure MT standards isolated from rabbit liver which can be bought from

chemical companies such as Sigma-Aldrich (Alhama et al., 2006:53; Chassaigne & Łobiński, 1999:111; Erk et al., 2002:57; Nischwitz et al., 2003:151; Sanz-Nebot et al., 2003:796). These isolated commercial standards contain many MT isoforms and subisoforms (Álvarez-Llamas et al., 2001:114; Chassaigne & Łobiński, 1999:111; Nischwitz et al., 2003:375; Schaumlöffel et al., 2002:161) and are not suitable for optimization of any isoform-specific detection method such as mass spectrometry.

After extensive searching, a company (Bestenbalt, Tallinn, Estonia) offering pure commercial rabbit MT standards, was found. Relatively pure (98 % as determined using HPLC) rabbit MT-2A was purchased from this company to be used as the *target protein* (MT-2A) to optimize the mass spectrometric detection of the protein. MT-2A from rabbit and humans are highly similar in primary structure with 92 % homology existing between their sequences and therefore it was considered a suitable standard for this purpose.

However, a commercial MT standard was not considered as *internal standard* for quantification of the target protein at the start of this study, because these commercial standards are very expensive (up to ~R6000 per mg). As this new MT quantification technique will probably be used frequently or perhaps routinely, such expensive MT standards were considered as an expensive second option to using a self-produced recombinant standard. The first (preferred) option was using a modified recombinant human MT-2A standard which can be cloned, expressed, isolated and used as the internal standard with known concentration and mass, to quantify the target protein. As mentioned before, the prerequisite for this standard was that it had to be produced at a sufficient amount and in a relatively pure form. In this chapter the cloning of the human MT-2A gene into an expression vector will be discussed.

3.1.2 Cloning and expression of recombinant modified MT-2A

The use of gene cloning and -expression in micro-organisms is now an established approach to produce recombinant proteins. It involves the incorporation of a DNA fragment (gene) of interest into a suitable bacterial plasmid (circular DNA) and the introduction of the recombinant plasmid into appropriate host bacteria to multiply the plasmid and express the (recombinant) protein of interest. There are many

expression systems for both prokaryotic and eukaryotic cells. When correct structure (especially tertiary) and post translation modifications are crucial for proper function of recombinant proteins, then eukaryotic expression would be more appropriate to express mammalian proteins. Since only primary structure and not function was crucial for the recombinant MT-2A, it was decided that bacterial expression of a modified MT-2A (MT-2A$^{\Delta}$), to be used as internal standard for quantification, would be adequate. Fortunately, literature exists on MT expression in bacterial cells which describes relative yield of expression with little practical complications (Eriste et al., 2003:162; Park et al., 2007:86; Yang et al., 2007:186-194; You, 1999:50).

Many commercial vectors are available for expression and every one is different. Most of these vectors have the following properties that should be kept in mind when choosing one for expression (Garrett & Grisham, 1999:397,413). These include a:

- *specific antibiotic coding region* for antibiotic selection. Many expression strains already have a plasmid incorporated into them which execute a certain function. These plasmids also give antibiotic selection which means that when you want to use these strains for expression, a plasmid which gives different antibiotic selection should be used to transform the DNA fragment into these cells. Otherwise, it would be difficult to discriminate between transformed and non-transformed cells as both would grow.

- *multiple cloning site* (MCS) containing a sequence of restriction endonuclease sites close together. The cloned DNA fragment and MCS should preferably have similar restriction sites to allow ligation.

- *promoter region* upstream (5') of the cloned fragment which allows constant expression or expression in the presence of an inducer. Most researchers would prefer the latter especially when expressing a protein toxic to the cells. The cells can be grown to a high density and then induced by the addition of a specific inducer.

- *specific tag* (chimeric peptide added to N- or C-terminal sequence of expressed protein) which facilitates purification of the recombinant protein. Large tags such as the GST-™ and Trx-tag™ is used to help the expression of small proteins. Purification using these tags is much "cleaner" when compared to purification of histidine-tagged proteins. The His-tag® is a small tag consisting of 10, 8 or 6 Histidines in tandem. The His-tag has a high affinity to bind metals which means a fused protein can be purified via metal chelating chromatography, which is cost-effective, easy to use and relatively quick (Woestenenk et al., 2004:217). The disadvantage of this however is that contamination can occur, as other metal binding proteins (such as FKBP-type peptydyl prolyl isomerase) is also purified (Hengen, 1995:285).

- *tag removal (protease cleavage) site* which can be used to remove the tag from the target protein after purification. Some common tag removal sites in plasmids include thrombin- and enterokinase cleavage sites. To ensure successful tag removal, the target protein should never contain the same cleavage site as that of the tag removal site elsewhere in the sequence. Thrombin is not always the best choice since unspecific cleavage of target protein might occur (from other proteases present in the purchased thrombin) (Christodoulou, 2000).

The choice of expression system was the first decision made followed by expression vector type. Bacterial expression was selected for reasons revealed in Section 4.1. It was also decided to add a histidine tag (His-tag) to the recombinant metallothionein as the system and experience to express and purify His-tagged proteins were already present in our laboratory. This relatively small tag could also be of value to add mass to the protein and thus make it different to native MT-2A (target protein). The next step was to decide at which terminal of the protein the tag should be added to improve purification. The terminal of choice should not be enclosed in the tertiary structure of the protein but exposed for affinity binding. Metallothionein's tertiary

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structure was investigated through literature and database research and it was found that the N-terminal of human MT-2A was exposed. Hence, the expression vector of choice needed to add an N-terminal His-tag. Other criteria such as endonuclease restriction sites and tag removal, as mentioned above, were further used to assist in the decision of expression vector as well as availability to our laboratory. The available expression vector which satisfied most of the criteria was pET28 (Novagen®).

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3.2 METHODOLOGY, RESULTS AND DISCUSSIONS

Most of the materials and methods used to clone human MT-2A are according to the manufacturer's specifications or commonly used and established protocols. These protocols will not be discussed in much detail unless where necessary steps were taken or modifications were made to overcome certain obstacles. A complete list of reagents and materials as well as the suppliers is available in Appendix A.

3.2.1 Expression construct design and restriction endonuclease optimization

3.2.1.1 Materials and Methodology

The limited restriction sites within the MT-2A cDNA complicated its direct use. The MT-2A gene was previously (Reinecke, 2004:41) incorporated into the pIRESneo2 plasmid via the use of PCR-generated restriction sites within the intronic regions (Reinecke, 2004:41). An *Eco*R I restriction site was created 40 bp away from the coding region towards the 3' end by PCR and was used along with *Eco*R V at the 5' end to incorporate a 273 bp cDNA fragment into the pIRESneo2 plasmid (Clontech®, BD Biosciences). Restriction sites of the pIRESneo-MT2 vector was compared with those of the pET28 plasmid to find matching enzyme restriction sites. Two restriction sites, a 5' *Bam*H I and 3' *Eco*R I restriction site, were selected to cut the MT-2A fragment from the pIRESneo-MT2 vector and insert it into the pET28 expression vector. The complete strategy is shown in Figure 3.1.

One important aspect of this *Bam*H I site has to be noted. The *Bam*H I restriction site is located *within* the coding region of the MT-2A sequence, just after the initiation codon. The use of this site would lead to the loss of the N-terminal methionine of the MT-2A. Since it was intended to express a modified MT-2A with a different mass to native MT-2A, either as a fusion protein with additional amino acids (His-tag) or with different mass after removal of the His-tag, this loss of the N-terminal methionine fits the purpose of the internal MT standard, which is to have a protein similar to native

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MT-2A but with a different mass. In addition, as mentioned in Section 3.1.2, the integrity of the protein's function is not crucial for the quantification procedure.

Most commercial bacterial expression vectors consist of three forms (A, B and C) which differ from each other only by one base pair, usually after the initiation codon and in front of the MCS, to allow cloning of genes in different reading frames (translation of codon sequences). With the 5'-end enzyme (*Bam*H I) known, the correct form of the selected expression vector, pET28B, was selected to give the correct reading frame and thus translational sequence (see Table 3.1).

Table 3.1: Translation of cloned MT-2A cDNA in pET28A-C vectors. When *Bam*H I (recognition site underlined) is used to incorporate the MT-2A cDNA fragment (first row) into pET28A-C vectors, the following possible translation possibilities exist. The MT-2A coding region and correct MT-2A translated sequence are displayed in bold.

	Partial sequence and translation
Human MT-2A	*Bam*H I --- GCC **ATG GAT CCC AAC TGC TCC TGC GCC GCC GGT GAC TCC** --- **M D P N C S C A A G D S** ---
pET-28A(+)	--- ATG GGT CGC GGA TCC **CAA CTG CTC CTG CGC CGC CGG TGA** --- --- M G R G S Q L L L R R R * ---
pET-28B(+)	--- ATG GGT CGG GAT CCC **AAC TGC TCC TGC GCC GCC GGT GAC** --- --- M G R **D P N C S C A A G D** ---
pET-28C(+)	--- ATG GGT CGG ATC CCA **ACT GCT CCT GCG CCG CCG GTG ACT** --- --- M G R I P T A P A P P V T ---

The *Bam*H I and *Eco*R I restriction sites on the pET28B plasmid was separated by only one base pair and hence concern rose whether both enzymes would cut the plasmid. As both enzymes were purchased from Promega®, their capability to recognize base pairs near the end of linearized DNA was investigated and compared using the Promega Life Science Catalogue (2006:353). The fact that these vectors are engineered and commercially sold like this also reduced the doubts there were.

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The compatibility of the cleaved ends given by *Bam*H I and *Eco*R I was also compared by means of available data given in the New England BioLabs® Inc. Catalogue (2005:265). Theoretically, no ligation is possible between the cleaved ends of *Bam*H I and *Eco*R I. This ensures that when the vector is digested with both enzymes, it would not close on itself again when DNA ligation is performed. Only when the insert is available would conjugation take place and the plasmid close.

The Promega Life Science Catalogue (2006:354) was also consulted to compare the enzyme activity of both enzymes in different reaction buffers and it was noted that both *Bam*H I and *Eco*R I have ~100 % activity in the MULTI-CORE™ buffer and ~75 % activity in 4-CORE® Buffer B. Therefore, it was possible to digest the target DNA with both enzymes simultaneously (double digestion). The double digestion was, however, first tested especially as *Eco*R I could show potential star activity (unspecific digestion) in the MULTI-CORE buffer. The pIRESneo-MT2 plasmid (558 ng), previously cultured and isolated by Reinecke (2004:52), was digested with *Bam*H I (10 U) and *Eco*R I (14 U) using the MULTI-CORE buffer or Buffer B (1x) as provided by Promega. All reaction mixtures in volumes of 50 µl were incubated for 30 min at 37 ℃ after which the samples were analyzed using elec trophoresis. Electrophoresis was carried out on a 1.5 % (m/v) agarose gel containing 1.3 µM ethidium bromide (EtBr). The gel was visualized and photographed with the Chemi Genius Bio Imaging System (Syngene, Cambridge, England) using GeneSnap (version 6.05, Synoptics). Figure 3.2 shows the photographed electrophoresis result of the restriction digestion of pIRESneo-MT2 with *Bam*H I and *Eco*R I using the MULTI-CORE buffer or 4-CORE Buffer B. This was done to confirm high enzyme activity and no star activity when using these buffers. A low range DNA ladder was used to confirm the 220 bp fragment rather to confirm the plasmid size.

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MULTICORE™ is a trademark of Promega Corporation (Madison, WI, USA)

4-CORE® is a registered trademark of Promega Corporation (Madison, WI, USA)

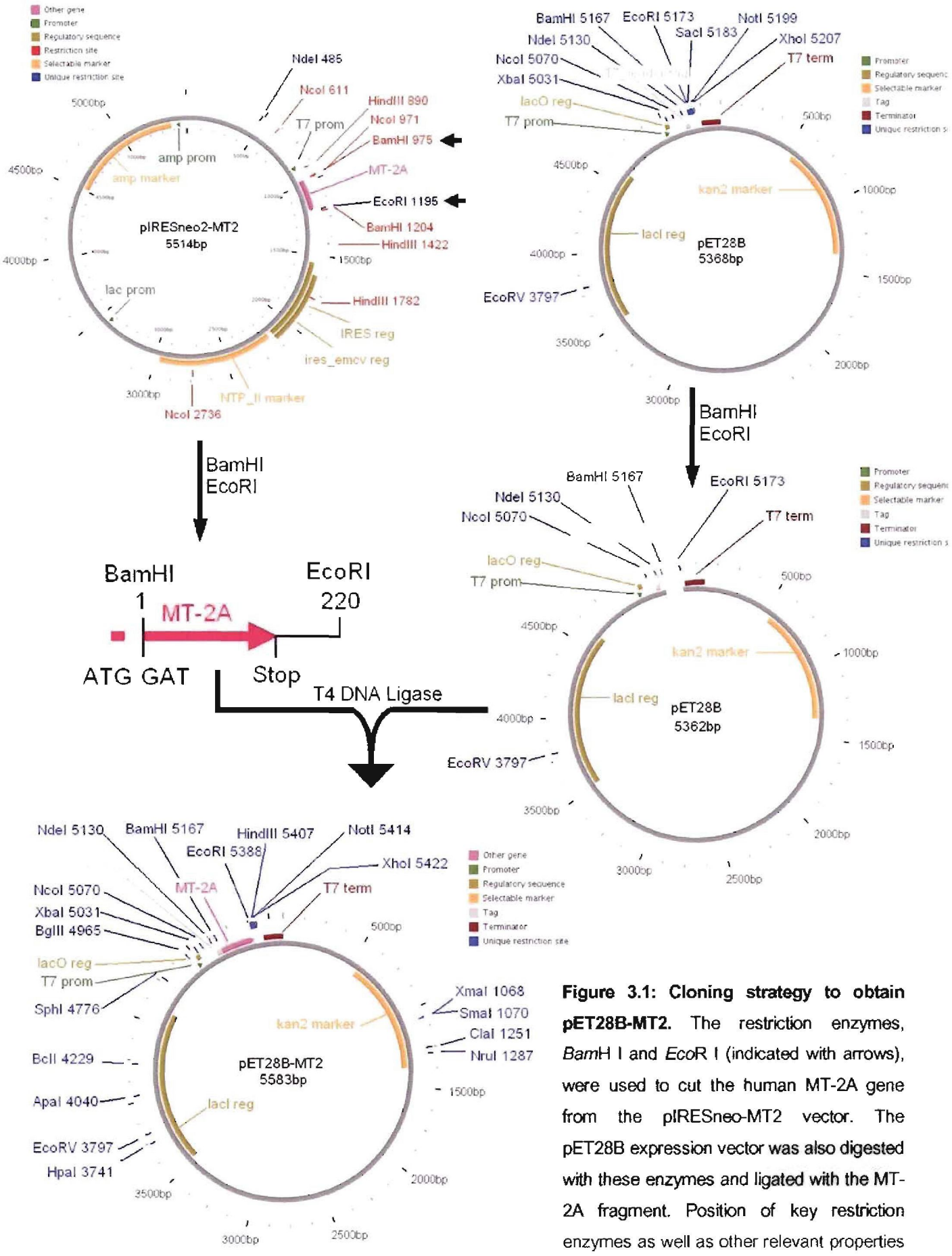

Figure 3.1: Cloning strategy to obtain pET28B-MT2. The restriction enzymes, *Bam*H I and *Eco*R I (indicated with arrows), were used to cut the human MT-2A gene from the pIRESneo-MT2 vector. The pET28B expression vector was also digested with these enzymes and ligated with the MT-2A fragment. Position of key restriction enzymes as well as other relevant properties of the plasmids are indicated.

3.2.1.2 Results and Discussion

The second and third lanes of Figure 3.2 show the result of the restriction endonuclease digestion of pIRESneo-MT2 digested with both *Bam*H I and *Eco*R I in MULTI-CORE buffer and Buffer B, respectively. Digestion of pIRESneo-MT2 with *Bam*H I and *Eco*R I resulted in formation of the two expected DNA fragments, which are the 5237 bp linear pIRESneo2 vector and 220 bp MT-2A insert. The 220 bp fragment is clearly visible in the second lane, but not in the third (pIRESneo-MT2 digested in Buffer B). Lanes 4 and 6 contain the linearized pIRESneo-MT2 fragment after it was digested with only *Eco*R I. The third last lane contains undigested, supercoiled pIRESneo-MT2 in the MULTI-CORE buffer that migrates faster through the gel than linear pIRESneo-MT2 (evident in the other lanes). Two heavier bands are observed in the same lane and are possibly plasmid-chains and are common signs of plasmids.

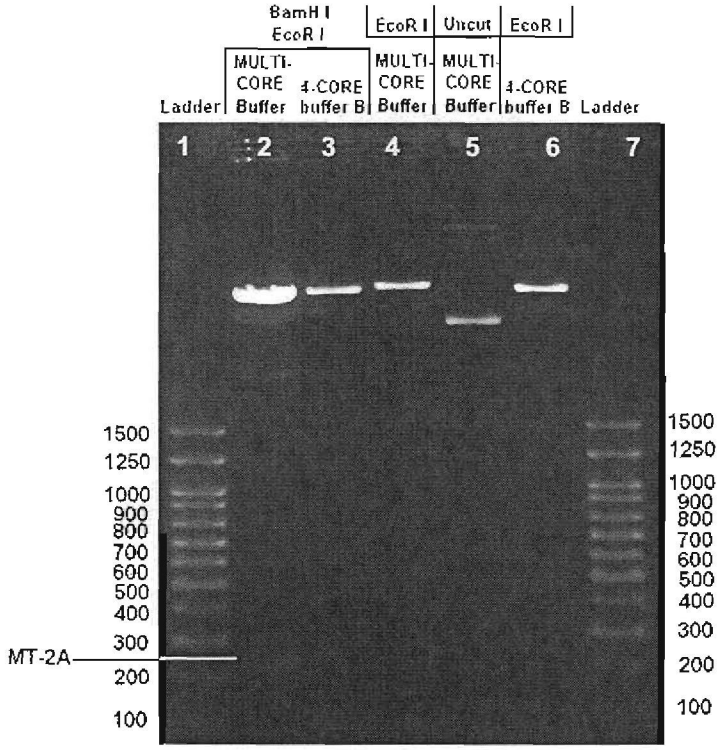

Figure 3.2: Restriction digestion of pIRESneo-MT2 with *Bam*H I and *Eco*R I using different reaction buffers. Enzyme activity using the MULTI-CORE buffer was compared with enzyme activity in the 4-CORE Buffer B. The ladder that was used in this gel and following results were self-created by PCR in the following way: specific primers were designed to use with the pIRESneo vector (as template for PCR). PCR using different combinations of the primers resulted in amplicons of different length which was used as the DNA ladder with lengths as indicated.

No star activity was visible since no other intermediate fragments are seen in any of the lanes containing digested plasmid. If one enzyme's activity is not high enough, the vector would predominantly be cut with only one enzyme and thus appear linear on the gel but with the insert still attached. A 220 bp fragment band was not observed in the third lane (pIRESneo-MT2 digested in Buffer B) which indicates that the activity in Buffer B is not ideal to cut the MT-2A insert from the pIRESneo-MT2 vector. Unfortunately, this statement was not valid as the second lane contains more vector (visible as the brighter band) which would automatically increase the visibility of the MT-2A fragment on the gel. The 220 bp fragment in the third lane may thus only be invisible due to lower concentration and was it not viable to choose the more appropriate reaction buffer for digestion merely on observed difference.

However, the MULTI-CORE buffer was still selected for further digestions merely because no star activity (mainly by *Eco*R I) was observed. As *Eco*R I was the possible determining factor when choosing the buffer giving higher enzyme activity (MULTI-CORE buffer), it was necessary to test its activity alone in the respected buffers (lanes 4 and 6). Both buffers seemed to give appropriate *Eco*R I activity and no star activity since only the linear vector fragments are visible. From this result it was decided to use the MULTI-CORE buffer for further digestions on both pIRESneo-MT2 and the pET28B plasmids.

3.2.2 Preparation of MT-2A cDNA insert for cloning into pET28 vector

3.2.2.1 Materials and Methodology

To prepare for ligation, pET28B and pIRESneo-MT2 were incubated with both *Bam*H I and *Eco*R I for 30 min at 37 ℃ as described before. Uncut pET28B and pET28B incubated with one restriction enzyme (*Bam*H I) were used as controls. The reaction mixtures were analyzed on a 1.5 % agarose gel containing 1.3 µM EtBr. The DNA bands, which can be seen in Figure 3.3, were subjected to long-wavelength UV light to visualize the bands. Exposure of the gel to this low-wavelength UV light was kept to a minimum in order to minimize base modifications. The linearized pET28B (5362 bp) and the MT-2A insert (220 bp) where extracted from the gel using the Wizard® SV

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Gel and PCR clean-up kit according to the instructions of the kit. The linearized pET28B and the MT-2A fragment were cut from the gel with a sharp, clean blade. Linearized pET28B digested with only *Bam*H I was also extracted from the gel which would act as a positive control for ligation.

3.2.2.2 Results and Discussion

As described the pIRESneo-MT2 and pET28B vectors were prepared for ligation with *Bam*H I and *Eco*R I using the MULTI-CORE buffer. Two gels were run from the same reaction mixtures, one gel for visualisation (Figure 3.3) and the other to extract the target fragments.

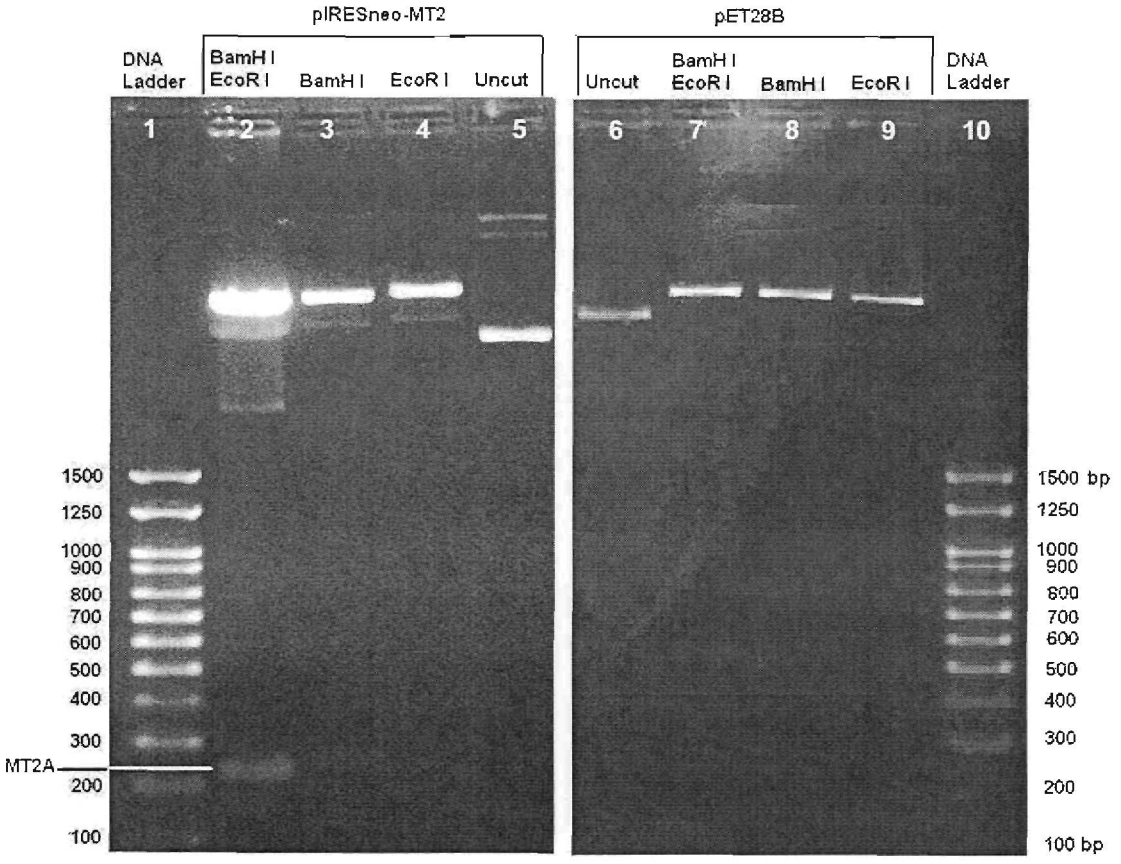

Figure 3.3: Preparation of pET28B and MT-2A insert for ligation. The MT-2A insert was cut from the pIRESneo-MT2 vector with *Bam*H I and *Eco*R I. The expression vector, pET28B, was also digested with *Bam*H I and *Eco*R I in order to incorporate the human MT-2A gene into the vector.

The human MT-2A insert (220 bp) was removed from the pIRESneo-MT2 vector and is clearly visible in lane 2. pIRESneo-MT2 incubated with only one enzyme (*Bam*H I

and *Eco*R I individually) and uncut pIRESneo-MT2 were controls (lanes 3, 4 and 5 respectively). Lane 7 contains the linear pET28B incubated with *Bam*H I and *Eco*R I. Since only 6 bp were lost when the plasmid was digested with both enzymes, no difference can be seen between it and pET28B digested with only one enzyme (lanes 8 and 9). The uncut plasmids (lanes 5 and 6) were negative controls to show absence of enzyme activities and clearly show the typical super coiled pattern of uncut plasmid DNA on the agarose gel. The 220 bp band in lane 2 and the DNA bands in lanes 7 and 8 were extracted from the gel via the Wizard SV Gel and PCR clean-up kit. The following section describes the construction of the pET28B-MT2 expression vector.

3.2.3 Construction of the MT-2A expression vector, pET28B-MT2

3.2.3.1 Ligation of MT-2A insert and pET28B to form pET28B-MT2

3.2.3.1.1 Materials and Methodology

The purity and concentration of the linear pET28B plasmid and MT-2A fragment was measured by a NanoDrop® ND-1000 spectrophotometer. Milli-Q® water (process explained in Appendix E) was used to blank the system before the samples were analysed. Since the NanoDrop ND-1000 spectrophotometer require only one micro-litre sample to determine absorbance, it was not necessary to dilute a specific volume of the sample to obtain larger volumes. The nucleic acid concentration (absorption at 260 nm wavelength), relative protein content (280 nm) and detergent (230 nm) measurements were done simultaneously to obtain an absorption curve. Absorbance at 320 nm was used as baseline measurement. The DNA concentrations were determined by the software by means of an adapted formula from Maniatis et al. (1982:249):

Equation 3.1: DNA concentration calculation.

$$[\text{DNA}] \text{ in ng/µl} = (A_{260} - A_{320}) \times 50 \text{ ng/µl}$$

Where [DNA] = DNA concentration; A = absorbance at the specified wavelength (nm)

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The concentrations obtained were given in ng/µl units. An estimated purity of the DNA samples was provided by the ratio of the DNA reading (260 nm) and protein reading (280 nm) as well as the DNA reading to detergent (230 nm) ratio. Samples that gave ratio values above 1.8 were considered as pure enough for further manipulation.

The isolated linear fragments were ligated as follows. A reaction containing MT-2A (10 ng) and pET28B DNA (120 ng) fragments to give a molar ratio of 2:1, T4 DNA ligase (1 U), ligation buffer (10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 30 mM Tris-HCl, pH 7.8) in a volume of 50 µl were incubated for 16 h at 4 ℃. Linear pET28B digested with only *Bam*H I was also incubated with T4 DNA ligase as a positive control for ligation. pET28B digested with both enzymes were incubated with T4 DNA ligase without the MT-2A insert as a negative control. One thing to note is that the ligation reactions never contained more than 1 µg (total) DNA as it would inhibit the ligase.

3.2.3.1.2 Results and Discussion

The concentrations obtained were acceptable for ligation as well as the estimated DNA purity (given by the A_{260}/A_{280} and A_{260}/A_{230} ratios). A full size plasmid map of the new construct (pET28B-MT2) is shown in Figure B.1. The ligation procedure was followed by transformation of the ligation products into DH10B cells.

3.2.3.2 Transformation of pET28B-MT2 into *E.coli* DH10B cells

3.2.3.2.1 Materials and Methodology

Ligation products were transformed into *E.coli* DH10B cells essentially as described before (Inoue et al., 1990:24). For preparation of competent cells for plasmid transformation, an overnight culture of *E.coli* DH10B was prepared. This overnight culture was then used to inoculate 50 ml Luria Bertani medium (LB, 10 g/l Tryptone, 5 g/l NaCl, 5 g/l yeast extract, 1 g/l D-glucose, pH 7.5, autoclaved) which was incubated in an INFORS HT (Bottmingen, Switzerland) Multitron incubator at 37 ℃ whilst shaking at 250 rpm. The cells were harvested as soon as the absorbance at 600 nm reached 0.6 – 1.0, by centrifugation at 6000 *g*, 4 ℃ for 10 min in a Beckman® Model J2-21 centrifuge using a JA14 rotor. Bacterial cells were kept on ice throughout the

entire procedure. The pellet was suspended in 2 ml pre-cooled transformation buffer (TB, 10 mM PIPES, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl, pH 6.7, autoclaved). Another 8 ml TB was added and left on ice for 30 min. The suspension was centrifuged at 6000 *g*, 4 ℃ for 10 min. The pellet was resuspended in 3 ml TB and an additional 6 ml TB added. DMSO (dimethyl sulfoxide) was added to a final concentration of 7 % (v/v) and aliquots of 100 µl volumes stored at -80 ℃ until use.

The ligation products were transformed into the newly prepared competent cells. Five microlitres of the ligation mixtures were added to 100 µl competent DH10B cells and kept for 30 min on ice. The cells were heat shocked for 30 seconds at 42 ℃ and then put on ice again. The volumes of cell suspensions were adjusted to 1 ml by the addition of 900 µl SOC medium (20 g/l Tryptone, 5 g/l yeast extract, 50 mg/l NaCl, 2.5 mM KCl, 20 mM glucose, pH 7.0, autoclaved) and incubated at 37 ℃ for one hour whilst shaking at 250 rpm. Half of the cell suspension was then streaked, using an L-tube, onto LB agar plates containing 50 µg/ml kanamycin. pIRESneo-MT2 was used as positive control for transformation and was streaked onto LB agar plates containing 100 µg/ml ampicillin. Non-transformed cells were used as controls for the antibiotics and streaked on similar plates, one plate containing 50 µg/ml kanamycin and the other containing 100 µg/ml ampicillin. All plates were incubated overnight at 37 ℃ in an INFORS HT Multitron incubator.

The plates were named according to the ligation product used to transform the cells and are further referred to in italics for easy reading. These are:

-"*pET28B-MT2*"

-"*pET28B (BamH I & EcoR I)*" digested with *Bam*H I and *Eco*RI

-"*pET28B (BamH I)*" digested only with *Bam*H I

-"*pIRESneo-MT2*"

-"*non-transformed cells*"

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Table 3.2: Prediction and explanation of plate growth. The sample plate (pET28B-MT2) is the plate of interest containing the experimental products necessary to reach the aim of this study. The control plates used to monitor ligation, transformation and antibiotic selection are also discussed.

Plate	Growth expected?	Comments
pET28B-MT2	Yes	This served as the sample plate that contained the ligation product necessary to reach the aim of this study. Ligation could take place as both vector and insert were digested with the same enzymes and thus contain matching cleaved sticky ends that can join.
pET28B (BamH I & EcoR I)	No	This plate served as the negative control for ligation. The plasmid was digested with different enzymes giving different cleaved sticky ends which cannot be joined.
pET28B (BamH I)	Yes	This plate served as the positive control for ligation. Since the vector was digested with only one enzyme which results in similar sticky ends, it should therefore close again in the presence of DNA ligase
pIRESneo-MT2	Yes	This plate served as the positive control for transformation. The plasmid wasn't modified in any way (such as digestion), thus no other factors except cell competence could determine transformation efficiency.
Non-transformed cells	No	This plate served as the negative control for antibiotic selection. As these cells were not transformed with a vector, they therefore do not contain antibiotic resistance and should not grow.

3.2.3.2.2 RESULTS AND DISCUSSION

All the plates grew more or less as were expected (Table 3.2). *E.coli* DH10B cells transformed with pIRESneo-MT2 were used as positive control for transformation and to determine transformation efficiency (# colonies on plate / ng of DNA plated x 1000 ng/µg). This plate delivered many clones and a relatively good transformation efficiency was determined (6.5 x 10^5 colonies per µg DNA). DH10B cells containing no plasmid were used as negative control for antibiotic selection with no visible colonies on either the kanamycin or ampicillin containing plate. Therefore, antibiotic selection also worked well. Linear pET28B which was incubated with only *Bam*H I was the positive control for ligation and as colonies grew, it was concluded that the

ligase enzyme and thus ligation procedure did work. If ligation had not worked, then the pET28B would remain linear and not be imported into the cells, which of course would not have been able to grow on the kanamycin-containing plates.

Linear pET28B incubated with both enzymes (*Bam*H I and *Eco*R I) were used as negative control for ligation and positive control for *Bam*H I and *Eco*R I digestion. The *Bam*H I and *Eco*R I overhangs are not able to ligate and in the absence of an insert, no ligation, transformation and hence no growth were expected. However, one colony did grow on the plate. As is also explained in Section 3.2.4.1.1, the linear pET28B band on the gel could contain pET28B cut with both or only one enzyme as the piece cut from the vector is too small to distinguish between the forms. Consequently, some of the extracted pET28B, incubated with both enzymes, was only cut by one enzyme and when ligase was added, it just closed again to form pET28B with no insert.

On the plate containing the potential pET28B-MT2 constructs, only ten clones grew. As ligation and antibiotic selection worked well and only one colony was found on the background plate it was expected that these clones would mainly contain the plasmid with the MT-2A insert. These clones were thus further evaluated to identify clones that contained the correct ligation product.

3.2.4 Identification and evaluation of clones containing the correct plasmid construct, pET28B-MT2

3.2.4.1 Screening for the pET28B-MT2 construct via restriction analysis

3.2.4.1.1 Materials and Methodology

As transformation and antibiotic selection gave expected results, it was expected that the bacterial colonies on the *pET28B-MT2* plate could only contain the correct ligation product. Unfortunately, this was not the case. Several steps were taken to increase the occurrence of the correct ligation product in transformed cells: 1) the use of two restriction enzymes giving distinct cleaved ends not able to self-ligate, 2) the separation and extraction of linearized pET28B from the undigested form, and 3) pET28B were incubated with twice the molar amount of MT-2A fragments during the ligation reaction. The main reason why some colonies might not have contained a

MT-2A insert was that some of the linear pET28B was only cut by one enzyme and not necessarily both. Thus, the plasmid could simply close again when ligase is added.

To identify a colony (clone) containing the correct ligation product, pET28B-MT2 (Figure 3.1), restriction analysis was done. Theoretically a restriction site(s) within the MT-2A insert can be used to create specific DNA fragment(s) which would identify the presence and orientation of the insert. However, none of the restriction sites within the MT-2A insert were used as these required rather expensive and uncommon restriction enzymes that were not available to us. An alternative approach was to use *Bam*H I and *Eco*R I that would give a 220 bp (MT-2A insert) and 5362 bp fragment (linearized pET28B) when the plasmid did contain the insert.

To obtain plasmid for restriction analysis, the colonies from *pET28B-MT2* and *pET28B (BamH I)* (as negative control) plates were picked separately and inoculated in 10 ml LB medium containing kanamycin (50 µg/ml). Each clone was then cultured to a density of 0.6 – 1.0 at 600 nm. A crude plasmid preparation of each clone was then isolated using a method called RAPS (rapid plasmid isolation) (Holmes & Quigley, 1981:194). The cells were collected by centrifugation of the culture for 2 min at 6 800 *g*. The cell pellet was suspended in 500 µl STET (8 % (m/v) sucrose, 5 % (v/v) Triton X100, 50 mM EDTA, 50 mM Tris-HCl, pH 8.0) and then transferred to a clean tube. Thirty microlitres lysozyme (10 mg in 1 ml STET) was added before boiling for 60 seconds and cooling on ice. The precipitate was separated from the mixture by means of centrifugation for 20 min at 12 000 *g* and 4 ℃. The supernatant was again transferred to a clean tube and mixed with an equal volume of isopropanol. This mixture was incubated at -20 ℃ for 30 to 60 m in after which it was centrifuged again for 20 min at 12 000 *g* and 4 ℃. The supernatant was discarded and the pe llet was further dried at room temperature for ~10 min before re-suspended in 50 µl TNE buffer (6 mM NaCl, 0.1 mM EDTA, 6 mM Tris-HCl, pH 7.5).

The concentrations of the RAPS plasmid preparation were determined spectrophotometrically with the NanoDrop ND-1000 spectrophotometer. An average concentration of 30 ng/µl was detected, although these preparations contain bacterial

RNA in addition to plasmid DNA. The RAPS products were incubated with both *Bam*H I and *Eco*R I as described before (Section 3.2.2.1). A third of the RAPS products were kept undigested to use as negative controls and to see whether target DNA was actually plasmid DNA. The same was done with the negative control (pET28B). The mixtures were then analyzed on a 1.5 % agarose gel containing 1.3 µM EtBr and visualized (as described before) with the Chemi Genius Bio Imaging System. For the identification of the small as well as large DNA fragments, bacteriophage Lambda DNA that was treated with *Hind* III (resulting in fragments ranging from 125 to 23 310 bp) was included as standard in addition to the self created pIRESneo DNA ladder (100 bp – 1500 bp) previously used.

3.2.4.1.2 Results and Discussion

Uncut super coiled plasmid DNA can clearly be seen in all lanes of Figure 3.4 where restriction enzymes were not used. This gives an indication of the presence and loading of plasmid DNA (but not its size). The bands presenting the cut, linear plasmids migrated between the 4361 and 6557 bp markers of the Lambda/*Hind* III ladder. In the lanes where restriction enzymes were used a visible 220 bp fragment could not be detected.

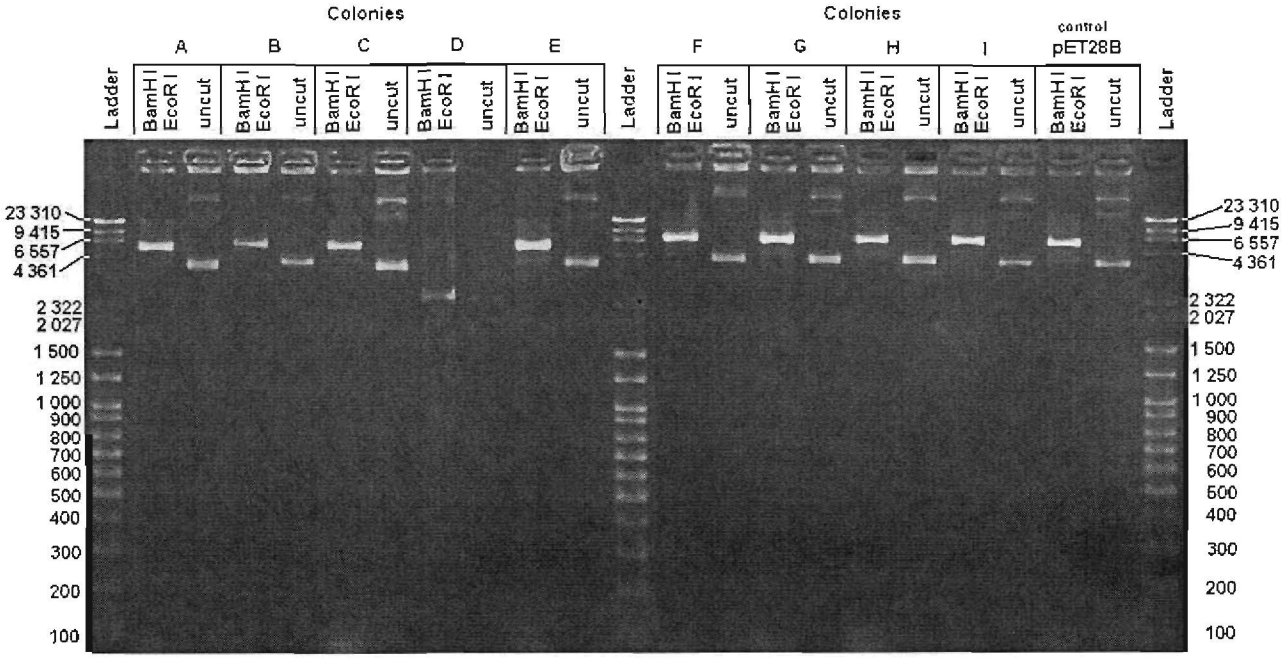

Figure 3.4: Restriction analyses of clones in order to screen for the pET28B-MT2 construct. Each clone was cultured and its plasmid isolated using the RAPS method.

Since no 220 bp insert was visible in any of the lanes containing digested plasmid, the first impression was that none of the constructs contained the MT-2A insert. But on inspecting the gel, it was clearly seen that contaminating nucleic acids, other than plasmid, were isolated with the RAPS method. Larger DNA was still visible near the pits of the gel. These contaminating nucleic acids were therefore responsible for a false plasmid concentration value obtained spectrophotometrically. The amount of plasmid digested with *Bam*H I and *Eco*R I were not enough to give a visible 220 bp fragment.

This approach, thus, did not work successfully. For DNA bands to be visualized clearly in an agarose gel (ethidium bromide stained) the band should contain about 100 ng (or more) DNA. Thus, to visualize the potential 200 bp fragment in the gel, about 2 µg of plasmid needed to be cut and loaded on the gel. The ~5 000 bp plasmids could be visualized successfully but not the 200 bp inserts. Consequently, a new approach was followed to confirm the insert in the plasmids. The use of PCR as an alternative method to screen for the pET28B-MT2 construct is discussed in the following section.

3.2.4.2 Screening for the pET28B-MT2 construct via PCR

3.2.4.2.1 Materials and Methodology

PCR (polymerase chain reaction) can be used to amplify the small insert before analysis via gel electrophoresis. Two primer sets were considered for screening of the correct construct. Reinecke (2004:42) designed and used human MT-2A primers in a previous study which could have been used to identify the MT-2A insert specifically. Unfortunately, it was realized that the *Eco*R I restriction site lies within the annealing site of the reverse primer and that the use of *Eco*R I would interfere with annealing. Only half of the reverse MT-2A primer would then be capable to anneal (to the MT-2A sequence) while the other half would be unable to anneal to the pET28B sequence. Therefore, this primer set was not used for screening. The pET28B vector contains sequences complementary to T7 sequencing primers, which are principally used for sequencing (see Figure B.1 which indicates the relative positions of these primers, *T7 term* and *T7 prom*). These T7 primers (obtained from Inqaba Biotechnical

Industries, Pretoria, South Africa) were used for screening clones using PCR. Primer information is given in the following table.

Table 3.3: Sequence of the T7 sequencing primers.

Primer	Sequence, annealing	Primer size	Amplicon size
T7 Promoter	5'-TAATACGACT CACTATAGGG-3'	20 bp	531 bp*
T7 Terminator	5'-GCTAGTTATT GCTCAGCGG-3'	19 bp	

* = amplicon size when the MT-2A in present otherwise the amplicon size would be 311 bp.

Established and published T7 primer PCR conditions (Novagen, 2006:17) were used which is summarized in Table 3.4. The T7 primers would result in a ~500 bp amplicon when the MT-2A insert is present while absence of the insert would result in a ~300 bp amplicon. The clones were again individually cultured and the plasmids isolated using the GeneJet™ Plasmid Miniprep Kit according the kit's instructions. Plasmids were not isolated via RAPS as the DNA polymerase used (TrueStart™ *Taq* DNA polymerase) is sensitive to contaminants and needs pure plasmid samples. These contaminants (probably macromolecules such as RNA, protein, carbohydrates) of the RAPS samples are clearly seen when looking at the absorption spectra obtained with the NanoDrop ND-1000 spectrophotometer (Figure C.1) and the results from the restriction analysis (Figure 3.4). Hence it was decided to do plasmid "minipreps" to obtain "cleaner" plasmid.

The concentration and absorption spectra of each miniprep product were obtained. The minipreps still did not deliver enough plasmid to use restriction analysis for screening but were clean enough for PCR. The minipreps were screened for an insert using the T7 primer set (with optimal PCR conditions and standard PCR mixtures containing 1.5 mM $MgCl_2$). Two PCR reactions containing pET28B and no template, respectively, were also assembled to serve as negative controls. No positive control was necessary to confirm whether the PCR process worked as each

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plasmid would be its own control. The PCR process was performed in a Thermo Hybaid® Multiblock System 0.2G thermal cycler. After PCR the samples were analysed by means of gel electrophoresis (1.5 % (m/v) agarose gel).

Table 3.4: Established PCR conditions for the T7 primer set as found in the literature (Novagen, 2006:17).

PCR Step	Number of Cycles	Action	Temperature (℃)	Duration
1	1	Initial denaturation	94	5 minutes
2	30	Denaturation	94	30 seconds
		Annealing	55	30 seconds
		Extension	72	30 seconds
3	1	Final extension	72	5 minutes
		Cooling	4	Hold

3.2.4.2.2 Results and Discussion

Minipreps was used to isolate the vectors from the individual clones, which included clones A-J from the *pET28B-MT2* plate, one clone from the *pET28B (BamH I)* plate and a clone containing pIRESneo-MT2. PCR was done with the T7 sequencing primers and conditions. The PCR products were analyzed on a 1.5 % (m/v) gel which is shown in Figure 3.5.

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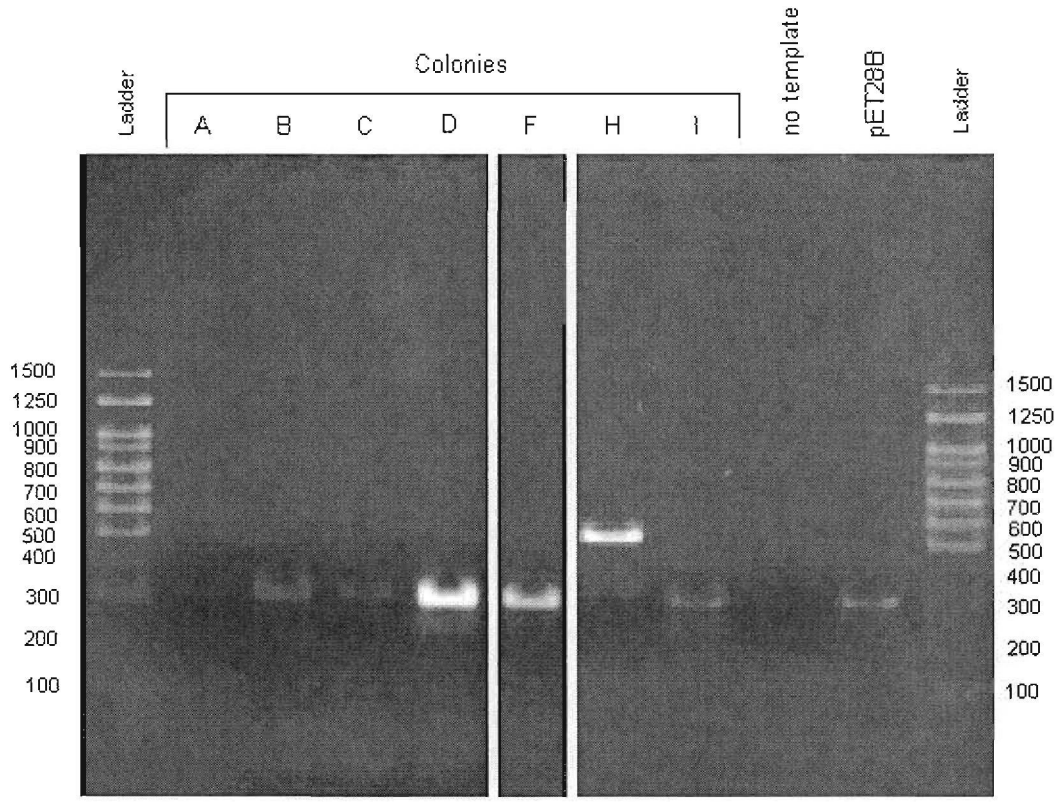

Figure 3.5: Screening for the pET28B-MT2 construct with PCR using the T7 primer set. Clones H-J was cultured, plasmids extracted and screened to confirm correct construct.

Figure 3.5 shows the PCR results obtained after the T7 primer set was used to screen for the pET28B-MT2 construct. PCR was successful as the controls gave the expected results. No contamination of the reagents and PCR mixtures occurred as no amplicons were synthesized in the absence of template. The pET28B vector only gave the ~300 bp amplicon which was the negative control. As mentioned, no positive controls were needed to confirm whether PCR worked as each vector was its own control. When the vector did not contain an insert, it gave a ~300 bp amplicon while the vector containing an insert gave the expected ~500 bp amplicon. From the results obtained, clone H were successfully identified via these primers to contain the MT-2A insert. All other clones gave negative results except clones E and G which did not give any amplicons. Section 3.2.5 describes the steps taken to ensure the identified construct were absolutely correct.

3.2.5 Confirmation of successful ligation via sequencing

3.2.5.1 Materials and Methodology

Clone H was successfully identified with the T7 primers to contain a presumably correct insert. However, the exact correctness of the sequence of the insert of clone H was still unknown from the initial evaluation. Any change in the sequence would result in the wrong translation (protein) sequence. Factors such as reading frame, correct orientation and mutations had to be confirmed. Clone H was cultured, the plasmid isolated with the GeneJet Plasmid Miniprep Kit and sent to Inqaba Biotechnical Industries (Pretoria) for sequencing as it could not be done on our campus. The T7 sequencing primer set was used and the chain termination (dideoxy) sequencing mechanism was used by Inqaba Biotechnical Industries essentially as described first by Sanger et al. (1977:5465). In order to verify the sequencing results, the plasmid was sequenced in both directions to obtain a forward sequence and reverse sequence. The results were processed using Chromas (version 2.31, Technelysium) and compared to the human MT-2A sequence (GenBank accession number NH_005953) using DNAMAN (version 4.13, Lynnon Biosoft).

3.2.5.2 Results and Discussion

The unprocessed forward sequence (electropherogram) is shown in Figure D.1 (see Appendix D) as example of the sequence results obtained from Inqaba Biotechnical Industries. The processed result is shown in Figure 3.6. The sequence and translation that was obtained from a reverse and forward sequence of the plasmid and as summarized in Figure 3.6 was exactly as predicted and corresponded to that of pET28B and human MT-2A. The translated sequence displayed in the figure also confirms the reading frame to be correct. Hence, this construct could be isolated and used to express histidine tagged human MT-2A.

#	Sequence, translation and restriction sites
1	TAGAAATAAT TTTGTTTAAC TTTAAGAAGG AGATATACC**A TGGGCAGCAG CCATCATCAT**
1	M G S S H H H
61	**CATCATCACA GCAGCGGCCT GGTGCCGCGC GGCAGCCATA TGGCTAGCAT GACTGGTGGA**
7	H H H S S G L V P R G S H M A S M T G G
	*Bam*H I
121	**CAGCAAATGG GTCGGGATCC CAACTGCTCC TGCGCCGCCG GTGACTCCTG CACCTGCGCC**
27	Q Q M G R D P N C S C A A G D S C T C A
181	**GGCTCCTGCA AATGCAAAGA GTGCAAATGC ACCTCCTGCA AGAAAAGCTG CTGCTCCTGC**
47	G S C K C K E C K C T S C K K S C C S C
241	**TGCCCTGTGG GCTGTGCCAA GTGTGCCCAG GGCTGCATCT GCAAAGGGGC GTCGGACAAG**
67	C P V G C A K C A Q G C I C K G A S D K
	*Eco*R I
301	**TGCAGCTGCT GCGCCTGA**TG CTGGGACAGC CCCGCTCCCA GATGTAAAGA ACGCGAATTC
87	C S C C A *
421	GAGCTCCGTC GACAAGCTTG CGGCCGCACT CGAGCACCAC CACCACCACC ACTGAGATCC
481	GGCTGCTAAC AAAGCCCGAA AGGAAGCTGA GTTGGCTGCT GCCACCGCTG AGCAATAAAC
541	TAGCAACCCC GTCCCTGTTT GTTTTTTTTT CTCTCTTCCC TTGGCCTCGG GGGTCGTGTG

Figure 3.6: The processed sequence result after sequencing pET28B-MT2 isolated from clone H with the T7 primer set. The processed plasmid sequence is given in comparison with the theoretical translation sequence. The plasmid sequence to be translated into the target protein is given in bold. The one letter abbreviations of the amino acids of the target protein sequence are also given. The *Bam*H I and *Eco*R I restriction sites, used to incorporate the MT-2A fragment into pET28B, are underlined. The asterisk indicates the stop codon.

3.3 SUMMARY AND CONCLUSIONS

For the production of an *internal standard* protein that was similar, but slightly different in mass, to native MT-2A or any other human MT isoform, a strategy was followed to produce a recombinant modified human MT-2A (MT-2A$^{\Delta}$). In this strategy the human MT-2A gene was cut from the pIRESneo-MT2 vector with *Bam*H I and *Eco*R I and incorporated into the pET28B expression vector (also digested with *Bam*H I and *Eco*R I) to obtain the pET28B-MT2 expression vector. The incorporation of MT-2A into pET28B was followed by the transformation of the ligation products into *E.coli* DH10B cells in order to confirm ligation and obtain sufficient stock of the ligated constructs. The acquired clones were screened via restriction analysis and PCR for the identification of the correct pET28B-MT2 construct. The correct pET28B-MT2 construct were successfully identified via PCR using a T7 primer set and the MT-2A sequence in the positive identified construct was confirmed via sequencing.

The incorporation of the human MT-2A gene into an expression vector of choice (pET28B) was therefore successful. The new construct (pET28B-MT2) could thus be isolated and used to express MT-2A$^{\Delta}$ – a MT variant that contains 32 extra amino acids (N-terminal methionine omitted). This variant includes an N-terminal His-tag that could be used for purification, in addition to increasing the mass of the protein. The expression of this MT-2A$^{\Delta}$ is discussed in the Chapter 4.

Chapter 4

Expression of recombinant MT-2A

4.1 INTRODUCTION

The previous chapter described the successful construction of a modified human MT-2A expression vector, pET28B-MT2. Chapter 4 will focus on the next step of this study which is the expression of recombinant modified human MT-2A (MT-2A$^{\Delta}$). MT-2A$^{\Delta}$ could theoretically be used as internal standard when quantifying human MT-2A in biological material after the tag is removed. Specific reference material is commonly used during quantification of molecules. Due to the purity and expense of most commercially available MT standards, it was initially decided to clone and express a modified human MT-2A in bacterial cells.

4.1.1 Bacterial protein expression

Bacterial protein expression is the easiest and least expensive expression system. There are a variety commercial bacterial expression vectors and host strains to choose from depending on application. The different expression strains can assist with expression optimization and regulation. Some well known and commonly used *E.coli* expression strains are the BL21(DE3) strains that carry a chromosomal copy of the T7 RNA polymerase gene under control of the *lacUV5* promoter and is compatble with T7 expression vectors (Novagen, 2004:113). The BL21 designation indicates that the host is deficient in both *lon* (cytoplasmic) and *ompT* (periplasmic) proteases resulting in higher level of intact recombinant proteins. The following strains were kindly provided by Dr. Oksana Levanets (Institute of Molecular Biology and Genetics, Kiev, Ukraine): BL21(DE3), BL21(DE3)pLysE, BL21(DE3)pLysS and BL21(DE3) CodonPlus®-RIL. These *E.coli* expression strains have features that assist with protein expression which are summarized in Table 4.1. The understanding of these features is essential to predict the expression results in Section 4.2.2.

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Table 4.1: Specific features and applications of the *E.coli* BL21 expression strains. Specific additional features not mentioned in the previous paragraph are listed in this table (Drechsel et al., 2000)

***E.coli* host strain**	**Additional features**	**Applications**
BL21(DE3)	none	Upon IPTG induction, T7 RNA polymerase is expressed by the host strain which expresses the target gene incorporated in the T7 expression vector. This strain has an inherited leakiness as the *lac* promoter allows some degree of transcription. Preferably, non-toxic proteins can be expressed with this strain.
BL21(DE3)pLysS	pLysS vector encodes T7 lysozyme which is a natural inhibitor of T7 RNA polymerase.	More stringent control of expression and reduction in protein expression in non-induced cells. Proteins normally toxic to the host strain can be expressed using this strain. Since no leaky protein expression occurs, the cells can be easily grown to a high density before induction.
BL21(DE3)pLysE	pLysE vector produces more T7 lysozyme than the pLysS plasmid	Even more stringent control of expression than the BL21(DE3)pLysS strain. Expression of toxic proteins in a controlled environment.
BL21(DE3) CodonPlus-RIL	This strain co-expresses the tRNAs of three rare *E.coli* codons which are: AGG/AGA (R, arginine), AUA (I, isoleucine) & CUA (L, leucine).	Proteins containing high number of rare *E.coli* codons can be expressed in this strain which co-express the tRNAs for these rare codons – in this case AGG/AGA (arginine), AUA (isoleucine) & CUA (leucine).

4.2 METHODOLOGY, RESULTS AND DISCUSSION

The materials and methods used to express and prepare collected samples will be discussed in detail. The general order for obtaining results is discussed only once in detail. This includes common expression method, sample preparation, protein determination and SDS-PAGE analysis. This general order of sample preparation and analysis will be referred to when discussing second and third time expression.

4.2.1 Transformation of pET28B-MT2 into *E.coli* expression strains

4.2.1.1 Materials and Methodology

As mentioned in Section 4.1.1, four of the most widely used *E.coli* BL21(DE3) expression strains were made available to our institution. They are: BL21(DE3); BL21(DE3)pLysE; BL21(DE3)pLysS; BL21(DE3) CodonPlus-RIL. These *E.coli* expression strains have features that assist with protein expression which are summarized in Table 4.1. From their features it was predicted that the BL21(DE3)pLysS or BL21(DE3)pLysE strains would express human MT-2A better than the other two strains. The reason for this prediction was simply the fact that most mammalian proteins are toxic to bacterial cells and "leaky" expression consequently impairs cell growth. The tight induction regulation given by the BL21(DE3)pLysS or BL21(DE3)pLysE strains means that the cells can be grown to a high density before induction (Woestenenk et al., 2004:227). For comparison, pET28B-MT2 was transformed into all four host strains to see which host strain expressed MT-2A the best.

Table 4.2: Prediction and explanation of colony growth. With transformation of pET28B-MT2 into the four host strains (designated as the plate name) the following results are expected. The control plates (- *cam* and - *kan*) were used to monitor antibiotic selection.

Plate	Growth expected?	Discussion
BL21(DE3)pET28B-MT2	Yes	When successfully transformed, this host strain should contain the pET28B-MT2 vector, which would give the cells resistance to kanamycin.
BL21(DE3)pLysS/pET28B-MT2	Yes	When successfully transformed, this host strain should contain the pET28B-MT2 and co-expressing pLysS vector, which would make the cells resistant to kanamycin and chloramphenicol.
BL21(DE3)pLysE/pET28B-MT2	Yes	When successfully transformed, this host strain should contain the pET28B-MT2 and co-expressing pLysE vector, which would make the cells resistant to kanamycin and chloramphenicol.
BL21(DE3) CodonPlus-RIL/pET28B-MT2	Yes	When successfully transformed, this host strain should contain the pET28B-MT2 and co-expressing pACYC vector, which would make the cells resistant to kanamycin and chloramphenicol. These cells also contain the pSC101 vector, which makes the cells resistant to streptomycin. This was however not used for selection.
- *cam*	No	Although the BL21(DE3) cells were transformed with pET28B-MT2, it did not contain any other vector which could give the cells resistance to chloramphenicol. This plate acted as negative control for chloramphenicol, to verify the selection.
- *kan*	No	Although the BL21(DE3)pLysS cells contain the pLysS vector which makes the cells chloramphenicol resistant, it were not transformed with pET28B-MT2 and do not contain any resistance to kanamycin. This plate acted as negative control for kanamycin, to verify the selection.

- *cam* = negative control for chloramphenicol selection; - *kan* = negative control for kanamycin selection

These host cells were made competent using the same method as described in Section 3.2.3.2.1. pET28B-MT2 were transformed into all four the expression strains which was then streaked onto LB plates containing 50 µg/ml kanamycin and 20 µg/ml chloramphenicol, except for the transformed BL21(DE3) cells, which were streaked onto LB plates containing only 50 µg/ml kanamycin. As a negative control for antibiotic selection, non-transformed BL21(DE3)pLysS and transformed BL21(DE3) cells were streaked onto LB plates containing kanamycin and chloramphenicol respectively. The following names were designated to the respective plates:

"BL21(DE3)pET28B-MT2"

"BL21(DE3)pLysS/pET28B-MT2"

"BL21(DE3)pLysE/pET28B-MT2"

"BL21(DE3) CodonPlus-RIL/pET28B-MT2"

"- cam"

"- kan"

4.2.1.2 Results and Discussion

All plates grew as was expected (Table 4.2) with no visible growth detected on the negative control plates. Therefore, transformation and antibiotic selection were successful. The antibiotic selection ensured and confirmed that the colonies on the sample plates did contain the pET28B-MT2 vector. Hence, these colonies were cultured and induced to express MT-2A$^{\Delta}$.

4.2.2 Evaluation of *E.coli* BL21(DE3) strains for MT-2A$^{\Delta}$ expression

It would have been ideal to optimize the expression of each of the four transformed strains to exploit and compare their capability to express MT-2A$^{\Delta}$ in order to select the best expression strain for further experimentation. Unfortunately, it was not the aim of this study to perform an elaborate and detailed optimization of all four strains. Therefore, only two factors known to influence expression significantly were varied for the strains. These are the promoter inducer (IPTG) concentration and expression time. The strain which gave the best MT-2A$^{\Delta}$ expression with these variations would be selected for further optimization.

4.2.2.1 Preliminary expression in four *E.coli* BL21(DE3) strains

4.2.2.1.1 Materials and Methodology

A common expression procedure (Drechsel et al., 2000) was used to express MT-$2A^{\Delta}$ in all four host strains for comparison. After successful transformation of pET28B-MT2 into competent strains (Section 4.2.1), colonies from each plate were picked to inoculate 10 ml LB medium containing the appropriate antibiotics and grown at 37 ℃ until the absorbance at 600 nm had reached 1.0. This inoculation culture was used to inoculate 100 ml LB medium containing the appropriate antibiotics (main culture). Cells were grown in an INFORS HT Multitron incubator at 37 ℃, whilst shaking at 200 rpm until the absorbance at 600 nm had reached 1.0.

The main culture of each host strain was divided into three separate cultures (of about 30 ml). Expression of the three individual cultures per strain was induced by the addition of IPTG (isopropyl β-D-thiogalactopyranoside) to final concentrations of 0.5, 1 and 2 mM, respectively. It must be pointed out that some research groups also add $CdCl_2$ (cadmium chloride) to a final concentration of 0.3 mM to the expression medium to facilitate metal binding (You, 1999:46). This step probably helps stabilize the expressed MT (You, 1999:46), especially against proteolytic degradation (Ma, 2005:31; Vallee, 1995: 30). Other research groups use $ZnSO_4$ to a final concentration of 0.2 mM (Ding et al., 2006:675). Since MT binds cadmium more effectively than zinc (Romero-Isart & Vasak, 2002:392; Sato & Kondoh, 2002:10; Stillman, 1995:473), it was decide to add $CdCl_2$ to the medium. Given that cadmium is toxic and impairs growth, $CdCl_2$ was only added to the medium at the same time IPTG were added (at the stage where exponential growth occurs). The cultures were incubated overnight (~16 h) at 25 ℃ whilst shaking at 200 rpm. Sample collection and preparation are discussed in the following section.

4.2.2.2 Sample collection and preparation for electrophoresis

4.2.2.2.1 Materials and Methodology

A 5 ml sample was collected from each culture at 0 (pre-induction), 1, 2, 4, 8 and 16 h after induction, respectively, and kept at –80 ℃ until electrophoresis. The samples

were centrifuged at 6 000 *g*, 4 ℃ for 10 min in a Heraeus ® Labofuge® 400R general purpose centrifuge using a swinging bucket rotor. Samples of the culture medium was stored for gel analysis to monitor whether MT-2A$^{\Delta}$ was present. MT-2A$^{\Delta}$ can be found in the culture medium if the expressed protein is exported from the cell or as a consequence of cell lysis. The cell pellet was suspended in binding buffer (0.5 M NaCl, 5 mM imidazole, 20 mM Tris-HCl, pH 7.9) and sonicated to break the cell walls and membranes. Sonication was performed with the Mistral Soniprep 150 Ultrasonic Disintegrator (MSE, London, UK) using two bursts of 5 seconds at 10 kHz, whilst keeping the suspension on ice. The suspension was centrifuged for 10 min at 6 000 *g* to discard cell debris. The resulting supernatant (total fraction) contained all soluble cytosolic proteins. A few microlitres of each total fraction were kept for gel electrophoresis. Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyse the culture medium and total fraction samples collected at each time period (methodology discussed in Section 4.2.2.4.1).

After the medium and total fraction samples were analysed and no MT expression was observed, the total fraction samples were heat treated (85 ℃ for 15 min) to remove interfering proteins. The use of heat treatment to partially isolate MT from a large number of proteins is discussed in the next chapter (Section 5.2.2.1). Erk et al. (2002:1213) proposed heat treatment to be the best MT extraction method; hence heat treatment with similar conditions was used. The heat resistant protein fractions (heat fractions) were also analysed with SDS-PAGE as was done with the total fractions. The results obtained are discussed in Section 4.2.2.4.2.

4.2.2.3 Protein determination via the BCA method

4.2.2.3.1 Materials and Methodology

It was obvious that after the respective preparations the resulting samples contained various amounts of protein. To compare the amount of protein expressed by each strain and during different conditions, each lane needed to contain the same amount of protein. Over-estimation can occur if the protein content is not normalised/ equalised between samples. The commonly used and already optimized BCA

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(bicinchoninic acid) protein determination method (Smith et al., 1985:77) was used to determine the protein concentration of each sample before it was analysed with SDS-PAGE. This method is based on the reaction of the protein peptide bonds with Cu^{2+} (under alkaline conditions) to produce Cu^{+} which in return react with bicinchoninic acid to give a purple colour. The colour changes and intensities are spectrophotometrically measured at 560 nm (Smith et al., 1985:79; Wilson & Walker, 2003:321).

The protein determination was done in a microtiter-plate (Nunc®) format as follows: a reaction solution (BCA:$CuSO_4 \cdot 5H_2O$ at 50:1 (v/v)) was prepared. BSA (bovine serum albumin) standard was used to prepare a concentration series between 0 and 10 µg. Volumes were corrected using Milli-Q water. The protein samples (test samples) were diluted by a factor of 10 and 10 µl of these diluted samples were loaded into the wells of the plate. All samples were analysed in triplicate. Two-hundred microlitres of the BCA reaction mixture were added to the wells with a multi-channel pipette and incubated for 20 min in a BIO-TEK® FL600™ Fluorescence Plate Reader, which was preheated to 37 ℃. After incubation the absorbance at 560 nm was measured. Data from the standard series were used to determine the protein content of samples using linear regression and only when a R^2 value above 0.99 was obtained. The concentration of samples were then determined according to Equation 4.1.

Equation 4.1: Calculation of protein concentration.

[protein] (µg/µl) = µg (determined from standards)/ volume (µl) x dilution factor

[protein] = protein concentration

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4.2.2.4 SDS-PAGE analysis of total- and heat fractions from collected samples

4.2.2.4.1 Materials and Methodology

It was decided, based on past experience to load approximately 30 µg of protein per lane when the gel was to be stained with Coomassie® Brilliant Blue R-250 (CBB). When Silver Stain Plus® was used only 1 µg protein was loaded per lane.

Appropriate amounts of sample as loaded on the gel, to ensure that each lane contained the same amount of protein. For all SDS-PAGE, a 5 % (m/v) stacking- and 15 % (m/v) resolving gel were used with Tris-glycine buffer essentially as described before (Laemmli, 1970:681). PageRuler™ Prestrained Protein Ladder Plus was used to estimate molecular size and identify target proteins. All gels were run at a constant current (25 mA) until the dye front reached the bottom of the gel. To select the expression strain, that express MT-2A$^{\Delta}$ the best, all the total fractions sampled were analysed. Detection of the separated proteins was performed with CBB staining. The gel was fixed with fixative solution (25 % (v/v) isopropanol, 10 % (v/v) acetic acid) for 20 min whilst gently shaking. After the fixative solution was removed, the gel was stained with CBB (10 % (v/v) acetic acid, 0.006 % (m/v) Coomassie Brilliant Blue R-250) for 20 min, followed by destaining of the gel with 10 % (v/v) acetic acid until sufficient background were removed. Due to low resolution with the Chemi Genius Bio Imaging System (Syngene, Cambridge, UK), the gels were put between two transparent papers and scanned with a high-resolution scanner (Lexmark® X6170) as was also done by others (Yu et al., 2001:38).

As will be discussed in the following section, the initial attempt to express MT-2A$^{\Delta}$ seemed to have failed. This was deduced as no clear, pertinent protein bands were visible in the 11 kDa region in all of the total fractions collected over time and at varying IPTG concentrations. The stored total fractions were heat treated (Section 4.2.2.2.1) to discard many of the large interfering proteins and thus to visualise MT-

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Silver Stain Plus® is a registered trademark of Bio-rad Laboratories, Inc. (Hercules, CA, USA)

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$2A^{\Delta}$ expression more effectively. The heat resistant protein fractions (heat fractions) were also analysed with SDS-PAGE as was done with the total fractions. The results are given and discussed in Section 4.2.2.4.2.

4.2.2.4.2 Results and Discussion

After the cells were broken, the total fraction of each sample was analysed via SDS-PAGE (15 % (m/v) separating gel) along with the respective medium samples. The gels were stained with CBB to visualise the proteins. Due to the large number of gels (eight) that were analysed it is impractical to display them all. Specific results will be shown here and the results of most others, especially when no expression occurred, will only be reported and discussed. Samples that gave the best (expression) results were afterwards analysed together with SDS-PAGE to obtain a "summarized" result rather than to display all eight gels. This was done with all the following results.

The bands on the gel (e.g. Figure 4.1) represent the stained proteins. The higher the amount of a specific protein, the more visible the particular band on the gel. The lack of a clear protein band in the 11 kDa region of either the medium and total fractions (as visualised on the CBB stained gel) meant that no detectable expression of MT-$2A^{\Delta}$ occurred. Hardly any bands were visible below the 11 kDa marker and as the theoretical mass of MT-$2A^{\Delta}$ is about 9.3 kDa, it seemed that MT-$2A^{\Delta}$ were not expressed (results not shown). To confirm whether some expression actually did occur, MT-$2A^{\Delta}$ was partially purified via heat treatment. The precipitation of numerous interfering proteins would result in a higher MT-$2A^{\Delta}$ to total protein ratio which in return would result in a more visible MT-$2A^{\Delta}$ band on the gel. The heat fractions were also analysed with SDS-PAGE. Again, it is impractical to display every gel and since the gels gave essentially the same result for every varied time, IPTG concentration and strain, it was decided to rather display a representative gel (Figure 4.1).

The gel in Figure 4.1 contains the total and heat fraction (collected after overnight expression) of each strain after expression was induced by 1 mM IPTG. The absence of a prominent $^{MT\text{-}2A\Delta}$ band (in the 11 kDa region) is common in all the total fractions analysed, as can be seen in this representative gel. It is well-known that *E.coli* does not express small proteins well and most of the time proteins smaller than 10 kDa are hardly expressed (Sommer et al., 2004:10).

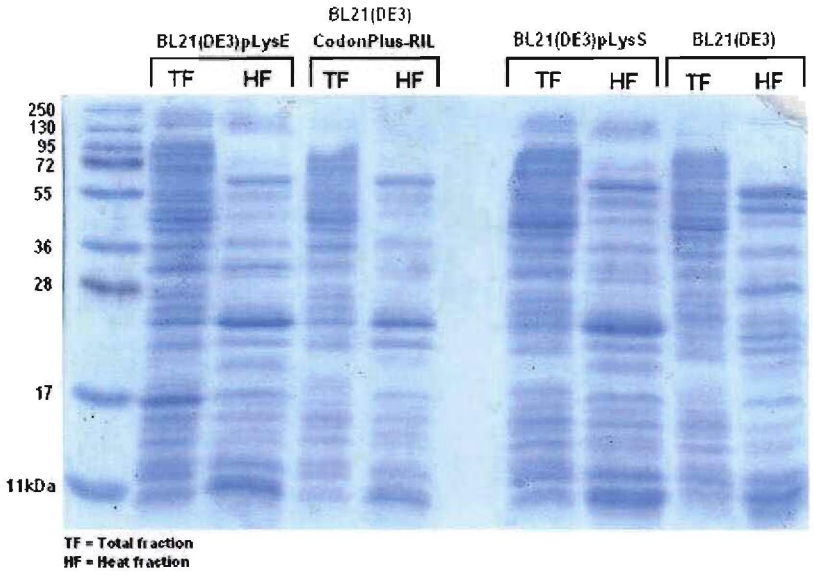


Figure 4.1: SDS-PAGE analysis of total and heat resistant protein fractions after overnight induction with 1 mM IPTG. A 15 % (m/v) separating gel (stained with CBB) was used to analyse the total and heat fractions of each strain after overnight expression. Sample names are indicated at the top as well as their treatments (TF = total fraction, HF = heat fraction). Molecular weight marker sizes are indicated in the first lane.

The histidine tag, along with the additional amino acids fused to the MT-2A, increased the size of the native protein from ~6.0 to 9.3 kDa. Although the size of the protein are still relatively small (< 10 kDa) and on the lower mass limit for good expression (Pazirandeh et al., 1998:4068; Sommer et al., 2004:10), it was thought that with enough optimization it could be successfully expressed. Many proteins were denatured and removed by heat treatment to consequently increase the MT-2A$^{\Delta}$ concentration. A slightly darker band is visible in the 11 kDa region of all the heat fractions. Although this band is slightly above the 11 kDa marker, it was assumed to be MT-2A$^{\Delta}$, but confirmation was needed.

4.2.2.5 SDS-PAGE analysis of total- and heat fractions of overnight induced and un-induced transformed cells

4.2.2.5.1 Materials and Methodology

The total protein fractions (total fractions) and heat resistant protein fractions (heat fractions) were again analysed but along with the total and heat fractions of respective non-induced samples to confirm the position of the MT-2A$^{\Delta}$ band.

Metallothionein standard (containing both forms I and II) was also analysed to confirm the relative position of the recombinant MT. The use of CBB to visualise MT were questioned especially since it is known that Coomassie interacts mostly with the hydrophobic (aromatic) areas of proteins in order to stain them. Since MT does not have aromatic residues, it should not effectively be stained by CBB (Alhama et al., 2006:56; Mizzen et al., 1996:80). This meant that even though large amounts of MT-2A$^{\Delta}$ may indeed be expressed, it could not be clearly detected with CBB at common concentrations. Therefore the gels were stained with the Silver Stain Plus kit from Bio-Rad, which interact with the peptide bonds (Park et al., 2007:84; Simes et al., 2003:309). Silver staining was performed according to the kit's specifications. The results of the silver stained gels containing the total and heat fractions are given and discussed in Section 4.2.2.5.2.

4.2.2.5.2 Results and Discussion

Figure 4.2 shows the total and heat fractions of each overnight induced and un-induced strain as visualised with silver staining. The use of silver staining resulted in the absence of the previously observed clear protein bands (in the heat fractions) just above the 11 kDa region which was assumed to be MT-2A$^{\Delta}$. No other major protein bands were visible below the 11 kDa marker. Silver staining (which is more sensitive than CBB staining) resulted in other major protein bands, which meant that any of these dark bands could potentially represent MT-2A$^{\Delta}$. The commercial MT standard (Figure 4.2 B) too gave confusing results. Two apparent protein bands (smeared over a large area) were visible in the 28 kDa region which did not represent the theoretical size of MT (~6 kDa). Although it is known from literature that the MT standards from Sigma-Aldrich contain many MT isoforms, it still could not explain the various bands and smearing, especially since the sizes of the isoforms differ by only a few hundred Dalton.

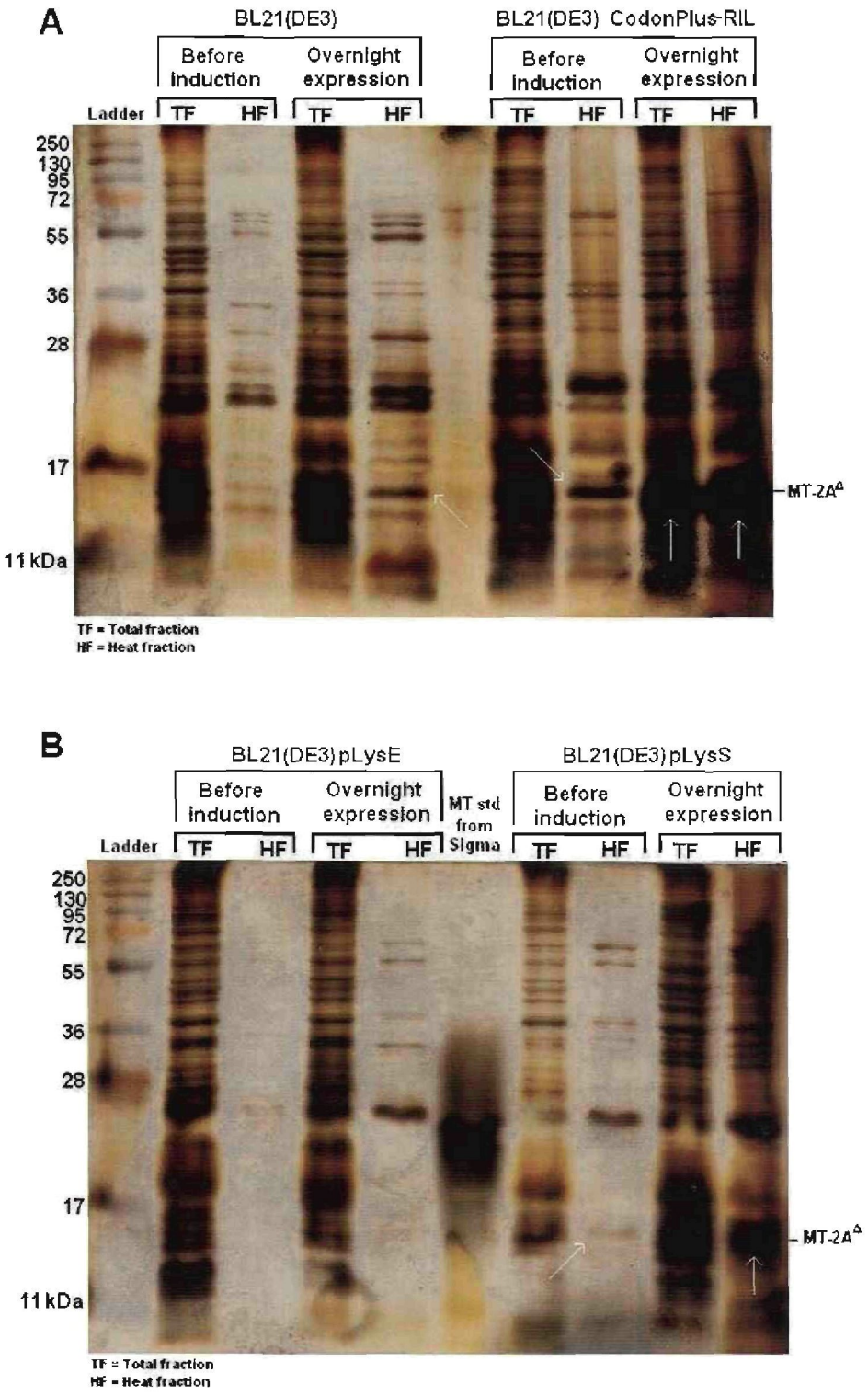


Figure 4.2: SDS-PAGE analysis of the total- and heat resistant protein fractions of each induced and un-induced strain. A 15 % (m/v) separating gel (silver stained) was used to analyse the total and heat fractions of each induced and un-induced strain (A for BL21(DE3) & - CodonPlus-RIL; and B for BL21(DE3)pLysS & - pLysE) after overnight expression. Sample names are indicated at the top as well as their treatments.

The literature was consulted in an attempt to explain the results obtained. Besides the generally low detection sensitivity of MT with common laboratory methods like SDS-PAGE and immuno staining (Western blot), the detection of MT is also associated with reduced electrophoretic mobility and band broadening in SDS-PAGE (Hong et al., 2001:247; Meloni et al., 2005:77). Thus, the position and smear of the MT standard on SDS-PAGE are a natural phenomenon and possible, although it appears not even close to where it is expected or near to where (presumably) expressed MT-2A$^{\Delta}$ appears. The exact position of the MT-2A$^{\Delta}$ was thus still not confirmed but was thought also to be in the 20 kDa region as indicated with arrows in Figure 4.2. Since the location of the recombinant MT-2A$^{\Delta}$ band on the gel was still unknown, it was decided to use the heat fractions of each strain to isolate the possible MT-2A$^{\Delta}$ from it, in the hope to see a large isolated band. The position of this band could also indicate the position of the MT-2A$^{\Delta}$ in the total fraction.

4.2.2.6 SDS-PAGE analysis of total-, heat- and isolated fractions collected from overnight expressed transformed cultures

4.2.2.6.1 Materials and Methodology

Small scale metal chelating chromatography was done to isolate his-tagged MT-2A$^{\Delta}$ (as described in Chapter 5, Section 5.2.3.1.1). The heat fractions (of each time period and IPTG concentration) were used to lessen the protein load on the column. A few microlitres of the heat fractions were kept for SDS-PAGE analysis. The rest was applied individually to a metal chelating column (Section 5.2.3.1.1). A few microlitres of the column flow through (after each application) were also collected to monitor whether MT-2A$^{\Delta}$ did bind to the column or not. The bound his-tagged protein was eluted by two volumes imidazole (1 M) which was collected separately. These collected samples were analysed with SDS-PAGE. Protein determination was done on all the samples. The same amount of protein (1 µg) was loaded per lane except for many of the elution samples, which did not even contain 1 µg protein after total concentration. In such cases, the entire elution fraction was loaded per lane. The gels were stained with silver staining. Again, not all the gels are displayed. The best expression results of each strain (regardless of the IPTG concentration and time of sampling) where selected to display in Figure 4.3.

4.2.2.6.2 RESULTS AND DISCUSSION

In Figure 4.3 the expression of MT-2A$^{\Delta}$ in the four *E.coli* strains in heat fractions and metal chelating purified fractions can be seen. Isolation of putative MT-2A$^{\Delta}$ with metal chelating chromatography resulted in an apparent single protein band just below the 17 kDa marker. This was visible only in the BL21(DE3)pLysS, -pLysE and –CodonPlus-RIL strain samples. In the BL21(DE3) CodonPlus-RIL strain, expression of this protein is also visible in the heat fraction. This protein (~15 kDa) was believed to be the expressed MT-2A$^{\Delta}$. The unexpected position of the apparent MT-2A$^{\Delta}$ on the gel could be explained by the natural reduced electrophoretic mobility of all metallothioneins (Hong et al., 2001:247; Meloni et al., 2005:77), but it was also necessary to consider other factors such as possible polymerization or faulty translation sequence. These mentioned factors, however, seemed unlikely. Since the theoretical size of MT-2A$^{\Delta}$ is 9.3 kDa, a dimer would theoretically be ~18.6 kDa, more than 3 kDa higher than the observed band. The sequence of the vector was also confirmed with sequencing (Section 3.2.5) and later this isolated protein was confirmed to be MT-2A$^{\Delta}$ with mass spectrometry (Section 5.2.1.3.2).

All the host strains expressed MT-2A$^{\Delta}$ best overnight (comparative results not shown) and so the prepared (isolated) overnight samples of each strain were used to compile the result shown in Figure 4.3. No isolated MT-2A$^{\Delta}$ (15 kDa) band were present in any of the samples collected from the BL21(DE3) strain, which meant that no MT-2A$^{\Delta}$ were expressed at any time regardless of the IPTG concentration. A faint 15 kDa band is visible in the overnight samples collected from the BL21(DE3)pLysS and –pLysE strains. Both gave optimal expression when 2 mM IPTG was used to induce expression. The BL21(DE3) CodonPlus-RIL strain clearly expressed MT-2A$^{\Delta}$ the best. This expression results were obtained when expression was induced with 1 mM IPTG and expressed overnight. Such a (relative) large amount of MT-2A$^{\Delta}$ was expressed that it is even visible in the heat fraction. The flow through does not contain MT-2A$^{\Delta}$, which means that it did bind successfully to the metals loaded onto the column.

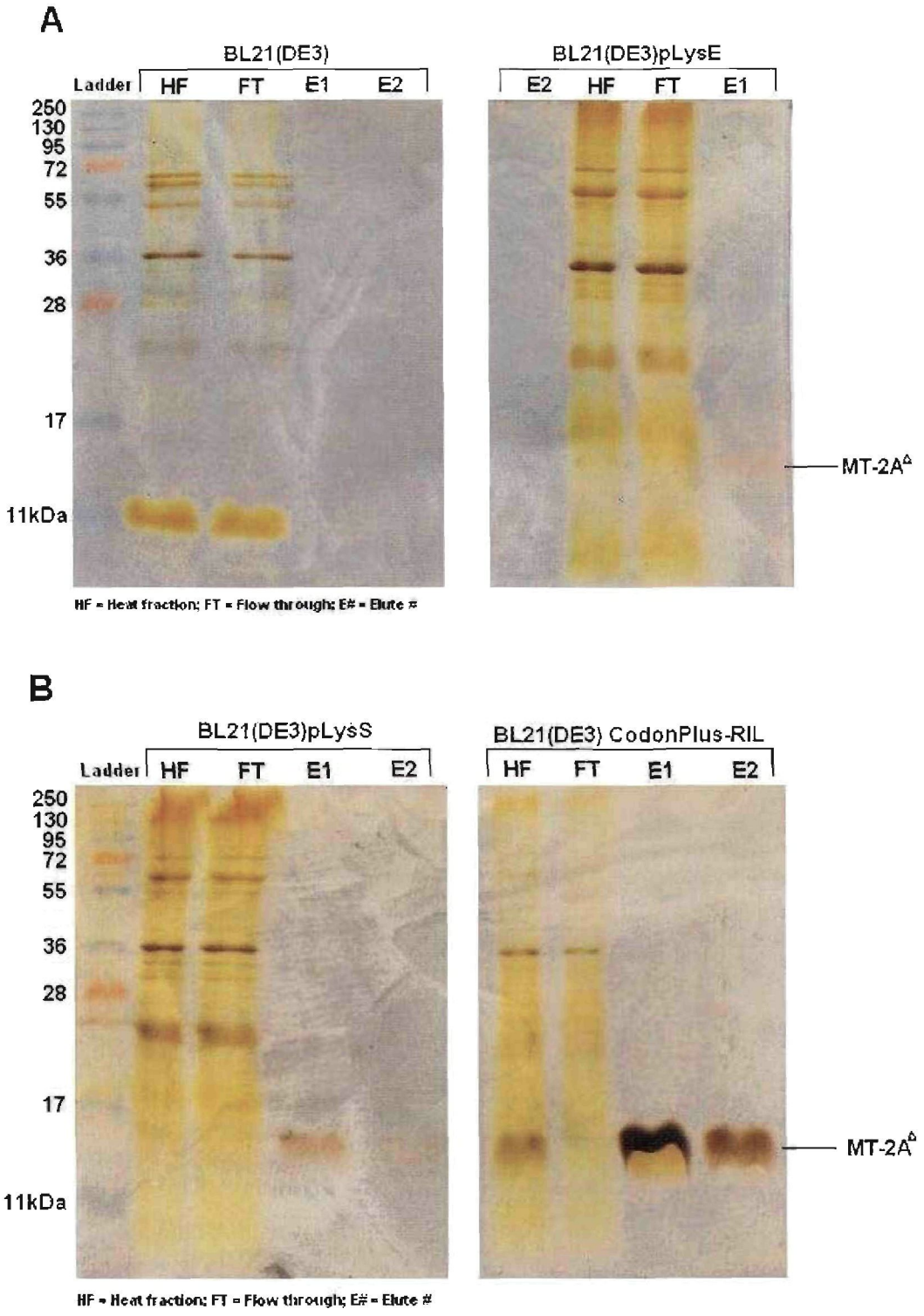


Figure 4.3: SDS-PAGE analysis of the heat fractions, column flow through and elute of pET28B-MT-2A transformed *E.coli* strains. The heat fractions were used to isolate MT-2A$^{\Delta}$ with affinity chromatography. The column flow through were analysed for MT-2A$^{\Delta}$ binding. The 1st (E1) and 2nd (E2) elutes represent the two volumes that were collected after imidazole was added to the column.

After these results were obtained and fully investigated, the previously analysed gels were again investigated to confirm MT-2A$^{\Delta}$ expression. The white arrows in Figure 4.2 show the bands which were identified afterwards to be MT-2A$^{\Delta}$. The previous gels did not add any new insight, but merely confirmed the optimal expression conditions of each strain, which was used to finally select the best strain for MT-2A$^{\Delta}$ expression.

4.2.2.7 MT-2A$^{\Delta}$ expression in four transformed and non-transformed *E.coli* BL21(DE3) strains in partly optimized conditions

4.2.2.7.1 Materials and Methodology

The previous results indicated that overnight expression gave the most visible MT band every time (when compared with the other time samples) regardless of the IPTG concentration, it was decided to perform IPTG optimization in overnight expressing cultures only. The IPTG concentration of each strain that resulted in the highest expression was further used to compare MT-2A$^{\Delta}$ expression among the strains. The BL21(DE3)- pLysS/pET28B-MT2 and –pLysE/pET28B-MT2 strains expressed MT-2A$^{\Delta}$ best at an IPTG concentration of 2 mM (described in Section 4.2.2.6.2). The BL21(DE3) CodonPlus-RIL/pET28B-MT2 strain expressed MT-2A$^{\Delta}$ at the same relative level when induced with IPTG to a final concentration of 0.5 and 1 mM but it was decided to use the latter for further comparison. No difference in MT-2A$^{\Delta}$ expression was found when the BL21(DE3)pET28B-MT2 cultures were induced by various concentrations of IPTG, hence it was decided to use 1 mM IPTG (final concentration) for further comparison.

A 5 ml starter culture was prepared for each transformed host strain and used to inoculate 50 ml LB medium containing the appropriate antibiotics. The same were done with all four untransformed host strains as negative control for MT-2A$^{\Delta}$ expression. These main cultures were grown at 37 ℃ until the density reached 1.0 at 600 nm. The IPTG concentration that gave the best MT-2A$^{\Delta}$ expression for each strain was used to induce expression. The cultures were kept overnight at 25 ℃ whilst shaking at 200 rpm. Before expression was induced, a pre-induction sample of the host strains containing the pET28B-MT2 vector was taken and prepared to obtain

the total and heat fractions. After overnight expression the cells were harvested and prepared as before to obtain the total and heat fractions of the transformed and non-transformed strains for SDS-PAGE analysis. Some of the heat fractions of the pET28B-MT2 vector containing cells were also used to isolate MT-2A$^{\Delta}$ via affinity chromatography. After electrophoresis the gels were stained with CBB and digitally scanned. After each gel was scanned, it was completely destained and then stained again with silver to compare the staining efficiency and sensitivity.

4.2.2.7.2 Results and Discussion

Figure 4.4 displays the CBB stained gel results of all four host strains and Figure.4.5 displays the same gels after silver staining. The MT standard from Sigma-Aldrich was included in the first gel (A) to confirm that CBB did not (or poorly) stain MT (Figure 4.4). About 50 µg standard were loaded in this case, which is relatively high for one single protein using CBB staining. When this gel, where no protein is detected, is compared with the silver stained gel in Figure 4.2, it is clearly visible that MT is more effectively detected with silver staining than CBB. Two factors usually separate silver from CBB staining: silver interact with the peptide bonds of proteins rather than certain amino acid residues, which result in more sensitive detection. These results correlates with those of other investigators, especially since more MT standard were loaded than were needed for detection. Thus, MT is not visible with CBB staining merely due to low amounts but simply due to the lack of certain amino acids (especially aromatic residues) and its small size.

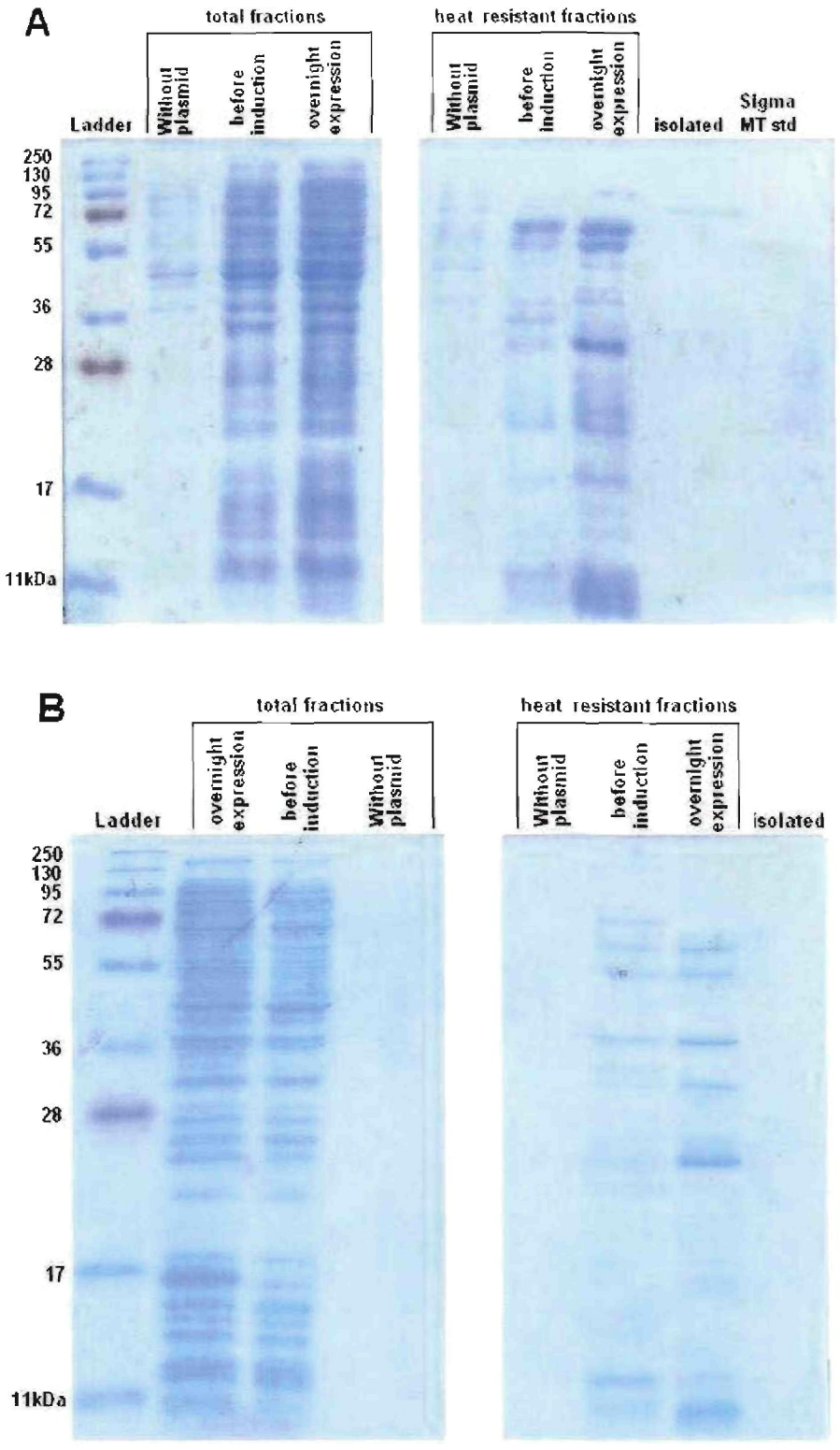


Figure 4.4 (A-B): The total-, heat resistant-, and metal-chelate binding isolated protein fractions of each pET28B-MT2 transformed and non-transformed stain after overnight expression as visualized with CBB staining. Gels A - D represent expression in BL21(DE3), BL21(DE3)pLysE, BL21(DE3) CodonPlus-RIL and BL21(DE3)pLysS, respectively. Expression between transformed and non-transformed strains was compared to confirm MT-2A$^{\Delta}$ expression.

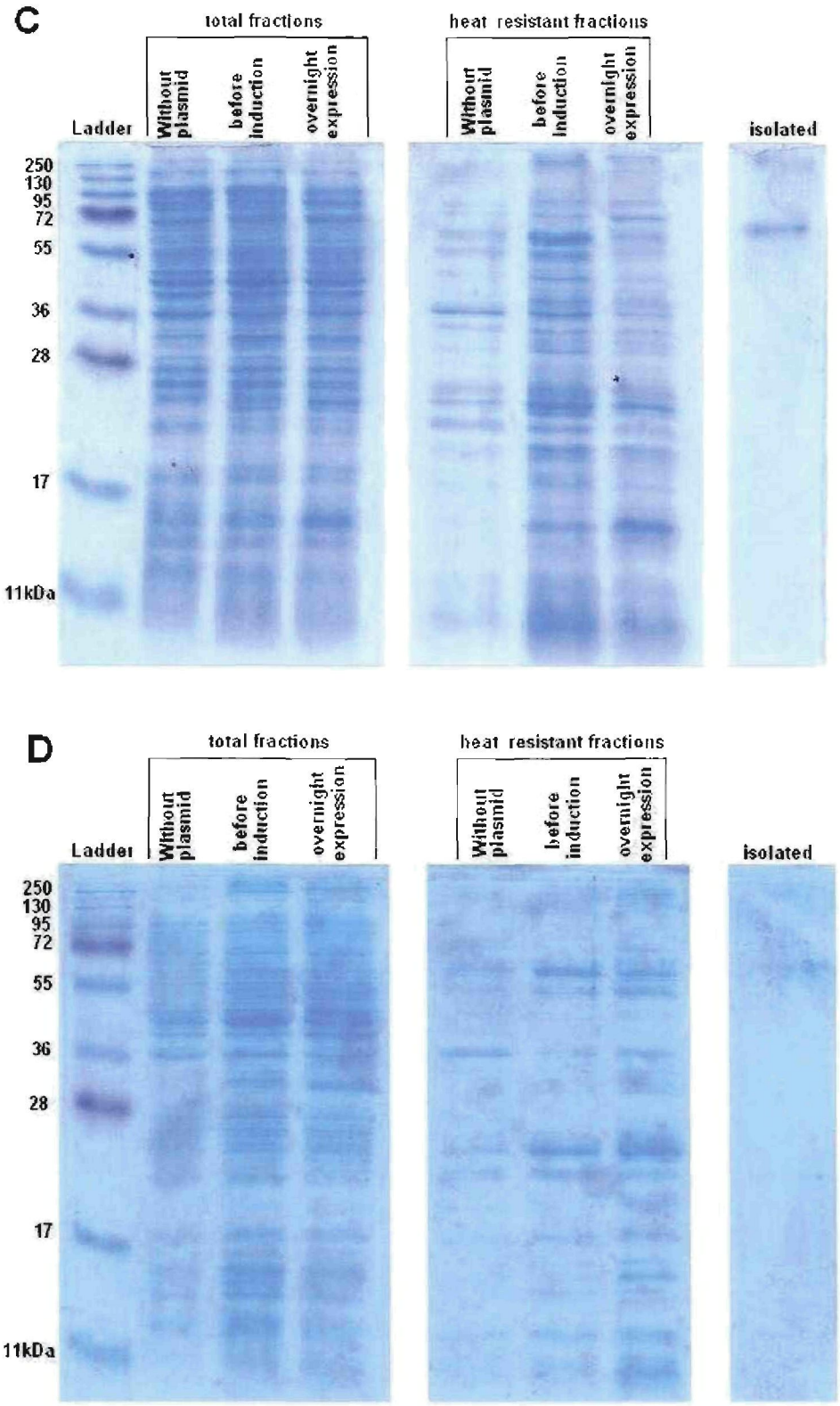


Figure 4.4 (C-D): The total-, heat resistant-, and metal-chelate binding isolated protein fractions of each pET28B-MT2 transformed and non-transformed stain after overnight expression as visualized with CBB staining. Gels A - D represent expression in BL21(DE3), BL21(DE3)pLysE, BL21(DE3) CodonPlus-RIL and BL21(DE3)pLysS, respectively. Expression between transformed and non-transformed strains was compared to confirm MT-2A$^{\Delta}$ expression.

While the position of MT-2A$^{\Delta}$ was known from the previous results, it was required to express the respective non-transformed strains to confirm expression and position in the fractions. The total and heat fractions of all the strains (transformed and non-transformed) were screened for the MT-2A$^{\Delta}$ band just below the 17 kDa region. However, as expected, the absence of a clear MT-2A$^{\Delta}$ band was common in the CBB stained gels. In these gels, the isolated fractions also lacked the MT-2A$^{\Delta}$ band (in contrast to the silver stained gels in Figure 4.5).

A prominent ~15 kDa band in the total and heat fractions of the transformed BL21(DE3) CodonPlus-RIL strain, however, is visible (Figure 4.4 C). Whether this is MT-2A$^{\Delta}$ or not might be disputed as no band was visible in the isolated fraction (last lane of Figure 4.4 C). From the previous results (Figure 4.3) it was clear that the BL21(DE3) CodonPlus-RIL strain expressed MT-2A$^{\Delta}$ the best and would it not be surprising if the major band was actually a relative large amount of MT-2A$^{\Delta}$. The absence of this band in the total and heat fractions of the expression samples collected from the non-transformed strain would confirm the band to be MT-2A$^{\Delta}$ but due to its absence in the isolated fraction, this could not be confirmed. The gels obtained with silver staining, however, gave better results (Figure 4.5).

The gels shown in Figure 4.5 are exactly the same gels shown in Figure 4.4 except that it was stained with silver after they were completely destained. Something to note is that it seems as if there were not the same amount of protein in each lane as some lanes did not contain any stained protein bands. This might have been caused by protein loss from the gel during re-staining especially since all lanes were loaded the same amount of protein. The MT standard gave the same results with silver staining as before, but since more standard was loaded, more bands were observed (Figure 4.2). A relatively thick band is visible just below the 17 kDa marker (~15 kDa) in the total and heat fractions of all the transformed strains except in the BL21(DE3)pLysE strain (Figure 4.5 B). The absence of similar bands in the samples collected from the non-transformed strains as well as the samples collected before expression was induced, confirms it to be the target protein (MT-2A$^{\Delta}$) expressed from the transformed vector (pET28B-MT2). Although the sequence of the MT-2A insert in the pET28B-MT2 vector was correct in every sense (orientation, sequence & frame),

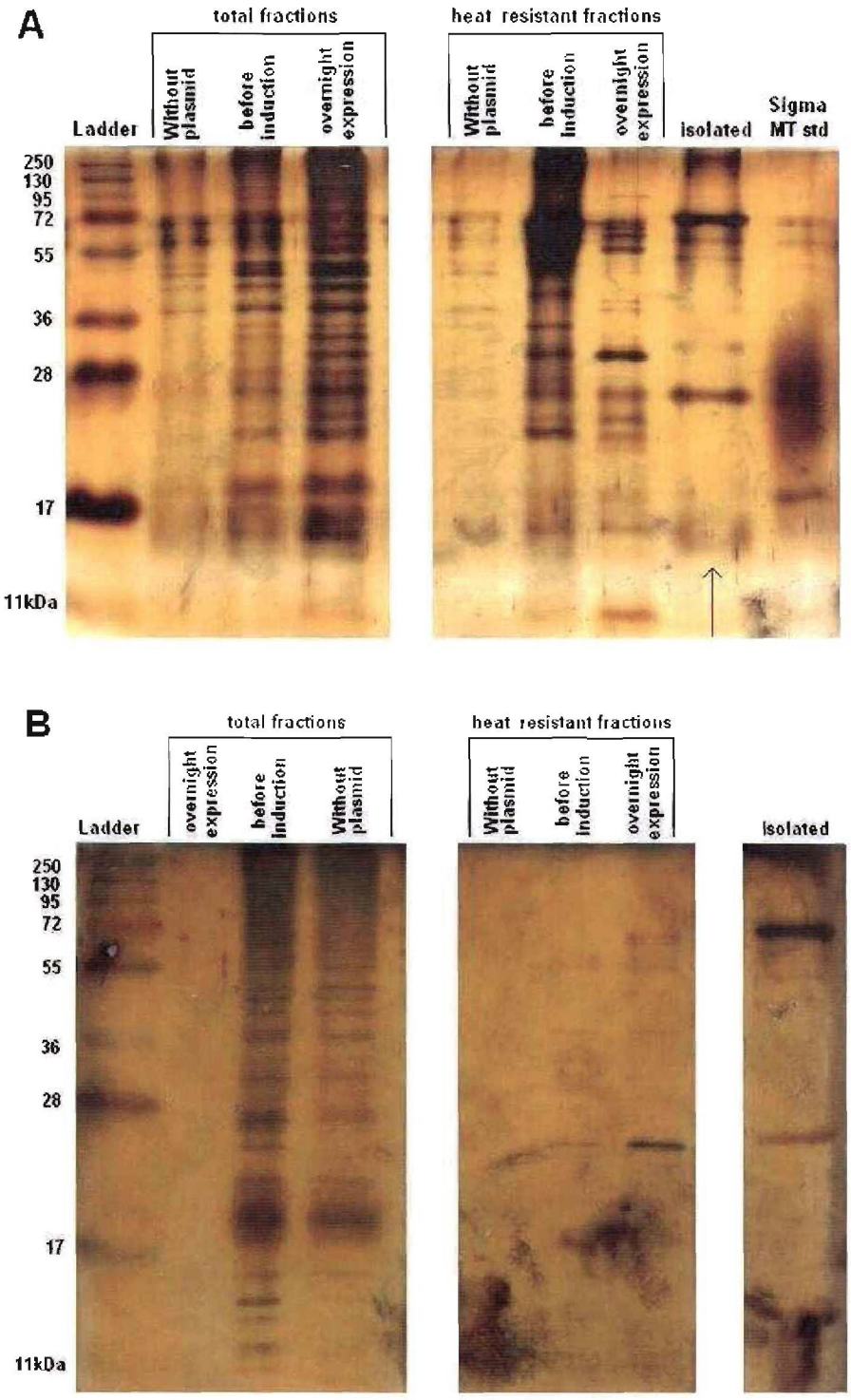


Figure 4.5 (A-B): The total-, heat resistant-, and metal chelate binding isolated protein fractions of each pET28B-MT2 transformed and non-transformed stain after overnight expression as visualized with silver staining. Gels A - D represent expression in BL21(DE3), BL21(DE3)pLysE, BL21(DE3) CodonPlus-RIL and BL21(DE3)pLysS, respectively. Expression between transformed and non-transformed strains was compared to confirm MT-2A$^{\Delta}$ expression. Arrows indicate presumed MT-2A$^{\Delta}$ band.

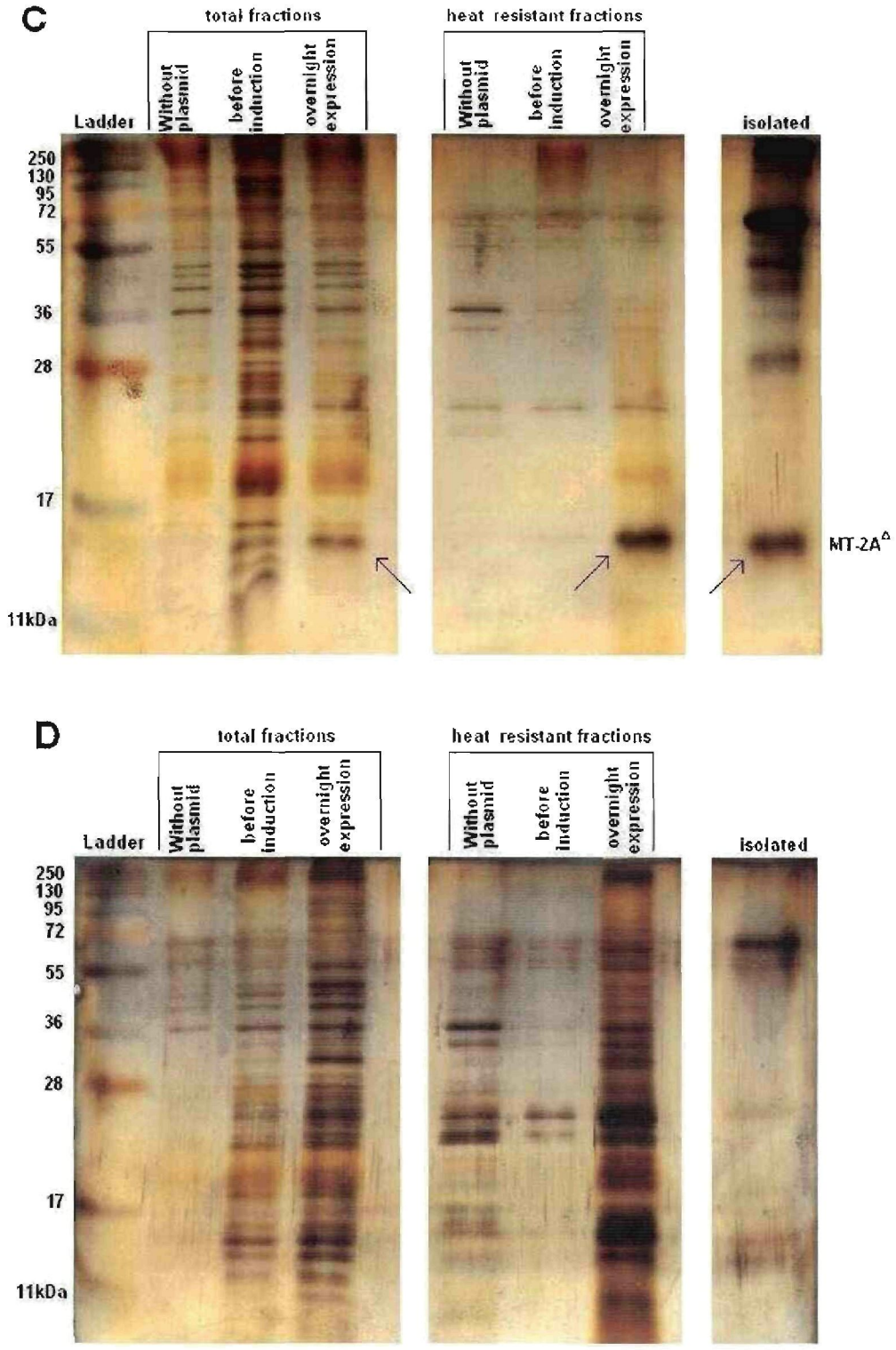


Figure 4.5 (C-D): The total-, heat resistant-, and metal chelate binding isolated protein fractions of each pET28B-MT2 transformed and non-transformed stain after overnight expression as visualized with silver staining. Gels A - D represent expression in BL21(DE3), BL21(DE3)pLysE, BL21(DE3) CodonPlus-RIL and BL21(DE3)pLysS, respectively. Expression between transformed and non-transformed strains was compared to confirm MT-2A$^{\Delta}$ expression. Arrows indicate presumed MT-2A$^{\Delta}$ band.

it was still necessary to confirm whether MT-2A$^{\Delta}$ was translated correctly from the pET28B-MT2 vector.

The isolated fractions of all the strains, except the BL21(DE3)pLysE (Figure 4.5 B), contained the presumed MT-2A$^{\Delta}$ band. Since this is also proof that the ~15 kDa band can be isolated with metal chelating chromatography, it supports the argument that this band is MT-2A$^{\Delta}$. The presence of these isolated bands in silver stained gels, also confirms the inability of CBB to stain metallothionein (absence of similar bands in the CBB stained gels). The apparent MT-2A$^{\Delta}$ bands (indicated by the arrows) are particularly present in the samples collected from the transformed BL21(DE3) CodonPlus-RIL strain (Figure 4.5 C) especially after overnight expression. Considering this as well as the previous results, BL21(DE3) CodonPlus-RIL strain is the best of the four tested strains to express MT-2A$^{\Delta}$ in large quantities. Therefore, the BL21(DE3) CodonPlus-RIL strain was selected for further expression optimization in order to express large amounts of MT-2A$^{\Delta}$.

4.2.3 Expression optimization of MT-2A$^{\Delta}$ in BL21(DE3) CodonPlus-RIL

4.2.3.1 Materials and Methodology

From the results obtained, it was concluded that the BL21(DE3) CodonPlus-RIL strain expressed MT-2A$^{\Delta}$ the best. This transformed host strain was therefore selected for further optimization to ultimately express large quantities of MT-2A$^{\Delta}$. Factors which are known to influence expression were varied individually. These included the following:

- *Culture media*: Other studies reported MT expressed in LB medium rather than rich medium such as 2YT (Park et al., 2007:86). The difference in expression when using different media (LB and 2YT) was compared in this study.

- *Temperature*: the temperature at which expression occur vary between proteins and host strains. Higher temperature result in an increasing translation rate but also in higher proteolytic degradation. Lower expression temperature is commonly used to facilitate hydrophilicity and reduction in the

formation of inclusion bodies of the recombinant protein. Hence, the difference in MT-2A$^{\Delta}$ expression at temperatures 18, 20, 25, 30, 37 and 40 ℃ was also compared in this study.

- *Time*: the time needed to express large quantities of recombinant protein may vary as a result of translation and degradation rate. Although, overnight expression resulted in larger amounts of MT-2A$^{\Delta}$, it still needed to be confirmed as well as compared with an additional period, the so called "weekend expression". With weekend expression, expression occurs over two days normally at low temperature (18 ℃).

- *Inducer concentration*: according to Drechsel et al., (2000) the normal IPTG final concentration to induce expression ranges from 0.05 – 2 mM. Although it seems that more IPTG would result in more expression, it is hardly the case. The IPTG concentration used to induce expression is a very important focus point which needs fine-tuning for optimal expression. Expression of MT-2A$^{\Delta}$ was compared when 0.05, 0.25, 0.5, 0.75, 1.0, 1.5 and 2.0 mM IPTG were used to induce expression.

Other factors commonly influencing expression, but to a lesser degree, were also compared which included expression induction during different *culture densities* and various *culture volumes*. As already mentioned, when expressing MT, investigators add $CdCl_2$ to a final concentration of 0.3 mM to the expression medium to facilitate metal binding and consequently stabilize the expressed MT (You, 1999:46). Since $CdCl_2$ is toxic it was also necessary to optimize the *$CdCl_2$ concentration*. Only one factor was varied at a time while the other factors were kept constant so that secondary influence could not occur. The factors which theoretically had the most influence on expression were varied first followed by factors with less influence to "fine-tune" expression. The same procedures were used to prepare the various culture samples. All optimization steps were monitored with SDS-PAGE. The results are summarized in Table 4.3.

These optimized conditions (Table 4.3) were verified by expressing MT-2A$^{\Delta}$ using the established conditions. A transformed and non-transformed BL21(DE3) CodonPlus-

RIL strain was cultured in 2YT medium at 37 ℃ whil st shaking at 200 rpm. When the absorbance at 600 nm reached 1.0, IPTG and $CdCl_2$ were added to a final concentration of 1 mM and 0.3 mM, respectively. The temperature of the cultures was decreased to 30 ℃ (not 25 ℃ as previously use d) which was then kept overnight (~16 h) for expression. After overnight expression the cells were harvested by means of centrifugation at 6 000 *g*, 4 ℃ for 10 min. The two cell pellets, obtained from the transformed and non-transformed culture, were suspended in binding buffer and sonicated to disrupt the cells. The cell debris was separated from the cytosol-containing fraction (total fraction) by centrifugation at 6 000 *g*, 4 ℃ for 10 min.

A small amount of the supernatant (total fraction) was kept for electrophoresis. The pellet containing the cell debris (pellet 1) was re-suspended in 8 M urea to confirm the absence of especially inclusion bodies. The rest of the total fraction was incubated at 85 ℃ for 15 min and centrifuged for 10 min at 10 0 00 *g*, 4 ℃. The resulting pellet (pellet 2) containing precipitated proteins where also re-suspended in 8 M urea to confirm the absence of denatured MT-2A$^{\Delta}$. A small amount of the supernatant (heat fraction) containing the heat resistant proteins were also kept for electrophoresis. The rest of the heat fraction was used to isolate MT-2A$^{\Delta}$ via metal chelating chromatography. The heat fraction was applied to the metal chelating column after which the flow through (proteins which did not bind to the column) was collected and kept for analysis to confirm the absence of MT-2A$^{\Delta}$. The isolation procedure was according to the manufacturer's specifications (discussed in the next chapter, Section 5.2.3.1.1). Samples of the 1st, 2nd and 3rd elution steps were also kept for electrophoresis.

The protein concentrations of all the collected and prepared samples were determined as previously described in order to load the same amount of protein per lane. When the total amount of protein in a sample was below required amount, (as was the case with the elution samples), the entire sample was loaded. After SDS-PAGE, the proteins were visualised with CBB and silver staining, respectively. As mentioned before, due to the large amount of gels used to monitor the optimization process, the optimal expression conditions will only be summarized in table format (Table 4.3).

4.2.3.2 RESULTS AND DISCUSSION

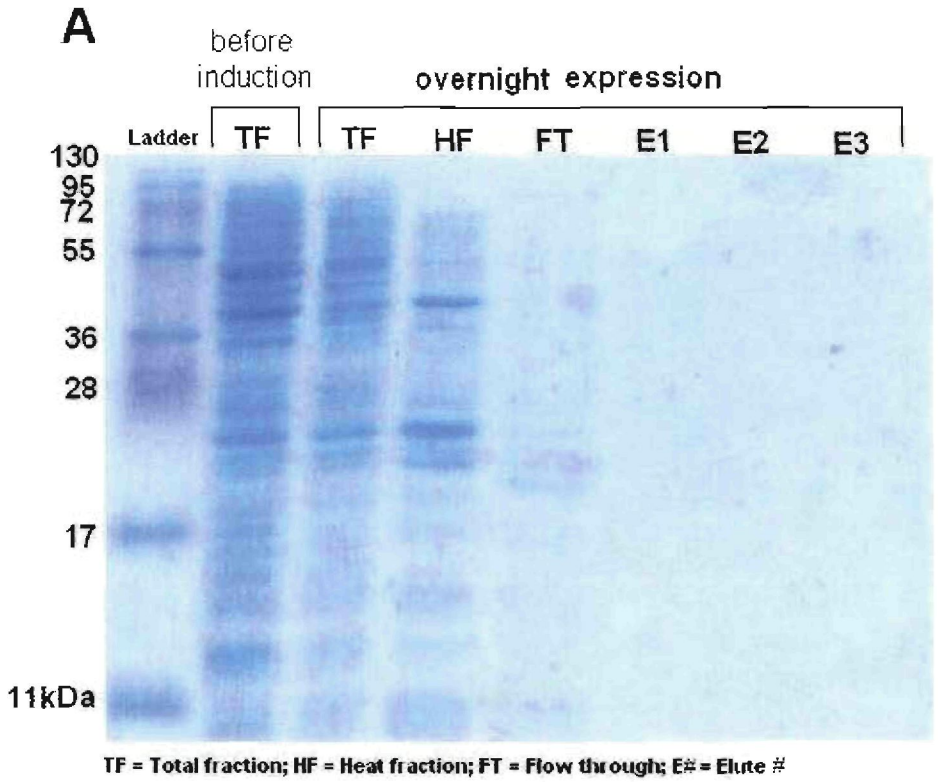
All optimization steps were visualised with SDS-PAGE. The results (not shown) obtained are summarized in table 4.3.

Table 4.3: Optimized MT-2A$^{\Delta}$ expression conditions in *E.coli* BL21(DE3) CodonPlus-RIL. Factors influencing expression were varied and compared to obtain optimal expression conditions. The conditions which were compared are listed below with the result obtained.

Variable	Conditions compared	Results
Culture medium	LB vs. 2YT	The use of 2YT resulted in the best (most) growth and expression.
Temperature	18, 20, 25, 30, 37 & 40 ℃	MT-2A$^{\Delta}$ was best expressed at 30 ℃. Expression at 37 ℃ is also acceptable but temperatures lower than 30 ℃ gave less expression. It was however decided to express at 30 ℃.
Time	Overnight expression vs. Weekend expression	Low expression was seen when the cells expressed for two days. The reason for this is not known. Therefore, all expression was further done overnight.
Inducer concentration	0.05, 0.25, 0.5, 0.75, 1.0, 1.5 & 2 mM	Although, both the 0.5 and 1 mM concentrations gave the same expression result, 1 mM were selected for further expression work
Culture density (OD at 600 nm)	0.6, 1.0 & >1.0	There was no difference in expression when the cultures were induced at density 0.6, 1.0 or > 1.0, thus it was decided to constantly induce expression when the culture densities reached 1.0.
Culture volume	100, 500 & 1000 ml	No visible difference in expression rate was observed in different culture volumes. Thus, large culture volumes could be used to express large amounts of MT-2A$^{\Delta}$.
$CdCl_2$ concentration	0.1, 0.3, 0.6 & 1.0 mM	As previously shown (You, 1999:46), 0.3 mM gave the best expression results. Higher concentrations resulted in almost immediate cell death resulting in less expression.

The optimized conditions were verified by culturing and expressing MT-2A$^{\Delta}$ using the established conditions. A transformed and non-transformed BL21(DE3) CodonPlus-RIL strain were cultured in 2YT medium at 37 ℃ whi lst shaking at 200 rpm. When the absorbance at 600 nm reached 1.0, IPTG and $CdCl_2$ were added to a final concentration of 1 mM and 0.3 mM, respectively. The temperature of the cultures was decreased to 30 ℃ (not 25 ℃ as previously) fo r overnight culturing and expression. After overnight expression the cells were harvested by means of centrifugation at 6 000 *g*, 4 ℃ for 10 min. The two cell pellets, obtained f rom the transformed and non-transformed culture, were prepared as described in Section 4.2.3.1 and analysed with SDS-PAGE. The following samples were used for electrophoresis: total fraction, pellet 1, heat fraction, pellet 2, flow through, elute 1 – 3. After SDS-PAGE, the gels were stained with CBB and silver staining, respectively. Separate gels were used for CBB and silver staining. These gels are displayed in Figure 4.6.

The difference between the pET28B-MT2 transformed and non-transformed gels is clearly visible. MT-2A$^{\Delta}$ was not expressed in the non-transformed strain which resulted in the absence of MT-2A$^{\Delta}$ in both CBB and silver stained gels. The transformed strain, on the other hand, expressed MT-2A$^{\Delta}$ successfully. The absence of MT-2A$^{\Delta}$ in pellet 1 indicates that it was not expressed in inclusion bodies and was thus not lost with the cell debris after cell lyses. MT-2A$^{\Delta}$ was also absent in the second pellet which confirms its heat stability. The column flow through also lacked MT-2A$^{\Delta}$ which indicated its successful binding to the nickel (metal used on the affinity column resin) and thus isolation. The optimized expression conditions resulted in relative large amounts of MT-2A$^{\Delta}$ as is visible in silver stained and, interestingly CBB stained gels as well. This is the second time in this study that MT-2A$^{\Delta}$ could clearly be detected by CBB and implies that CBB has a weak but not absent detection capacity for MT staining (also see Figure 4.4 C).



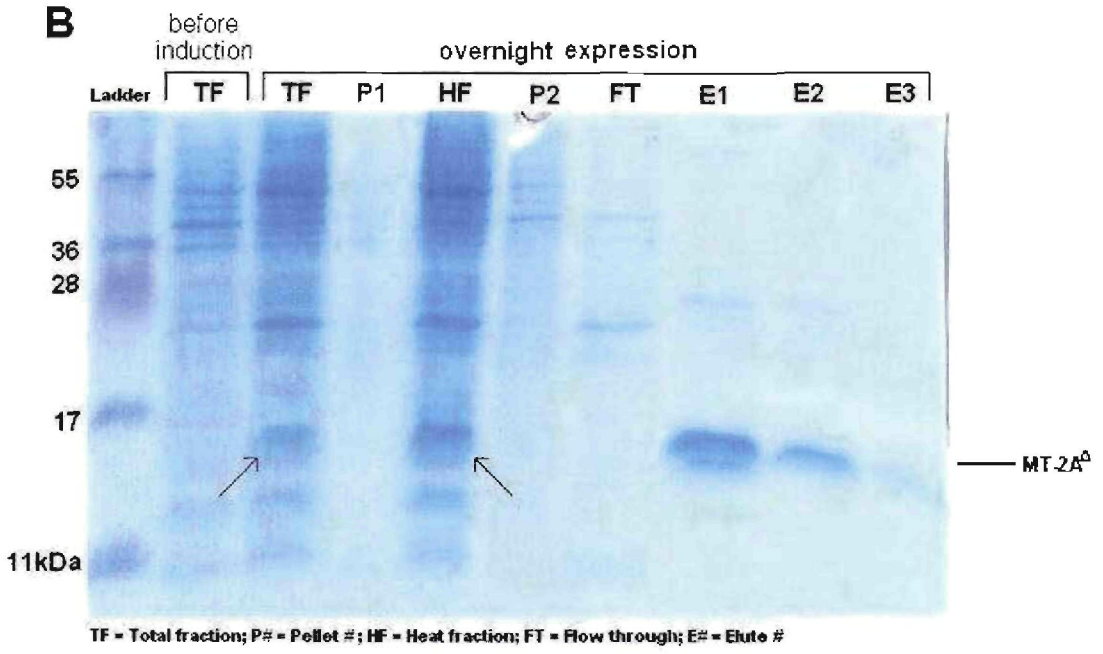
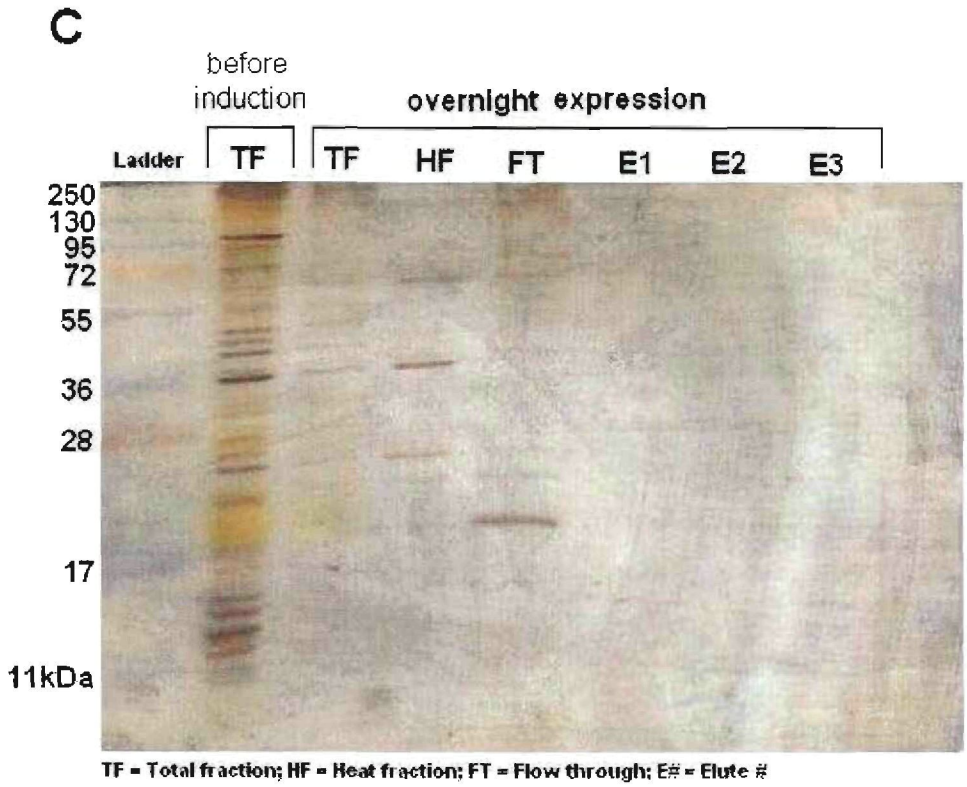


Figure 4.6 (A-B): Expression of MT-2A$^{\Delta}$ under optimized conditions in *E.coli* BL21 (DE3) CodonPlus-RIL. Gels A & C represent samples obtained from the non-transformed strain as visualised with CBB (A) and silver (C) staining. Gels B & D represent the samples obtained from the pET28B-MT2 transformed cells after the respective stainings. Arrows indicate position of MT-2A$^{\Delta}$.



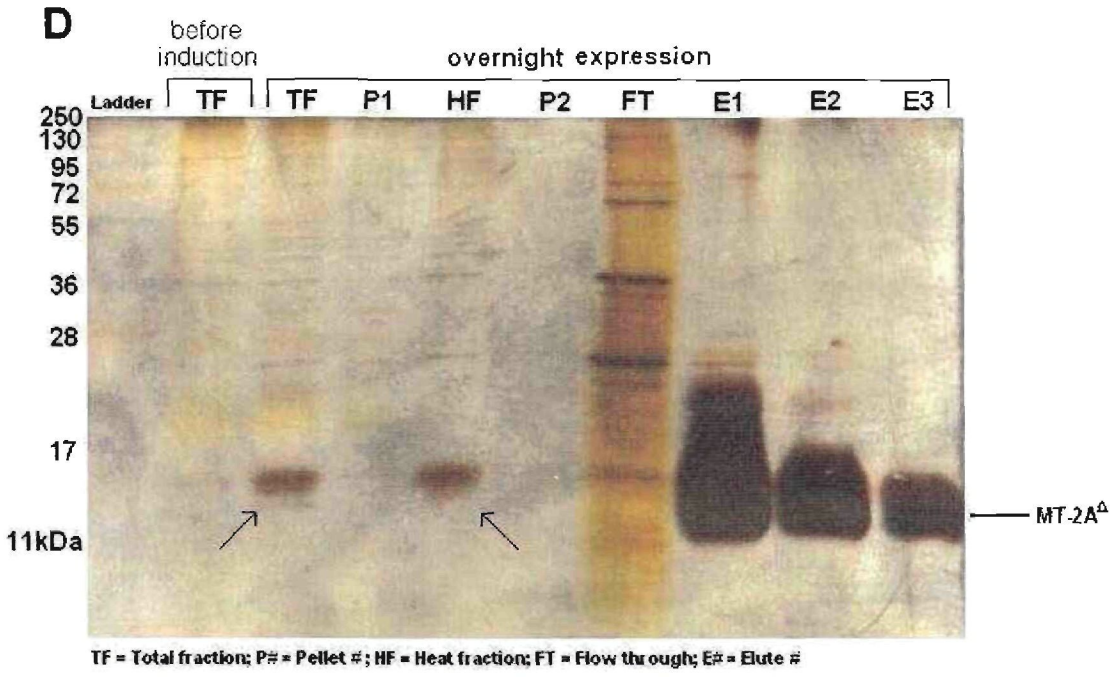


Figure 4.6 (C-D): Expression of MT-2A$^{\Delta}$ under optimized conditions in *E.coli* BL21 (DE3) CodonPlus-RIL. Gels A & C represent samples obtained from the non-transformed strain as visualised with CBB (A) and silver (C) staining. Gels B & D represent the samples obtained from the pET28B-MT2 transformed cells after the respective stainings. Arrows indicate position of MT-2A$^{\Delta}$.

These results represent the best expression that could be obtained after strain selection and optimization. The expression levels of MT-2A$^{\Delta}$, however, remained low when compared with the expression levels of other recombinant proteins in *E.coli*. The expression levels of MT-2A$^{\Delta}$ were measured and compared to the total amount of protein. After optimization, only 6 µg MT-2A$^{\Delta}$ was expressed per milligram total protein. This means that 200 µg MT-2A$^{\Delta}$ is expressed in 100 ml culture which is equivalent to ~800 mg cells. It is generally accepted that under optimized conditions, more than 5 mg recombinant protein (MT) can be expressed in one litre *E.coli* (You et al., 1999:50), as is normally the case with other recombinant proteins (Murooka & Nagaoka, 1987:206). Although expression was not high compared to the expression of other proteins, these conditions were used to express relative large (mg) amounts of MT-2A$^{\Delta}$. The isolation and purification of these large amounts MT-2A$^{\Delta}$ are discussed in the following chapter.

The conditions used to express relative large amounts of MT-2A$^{\Delta}$ (using large culture volumes) were as follows: one litre 2YT medium containing kanamycin and chloramphenicol was inoculated with BL21(DE3) CodonPlus-RIL/pET28B-MT2 cells and grown at 37 ℃ whilst shaking at 200 rpm until the density reached 1.0 at 600 nm. Expression was induced by the addition of IPTG to a final concentration of 1 mM. Cadmium chloride was also added to a final concentration of 0.3 mM in order to stabilize expressed MT. Expression was done overnight (~16 h) at 30 ℃ whilst shaking at 200 rpm and then harvested by means of centrifugation in a Beckman Model J2-21 centrifuge using a JA14 rotor (6 000 *g*, 10 min, 4 ℃). These large scale expression cultures were used to isolated relative large amounts of MT-2A$^{\Delta}$ and were repeated numerous times until about 2 mg MT-2A$^{\Delta}$ were obtained. The isolation and purification of MT-2A$^{\Delta}$ are discussed in Chapter 5.

4.3 SUMMARY AND CONCLUSIONS

The pET28B-MT2 expression vector was transformed into four *E.coli* expression strains in an attempt to find the strain that express MT-2A$^{\Delta}$ the best. The transformed strains were partially optimized to compare MT-2A$^{\Delta}$ expression. BL21(DE3) CodonPlu-RIL was selected to express MT-2A$^{\Delta}$ and was further optimized. CBB and silver staining was used when expression was monitored with SDS-PAGE.

In accordance with reports by others (Meloni et al., 2005:77), the apparent molecular weight of MT-2A$^{\Delta}$ estimated by SDS-PAGE was much higher than its true molecular mass of ~9.3 kDa. MT was detected more effectively with silver staining than CBB stairing, also as suggested by others (Alhama et al., 2006:56). The expression levels of MT-2A$^{\Delta}$ remained relatively low when compared with expression of other recombinant proteins in *E.coli.* The relatively low expression levels of mammalian MT in *E.coli* remains an obstacle despite numerous attempts to solve this problem during the past two decades (Hong et al., 2001:247). Nevertheless, the objective to express MT-2A$^{\Delta}$ was apparently (later to be confirmed) successful and could be further evaluated and used in the study.

Chapter 5

Purification of recombinant MT-2A

5.1 INTRODUCTION

The human MT-2A gene was successfully cloned into the pET28B expression vector as described in Chapter 3. This MT-2A expression vector (pET28B-MT2) was transformed into an *E.coli* host strain that was induced to express recombinant modified MT-2A (MT-2A$^{\Delta}$) successfully. Strain selection and expression optimization are described in Chapter 4. After the MT-2A$^{\Delta}$ expression was optimized, large cultures were repeatedly used to express relatively large quantities of MT-2A$^{\Delta}$. The total protein fractions were obtained and stored at -80 ℃ for MT-2A$^{\Delta}$ isolation. Isolated MT-2A$^{\Delta}$ would eventually be used as internal standard when quantifying human MT-2A in biological material. This chapter will focus on the required procedures to isolate MT-2A$^{\Delta}$. Removal of the tag from the recombinant protein will also be discussed as well as additional purification.

For MT-2A* (without His-tag) to be used as *internal standard* in a mass spectrometric quantification approach, it is required to be of high purity (95 %) and to be uniform. The *internal standard* can in principle be used to estimate/calculate the concentration of metallothionein (MT-2A) in biological samples via linear regression while the purchased standard (rabbit MT-2A from Bestenbalt) can be used to optimize the detection of MT-2A. To do this, it is important that the concentration of the *internal standard* should always be known. Purified recombinant standards are usually dried and weighed to determine the amount, but protein determination can also be used to determine the concentration (and total amount) of the protein standard in solution. However, when the sample is contaminated by other proteins, the "known concentration/amount" would actually be of the whole protein sample (not only the MT standard). The isolation and purification of MT-2A$^{\Delta}$ are discussed in this chapter as well as the removal of the tag to obtain a MT standard (MT-2A*) which is very similar to the biological isoforms.

5.2 METHODOLOGY, RESULTS AND DISCUSSION

5.2.1 Mass spectrometric analysis of isolated MT-2A^{A} and rabbit MT-2A

To understand the following section where mass spectrometry was used to identify and evaluate the purity of protein samples, it is necessary to give a quick overview of electrospray ionisation mass spectrometry (ESI-MS).

Mass spectrometry involves the separate propulsion of ions, each with specific velocity, in the gas phase by means of electric or magnetic fields into a suitable mass analyser (detector) (Wilson & Walker, 2003:528). Large biomolecules (such as proteins) in solution (liquid) can be vaporized to detect them. When liquid is passed through the ESI source, the high temperature and electrical charge cause the liquid to become gaseous and result in intact multiple charged ions (each species of the same biomolecule carries a different charge) (Łobiński et al., 1998:46; Prange & Schaumlöffel, 2002:451; Rosenberg, 2003:844; Vestling, 2003:122). The basic amino acids (lysine, arginine and histidine) as well as the N-terminal play an important role in protonation (Vestling, 2003:123; Wilson & Walker, 2003:553).

Proteins containing more than one basic amino acid can theoretically attain more than one charge. Identical proteins, however, do not always attain the same charge when passing through the ESI source but can exist in various charged states when passing through the mass spectrometer, resulting in a spectrum of *m/z* (relative mass to charge) peaks. Each peak represents a different charged state of the same chemical entity (protein). This spectrum is generally referred to as the convoluted spectrum.

The following equation can be used to calculate the charged state each peak represents:

Equation 5.1: Calculation of the charge state of a peak from a multiple charged protein.

$$n_2 = (m_1 - 1)/ (m_2 - m_1)$$

In this equation n_2 (charge or z) represents the charge of the second peak (m_2); m_1 represents the m/z value of the first peak and m_2 the m/z value of the second (related) peak. A deconvoluted spectrum (zero-charge state) can be created when the various charge states (represented by specific peaks) are known. Although mass analysis software can reconstruct accurate deconvoluted spectra of proteins, it is still necessary to be familiar with the principals. When the charged states are known, the following equation can be used to determine the average mass of the protein represented by the spectrum of peaks:

Equation 5.2: Calculation of the relative mass of the multiple charged proteins.

$$M = n_2 (m_2 - 1)$$

In this equation M is the relative mass of the neutral molecule. The more multiple-charged peaks a protein gives, the more accurate the molecular mass can be determined (McSheehy & Mester, 2003:316).

5.2.1.1 Calibration and settings of the mass analyser.

5.2.1.1.1 Materials and Methodology

The Waters® VB Quatro Micro® MS/MS was calibrated with 1 mg/ml horse heart myoglobin bound to heme b (Sigma-Aldrich) dissolved in (Milli-Q) water. A few microlitres of the mixture were injected directly into the ionization source (electrospray ionisation, ESI) which carried a charge of 3.2 kV and was at 90 ℃. A flow rate of 10 µl/min was maintained by the Waters 1525 binary HPLC pump. Each run took approximately three minutes, attaining a spectrum every two seconds from which an average spectrum of all captured spectra was created afterwards. MS1 was set to scan from 500 – 2000 amu (in the positive ion mode). The convoluted spectrum of the horse heart myoglobin was compared and calibrated to fit the theoretical spectrum. The convoluted spectrum is displayed in Figure 5.1.

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5.2.1.1.2 RESULTS AND DISCUSSION

The various charged states of myoglobin are represented by the series of mass to charge (*m/z*) peaks in the given convoluted spectrum in Figure 5.1. The charge state each peak represent are also given as was determined by Equation 5.1. The average mass of horse heart myoglobin (without heme b) is 16 971.02 ± 20.96 Da as determined with Equation 5.2 which corresponds to the theoretical mass of 16 955 Da. The less charge a molecule carries the more error when calculating its mass. More than 0.1 % error occurs when peaks with charged states less than 15+ are used to calculate the molecular mass. Heme b, which dissociated from myoglobin under the acidic conditions, is represented in this figure by the 616.49 *m/z* peak.

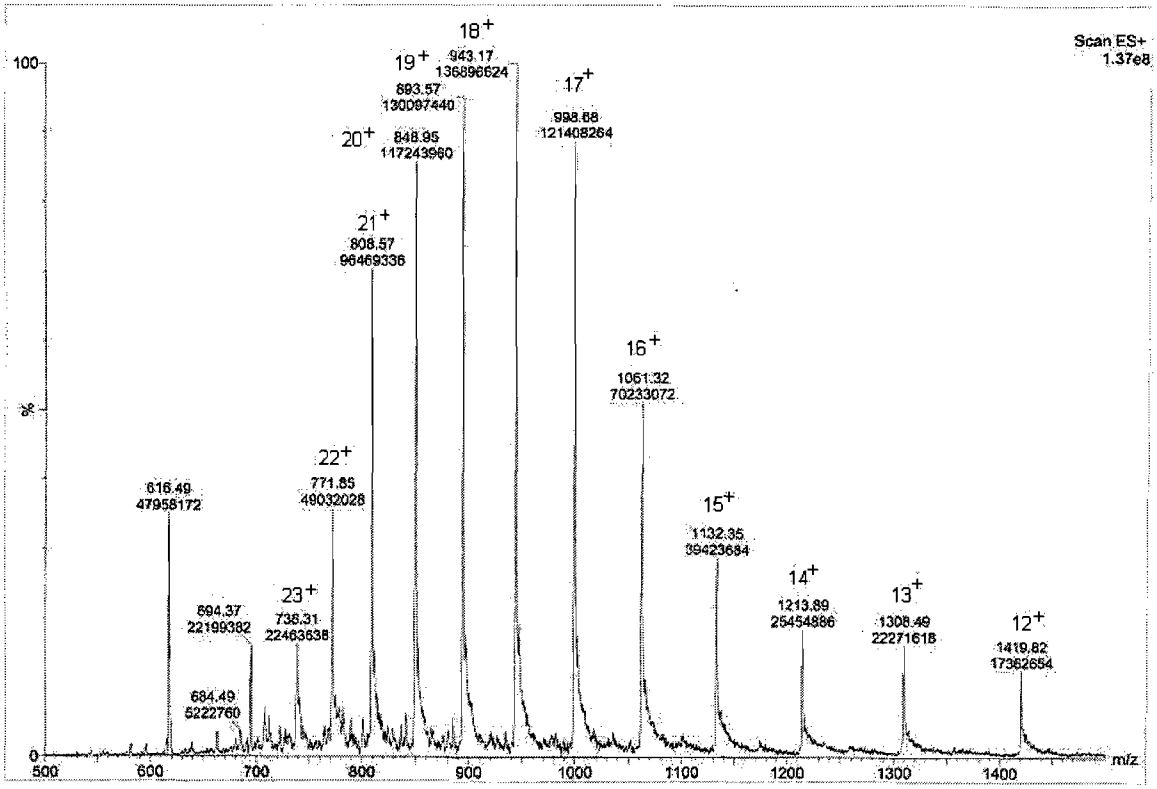

Figure 5.1: Convoluted mass spectrum of horse heart myoglobin. Horse heart myoglobin was used to calibrate the mass analyser. The charge state (e.g. 12^+) as well as the *m/z* (e.g. 1419.82) each peak represent is indicated in this and following spectra in this chapter. Each peak's intensity is given beneath the respective *m/z* values (e.g. 17362654).

5.2.1.2 Mass spectrometric analysis of rabbit MT-2A to confirm purity and calibration.

5.2.1.2.1 Materials and Methodology

After calibration, rabbit MT-2A standard (Bestenbalt, Tallinn, Estonia) was analysed to confirm the mass analyser's ability to detect the protein correctly (after calibration) and confirm relative sensitivity. Since most commercial MT standards contain some kind of contamination such as more than one isoform, it was also required to confirm the purity of this standard which the supplier declared to be of high (98 %) purity. The standard was suspended in a 50 % (v/v) acetonitrile, 1 % (v/v) formic acid solution which is commonly used to facilitate ionisation (protonation) of compounds (Miranker, 2000:14025). The high percentage formic acid was also necessary to lower the pH to consequently dissociate possible bound metals from the protein. Fifty micro-litres of a 1 mg/ml mixture were injected directly (without chromatography) into the ESI source. Ninety spectra were captured to obtain an average spectrum containing the MT-2A fingerprint (of peaks representing multiple charged rabbit MT-2A ions). The convoluted spectrum is displayed in Figure 5.2.

5.2.1.2.2 Results and Discussion

Three molecular ions of rabbit MT-2A were observed, namely $[M+6H]^{6+}$ (*m/z* 1021.7), $[M+5H]^{5+}$ (*m/z* 1225.87) and $[M+4H]^{4+}$ (*m/z* 1532.26) in the spectrum shown in Figure 5.2. By using Equations 5.1 and 5.2 the charges of related peaks and the average mass of the protein can be determined. The largest peak represents the charged state of MT-2A that was detected the most (Miranker, 2000:14025). The specific charges as determined with Equation 5.1 are given in the figure. The average mass of rabbit MT-2A, as determined with Equation 5.2, obtained from the spectrum is 6124.53 ± 0.45 Da which corresponds to the theoretical mass of 6125.47 Da. This result indicated that calibration was successful and also confirmed the high purity of the standard. A few small peaks are, however, visible on the left-hand side of the 6+ peak. These peaks also represent a protein and a possible contaminating isoform. As no isoform of MT has a mass around 5951.17 Da, it was more likely a fragment of the rabbit MT which can result in harsh ionization conditions. The mass spectrometer's ability to detect the metallothionein was also confirmed by analysing this standard.

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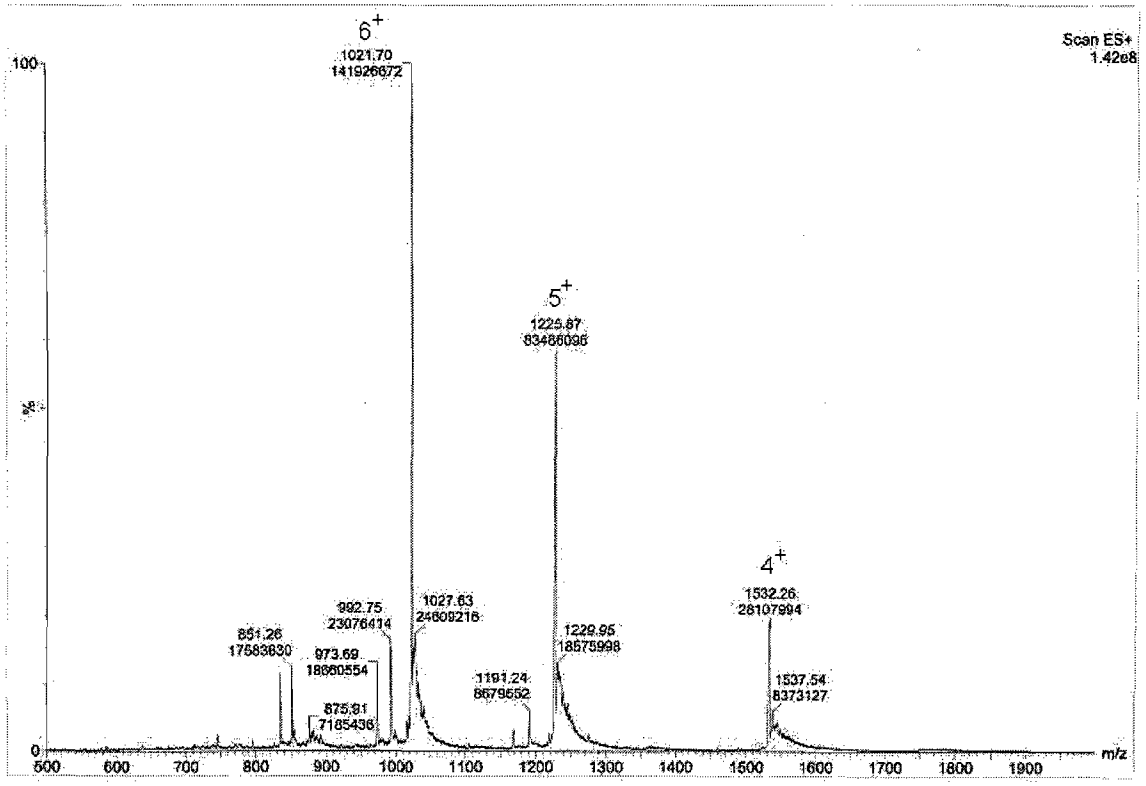

Figure 5.2: Convoluted mass spectrum of 98 % pure rabbit MT-2A standard from Bestenbalt. The charge states (e.g. 6^+) as well as the *m/z* (e.g. 1021.70) each peak represent is indicated. Each peak's intensity is given beneath the respective *m/z* values (e.g. 141926672).

5.2.1.3 Mass spectrometric analysis of MT-2A$^{\Delta}$ to confirm expected size and determine purity.

5.2.1.3.1 Materials and Methodology

After the purity of the rabbit MT-2A standard was confirmed as well as the machine's ability to detect the various charged ions accurately were investigated, MT-2A$^{\Delta}$ was analysed using the same solutions and settings. A small amount of the stored total fractions were used to isolate MT-2A$^{\Delta}$ with metal chelating chromatography according to the manufacturer's specifications as described in Section 5.2.3. This was done to observe whether metal chelating chromatography was capable to give (95 %) pure MT-2A$^{\Delta}$ especially since the results in Chapter 4 contained visible contamination. The isolated recombinant protein concentration was determined with the BCA method (Section 4.2.2.3.1) in order to analyse the same concentration of recombinant protein. A final concentration of 1 mg expressed protein per millilitre solution was prepared for analysis.

5.2.1.3.2 RESULTS AND DISCUSSION

The convoluted spectrum obtained after analysis is displayed in the following figure. Some areas of the spectrum were enlarged to show the target peaks among the noise.

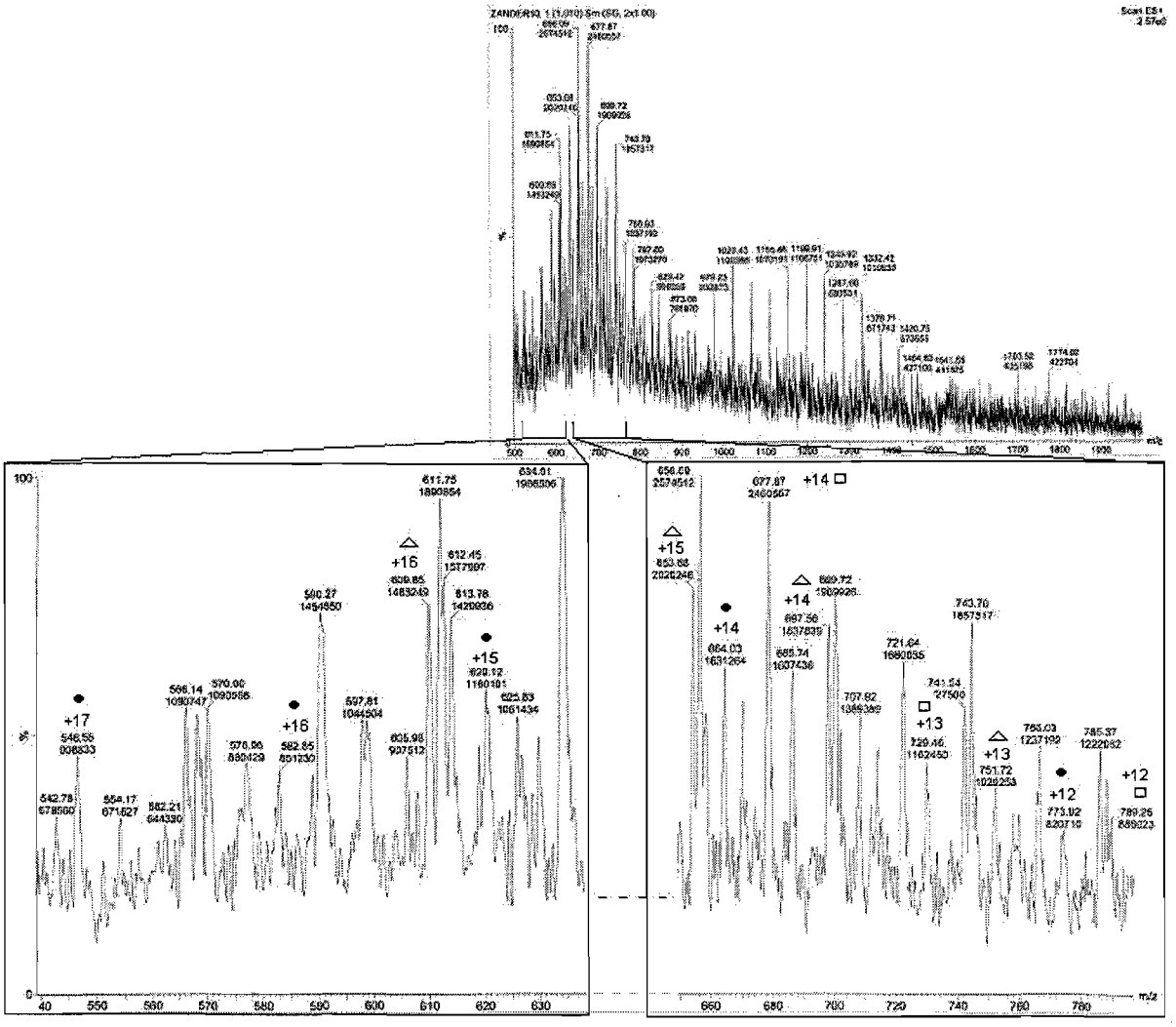

Figure 5.3: The convoluted spectrum of MT-2A$^{\Delta}$. The enlarged areas of the spectrum are displayed as well, to show target peaks among the noise. MT-2A$^{\Delta}$ in the native form is shown as closed circles while zinc saturated MT-2A$^{\Delta}$ (Zn_7-MT) are shown as open triangles. One contaminant protein is indicated by open squares.

Interpretation of the obtained spectrum (Figure 5.3) was very difficult because of the complexity of the mixture and poor signal-to-noise ratio. This complex spectrum may be ascribed to two factors: contaminants in the isolated mixture and various forms of MT-2A$^{\Delta}$. The spectrum obtained from the isolated MT-2A$^{\Delta}$ sample showed that it contained numerous protein contaminants as well as numerous other non-protein like

molecules (not specifically identified). Since the MT-2A$^{\Delta}$ band on SDS-PAGE gels were regularly seen at the 15 kDa region as displayed and discussed in Chapter 4 (Section 4.2.2.6.2), it was also necessary to confirm the mass of the expressed MT-2A$^{\Delta}$. This phenomenon is due to the reported reduced electrophoretic mobility of MT (Meloni et al., 2005:77) but size (mass) confirmation was required.

An *average* MT-2A$^{\Delta}$ mass of 9294.12 ± 18.7 Da was obtained in the convoluted spectrum (corresponding to the theoretical mass of 9303.66 Da) which confirmed the presence of MT-2A$^{\Delta}$ in the analyzed sample. Since both the peaks of oxidized and reduced MT-2A$^{\Delta}$, were used to determine the protein mass, it resulted in a slightly lower mass than the theoretical mass. Another factor that influences the observed mass is the isotopic variance between protein molecules. The larger the protein, the more variance is detected. Therefore *average* mass is used when referring to large biomolecules rather than *mono-isotopic* mass. Other proteins were also identified by Equations 5.1 and 5.2 (only one is shown Figure 5.3).

Not only did the isolated sample contain contaminant proteins but various metal bound species of MT-2A$^{\Delta}$ were also identified in the spectrum (zinc bound MT is shown as an example). This contributed to the complex spectrum and also overall low intensity of the MT-2A$^{\Delta}$ representing peaks. This result did not only mean that more purification was needed but also a procedure to ensure unity among metallothionein species. Therefore, it was required to optimize the whole isolation procedure to isolate MT-2A$^{\Delta}$ without contaminating proteins and obtain unity before analysis.

The "size" and "relative molecular mass" of a protein is generally accepted as the same, which is technically not absolutely correct. These two terms are, however, very similar especially when read in the correct context. Technically, the "size" of a protein refers to its shape (3D structure). However, when proteins are separated with SDS-PAGE only via their primary structure (and not charge or shape), the term "size" is also commonly used to refer to its "relative molecular mass". In this case the two terms are related. In this dissertation when referring to the "size" of the protein, it relates to "relative molecular mass" as it is used in context with SDS-PAGE.

5.2.2. Extraction of MT-containing fractions from biological material

5.2.2.1 Selection and optimization of various MT extraction methods.

As already mentioned (Section 3.1.2), metal chelating chromatography is not the most specific isolation procedure for recombinant proteins especially since other metal binding proteins (rich in cysteine or histidine residues) are also isolated with this technique. FKBP-type peptydyl prolyl isomerase (~25 kDa) found in *E.coli* is commonly isolated with metal chelating chromatography and almost constantly present in the isolated fraction (as can be seen in the SDS-PAGE results from the previous chapter) (Hengen, 1995:285). Reduction of the protein load on the metal chelating column would also result in less non-specific binding as well as less contamination of other metal binding proteins. A preliminary extraction step was needed to discard many interfering proteins before metal chelating chromatography.

There are four MT extraction techniques commonly used to extract the MT-containing fraction from the other proteins. These are:

- *SEC (Size exclusion chromatography):* To separate MT from most other proteins, Sephadex® G-75 is commonly used which separates the low molecular weight (LMW) proteins from the high molecular weight (HMW) proteins (Eriste et al., 2003:162; Erk et al., 2002:1212; Poleć et al., 2002:201; Temara et al., 1997:21; Wan et al., 1993:610). Sephadex G-50 was also used by Hunziker and Kagi (1985:376) and gave satisfactory results. However, SEC has poor resolution capacity in general and cannot effectively separate proteins of similar mass, such as the 25 kDa contaminating protein from the 9.3 kDa MT-2A$^{\Delta}$ protein.

- *Organic solvent extraction:* MT is known to be stable in 50 % (v/v) organic solvents while other interfering proteins precipitate (Beattie, 1998:261; Cartel, 1996:161; Erk et al., 2002:1213; Geret et al., 1998: 546; Richards & Beattie, 1995:29). After discarding the precipitated proteins, the resulting supernatant containing MT can be further utilised; but this supernatant can also be used to precipitate MT in 80 % (v/v) solvent followed by re-suspension.

Sephadex® is a registered trademark of GE Healthcare Ltd. (Buckinghamshire, UK)

- *Heat treatment:* Since metal-bound MT is heat resistant, heat treatment is more often used to clear most of the large interfering proteins. Temperatures ranging to 100 ℃ have been used to clear the MT fraction from many interfering proteins (Beattie, 1998:261).

- *Acid precipitation:* Since MT is known to be tolerant of acidic conditions including perchloric acid, it is logical to attempt to exploit this characteristic (Richards & Beattie, 1995:35). Metal-bound MT is stable in solutions with a pH of < 4 (Beattie, 1998:261; Cartel, 1996:161; Erk et al., 2002:1213; Geret et al., 1998: 546; Richards & Beattie, 1995:29). The acid environment results in the denaturing of other proteins (especially enzymes) and also limits oxidation as all sulphur groups are protonated (Rigby Duncan & Stilman, 2006:2103).

Although these methods are very different, it more or less extracts similar (type) proteins because of the fact that most heat-, pH- and organic solvent stable proteins are those bound to metals. Only SEC does not give similar proteins as it does not use their chemical characteristics but only their size/shape. Therefore, SEC can be used along with heat treatment, acid precipitation or organic solvent extraction to obtain cleaner MT fractions. Since these methods gave more or less the same protein-containing fraction, it was decided to only use one method: the method that would result in the loss of most interfering proteins by precipitation and least MT-2A$^{\Delta}$ co-precipitation. Erk et al. (2002:1213) tested and compared these techniques and concluded heat treatment to be the best way to extract MT from many interfering proteins. The conditions of heat treatment that gave the best results were: 85 – 70 ℃ for 15 min which also results in the breakage of disulfide bridges of potential MT dimers (Erk et al., 2002:1216). These conditions were initially used when preparing heat treated fractions for gel analysis as described in the previous chapter. Since bacterial cultures were used, with different protein content than eukaryotic material as well as a modified MT-2A, it was necessary to compare and optimize these extraction techniques.

5.2.2.1.1 Materials and Methodology

Each technique was first optimized to obtain the best conditions for that specific technique. Heat treatment at various temperatures and durations were compared,

acid precipitation with various pH's and acids, and the percentage and type of organic solvent were compared to obtain the best conditions for each method. The following temperatures were investigated to partially isolate MT from the other proteins: 60, 65, 70, 75, 80, 85, 90, 95 and 100 ℃. After the optimal temperature was obtained, the duration of the heat treatment step was also investigated. Heat treatment of samples for 1 to 15 min is common in the literature and thus the following durations were tested: 1, 5, 10, 15 and 30 min.

A *perchloric acid (PCA) extraction method from* Richards & Beattie (1995:30) was investigated. One tenth of 70 % (v/v) PCA was added to (9/10) total fraction. The 7 % (v/v) PCA (final concentration) would precipitate many proteins except MT. Another acid precipitation method was investigated: trichloroacetic acid (TCA) extraction method from Hussain et al. (1996:146). A final concentration of 2 % (m/v) TCA was used to precipitate interfering proteins in the total fraction. "pH precipitation" was also investigated by lowering the pH of the total fraction to precipitate interfering proteins. A few drops of concentrated HCl (11.7 M) were added to the total fraction in order to lower the pH.

Three organic solvents were experimentally tested by Erk et al. (2002:1212) to extract MT, namely ethanol (EtOH), acetone and acetonitrile (ACN). These organic solvents were each separately added to the total fraction to obtain 50 % (v/v) final concentration. The precipitate was discarded after centrifugation (10 000 *g*, 10 min, 4 ℃). A few micro-litres of the supernatant were kept for SDS-PAGE analysis. The same organic solvent was added to obtain a final concentration of 60 % (v/v). The precipitate was discarded and some of the supernatant were kept for analysis. This procedure was repeated until the organic solvent concentration was 80 % (v/v) (which is the concentration where MT should precipitate according to Erk et al. (2002:1213)).

SEC was, however, not optimized but merely tested as only Sephadex G-25 and G-100 were available at that time. The best results obtained from each method were compared with the other to acquire the best MT extraction method. Each MT extraction method was done with 1 ml total fraction taken from a single 30 ml culture, to eliminate the possibility that MT-2A$^{\Delta}$ was expressed in different amounts in different cultures. After each method, the extracted samples were desalted and

concentrated in order for the samples to contain similar final volumes (eliminating the diluting factor when loading the gels). Fifteen microlitres of each sample were used to analyse by SDS-PAGE, according to the methodology described in Section 4.2.2.4.1, regardless of the protein concentration. Since every extraction sample had similar starting MT-2A$^{\Delta}$ concentrations and each sample was concentrated to contain similar volumes, protein concentration was not equalized/ normalized before SDS-PAGE analysis. The gels were stained with the Silver Stain Plus kit.

5.2.2.1.2 Results and Discussion

Due to the great number of data (gels) generated, not all the results are presented in this chapter. The best extraction result each extraction method gave based on amount of contaminants discarded and MT-2A$^{\Delta}$ reserved, were analysed afterwards together and are shown in the following summarizing figure.

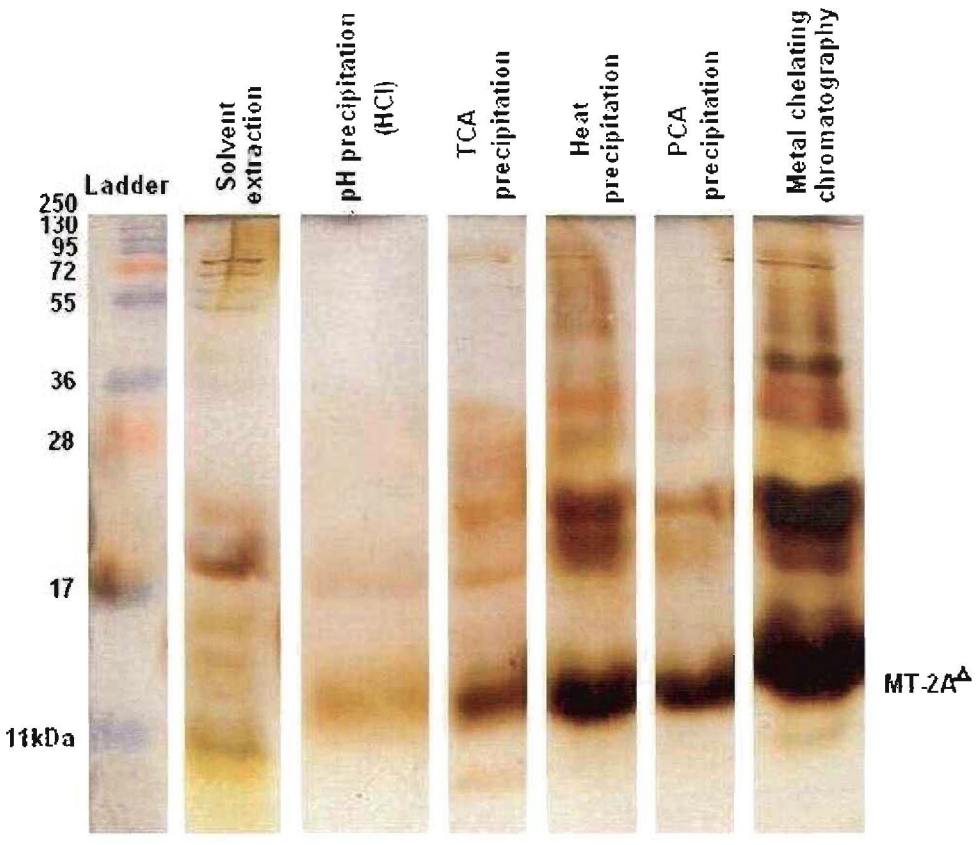

Figure 5.4: SDS-PAGE analysis of extracted MT-2A$^{\Delta}$ from biological material using heat, acid and solvent extraction methods. Commonly used organic solvent (acetone) extraction, heat- (80 ℃) and acid precipitation methods (TCA, PCA and HCl) were tested to extract MT from biological material before isolating it further with metal chelating chromatography. A 15 % (m/v) separating gel (stained with silver) was used.

Figure 5.4 compares the best results that resulted from each extraction method (starting with the same amount of MT2A$^{\Delta}$). The total fraction (starting material) was not used to compare the extraction results in Figure 5.4 and was thus not included. The problem to compare the extracted MT-2A$^{\Delta}$ with the total fraction is that in the total fraction, the MT-2A$^{\Delta}$ band would be unclear due to the many interfering proteins. To confirm the extraction method that worked the best, the results were compared them with the metal affinity isolated fraction, which contain all MT-2A$^{\Delta}$ with less interference.

Using visual comparison, the SDS-PAGE analysis indicated that heat treatment gave the least MT co-precipitation and consequently the best improvement of purity of MT-2A$^{\Delta}$. Although the other extraction methods resulted in less contamination, it did however, result in some co-precipitation of MT-2A$^{\Delta}$, reducing the yield. PCA precipitation seemed to be the second best option which resulted in little contamination and slightly lower MT-2A$^{\Delta}$ yield. The solvent (acetone) extraction and pH precipitation methods, however, resulted in the loss of relatively large amounts of MT-2A$^{\Delta}$. The other solvents tested (EtOH and ACN) gave similar losses of MT-2A$^{\Delta}$ and therefore only the acetone extraction are shown.

The heat treatment results that gave the best MT-2A$^{\Delta}$ yield and less contamination were obtained when the total fractions were incubated for 15 min at 80 ℃ (in a water bath) especially when large volumes of total fraction was heated (e.g. result in Figure 5.5). Hence, it was decided to use these conditions to extract MT-2A$^{\Delta}$ from the cytosolic proteins before isolating MT-2A$^{\Delta}$ further with metal chelating chromatography.

5.2.2.2 Extraction of MT-2A$^{\Delta}$ from biological material by means of heat treatment.

5.2.2.2.1 Materials and Methodology

As described in Chapter 4, following harvesting of cultured bacterial cells, the cells were broken by means of sonication and then centrifuged (6 000 *g*, 10 min, 4 ℃) to discard cell debris. One requirement to use heat treatment is that all MT-2A$^{\Delta}$ needed to be saturated with metals to make it heat resistant. The addition of CdCl2 when

inducing expression insured the saturation of all expressed MT-2A$^{\Delta}$. The resulting supernatant (total fraction) was then used to extract the MT-fraction from various cytosolic proteins. The total fraction was "heat treated" by incubating it at 80 ℃ for 15 min in a water bath. The precipitated proteins were then separated from the rest (heat fraction) by centrifugation at 10 000 *g* for 10 min at 4 ℃ and discarded. The resulting heat fraction contained all the heat resistant proteins including MT-2A$^{\Delta}$. These heat fractions were further used to isolate MT-2A$^{\Delta}$.

5.2.2.2.2 Results and Discussion

The heat treatment conditions used to extract MT-2A$^{\Delta}$ from as many interfering proteins as possible gave results similar to that shown in Figure 5.5.

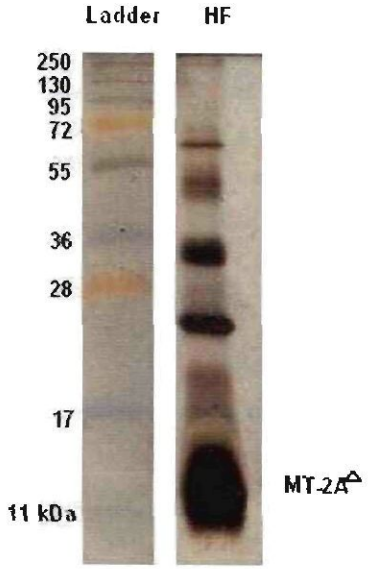

Figure 5.5: SDS-PAGE analysis illustrating the heat fraction obtained with optimized conditions. This heat fraction was used for metal chelating chromatography. A 15 % (m/v) separating gel (stained with silver) was used.

The heat fraction obtained after heat treatment of the total fractions resulted in the isolation of heat resistant proteins as shown in Figure 5.5. These heat resistant proteins are normally the proteins that are able to bind metals. Among the heat resistant proteins is FKBP-type peptydyl prolyl isomerase (~25 kDa) and MT-2A$^{\Delta}$ (clearly visible in Figure 5.5). Therefore, heat treatment was used successfully to lessen the protein load before isolating MT-2A$^{\Delta}$ via metal chelating chromatography.

5.2.3 Isolation of MT-2A$^{\Delta}$ by means of metal chelating chromatography

Metal chelating (affinity) chromatography is based on the selective binding of proteins containing certain residues (such as histidine, cysteine or tryptophan) to immobilised metal ions. Non-bound proteins are then separated from bound proteins which are later released from the metal ions in order to collect it. The His-tag consisting of 6, 8 or 10 histidine residues in tandem has a high affinity for metal binding (especially nickel, Ni^{2+}). Fusion of this tag to recombinant proteins means that the protein can be isolated from other interfering proteins by means of metal affinity chromatography (Champbell, 1997:1; Wilson & Walker, 2003:180).

5.2.3.1 Optimization of metal chelating chromatography to minimize contaminants in isolated MT-2A$^{\Delta}$ fraction.

5.2.3.1.1 Materials and Methodology

All metal affinity isolation procedures described in the previous chapter was done according to the manufacturer's specifications. A HiTrap™ chelating HP column, loaded with His-Bind® Resin, was used to perform metal chelating chromatography. The isolated fractions obtained with this method (without the additional extraction method) were contaminated, as was seen in the MS spectrum (Figure 5.3) and SDS-PAGE gel from Section 5.2.2.1.2 (Figure 5.4, last lane). The protein load on the column was reduced by performing an extraction (heat treatment) step prior to metal chelating chromatography. However there was still contamination visible (results not shown). Therefore, it was required to investigate various metal binding conditions in order to optimize the isolation procedure. In addition to the fused His-tag, MT-2A$^{\Delta}$ also contains a high cysteine content which was exploited to isolate it more specifically. The high cysteine content meant that MT-2A$^{\Delta}$ was theoretically able to bind more firmly to the immobilized metal ions loaded onto the column (Honda et al., 2005:206) which would consequently require more extreme conditions to release it.

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His-Bind® is a registered trademark of EMD Biosciences, Inc. (San Diego, CA, USA)

Factors such as an additional wash step, increased imidazole concentration in the wash buffer and pH elution were investigated to reduce the contaminants when isolating MT-2A$^{\Delta}$. Normally, after the total fraction was applied to the column, it was washed with six times the column volume (or twice with three times the resin bed volume when using the batch purification method) of wash buffer to discard non-bound and slightly bound proteins. An additional wash step (nine times the column volume similar to trice with three times the resin bed volume) was tested to confirm the removal of all the non-bound and slightly bound proteins. However, the isolated fractions still contained contaminant proteins (Figure 5.6).

Since MT-2A$^{\Delta}$ contains additional metal binding (cysteine) residues other than the His-tag, it was thought to bind more firmly to the metal ions on the column. Elution conditions could therefore, be gradually increased to release the less firmly bound proteins before eluting MT-2A$^{\Delta}$. The pH was gradually lowered until it reached ~2. The fractions were collected and analysed to confirm the pH at which MT-2A$^{\Delta}$ were released as well as the purity of the eluted MT-2A$^{\Delta}$ fraction (see Figure 5.7). This approach, however, resulted in poor purification because no MT-2A$^{\Delta}$ was visible in any of the collected samples as visualised with SDS-PAGE (CBB stained). The same principal was applied by gradually increasing the imidazole concentration until MT-2A$^{\Delta}$ was released from the column. The samples were desalted and analysed with SDS-PAGE. The results obtained are shown in Figure 5.8.

According to the manufacturer, 1 ml nickel loaded His-Bind resin can bind approximately 8 mg His-tagged recombinant protein. If 5 mg MT-2A$^{\Delta}$ can be expressed in one litre culture in ideal conditions, it is possible to isolate all MT-2A$^{\Delta}$ (one litre culture's) in a single run (using the 1 ml HiTrap chelating HP column). Unfortunately, the one millilitre column used could not be coupled to a HPLC system but was operated with a compatible 10 ml syringe. This prolonged the procedure especially as the lysate of one litre culture (100 ml) had to be applied in ten fractions. Since large volumes culture were repeatedly used to express sufficient MT-2A$^{\Delta}$, it would be unpractical to use this approach. No other larger capacity columns were available at that time, so an alternative approach was tested. The batch purification method was compared to the column purification method (both according to the manufacturer's specifications but with adapted volumes). The batch method requires

a tube filled with a certain volume His-bind resin and centrifugation forces (rather than pressure) to separate the resin from the liquid after each step.

Two 50 ml BD Falcon™ tubes were filled with His-bind Resin to attain a bed volume of 1 ml. The resin was washed once with 4 ml water and then loaded three times with 2 ml nickelsulfate (50 mM). The resin was inverted several times to mix and then collected by means of centrifugation (1 min at 1 000 *g*). The liquid separated from the resin was then discarded or collected (as necessary). After the resin was loaded with nickel, it was washed with 4 ml binding buffer (0.5 M NaCl, 5 mM imidazole, 20 mM Tris-HCl, pH 7.9). Fifty millilitres of the heat-treated fraction was mixed with the resin and incubated for 15 min whilst mixing. The "flow through" (containing the non-bound proteins) were separated from the resin. The resin was washed three times with 3 ml binding buffer and twice with 3 ml wash buffer (0.5 M NaCl, 60 mM imidazole, 20 mM Tris-HCl, pH 7.9). The protein was eluted twice with 3 ml elution buffer (0.5 M NaCl, 1.0 M imidazole, 20 mM Tris-HCl, pH 7.9). The resin was stored at 4 ℃ with the nickel attached when it was to be used soon afterwards. When it was stored for long periods without use, the nickel was striped from the resin with 3 ml strip buffer (0.5 M NaCl, 100 mM EDTA, 20 mM Tris-HCl, pH 7.9) and kept in this solution at 4 ℃.

Except for the considerations of the technique as described before there are also other problems associated with purification of MT specifically. One major factor is oxidation of MTs which results in the formation of disulfide bridges and thus polymerization (Dabrio et al., 2002:130; Minkel et al., 1980:476). Care had to be taken to avoid oxidation and polymerization. Dithiothreitol (DTT) was therefore added to all the isolated fractions to a final concentration of 50 mM.

BD Falcon™ is a trademark of BD Biosciences (Franklin Lakes, NJ, USA)

5.2.3.1.2 RESULTS AND DISCUSSION

The results from the additional wash step (Figure 5.6), pH elution (Figure 5.7) and imidazole elution (Figure 5.8) are shown next.

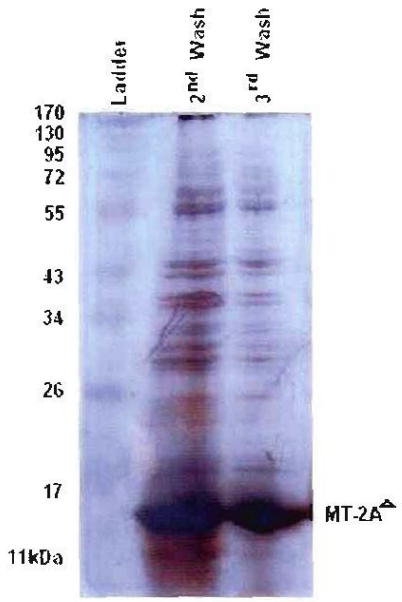

Figure 5.6: SDS-PAGE analysis to evaluate the addition of an extra wash step during elution of MT-2A$^{\Delta}$. This gel represents the eluted MT-2A$^{\Delta}$ fractions before and after the additional wash step. A 15 % (m/v) separating gel (stained with silver) was used.

Figure 5.6 represents the collected eluted MT-2A$^{\Delta}$ fractions before and after the additional wash step. The use of silver staining and high protein load (per lane) of the collected samples shows the grade of contamination that occurred with normal metal chelating procedures as visualised in Figure 5.6. The extra wash step did remove more unbound or slightly bound proteins as was expected, but the elute was still not pure enough to use as standard. A fourth wash step did not remove more contaminants and was therefore not used. The following figure illustrates the next step that was investigated to purify MT-2A$^{\Delta}$.

Figure 5.7 shows the elution of MT-2A$^{\Delta}$ from the column with decreasing pH. No visible MT-2A$^{\Delta}$ can be seen in the lanes which meant that it probably eluted from the column even when the pH were above 5 or eluted gradually (regardless of the pH) as seems to have happened to other proteins (FKBP-type peptydyl prolyl isomerase). If MT-2A$^{\Delta}$ eluted (gradually) it would not be visible with CBB staining as CBB can only stain a large amount of this protein. The theory that MT-2A$^{\Delta}$ should

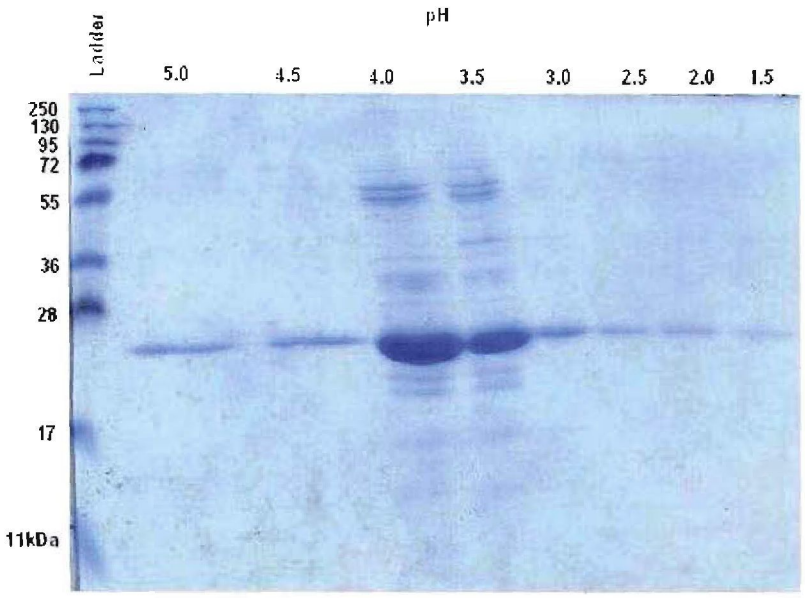

Figure 5.7: SDS-PAGE analysis illustrating the use of decreasing pH to elute MT-2A$^{\Delta}$ from the column. A 15 % (m/v) separating gel (stained with CBB) was used to visualise the collected elution fractions.

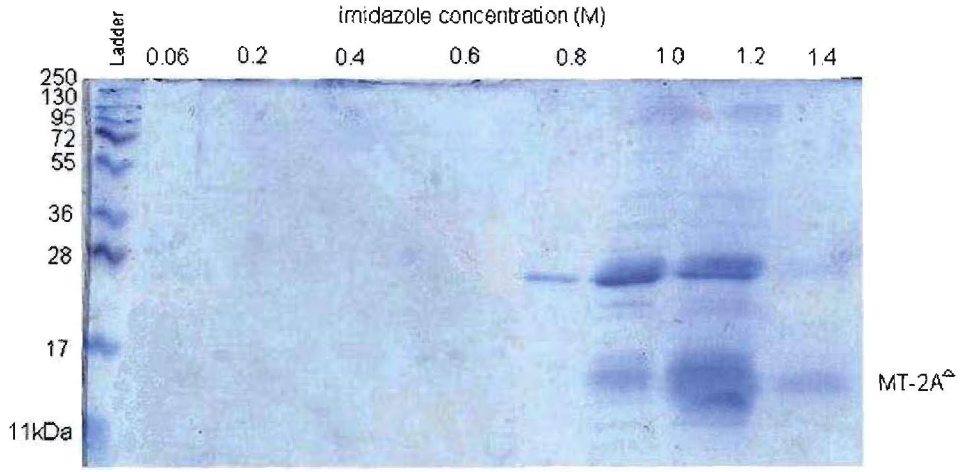

Figure 5.8: SDS-PAGE analysis illustrating the elution of MT-2A$^{\Delta}$ with increased imidazole concentration. A 15 % (m/v) separating gel (stained with CBB) was used to visualise the collected elution fractions.

bind tighter to the resin was, however, still investigated by using increasing imidazole concentration in the washing buffer.

Figure 5.8 shows the SDS-PAGE analysis of the elution of MT-2A$^{\Delta}$ with gradually increased imidazole concentrations as visualised with CBB. Although most MT-2A$^{\Delta}$ remained bound when the imidazole concentration reached 1.0 M, it seemed that some was released from the column since a ~15 kDa band was increasingly visible on the SDS-PAGE gel. The imidazole concentration that gave the least contaminant species was 1.2 M, compared with the suggested optimal elution concentration of 1.0 M given by the manufacturer. Therefore, it was decided to increase the imidazole concentration in the wash buffer from 60 mM to 1.0 M to isolate MT-2A$^{\Delta}$ more purely. The increased imidazole concentration in the wash buffer would elute (and therefore wash away) many interfering proteins before eluting MT-2A$^{\Delta}$. Some MT-2A$^{\Delta}$ were lost with this approach, but more pure sample was obtained. This approach was especially incorporated when the batch purification method was used.

5.2.3.2 Removal of imidazole from isolated samples via dialysis

5.2.3.2.1 Materials and Methodology

The high imidazole concentration in the collected samples interfered with protein determination and SDS-PAGE analysis. The isolated fractions also crystallized when it was attempted to concentrate it. The crystals most probably were the imidazole which therefore had to be removed from every isolated fraction. Two types of desalting techniques were tested: SEC and dialysis. A 5 ml column loaded with Sephadex G-25 (medium) from Pharmacia was tested with various solutions, ranging from water, 10 mM ammonium acetate, 5 mM sodium chloride or 0.1 mM Tris-HCl, pH 7.0. Although the procedure was successful, it did not desalt enough isolated fraction per run and a lot of protein was lost because of the protein - imidazole peak overlap (results not shown).

The alternative was to use dialysis, which is capable of removing almost all the imidazole from large volumes samples (isolated fractions) with minimum loss of protein. SnakeSkin™ pleated dialysis tubing from Pierce (Rockford, IL, USA) with a molecular cut-off of 3.5 kDa was used for dialysis. An advantage of snakeskin dialysis tubing is that the optimal length tubing can be used to desalt large sample volumes.

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Tubing was filled with the isolated protein solution which was then put in (Milli-Q) water whilst stirring overnight (according to the manufacturer's specifications). The water was replaced three times with fresh water. This ensured that most of the salts, interfering with SDS-PAGE and protein determination, would be discarded. However, not all salts were removed as was seen in the chromatograms in the following section but the lower concentrations did not interfere with analysis or vacuum concentration. Each isolated MT-2A$^{\Delta}$ fraction was therefore desalted in this way and then concentrated (via the Savant SpeedVac® SVC 100 concentrator) before further steps were taken.

5.2.4 Purification of MT-2A$^{\Delta}$ with reverse phase HPLC

Even after metal chelating chromatography optimization and the addition of a MT extraction procedure, the optimal purity (95 %) was not reached. Not only was the MT-2A$^{\Delta}$ sample contaminated with other proteins, but the recombinant MT was probably bound to various metals or perhaps oxidized. The protein needed to be uniform before it could be used as internal standard especially since all these factors (metals bound or oxidized) would result in different masses. As a result, a third purification step was required to purify MT-2A$^{\Delta}$ even more and to obtain a uniform protein fraction.

It is known from the literature that reverse phase HPLC and capillary electrophoresis (CE) are the best separation techniques for MT isoform separation (Beattie, 1998:266; Chassaigne & Łobiński, 1999:111). It was decided to use reverse phase HPLC (which was available at that time) to purify MT-2A$^{\Delta}$. Since MT is water soluble, its elution is achieved by decreasing the polarity of the mobile phase (Chassaigne & Łobiński, 1999:111; Prange & Schaumlöffel, 2002:451). The low pH mobile phases normally used would also aid in the removal of metals from MT-2A$^{\Delta}$. This is especially true for mobile phases containing 0.1 % (v/v) TFA (Alhama et al., 2006:53; Hong et al., 2001:247; Sanz-Nebot et al., 2003:383)

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5.2.4.1 Confirming the ability of available columns to separate MT-2A$^{\Delta}$ from interfering proteins

5.2.4.1.1 Materials and Methodology

The literature was consulted to investigate which columns and mobile phases were commonly used when separating MT isoforms. McSheehy & Mester (2003:313) as well as Prange & Schaumlöffel (2002:450) summarized the columns and mobile phases used to separate MT isoforms and other proteins in table format. The columns that are used the most are the C_{18} and C_8 columns also confirmed by Chassaigne & Łobiński (1999:111). A C_{18} (Phenomenex Luna® 5u, 250 x 2.0 mm, 5 µm particle size) and C_8 column (Waters Nova-Pak®, 3.9 x 150 mm, 4 µm particle size) were available at our institution. Łobiński et al. (1998:276) also summarized commonly used columns with attention to particle size. The columns used by other researchers varied in particle size. Although MT was successfully separated in columns containing a particle size as low as ~4 µm, it was assumed that a larger particle size (5 – 10 µm) would be more ideal.

The available columns fortunately had the same size particles as some columns used in the literature. It was therefore thought that the available columns could separate MT-2A$^{\Delta}$ successful from the other, mostly larger sized, proteins. Mobile phases containing TFA (trifluoroacetic acid) were more frequently used than mobile phases containing acetic acid or ammonium acetate. A 0.1 % (v/v) TFA mobile phase (Mobile phase A) and 0.1 % TFA (v/v) and 60 % ACN mobile phase (Mobile phase B), seemed to be the better choice as it was mentioned by a few researchers to dissociate metals bound to MT (Alhama et al., 2006:53; Hong et al., 2001:247; Sanz-Nebot et al., 2003:383).

The available C_{18} and C_8 columns were attached to a Dionex® HPLC system to test and compare its protein separation abilities. The Dionex HPLC system consisted of a Dionex model P580A HPG pump equipped with an online degasser (Model DG-2410), an automated ASI-100® sample injector and a Dionex UV/Vis detector (UVD

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Nova-pak® is a registered trademark of Waters Corporation (Milford, MA, USA)

Dionex® and ASI-100® are registered trademarks of Dionex Corporation (Sunnyvale, CA, USA)

170S/340S). Since MT (as well as MT-2A$^{\Delta}$) does not contain any aromatic residues it cannot be detected at 280 nm, which is normally used to detect proteins. Despite this fact a few reports describes detection of MT by means of spectrophotometry. The wavelengths and absorbing factor measured are summarized in Table 5.1.

Table 5.1: Wavelengths used to detect MT as described in literature.

Wavelength (nm)	Absorbing factor	Reference
200, 214 or 220	Peptide bonds are easily detected by the lower UV wavelengths (200 nm). Unfortunately, various other compounds absorb at 200 nm which means that non-specific detection occur. Therefore, many researchers use 214 – 220 nm which lessen non-specific detection.	Dallinger et al., 2001:4127; Hong et al., 2001:245; Richards & Beattie, 1995:29; Simpkins et al., 1998:17; Wan et al., 1993:610
343	Determination of thiols after incubation with 2,2'-dithiodipyridine	Hong et al., 2001:245
235	Detect Cu-MT which is not stable when DTT or 2-ME is added	Sayers et al., 1999:859
250	Cd-S bonds	Ding et al., 2006:676

Cu-MT = copper bound metallothionein; 2-ME = 2-mercaptoethanol; Cd-S = cadmium-sulphur bonds

The following is a quote from Rigby Duncan & Stillman (2006:2103): "Spectroscopic analysis of apo-MT shows no absorption above 210 nm by UV absorption spectroscopy since the protein is devoid of aromatic amino acids". Since the detector used by our apparatus is able to detect MT using multiple UV wavelengths, it was decided to use 214 and 280 nm simultaneously when detecting MT elution. The use of both wavelengths simultaneous meant that MT would be represented by only one peak (214 nm), lacking detection at 280 nm. The contaminant proteins would (probably) be detected with both wavelengths and would thus be presented by two peaks (as detected by 214 and 280 nm respectively).

Cytochrome c (molecular mass of ~13 kDa) and rabbit MT-2A was used to predict the relative retention time of MT-2A$^{\Delta}$ in order to recover it after elution. These proteins are also water soluble and small in size. The absence of an automated fraction collector meant that all MT-2A$^{\Delta}$ recoveries had to be done by hand and was the exact time of elution critical to avoid collecting other contaminating proteins (represented by

other peaks near the MT-2A$^{\Delta}$ peak on the chromatogram, Figure 5.9). When MT-2A$^{\Delta}$ passes through the photocell of the detector and a resulting peak appears on the chromatogram, it still has to travel a distance until it elutes from the tube exiting the UV detector. This precise volume (dead volume) also had to be measured in order to collect the eluted MT-2A$^{\Delta}$ without contaminants. One milligram per millilitre cytochrome c and rabbit MT-2A was injected together and eluted with a 0 - 100 % gradient over ~20 min (with 1 ml/min flow rate). The following mobile phases were also used: Mobile phase A, 0.1 % (v/v) trifluoroacetic acid (TFA) in water; Mobile phase B, 0.1 % (v/v) TFA and 60 % acetonitrile (ACN). The C_8 column separated the two proteins more effectively than the C_{18} columns therefore it was decided to use the C_8 column in further optimization and purification.

After the position of elution of both proteins was identified and successfully recovered (from the C_8 column), a relative small amount of isolated MT-2A$^{\Delta}$ was injected to confirm the exact position of the MT-2A$^{\Delta}$ peak (elution time) in order to collect it afterwards. The MT-2A$^{\Delta}$ sample did not give a tall, sharp peak as was expected and eluted as one of numerous "small" peaks (Figure 5.9). Detection of MT-2A$^{\Delta}$ with the selected wavelengths was also questioned. Due to the absence of a prominent peak it was decided to collect elution fractions every minute after injection of sample and analyse it with SDS-PAGE. This would also indicate whether MT-2A$^{\Delta}$ elutes gradually over time and appear merely as background or elutes with the salts. The collected fractions were desalted as described in Section 5.2.3.2.1 and further prepared according to the same methodology as described in Chapter 4, Sections 4.2.2.3 to 4.2.2.4. The chromatogram and SDS-PAGE results are shown in Figure 5.9 and Figure 5.10, respectively.

5.2.4.1.2 Results and Discussion

Figure 5.9 shows the chromatogram after metal chelating isolated MT-2A$^{\Delta}$ were analysed with reverse phase chromatography. Water was first analysed to obtain a base line for reference. Afterwards the metal chelating isolated fraction was injected. The gradient used is indicated by the dotted line. The gradient (% Mobile phase B) was kept at 0 % for three minutes after the sample was injected to elute salts (represented by the tall peak in the beginning of the chromatogram). After three minutes, the gradient increased linearly to 100 % (Mobile phase B) for 20 min and

remained at 100 % Mobile phase B for 2 – 3 min to ensure the removal and elution of all bound compounds from the column. The column was then washed for 10 min with Mobile phase A to obtain a base line before the next run.

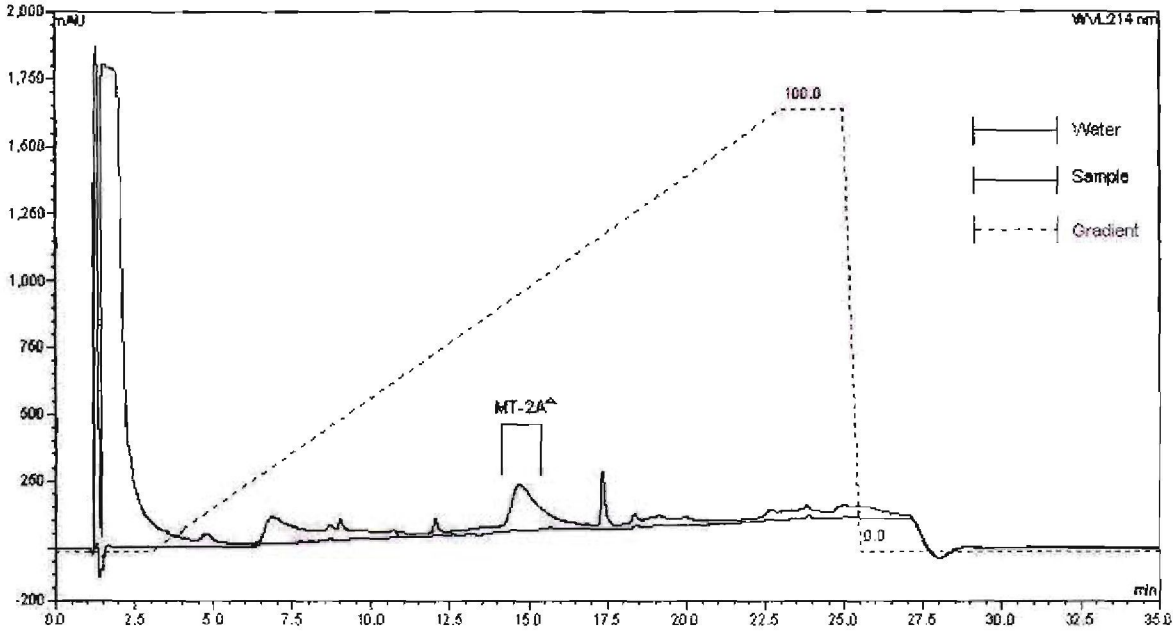

Figure 5.9: Overlaid chromatogram of the isolated fraction (after metal chelating chromatography) and water (blank). After sample injection, all elute were captured in 1 ml fractions to confirm the elution position and thus retention time of MT-2A$^{\Delta}$.

The MT-2A$^{\Delta}$ sample (unlike the cytochrome c and rabbit MT-2A mixture) did not give a tall, clear peak as was expected and eluted as one of numerous "small" peaks. Since cytochrome c and rabbit MT-2A eluted at 18 and 12 min respectively, it was assume that MT-2A$^{\Delta}$ would also elute during this time (12 – 18 min). However, since only a series of small peaks was visible, confirmation was needed.

The samples collected each minute after sample injection was analysed with SDS-PAGE (Figure 5.10) to confirm the elution time (and peak) of MT-2A$^{\Delta}$. From the SDS-PAGE results, it is clear that MT-2A$^{\Delta}$ eluted after 14 min which correspond to a relative large elution peak (14-15 min peak) as indicated in the chromatogram (Figure 5.9). This meant that MT-2A$^{\Delta}$ eluted from the column at about 50 % Mobile phase B. When investigating the chromatogram, it is clear that the MT-2A$^{\Delta}$ peak is broadened (severe tailing) and it appeared that MT-2A$^{\Delta}$ eluted gradually over about 3 minutes. The MT-2A$^{\Delta}$ peak tailing could be a co-eluting (overlapped) protein not fully

separated from MT-2A$^{\Delta}$. This assumption was confirmed when the chromatograms obtained with 214 and 280 nm detection were compared. The beginning of the peak lack absorbance at 280 nm while the latter part (tail) absorbs at 280 nm. However, no other (clear) protein band is visible in the MT-2A$^{\Delta}$ lanes which meant that the concentration of the possible co-eluting protein was too small to visualise on the gel.

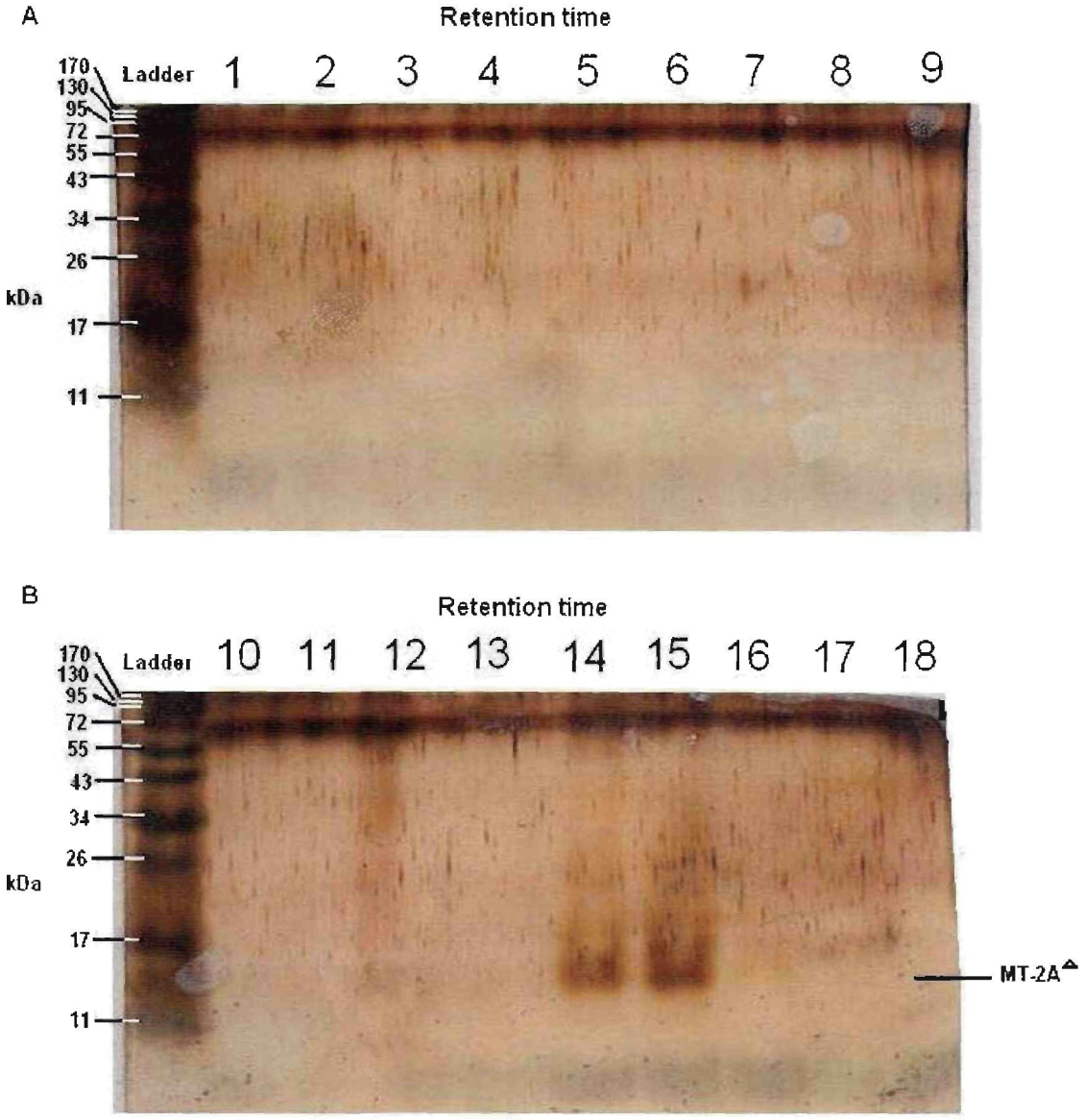

Figure 5.10: SDS-PAGE analyses of the reverse phase HPLC fractions collected each minute after sample injection. Fifteen percent (m/v) separating gels were used. The gels were stained with silver for optimal sensitivity.

5.2.4.2 Optimization of reverse phase HPLC to purify MT-2A$^{\Delta}$.

5.2.4.2.1 Materials and Methodology

After the MT-2A$^{\Delta}$ representing peak (time of elution) was identified, it was necessary to further optimize the chromatographic conditions. Figure 5.9 shows the broadened MT-2A$^{\Delta}$ peak which meant that it was released (eluted) gradually and that the fraction probably contained other proteins/ compounds. Therefore, it was aimed to concentrate the peak (fraction) more to obtain a sharper MT-2A$^{\Delta}$ peak. Factors that were investigated were column temperature, mobile phases and gradient. The warmer the temperature of the column the better compounds elute from it. However, no difference was detected in the manner of elution of MT-2A$^{\Delta}$ in any of the tested temperatures within the column's working window (data not shown). Only acidic mobile phases were tested since it was aimed to dissociate all metals from the MT-2A$^{\Delta}$ to obtain uniformity. The mobile phases initially used (containing 0.1 % TFA) gave the best results compared to ammonium acetate and acetic acid containing mobile phases (Appendix F). Therefore, the gradient was the only other factor considered to improve the chromatographic separating conditions to obtain a sharper, concentrated MT-2A$^{\Delta}$ elution profile.

The mobile phase gradient is normally flattened (referring to the gradient as seen on the chromatogram) when better resolution (separation of peaks near each other) is required. This also meant that where two different proteins gave one overlapping peak, they could be separated with a flatter gradient. The disadvantage of a flatter gradient is that the run time increases as well. The previous results (Figure 5.9) showed that MT-2A$^{\Delta}$ eluted at approximately 50 % Mobile phase B, thus the gradient was flattened (over 10 min) between 45 and 50 %. Between minutes 2 to 5 the gradient increased from 0 to 45 % allowing salts and other proteins not binding strongly to the column to be eluted. From minutes 5 to 15 the gradient only increased to 50 % releasing MT-2A$^{\Delta}$ expectedly separate from other proteins. After the 15th min the gradient was rapidly increased to 100 % within 3 min to ensure the elution of all retained compounds before the next run.

5.2.4.2.2 RESULTS AND DISCUSSION

As mentioned, the use of different temperatures (results not shown) and mobile phases did not improve the separation. In most cases, the other mobile phases resulted in worse separation and multiple MT peaks as a result of metals that were probably still bound to it (Appendix F). The mobile phases initially used (A: 0.1 % TFA; B: 0.1 % TFA, 60 % ACN) gave the best, reproducible results. The gradient was flattened to separate MT-2A$^{\Delta}$ from other co-eluting proteins and compounds. The following chromatogram (Figure 5.11) shows the final gradient used to elute MT-2A$^{\Delta}$ from the column.

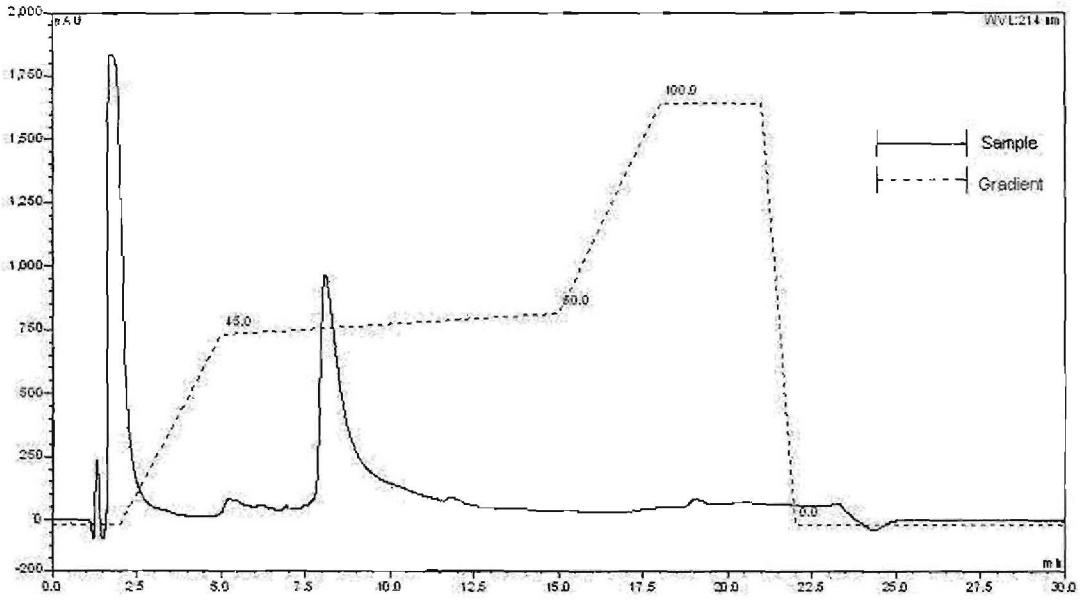

Figure 5.11: Chromatogram to illustrate optimized separating conditions. The gradient was flattened to increase the resolution of the peaks on the chromatogram and consequently better separation of MT-2AΔ from surrounding proteins (in the chromatogram).

It was observed from the results obtained (Figure 5.11) that the MT-2A$^{\Delta}$ elution peak still remained broadened and tailing despite the flattened gradient. The lack of many other protein peaks (such as in Figure 5.9) is due to the sudden increase of the gradient before and after MT-2A$^{\Delta}$ elution which resulted that many proteins possibly eluted together and appeared as overlapped peaks. Except for this and the gradient difference, the only other difference between the chromatograms in Figures 5.9 and 5.11 is the amount of MT-2A$^{\Delta}$ injected. The latter chromatogram clearly represents more MT-2A$^{\Delta}$ which was concentrated before HPLC purification. This concentration also resulted in the precipitation of many of the other isolated proteins and was not re-

suspended in water before injection. This resulted that MT-2A$^{\Delta}$ appears as the dominant peak (Figure 5.11). This additional step is described in the following section.

5.2.4.3 Purification of MT-2A$^{\Delta}$ with optimized reverse phase HPLC

5.2.4.3.1 Materials and Methodology

Desalted samples were concentrated with the Savant SpeedVac SVC 100 concentrator before being subjected to reverse phase chromatography. This was done to purify as much protein as possible in the least amount of runs and to precipitate more of the other interfering proteins. Only 100 µl sample could be injected per run which took approximately 30 min to complete. With the conditions (gradient) used, MT-2A$^{\Delta}$ eluted after approximately 8 min as confirmed with SDS-PAGE (gel not shown). The region of the peak (the apex) that was collected is indicated in Figure 5.12. The programmed functions included a column cleanup step (100 % Mobile phase B for 3 min) and a step to attain a baseline (10 min running only Mobile phase A). This whole procedure was repeated several times until a sufficiently "pure" MT-2A$^{\Delta}$ stock (of 2 mg) was obtained. Collected samples were pooled and stored at -80 ℃. The purity of the pooled samples was confirmed with SDS-PAGE analysis (not shown) and mass spectrometry. The same procedures was used as described previously which included desalting as TFA is known to interfere with SDS-PAGE analysis and especially mass spectrometry (ionization) (Apffel et al., 1995: 181). The Agilent (Santa Clara, CA, USA) 6210 Time-Of-Flight LC/MS was used to confirm purity essentially as described in Section 5.2.1.1.1

5.2.4.3. Results and Discussion

The area of the peak that was collected is indicated in Figure 5.12. The tail of the peak was not collected as it contained possible interfering proteins. After MT-2A$^{\Delta}$ was repeatedly HPLC purified, it was analysed with SDS-PAGE and mass spectrometry to confirm purity. The SDS-PAGE results are not shown as virtually the same results were obtained as shown in Figure 5.10. Only a MT-2A$^{\Delta}$ band was visible. The absence of other protein bands did, however, not mean the total absence of contaminant proteins, but rather indicated that the concentration of any possible contaminant proteins was low.

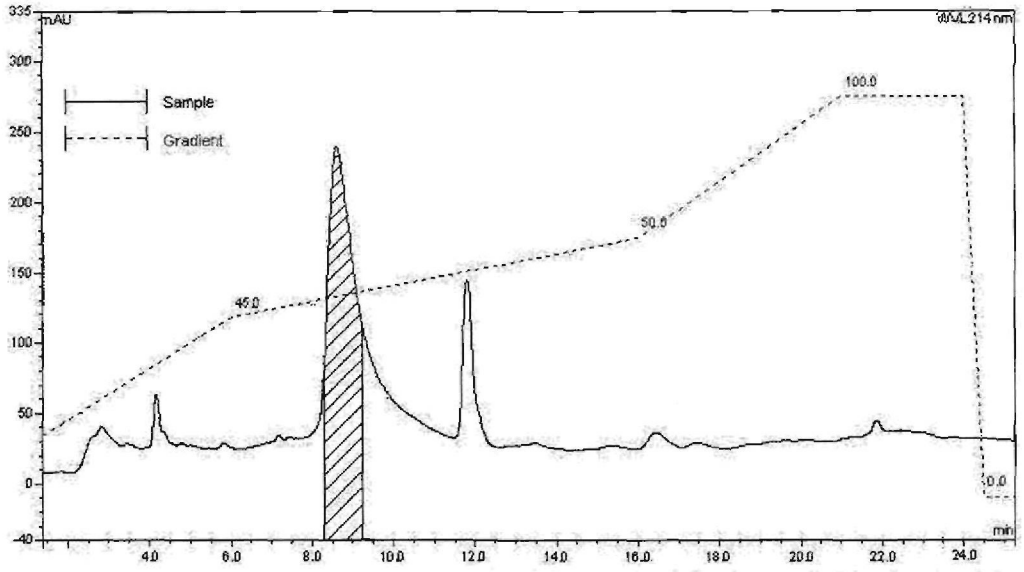

Figure 5.12: Chromatogram illustrating the fraction of the MT-2A$^{\Delta}$ peak collected. The tail area was not recovered as it was assumed to be other proteins.

The deconvoluted spectrum of the purified sample is shown in Figure 5.13. MT-2A$^{\Delta}$ is represented by the dominant peak (9.3 kDa) which corresponds to its theoretical *mono-isotopic* mass (Figure 5.14). After the additional HPLC purification, the MT-2A$^{\Delta}$ standard is therefore much purer compared to the sample after metal chelating chromatography. This sample, however, still contained some contaminating proteins as can be seen by the small peaks on the spectrum (Figure 5.13). This result also confirms the SDS-PAGE results that MT-2A$^{\Delta}$ is by far the more abundant protein in the sample. After HPLC purification the MT-2A$^{\Delta}$ sample had a relative purity of 70 % (as determined using the deconvoluted mass spectrum). Since the purity was obtained, all other isolated samples were purified with reverse phase HPLC, pooled and kept at -80 ℃.

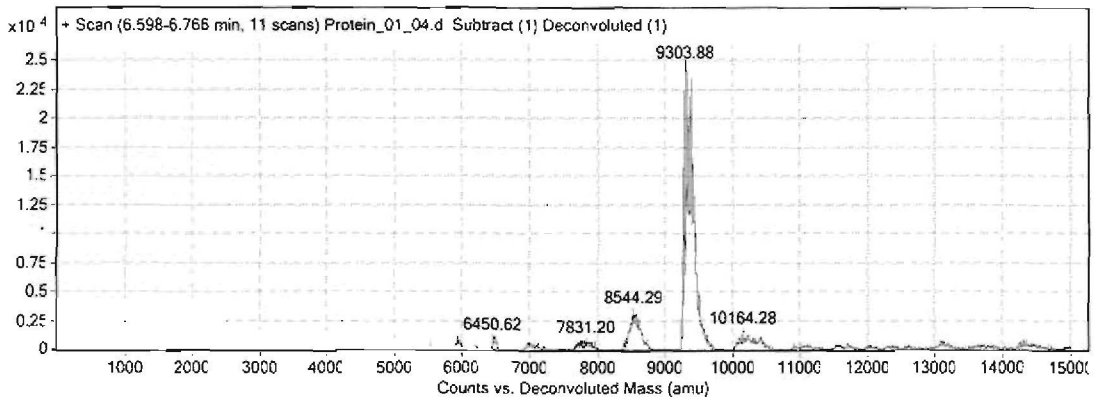

Figure 5.13: Deconvoluted mass spectrum of MT-2A$^{\Delta}$ after additional purification.

5.2.5 Removal of the His-tag from recombinant MT-2A

Although the purity (~70 %) of the purified MT-2A$^{\Delta}$ was still not sufficient (>95 %) to use as standard, it was decided to proceed with the following steps of the formulated strategy to reach the aim of this study. As described before, the aim of this study was to construct a recombinant MT-2A slightly modified to have a different mass than other natural MT isoforms (human MT-2A), but similar ESI-MS ionisation properties. The removal of the His-tag and some additional amino acids would result in a 7.42 kDa modified human MT-2A (MT-2A*) containing 15 N-terminal added amino acids in the place of the N-terminal methionine. The following figure illustrates the fused recombinant protein and end product expected after thrombin cleavage.

#	Primary sequence, Thrombin cleavage site, histidine tag	Calculated mono-isotopic masses
	Thrombin	
1	MGSSHHHHHH SSGLVPRGSH MASMTGGQQM GRDPNCSCAA	
41	GDSCTCAGSC KCKECKCTSC KKSCCSCCPV GCAKCAQGCI	9303 Da
81	CKGASDKCSC CA	
1	GSHMASMTGG QQMGRDPNCS CAAGDSCTCA GSCKCKECKC	7422 Da
41	TSCKKSCCSC CPVGCAKCAQ GCICKGASDK CSCCA	

Figure 5.14: The sequence of MT-2A$^{\Delta}$ with and without the His-tag. The His-tag and thrombin cleavage site is indicated in the figure. The fused protein consists of 92 amino acids while the cleaved protein consists of 75 amino acids. The human MT-2A sequence in indicated in bold.

It is obvious that the standard would contain more contaminants after thrombin cleavage. Not only would thrombin be present in the sample, but the removed His-tag as well. Although the thrombin concentrations may be negligible, the tag will not be and requires removal from MT-2A*. A quick and easy way to separate the cleaved His-tag from target recombinant protein would be to use metal chelating chromatography. The tag would bind to the immobilized metals while the recombinant protein would not (and could be collected as the flow through). Unfortunately, MT-2A* was also theoretically able to bind to the immobilized metal and so this approach was not considered. An alternative method would be to separate them with reverse phase chromatography. The possibility that the cleaved MT-2A* standard might be

separated from the previous contaminants due to its smaller size (and possible increased hydrophilicity) also supported the decision to use reverse phase HPLC again to finally purify the expected standard. The following sections describe thrombin optimization and cleavage to remove the His-tag.

5.2.5.1 Thrombin cleavage optimization and cleavage to remove the His-tag

5.2.5.1.1 Materials and Methodology

Thrombin is a protease which recognizes the LVPRGS sequence and cleaves after LVPR. Although commercial thrombin is commonly associated with unspecific cleavage (due to other proteins in the sample) (Christodoulou, 2000), it is also known to lose enzymatic activity at the slightest change from optimal conditions – resulting in undigested products. Two factors were investigated in order to optimize conditions: cleavage time and thrombin concentration. The reaction mixtures were prepared and incubated according the manufacturer's specifications except for the variables (cleavage time and thrombin concentration) that were tested. A negative control containing no thrombin was also included in every digestion series. Due to the low yields of MT-2A$^{\Delta}$, only 1 ng MT-2A$^{\Delta}$ was used for each reaction.

The purchased Restriction Grade Thrombin came with a cleavage monitoring (control) protein to use as control for cleavage. One nanogram of this protein was also incubated in the same reaction mixtures and conditions to serve as positive control for thrombin cleavage. The reaction mixtures were incubated in PCR blocks (Thermo Hybaid Multiblock System 0.2G thermocycler) to ensure the correct temperatures. Afterwards the reactions were analysed with SDS-PAGE as described previously.

5.2.5.1.2 Results and Discussion

Figure 5.15 shows the SDS-PAGE results, monitoring the cleavage process with various amounts of thrombin in the reactions.

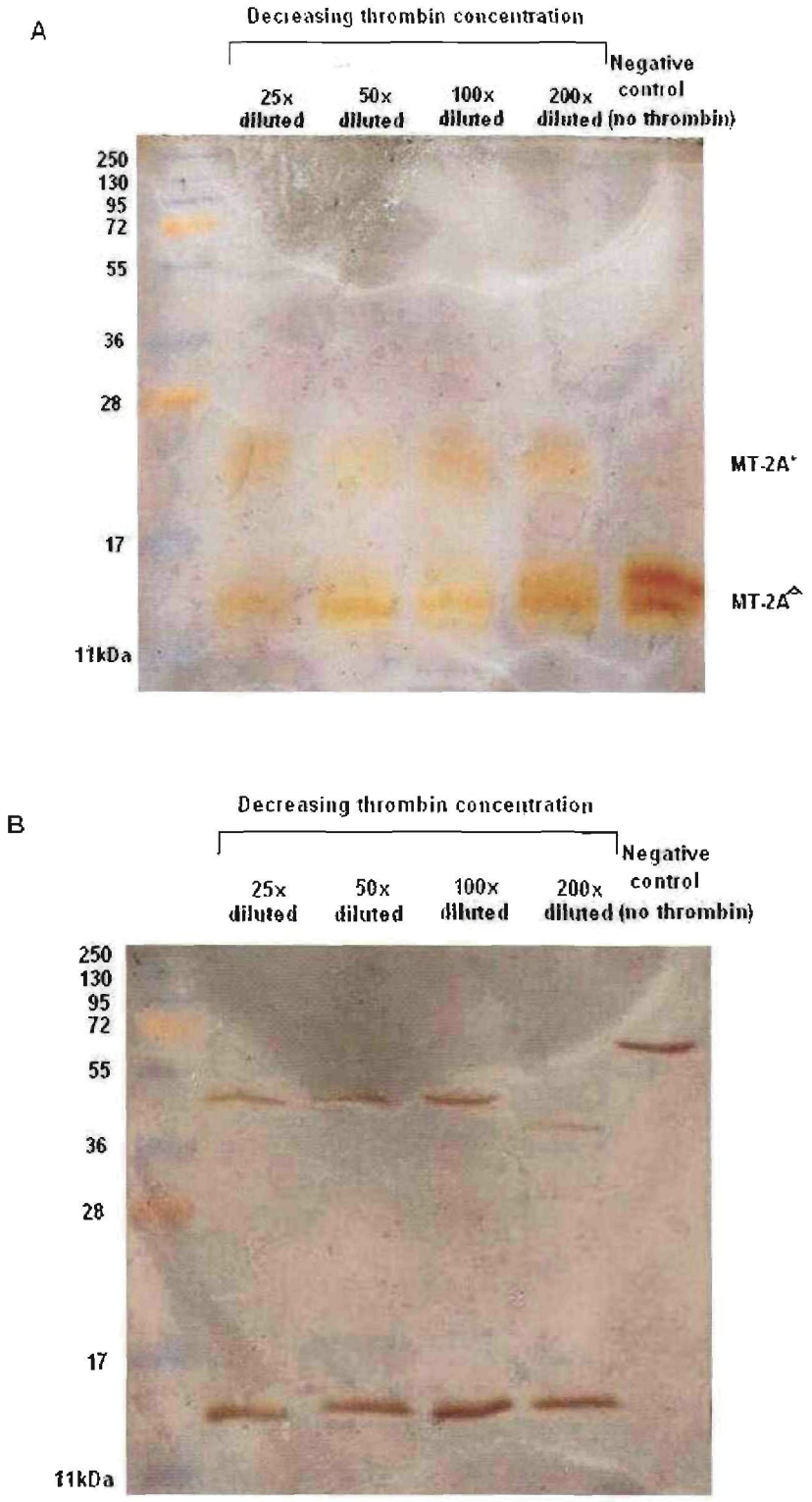

Figure 5.15: SDS-PAGE analysis to monitor thrombin cleavage with varying enzyme concentrations. Thrombin cleavage of MT-2A$^{\Delta}$ (A) and cleavage control protein (B) with varying enzyme concentrations. A 15 % (m/v) separating gel (stained with silver) was used to monitor thrombin cleavage at various concentrations.

Figure 5.15 B shows the thrombin cleavage of the cleavage control protein. Although it is clear from this result that all the protein has been cleaved with low amount of thrombin, the same was not visible in the gel containing MT-2A$^{\Delta}$. The lane containing the uncut negative control sample only has the single band while the lanes containing the cleaved samples clearly contains two bands and the absence of the uncut protein band. Since the same conditions and reaction mixtures were used to digest MT-2A$^{\Delta}$, it can be concluded that all thrombin cleavage worked as expected.

A 7.4 and 1.8 kDa fragment were expected after the thrombin cleavage of MT-2A$^{\Delta}$. The 7.4 kDa protein fragment was expected to be visible on the gel but not the 1.8 kDa as it would probably migrate with the dye front. Instead a ~15 kDa (probably uncleaved MT-2A$^{\Delta}$) and a ~ 25 kDa fragment (probably cleaved MT-2A$^{\Delta}$ – see explanation that follows) were visible in Figure 5.15 A. The uncut negative control only had the one ~15 kDa protein band (MT-2A$^{\Delta}$). This meant that the ~15 kDa bands visible in the other lanes containing cleaved samples is MT-2A$^{\Delta}$. Thus, it seems that not all the MT-2A$^{\Delta}$ were cleaved within 16 hours (as specified by the manufacturer) regardless the thrombin concentration. It is, however, clear that the higher the thrombin concentration the more MT-2A$^{\Delta}$ were cleaved which gave a less clear band on the gel. The lane containing the uncut negative control sample has a very clear , but broad band representing a large amount of uncut MT-2A$^{\Delta}$.

By comparing the other lanes to the uncut negative control it was easy to assign the ~15 kDa band to MT-2A$^{\Delta}$. The ~25 kDa protein band was more difficult to explain. Since the concentration of thrombin was undetectably small and its theoretical size is 36 kDa, this band could not represent thrombin. The only conclusion that was made was that with the removal of the tag, the natural characteristics of MT-2A* became more apparent which include reduced electrophoretic mobility. Therefore, it was assumed that this band is cleaved MT-2A$^{\Delta}$ (MT-2A*). This was a speculative possibility which had to be confirmed. Since this cleaved sample had to be again purified by reverse phase HPLC, it was thought that HPLC could be used to confirm the cleavage (Section 5.2.5.2).

Thrombin digestion was monitored over time with SDS-PAGE and is shown in Figure 5.16. The results obtained (Figure 5.16) are different from the results in Figure 5.15 A

as there is no second protein band (clearly) visible in the lanes containing the digested products and which was assumed to be MT-2A*. Nevertheless, from this result it was observed that most MT-2A$^{\Delta}$ was cleaved after 8 hours (represented by the less clear MT-2A$^{\Delta}$ band). Thus, the incubation time, as given by the manufacturer, gave the best results and was used for further digestion.

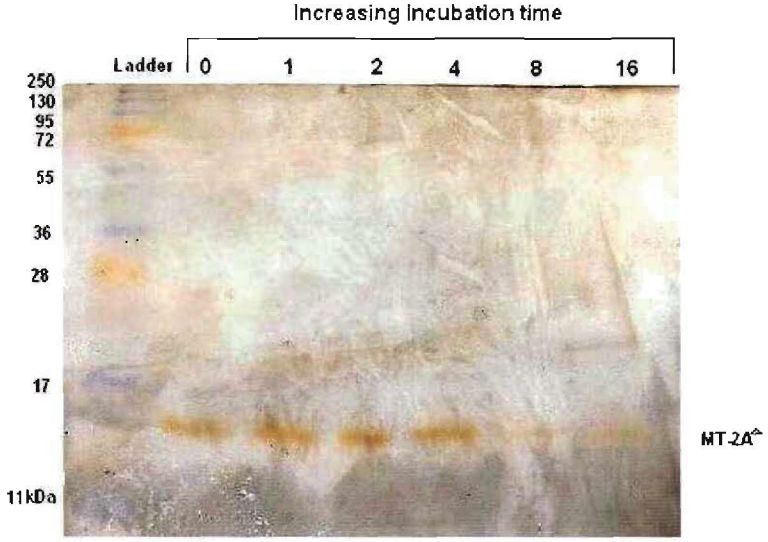

Figure 5.16: SDS-PAGE analysis to monitor thrombin cleavage with varying reaction times. The incubation time is given in hours. A 15 % (m/v) separating gel stained with silver was used.

5.2.5.2 Confirmation of thrombin cleavage with reverse phase HPLC

5.2.5.2.1 Materials and Methodology

From the results obtained, it was clear that the conditions as described by the manufacturer were suitable for cleavage. These conditions were again used to cleave MT-2A$^{\Delta}$ to analyse it with reverse phase HPLC before large batch cleavage and purification. A negative control was also prepared which was MT-2A$^{\Delta}$ incubated in the same cleavage buffer but without thrombin. After cleavage the samples were analysed with RP-HPLC as described before, using the same mobile phases and conditions. Since the MT-2A$^{\Delta}$ sample was relatively pure (Figure 5.13), it was expected that the cleaved sample would give at least two clearly distinguishable chromatographic fractions namely the removed tag and MT-2A*. The negative control

would result in the same peak as visible in Figure 5.11. A third peak was also expected which would represent the uncut protein.

5.2.5.2.2 Results and Discussion

Figure 5.17 shows the chromatogram obtained after the thrombin cleaved sample was analysed. The negative control gave the same result (a single peak) as that in Figure 5.11 and is therefore not shown.

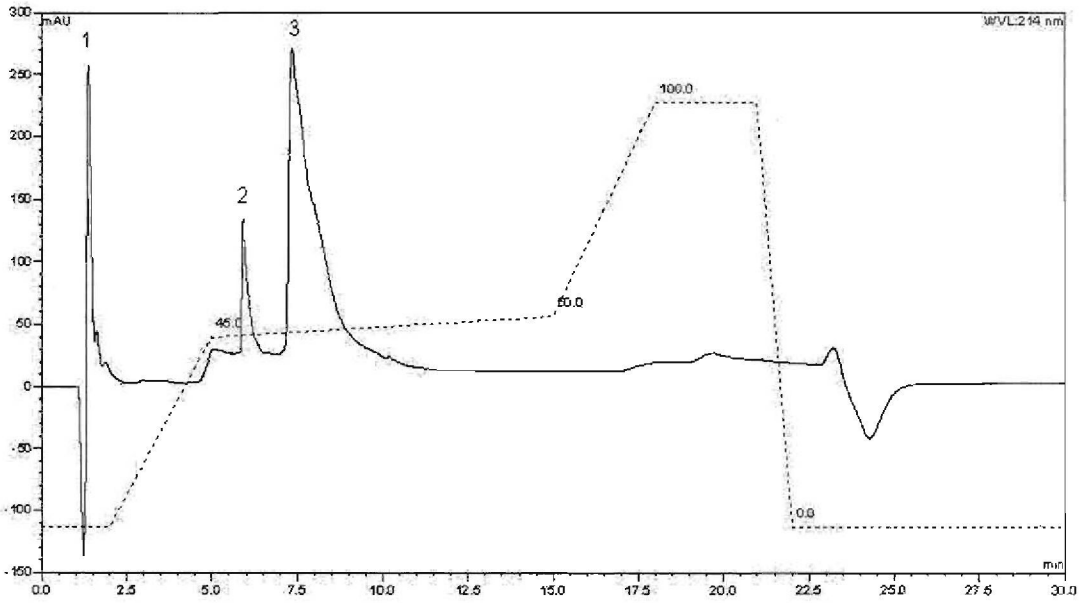

Figure 5.17: Chromatogram of thrombin cleaved MT-2A$^{\Delta}$. The target peaks are indicated with the numbers 1 to 3.

On this chromatogram two separate and clear peaks were observed as was expected - a small peak and a larger peak. It was first assumed that the smaller peak represents the removed tag and the larger peak to represent MT-2A*. According to our first assumption, the absence of a third clear peak also meant that all MT-2A$^{\Delta}$ were cleaved which was unlikely and also unexpected (Section 5.2.5.1.2). Since the larger peak (# 3) appeared at the exact position (elution time) as uncut MT-2A$^{\Delta}$ (Figure 5.11) it was therefore reassumed that this peak was the uncut MT-2A$^{\Delta}$ while the smaller peak (# 2) probably represented MT-2A*. Since the tag was small and probably very hydrophilic, it was though to elute with the salts (peak # 1 as indicated in Figure 5.17). These peaks (fractions) were collected separately to confirm with mass spectrometry.

5.2.5.3 Confirmation of thrombin cleavage with mass spectrometry

5.2.5.3.1 Materials and Methodology

The two peaks at 6 and 8 min (Figure 5.17) was collected and prepared for mass spectrometry as described previously (Section 5.2.4.3.1). It was expected that the small peak (# 2 as indicated in Figure 5.17) would contain an overwhelming 7.4 kDa peak in the deconvoluted mass spectrum (representing MT-2A*). The second peak was assume to be uncut MT-2A$^{\Delta}$ which was expected to give the same 9.3 kDa peak as previously obtained (Figure 5.13). The samples were analysed with the Agilent 6210 Time-Of-Flight LC/MS as previously described (Section 5.2.4.3.1).

5.2.5.3.2 Results and Discussion

Figure 5.18 shows the spectra obtained after peaks number 2 and 3 were respectively analysed with mass spectrometry. Figure 5.18 shows the deconvoluted mass spectra obtained of fractions # 2 and # 3 (as indicated in Figure 5.17). Fraction # 2 was expected to be MT-2A* but did not contain the expected (7422 Da) protein. Many other peaks were, however, observed but was thought to be background noise rather than noteworthy compounds. The expected peak (9.3 kDa) from fraction # 3 was not present and the spectrum also contained many other (what was though to be) irrelevant peaks.

The cleavage procedure was repeated (with the HPLC purified MT-2A$^{\Delta}$ kept at -80 ℃) followed by HPLC purification. After basically the same noisy spectra were obtained (after numerous attempts) it was decided to analyse a small amount of the HPLC purified sample in its undigested form. This was done to confirm the protein's integrity and purity (and ESI-MS ionisation previously optimized). Figure G.1 (Appendix G) shows the deconvoluted mass spectrum obtained. Instead of getting the 9303.66 Da peak, a 9208.47 Da peak was found which corresponded to the mass of MT-2A$^{\Delta}$ after the C-terminal alanine was lost. This meant that the protein was unstable or that some contaminated proteins (with enzymatic activity) cleaved the metal free protein.

The spectra obtained from the thrombin cleaved proteins were again investigated with the main objective: to identify certain peaks. Figure 5.18 A and B show some of the identified peaks with the respective fragments of MT-2A$^{\Delta}$. Spectrum A (which was thought to be MT-2A*) mainly contains fragments of MT-2A* (rather than MT-2A$^{\Delta}$) which can be seen by the starting sequence of the fragments. Most of the identified fragments started with "GSH—" which refers to MT-2A* (see Figure 5.14). From this it seems that the protein was indeed cleaved with thrombin, but due to unspecific cleavage or instability of the protein it resulted in "bits and pieces" of the target protein. Spectrum B (which was thought to be uncut MT-2A$^{\Delta}$) contains fragments of MT-2A$^{\Delta}$ (rather than MT-2A*).

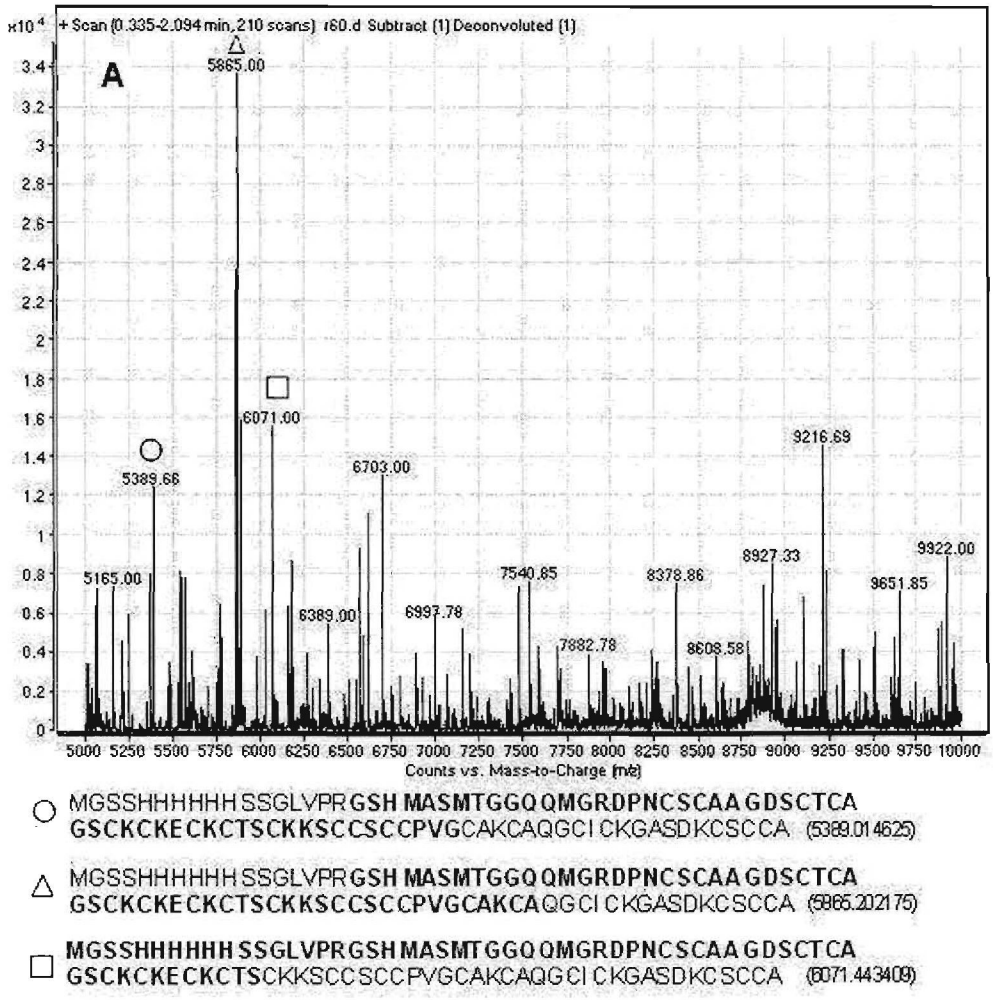

Figure 5.18 (A): Deconvoluted mass spectra of the thrombin cleaved MT-2A$^{\Delta}$ fractions. A is the spectrum of peak # 2 and B is the mass spectrum of peak # 3 as indicated in Figure 5.17. Some of the identified peaks (indicated with a circle, triangle and square) are explained by referring to the MT-2A$^{\Delta}$ primary sequence. The respective peaks represent certain fragments of MT-2A$^{\Delta}$ (indicated in bold). The theoretical mass of the respective fragments are also given (in brackets after the sequence).

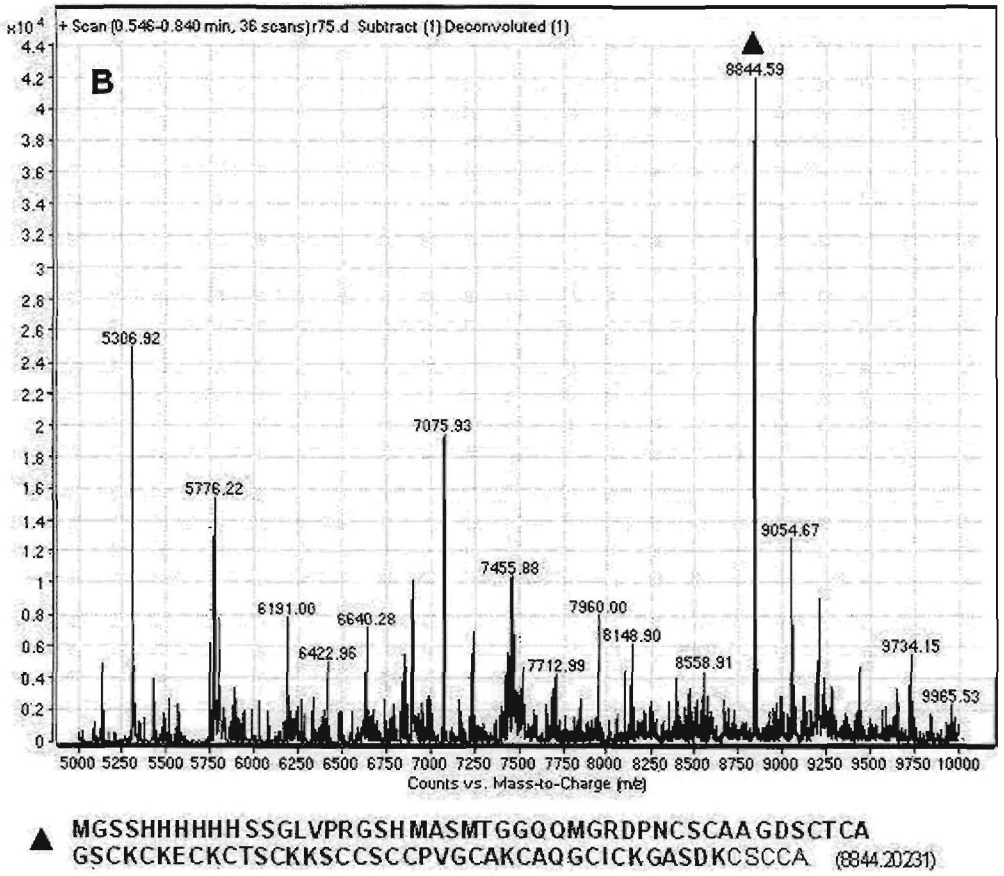

Figure 5.18 (B): Deconvoluted mass spectra of the thrombin cleaved MT-2A$^{\Delta}$ fractions. A is the spectrum of peak # 2 and B is the mass spectrum of peak # 3 as indicated in Figure 5.17. Some of the identified peaks (indicated with a circle, triangle and square) are explained by referring to the MT-2A$^{\Delta}$ primary sequence. The respective peaks represent certain fragments of MT-2A$^{\Delta}$ (indicated in bold). The theoretical mass of the respective fragments are also given (in brackets after the sequence).

Although these protein fragments were not separated mainly according to size (e.g. with SEC) but according to their hydrophilicity (with reverse phase HPLC), it does not explain the two large peaks obtained in the chromatogram. Even though these fragments are from the same protein, the loss of certain residues (amino acids) from its sequence should have resulted in different interaction with the chromatographic phases and more peaks. Therefore, another explanation would be that the fragmentation occurred after HPLC during the concentration procedure or lag time (between HPLC purification and MS analysis).

5.3 SUMMARY AND CONCLUSIONS

As mentioned before, metal chelating chromatography alone did not lead to the necessary purity (95 %) and also failed to produce uniform MT-2A$^{\Delta}$. Various properties of MT were exploited in an attempt to obtain pure MT-2A$^{\Delta}$. A three step purification procedure was used to obtain the necessary purity. The use of three purification steps is commonly used by other researchers to purify MT from recombinant expression systems or animal homogenates (You et al., 1999:50). MT-2A$^{\Delta}$ was successfully purified from many other contaminants but was not of analytical purity (95 %).

To reach the objective of this study, it was necessary to remove the His-tag from the recombinant protein in order to obtain a modified MT-2A (MT-2A*) standard with slightly different mass than most other native human MT isoforms and subisoforms. The His-tag was, however, unsuccessfully removed and (what is believe to be) unspecific cleavage resulted in various fragments of the recombinant protein. Furthermore, it was also found that HPLC purified MT-2A$^{\Delta}$ was unstable (even at -80 ℃) and resulted in the loss of certain residues from its primary structure. The reason for the instability is still unknown. Therefore, it is concluded that, although it was possible to produce a modified recombinant MT-2A protein, which was the objective of this study, it was unstable and did not result in reproducible stable sample (MT standard) for use in a highly sensitive detection and quantification method such as mass spectrometry. Several factors support this conclusion and are discussed in more detail in Chapter 6.

Chapter 6

Conclusions and future perspectives

6.1 OBJECTIVE, AIMS AND STRATEGY

The induction of metallothioneins in mitochondrial disorders was studied by means of RNA level measurements (Olivier, 2004:42; Pretorius, 2006:44). This was done in relation to oxidative stress measurements and damage or phenotypic observation (Reinecke, 2004:89). Reinecke et al. (2004:86) showed that the levels of induction of MT mRNA expression differ from protein levels. *From these and other studies it is clear that MT should be measured on protein level to be able to investigate the expression and role of MT more accurately. A method to quantify metallothionein quickly and accurately in biological material needs to be established, which can be used to measure and compare the MT levels in biological samples of various types.*

The *objective* of this study was to produce a pure recombinant modified MT-2A standard which will be suitable to use as *internal standard* in a mass spectrometric quantification method for human MT-2A.

For the production of recombinant modified MT-2A, a *strategy* was formulated with specific aims. The formulated strategy consisted of three main sections and aims (see Figure 6.1) each containing sub-sections and aims which were reported on in detail in Chapters 3 – 5.

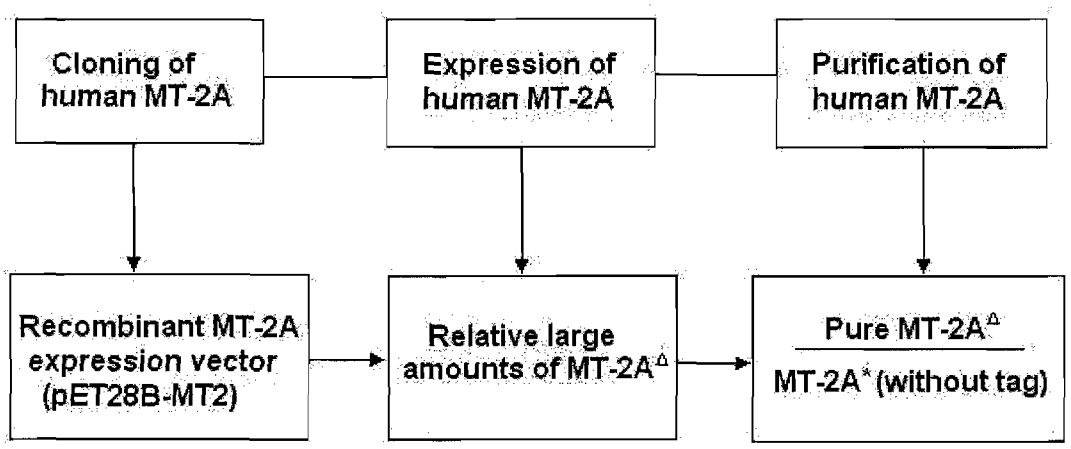

Figure 6.1: Concise experimental strategy and aims. The strategy of this study consisted of three main sections which were also the titles of Chapters 3 – 5, respectively. The main aim of each section had to be reached before the next section could have been started (as indicated by the arrows).

The respective sections and aims of this strategy were formulated to reach the objective of this study, which is to obtain pure recombinant MT-2A*, in the least amount of time, effort and cost. The strategy consisted of cloning, expression and purification of recombinant modified MT-2A. The aim of each section in the strategy had to be reached before it was possible to progress to the next section.

The rationale behind each step and aim is discussed in detail in the previous chapters. Chapter 3 discussed the cloning of human MT-2A to obtain a recombinant MT-2A expression vector (pET28B-MT2). Chapter 4 discussed the expression of recombinant human MT-2A to obtain relative large amounts of MT-2A$^{\Delta}$. Chapter 5 discussed the purification of MT-2A$^{\Delta}$ to obtain pure MT-2A$^{\Delta}$ before removal of the His-tag to obtain MT-2A*.

6.2 CONCLUSIONS

By following the strategy that was put forth, the following conclusions (regarding the main aim of each section) was made:

6.2.1 Cloning of human MT-2A (Chapter 3)

The aim of this section was to design and construct a modified recombinant MT-2A expression vector, pET28B-MT2. The human MT-2A gene was successfully sub-cloned into the base expression vector, pET28B. This vector was characterized by sequencing and a sufficient amount and appropriate quality plasmid preparation for transformation was produced.

6.2.2 Expression of recombinant MT-2A (Chapter 4)

The aim of this section was to investigate MT-2A$^{\Delta}$ expression in E.coli BL21(DE3) strains and optimize expression to produce relatively large amounts of MT-2A$^{\Delta}$. This aim was successfully reached with one specific strain, BL21(DE3) CodonPlus®-RIL, although the expression of MT-2A$^{\Delta}$ remained relatively low (2 mg per litre culture), even after optimization of expression conditions. This was in accordance with other reports. Hong et al. (2001:248) summarized the common low expression values of recombinant MT from other reports with an average of ~5 mg/l. Despite numerous attempts to solve the low expression of recombinant MT in bacterial cells over the past decades, it still remains a common problem (Hong et al., 2001:248). The instability of the apoprotein, toxicity of the (mammalian) protein to the host cells and susceptibility of the bacterial cells to added metals add to the difficulty of expressing these proteins (Hong et al., 2001:248). MT's small size and repetitive sequence are also responsible for the low yields (Paziranden et al., 1998:4068). Even with the use of large tags (such as intein and GST) the general yield stays low (Hong et al., 2001:248).

6.2.3 Purification of recombinant MT-2A (Chapter 5)

The aim of this section was to isolate and purify expressed MT-2A$^{\Delta}$ followed by the removal of the His-tag to obtain MT-2A.* MT-2A$^{\Delta}$ was successfully purified using a three step purification procedure. Even though the purity (~70 %) was not sufficient for use as internal control in a mass spectrometric quantification method, the procedure did increase the purity from ~0.61 % (equivalent to 2 mg MT-2A$^{\Delta}$ per 327.4 mg total protein) to ~70 % (see Chapter 5). Although this aim was successfully reached when regarding the significant increase in purity, it was not reached without using extensive effort (metal chelating chromatography, dialysis and reverse-phase

HPLC) and time. The low expression conditions and three step purification procedure also did not deliver a highly pure (>95 %) or large amount of MT-2A$^{\Delta}$.

The removal of the His-tag and some additional amino acids by thrombin cleavage along with additional purification of MT-2A* were, however, unsuccessful. Since MT-2A$^{\Delta}$'s mass and primary structure was significantly higher (>2 kDa) than the masses and structure of the human MT isoforms, it was regarded unsuitable to be used as *internal standard* with the tag in place. The results in Chapter 5 indicated that unspecific cleavage probably occurred (Christodoulou, 2000) which resulted in useless fragments of the target protein. Native MT-2A is known to be relatively stable (from commercial brochures) and, despite the inability to remove the His-tag, it was also found that MT-2A$^{\Delta}$ was comparatively unstable. Although it is not clear whether protease activity or other factors is responsible for the degradation (instability) of the protein, one certainty is that this degradation cannot be reduced or stopped without compromising the contents and purity of the protein sample.

6.3 ALTERNATIVE APPROACHES

For reasons revealed in Chapter 3, the strategy that was put forth at the onset of this study was considered to be of high merit based on literature and an appropriate approach, considering the capacity available at this institution, to obtain an *internal standard*. After completion of mostly all aims of the formulated strategy and considering the results that was obtained, it can be concluded in hind sight that a few changes might have assisted the expression and purification of the recombinant protein. These alternative approaches to the strategy are the following:

- To avoid extremely low expression levels of MT in bacterial cells, the small protein could have been fused to a larger tag such as a glutathione S-transferase (GST) tag. Although it is mentioned that larger tags do not result in greater expression of MT (Hong et al., 2001:248), the general yield of MT using the larger tags might have been greater in comparison with the use of the small His-tag, especially since MT is already a small protein.

- Expression of MT in eukaryotic systems has the potential to produce relative high yields of recombinant protein with the necessary post translational modifications and folding to obtain stable MT. Although expression in bacterial cells such as done in this study generally produces higher expression and is easier and less expensive to culture, the stability of the recombinant protein may be better due to post-translational modification that occurs in eukaryotic cells. The N-terminal of mammalian MT is usually acetylated which is believed to keep the protein from degradation (Ghoshal & Jacob, 2001:359; Orga & Suzuki, 1999:23; Sanz-Nebot et al., 2003:389).

- The use of other tags such as the GST-tag as ligand targets for affinity purification might also have resulted in better isolation of the recombinant protein with less interference of other proteins. More specific isolation of the fused recombinant protein would consequently have reduced the amount of purification steps (and thus time) needed to obtain pure protein.

- The use of a smaller tag fused closer to the protein is another option that was considered during the study. In theory it may have been more ideal since no cleavage would have been necessary as the protein's mass and structure would be more similar to the native protein. This alternative approach was indeed explored by Dr. Oksana Levanets (post-doctoral fellow at the Mitochondrial Research Laboratory, NWU) during the duration of this study. Here, the His-tag was fused to human MT-2A without the 22 additional amino acids between the His-tag and the N-terminal D (including the thrombin recognition sequence). After several attempts, expression of this small recombinant protein was not successful (personal communication Dr Levanets) and supports literature reports that expression of such small proteins (<10 kDa) in *E.coli* can rarely be achieved (Sommer et al., 2004:10).

From these conclusions and reports it is clear that producing and purifying bacterially produced recombinant MTs for the purpose of using highly pure forms as internal standards in MS-based quantification methods have limitations. The use of commercially available highly pure (tissue purified) rabbit MT isoforms, which is highly

similar to human MT isoforms but with slight mass differences, was considered as an alternative approach to optimize and quantify human MT-2A using a mass spectrometry technique.

From the onset of this study it was decided to use rabbit MT-2A to optimize the mass spectrometric based quantification technique in a future study as no (pure) human MT-2A was commercially available. It was, however, planned to use MT-2A* as the internal standard when optimizing the technique as well as when MT in biological material needed to be quantified. The alternative to this approach then is to use rabbit MT-2E as internal standard, which has a slightly different mass (>100 Da) than both the rabbit MT-2A and human MT isoforms, but still is highly similar in structure. Thus, in addition to the rabbit MT-2A standard used to calibrate the mass spectrometer (along with myoglobin) and optimize ionisation, another MT isoform which is not found in humans, rabbit MT-2E (Bestenbalt), was purchased for preliminary evaluation of its use as *internal standard* as an alternative to MT-2A* in future studies.

Both rabbit MT-2A and MT-2E were analysed with the Agilent 6210 TOF LC/MS (methodology in Chapter 5) in order to confirm its purity and the apparatus' ability to detect it accurately. The analysis of these purchased standards was repeated a few months later to confirm integrity and stability. The standards were found to be stable and gave the same spectra over time. The spectra showed in Figure 6.2 were obtained. Rabbit MT-2A gave a 6123.4 Da peak which corresponded well to its theoretical average mass of 6125.23 Da. Rabbit MT-2E gave a 6240.93 Da peak which corresponded to its theoretical average mass of 6241.43. The absence of other peaks in both spectra confirmed that the purchased rabbit MT standards was indeed pure and clearly proved to be a promising alternative approach in future development of a MS-based quantification method.

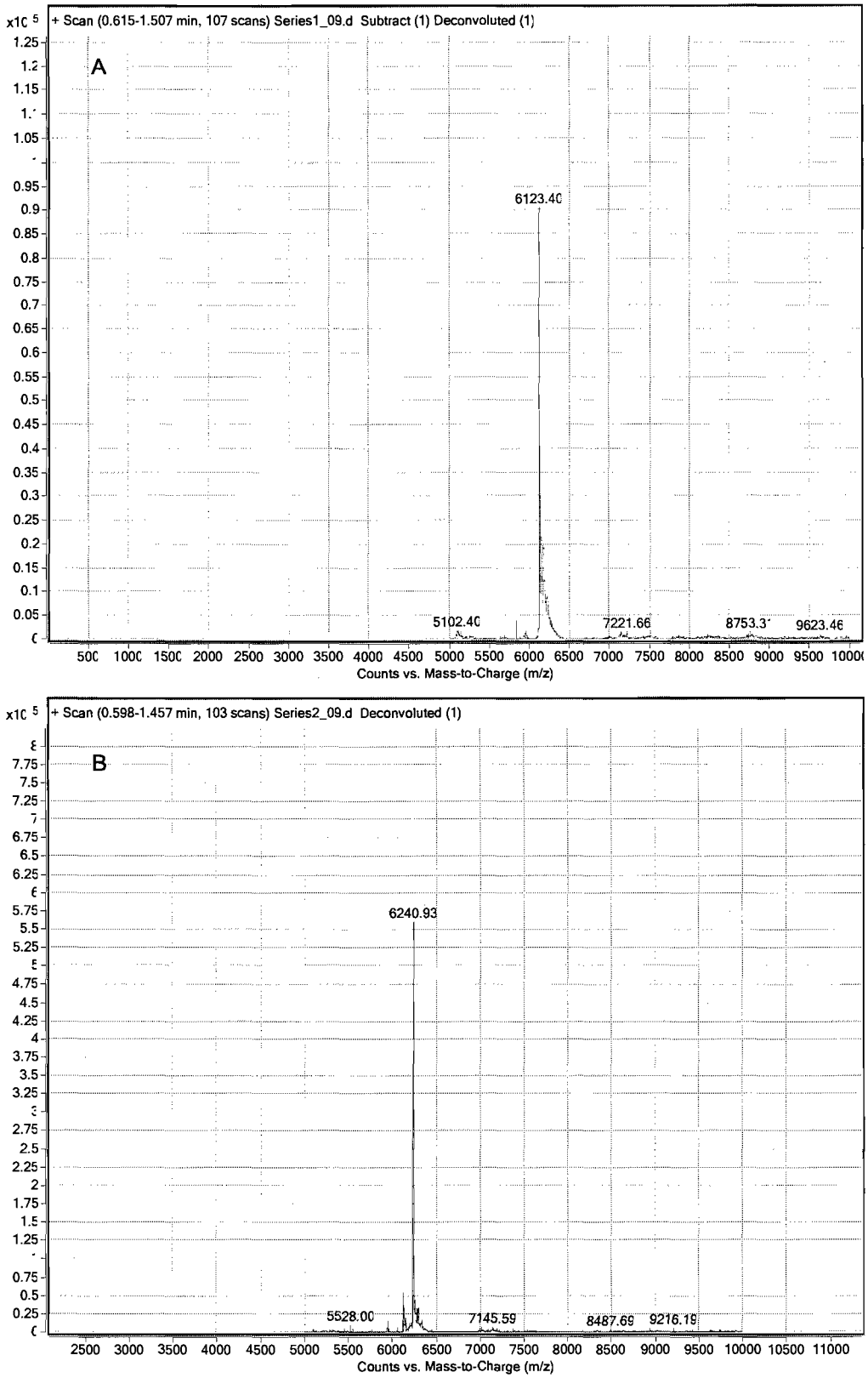

Figure 6.2: Deconvoluted mass spectra of rabbit MT-2A (A) and MT-2E (B). These two purchased standards was analysed to confirm purity and the apparatus' ability to detect them accurately, especially when injected together.

A preliminary concentration range for each standard was created and analysed to confirm their detection at various concentrations and to compare their response factor and detection sensitivity. It was not intended to as yet obtain the detection limits of linear regions but to briefly explore the response in peak intensity at different concentrations of the proteins. These explorative results (not shown) showed a linear increase in detection response (peak intensity) as the concentrations of the standards increased. From these results it appears that these rabbit MT standards could, once better optimization is done, be used to develop the MS-based method.

6.4 FINAL CONCLUSIONS

The main reason why a recombinant MT-2A standard was initially selected above a commercially obtained rabbit MT protein as *internal standard* was the expected cost-to-yield ratio (especially on the long run) as well as the fact that at the onset of the study such a standard (non MT-2) was not available. It was thought that it would be more cost-effective to express and purify modified MT-2A (when needed) than to purchase MT standard. Unfortunately, the effort, time and costs that went into the expression and purification of the recombinant MT were unexpectedly high, in addition to the fact that MT2A* were not successfully produced. In this study approximately 2 mg MT-2A$^{\Delta}$ was expressed per litre culture which is equivalent to ~7.6 g cells and ~327.4 mg total protein. Potentially, ~2 mg MT-2A* could have been obtained per one litre culture after all purification and tag removal steps were completed (if successful). The low yield and various procedures to obtain the target protein meant that it was just as expensive (~R 4500) to obtain an *internal standard* via this route (but with a great deal effort and time) than using a commercial standard. Commercial rabbit MT from Bestenbalt has an average cost of R 6000 per milligram and a purity of 98 %. In addition, the recombinant protein was (in our laboratory under the same storage conditions to MT-2A$^{\Delta}$) stable over relative long periods (~12 months). *Thus, in comparison with producing a recombinant protein as described in this study, it is more cost-effective and sensible with regard to time and effort to use commercial MT standards than a self produced recombinant standard.*

6.5 FUTURE PERSPECTIVES

As mentioned before, a standard such as the commercial rabbit MT standard, MT-2E, holds much promise for developing a MS-based MT quantification method. A mass spectrometric based quantification method can now be further developed and optimized. After optimization of MT detection and response factors on an LC-ESI-(TOF)MS, it is likely that human MT-2A, as well as other isoforms, can be measured in biological material using this approach. The quantification of MT isoforms in biological material will require developing and refining sample preparation, optimizing detection of target proteins and standards (such as rabbit MT-2E) and the use of hyphenated techniques to ensure specific detection. This institution is equipped with the latest mass spectrometers and separation systems which include both HPLC and CE. In addition to this studies' outcomes, the necessary expertise available at this institution means that this study can now be undertaken at the School of Biochemistry at the North-West University.

An established mass spectrometric based quantification method can potentially be useful in studies where MT levels can be measure as part of the diagnosis of diseases (such as Alzheimer's – see Chapter 2), oxidative stress status or for therapeutic monitoring programmes when using MT as therapeutic agent (Chapter 2). The measurement of MT isoforms in plant and aquatic animals can also be performed with an established MS technique to monitor (heavy metal) pollution.

In conclusion, from previous studies at this institution and current literature, a problem was identified and a study formulated to meet the requirements for the degree Magister Scientiae (Biochemistry). Specific aims, as stated in the strategy (Chapter 2), were completed (Chapters 3-5) using appropriate methodologies in an attempt to reach the objective of this study. Results were obtained, presented (Chapters 3-5) and critically evaluated (Chapter 6). Limitations to the strategy were identified and an alternative strategy for further development of this study in the future was proposed.

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Appendix A: List of Materials

MATERIALS (IN ALPHABETICAL ORDER)	MANUFACTURER
A	
Acetic acid (glacial)	Saarchem
Acetonitrile	Acros Organics
Acryl amide	Promega
Agar	Biolab
Agarose	Bioline
Ammonium acetate	Saarchem
Ammonium peroxodisulfate	Sigma-Aldrich
B	
*Bam*H I (including buffers)	Promega
Bicinchoninic acid	Sigma-Aldrich
Bromophenol blue	BDH
C	
Cadmium chloride	Merck
Calcium chloride	Merck
Chloramphenicol	Calbiochem
Coomassie Brilliant Blue R-250	Merck
Copper (II) suphate solution	Sigma-Aldrich
Cytochrome c	Sigma-Aldrich
D	
Dimethyl sulphoxide	Saarchem
Dithiothreitol	Biosolve Ltd
E	
*Eco*R I (including buffers)	Promega
Ethanol (absolute)	Merck
Ethidium bromide	Fluka
Ethylenediaminetetraacetic acid dipotassium	Fluka

G

GeneJET Plasmid Miniprep Kit ..... Fermentas

Glucose ..... Holpro Analytics

Glycerol ..... Sigma-Aldrich

Glycine ..... BDH

H

His-Bind Resin ..... Novagen

Hydrochloride ..... Bio-zone chemicals

I

Imidazole hydrochloride ..... Sigma-Aldrich

IPTG ..... Bioline

Iso-2-propanol ..... Labchem

K

Kanamycin Sulfate ..... Fluka

M

Magnesium chloride ..... Sigma-Aldrich

Manganese chloride ..... Merck

Metallothionein ..... Sigma-Aldrich

Methanol ..... Acros Organics

Myoglobin (from horse heart) ..... Sigma-Aldrich

N

Nickel (II)-sulfate ..... Fluka

N, N' – Methylene bis acrylamide ..... Serva

TEMED ..... Merck

P

PageRuler Prestained Protein ladder ..... Fermentas Life Sciences

pET28 ..... Novagen

PIPES ..... Merck

Potassium chloride ..... Sigma-Aldrich

R

Rabbit MT-2A ..... Bestenbalt LLC

Rabbit MT-2E ..... Bestenbalt LLC

S

Sephadex G-25 ..... Pharmacia

Sephadex G-100 ..... Pharmacia

Silver Stain Plus ..... Bio-rad

Snakeskin Dialysis tubing ..... Pierce

Sodium chloride ..... BDH

Sodium dodedyl sulphate ..... Sigma-Aldrich

Sucrose ..... Saarchem

T

T4 DNA Ligase ..... Promega

T7 Promotor primer ..... Inqaba

T7 Terminator primer ..... Inqaba

Thrombin (restriction grade) ..... Novagen

Trichloroacetic acid ..... Saarchem

Trifluoroacetic acid ..... Sigma-Aldrich

Tris ..... Roche

Tris-hydrochloride ..... Roche

Triton X-100 ..... BDH

TrueStart *Taq* DNA Polymerase ..... Fermentas Life Sciences

Tryptone ..... Biolab

W

Wizard SV Gel and PCR

Clean-up System ..... Promega

X

Xylene cyanol ..... BDH

Y

Yeast extract ..... Biolab

Appendix B: pET28B-MT2 map

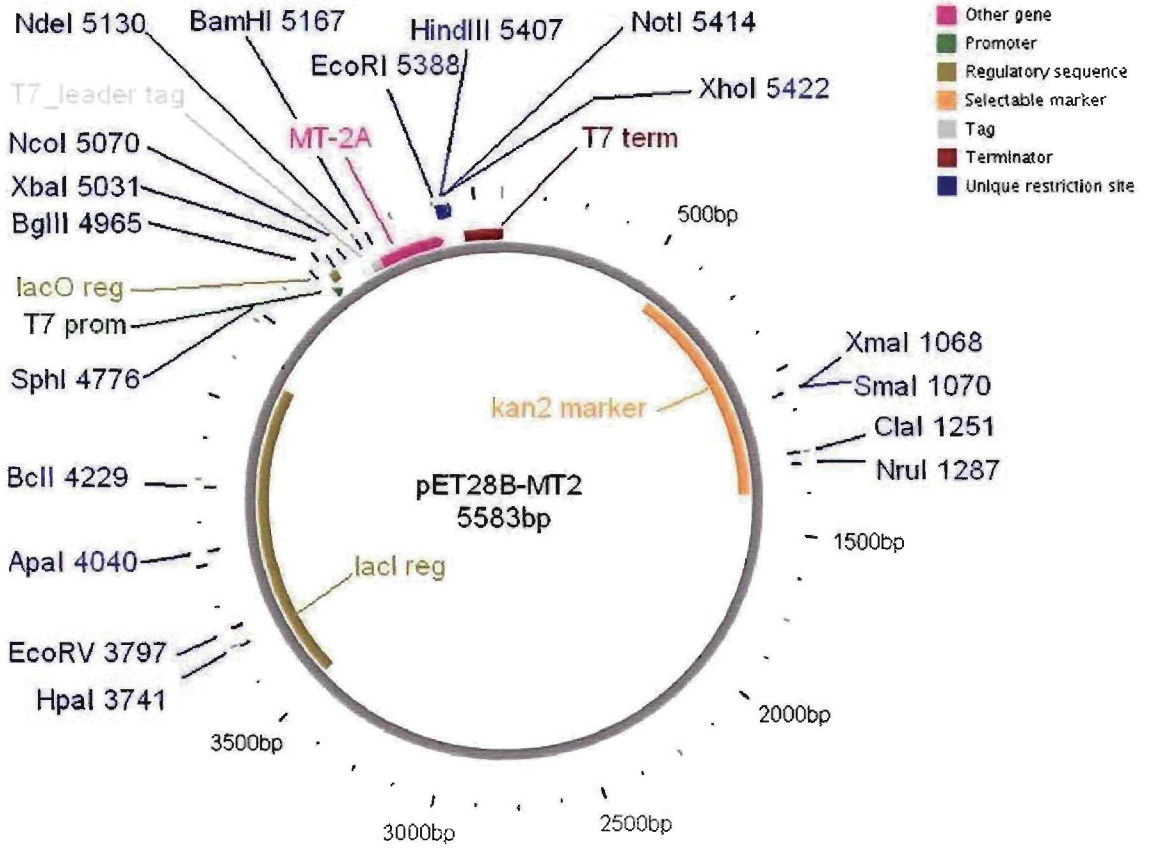

Figure B.1: The plasmid map of the MT-2A expression vector, pET28B-MT2. Key features of the plasmid such as restriction sites, antibiotic selection, etc. are also given in this figure. The legend on the top-right corner explains the colour coding. This plasmid map was created by the web-based program, PlasMapper.

Appendix C: RAPS & Miniprep wavelength scan spectra

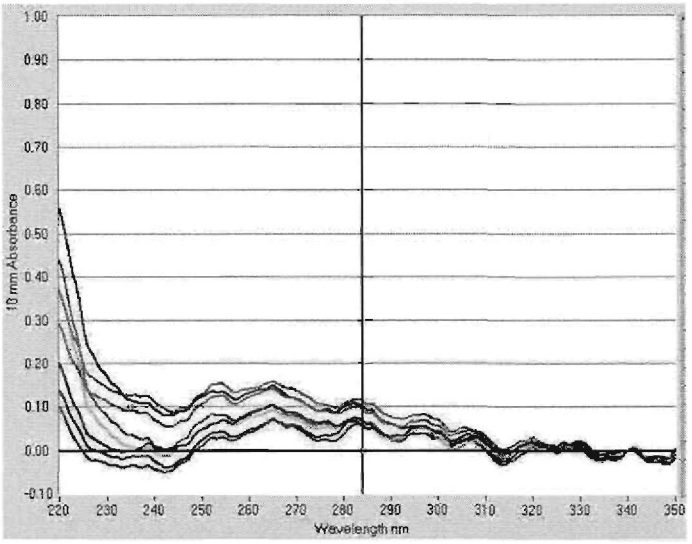

Figure C.1: Absorbance spectra of plasmid isolated via RAPS as measured with the Nanodrop ND-100 spectrophotometer. The different coloured lines indicate the different RAPS products measured.

The overall DNA concentration (260 nm) of RAPS samples were low and in relation with protein content (280 nm) also relatively low (~ 1.2) (Figure C.1). Furthermore, from the wavelength scan it is clear that the spectra is jagged and the sample therefore not pure. Comparatively, the DNA concentration as well as the 260:280 ratio obtained using the miniprep (GeneJET) kit is much higher (~2.0) and the spectrum much more smooth (Figure C.2).

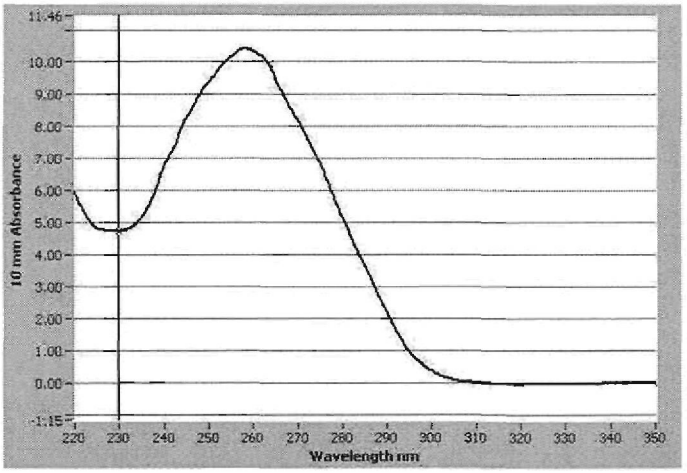

Figure C.2: Absorbance spectrum of plasmid isolated via miniprep kit as measured with the Nanodrop ND-100 spectrophotometer.

Appendix D: DNA sequencing results

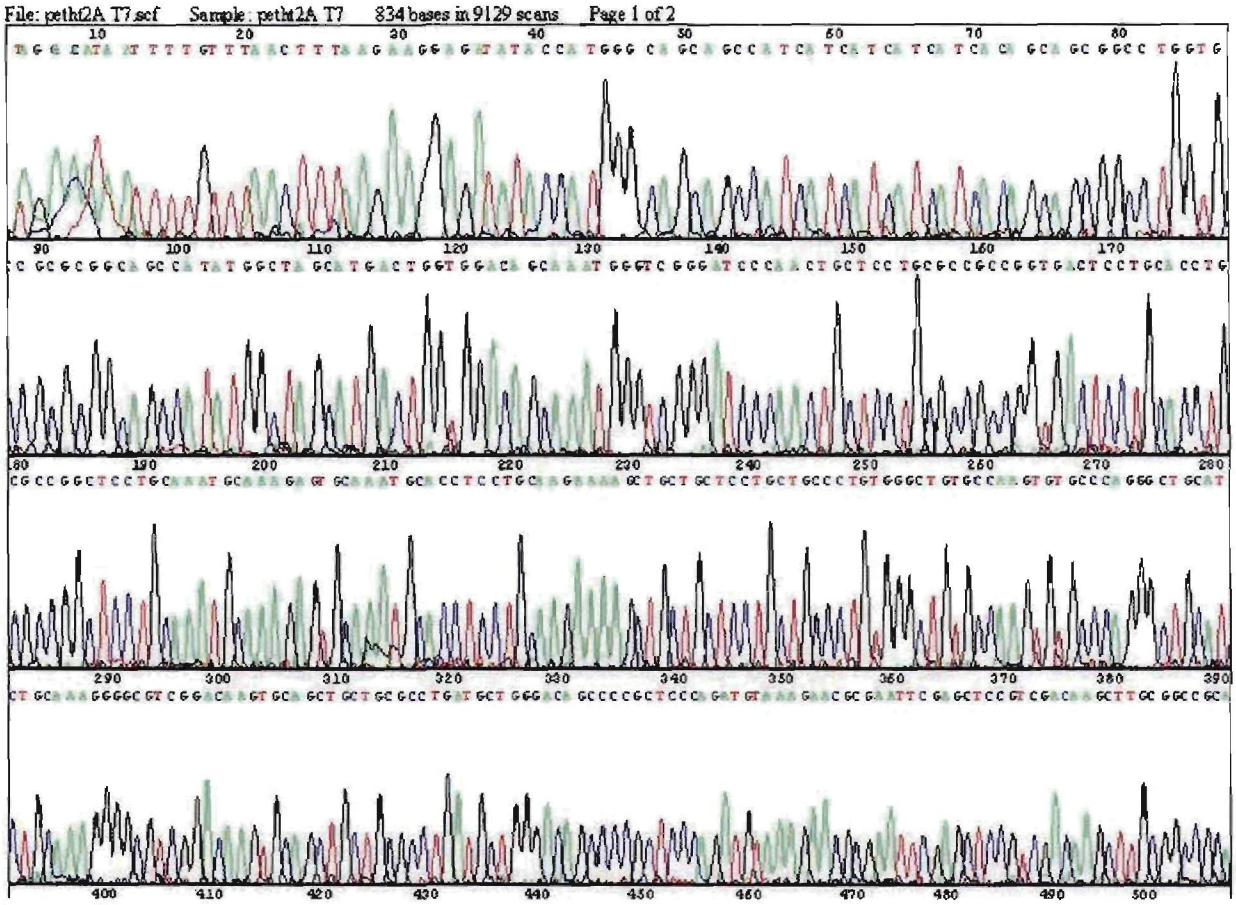

Figure D.1: Electropherogram of dideoxysequencing of pET28B-MT2 using the T7 promoter primer. This figure shows the unprocessed sequence results obtained from Inqaba Biotechnical Industries as presented by Chromas (version 2.31, Technelysium) after pET28B-MT2 was sequenced. The sequence given at the top of each row was processed and compared to the human MT-2A gene. Only part of the results is shown for an example of the unprocessed sequencing results. The processed sequence result is given and discussed in Chapter 3.

Appendix E: Milli-Q purification process

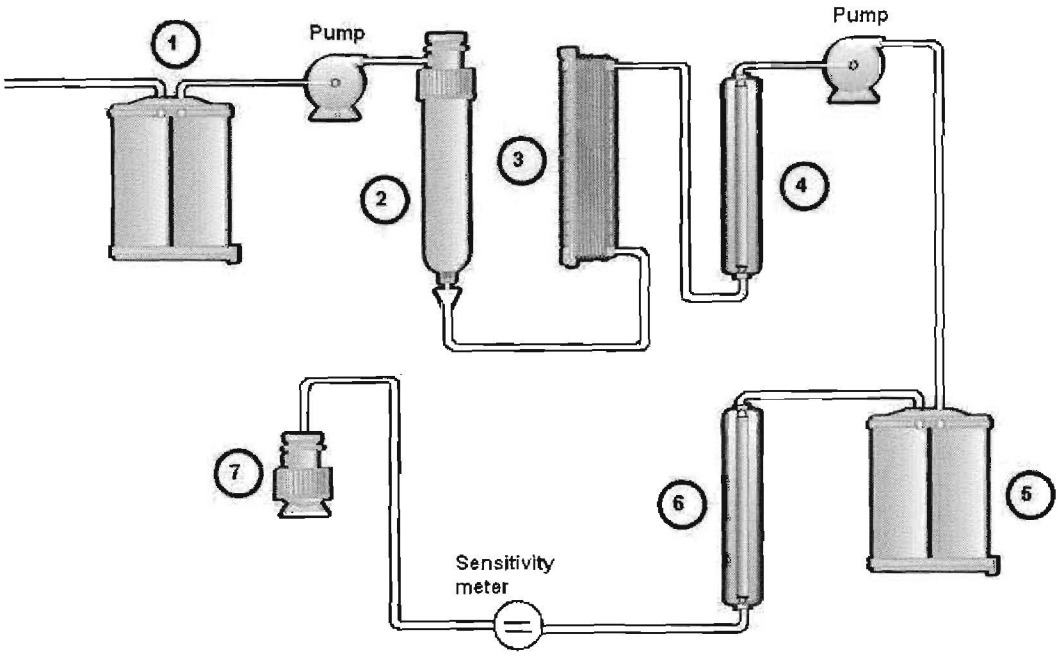

Figure E.1: The Elix® 10 and Milli-Q® Gradient purification process to produce ultra pure water (referred to Milli-Q water in the text). Figure modified from Elix 10 and Milli-Q brochures provided by Millipore Corporation, Billerica, MA, USA.

Elix 10 purification procedure:

1. Tap water enters the Progard® pre-treatment pack which removes particles (with 1 µm filter), free chlorine and colloids (with carbon filter). This also prevents mineral scaling of the reverse osmosis (RO) membrane in hard water areas.
2. The temperature controlled booster pump pressurise the water and ensure a constant flow rate to the RO cartridge. This unit removes 94 – 99 % of inorganic ions, 99 % of all dissolved organic substances with a molecular weight > 100 Da as well as micro-organisms and particles.
3. The water exiting the RO cartridge enters the Elix module (electrodeionisation) to remove remaining ions. The ion exchange resin in the module is constantly regenerated by means of an electrical current.

4. The final step in the Elix 10 system is the sanitisation of the water through a 254 nm UV lamp in a stainless steel cartridge. Organic compounds are oxidised and bacteria killed.

The Milli-Q Gradient purification procedure:

5. This system is coupled to the Elix 10 system. The water passes through the Q-Gard® cartridge containing Jetpore® ion-exchange resin and Organex®.
6. The water enters the double wave UV lamp to oxidize organic molecules and kill bacteria.
7. The ultra pure water is directed through a final filter made up of a 0.22 µm membrane which removes particles and bacteria greater than 0.22 µm.

Elix, Jetpore, Milli-Q, Organex, Progard, Q-Gard are all registered trademarks of Millipore Corporation, Billerica, MA, USA.

Appendix F: RP-HPLC with various solvents

Ammonium acetate (5 mM, pH 6) and 1 % (v/v) acetic acid containing mobile phases was tested along with the 0.1 % (v/v) TFA containing mobile phase to purify MT-2A$^{\Delta}$ with reverse phase HPLC. The TFA containing mobile phase gave the best results (as described in Chapter 5, Section 5.2.4.1.2). Both the acetate containing mobile phases failed to separate MT-2A$^{\Delta}$ from other proteins and to obtain uniformity. Figure F.1 shows the chromatograms obtained using these mobile phases.

The respective mobile phases were tested individually after the HPLC system was thoroughly "cleaned" and saturated with the respective mobile phase. Metal affinity chromatographic isolated MT-2A$^{\Delta}$ was injected and all the fractions collected to confirm the exact elution time (position on chromatogram) of MT-2A$^{\Delta}$. The collected fractions were prepared as described in Chapter 4 (Section 4.2.2).

Both the acetic acid and ammonium acetate mobile phases resulted in the unspecific elution of MT-2A$^{\Delta}$. The protein eluted with other proteins and also as different peaks in the same run. This was confirmed by the MT-2A$^{\Delta}$ band on the gels (not shown) corresponding to the peaks indicated on the chromatograms. The relatively high pH of the acetate mobile phases probably failed to remove the various metals bound to MT-2A$^{\Delta}$, which meant that the various MT-2A$^{\Delta}$ presenting peaks was possibly various metal bound MT-2A$^{\Delta}$ species (Nischwitz et al., 2003:148).

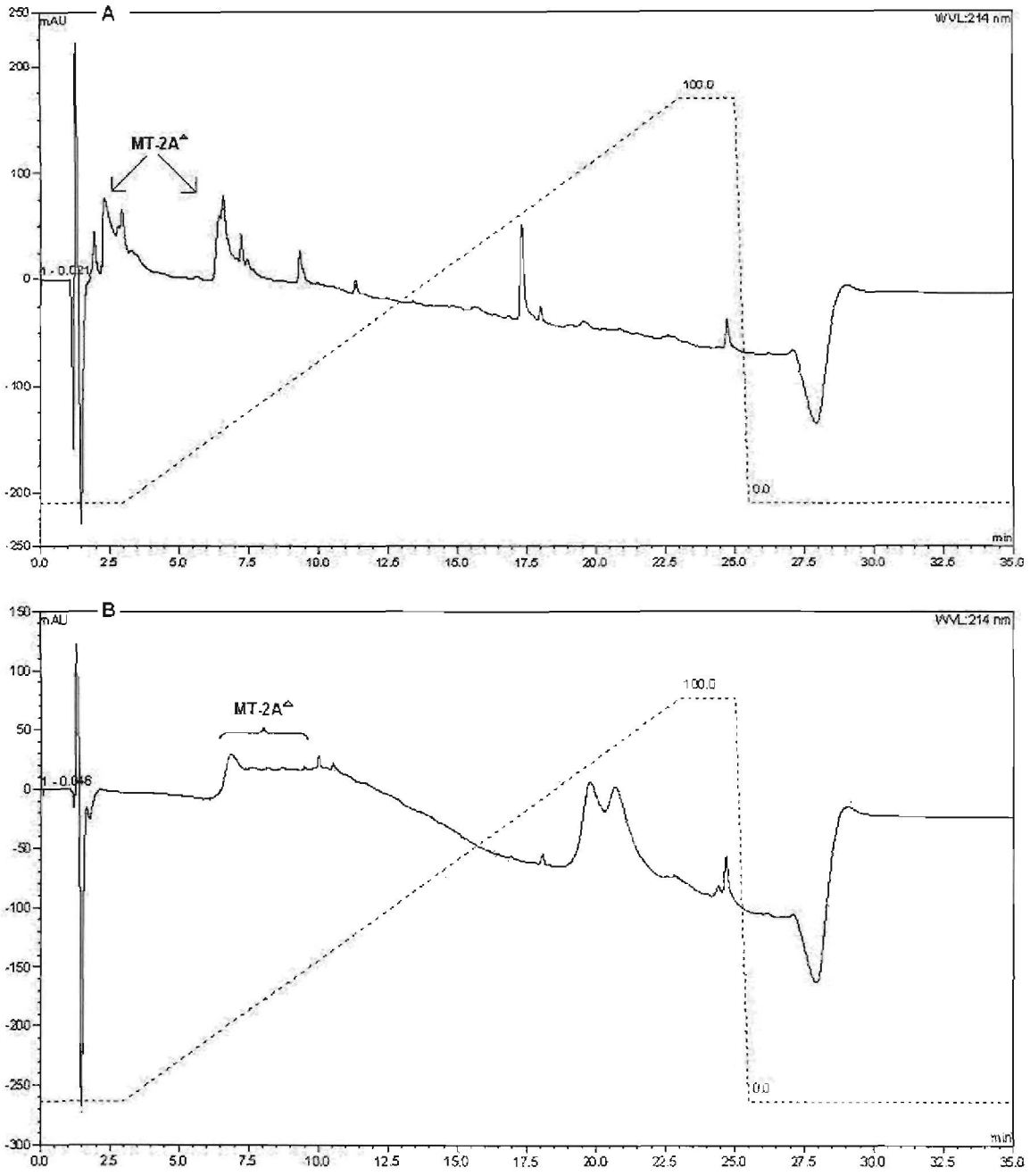

Figure F.1: Chromatogram illustrating MT-2A$^{\Delta}$ separation with 1 % (v/v) acetic acid (A) and 5 mM ammonium acetate (B). The elution positions of MT-2A$^{\Delta}$ (as determined by SDS-PAGE) are also indicated.

Appendix G: Instability of MT-2A$^{\Delta}$

All RP-HPLC purified MT-2A$^{\Delta}$ was pooled and concentrated to evaporate TFA and ACN in the sample. After total concentration, the pooled sample was re-suspended in Milli-Q water and stored at -80 ℃. Before storage, the sample was analysed with mass spectrometry to confirm expected mass and purity (Figure 5.13). After several months the stored protein was again analysed with mass spectrometry to confirm integrity of the protein. The following spectrum was obtained.

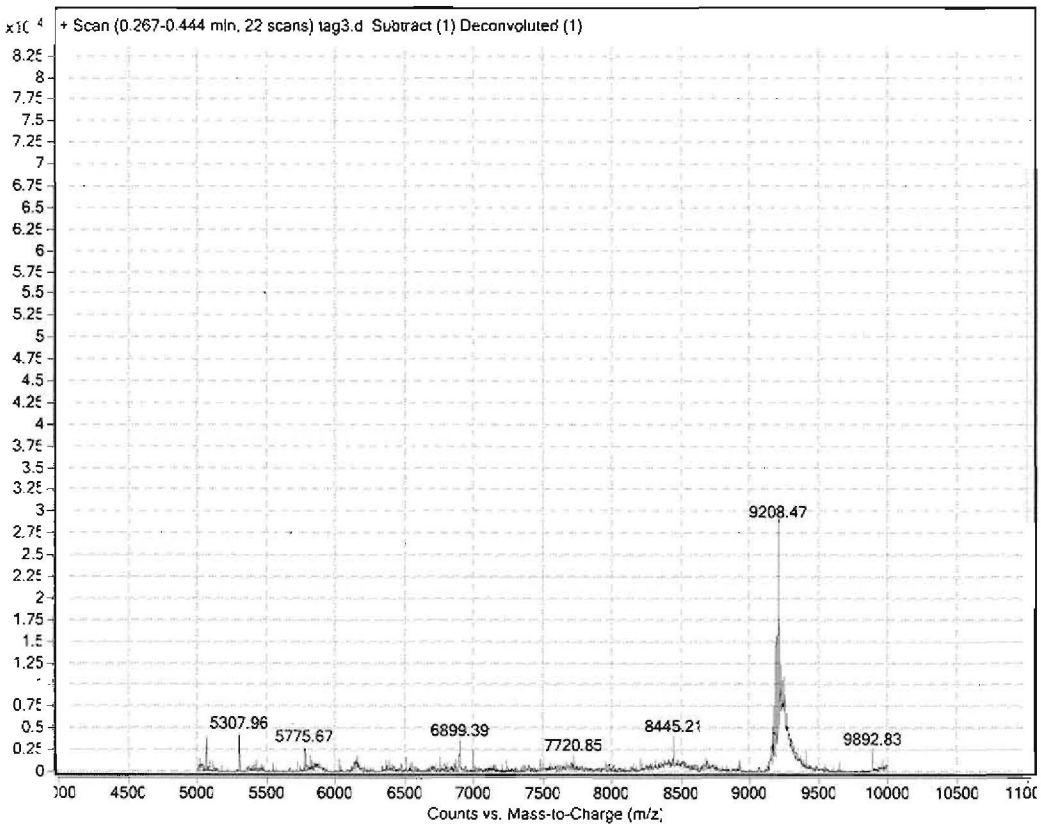

Figure G.1: Deconvoluted mass spectrum of MT-2A$^{\Delta}$ after several months' storage at -80 ℃.

Instead of obtaining a 9303 Da peak, a 9208.47 Da peak was obtained. This mass corresponded to MT-2A$^{\Delta}$, but without its C-terminal alanine. The loss of the C-terminal alanine was unexpected and unexplainable, but protease activity was a probable cause. No MS fragmentation was expected since the same ionisation conditions was used as previous (Section 5.2.4.3.1).