

Regulation of the thioredoxin system in *Saccharomyces cerevisiae*

by

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As the candidate's supervisor I have approved this dissertation for submission.

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Preface

The experimental work described in this dissertation was carried out in the Discipline of Genetics, School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, from February 2011 to February 2013 under the supervision of Dr C.S Pillay.

These studies represent original work by the author and have not otherwise been submitted in any form to another University. Where use has been made of the work by other authors it has been duly acknowledged in the text.

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List of publications and presentations

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- Padayachee, L. and Pillay, C. S. 2013. Thioredoxin should be modeled as a redox couple and not an enzyme in computational systems biology models, FEBS Letters. *Manuscript in preparation.*

Presentations:

- South African Genetics and Bioinformatics Society conference, University of Stellenbosch, Cape Town (2012). *Best MSc poster presentation- Computational Biology and Bioinformatics.*
- School of Life Sciences, Postgrad Research day 2012.

Abstract

The thioredoxin system consisting of thioredoxin (Trx), thioredoxin reductase and NADPH plays a significant role in a large number of redox-dependent processes such as DNA synthesis and anti-oxidant defense. Elevated levels of this system have been associated with a number of diseases including cancer and HIV. Understanding the regulation of this network from a systems perspective is therefore essential. However, contradictory descriptions of thioredoxin as both an enzyme and redox couple have stifled the adoption of systems biology approaches within the field. Using kinetic modeling, this discrepancy was resolved by proposing that saturation of Trx activity could be due to the saturation of the Trx redox cycle which consequently allowed development of the first computational models of the thioredoxin system in Jurkat T-cells and *Escherichia coli*. While these models successfully described the network properties of the thioredoxin system in these organisms, further confirmatory studies were required before this modeling approach could be generally accepted. The aim of this study was to utilize computational and molecular methods to confirm or reject this proposed mechanism for thioredoxin activity. To determine if there is any difference in the kinetic models obtained when thioredoxin was modeled as an enzyme or as a redox couple, representative core models were developed. The data showed that when modeling Trx as a redox couple, the system was able to achieve steady state, there was a re-distribution of Trx into its oxidized form and, thioredoxin reductase affected the rates within the system. On the other hand, when Trx was modeled as an enzyme, the system could not reach a steady state, Trx remained in the reduced form and thioredoxin reductase concentration had no effect on the rates within the system. As these properties could be directly tested *in vitro*, we sought to directly confirm which model was correct. The thioredoxin system from *Saccharomyces cerevisiae* was cloned, expressed and purified and substrate saturation curves were generated using insulin as a model substrate. The data showed that the system reached steady state and with increasing concentrations of insulin, the system saturated with a progressive re-distribution of the thioredoxin moiety into its oxidized form. Further, increasing the thioredoxin reductase concentration increased the flux through the system. Collectively, the results obtained through *in vitro* analyses provided unambiguous support for the thioredoxin redox couple model. These results will enable the construction of a complete computational model of the yeast thioredoxin system and provide a basis for the analysis of this network in a number of pathologies.

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List of abbreviations

AEBSF	4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride
AgNO ₃	Silver nitrate
BER	Base excision repair
BSA	Bovine serum albumin
ddH ₂ O	Milli-Q-water
dH ₂ O	Distilled water
DTNB	5, 5'-dithiobis(2-nitrobenzoic acid)
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
H ₂ O ₂	Hydrogen peroxide
IAA	Iodoacetic acid
IAM	Iodoacetamide
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria Bertani
Na ₂ CO ₃	Sodium carbonate
Na ₂ S ₂ O ₃ .5H ₂ O	Sodium thiosulfate
NADPH	β-nicotinamide adenine dinucleotide phosphate
(NH ₄) ₂ SO ₄	Ammonium sulphate
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PySCeS	Python Simulator of Cellular Systems
Ref-1	Redox factor-1
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SGD	<i>Saccharomyces</i> genome database
TAP	Tandem Affinity Purification
TCA	Trichloroacetic acid
TEMED	N,N,N', N'-tetramethylethylenediamine
Trx	Thioredoxin
YPD	Yeast peptone dextrose

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Chapter 1

1.1 Introduction

Oxygen in its ground state is relatively unreactive and harmless but upon excitation or partial reduction, forms a number of reactive species including the superoxide anion (O_2^-), singlet oxygen (O_2^1), ozone (O_3), and the hydroxyl radical ($\cdot OH$) that can damage a range of intracellular components (Jamieson, 1998). Oxygen-utilizing cells have consequently evolved defense mechanisms to detoxify reactive oxygen species (ROS) (Grant, 2001), reduce the rate of their production, and repair the damage caused by them (Ross *et al.*, 2000). Oxidative stress is a state within the cell in which the level of ROS exceeds the available antioxidant defenses that scavenge and inactivate them (Ross *et al.*, 2000, Sheehan *et al.*, 2010). The thioredoxin system is an important conserved system that protects the cell against oxidative stress by aiding in the reduction of peroxides into harmless products and repairing oxidatively damaged proteins (Toledano *et al.*, 2007).

Thioredoxin (Trx), nicotinamide adenine dinucleotide phosphate (NADPH) and thioredoxin reductase comprise the thioredoxin system which is widely distributed among prokaryotes and eukaryotes (Arnér and Holmgren, 2006). This system was first discovered by Reichard and coworkers in 1964 as a hydrogen donor for the enzymatic synthesis of cytidine deoxyribonucleoside diphosphate by ribonucleotide reductase in *Escherichia coli* (Laurent *et al.*, 1964). Since then, the thioredoxin system has been implicated in many cellular functions (Figure 1.1) including the synthesis of the other deoxyribonucleotides (Toledano *et al.*, 2007), maintaining the reduced environment of the cell, redox control of transcription factors and stimulating cell growth (Karlenius and Tonissen, 2010). However, elevated levels of this system have been associated with a number of pathologies (Figure 1.1) including HIV (Nakamura *et al.*, 1996, Nakamura *et al.*, 2001), asthma (Yamada *et al.*, 2003), diabetes (Thirunavukkarasu *et al.*, 2007) and malaria (Nickel *et al.*, 2006). Studies on human lung cancer have also shown that Trx and its downstream antioxidants, peroxiredoxin 1 and 2, are upregulated in cancerous tissue and may represent an adaptation by tumour cells for increased proliferation (Kim *et al.*, 2003, Park *et al.*, 2006, Xu *et al.*, 2012). A study involving the analysis of the plasma Trx1 levels in first episode schizophrenic patients

showed increased levels of Trx1 in comparison to healthy control individuals (Zhang *et al.*, 2009).

Therefore understanding the regulation of the thioredoxin system is essential for the treatment of these pathologies.

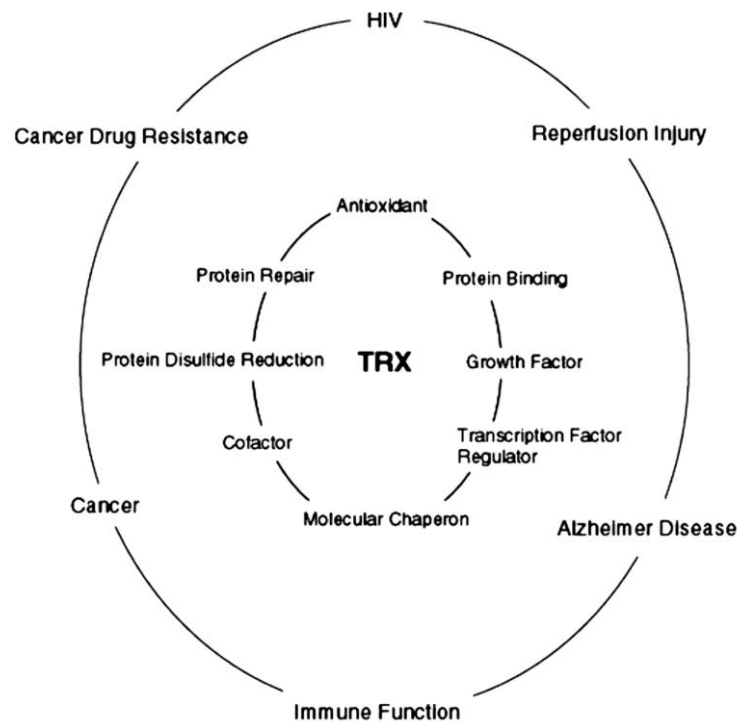


Figure 1.1 Roles of thioredoxin. Trx participates in essential processes (inner circle) and is involved in either pathophysiological processes or in major disease states (outer circle) (Hirota *et al.*, 2002).

1.2 The thioredoxin system

1.2.1 Thioredoxin reductase

Thioredoxin reductase is a member of a family of pyridine nucleotide-disulfide oxidoreductases (Williams, 1995, Arnér and Holmgren, 2000b). Members of this family are homodimeric flavoproteins, in which each subunit has an NADPH binding site, a redox-active disulfide bond and a tightly bound flavin adenine dinucleotide (FAD) prosthetic group

(Williams, 1995, Zhong *et al.*, 2000). This FAD group mediates the transfer of reducing equivalents from NADPH to its own disulfide bond, and then to the disulfide bond of the substrate (Holmgren and Bjornstedt, 1995, Williams, 1995). Thioredoxin reductase is the only known enzyme to reduce oxidized Trx (Figure 1.2). It is also able to reduce non-disulfide substrates such as hydrogen peroxide (Takemoto *et al.*, 1998).

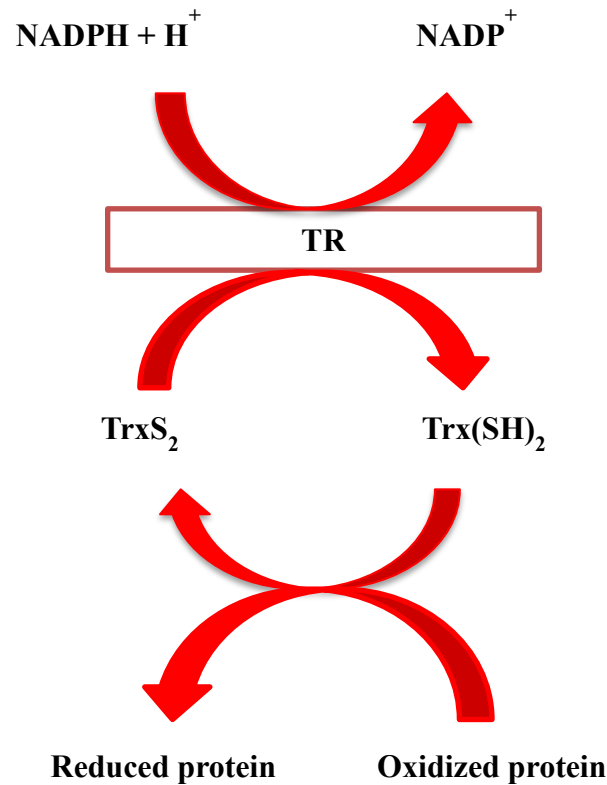


Figure 1.2 The thioredoxin system. Reduced Trx (Trx(SH)₂) directly reduces oxidized proteins and is oxidized (TrxS₂). The regeneration of Trx from its oxidized form is catalyzed by thioredoxin reductase (TR) by using NADPH + H⁺ (Lillig and Holmgren, 2007).

Thioredoxin reductases can be classified into two types, based on size. The first type has a higher molecular mass of approximately 55 kDa and is generally found in animals (Gasdaska *et al.*, 1995) and protozoa such as the malaria parasite (Krnajski *et al.*, 2001). The second type, present in archaea, bacteria, and lower eukaryotes (such as yeast), has a lower molecular mass of 35 kDa (Williams, 1995, Hirt *et al.*, 2002). The larger thioredoxin reductase found in complex eukaryotic organisms is more closely related to glutathione reductase than to bacterial thioredoxin reductase and has a broader substrate specificity

(Mustacich and Powis, 2000) and an additional selenium redox active motif (Arnér and Holmgren, 2000b, Zhong *et al.*, 2000).

1.2.2 Thioredoxin

Trx is a low molecular weight (12 kDa), thermostable and ubiquitous protein that can be localized in the cytoplasm, in the membranes, in the mitochondrial eukaryotic cell fractions as well as in the extracellular space (Das *et al.*, 1999). This redox-active protein has a conserved Cys-Gly-Pro-Cys catalytic site that undergoes reversible oxidation/reduction of the two cysteine (Cys) residues (Štefanková *et al.*, 2005) and Trx can exist either in a reduced (thioredoxin-(SH)₂) or oxidized form (thioredoxin-S₂). The overall difference between the oxidized and reduced forms of Trx is subtle (Holmgren, 1995) and simply involves a local conformational change in and around the redox-active disulfide (Arnér and Holmgren, 2000b). In its oxidized form, Trx is more stable than reduced Trx (Figure 1.3) (Collet and Messens, 2010) and contains a single-redox-active disulfide that is formed from the two half-cysteine residues of the protein. In the presence of thioredoxin reductase and NADPH, this disulfide is opened and the reduced form of Trx is subsequently formed (Collet and Messens, 2010). Therefore, reduced Trx has a dithiol whereas oxidized Trx has a disulfide (Holmgren and Bjornstedt, 1995). The active form of Trx (thioredoxin-(SH)₂) becomes reoxidized whilst providing reducing equivalents to target molecules.

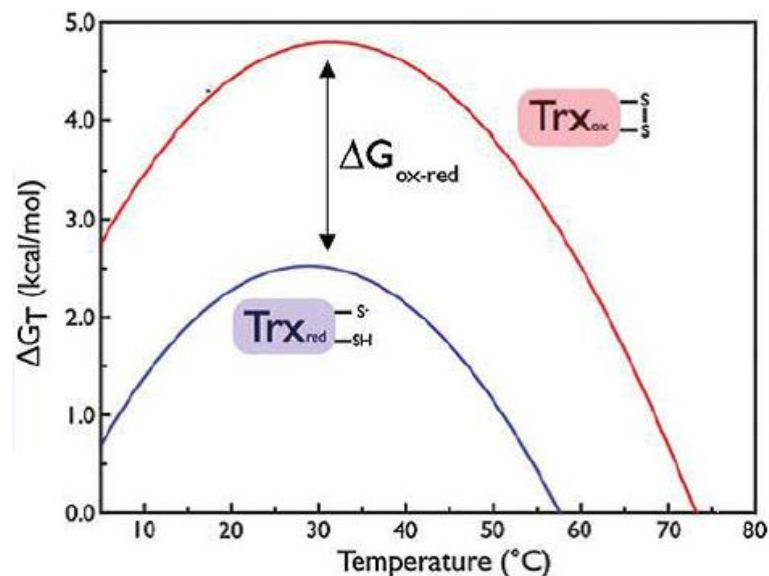


Figure 1.3 Thermodynamic stability of Trx as a function of temperature. Oxidized Trx (red curve) is more stable than reduced Trx (blue curve) (Collet and Messens, 2010).

Saccharomyces cerevisiae contains three isoforms of Trx. Trx1 and Trx2 are located in the cytosol (Gan, 1991, Xu and Wickner, 1996, Trotter and Grant, 2005) whereas Trx3 is located in the mitochondrial matrix (Pedrajas *et al.*, 1999, Herrero *et al.*, 2008).

1.2.2.1 Reduction mechanism of Trx

Trx has a hydrophobic surface patch that enables it to make a complex with an oxidized substrate. Upon the formation of this complex, nucleophilic attack by the thiolate of Cys32 found in Trx results in the formation of a transient mixed disulfide with substrate. This is followed by a nucleophilic attack of the deprotonated Cys35 which generates oxidized Trx and the reduced substrate (Holmgren, 1985). Oxidized Trx, in turn, needs to be reduced by thioredoxin reductase to become active again (Figure 1.2).

1.2.3 Connectivity of the thioredoxin system

The Trx system participates in multiple reactions and several omic techniques and methods have been developed to identify the proteins that interact intra-cellularly with Trx. These methods have led to the identification of a large variety of potential Trx substrates. An example of such an approach is one in which the C-terminal cysteine of the catalytic site of Trx is mutated to an alanine (Balmer *et al.*, 2004). The C-terminal cysteine is essential for the dissociation of the mixed disulfide complex that results in the release of a reduced substrate protein and oxidized Trx. Mutation of the C-terminal cysteine prevents this dissociation, thereby allowing the formation of stable complexes between Trx and its substrates. The mutant protein can be expressed *in vivo* and the complexes are then purified by affinity chromatography (Depuydt *et al.*, 2009).

An alternate approach involves differential thiol-labeling (Figure 1.4) that identifies the proteins kept reduced by Trx. The *in vivo* differential thiol trapping technique was developed by Leichert and Jakob (2004). In this approach, the accessible thiol groups are carbamidomethylated (CAM) with iodoacetamide (IAM) and blocked for the subsequent reduction and alkylation steps. DTT is used to reduce the disulfide bonds and the newly accessible thiols are then labeled with radioactive iodoacetamide (¹⁴C-IAM). A radioactive label is therefore incorporated into the proteins that originally contained disulfide bonds.

Proteins that require Trx for reduction can be identified through a comparison of cellular extracts prepared from wild-type and Trx-knockout strains.

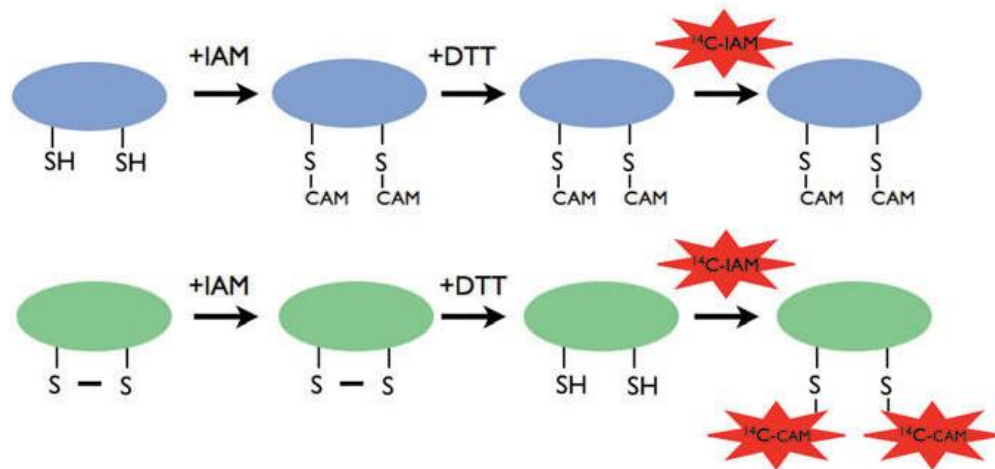


Figure 1.4 Trapping Trx substrates. Extracts were quenched under acid conditions and cysteines in their thiol form were alkylated using unlabeled iodoacetamide (IAM). Disulfide bonds were reduced with dithiothreitol (DTT) and newly accessible thiol groups were modified with [¹⁴C]-labeled IAM. A radioactive label was therefore incorporated into the proteins that originally contained disulfide bonds (Collet and Messens, 2010).

Another approach in which Trx substrates have been identified is through the purification of proteins bound to Trx using Tandem Affinity Purification (TAP) followed by MS/MS mass spectrometrical analysis (Kumar *et al.*, 2004). The procedure involved a TAP-tag being appended to the C-terminus of *E. coli* Trx1 and thereafter the Trx-substrate complexes were purified using two affinity chromatography steps. Through this method, a total of 80 proteins have been identified, implicating *E. coli* Trx1 in approximately 26 cellular processes (Kumar *et al.*, 2004).

The biosynthetic reactions in which Trx has been implicated include providing reducing equivalents to ribonucleotide reductase, thioredoxin peroxidase (Pigiet and Conley, 1977) and methionine sulfoxide reductases (Sengupta and Holmgren, 2012), the regeneration of oxidatively damaged proteins and scavenging reactive oxygen species as well as other free radicals (Das and Das, 2000). Through redox regulation, several transcription factors are activated by Trx which modulates their DNA binding activities (Holmgren and Lu, 2010, Karlenius and Tonissen, 2010). In mammalian cells, Trx directly modulates the activity of

various transcription factors such as AP-1 and p53 (Mukherjee and Martin, 2008). Some transcription factors can be indirectly reduced by Trx which reduces Ref-1 (redox factor-1) which in turn reduces other transcription factors (Dempfle *et al.*, 1991, Robson and Hickson, 1991, Robson *et al.*, 1991).

Transcription factors dependent on the Trx/Ref-1 interaction are responsible for the activation of many genes that have the overall effect of promoting cell viability in response to adverse conditions including oxidative stress and hypoxia (Karlenius and Tonissen, 2010). Ref-1 functions not only as a major redox-signaling factor but is also a DNA-repair endonuclease, and is involved in the base excision repair (BER) pathway (Dempfle *et al.*, 1991). This pathway is responsible for restoring apurinic/aprimidinic (AP) sites in DNA, which are a major end product in reactive oxygen species (ROS) damage (Karlenius and Tonissen, 2010).

In plants, the thioredoxin system provides a crucial link between the activities of electron transport and carbon assimilation (Meyer *et al.*, 2008). Plant Trxs are involved in multiple processes such as photorespiration, lipid and hormone metabolism, membrane transport, ATP synthesis (Balmer *et al.*, 2004) and seed germination (Joudrier *et al.*, 2005). In the chloroplast, the activities of a number of enzymes including four enzymes of the Benson-Calvin cycle, fructose biphosphatase, sedoheptulose biphosphatase, ribulose-5-P kinase and glyceraldehyde-P dehydrogenase, are all regulated by Trx (Dey and Harborne, 1997). Other chloroplastic enzymes regulated by Trx include NADP-malate dehydrogenase, phenylalanine ammonia lyase and glucose-6-phosphate dehydrogenase (Dey and Harborne, 1997). A Trx isoform, Trx h has been associated in the reduction of seed α -amylase and trypsin inhibitors from several sources. In soybean, the Kunitz and Bowman-Birk trypsin inhibitors lose their ability to inhibit trypsin after reduction by Trx h (Jiao *et al.*, 1992). In 2009, it was reported that approximately 500 proteins had been identified as potential or established Trx targets in land plants and oxygenic photosynthetic microorganisms (Montrichard *et al.*, 2009).

The Trx system also participates in multiple reactions in *Escherichia coli*, including the reduction of sulfate which is catalyzed by an enzyme called 3'-phosphoadenosine 5'-phosphosulfate (PAPS) reductase (Tsang, 1981). Another Trx-dependent reaction in *E. coli* is the reduction of methionine sulfoxide by methionine sulfoxide reductase (Lillig and Holmgren, 2007). In addition to this, Trx also provides reducing equivalents to the inner membrane protein DsbD which transfers electrons to the periplasm (Rietsch *et al.*, 1997,

Chung *et al.*, 2000). In *S. cerevisiae*, the Trx system is also an important defense mechanism against cadmium (Vido *et al.*, 2001) and has also been identified as a reducing agent in the reduction of methionine sulfoxide to sulfate (Wilson *et al.*, 1961, Gan, 1991).

The cited literature merely provides a brief description of the scale of the Trx system. It is evident that this system has diverse interactions with various protein and non-protein substrates.

1.3 Discussion

Many diseases, including those mentioned above (Section 1.1), involve a large variety and number of elements that interact via complex networks and nonlinear interactions (Wang, 2010). Therefore, knocking out a single target molecule in a biochemical pathway may be insufficient for treating a disease such as cancer, because the cells find alternate molecular pathways to escape the blockage (Wang, 2010). For this reason, a number of current drug design strategies are often ineffective. It is increasingly believed that a systems approach, rather than the current gene-centric view could solve these problems (Wang, 2010, Loscalzo and Barabasi, 2011). Systems biology helps to provide an understanding of complex phenomena by generating detailed interaction maps of various cellular networks and by developing sophisticated mathematical and computational methods and tools with which to analyze these networks. Understanding the complex systems involved in various pathologies will make it possible to develop smarter therapeutic strategies which could lead to significant advances in the treatment of disease (Wang, 2010).

The study of the complete, integrated Trx network (Figure 1.1) was difficult and therefore the individual reactions within the network have usually been studied in isolation. Although such approaches have produced a significant amount of information and understanding, they have been limited in their ability to predict the effects of alterations in single or multiple components upon the dynamics of the whole network. Therefore analysis of the redox regulation of this network using a systems biology approach would represent a significant advance. A first step in any systems biology approach, such as kinetic modeling, is to define clearly the components under study (Pillay *et al.*, 2013). However, with Trx, it was not clear how this protein should be modeled and due to contrasting *in vivo* and *in vitro* descriptions, construction of models of the thioredoxin system have been complicated (Pillay

et al., 2011). Redoxins have been described as both redox couples with redox potentials (Figure 1.5 A), and as enzymes with Michaelis–Menten parameters (Figure 1.5 B) as they exhibit properties of both. For computational modeling studies, if redoxins were treated as redox couples, then the couples would be variables within computational models of these systems and each member of a couple would be described with an ordinary differential equation. Alternatively, if redoxins were modeled as enzymes, then their rate constants and concentrations would be parameters within the rate vectors of these models (Pillay *et al.*, 2013).

There are a number of inconsistencies in the description of redoxins as enzymes (Pillay *et al.*, 2009). Using computational systems biology tools, our lab proposed an approach to describe redoxin activity in systems biology applications (Pillay *et al.*, 2009). In contrast to the long held view that Trx is an enzyme, it was suggested that the enzymatic properties attributed to Trx resulted from the saturation of the Trx redox cycle. With increasing concentrations of substrate, the thioredoxin reductase concentration became rate-limiting and therefore the entire system becomes saturated with the consequent re-distribution of oxidized and reduced Trx (Figure 1.5 C). However, further confirmatory studies are required before this modeling approach can be generally accepted. The main aim of this study was to utilize computational and molecular methods to confirm or reject the proposed model for thioredoxin activity.

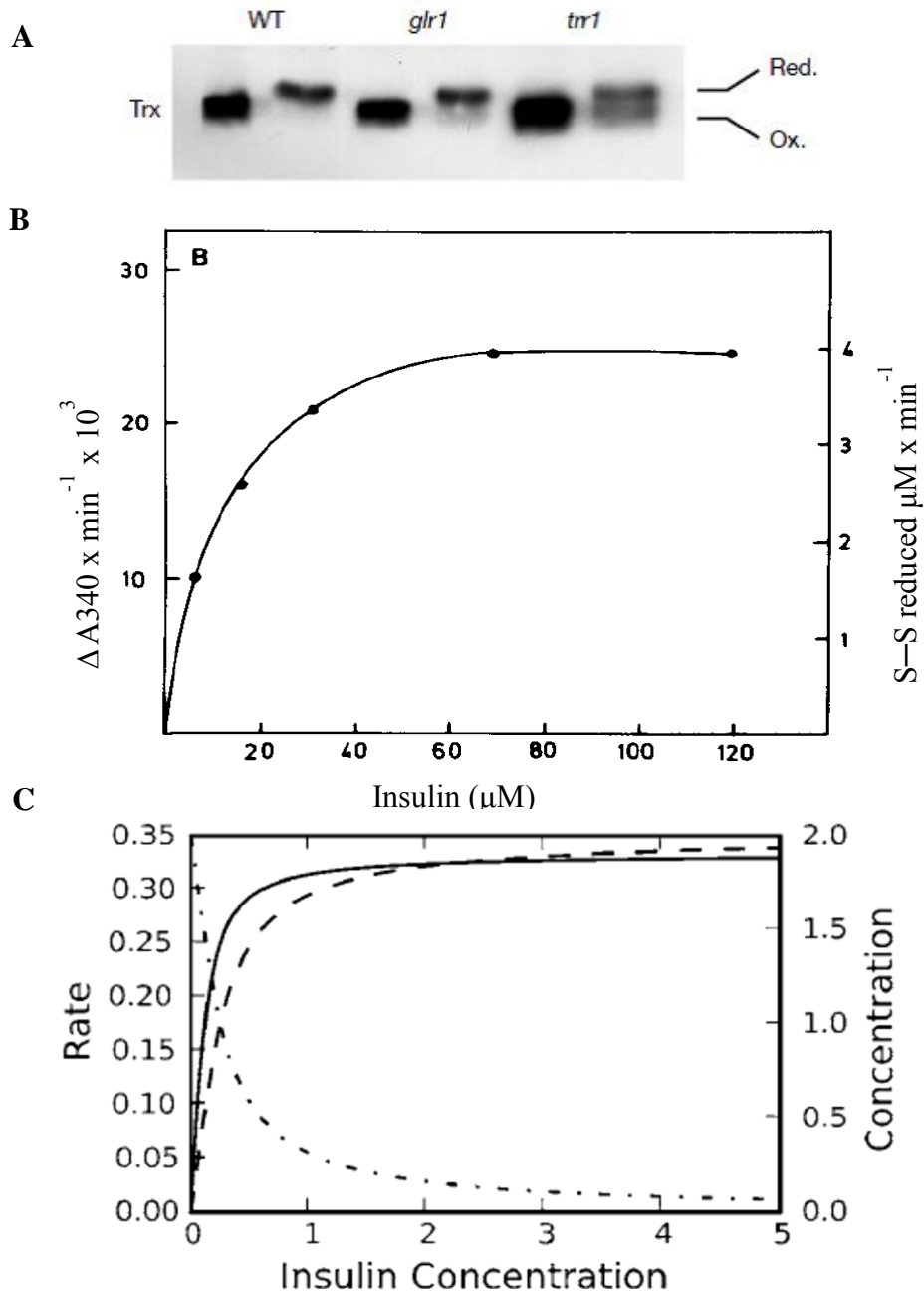


Figure 1.5 Differing descriptions of Trx activity. Redoxins have been described as redox couples (with redox potentials) (A) (Trotter and Grant, 2003) and as enzymes (with Michaelis–Menten parameters) (B) (Holmgren, 1979a). Due to the number of inconsistencies in the description of redoxins as enzymes, a new approach for describing redoxin activity was proposed (C) (Pillay *et al.*, 2009) where it was suggested that with increasing concentrations of substrate, the thioredoxin reductase concentration became rate-limiting and therefore the entire system becomes saturated with the consequent re-distribution of oxidized (broken line) and reduced (dash-dot line) Trx.

Chapter 2: Materials and basic methods

This chapter describes all the methods that were routinely carried out during the course of this research project.

2.1 Materials

Acrylamide-Bis ready to use solution (37.5:1) and Polyethylene glycol (PEG) 20 000 were obtained from Merck (South Africa) while TEMED, ammonium persulfate, Coomassie brilliant blue G-250 powder, Coomassie brilliant blue R-250 powder, *t*-butanol and BSA were obtained from Sigma (Capital Labs, South Africa). Agarose for gel electrophoresis was purchased from Whitehead Scientific (Pty) Ltd (Cape Town, South Africa). All other common reagents were obtained from Saarchem (Merck, South Africa).

2.2 Bradford assay

The Bradford assay is based on the binding of Coomassie brilliant blue G-250 to protein. The dye exists in three forms, an anionic blue form, a neutral green form and a cationic red form. The binding of the cationic form of the dye to the protein causes a shift in the absorption maximum of the dye from 365 nm to 595 nm and this allows for spectrophotometric quantification of the protein. The protein-dye complex has a high extinction coefficient thus leading to great sensitivity in measurement of the protein (Bradford, 1976).

This assay is reproducible, accurate and rapid. Unlike other protein assays, this assay is less susceptible to interference by various chemicals such as Tris and EDTA that may be present in protein samples (Bradford, 1976).

2.2.1 Preparation of reagents

2.2.1.1 Dye reagent

Coomassie brilliant blue G-250 (0.6 g) was dissolved in 1 liter of perchloric acid (2% (v/v)). The solution was stirred for 1 hour and thereafter filtered through Whatman No. 1 filter paper. The resulting solution was stored in an amber coloured bottle. Visual checks for

precipitation were made prior to use. If precipitation was present, a new batch of reagent was made.

2.2.1.2 Standard protein solution

A 1 mg/ml bovine serum albumin solution was made up in dH₂O.

2.2.2 Method

Samples were diluted to 100 µl with dH₂O, dye reagent (900 µl) was added and the solution was briefly vortexed. The solution was allowed to stand for 5 minutes for colour development and thereafter transferred to plastic cuvettes (1.5 ml). The absorbance was read at 595 nm against appropriate blanks. A standard curve, relating absorbance at 595 nm to 0-100 µl (0-100 µg) of the standard protein was constructed.

Beyond 50 µg of the standard protein, the curve began to plateau making the readings at these points unreliable therefore a second curve was generated using the linear portion of the initial curve (0-50 µg).

2.3 Tris-Tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Tricine SDS-PAGE is commonly used to resolve smaller proteins in the mass range 1-100 kDa which are sometimes poorly resolved by the conventional Laemmli SDS-PAGE system (Schägger, 2006). In this system, tricine is used as the trailing ion in the stacking phase and allows a resolution of small proteins at lower acrylamide concentrations than in glycine SDS-PAGE systems (Schägger and von Jagow, 1987).

2.3.1 Preparation of reagents

2.3.1.1 Gel buffer (3 M Tris-HCl, 0.3% (m/v) SDS, pH 8.45)

Tris (72.7 g) was dissolved in 200 ml dH₂O, and adjusted to pH 8.45 with HCl. 10% (m/v) SDS (6 ml) was added and the solution was made up to 250 ml.

2.3.1.2 SDS (10% (m/v))

SDS (10 g) was dissolved in dH₂O and made up to 100 ml.

2.3.1.3 Anode buffer (0.2 M Tris-HCl, pH 8.9)

Tris (24.22 g) was dissolved in 950 ml of dH₂O, adjusted to pH 8.9 with HCl and made up to 1 liter.

2.3.1.4 Cathode buffer (0.1 M Tris-HCl, 0.1 M Tricine, 0.1% (m/v) SDS, pH 8.25)

Tris (12.1 g), Tricine (17.9 g) and 10% (m/v) SDS (10 ml) were made up to 800 ml with dH₂O and the pH adjusted if necessary. The buffer was made to a final volume of 1 liter.

2.3.1.5 Initiator (10% (m/v))

Ammonium persulfate (0.2 g) was made up to 2 ml with dH₂O just before use.

2.3.1.6 Treatment buffer (125mM Tris-HCl, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2- mercaptoethanol, 0.01% (m/v) bromophenol blue, pH 6.8)

Stacking gel buffer (2.5 ml), glycerol (2 ml), 10% (m/v) SDS (4 ml), 2-mercaptoethanol (1 ml) and bromophenol blue (0.01% (m/v)) were made up to 10 ml with dH₂O. Samples were boiled for 5 minutes in treatment buffer before electrophoresis.

2.3.2 Method

The compositions of the stacking and resolving gels used are described in Table 2.1. The gels were run at 42 mA with unlimited voltage and were stopped when the tracking dye reached the bottom of the gel. The gels were usually stained with Coomassie (Section 2.4) but when low concentrations (nanogram range) of proteins were loaded onto the gel, the gel was stained by silver staining (Section 2.5).

2.4 Coomassie stain

Coomassie Blue R-250 is an anionic dye that binds to proteins. Formation of the protein/dye complex stabilises the negatively charged anionic form of the dye producing the blue colour which may then be seen in the gel. Although 50-fold less sensitive than silver staining, Coomassie Blue staining is a relatively simple and more quantitative method (Dennison, 2003).

Table 2.1 Preparation of the resolving and stacking gels for Tris-tricine SDS-PAGE

Reagents	Resolving gel (ml)	Stacking gel (ml)
Monomer	8.0	0.67
Gel Buffer	4.0	1.25
dH ₂ O	3.71	3.0
Ammonium persulfate	0.240	0.050
TEMED	0.016	0.010

2.4.1 Preparation of reagents

2.4.1.1 Coomassie blue stain (0.125% (m/v) Coomassie brilliant blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid)

Coomassie brilliant blue R-250 powder (0.125 g), methanol (50 ml) and acetic acid (10 ml) were made up to 100 ml with dH₂O.

2.4.1.2 Destain I (50% (v/v) methanol, 10% (v/v) acetic acid)

Methanol (500 ml) and acetic acid (100 ml) were made up to 1 liter with dH₂O.

2.4.1.3 Destain II (5% (v/v) methanol, 7% (v/v) acetic acid)

Methanol (50 ml) and acetic acid (70 ml) were made up to 1 liter with dH₂O.

2.4.2 Method

All steps were carried out at room temperature in clean containers. Glassware and plasticware were thoroughly washed with ddH₂O. Upon completion of electrophoresis, gels were soaked in Coomassie blue stain overnight at room temperature with gentle agitation. The stain was then removed and the gels were soaked in destain I on a shaker until background staining was reduced. Gels were then transferred to destain II and thereafter photographed under white light.

2.5 Silver staining

Silver staining has a high sensitivity and can detect protein in the low nanogram range. This method involves binding of silver ions to amino acid residues followed by reduction to metallic silver (Chevallet *et al.*, 2006). This procedure can be divided into five main phases. In the first phase, the gel is fixed to eliminate interfering substances such as SDS, amino acids and Tris that cause a high background and therefore poor contrast. In the second phase, the gel is sensitized. In the third phase, the gel is impregnated with the silvering agent. Two main families of silver staining methods can be distinguished at this step, those using silver nitrate solutions as the silvering agent (acidic methods) and those using a basic silver-ammonia or less frequently a silver-amine complex (basic methods). In the fourth phase, the image is developed, generally by a dilute formaldehyde solution and in the final phase, the reaction is stopped to prevent overdevelopment (Rabilloud *et al.*, 1994).

2.5.1 Preparation of reagents

2.5.1.1 Fixing solution (30% (v/v) ethanol, 10% (v/v) acetic acid)

Ethanol (60 ml) and acetic acid (20 ml) were made up to 200 ml with dH₂O.

2.5.1.2 Wash solution (20% (v/v) ethanol)

Absolute ethanol (40 ml) was made up to 200 ml with dH₂O.

2.5.1.3 Impregnation solution (0.2% (m/v) AgNO₃, 0.075% (v/v) formaldehyde)

AgNO₃ (0.4 g) and 37% formaldehyde (0.15 ml) were made up to 200 ml with dH₂O and stored in a dark cupboard.

2.5.1.4 Reduction solution (0.02% (m/v) Na₂S₂O₃·5H₂O)

Na₂S₂O₃·5H₂O (0.1 g) was made up to 500 ml with dH₂O.

2.5.1.5 Development solution (3% (m/v) Na₂CO₃, 0.0005% (m/v) Na₂S₂O₃·5H₂O, 0.05% (v/v) formaldehyde)

Na₂CO₃ (30 g), Na₂S₂O₃·5H₂O (0.005 g) and 37% formaldehyde (0.5 ml) were made up to 1 liter with dH₂O.

2.5.1.6 Stop solution (5% (v/v) acetic acid)

Acetic acid (5 ml) was made up to 100 ml with dH₂O.

2.5.2 Method

All steps were carried out at room temperature in clean containers. All containers and volumetric flasks were washed with ddH₂O. Upon completion of electrophoresis, gels were soaked in fixing solution overnight. The gels were thereafter rinsed with wash solution (2 x 10 min) and ddH₂O (2 x 10 min). The gels were treated with reduction solution (1 min), rinsed with ddH₂O (3 x 30 sec) and soaked in impregnation solution (20 min). After rinsing with ddH₂O (3 x 30 sec), the gels were immersed in development solution until the bands became visible. The gels were rinsed with ddH₂O (30 sec), treated with stop solution (10 min) and thereafter stored in dH₂O.

2.6 Agarose gel electrophoresis

2.6.1 Preparation of reagents

2.6.1.1 Loading buffer (30% (v/v) glycerol, 0.25% (m/v) bromophenol blue)

Bromophenol blue (0.01 g) was added to 80% glycerol (1.5 ml) and the volume was made up to 4 ml with dH₂O.

2.6.1.2 50 x TAE

A 50 x TAE stock solution was made by dissolving Tris (24.2 g), glacial acetic acid (5.71 ml) and 10 ml 0.5 M EDTA (pH 8.0) in 1 liter dH₂O.

2.6.2 Method

Analysis of isolated plasmid DNA was carried out on 1 % (w/v) agarose gels while restriction digestion and PCR products were carried out on 2 % (w/v) agarose gels. Gels were prepared by dissolving agarose in 50 ml 1 x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA (pH 8.0)) by gentle heating over a bunsen burner. Once cooled, 2 µl ethidium bromide (10 mg/ml) was added, the gel was poured into the casting tray and allowed to polymerize (30 min). Gels were run at 90 V until the bromophenol blue in the loading dye had migrated approximately $\frac{3}{4}$ down the gel and thereafter photographed under ultraviolet

light. A standard curve, relating log molecular weight to the distance travelled by each fragment (mm) was constructed and used to determine the size of the bands of interest, through extrapolation.

2.7 DNA quantification

The NanoDrop™ 2000 UV-Vis Spectrophotometer (Thermo Scientific, South Africa) was used to determine the purity and concentration of the DNA. The concentration was measured in ng/μl.

2.8 Concentration of protein samples

Dilute protein samples were concentrated prior to kinetic analysis. A simple method of concentrating samples was by dialysis against polyethylene glycol (PEG). Trx and thioredoxin reductase samples were placed in dialysis tubing with a cut-off of 3.5 kDa and 10 kDa, respectively and dialyzed against PEG (20 kDa).

Chapter 3: Purification of the yeast thioredoxin system

3.1 Introduction

The initial step in the isolation of a protein is to choose a method of measuring the presence of the protein and of distinguishing it from all other proteins that might be present in the same material. This is achieved by an activity assay which simply measures the unique activity of the protein and allows various materials to be analyzed so that the one containing the largest amount of the desired protein, can be used as the starting material (Dennison, 2003). Usually, the more specific the assay, the more effective the purification is (Berg *et al.*, 2002).

Once a source material has been selected, the protein must be extracted in a soluble form suitable for manipulation. The object of extraction is to get the target protein out of the cellular material where it is located and into solution so that it can be manipulated. This can be achieved by homogenisation, which disrupts the tissues and breaks open the cells to release their contents. The extract can then be clarified by either filtration or centrifugation. The crude extracts containing the desired protein are thereafter purified on the basis of characteristics such as solubility, size, charge and specific binding affinity (Dennison, 2003).

Various methods have been used in the purification of Trx and thioredoxin reductase. The initial methods employed were tedious and time consuming and were replaced with cloning technologies which were simpler and faster (Buchanan *et al.*, 2012). The sections below describe the materials and methods used to isolate the Trx system. The methods section is divided into separate sub-sections with descriptions because of the range of techniques covered. All the results are presented in a single section (Section 3.13).

3.2 Materials

Dithiothreitol (DTT), bovine pancreas insulin, 5, 5'-dithiobis(2-nitrobenzoic acid) (DTNB), β -nicotinamide adenine dinucleotide phosphate (NADPH), bovine serum albumin (BSA), isopropyl β -D-1-thiogalactopyranoside (IPTG), kanamycin, ampicillin, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) and the diethylaminoethanol (DEAE) Sepharose were obtained from Sigma (Capital Labs, South Africa) while RNase A was obtained from Roche (South Africa). A Rapid DNA Ligation Kit, the Fermentas Gel

Extraction Kit, the InsTAclone™ PCR cloning kit, TransformAid™ Bacterial Transformation Kit and Taq™ DNA polymerase were obtained from Inqaba Biotech (Johannesburg, South Africa) while the QIAquick™ Gel Extraction Kit and Ni-NTA agarose were obtained from Whitehead Scientific (Pty) Ltd (Cape Town, South Africa). The New England Biolabs HindIII, NdeI and BamHI restriction enzymes were obtained from The Scientific Group (Midrand, South Africa) and all other common chemicals were obtained from Saarchem (Merck, South Africa) and were of the highest purity available. pET28a was generously donated by Professor Dean Goldring (Biochemistry, UKZN) and the thioredoxin reductase clones (pMPTRRA, B, C and D) were generously supplied by Miss M.M Photolo (MSc candidate, UKZN).

3.3 Preparation of common reagents

The preparation of all common reagents has been described in the text below whilst the preparation of specialized reagents will be described later in the chapter.

3.3.1 DTT

A fresh stock solution of DTT (1 M) was prepared at the time of use by dissolving DTT powder (0.154 g) in ddH₂O (1 ml). 200 µl of this stock solution was made up to 2 ml with ddH₂O and the resulting DTT solution (final concentration 100 mM) was used in the Trx activity assay.

3.3.2 Bovine pancreas insulin

A 1.6 mM stock solution of insulin was prepared by suspending insulin (50 mg) in 0.5 ml 100 mM potassium phosphate buffer (pH 6.5). The pH was adjusted to 2-3 with 1 M HCl to dissolve the protein completely and was thereafter titrated back to the original pH of the buffer (pH 6.5) with NaOH (1 M). Finally, the volume was adjusted to 5 ml with ddH₂O. The clear insulin solution was stored at – 20°C.

3.3.3 DTNB

A stock solution of DTNB (63.1 mM, final concentration) was freshly prepared by dissolving DTNB powder (0.025 g) in 99% ethanol (1 ml). The resulting solution was stored in the dark as DTNB is light sensitive.

3.3.4 NADPH

Stock vials were prepared by keeping 10 mg portions of NADPH (dry) in separate small microcentrifuge tubes at -20°C. At the time of use, a single tube was dissolved in ddH₂O (250 µl). The stock solution (final concentration 50 mM) was stored at -20°C.

3.3.5 IPTG stock solution

A stock solution (100 mM) was prepared by dissolving IPTG powder (0.238 g) in dH₂O and the volume was made up to 10 ml. This suspension was sterilized by passing it through a 0.2 µm filter.

3.3.6 Kanamycin stock solution

A stock solution (30 mg/ml) was prepared by dissolving kanamycin sulphate (0.3 g) in dH₂O. The volume was made up to 10 ml. This suspension was sterilized by passing it through a 0.2 µm filter and stored in 1 ml aliquots at -20°C.

3.3.7 Ampicillin stock solution

A stock solution (25 mg/ml) was prepared by dissolving ampicillin sodium salt (0.25 g) in dH₂O. The volume was made up to 10 ml. This suspension was sterilized by passing it through a 0.2 µm filter and stored in 1 ml aliquots at -20°C.

3.3.8 Bacterial growth media

3.3.8.1 Luria Bertani (LB) broth

Tryptone (1% (w/v)), yeast extract (0.5% (w/v)) and NaCl (0.5% (w/v)) were dissolved in dH₂O and the solution was made up to the desired volume. The solution was autoclaved and stored at room temperature. When necessary, the appropriate antibiotic, either

kanamycin (30 µg/ml, final concentration) or ampicillin (50 µg/ml, final concentration) was added.

3.3.7.2 Luria Bertani (LB) agar

Tryptone (1% (w/v)), yeast extract (0.5% (w/v)), NaCl (0.5% (w/v)) and bacteriological agar (1.5% (w/v)) were dissolved in dH₂O and the solution was made up to the desired volume. The solution was autoclaved and upon cooling, media was poured into petri dishes. When necessary, the appropriate antibiotic, either kanamycin (30 µg/ml, final concentration) or ampicillin (50 µg/ml, final concentration) was added. The plates were stored at 4°C.

3.3.7.3 Yeast Peptone Dextrose (YPD)

Yeast extract (1% (w/v)), peptone (2% (w/v)) and dextrose (2% (w/v)) were dissolved in dH₂O and the solution was made up to the desired volume. The solution was autoclaved and stored at room temperature.

3.3.7.4 SOC media

Yeast extract (0.5% (w/v)), tryptone (2% (w/v)), NaCl (10 mM), KCl (2.5 mM) and MgCl₂ (10 mM) were dissolved in dH₂O and the solution was made up to the desired volume. The solution was autoclaved and once cooled, glucose (20 mM, final concentration) was added. The glucose solution (1 M) was sterilized by passing it through a 0.2 µm filter. The SOC media was then stored at 4°C.

3.3.7.5 2xYT media

Tryptone (1.6% (w/v)), yeast extract (1% (w/v)) and NaCl (0.5% (w/v)) were dissolved in dH₂O. The pH was adjusted to 7.0 with NaOH and the volume was made up with dH₂O. The solution was sterilized by autoclaving and thereafter stored at room temperature.

3.4 The yeast thioredoxin activity assay

3.4.1 Introduction

There are a number of assays that have been developed to test the activity of Trx. Trx activity was measured using insulin as a substrate where the reduction of insulin by Trx was followed spectrophotometrically at 650 nm (Arnér and Holmgren, 2000a). Reduced Trx was then regenerated by thioredoxin reductase and NADPH. Mark and Richardson (1976) utilized an alternate activity assay that measured the reduction of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) by Trx to form a yellow product measured at 412 nm and reduced Trx was again regenerated by thioredoxin reductase and NADPH. However, both methods possessed a significant limitation as purified thioredoxin reductase was required. Although commercial kits, such as the fluorescent thioredoxin assay available from IMCO Corporation Ltd, have all the necessary components for the assay, they are relatively expensive. In conclusion, current assays for Trx are constrained by the cost and availability of pure enzymes and therefore it was important to develop a novel Trx activity assay that was inexpensive and allowed for the quick identification of the target molecules from crude extracts.

Holmgren (1979b) performed an experiment to determine the rate of the reduction of insulin disulfides by DTT. He showed that the addition of *E. coli* Trx to the reaction mixture increased the rate of reduction significantly. Based on these findings, we deduced that if Trx increased the rate of insulin disulfide reduction in the presence of DTT, this could be used as the basis for an activity assay for Trx. As pure thioredoxin reductase was no longer needed, the cost of the assay was greatly reduced. However, a potential problem with this assay and the above mentioned assays was that they took over 20 min before a result was obtained.

3.4.2 Methods

To determine the activity of Trx, a number of assay conditions were tested based on data in Holmgren (1979b). These conditions included variations in insulin (0.01 mM and 0.13 mM) and DTT (0.33 mM and 1 mM) concentrations as well as the pH of the potassium phosphate buffer (pH 6.5 and 7.0). Each reaction mixture contained 2 mM EDTA and proceeded at 25°C (Holmgren, 1979b). A modified version of the original assay (Holmgren,

1979b) involving the incubation of all components for 20 min at 25°C prior to absorbance readings being taken, was also attempted (Sigma-Aldrich, 1994). A method involving the reduction of the Trx by preincubating 0.1 M DTT (10 µl) with the Trx containing crude extract for 10 min at 25°C was developed and used in this study. A master mix containing 84 mM potassium phosphate buffer (pH 7.0), EDTA (2.67 mM) and insulin (0.21 mM) was made into a final volume of 9 ml. Thereafter the reaction was initiated by addition of the preincubated suspension (250 µl) to the master mix (750 µl). The final reaction mixture contained 63 mM potassium phosphate buffer (pH 7.0), EDTA (2 mM), insulin (0.01 mM) and DTT (1 mM) in a final volume of 1 ml. The change in absorbance at 650 nm was directly monitored using a sample without the addition of the crude extract as the reference cuvette. Absorbance measurements were made with a UV-1800 Shimadzu Spectrophotometer.

3.5 The yeast thioredoxin reductase activity assay

3.5.1 Introduction

The thioredoxin reductase assay involves monitoring either NADPH oxidation or direct reduction of a substrate such as 5, 5'-dithiobis(2-nitrobenzoic acid) (DTNB). In the first assay, the reduction of oxidized Trx is measured in a mixture containing potassium phosphate, KCl, EDTA and NADPH as well as oxidized Trx. After addition of the thioredoxin reductase source, the oxidation of NADPH is monitored spectrophotometrically. However, the limitation with this method is that purified Trx is required. Trx, the natural substrate of thioredoxin reductase, is very expensive and difficult to obtain so the activity of thioredoxin reductase is usually assayed using an alternate substrate.

The alternative assay to measure the activity of thioredoxin reductase is by monitoring the change in absorbance at 412 nm occurring as a result of the reduction of DTNB to TNB. The use of DTNB as a substitute for Trx has proved to be sufficiently specific (Arnér and Holmgren, 2000a, Štefanková *et al.*, 2006). It was shown that the use of univalent cations in the thioredoxin reductase activity assay significantly increased the rate of reduction (Lim and Lim, 1995). The role played by the univalent cations is still unknown but it has been proposed that these cations may induce a conformational change in *E. coli* thioredoxin reductase, in which its closed conformation is converted to an open conformation thereby

exposing the active site. Based on this mechanism the exposed active site of thioredoxin reductase can directly interact with DTNB (Lim and Lim, 1995).

3.5.2 Methods

To determine the activity of thioredoxin reductase, a number of assay conditions were tested including DTNB (1 mM - 5 mM) and NADPH (0.3 mM - 0.5 mM) concentrations as well as the pH of the potassium phosphate buffer (pH 7.0 and 7.2) (Arnér and Holmgren, 2000a, Štefanková *et al.*, 2006). The effect of including univalent cations, NaCl, within the reaction was also tested (Lim and Lim, 1995). The final reaction mixture used in this study contained 0.1 M Tris-Cl (pH 8.0), 0.5 mM DTNB, 0.24 mM NADPH, 0.01% BSA, 0.5 M NaCl and crude extract containing TrxR in a final volume of 1 ml. The reaction was initiated by the addition of the crude extract containing thioredoxin reductase and proceeded at 25°C. The change in absorbance at 412 nm was directly monitored using a sample without the addition of the crude extract as the reference cuvette. Absorbance measurements were made with a UV-1800 Shimadzu Spectrophotometer.

3.6 Purification of Trx

3.6.1 Introduction

In order to shorten the extraction time and increase the extraction yield, various extraction techniques for Trx have been developed including ultrasound-assisted extraction, supercritical fluid extraction, hot-water extraction and solvent extraction. Among these, ethanol extraction was promoted as an inexpensive, simple and efficient alternative to conventional extraction methods (Xiong *et al.*, 2009).

An ethanol extraction method was developed in which Trx was excreted directly into the medium by treating *S. cerevisiae* cells with ethanol (Inoue *et al.*, 2007). This extraction was carried out on a large scale (45,000-liter tank) and the ethanol in the extracts was subsequently removed with an evaporator followed by lyophilization. The effect of a number of conditions including ethanol concentration (0 – 20% (v/v)) and temperature (4-37°C) on the release of Trx from *S. cerevisiae* were tested. Trx release was monitored by Western blot

analysis and it was shown that at 37°C, almost the maximum amount of Trx could be extracted in 2 hours with 20% ethanol (Inoue *et al.*, 2007).

Usually solvent disruption of cells results in the release of various water-soluble proteins which can complicate purification. However, if ethanol extraction was performed under appropriate conditions (Inoue *et al.*, 2007), the cells preserved their membranes and most intracellular proteins were retained within the intact plasma membranes. In addition, ethanol offered several advantages including lower protein concentration in the extraction broth which ultimately may reduce the cost and complexity of the purification process (Xiong *et al.*, 2009). This method was proposed to be efficacious for the preparation of the starting material for the purification of Trx (Inoue *et al.*, 2007).

Earlier methods for the purification of thioredoxin were extremely extensive and time consuming as it involved several steps including multiple centrifugation steps, RNase, protamine sulfate and ammonium sulfate treatment (Williams *et al.*, 1967). However, Harms *et al.* (1998) were able to purify Trx from anaerobic, amino-acid-utilizing bacteria in four steps involving anion-exchange chromatography, acid treatment, affinity chromatography with Procion Red and finally gel permeation chromatography. As Trx is thermostable, most techniques make use of a heat treatment step (Porqué *et al.*, 1970, Kim *et al.*, 2005) which results in the denaturation of most of the undesired proteins.

In contrast, Bao (2006) utilized recombinant purification techniques to acquire pure Trx. Their method involved the PCR amplification of the *TRX3* gene using *S. cerevisiae* genomic DNA as a template. The amplified fragments were cloned into a pET28a-derived expression vector (pET28ad) encoding a hexahistidine (6×His) tag and thereafter transformed into the host strain *E. coli* BL21 (DE3). These cells containing the plasmid were grown and were subsequently lysed using both the freeze-thaw and sonication method. The protein was then purified by nickel affinity chromatography.

Wang *et al.* (2009) also utilized recombinant expression for the purification of Trx. This was achieved by cloning the Trx gene from *Acidothiobacillus ferrooxidans*. The gene was amplified by PCR, utilizing primers that were designed to add six continuous histidine codons to the 5' primer. Genomic DNA from *A. ferrooxidans* was used as the template. PCR was performed and the product was gel purified, double-digested and ligated into an expression vector, resulting in a plasmid which encoded a fusion protein containing a hexa-

His-tag. The recombinant plasmid was then transformed into *E. coli* competent cells for expression purposes. These cells containing the plasmid were grown and were subsequently lysed and centrifuged. The final step of purification involved the clear supernatant being applied to a Hi-Trap column.

To prepare purified Trx for the purpose of this study, both conventional and molecular cloning techniques were attempted. For both methods, a growth curve for *S. cerevisiae* was used to estimate the time required for the yeast to reach a given optical density.

3.7 Generation of *S. cerevisiae* growth curve

3.7.1 Method

YPD medium (25 ml) was inoculated with a single *S. cerevisiae* BY4743 (*MATa/MATa his3Δ0/his3Δ0 leu2Δ0/leu2Δ0 MET15/met15Δ0 LYS2/lys2Δ0 ura3Δ0 /ura3Δ0*) colony. The yeast inoculated medium was incubated overnight (16 hours) in a shaking incubator (30°C, 200 rpm). The overnight culture (1 ml) was transferred into fresh YPD medium (199 ml). This culture was incubated (8 hours, 30°C) with shaking (200 rpm) and the OD₆₀₀ values of the culture were recorded at 0 minutes, 120 minutes and thereafter at 60 minute intervals. This experiment was performed in duplicate and growth curves were generated by plotting ln (OD₆₀₀) values of the exponential growth phase against time (min). The doubling time (t_d) was calculated by equation 3.1.

$$t_d = \ln 2 / \text{gradient} \quad (3.1)$$

3.8 Ethanol extraction of Trx

3.8.1 Method

S. cerevisiae cells were cultured in YPD (30°C, 200 rpm, 18 hours) and 30 A₆₀₀ units of culture was centrifuged (20 000 x g, 5 min, 4°C). After the pellet was washed with dH₂O, it was resuspended in 1 ml dH₂O containing 20% (v/v) ethanol and incubated at 37°C for 2 hours. As a control for the effect of ethanol, an additional cell pellet was resuspended in dH₂O and thereafter similarly incubated (37°C, 2 hours). The cell suspensions were centrifuged (20 000 x g, 5 min, 4°C) and both the cell pellet and the supernatant were

analyzed by SDS-PAGE (Section 2.3). For these analyses, an equal volume of 2 × sample buffer (125 mM Tris-HCl, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v) mercaptoethanol, 0.01% (m/v) bromophenol blue, pH 6.8) was added to 20 µl of the re-suspended pellet as well as the supernatant.

3.9 Cloning of Trx

3.9.1 Methods

3.9.1.1 Isolation of genomic DNA from *S. cerevisiae*

Yeast genomic DNA was isolated utilizing the Bust 'N Grab method (Harju *et al.*, 2004). Briefly, two 1.5 ml samples of a 16 hour yeast culture grown in YPD were pelleted by centrifugation (20000 x g, 5 min, room temperature). The pellet was completely resuspended in 200 µl of lysis buffer (2% (v/v) Triton X-100, 1% (w/v) SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0)) by gentle inversion of the tubes. The tubes were then incubated at -75°C until the contents was completely frozen (5 min). These tubes were then rapidly transferred to a 95°C water bath (1 min) to allow the contents to thaw. This process was repeated three times and thereafter the tubes were vortexed for 30 seconds. Chloroform (200 µl) was added to each tube followed by vortexing (2 min). Upon centrifugation (20 000 x g, 5 min, room temperature), three distinct layers could be seen and the upper aqueous phase was transferred to a clean eppendorf tube containing 400 µl ice-cold 100% ethanol. The tubes were gently inverted and incubated for 5 min at -20°C for precipitation of the DNA. The precipitated DNA was pelleted by centrifugation (20 000 × g, 5 min, room temperature), washed with 70% (v/v) ethanol (0.5 ml), air-dried and re-suspended in 50 µl TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)). These samples were assayed at 260 nm and 280 nm to determine DNA concentration and purity (Section 2.7) and were also analyzed by agarose gel electrophoresis (Section 2.6).

3.9.1.2 PCR

To amplify the *TRX1* gene, PCR was performed using yeast *TRX1* specific primers, 5'-AGCCCATATGGTTACTCAATTCAA^{AA}ACTGCC-3' and 5'-ACGAAGCTTAAGCATTAGCAGCAATGGC-3' (NdeI and HindIII sites are underlined, respectively). These primers were designed using Primer3 (<http://frodo.wi.mit.edu>) and the Trx sequence was obtained

from the *Saccharomyces* genome database (SGD) (<http://www.yeastgenome.org>, Gene ID 850732). The primers were designed so that there was an overlap between the start codon and the restriction site of NdeI whilst the HindIII restriction site overlapped with the stop codon. This design ensured that more of the coding sequence was included in the primer, increasing the accuracy of the PCR. The PCR reaction mixture consisted of Taq[™] DNA polymerase (0.5 U), reaction buffer (1X), a dNTP mix (0.2 mM), the forward and reverse primers (250 nM each) and DNA (0.01-1 µg) in 25 µl. The following PCR cycling conditions were used: 95°C, 3 min (initial denaturation); 95°C, 30 sec; 55°C, 30 sec; 72°C, 30 sec (30 cycles) and a final extension at 72°C, 7 min. For all PCR experiments a no template control was included in order to check for contamination and the PCR products were analyzed by gel electrophoresis (Section 2.6).

3.9.1.3 Mini-prep procedure for purification of plasmid DNA

Plasmid DNA was purified using the standard mini-prep procedure (Sambrook *et al.*, 1989). Two 10 ml samples of an overnight culture grown in LB (containing the appropriate antibiotic) were centrifuged (7250 x g, 5 min, 4°C). The pellet was resuspended in 200 µl GTE solution (25 mM Tris-Cl (pH 8.0), 10 mM EDTA, 50 mM glucose), RNase A (2 µl) was added and the suspension was incubated at room temperature for 5 min before being transferred to a sterile eppendorf tube. 400 µl NaOH/SDS solution (0.2 M NaOH, 1% (w/v) SDS) was added to lyse the cells and the suspension was mixed by gentle finger tapping followed by incubation on ice for 5 min. 3 M potassium acetate solution (300 µl) was added and the suspension was incubated on ice for 5 min before being centrifuged (12 000 x g, 5 min, room temperature). Isopropanol (600 µl) was added to the supernatant (800 µl) and incubated at -20°C for 30 min. The suspension was centrifuged (12 000 x g, 5 min, room temperature) and the pellet was washed with ice-cold 70% ethanol (500 µl) to remove salt. The pellet was then resuspended in 50 µl TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)).

3.9.1.4 Restriction Digestion

Plasmid DNA was routinely restricted with either BamHI or HindIII to linearise the DNA for sizing. In these reactions, plasmid DNA (1 µg), restriction enzyme (2 U), buffer (1 X) and for a BamHI digest, BSA (0.1 µg/ µl) were incubated at 37°C for 2 hours. For cloning experiments, plasmid DNA was restricted with HindIII and NdeI to liberate the

TRX1 fragment. In these reactions, plasmid DNA (37.5 µg), restriction enzyme (2 U each) and nuclease free water were all incubated with 1X NEBuffer 2 at 37°C for 4 hours.

3.9.1.5 Gel purification and Ligation

The amplified *TRX1* fragment was gel purified using the QIAquick™ Gel Extraction Kit and thereafter ligated with the pTZ57R/T vector using the InsTAclone™ PCR cloning kit to yield the pTrxA, pTrxB and pTrxC clones. A typical reaction mixture contained the pTZ57R/T vector DNA (0.165 µg), purified PCR fragment (at 3: 1 molar excess over vector), Ligation buffer (1 X), T4 DNA Ligase (5U/µl) and nuclease free water. The pTZ57R/T vector was transformed into *E. coli* JM109 cells using a TransformAid™ Bacterial Transformation Kit according to the manufacturer's instructions. As a positive control for competent cell growth, *E. coli* JM109 competent cells were plated onto a LB agar plate with no antibiotic and as a negative control, untransformed cells were plated onto LB plates containing ampicillin (50 µg/ml). To purify the recombinant pTZ57R/T DNA, a plasmid mini-prep procedure was performed (Section 3.9.1.3). PCR (Section 3.9.1.2) using the purified recombinant plasmid as a template was done to confirm the presence of the *TRX1* insert. A restriction digestion using BamHI was also performed to linearise the vector for sizing and the vector was digested with HindIII and NdeI to liberate the *TRX1* fragment.

3.9.1.6 Plasmid DNA extraction of pET28a

The expression vector, pET28a, was isolated from *E. coli* BL21 (DE3) cells using a plasmid DNA extraction procedure (Section 3.9.1.3). A₂₆₀ and A₂₈₀ readings of the samples were taken to determine DNA concentration and purity and these samples were also analyzed by agarose gel electrophoresis (Section 2.6).

3.9.1.7 Gel purification and Ligation

Restriction digested pET28a (section 3.9.1.4) and the liberated *TRX1* fragment were both gel purified using a Fermentas Gel Extraction Kit. The gel-extracted *TRX1* fragment and restricted pET28a expression vector were subsequently ligated using a Rapid DNA Ligation Kit (Fermentas) to form the expression plasmids pLPTrxA, pLPTrxB and pLPTrxC. A typical ligation mixture contained vector DNA (10 – 100 ng), insert DNA (at 3: 1 molar

excess over vector), 1X Rapid Ligation Buffer, T4 DNA Ligase (5U/ μ l) and nuclease free water. The mixture was incubated at 4°C overnight and was then used for transformation.

3.9.1.8 Preparation of competent cells using the calcium chloride method

A calcium chloride (CaCl_2) method (Sabel'nikov *et al.*, 1977) was used to make *E. coli* BL21 (DE3) competent cells. *E. coli* BL21 (DE3) cells were cultured in 2xYT medium until an OD_{600} (0.3-0.4) was reached. Two 10 ml samples were transferred to ice-cold sterile tubes, incubated on ice for 10 min and thereafter centrifuged (4500 x g, 10 min, 4°C). The pellet was resuspended in ice-cold 0.1 M calcium chloride (10 ml). The suspension was centrifuged (4500 x g, 10 min, 4°C) and the pellet was resuspended in ice-cold 0.1 M calcium chloride (2 ml) followed by a 30 min incubation on ice. These competent cells were then used for transformation.

3.9.1.9 Transformation of *E. coli* BL21 (DE3) with pET28a

The ligation mix (1 μ l) was added to the competent cells (20 μ l) and incubated on ice (30 min). This mixture was then heat shocked (42°C, 90 sec) and immediately placed on ice (2 min). Pre-warmed (37°C) SOC media (80 μ l) was added to the cells and the cells were incubated for 1 hour at 37°C in a shaking water bath. The transformation mix (50 μ l) was plated onto LB agar plates containing kanamycin (30 μ g/ml) and incubated at 37°C overnight. As a control for competent cell growth, *E. coli* BL21 (DE3) competent cells were plated onto a LB agar plate (no antibiotic) whilst in the control for antibiotic activity, untransformed *E. coli* BL21 (DE3) competent cells were plated onto the selective medium. As a control for the competency, *E. coli* BL21 (DE3) cells were transformed with the pET28a expression vector (70 ng). To confirm the identity of the clones obtained, a colony PCR using the reaction conditions described above (Section 3.9.1.2) was done and the plasmid DNA was purified and restricted with HindIII to linearize the vector for sizing.

3.9.1.10 Colony PCR

Sections of single colonies from the transformation plate were selected, dissolved in autoclaved distilled water (25 μ l) and incubated in a water bath (2 min, 100°C). This mixture was centrifuged (13 000x g, 2 min, room temperature) and 2 μ l of the supernatant was used to perform a PCR using the conditions described above (Section 3.9.1.2).

3.9.1.11 Induction and optimization of expression

Protein expression of the pET28a clones (pLPTrxA, B, C) was induced by culturing selected clones in LB media (30 µg/ml of kanamycin) until an OD₆₀₀ (0.3-0.4) was reached. Isopropyl β-D-1-thiogalactopyranoside (IPTG, final concentration 0.5 mM) was added to cultures which were incubated at 37°C in a shaking waterbath. Following IPTG addition, samples were taken every 30 min and an OD₆₀₀ was measured. To optimize expression, samples were also taken at 2, 4, 6, 17 and 19 hours and an OD₆₀₀ was measured. All samples were centrifuged (12 000 × g, 10 min, 4°C) and re-suspended in water to a final OD₆₀₀ of 10. This step ensured that approximately equal concentrations of protein were compared over the course of the induction experiment. An equal volume of 2 × sample buffer (125mM Tris-HCl, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v) mercaptoethanol, 0.01% (m/v) bromophenol blue, pH 6.8) was added to 20 µl of the re-suspended samples and the samples were analyzed by SDS-PAGE (Section 2.3). For the optimization experiments, cells were also sonicated, centrifuged (15 000 × g, 20 min, 4°C) and thereafter the supernatant was subjected to the insulin activity assay (Section 3.4.2). The activity of each sample was calculated and this was used to determine the optimal expression time for purification.

3.10 Purification of thioredoxin reductase

3.10.1 Introduction

The original purification methods involved disruption of yeast cells to release thioredoxin reductase into the medium. These methods were tedious and time consuming as they involved repetitive steps of ammonium sulfate precipitation, anion-exchange and gel filtration chromatography (Williams *et al.*, 1967). Seo and Lee (2010) were able to purify thioredoxin reductase in 3 basic steps namely ammonium sulfate fractionation, 2' 5' ADP-sepharose affinity chromatography and finally sephadex G-100 column chromatography.

In contrast Pedrajas *et al.* (1999) utilized recombinant expression for the purification of thioredoxin reductase. This procedure involved PCR using *TRR2* primers and the genomic DNA from *S. cerevisiae* as a template. The PCR products were cloned into the pGEM-T Easy Vector System and thereafter sequenced. For expression of recombinant thioredoxin reductase proteins, a mutagenic forward primer was used and PCR amplification was performed. The amplified fragment was cloned into a pET15b expression vector, fusing the

cloned fragments to a poly HIS sequence. The constructed plasmid was then transformed into *E. coli* BL21 (DE3) competent cells for expression purposes. These cells containing the plasmid were grown and were subsequently lysed and the protein was purified by nickel affinity chromatography (Pedrajas *et al.*, 1999).

As with the Trx purification, both native and recombinant methods were explored to purify thioredoxin reductase.

3.10.2 Native purification of thioredoxin reductase

3.10.2.1. Preparation of thioredoxin reductase crude extract for purification

Saccharomyces cerevisiae was cultured in YPD overnight (30°C, 200 rpm). The cells were centrifuged (10 000 rpm, 10 min, 4°C) and the pellet was resuspended in half its volume of 0.05 M Tris buffer (pH 7.5). A fraction of the suspension was lysed by either the freeze-thaw or bead beat method while the remaining cells remained unlysed. Using the freeze-thaw method, the cells were incubated at -75°C, until the contents was completely frozen (5 min), and thereafter rapidly transferred to a 95°C water bath (1 min) to allow the contents to thaw. This process was repeated three times. Disintegration by bead beating was effected twice (2 min) using a bead beater with intermittent cooling on ice (2 min). All lysed cells were centrifuged (20 000 x g, 20 min, 4°C) and the supernatants as well as the unlysed cells were subjected to the activity assay (Section 3.5.2) to determine which method resulted in the least loss of activity. As the bead beat samples provided promising results, these samples were subjected to the TPP procedure (Section 3.10.2.2), analyzed by SDS-PAGE (Section 2.3) and the activity of each fraction was then determined using the activity assay (Section 3.5.2). TPP fractions of the bead beat samples were pooled and dialyzed overnight at 4°C against potassium phosphate buffer (pH 7.0). This suspension was then mixed and centrifuged (7500 rpm, 5 min, 4°C). This final supernatant was used as crude extract for ion-exchange chromatography.

3.10.2.2 Three phase partitioning (TPP)

The three-phase partitioning technique is a simple and efficient procedure to purify proteins that has been used both for upstream and downstream protein purification processes (Dennison and Lovrien, 1997) and has sometimes been used for direct one-step purification (Sharma and Gupta, 2001). This method uses *t*-butanol and ammonium sulfate to precipitate

enzymes and proteins out of aqueous solutions. Under normal conditions, *t*-butanol is completely miscible with water but upon the addition of enough ammonium sulfate, the solution separates into two phases. If proteins are present within the solution, they may, depending on the concentration of ammonium sulfate added, separate into a third phase between the lower aqueous and upper *t*-butanol phase (Dennison and Lovrien, 1997).

3.10.2.2.1 Method

t-Butanol was mixed with the crude extract to a final volume of 30% (v/v). The volume of *t*-butanol required was calculated from the following equation:

$$y = (0.3/0.7)x$$

where y = volume of *t*-butanol

x = volume of extract

t-Butanol was warmed to 30°C prior to use, as it crystallizes below 25°C. Solid (NH₄)₂SO₄ (10% (m/v)) was added to the mixture and dissolved by stirring. This mixture was centrifuged (8000 x *g*, 10 min, 4°C) in a swing-out rotor resulting in a firm layer of precipitate between the *t*-butanol and aqueous phases. The procedure was followed until the solution reached a saturation point. The precipitate was resuspended in an appropriate buffer.

3.10.2.3 DEAE Sepharose chromatography

The DEAE sepharose resin was equilibrated with 400 ml buffer A (20 mM Tris-HCl (pH 7.5)) containing NaCl (2 M) and EDTA (1 mM) and thereafter washed with buffer A (400 ml) at a flow rate of 0.24 cm/min. The thioredoxin reductase containing crude extract was loaded onto the column of DEAE-Sepharose (3 cm x 9 cm) and washed with buffer A (200 ml) containing EDTA (1 mM). Proteins were eluted with a step gradient of 0 to 500 mM NaCl each in buffer A (200 ml). Fractions (9.4 ml) were collected, analyzed by SDS-PAGE (Section 2.3) and the activity of each fraction was determined (Section 3.5.2).

3.11 Cloning of thioredoxin reductase

3.11.1 Methods

3.11.1.1 Colony PCR

Clones of thioredoxin reductase (pMPTRRA, B, C and D) were obtained from Miss M.M Photolo (MSc candidate, UKZN). These clones had been sequenced confirming that they were native *TRR1*. These were then tested to confirm the presence of the *TRR1* gene by performing a colony PCR (Section 3.9.1.10) using yeast *TRR1* specific primers, 5'-AGCCCATATGGTTTACAACAAGTTAC-3' and 5'-ACGAAAGCTTATTCTAGGGAAGTAAAGT-3' (NdeI and HindIII sites are underlined, respectively). The PCR reaction mixture consisted of TaqTM DNA polymerase (0.625 U), reaction buffer (1X), a dNTP mix (0.2 mM), the forward and reverse primers (250 nM each) and DNA (0.01-1 µg) in 25 µl. The following PCR cycling conditions were used: 94°C, 1 min (initial denaturation); 94°C, 30 sec; 46°C, 30 sec; 72°C, 1 min (35 cycles) and a final extension at 72°C, 5 min. For all PCR experiments a no template control was included in order to check for contamination and the PCR products were analyzed by gel electrophoresis (90 V).

3.11.1.2 Induction and expression

Protein expression of the pET28a clones (pMPTRRA, B, C, D) was induced as described in Section 3.9.1.11. Optimization of protein expression was conducted with samples being taken every 60 minutes for 6 hours and an OD₆₀₀ being measured. These samples were centrifuged (12 000 × g, 10 min, 4°C) and re-suspended in water to a final OD₆₀₀ of 10. An equal volume of 2 × sample buffer (125 mM Tris-HCl, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v) mercaptoethanol, 0.01% (m/v) bromophenol blue, pH 6.8) was added to 20 µl of the re-suspended samples and the samples were analyzed by SDS-PAGE (Section 2.3). The cells were then sonicated, centrifuged (15 000 × g, 20 min, 4°C) and thereafter the supernatant was subjected to the DTNB activity assay (Section 3.5.2) (Lim and Lim, 1995). The activity of each sample was calculated and this was used to determine the optimal expression time for purification.

3.12 Recombinant purification of Trx and thioredoxin reductase

3.12.1 Methods

3.12.1.1 Preparation of Trx and thioredoxin reductase crude extract for purification

To purify recombinant proteins, transformed *E. coli* (DE3) cells containing the appropriate plasmid were cultured in LB media (30 µg/ml of kanamycin) until an OD₆₀₀ (0.6-0.7) was reached. Protein expression was induced by adding IPTG (final concentration 0.5 mM) and thereafter the Trx cultures were incubated at 37°C in a shaking water bath for 6 hours whilst the thioredoxin reductase cultures were incubated for 1 hour. Cells were harvested by centrifugation (12 000 x g, 10 min, 4°C), resuspended in 10 volumes of extraction buffer (20 mM Tris-HCl (pH 7.5), 10 mM NaCl, 1 mM EDTA, 0.2 mM AEBSF and 5 mM DTT) and thereafter disrupted by sonication for 5 min. The samples were then centrifuged (12 000 x g, 30 min, 4°C) and the Trx containing supernatant was heat-treated (75°C, 30 min) followed by centrifugation (12 000 x g, 30 min, 4°C). The heat treatment step was omitted for thioredoxin reductase as this protein was not heat stable. The final supernatant of each protein was used as crude extract in Ni-NTA affinity purification.

3.12.1.2 Ni-NTA affinity purification

Ni-NTA agarose beads (2 ml) were packed into a column (2 ml bed volume, 0.8 x 4 cm), equilibrated with 5 volumes equilibration buffer (0.02 M imidazole, 0.5 M NaCl, 0.001 M mercaptoethanol, 0.02 M Tris-HCL (pH 8.0)) and thereafter incubated with the appropriate crude extract (4°C, 16 hours) in a Revolver™ 360° Sample Mixer. The unbound fraction was eluted by gravity and the resin was washed with 10 volumes of wash buffer (0.5 M NaCl, 0.02 M Tris-HCl (pH 8.0)). Two volumes of elution buffer (0.25 M imidazole, 0.5 M NaCl, 0.02 M Tris-HCL (pH 8.0)) was incubated with the resin (4°C, 30 min) and thereafter the bound protein was eluted. All fractions were analyzed by SDS-PAGE (Section 2.3). The resin was then washed with 0.5 M NaOH for 30 min and stored in 30% (v/v) ethanol at 4°C. When the resin changed from light-blue to brownish grey, it was regenerated according to the manufacturer's instructions.

3.13 Results

3.13.1 Developing a cheaper and faster Trx activity assay

Thioredoxin is known to reduce insulin which can be measured by the increase in turbidity due to the precipitation of the free insulin β -chain (Holmgren, 1979b). An assay was developed to detect the presence of Trx in crude extracts by measuring the insulin reduction by Trx with the use of DTT, instead of thioredoxin reductase. To develop this assay, various assay conditions were tested as shown below (Table 3.1). The same crude extract was used for all the assays.

Assays 1-3 showed no activity for Trx and changes in the reagent concentrations had no effect on the rate in these 3 assays (Table 3.1). An unexpected result observed was the lack of activity when using potassium phosphate buffer at pH 6.5 as this pH was expected to increase the sensitivity of the assay due to the lower solubility of reduced insulin at this pH value (Arnér and Holmgren, 2000a). In fact, it was evident that the DTT-dependent reduction of insulin in the control reaction occurred at a faster rate (Section 3.4.2) and these assay conditions were not suitable for the purpose of this study.

As a Trx activity assay was required for the purification process, it was necessary to try alternate methods. Assay 4 involved a 20 min incubation of all the components of the assay prior to absorbance readings being taken and the reduction of insulin was observed. It was unclear whether this result was obtained due to the change in potassium phosphate buffer pH (pH 6.5 to pH 7.0) (Table 3.1) or the inclusion of an incubation period prior to readings being taken. While a rate was observed, the 20 min incubation period posed a problem as this was time consuming. To minimize the time needed to complete the assay, Trx was reduced by preincubation with DTT for 10 min at 25°C prior to the start of the reaction (Table 3.1, Assay 5). The rate observed when using this assay was the highest of all the assays. Thus, the inclusion of a simple preincubation step, allowed for an assay that was relatively cheap and faster than published assays for Trx.

Table 3.1 Comparison of the rates obtained by the modification of two Trx activity assays.

Assay	Reference ^a	Assay Reagents					Total time (min) ^b	Rate (OD ₆₅₀ /min) x 10 ⁻² ± SE (n=2) ^c
		Buffer (pH 6.5) (mM)	Buffer (pH 7.0) (mM)	EDTA(mM)	Insulin(mM)	DTT(mM)		
1	(Holmgren, 1979b)	100	-	2	0.13	0.33	20	0
2	(Arnér and Holmgren, 2000a)	100	-	2	0.13	1	20	0
3	(Holmgren, 1979b)	100	-	2	0.01	1	20	0
4	(Sigma- Aldrich, 1994)	-	63	2	0.01	1	22	1.01 ± 0.0024
5	This study	-	63	2	0.01	1	10	2.26 ± 0.0048

^aThe assays used in this study were modified versions of the assays obtained from the references given.

^b Total time required before measurement could begin.

^c The same crude extract was used for all the assays tested.

3.13.2 The development of the thioredoxin reductase assay

The reduction of DTNB, a generic disulfide substrate, was used to measure thioredoxin reductase activity by the change in absorbance at 412 nm. Various published methods were attempted to obtain a feasible assay (Table 3.2). The same crude extract was used for all the assays.

It was evident that both Assay 1 and 2 could be used to determine the activity of thioredoxin reductase based on the reduction of DTNB (Table 3.2). However, the inclusion of NaCl in the assay dramatically increased the rate of DTNB reduction. Assay 3 was therefore chosen as the activity assay for thioredoxin reductase.

3.13.3 Native purification of Trx using ethanol was unsuccessful

For the native purification of Trx, a growth curve for *S. cerevisiae* was constructed. The growth curve was used to determine the doubling time for this particular yeast strain and was used to estimate the time taken for cells to reach their target optical density for downstream isolation procedures (Figure 3.1)

3.13.3.1 Generation of *S. cerevisiae* growth curve

The growth curve data was transformed (Figure 3.1) and a doubling time of 115 min was found. To determine the target optical density based on this doubling time, the following equation was used

$$y = 0.0065 x - 3.4679 \quad (3.2)$$

where y refers to the \ln value of the required optical density and x represents the time (hours) taken to reach this optical density.

3.13.3.2 Native purification of Trx

To circumvent the difficulty in isolating thioredoxin from the cellular components, Inoue *et al.* (2007) devised a method to extract thioredoxin from yeast without disrupting the cells which involved the treatment of yeast cells with 20% (v/v) ethanol at 37°C. A control (0% ethanol) and test study (20% ethanol) showed that significant concentrations of Trx were not released from this strain of *S. cerevisiae* (Figure 3.2). Recombinant expression and purification of Trx was then attempted.

Table 3.2 Comparison of the rates obtained by utilizing various thioredoxin reductase activity assays.

Assay	Reference ^a	Assay Reagents					Rate (OD ₄₁₂ /min) x 10 ⁻² ± SE (n=2) ^b
		Univalent cations: NaCl (mM)	Potassium phosphate buffer (pH 7.0) (mM)	Potassium phosphate buffer (pH 7.2) (mM)	DTNB (mM)	NADPH (mM)	
1	(Arnér and Holmgren, 2000a)	-	58	-	5	0.3	0.128 ± 0
2	(Štefanková <i>et al.</i> , 2006)	-	-	100	1	0.5	0.0622 ± 0
3	(Lim and Lim, 1995)	500	^c -	-	0.5	0.24	2.89 ± 0.006235

^a The assays used in this study were modified versions of the assays obtained from the references given.

^b The same crude extract was used for all the assays tested.

^c The buffer used in Assay 3 was Tris-Cl (pH 8.0).

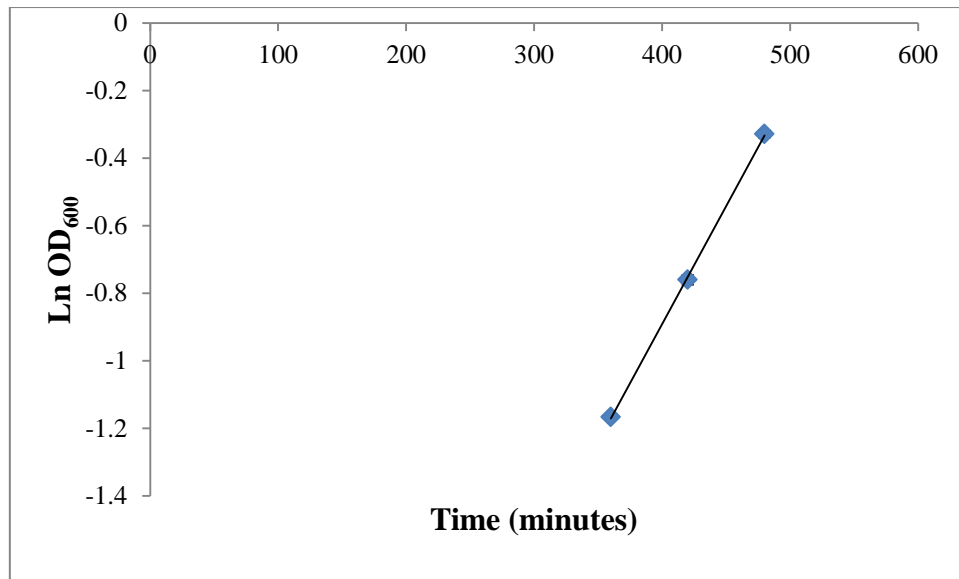


Figure 3.1 Growth curve generated for *S. cerevisiae*. Plot of $\ln(\text{OD}_{600})$ of the exponential growth phase against time ($n = 2$). Standard error bars are smaller than the symbols.

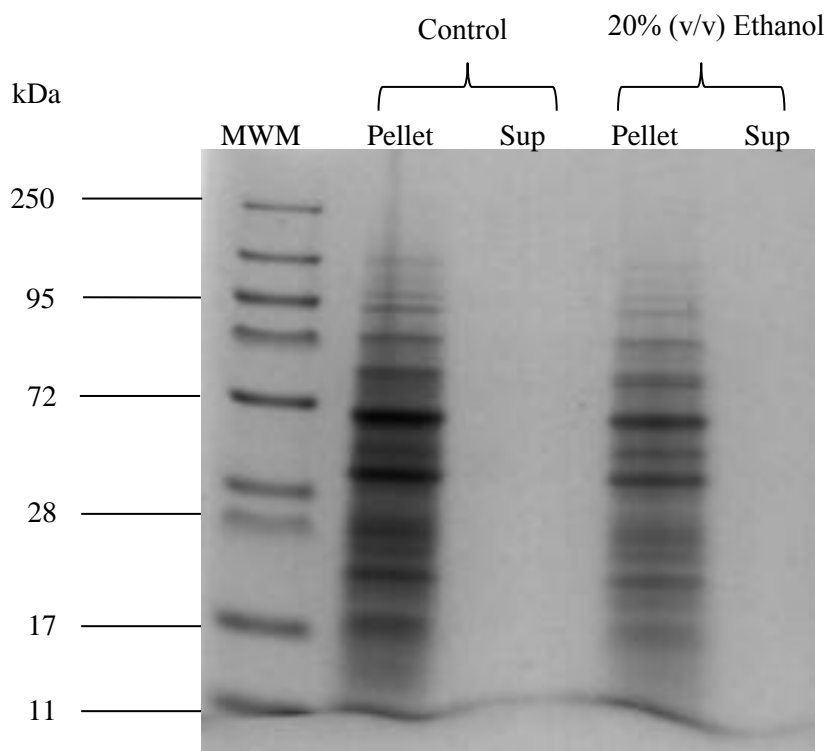


Figure 3.2 Tris-tricine SDS-PAGE of *S. cerevisiae* BY4743 cells incubated with 20% ethanol at 37°C for 2 hours. The treated and control cells were suspended in 20% (v/v) ethanol and dH₂O, respectively. After the 2 hour incubation period at 37°C, the cells were centrifuged and both the pellets and supernatants (Sup) from the treated and control cells were analyzed by SDS-PAGE.

3.13.4 The successful cloning and expression of *TRX1*

Genomic DNA was successfully isolated from *S. cerevisiae* using the Bst ‘N Grab method (Figure 3.3 A) (Harju *et al.*, 2004) and PCR amplification of the *TRX1* gene was performed using this DNA as a template and *TRX1* specific primers (Figure 3.3 B). Upon completion of the PCR reaction, the amplicons were gel purified (Figure 3.3 C). This was successful as the bands obtained were found to be 316 bp.

The gel purified *TRX1* fragment was thereafter ligated to the pTZ57R/T vector using the InsTAclone™ PCR cloning kit and transformed into *E. coli* JM109 cells to yield three clones. Plasmid DNA from these clones, pTrxA, pTrxB and pTrxC, was isolated using a mini-prep procedure. The plasmid DNA showed linear, nicked and closed coiled circular forms (Figure 3.4 A). The identities of the clones were confirmed by performing a single restriction digestion with BamHI to linearize the plasmid DNA (Figure 3.4 B). The size of the bands obtained was 3162 bp which was close to the expected size of 3211 bp. To further confirm that the pTrxA-C clones contained the *TRX1* fragment, a PCR was performed using the plasmid DNA as a template. The amplicons (316 bp) obtained clearly showed that *TRX1* was present in the pTrxA-C vectors (Figure 3.4 C). Bands appearing higher up are due to the plasmid template in the reaction. A final confirmation was achieved by digesting the pTrxA-C vectors with HindIII and NdeI to liberate the *TRX1* fragment (Figure 3.4 D). Both Molecular weight marker XIV and Molecular weight marker III were loaded so as to size the *TRX1* fragments and the restricted vectors, respectively.

pET28a was isolated from *E. coli* BL21 (DE3) cells using the standard mini-prep procedure (Figure 3.5 A) and a double digestion with NdeI and HindIII was performed (Figure 3.5 B) so that the equivalently cut *TRX1* fragment could be ligated into the expression vector.

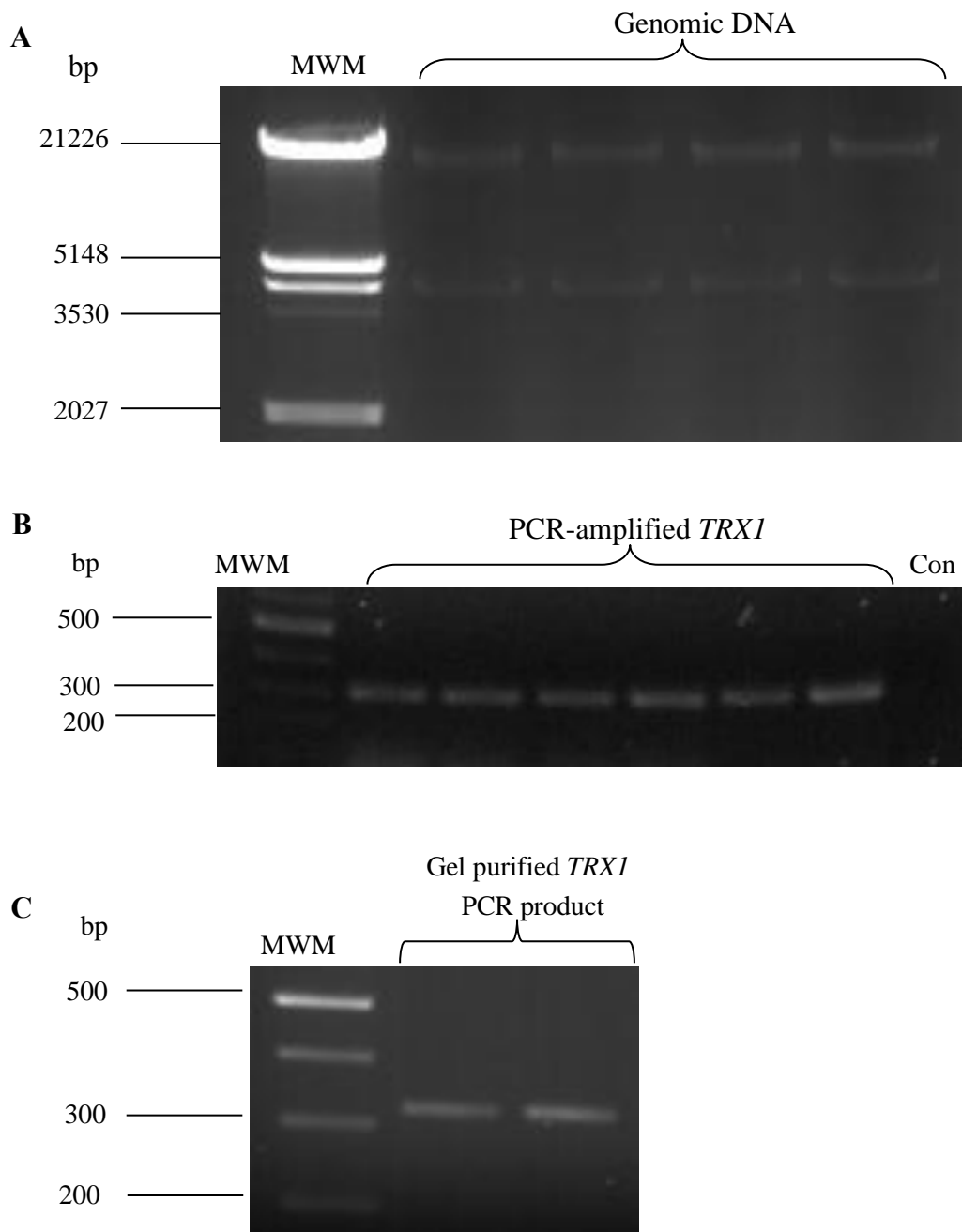


Figure 3.3 Genomic DNA isolation and PCR amplification of the *TRX1* gene. Genomic DNA was isolated from *S. cerevisiae* using the Bust ‘n Grab procedure (Harju *et al.*, 2004) (A). PCR of the DNA was undertaken using *TRX1*-specific primers (B). The PCR product was gel purified using the Fermentas gel purification kit (C). The gels have been cropped to fit the figures into this thesis but no other bands were observed in the non-MWM lanes.

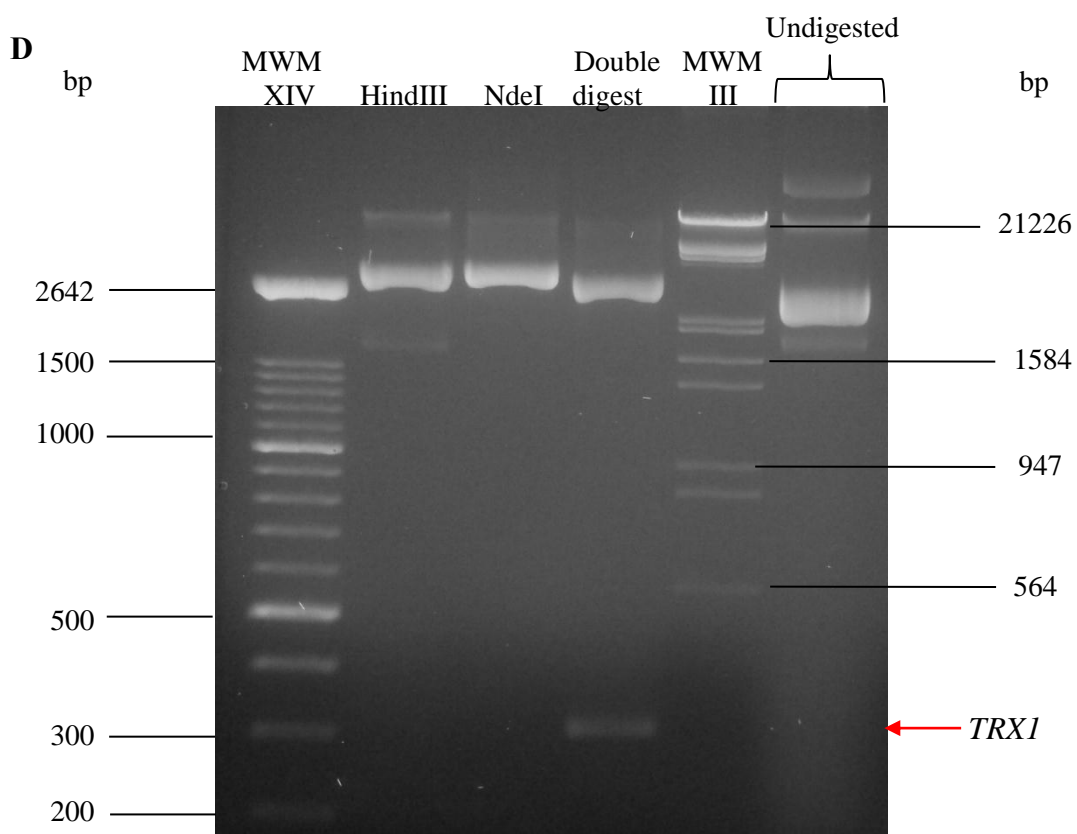
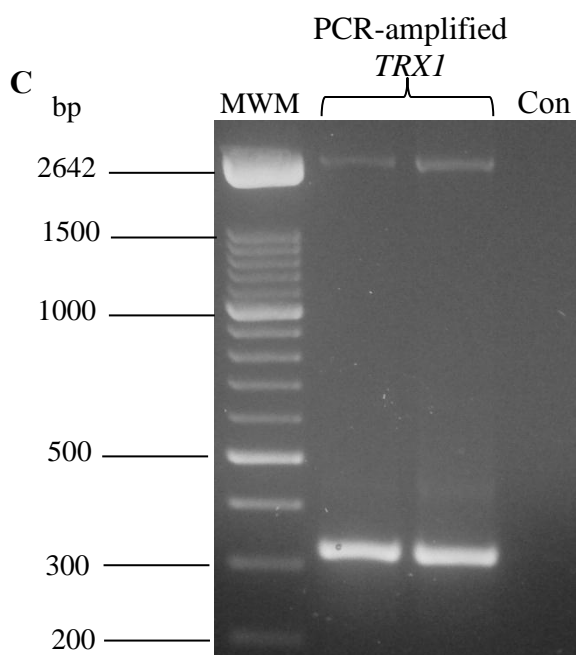
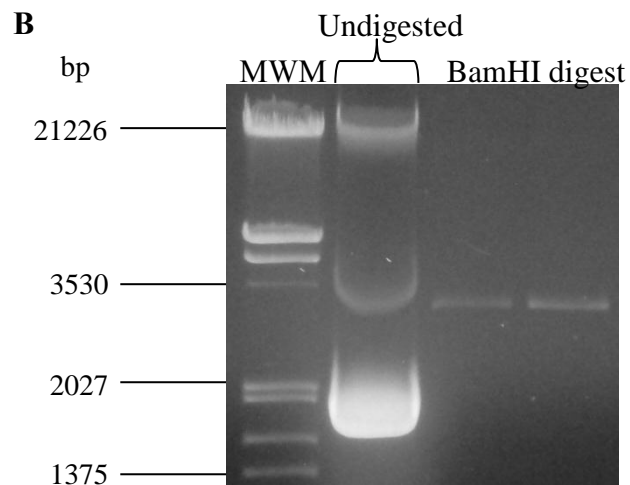
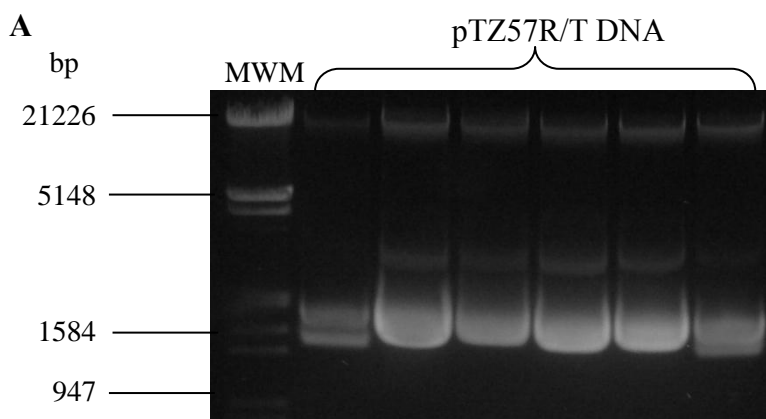


Figure 3.4 Confirming that the pTZ57R/T clones contained *TRX1*. pTZ57R/T DNA was isolated from *E. coli* JM109 cells using a standard mini-prep procedure (A). pTZ57R/T DNA was restricted with BamHI to linearize the plasmid (B). In addition, PCR of the plasmid DNA was undertaken using *TRX1*-specific primers to confirm the presence of the insert (C). Restriction digestion of the cloning vector pTZ57R/T-*TRX1* showing the release of the *TRX1* fragment (D). Some gels have been cropped to fit the figures into this thesis but no other bands were observed in the non-MWM lanes.

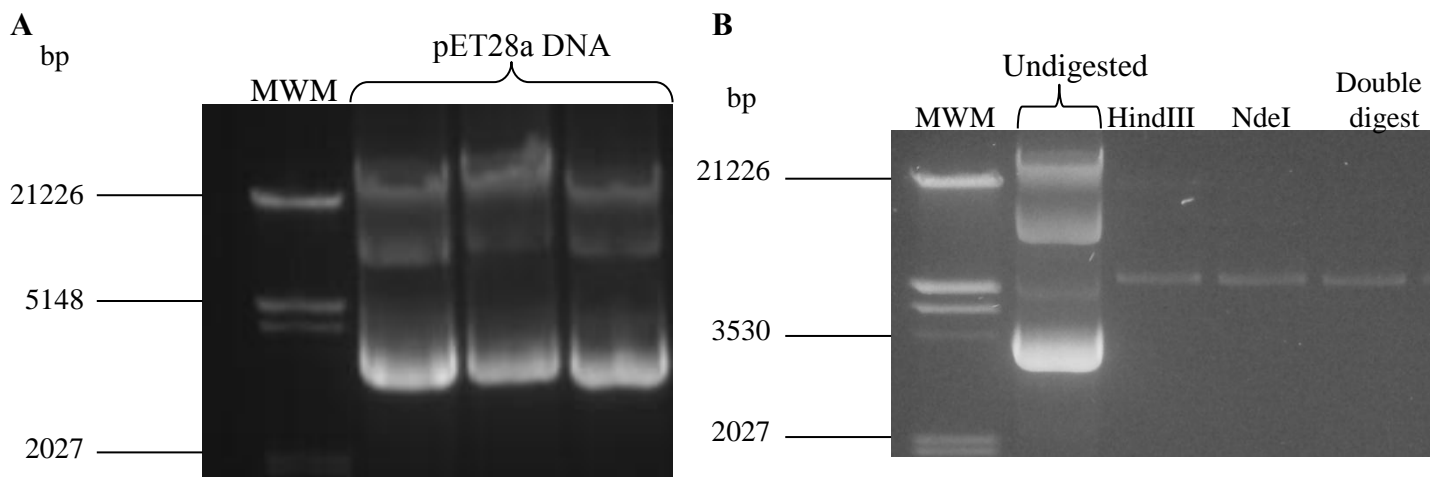


Figure 3.5 pET28a expression vector isolation and restriction endonuclease digestion. pET28a was isolated from *E. coli* BL21 (DE3) cells using a standard mini-prep procedure (A). Restriction digestion of the expression vector (B). The gels have been cropped to fit the figures into this thesis but no other bands were observed in the non-MWM lanes.

The restriction digested pET28a and the liberated *TRX1* fragment were both gel purified using a Fermentas Gel Extraction Kit. The gel-extracted *TRX1* fragment and restricted pET28a expression vector were subsequently ligated, transformed into *E. coli* BL21 (DE3) cells and a mini-prep was performed to isolate the plasmid DNA (Figure 3.6 A). A PCR was performed on the mini-prep to verify the success of the transformation (Figure 3.6 B). The band sizes of the mini-prep PCR (316 bp) indicated that *TRX1* was successfully cloned into the pET28a expression vector. A single restriction digest was performed on the pLPTrxA/B/C clones using HindIII and the resulting band size (5623 bp), which was very close to the expected band size (5620 bp), indicated that the transformation was successful (Figure 3.6 C). To further confirm a successful transformation the

pLPTrxA/B/C clones were double digested with HindIII and NdeI to liberate the *TRX1* fragment (Figure 3.6 D).

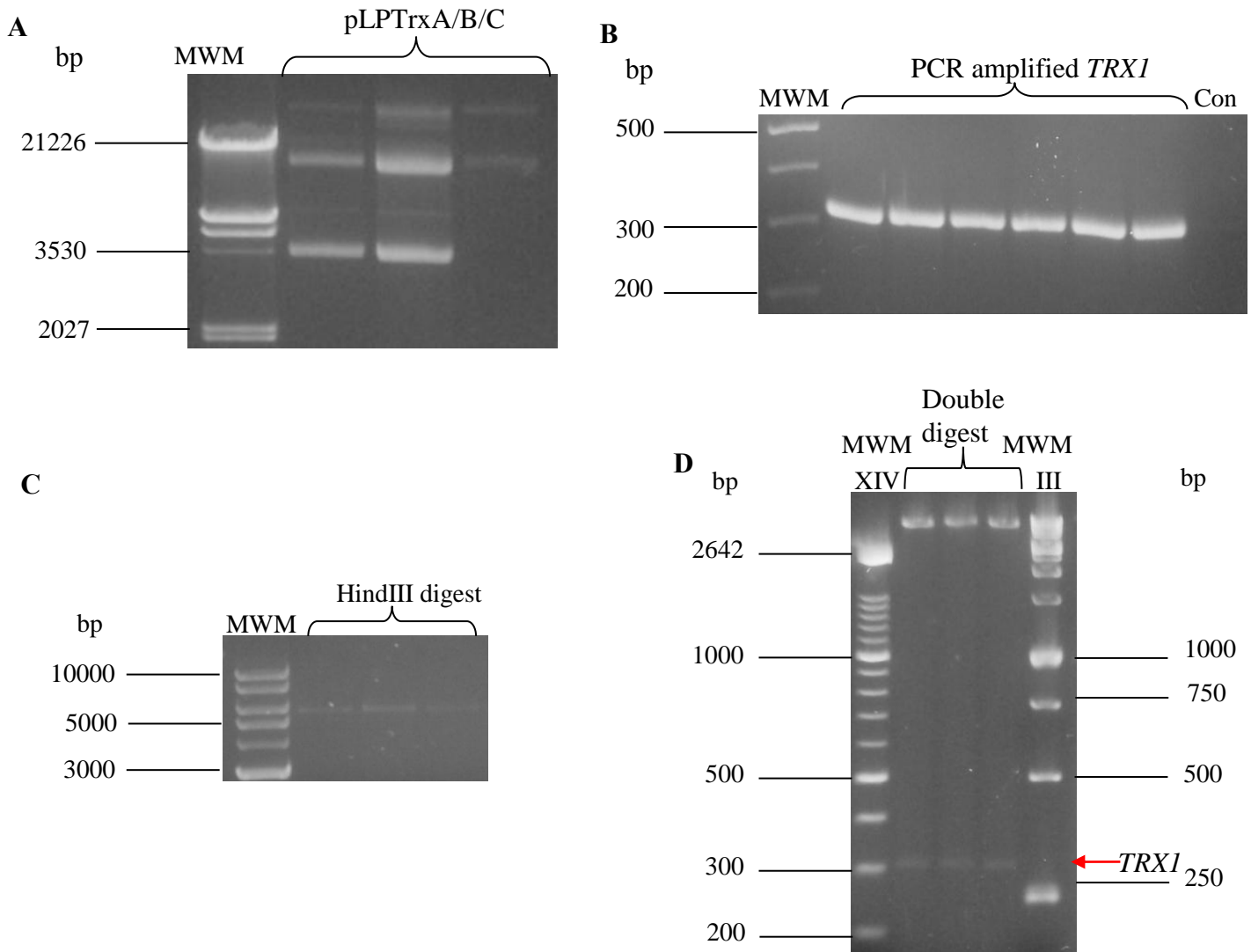


Figure 3.6 Transformation of *E. coli* BL21 (DE3) with pLPTrxA/B/C. pLPTrxA/B/C DNA was isolated from *E. coli* BL21 (DE3) cells using a standard mini-prep procedure (A). PCR performed on pLPTrxA/B/C clones to verify success of transformation (B). Single restriction digestion of pLPTrxA/B/C clones was undertaken with HindIII to size the clones (C) and double digestion with HindIII and NdeI was undertaken to release the *TRX1* gene (D). The gels have been cropped to fit the figures into this thesis but no other bands were observed in the non-MWM lanes.

The pLPTrxA/B/C clones were subsequently sequenced (Central Analytical Facilities, Stellenbosch University) in both directions and the sequencing traces were also manually evaluated. The sequences were aligned to the *TRX1* sequence from the SGD (<http://www.yeastgenome.org>) using ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>).

A

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gi | 330443681_c232013-231702      ATGGTTACTCAATTCAAAACTGCCAGCGAATTCGACTCTGCAATTGCTCA 50
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H10_pET_28a_LPTrxC_T7_promoter     ATGGTTACTCAATTCAAAACTGCCAGCGAATTCGACTCTGCAATTGCTCA 50
G10_pET_28a_LPTrxB_T7_promoter     ATGGTTACTCAATTCAAAACTGCCAGCGAATTCGACTCTGCAATTGCTCA 50
*****

gi | 330443681_c232013-231702      AGACAAGCTAGTTGTCGTAGATTTCACGCCACTTGGTGC GGTCATGTA 100
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H10_pET_28a_LPTrxC_T7_promoter     AGACAAGCTAGTTGTCGTAGATTTCACGCCACTTGGTGC GGTCATGTA 100
G10_pET_28a_LPTrxB_T7_promoter     AGACAAGCTAGTTGTCGTAGATTTCACGCCACTTGGTGC GGTCATGTA 100
*****

gi | 330443681_c232013-231702      AAATGATTGCTCCAATGATTGAAAAATTCCTGAAACAATACCCACAAGCT 150
F06_pET_28a_LPTrxA_T7_promoter    AAATGATTGCTCCAATGATTGAAAAATTCCTGAAACAATACCCACAAGCT 150
H10_pET_28a_LPTrxC_T7_promoter     AAATGATTGCTCCAATGATTGAAAAATTCCTGAAACAATACCCACAAGCT 150
G10_pET_28a_LPTrxB_T7_promoter     AAATGATTGCTCCAATGATTGAAAAATTCCTGAAACAATACCCACAAGCT 150
*****

gi | 330443681_c232013-231702      GATTTCTATAAATTGGATGTCGATGAATGGGTGATGTTGCACAAAAGAA 200
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H10_pET_28a_LPTrxC_T7_promoter     GATTTCTATAAATTGGATGTCGATGAATGGGTGATGTTGCACAAAAGAA 200
G10_pET_28a_LPTrxB_T7_promoter     GATTTCTATAAATTGGATGTCGATGAATGGGTGATGTTGCACAAAAGAA 200
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H10_pET_28a_LPTrxC_T7_promoter     TGAAGTTTCCGCTATGCCAACTTTGCTTCATTCAAGAACGGTAAGGAAG 250
G10_pET_28a_LPTrxB_T7_promoter     TGAAGTTTCCGCTATGCCAACTTTGCTTCATTCAAGAACGGTAAGGAAG 250
*****

gi | 330443681_c232013-231702      TTGCAAAGGTTGTTGGTGCCAACCCAGCGGCTATTAAGCAAGCCATTGCT 300
F06_pET_28a_LPTrxA_T7_promoter    TTGCAAAGGTTGTTGGTGCCAACCCAGCGGCTATTAAGCAAGCCATTGCT 300
H10_pET_28a_LPTrxC_T7_promoter     TTGCAAAGGTTGTTGGTGCCAACCCAGCGGCTATTAAGCAAGCCATTGCT 300
G10_pET_28a_LPTrxB_T7_promoter     TTGCAAAGGTTGTTGGTGCCAACCCAGCGGCTATTAAGCAAGCCATTGCT 300
*****

gi | 330443681_c232013-231702      GCTAATGCTTAA 312
F06_pET_28a_LPTrxA_T7_promoter    GCTAATGCTTAA 312
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G10_pET_28a_LPTrxB_T7_promoter     GCTAATGCTTAA 312
*****

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B

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G11_pET_28a_LPTrxA_T7_terminat    TTAAGCATTAGCAGCAATGGCTTGCTTAATAGCCGCTGGGTTGGCACCAA 50
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G11_pET_28a_LPTrxA_T7_terminat    CAACCTTTGCAACTTCCTTACC GTTCTTGAATAGAAGCAAAGTTGGCATA 100
H11_pET_28a_LPTrxB_T7_terminat    CAACCTTTGCAACTTCCTTACC GTTCTTGAATAGAAGCAAAGTTGGCATA 100
*****

gi | 330443681_231702-232013      GCGGAAACTTCATTCTTTTGTGCAACATCACCCAATTCATCGACATCCAA 150
G12_pET_28a_LPTrxC_T7_terminat    GCGGAAACTTCATTCTTTTGTGCAACATCACCCAATTCATCGACATCCAA 150
G11_pET_28a_LPTrxA_T7_terminat    GCGGAAACTTCATTCTTTTGTGCAACATCACCCAATTCATCGACATCCAA 150
H11_pET_28a_LPTrxB_T7_terminat    GCGGAAACTTCATTCTTTTGTGCAACATCACCCAATTCATCGACATCCAA 150
*****

gi | 330443681_231702-232013      TTTATAGAAATCAGCTTGTGGGATTGTTTCAGAGAATTTTCAATCATTG 200
G12_pET_28a_LPTrxC_T7_terminat    TTTATAGAAATCAGCTTGTGGGATTGTTTCAGAGAATTTTCAATCATTG 200
G11_pET_28a_LPTrxA_T7_terminat    TTTATAGAAATCAGCTTGTGGGATTGTTTCAGAGAATTTTCAATCATTG 200
H11_pET_28a_LPTrxB_T7_terminat    TTTATAGAAATCAGCTTGTGGGATTGTTTCAGAGAATTTTCAATCATTG 200
*****

gi | 330443681_231702-232013      GAGCAATCATTTTACATGGACCGCACCAAGTGGCGTAGAAATCTACGACA 250
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G11_pET_28a_LPTrxA_T7_terminat    GAGCAATCATTTTACATGGACCGCACCAAGTGGCGTAGAAATCTACGACA 250
H11_pET_28a_LPTrxB_T7_terminat    GAGCAATCATTTTACATGGACCGCACCAAGTGGCGTAGAAATCTACGACA 250
*****

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G11_pET_28a_LPTrxA_T7_terminat    ACTAGCTTGTCTTGAGCAATTCAGAGATCGAATTCGCTGGCAGTTTTGAA 300
H11_pET_28a_LPTrxB_T7_terminat    ACTAGCTTGTCTTGAGCAATTCAGAGATCGAATTCGCTGGCAGTTTTGAA 300
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gi | 330443681_231702-232013      TTGAGTAACCAT 312
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G11_pET_28a_LPTrxA_T7_terminat    TTGAGTAACCAT 312
H11_pET_28a_LPTrxB_T7_terminat    TTGAGTAACCAT 312
*****

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Figure 3.7 Alignment of the pLPTrxA/B/C (A) promoter sequences and (B) terminator sequences with the *TRX1* sequence and reverse complement of the *TRX1* sequence from

S. cerevisiae (NCBI Reference Sequence: NC_001144.5), respectively. Identical residues are denoted with an asterisk. The overall percentage identity between each promoter and reference sequence as well as each terminator and reference sequence was 100 %.

Sequence analysis revealed that the cloning of the *TRX1* gene was a success (Figure 3.7) and these clones could be used for expression. To determine whether Trx could be successfully expressed by the pLPTrxA/B/C clones, the clones were induced at different time intervals (Figure 3.8). The protein was successfully expressed as the calculated band size was 13 kDa for Trx which was very close to the expected size of 12 kDa (Figure 3.8).

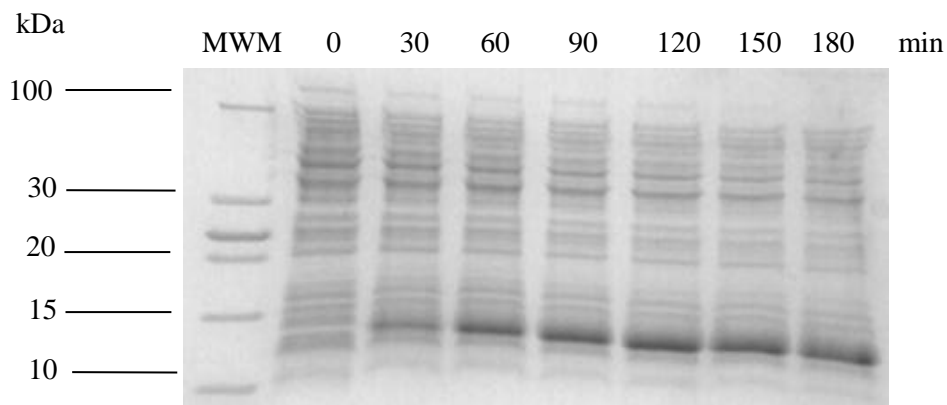


Figure 3.8 Induction of *TRX1*. pLPTrxA/B/C transformed cells were induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for various time intervals (0-180 min).

The aim of the next experiment was to determine the optimal induction time for harvesting Trx. Cultures were IPTG-induced for a number of time points (2-19 hours) and analyzed by SDS-PAGE (Figure 3.9 A) and activity assays (Figure 3.9 B) at each time point was performed. Optimum expression (Figure 3.9 A) and maximum specific activity (Figure 3.9 B) was observed after inducing the cells for 6 hours and this time point was therefore used in the purification of Trx.

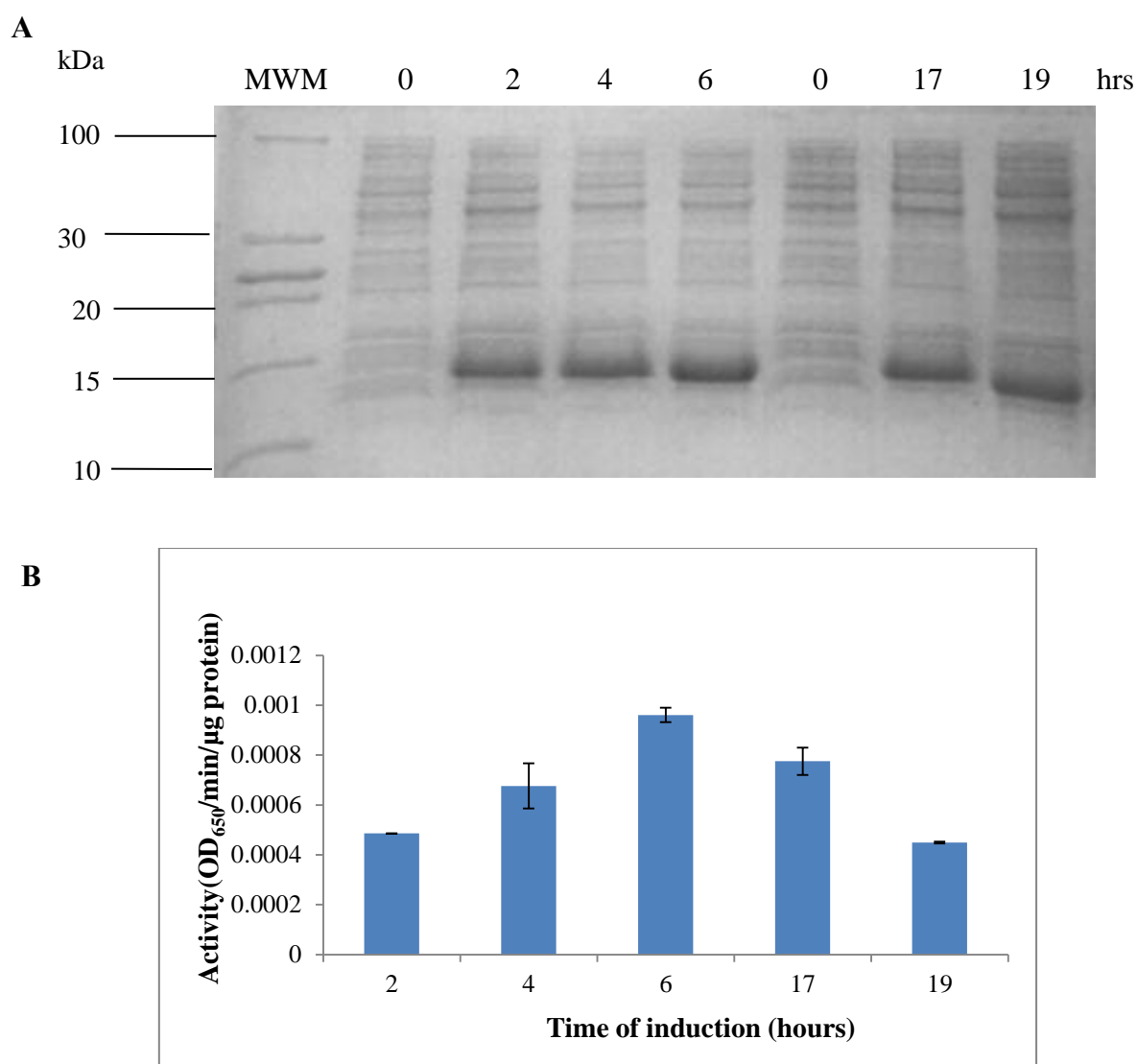


Figure 3.9 Expression and specific activity of *TRX1* at various time points (2-19 hours). Recombinant *TRX1* expression was induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for 0-19 hours and analyzed by SDS-PAGE (A). The specific activity of induced fractions (B) was also determined. Duplicate biological samples were assayed at a given time point and the standard error is indicated.

3.13.5 The native purification of thioredoxin reductase was unsuccessful

S. cerevisiae cells were lysed by bead beating and thereafter subjected to three-phase partitioning and the fractions obtained were analyzed by SDS-PAGE (Figure 3.10).

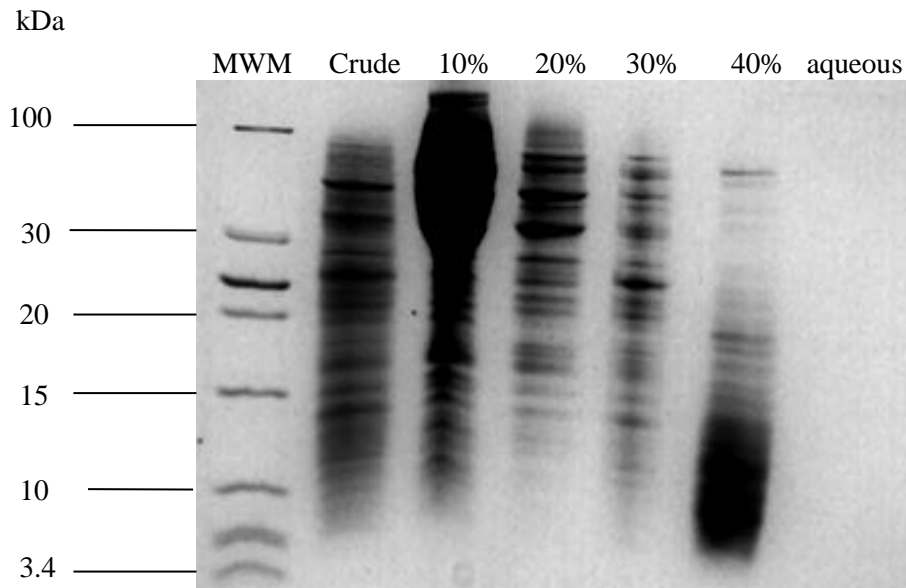


Figure 3.10 Three phase partitioning (TPP) fractions of *S. cerevisiae* BY4743. *S. cerevisiae* BY4743 cells were lysed by bead beating and thereafter treated with increasing concentrations of ammonium sulfate (10-40%).

Each fraction was thereafter assayed for thioredoxin reductase activity. Fractions containing activity were pooled, dialyzed and used as crude extract for ion exchange chromatography (Figure 3.11). A step gradient (50-500 mM NaCl) was used to elute thioredoxin reductase containing fractions (Kim *et al.*, 2005) which were analyzed by SDS-PAGE (Figure 3.11 B). Unfortunately, a 70 kDa band was observed which represented an unknown protein with DTNB activity. Recombinant purification of thioredoxin reductase was then attempted.

3.13.6 The purification of recombinant thioredoxin reductase and Trx by affinity chromatography

The pMPTRRA/B/C/D clones were obtained from Miss M.M Photolo (MSc candidate, UKZN) and were subjected to colony PCR to ensure that the *TRR1* gene was present. Upon confirmation (data not shown), these clones were IPTG-induced at different time intervals in order to confirm that this protein could be expressed by these clones. The protein was expressed successfully with an induced band at a calculated size of 36 kDa which was very close to the expected size of 35 kDa (Figure 3.12). Once successful induction was observed, it was necessary to determine the optimal induction time for harvesting thioredoxin

reductase. Cultures were IPTG-induced for a number of time points (1-6 hours) (Figure 3.12 A) and assayed for activity (Figure 3.12 B). Over the time points tested, optimum expression (Figure 3.12 A) and maximum specific activity (Figure 3.12 B) were observed after inducing the cells for 1 hour and this time point was used for the purification of thioredoxin reductase.

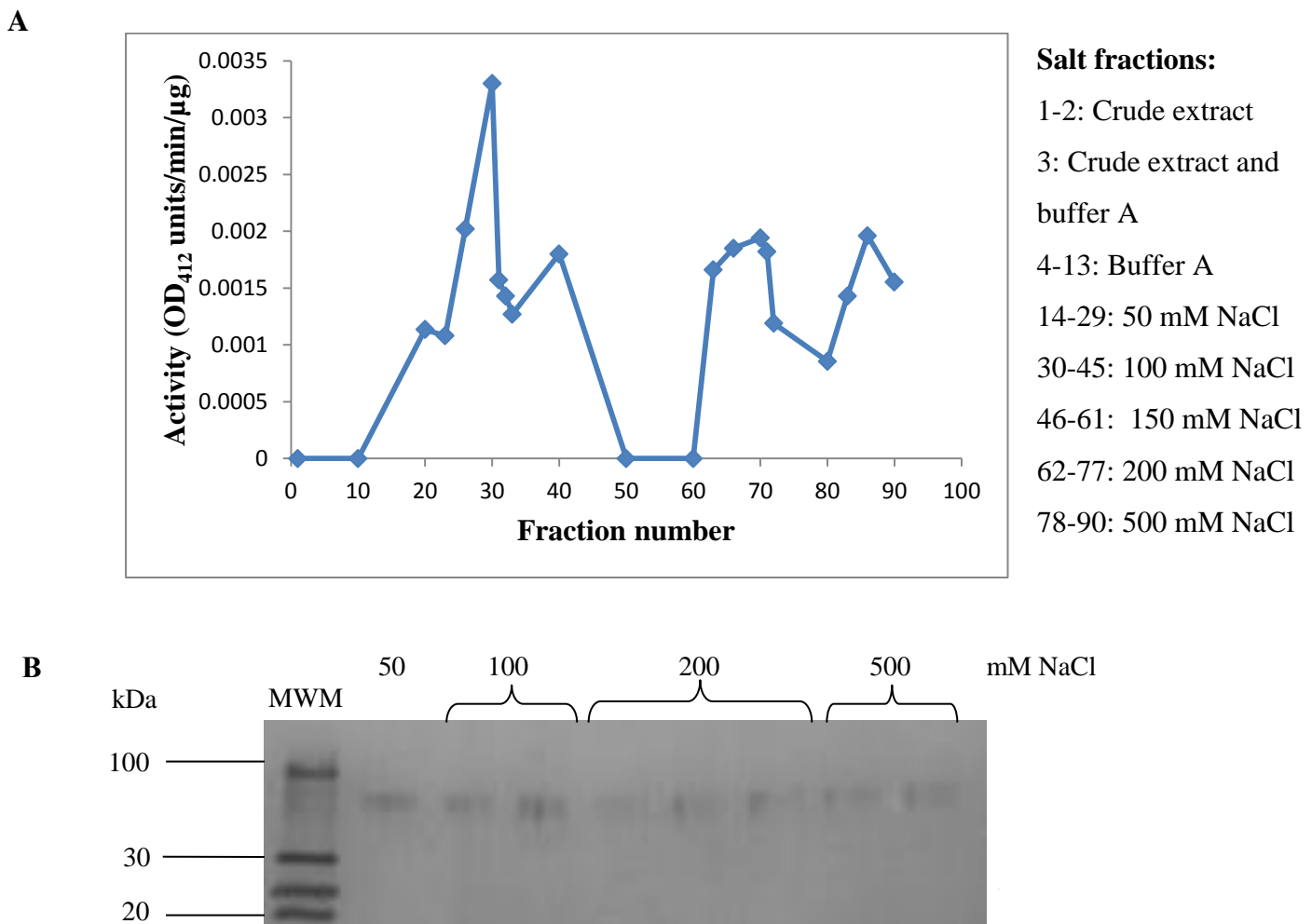


Figure 3.11 (A) Activity assay and (B) silver stain of NaCl eluates from the DEAE Sepharose column. Dialysed TPP fractions of thioredoxin reductase were separated by ion-exchange chromatography. Each eluate was subjected to the thioredoxin reductase activity assay (A) and the fractions displaying the highest activity were analyzed by SDS-PAGE (B). The gel has been cropped to fit the figure into this thesis but no other bands were observed in the non-MWM lanes.

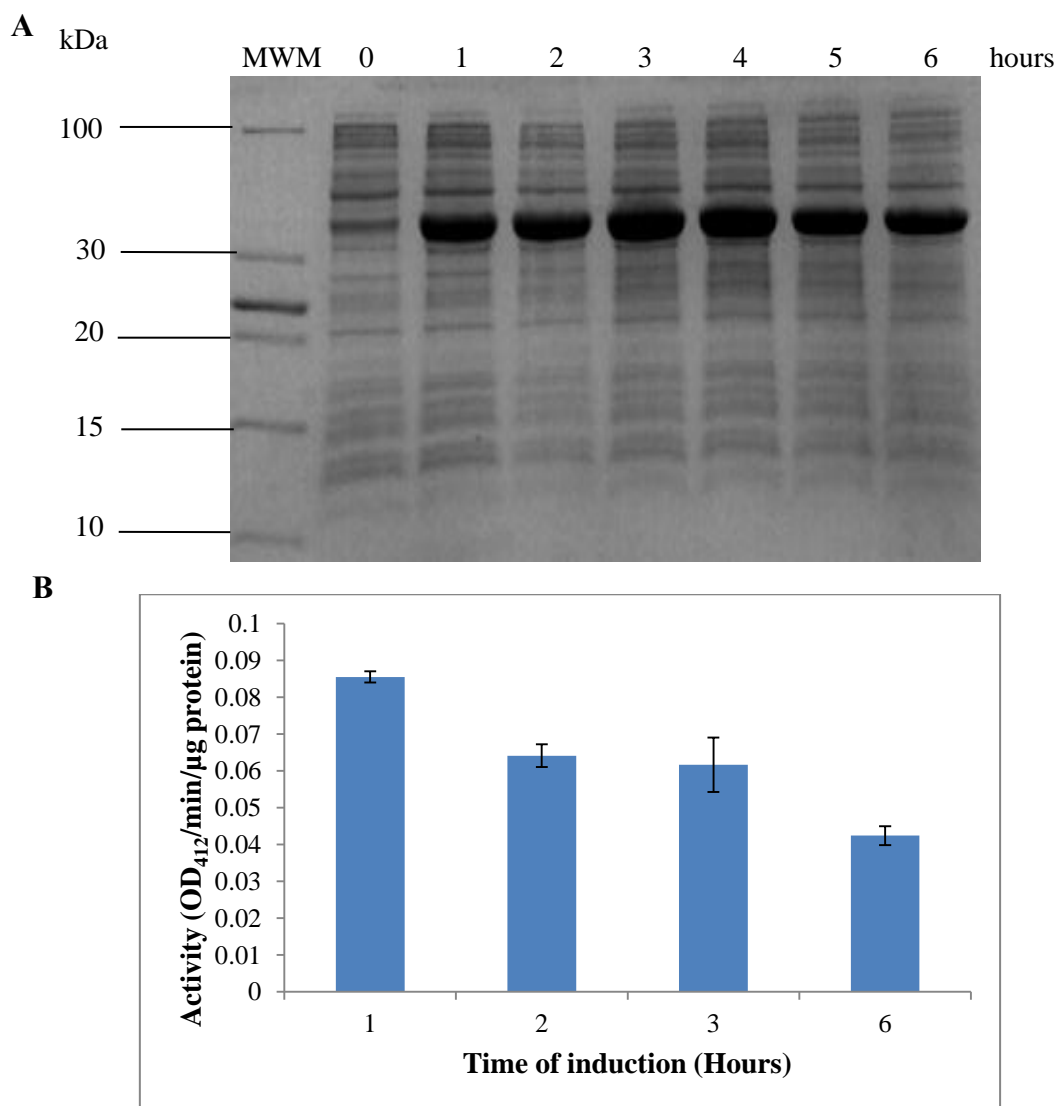


Figure 3.12 Induction of *TRRI*. Recombinant *TRRI* expression was induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for various time intervals (0-6 hours) and analyzed by SDS-PAGE (A) and the DTNB reduction assay (B). Duplicate biological samples were assayed at each time point and the standard error is indicated.

To purify the recombinant proteins, transformed *E. coli* (DE3) cells containing the appropriate plasmid were cultured and induced with IPTG for the appropriate time. These fractions were then treated as described above (Section 3.12.1.1) and used as crude extract for nickel affinity purification. The presence of single bands in the latter lanes indicated a successful purification of Trx and thioredoxin reductase with sizes of 13 kDa and 35 kDa respectively (Figure 3.13)

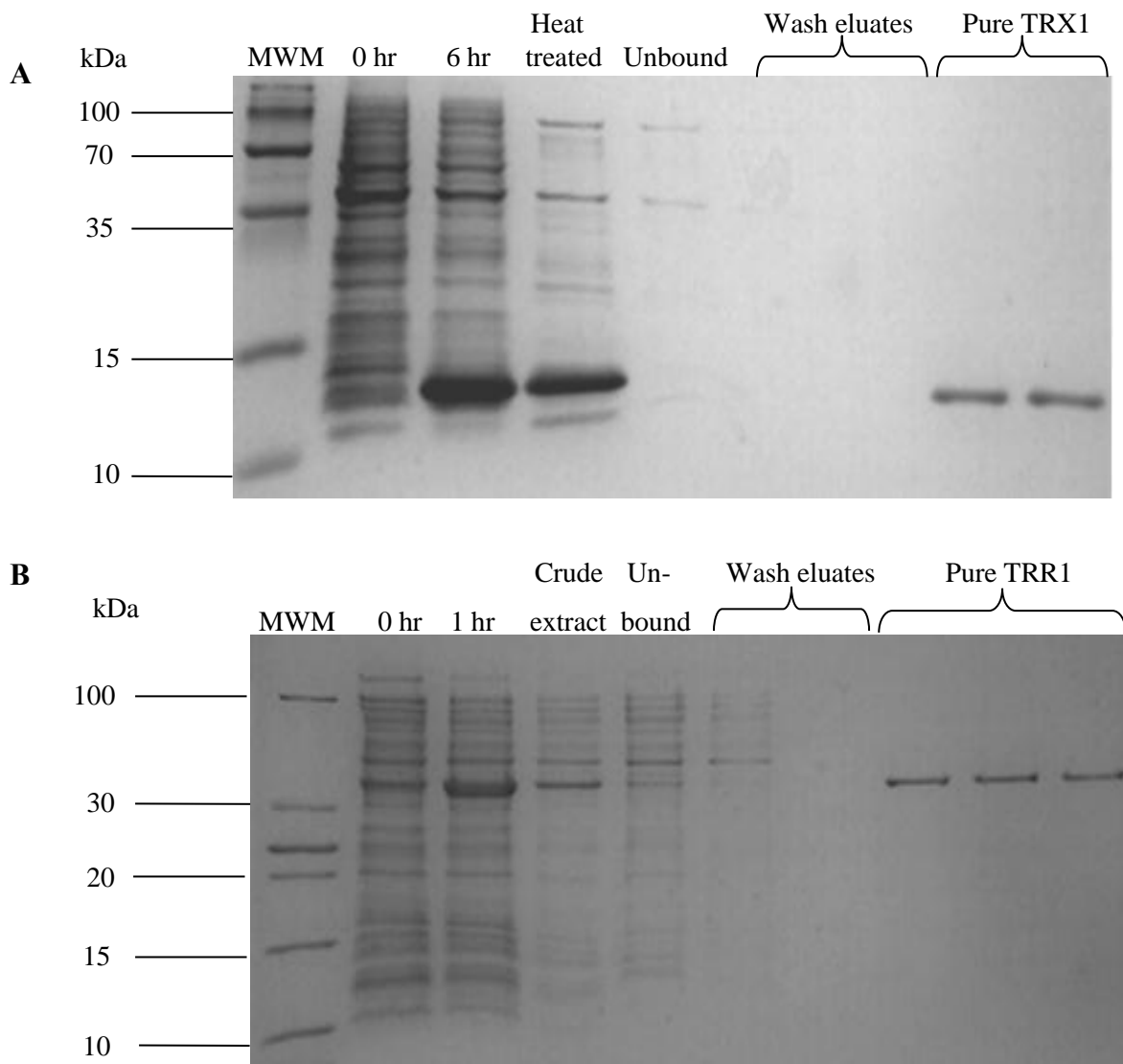


Figure 3.13 Ni-NTA affinity purification of recombinant TRX1 (A) and TRR1 (B). *TRX1* expression was induced for 6 hours and the cells processed as described in Section 3.12.1.1 to yield pure TRX1 (A). *TRR1* was induced for 1 hour and the cells processed as described in Section 3.12.1.1 to yield pure TRR1 (B). The gels were stained with Coomassie blue.

3.14 Discussion

To purify Trx and thioredoxin reductase, a number of hurdles had to be overcome. Firstly, a major problem with assaying coupled systems is that at least one of the components must be readily available. Both Trx and thioredoxin reductase are extremely expensive and therefore purchasing these proteins was not an option. For this reason, it was necessary to develop assays for each protein that did not require its counterpart.

The standard insulin assay (Arner and Holmgren, 2000a) for determining the activity of thioredoxin is both time-consuming and costly which makes it impractical for protein isolations. We tried to develop an assay based on the DTT-dependent reduction of insulin by Trx (Holmgren, 1979b). It was found that DTT could indeed be used instead of thioredoxin reductase which provided a considerable saving. Further, the inclusion of a preincubation step with DTT increased the rate of reduction and the assay could be completed in a shorter time interval (10 min) (Table 3.1). This assay was used for the subsequent purification steps.

Thioredoxin, the natural substrate of thioredoxin reductase is also very expensive and difficult to obtain so the activity of thioredoxin reductase is usually assayed using 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). For this study, assays described by Arner and Holmgren (2000a) as well as Štefanková *et al.* (2006) were attempted. These assays enabled the detection of thioredoxin reductase present in crude extracts in the absence of Trx, thereby greatly reducing the cost associated with the native assay. However, the rates obtained through both methods were not satisfactory (Table 3.2) and could have been caused by the NADPH-dependent inactivation of thioredoxin reductase (Štefanková *et al.*, 2006). However, by including univalent cations (Lim and Lim, 1995), we managed to significantly increase the sensitivity of the assay. It was shown that the use of univalent cations such as NaCl in the thioredoxin reductase activity assay significantly increased the rate of reduction of DTNB (Table 3.2). Interestingly, Lim and Lim (1995) also indicated that the optimum temperature for the direct reduction of DTNB was 10°C. This finding could make this assay very specific for the determination of thioredoxin reductase activity and may be useful in studies to determine the *in vivo* activity of thioredoxin reductase.

Once activity assays for both Trx and thioredoxin reductase were developed, the next step involved the purification of both proteins. To obtain thioredoxin, it was hoped that this protein would be harvested from the supernatants of yeast cells incubated with 20% (v/v) ethanol at 37°C for 2 hours (Inoue *et al.*, 2007). After this incubation period, it was expected that thioredoxin would be secreted directly into the medium (Inoue *et al.*, 2007) and rapidly isolated. According to Inoue *et al.* (2007), this method potentially offered several advantages over conventional extraction methods. However, for the analysis of the extracted Trx, they utilized Western blotting rather than SDS-PAGE which provided an indication that the yield of thioredoxin obtained by this method was extremely low. The results obtained in this study confirmed this (Figure 3.2). Therefore, the use of this method as an initial step in the

purification process was not feasible. For this reason, recombinant expression and purification became the method of choice.

Genomic DNA from *S. cerevisiae* was successfully isolated and the *TRX1* gene was successfully amplified and cloned into the pTZ57R/T cloning vector and then subcloned into the pET28a expression vector. The successful induction of Trx expression (Figure 3.8) and the 100% similarity observed with the sequence data alignments (Figure 3.7) showed that *TRX1* was successfully cloned and expressed.

To obtain thioredoxin reductase, native purification was also attempted. This involved lysing the cells, separation by TPP and thereafter subjecting the fractions to ion-exchange chromatography. This method was unsuccessful as the desired protein could not be isolated (Figure 3.11) which could have occurred as a result of a low concentration of thioredoxin reductase being present in the crude extract thereby allowing another protein with a similar charge, found in a higher concentration to bind to the DEAE resin. Recombinant expression and purification was then attempted. The thioredoxin reductase clones were tested and found to contain the *TRR1* gene. Thioredoxin reductase expression was induced (Figure 3.12) and both thioredoxin and thioredoxin reductase were successfully purified by nickel affinity chromatography (Figure 3.13). The purified thioredoxin system was then concentrated as described in Section 2.8 and was thereafter available for kinetic analysis experiments.

Chapter 4: Thioredoxin should be modeled as a redox couple and not an enzyme in computational systems biology models

4.1 Introduction

The central question that this thesis sought to answer was whether Trx should be modeled as an enzyme or redox couple in computational systems biology models. An equally valid question is whether it actually makes a difference to the model developed when Trx is modeled as an enzyme or redox couple. Initial kinetic assays showed that the DTT-dependent reduction of insulin increased in the presence of Trx, suggesting that Trx catalysed the rate of reduction (Holmgren, 1979a). Holmgren (1979a) developed the insulin reduction assay which showed substrate saturation behaviour (Figure 1.5 B) and it was assumed that this reflected the saturation of thioredoxin itself and Michaelis–Menten kinetic parameters were consequently assigned to this protein (Chapter 1). Various problems existed with this definition of Trx and these issues were resolved by a kinetic modeling study which suggested that Trx should be modeled as a redox couple (Pillay *et al.*, 2009). However, as Holmgren (1979a) provided what seemed to be a plausible description for Trx activity over 30 years ago, this new insight has not been readily accepted. For example, Lin (2010) utilized data fitting experiments and proposed that Trx does indeed function as an enzyme in a complete redox system. The activity of other redoxins (glutaredoxins and peroxiredoxins) have also been described using Michaelis–Menten kinetic parameters (see for example (Akerman and Müller, 2003, Akerman and Müller, 2005, Peltoniemi *et al.*, 2006)) and therefore more convincing data is needed before overturning decades of research. To resolve these disputes, the insulin assay system was analyzed using computational modeling and *in vitro* kinetic analyses.

To confirm the hypothesis proposed by our lab that with increasing concentrations of substrate, the thioredoxin reductase concentration becomes rate limiting and therefore the entire system becomes saturated with the consequent re-distribution of the Trx moiety (Pillay *et al.*, 2009), it was imperative to develop a method to track the oxidized and reduced forms of Trx.

When determining the redox state of a protein, samples have to be quenched to trap the prevailing thiol-disulfide status. Two approaches are commonly used. The first entails blocking free thiols with a cell-permeable alkylating agent and the second method involves the quenching of thiol-disulfide exchange with an acid. Both methods pose advantages and disadvantages (Figure 4.1), so the optimal procedure for quenching the thiol-disulfide state is a combination of the two strategies where samples are first quenched with TCA followed by alkylation of thiols (Hansen and Winther, 2009). This method allows for the separation of the reduced form from the oxidized form of thioredoxin on the basis of the difference in molecular mass by SDS-PAGE or the difference in charge by urea PAGE, depending on the alkylating agent used.

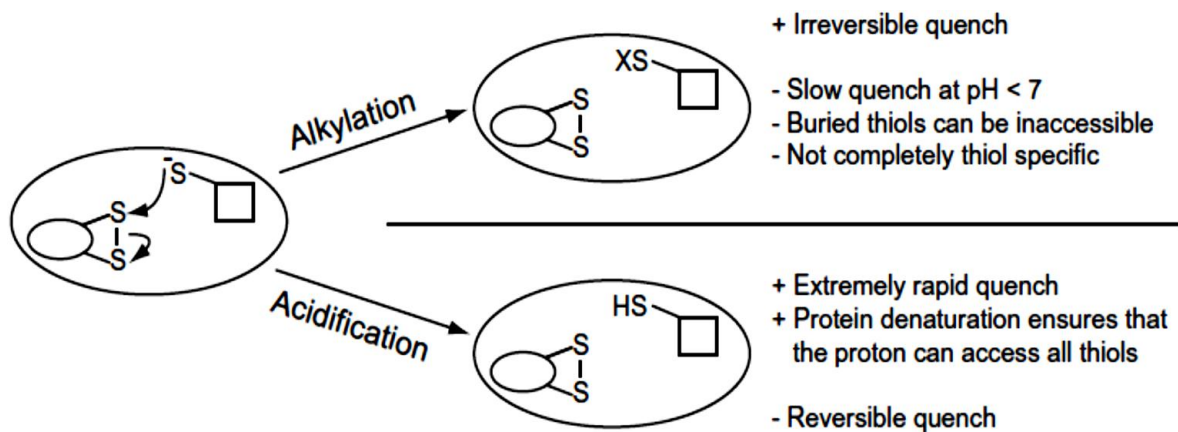


Figure 4.1 Two approaches for quenching the cellular thiol-disulfide status. Positive symbols (+) denote advantages and negative (-) symbols denote disadvantages (Hansen and Winther, 2009).

4.2 Materials and methods

4.2.1 Materials

Trichloroacetic acid (TCA) and Iodoacetic acid (IAA) were obtained from Sigma (Capital Labs, South Africa) while hydrogen peroxide (H₂O₂) and all other common chemicals were obtained from Saarchem (Merck, South Africa).

4.2.2 Preparation of reagents

The preparation of all common reagents has been described below whilst the preparation of specialized reagents has been described later in the chapter.

4.2.2.1 Iodoacetic acid (IAA)

A stock solution of IAA (1 M) was freshly prepared by dissolving IAA powder (0.0935 g) in dH₂O (0.5 ml).

4.3 Methods

4.3.1 Kinetic modeling

Kinetic modeling experiments were carried out using the open source Python Simulator for Cellular Systems (PySCeS) modeling software (Olivier *et al.*, 2005) (<http://pysces.sourceforge.net>). A basic set of parameters were chosen to create a core model of this system. Realistic models use realistic kinetic parameters and rate expressions whereas core models use default kinetic parameter sets and basic rate expressions making them extremely useful in studying the generic underlying behavior within systems (Pillay *et al.*, 2013). These models both contained a thioredoxin reductase reaction modeled with Michaelis-Menten kinetics and an insulin reduction reaction (Table 4.1). In the ‘thioredoxin enzyme’ model, insulin reduction was catalyzed by thioredoxin and modeled with a Michaelis-Menten expression while in the ‘thioredoxin redox couple’ model, insulin reduction was described using mass action kinetics (Table 4.1). The apparent second-order rate constant (k_{cat}/K_m) for this reaction in the thioredoxin enzyme model was equal to the second-order rate constant in the thioredoxin redox couple model allowing the models to be broadly comparable. These parameters are given in the text and model files are given in the appendix.

4.3.2 Insulin activity assay

For the purpose of kinetic analysis, the reaction mixture contained in a final volume of 0.5 ml, 100 mM potassium phosphate buffer (pH 7.0), 2 mM EDTA, 0.4 mM NADPH, 1.5 μ M Trx, insulin (20-200 μ M) and thioredoxin reductase (0.1-0.3 μ M) which initiated the reaction. Reactions proceeded at 25°C and the consumption of NADPH was monitored as a decrease in absorbance at 340 nm. A sample without the addition of thioredoxin reductase

was used as the reference cuvette. Absorbance measurements were made with a UV-1800 Shimadzu Spectrophotometer for 5 min. Initial studies showed that the system remained in steady state beyond the assay periods described below.

Table 4.1 Kinetic parameters and species concentrations used to compare the thioredoxin enzyme and redox couple models

Species	Value
NADPH	100 μM
NADP	1 μM
TrxSS	0.5 μM
TrxSH	0.5 μM
Insulin (oxidized)	5 μM
Insulin (reduced)	1 μM

Parameters	Thioredoxin Reductase
[TR]	0.1 μM
k_{cat}	100 min^{-1}
K_{nadph}	1.2 μM
K_{trxss}	2.8 μM

Insulin reduction

	Thioredoxin Enzyme	Thioredoxin Redox Couple
Rate expression	Michaelis-Menten	Mass action
k_{cat}	1 min^{-1}	-
K_{insulin}	1 μM	-
k_{insulin}	-	1 $\mu\text{M}^{-1} \text{min}^{-1}$

4.3.3 Determination of the redox state of Trx

The redox state of thioredoxin was determined by adding the activity assay components directly into 100% (w/v) ice-cold TCA (200 μ l) and incubating on ice for 20 min. The TCA-treated samples were centrifuged (22 065 x g, 25 min, 4°C) and the supernatant was removed. The pellet was resuspended in denaturing buffer (40 μ l, 6 M Urea, 10 mM EDTA, 0.5% (w/v) SDS and 200 mM Tris-HCl (pH 8.5)) supplemented with 100 mM IAA and incubated on ice for 10 min. The reaction was stopped by the addition of 20% (w/v) ice-cold TCA (40 μ l) to each sample. After a 20 min incubation period on ice, the alkylated proteins were centrifuged (22 065 x g, 25 min, 4°C) and the resulting pellet was resuspended in 100 μ l 2 \times sample buffer (125 mM Tris-HCl, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v) mercaptoethanol, 0.01% (m/v) bromophenol blue, pH 6.8). Samples were boiled (90°C, 5 min) and separated by SDS PAGE using a 17.5% acrylamide gel that initial studies showed could separate the oxidized and reduced forms of Trx. The controls for these experiments consisted of oxidized and reduced Trx. Oxidized Trx was obtained by incubating Trx (1.14 mM) with H₂O₂ (3 mM) on ice for 45 min while reduced Trx was obtained by incubating Trx (1.14 mM) with DTT (2.78 mM) on ice for 45 min. Both samples were then treated as described above.

4.4 Results

4.4.1 Computational modeling

To determine if there is any difference in the kinetic models obtained when thioredoxin was modeled as an enzyme or as a redox couple, representative core models were developed (Appendix 1). A basic set of parameters were used for both models so that their behaviour could be compared (Table 4.1).

A comparison of the models' sensitivity to insulin was performed and we found that over all the insulin concentrations tested, the thioredoxin enzyme model could not reach a steady state as the concentration of oxidized thioredoxin became depleted in the system (Figure 4.2 A). Without a reaction to regenerate oxidized thioredoxin, the activity of thioredoxin reductase and therefore the flux as measured by NADPH oxidation, decreased to zero, despite the presence of reduced thioredoxin in the system (Figure 4.2 A). In contrast, when thioredoxin was modeled as a redox couple, the system reached a steady state and the effect of increasing insulin concentrations on the system could be determined (Figure 4.2 B).

Further, with increases in the insulin concentration, the thioredoxin redox cycle saturated and there was a re-distribution of the thioredoxin moiety into the oxidized form (Figure 4.2 B) which was in contrast to the thioredoxin enzyme model (Figure 4.2 A).

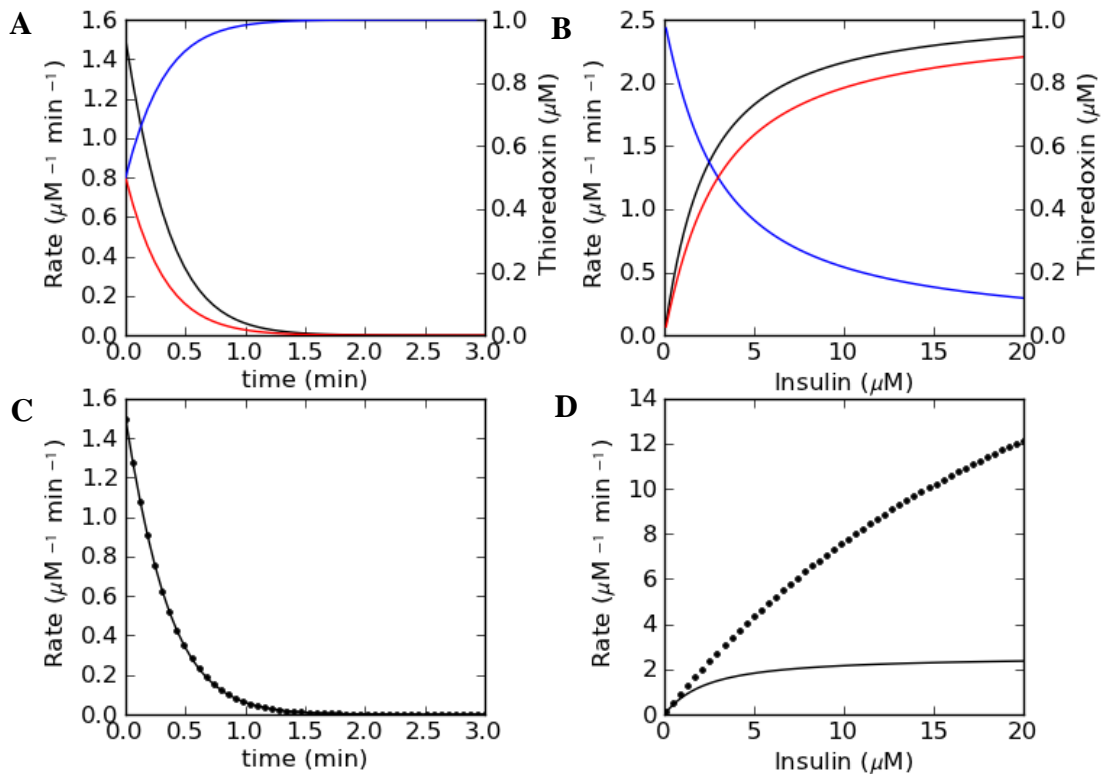


Figure 4.2 Computational models with thioredoxin modeled as an enzyme or redox couple have distinct kinetic properties. When thioredoxin was modeled as an enzyme, the system failed to reach steady state (A) with the flux (black) and concentration of oxidized thioredoxin (red) decreasing with time while the concentration of reduced thioredoxin (blue) increased with time. When thioredoxin was modeled as a redox couple (B), the system reached steady state and the effect of increasing insulin concentrations on the flux (black) and on the oxidized (red) and reduced (blue) thioredoxin concentrations could be monitored. Increasing the concentration of thioredoxin reductase from 0.1 μM (solid line) to 1 μM (dots) had no effect on the simulation results when thioredoxin was modeled as an enzyme (C) but the steady state fluxes increased in thioredoxin redox couple model (D).

The effect of increasing the thioredoxin reductase activity on the rates within the system was also tested. Based on Holmgren's (1979a) assumption that the thioredoxin

enzyme becomes saturated by increasing concentrations of insulin, it was expected and observed that changes in the thioredoxin reductase concentration have no effect on the rates within this model (Figure 4.2 C). However, increasing the thioredoxin reductase concentration increased the rate of insulin reduction in the thioredoxin redox couple model, confirming that its activity was limiting in this system (Figure 4.2 D). Thus, modeling thioredoxin as an enzyme or as a redox couple resulted in computational models with contrasting kinetic properties showing that this distinction is important. If the thioredoxin redox couple model is correct, the system should be able to achieve steady state, there should be a re-distribution of Trx into its oxidized form and thioredoxin reductase should affect the rates within the system. On the other hand, if Trx was an enzyme, reaching a steady state would be unachievable, Trx would remain in the reduced form and thioredoxin reductase concentration would have no effect on the rates within the system. As these properties can be directly tested *in vitro*, we sought to directly confirm which model was correct.

4.4.2 *In vitro* kinetic analysis

4.4.2.1 Prediction I: Steady state

According to the thioredoxin redox couple model, the system should be able to achieve steady state. To test this prediction, the rate of NADPH oxidation was monitored as a decrease in absorbance at 340 nm.

An initial decrease in the rate at 340 nm was observed within the first 2 min of the reaction and thereafter the system reached a steady state (Figure 4.3) over all concentrations of insulin tested (data not shown), supporting the Trx redox couple model. A 5 min reaction period was chosen for subsequent assays.

4.4.2.2 Prediction II: Re-distribution of the Trx moiety

With increases in the insulin concentration, the rate of reduction apparently saturated. According to the redox couple model, this is due to the saturation of the Trx redox cycle with the Trx moiety distributed into its oxidized form (Figure 4.2 B). However, according to the Trx enzyme model, Trx should be in its reduced form (Figure 4.2 A). To determine whether there was a re-distribution of the thioredoxin moiety into the oxidized and reduced forms, the cellular redox state was preserved by rapidly treating the reactions with TCA, which

protonates free thiol groups, preventing artificial oxidation during sample preparation (Hansen and Winther, 2009). This procedure is usually used in conjunction with Western blotting to probe the redox state within cells and is the first study, to our knowledge, to use it *in vitro*. This was advantageous as the need for Western blotting was eliminated. Extracts were thereafter alkylated with IAA which reacts irreversibly with thiols in a nucleophilic substitution reaction to form the corresponding carboxymethyl derivative (Hansen and Winther, 2009). Mobility standards corresponding to fully oxidized and reduced Trx were prepared by treating samples with H₂O₂ (3 mM) and DTT (2.78 mM) respectively prior to treatment with IAA (Figure 4.4).

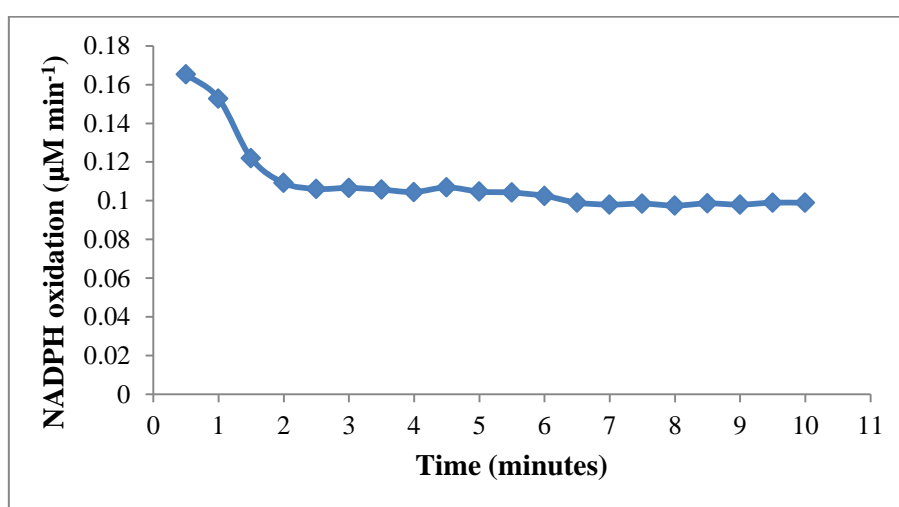


Figure 4.3 Representative graph of NADPH oxidation. The reaction mixture contained in a final volume of 0.5 ml, 100 mM potassium phosphate buffer (pH 7.0), 2 mM EDTA, 0.4 mM NADPH, 1.5 µM Trx, 20 µM insulin and 0.1 µM thioredoxin reductase. Experiments were performed in triplicate and proceeded at 25°C.

Alkylation of thiols with IAA adds a negatively charged carboxymethyl adduct to the thiol resulting in the dithiol (reduced) form of Trx migrating faster toward the anode than the disulfide (oxidized) form using native PAGE (Hansen and Winther, 2009). However, SDS-PAGE was used for the duration of this study and therefore migration and separation of protein bands depended on size. For this reason, reduced Trx, containing the carboxymethyl group, migrated slower than the oxidized form of Trx (Figure 4.4).

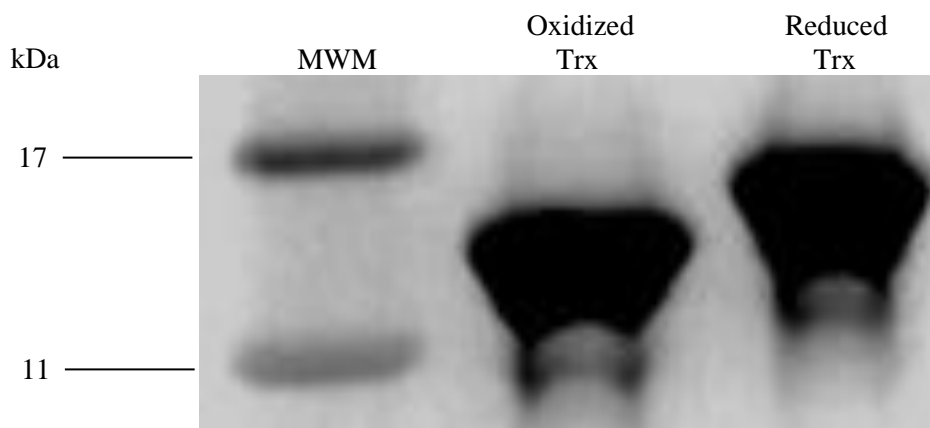


Figure 4.4 IAA-treated oxidized and reduced Trx show different mobility during SDS-PAGE. Trx was oxidized with H_2O_2 (3 mM) and reduced with DTT (2.78 mM). Samples were separated using SDS PAGE. The gel has been cropped to fit the figure into this thesis but no other bands were observed in the non-MWM lane.

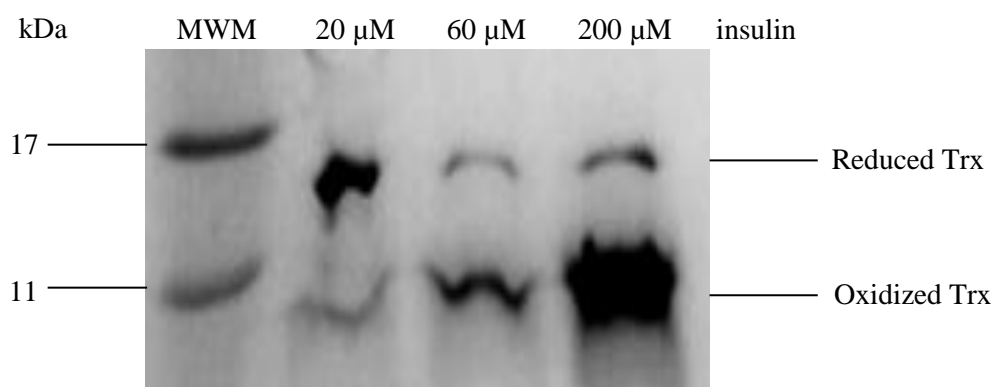


Figure 4.5 Re-distribution of the Trx moiety. The reaction mixture contained in a final volume of 0.5 ml, 100 mM potassium phosphate buffer (pH 7.0), 2 mM EDTA, 0.4 mM NADPH, 1.5 μM Trx, insulin (20-200 μM) and 0.1 μM thioredoxin reductase. Experiments were performed in triplicate and a representative gel is shown. The gel has been cropped to fit the figure into this thesis but no other bands were observed in the non-MWM lane.

Increases in the insulin concentration resulted in the progressive re-distribution of the thioredoxin moiety into its oxidized form (Figure 4.5) which was consistent with the saturation of the thioredoxin redox cycle due to the thioredoxin reductase limitation and this data therefore supports the thioredoxin redox couple model.

4.4.2.3 Prediction III: Influence of thioredoxin reductase

According to the thioredoxin redox couple model, increases in the thioredoxin reductase concentration should result in increases in the flux through the system and a higher insulin concentration would be needed to saturate the cycle (Figure 4.2 D). To test this prediction, the rate of NADPH oxidation was measured at 340 nm at various concentrations of insulin (20 μM , 60 μM and 200 μM) and thioredoxin reductase (TR) (0.1 μM and 0.3 μM). When the concentration of thioredoxin reductase was increased from 0.1 μM to 0.3 μM , there was a corresponding increase in the flux through the system (Figure 4.6) which supported the thioredoxin redox couple model and not the thioredoxin enzyme model (Figure 4.2 C).

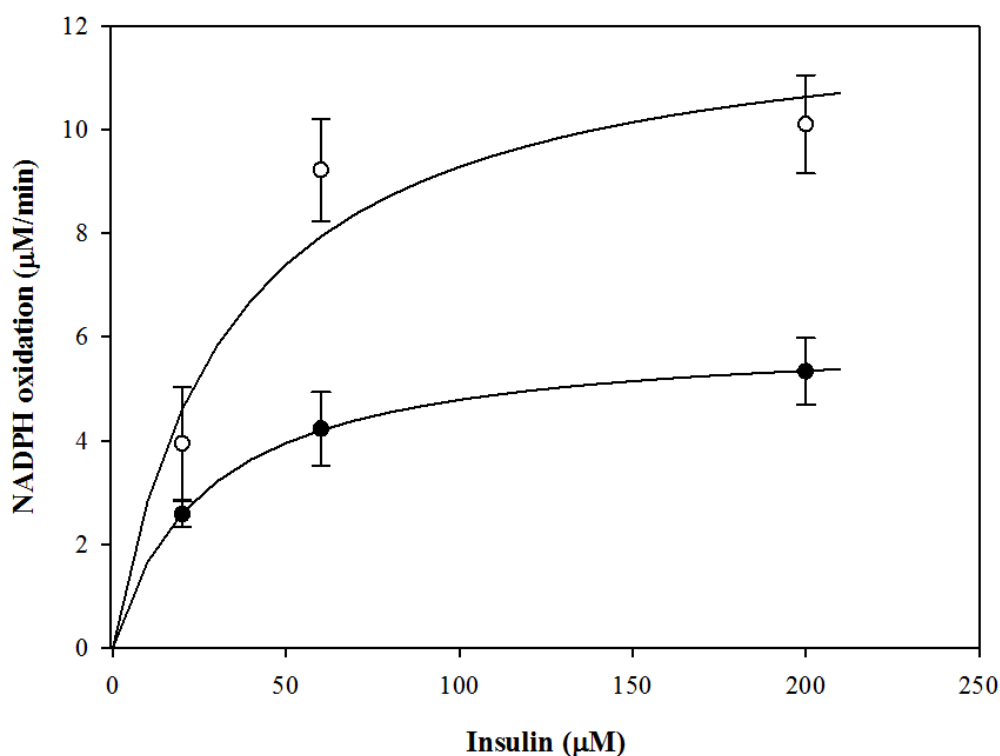


Figure 4.6 Reduction of insulin by the thioredoxin system. The rate of NADPH oxidation was measured at 340 nm at various concentrations of insulin (20 μM , 60 μM and 200 μM) and thioredoxin reductase (0.1 μM (●) and 0.3 μM (○)). Experiments were performed in triplicate and the standard error bars are indicated.

Increases in the insulin concentration were accompanied by the conversion of the Trx moiety into its oxidized form (Figure 4.7). However with the redox couple, it was expected that an increase in the thioredoxin reductase concentration should increase the steady state

(reduced) thioredoxin concentration at a given concentration of insulin (Figure 4.2 A-B). Our analysis of the oxidized and reduced concentrations of Trx confirmed the modeling result with a greater concentration of reduced Trx for a given insulin concentration at a higher thioredoxin reductase concentration (Figure 4.7 C-D). The reduced and oxidized forms of thioredoxin reductase can also be seen higher in the gel (Figure 4.7 D). In summary, thioredoxin reductase was limiting in the system and saturation was due to the saturation of the Trx redox cycle.

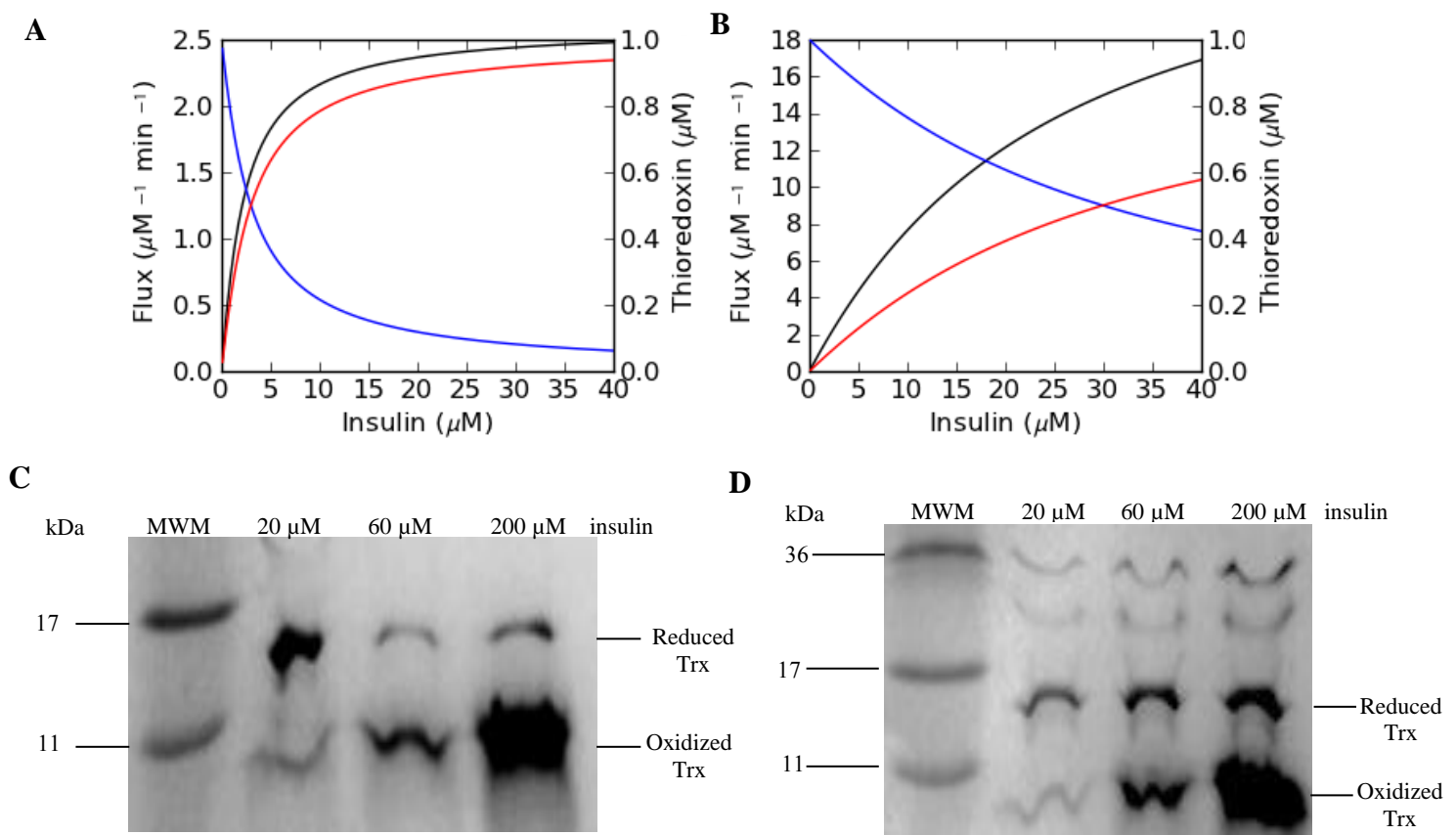


Figure 4.7 The thioredoxin redox couple model predicted that thioredoxin reductase is limiting in the Trx system. Increases in the insulin concentration in the redox couple model resulted in a decrease of the reduced Trx concentration (blue) using 0.1 μM thioredoxin reductase (A). In comparison, when the thioredoxin reductase concentration was increased to 0.3 μM, the steady state (reduced) thioredoxin concentration (blue) at a given concentration of insulin was higher (B). *In vitro* kinetic analysis confirmed this prediction where less reduced Trx was evident when using 0.1 μM thioredoxin reductase (C) as compared to using 0.3 μM thioredoxin reductase (D). The gels have been cropped to fit these figures into this thesis but no other bands were observed in the non-MWM lanes.

4.5 Discussion

Our data showed that describing redoxins as enzymes or redox couples is not merely a ‘semantic’ problem as thioredoxin models developed using either definition have distinct kinetic properties (Figure 4.2). Our modeling results showed that the thioredoxin enzyme model could not reach a steady state (Figure 4.2 A) which was in contrast to the well-established properties of the system *in vitro* (Holmgren, 1979a, Arnér and Holmgren, 2000a). In comparison, the thioredoxin redox couple model reached a steady state and showed saturation with increasing insulin concentrations and a subsequent re-distribution of the thioredoxin moiety into its oxidized form was observed (Figure 4.2 B). Both models also displayed distinct responses to changes in the thioredoxin reductase concentration with the thioredoxin enzyme model displaying no effect to these changes (Figure 4.2 C) while the fluxes in the thioredoxin redox couple model increased with increased thioredoxin reductase concentrations (Figure 4.2 D).

We used the classical insulin reduction assay for thioredoxin activity to test the predictions from these models *in vitro*. We specifically did not fit these kinetic models to our datasets as this approach had been previously used to both confirm and/or reject the thioredoxin enzyme and redox couple approaches (Holmgren, 1979a, Pillay *et al.*, 2009, Lin, 2010) and we wanted to resolve this dispute independently. Our results showed that the system reached steady state and with increasing concentrations of insulin, the system saturated with a progressive re-distribution of thioredoxin into its oxidized form. Further, increasing the thioredoxin reductase concentration increased the flux through the system (Figure 4.6). Collectively, the results obtained through *in vitro* analyses provide unambiguous support for the thioredoxin redox couple model.

Chapter 5: General discussion

In systems biology, the construction of kinetic models is becoming increasingly important in order to understand and quantify how complex systems such as the Trx system are regulated (Pillay *et al.*, 2013). However, for these approaches to succeed, precise definitions of the components within the system are a necessary first step which has been complicated by the description of redoxins as both enzymes and redox couples. While our lab had been able to resolve these contradictions into a consistent quantitative framework (Pillay *et al.*, 2009), further confirmatory studies were required before these results could be generally accepted.

To resolve these existing disputes, a number of hurdles had to be overcome. Firstly, it was imperative to develop cheap and fast activity assays for both Trx and thioredoxin reductase. A novel assay was developed for the detection of Trx activity involving the DTT-dependent reduction of insulin (Chapter 3). The use of DTT, rather than thioredoxin reductase and the inclusion of a preincubation step to reduce the Trx with DTT, made this assay both cheap and fast. With respect to the detection of thioredoxin reductase activity, an assay utilizing univalent cations in the reaction mixture to increase the rate of DTNB reduction by thioredoxin reductase was chosen. Once appropriate assays were found, the thioredoxin system had to be isolated from *Saccharomyces cerevisiae* and subsequently purified. *S. cerevisiae* was an ideal model eukaryote for studying the thioredoxin system because of the genetic and biochemical tractability of the organism and the availability of null mutants lacking components of the system (Grant, 2001, Wheeler and Grant, 2004). Apart from being easy to manipulate, *S. cerevisiae* also represents an excellent, well-established model system for understanding fundamental cellular processes relevant to higher eukaryotic organisms. This eukaryote is inexpensive to maintain and grow and its entire genome has been sequenced (Goffeau *et al.*, 1996, Galao *et al.*, 2007). All these attributes make *S. cerevisiae* a suitable candidate for the study of the regulation of the thioredoxin system.

To obtain Trx, cells were incubated with ethanol in the hope that Trx would be excreted into the surrounding medium whilst the cells remain intact (Inoue *et al.*, 2007). However, this method was unsuccessful. Native purification of thioredoxin reductase involving lysis of yeast cells, three-phase partitioning and ion-exchange chromatography was also unsuccessful. For this reason, recombinant purification was attempted and both proteins

were successfully cloned, expressed and purified using nickel affinity-chromatography (Chapter 3).

Computational modeling was used to determine if there was a difference in the models obtained when Trx was modeled as an enzyme or redox couple. Our results showed that distinct differences existed between the two models (Chapter 4). In contrast to the thioredoxin redox couple model, the thioredoxin enzyme model was unable to achieve steady state over all the insulin concentrations tested. Further, with increases in the insulin concentration, the thioredoxin redox cycle saturated and there was a re-distribution of the thioredoxin moiety into the oxidized form which was in contrast to the thioredoxin enzyme model. Finally, increasing the thioredoxin reductase concentration increased the rate of insulin reduction in the thioredoxin redox couple model whilst this increase in thioredoxin reductase concentration had no effect on the thioredoxin enzyme model (Chapter 4). To confirm each prediction of the redox couple model, *in vitro* kinetic analyses was implemented using the classical insulin reduction assay (Holmgren, 1979a).

Through the use of *in vitro* analyses, three independent pieces of evidence confirming the thioredoxin redox couple model were obtained. Firstly, the system reached a steady state. Secondly, with increases in the insulin concentration, the thioredoxin redox cycle saturated and there was a re-distribution of the thioredoxin moiety into its oxidized form. Finally, increasing the thioredoxin reductase concentration increased the rate of insulin reduction. This kinetic mechanism accounts for both the *in vitro* and the *in vivo* properties attributed to redoxins and has a number of implications for the field. Firstly, as we are now able to obtain consistent *in vitro* kinetic parameter sets for Trx reactions, it is envisioned that an integrated computational model of this system will precisely describe its role in redox regulation which has application for the treatment of a number of pathologies including HIV and cancer in which redoxin activity plays a key role. Secondly, a broader revision of redoxin activity is required as these results dismantled the well-established description of Trx activity. Finally, through these findings and a conclusive description of Trx activity, theoretical modeling studies can be viewed with a lot more confidence. This work serves as a good starting point for the further adoption of systems biology approaches in the study of redoxins and their respective systems.

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Appendix 1

Kinetic modeling experiments were carried out using the open source Python Simulator for Cellular Systems (PySCeS) modeling software (Olivier *et al.*, 2005) (<http://pysces.sourceforge.net>). The following modeling files were used to determine if there was a difference when Trx was modeled as an enzyme or redox couple.

File 1: Trx modeled as a redox couple

FIX: NADPH NADP PSS PSH

R1: NADPH + TrxSS = NADP + TrxSH

$(kcat1 * TR * (NADPH / Knadph) * (TrxSS / K1trxss)) / ((1 + NADPH / Knadph) * (1 + TrxSS / K1trxss))$

R2: TrxSH + PSS = TrxSS + PSH

$k2 * TrxSH * PSS$

#Kinetic Parameters = μ M and min

TR = 0.1

kcat1 = 100

Knadph = 1.2

K1trxss = 2.8

k2 = 1

#Species concentrations

NADPH = 100

NADP = 1

TrxSS = 0.5

TrxSH = 0.5

PSS = 5

PSH = 1

File 2: Trx modeled as an enzyme

FIX: NADPH NADP PSS PSH

R1: NADPH + TrxSS = NADP + TrxSH

$(kcat1 * TR * (NADPH / Knadph) * (TrxSS / K1trxss)) / ((1 + NADPH / Knadph) * (1 + TrxSS / K1trxss))$

R2: PSS = PSH

$(kcat2 * TrxSH * PSS) / (Kpss + PSS)$

#Kinetic Parameters = μ M min

TR = 0.1

kcat1 = 100

Knadph = 1.2

K1trxss = 2.8

kcat2 = 1

Kpss = 1

#Species concentrations

NADPH = 100

NADP = 1

TrxSS = 0.5

TrxSH = 0.5

PSS = 5

PSH = 1

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Olivier, B. G., Rohwer, J. M. & Hofmeyr, J.-H. S. 2005. Modelling cellular systems with PySCeS. *Bioinformatics*, 21, 560-561.