

The Structural and Functional Analysis of Peroxiredoxin 6 and Glutathione Transferase P1-1



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degree of Master of Science.

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Research Outputs

Conference Presentations

Name	Date	Location	Title	Authors
South African Society of Biochemistry and Molecular Biology 25 th Congress	July 10 th – 14 th 2016	East London International Convention Centre, East London, South Africa	The structural and functional analysis of peroxiredoxin 6 and glutathione transferase p1	Molaudzi Z. Dirr H.W.
Molecular Biosciences Research Thrust	Dec 8 th 2016	University of the Witwatersrand, South Africa	The structural and functional analysis of peroxiredoxin 6 and glutathione transferase p1	Molaudzi Z. Dirr H.W.

Abstract

Glutathione transferase P1-1 (GSTP1-1) is an enzyme belonging to the glutathione transferases superfamily of enzymes responsible for xenobiotic detoxification metabolism in the cells. It has been shown recently that GSTP1-1 performs a distinct function from its family members in that it acts as a carrier of the glutathione in the reactivation and glutathionylation of oxidised peroxiredoxin 6 (Prdx6). Prdx6 is a peroxidase belonging to the peroxiredoxin superfamily. The family functions to reduce organic peroxides which are sources of oxidative stress. Prdx6, however, differs from its family members as it is a bi-functional enzyme and it only contains one cysteine in its catalytic centre. The interaction of GSTP1-1 with Prdx6 has proven to be vital for the functioning of the Prdx6.

The recombinant Prdx6 and GSTP1-1 proteins have been over-expressed and purified to homogeneity. The secondary structure of the proteins was studied using circular dichroism which has shown that GSTP1-1 is predominantly alpha helical and Prdx6 is mainly alpha helical with aspects of a beta sheet. The tertiary structural analysis has been carried out using tryptophan fluorescence which revealed that in both proteins the tryptophans are partially exposed to solvent. Furthermore, the quaternary structure was analysed using size exclusion-HPLC which indicated that the proteins are homodimeric in solution (both ~50 kDa). This study will present the findings on the overall characterisation and the implications of the findings on the interaction of these proteins.

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List of Abbreviations

CD	Circular dichroism
CDNB	1-Chloro-2,4-dinitrobenzene
CSIR	Council for Scientific and Industrial Research
Cys	Cysteine
<i>E. coli</i>	<i>Escherichia coli</i>
GSH	Glutathione
GST	Glutathione transferase
GSTP1-1	Glutathione transferase P1-1
H ₂ O ₂	Hydrogen peroxide
hGSTP1-1	Human glutathione transferase P1-1
IMAC	Immobilised metal ion affinity chromatography
IPTG	Isopropyl-beta-D-1-thiogalactopyranoside
JNK	<i>c-Jun</i> NH ₂ -terminal kinase
LC-MS/MS	Liquid chromatography-mass spectrometry/mass spectrometry
MAPK	Mitogen activated protein kinase
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
Prdx6	Peroxiredoxin 6
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE-HPLC	Size exclusion-high pressure liquid chromatography
TNF	Tumour necrosis factor

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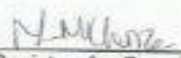
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BRIEF DESCRIPTION OF APPLICATION:

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APPLICANT: Professor HW Dirr

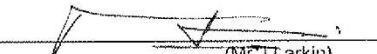
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DATE CONSIDERED: 22 June 2015

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CHAPTER 1: INTRODUCTION

1.1. Peroxiredoxin 6 (Prdx6)

Peroxiredoxins are a superfamily of important thiol, Se-independent (nonseleno) and nonheme peroxidases (Noguera-Mazon *et al.*, 2006; Fisher, 2011; Lien *et al.*, 2012). These ubiquitous enzymes function together with other reductants such as thioredoxins or sometimes with glutaredoxin-glutathione to reduce organic peroxides such as H₂O₂ (hydrogen peroxide) and peroxynitrite (Immenschuh and Baumgart-Vogt, 2005; Noguera-Mazon *et al.*, 2006; Lien *et al.*, 2012). They use their peroxidase activity ($\text{ROOH} + 2\text{e}^- \rightarrow \text{ROH} + \text{H}_2\text{O}$) to exert their protective antioxidant activity (Wood *et al.*, 2003; Noguera-Mazon *et al.*, 2006). The organic peroxides that these enzymes serve to reduce and eliminate are sources of oxidative stress. Oxidative stress causes damage to most cellular constituents and this may include DNA-breakage, cross-linking of protein and lipid peroxidation (Immenschuh and Baumgart-Vogt, 2005). The peroxidase activity of these enzymes allows them to function as physiological barriers against oxidative stress by reducing peroxide formation in cells. The peroxiredoxins use the conserved cysteine (Cys) residues within their active sites to cleave the peroxy bonds of the various peroxide substrates which they are removing (Zheng *et al.*, 2010).

The peroxiredoxins are classified according to the number of redox-active cysteine residues they have that are involved in their enzymatic catalytic cycle (Noguera-Mazon *et al.*, 2006). The peroxiredoxins can either be 1-Cys or 2-Cys depending on whether they have one redox-active cysteine residue (1-Cys) or two redox-active cysteine residues (2-Cys) conserved within their enzymatic active sites (Manevich and Fisher, 2005). The enzymes under the 2-Cys residue category contain the N-terminal and C-terminal Cys residues whereas the enzymes under the 1-Cys residue category contain only the N-terminal Cys residue (Tavender and Bulleid, 2010). All the members of this family function by having the conserved cysteine residues of their peroxidase activity oxidised into sulfenic acid by the substrate they are reducing (Choi *et al.*, 1998; Manevich *et al.*, 2007).

Peroxiredoxin 6 (Prdx6) is an exception to its family members in that it is a 1-Cys enzyme instead of a 2-Cys enzyme like the rest of the members of the family (Lien *et al.*, 2012). In addition, Prdx6 is a bifunctional enzyme because it exhibits glutathione (GSH) peroxidase and

phospholipase A₂ enzymatic activities (Manevich and Fisher, 2005; Jeong *et al.*, 2012) unlike the other members of this family which exhibit only the peroxidase activity. The homodimeric 50 kDa peroxiredoxin is also the only member of the family which does not use thioredoxin as an electron donor but uses GSH as its electron donor (Manevich *et al.*, 2004; Manevich and Fisher, 2005; Lien *et al.*, 2012). Moreover, Prdx6 has the ability to reduce H₂O₂ and phospholipid hydroperoxides (Manevich *et al.*, 2004).

1.1.1. Structure of Pdx6

The structure of the Prdx6 has been solved using X-ray diffraction crystallography and it has been observed that the homodimeric Prdx6 contains a thioredoxin fold at the N-terminus. The fold consists of a four-stranded anti-parallel beta sheet sandwiched between two alpha helices; this fold is important for the overall structure and therefore the function of the protein (Figure 1) (Zheng *et al.*, 2010; Fisher, 2011). The C-terminus on the other end is responsible for the dimerisation of the protein structure (Choi *et al.*, 1998).

The protein has a narrow catalytic pocket in which its catalytic residue (Cys47) is embedded (Fisher, 2011). The Cys47 is made accessible to the GSH during the reduction of the protein from the oxidised (inactive state) to the reduced state (the active state) with aid of glutathione transferase pi (GSTP1-1) (Ralat *et al.*, 2006). The reactivation requires the Prdx6 to form a heterodimer with GSTP1-1 (Ralat *et al.*, 2006). The buried nature of the catalytic pocket makes the protein unable to form dimers via disulfide bonds in its native configuration thus the protein dimerises through hydrophobic interactions (Fisher, 2011).

1.1.2. Catalytic functions of Prdx6

As aforementioned, Prdx6 is a bi-functional enzyme which possesses two enzymatic activities, these being the glutathione (GSH) peroxidase and phospholipase A₂ enzymatic activities (Manevich and Fisher, 2005; Jeong *et al.*, 2012).

1.1.2.1. The glutathione (GSH) peroxidase enzymatic activity

The peroxidase enzymatic activity of the Prdx6 is dependent on the functioning of the Cys47 catalytic residue and it also dependent on the conserved catalytic sequence: PVCTTE (Zheng *et al.*, 2010). In the 2-Cys peroxiredoxins, the catalytic cysteine forms a disulfide with a second

interacting cysteine (within the same subunit) also known as the resolving cysteine (Manevich *et al.*, 2007). This disulfide is reduced back to the sulfhydryl form by the electron donor thioredoxin (Manevich *et al.*, 2007). However, in Prdx6 the oxidised catalytic cysteine residue (sulfenic acid) is reduced back to a thiol (sulfhydryl) by GSH bound GSTP1-1 to complete the catalytic cycle of the peroxidase activity due to the absence of the C-terminal resolving cysteine (Manevich *et al.*, 2007; Lien *et al.*, 2012).

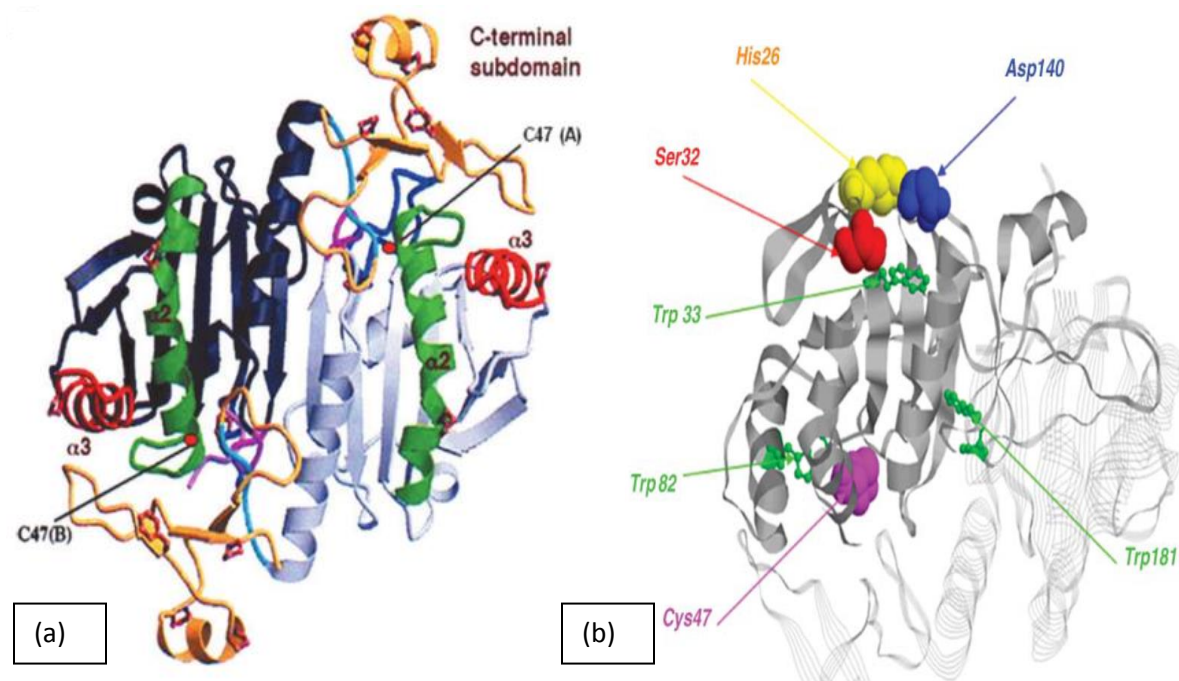


Figure 1: The tertiary structure of Prdx6. (a) Shows the thioredoxin fold which consists of the flanking two alpha helices ($\alpha 2$ (green) and $\alpha 3$ (red)) and a four-stranded beta sheet (navy blue). The Cys47 residues are shown for each monomer. (b) Depicts the homodimeric oxidised tertiary structure model of Prdx6. The model is based upon the structure of Prdx6 in the PDB data base (PDB ID: 1PRX). The model shows the tryptophan residues found in the homodimer (Trp33, Trp82 and Trp181) along with the oxidised Cys47 which is the peroxidase catalytic center of Prdx6. The residues His26, Ser32 and Asp140 are the residues forming the catalytic triad of the phospholipase A₂ activity. Images adapted from Fisher (2011) and Manevich *et al.* (2007), respectively.

1.1.2.2. The calcium-independent phospholipase A₂ enzymatic activity

The second enzymatic activity of Prdx6 is the calcium-independent phospholipase A₂. This activity is dependent on the catalytic triad of the protein: Ser32, His26, and Asp140. The activity is important for cell survival especially for cells that are undergoing oxidative stress (Chhunchha *et al.*, 2011; Lien *et al.*, 2012). The phospholipase A₂ catalyses the hydrolysis of the acyl group at the sn-2 position of glycerophospholipids and it has been implicated in the metabolism of lung surfactant phospholipids as well as activation of the NADPH oxidase (Lien *et al.*, 2012). This activity is maximal at pH 4 hence it is also referred to as the acidic calcium-independent phospholipase A₂ activity and it can be inhibited by serine protease inhibitors which provides evidence that it contains a serine active centre (Manevich and Fisher, 2005; Manevich *et al.*, 2007). Both the peroxidase catalytic activity and the phospholipase A₂ activity are required for Prdx6 to exhibit complete antioxidant functions (Lien *et al.*, 2012).

1.2. Glutathione transferase P1-1 (GSTP1-1)

The glutathione transferases (GSTs; EC 2.5.1.18) belong to a superfamily of enzymes that play an important role in cellular xenobiotic detoxification metabolism (Coles and Ketterer, 1990; Oakley *et al.*, 1997). They assist in protecting the cell from products and substances of oxidative stress and they are also involved in the catalysis of metabolic reactions that are not related to cellular detoxification (Oakley *et al.*, 1997; Laborde, 2010). They excrete noxious substances in the cell by conjugating some of these electrophilic substances to the GSH tripeptide, making these substances less toxic and excretable (Dirr *et al.*, 1994; Oakley *et al.*, 1997; Laborde, 2010). In addition, the GSTs are able to function as ligand-binding proteins known as ligandins (Reinemer *et al.*, 1991; Dirr *et al.*, 1994); these types of proteins function to facilitate intracellular transport of non-substrate substances in the cells (Dirr *et al.*, 1994). GSTs can also confer glutathione peroxidase activity in which they catalyse the organic hydroperoxides into their corresponding alcohols (Chow *et al.*, 2007). These enzymes have also been shown to be involved in the regulation of cell signalling pathways that control cell proliferation and cell death (apoptosis) (Laborde, 2010).

GSTs are classified under at least 8 gene classes referred to as Alpha, Mu, Pi, Theta, Sigma, Omega, Zeta and Kappa (Mannervik *et al.*, 1985; Meyer *et al.*, 1991; Lo Bello *et al.*, 2001).

Members of one gene class typically have an amino acid sequence similarity of about 70 % whereas the similarity is less than 30 % between members of different gene classes (Dirr *et al.*, 1994). Furthermore, members of the same gene class are able to dimerise but subunits of members from different gene classes are not able to dimerise showing that the interface of the enzymes possesses a class-specific recognition site (Dirr *et al.*, 1994). The GSTs family members exist as either homo- or heterodimeric species in which each monomeric subunit has a molecular mass of about 25 kDa with a single active site per monomer (Reinemer *et al.*, 1991; Dirr *et al.*, 1994; Oakley *et al.*, 1997). For the purposes of this study the focus is on the human GSTP1-1 from the pi gene class, this protein has been shown to play a role in the development of anti-cancer drug resistance of tumour cell lines (Tew, 1994; Oakley *et al.*, 1997; Vasieva, 2011).

The human GSTP1-1 isozyme, herein after referred to as GSTP1-1, is normally expressed in epithelial tissue such as the lungs, oesophagus and placenta, however in cancerous conditions the enzyme is over-expressed in the bladder, stomach, colon and lung compared to other normal tissue (Watson *et al.*, 1998). In some conditions GSTP1-1 is considered a pre-neoplastic marker. The enzyme has thus been of interest for chemotherapeutic drug research.

1.2.1. Structure of GSTP1-1

The structure of the enzyme has been elucidated using X-ray diffraction crystallography and these studies have shown that the enzyme is a homodimer composed of two subunits, each with two domains, these being the N-terminal domain and the C-terminal domain (Reinemer *et al.*, 1991; Dirr *et al.*, 1994). The N-terminal domain is made of residues 1 to 74 and the C-terminal domain is made of residues 81 to 207 (Reinemer *et al.*, 1991). Each subunit contains a catalytically independent active site that consists of a glutathione binding site which is referred as the G-site; this site is composed mainly of the N-terminal domain residues (Danielson and Mannervik, 1985; Dirr *et al.*, 1994). There is also the electrophilic substrate binding site referred to as the H-site; this site is predominantly composed of the C-terminal domain residues (Mannervik *et al.*, 1985; Reinemer *et al.*, 1991). The N-terminal domain (domain 1 in Figure 2) has a thioredoxin fold and the C-terminal domain (domain 2 in Figure 2) is a strictly alpha helical structure (Dirr *et al.*, 1994). The crystal structure of the isozyme is presented as Figure 2.

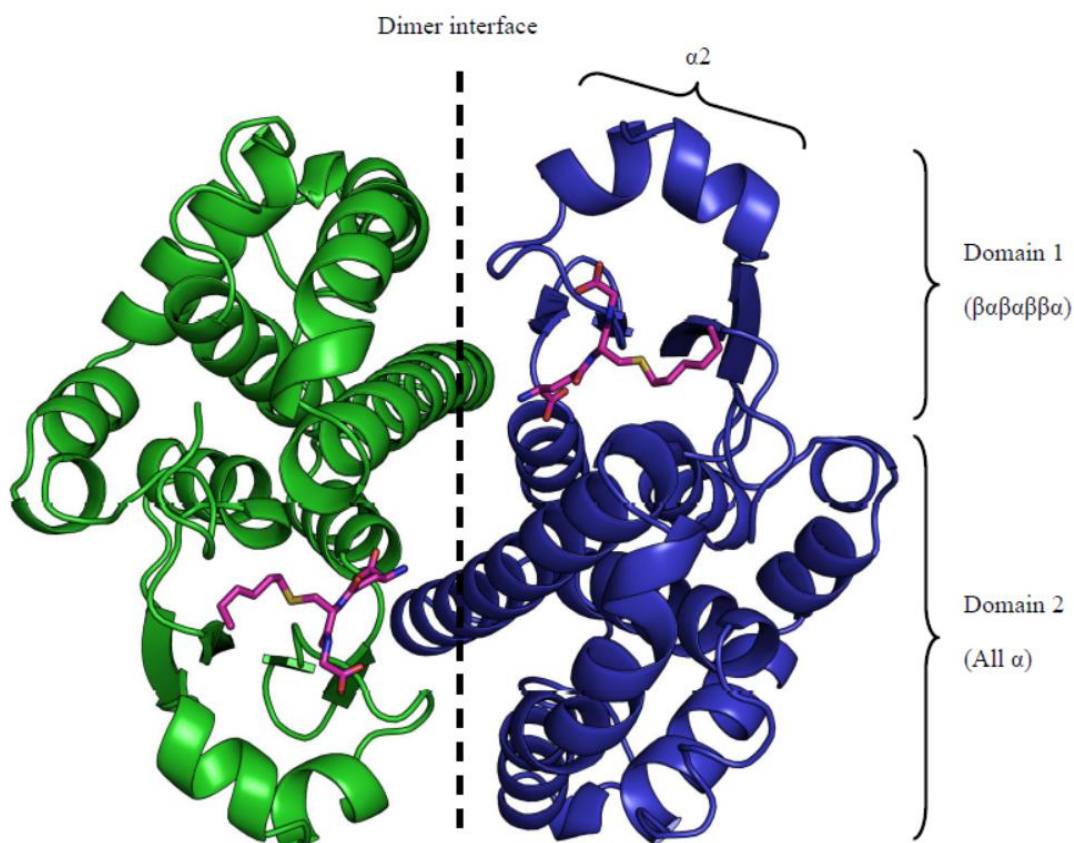


Figure 2: The quaternary structure of the hGSTP1-1 protein bound to S-hexylglutathione. PDB ID: 9GSS. The location of the active site of the protein is shown by the position of the S-hexylglutathione (coloured magenta in the image). The image is adapted from a PhD thesis submitted by David Balchin (2013) [http://wiredspace.wits.ac.za/bitstream/handle/10539/14759/PhD%20Thesis_David%20Balchin%200600215W_Final%20submission.pdf?sequence=1]. The image was generated using PyMol (www.pymol.sourceforge.net).

1.2.2. Catalytic functions of GSTP1-1

The GSTP1-1, like the rest of its family members, confers glutathione activity in which it catalyses the conjugation of reduced GSH to an electrophilic substrate (Reinemer *et al.*, 1991). GSTP1-1 specifically catalyses the detoxification of electrophilic epoxides, which are produced upon the metabolism of polycyclic aromatic hydrocarbons (Chow *et al.*, 2007; Vasieva, 2011). The enzyme also carries out a variety of regulatory functions within the cell; these include the regulation of the

signalling kinases and thus the cell signalling pathways (Adler *et al.*, 1999). GSTP1-1 modulates the c-*Jun* NH₂-terminal kinase (JNK) by using its C-terminal region to bind the JNK and its active centre to regulate the JNK (Adler *et al.*, 1999; Elsby *et al.*, 2003). JNK is a mitogen activated protein kinase (MAPK) which plays a key role as a regulator of apoptosis, stress response, inflammation, and cell differentiation and proliferation; in this manner, the enzyme is able to exhibit anti-apoptotic activity (Laborde, 2010). There are other signalling molecules which the GSTP1-1 regulates including the TNF receptor factor 2 and the TNF- α of which it suppresses the activity of both (Vasieva, 2011).

GSTP1-1 also functions within the cell to reactivate Prdx6 and it also functions in the regulation of nitric oxide (NO). Exposure to NO can lead to nitration and/or nitrosylation which are a very important form of post-translational modifications on target proteins (Liu *et al.*, 2004). NO homeostasis imbalance due to GSTP1-1 knock out has been implicated in pulmonary and critical care medicine disorders such as apnoea, asthma and cystic fibrosis (Liu *et al.*, 2004; Vasieva, 2011)

The structural and functional study of Prdx6 and GSTP1-1 is essential to elucidate the structural and functional characteristics of the proteins, this will allow for a fundamental understanding of the biochemical properties of the proteins and their physiological importance.

1.3. Aim and objectives

1.3.1. Aim

The aim of this study was to structurally and functionally characterise the recombinant Prdx6 and GSTP1-1 proteins.

1.3.2. Objectives

- 1.3.2.1. Over-expression of the Prdx6 and GSTP1-1 proteins using *Escherichia coli* (*E. coli*) cells as the expression system.
- 1.3.2.2. Purification of His-tagged Prdx6 and GSTP1-1 using immobilised metal ion affinity chromatography (IMAC).
- 1.3.2.3. Protein identity confirmation using mass spectrometry.
- 1.3.2.4. Structural analysis of the pure proteins using circular dichroism (CD) spectrometry (secondary structure), fluorescence spectroscopy (local tertiary structure) and size exclusion-high pressure liquid chromatography (SE-HPLC) (quaternary structure).
- 1.3.2.5. Functional characterisation of the proteins by analysing the peroxidase activity using the GSH reductase/NADPH-coupled GSH peroxidase assay for Prdx6 and analysing the GST activity of GSTP1-1 using the 1-chloro-2,4-dinitrobenzene (CDNB)/GSH assay.

CHAPTER 2: EXPERIMENTAL PROCEDURES

2.1. Materials

The plasmids used in this study were synthesised by GenScript (New Jersey, USA). The gel filtration standards used for SEC-HPLC (catalog number MWGF70) and the chemicals GSH, NADPH as well as the glutathione reductase enzyme were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the other reagents used were of analytical grade.

2.2. Expression plasmids and transformation of *Escherichia coli* cells

The expression system used for this investigation was the *E. coli* which is the model organism commonly used for mammalian protein expression. This expression system has been used in literature and has proven to be efficient at expressing human proteins. *E. coli* cells were transformed for expression of Prdx6 using a pET-11a vector while a pET-15b vector was used to transform *E. coli* cells for expression of GSTP1-1. The target gene sequences for the proteins were harmonised for expression in the *E. coli* T7 Express bacterial strain. The DNA insert for the Prdx6 was designed (Dr I. Achilonu, University of the Witwatersrand) with an N-terminus hexa-His-tag coding sequence for purification as well as a thrombin cleavage site. The plasmid was introduced into chemically competent T7 Express *E. coli* cells using the standard heat-shock transformation method (depicted in Figure 3) (<http://www.addgene.org/plasmid-protocols/bacterial-transformation/>). The T7 Express cells with the plasmid for GSTP1-1 were a generous gift from Ms Kimberly Wiid (University of the Witwatersrand). The plasmid carries an N-terminal His-tag and a thrombin cleavage site.

The pET family of vectors contain a T7 RNA polymerase; this polymerase has high levels of activity with high transcription efficiency such that the expression of target proteins is above 50 % within hours after induction of protein synthesis (Jia and Jeon, 2016). The polymerase is able to direct such high levels of transcription because it is highly selective for its own promoters (the T7 promoters) which do not occur naturally in *E. coli* and its transcription can proceed without termination (Studier and Moffatt, 1986). The high levels of transcription also result in a great decrease of “in-house” *E. coli* RNA polymerase transcription levels therefore decreasing “in-house” protein synthesis and increasing target protein synthesis (Studier and Moffatt, 1986; Jia

and Jeon, 2016). The pET vector expression system thus not only provides stability but also provides high levels of expression of target proteins within a short period of time. The *E. coli* strain T7 Express was selected based also on that it lacks the *lon* and *ompT* proteases and it is compatible with the T7 *lacO* promoter which is part of the vectors used (Jiang *et al.*, 2002). The absence of *lon* and *ompT* proteases helps in preventing target protein degradation by endogenous proteases of the cell upon synthesis (Jiang *et al.*, 2002).

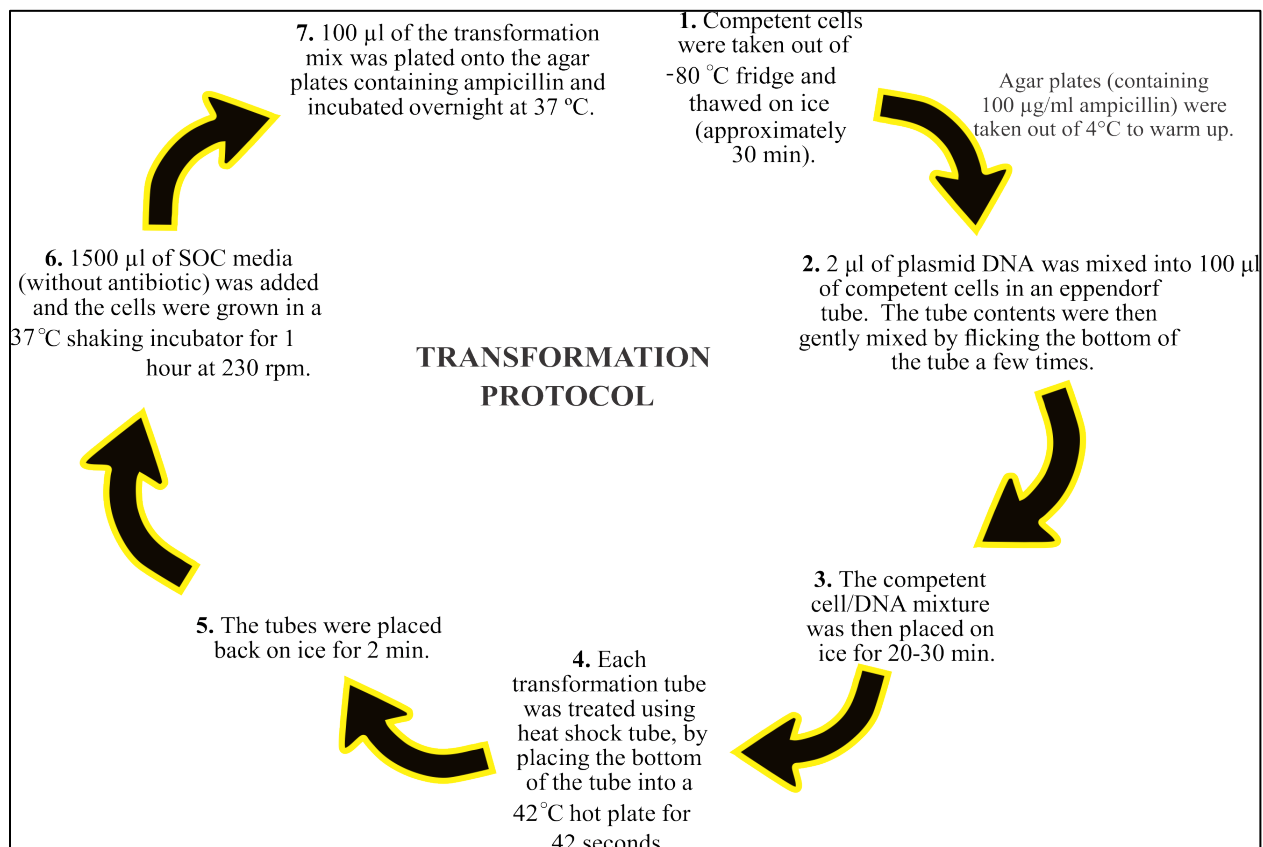


Figure 3: The standard heat-shock transformation protocol followed to transform the T7 Express *E. coli* cells.

2.3. Plasmid purification and verification

The pET-11a and pET-15b vectors encode a gene for ampicillin resistance which was used as a selective measure to choose successfully transformed cells for plasmid extraction. The Prdx6 and GST1-1 plasmids were extracted from transformed cells using the same protocol. Glycerol stocks of T7 Express *E. coli* cells were prepared following transformation with either the pET-11a (Prdx6) or pET-15b (GSTP1-1) plasmid and stored at -80 °C; these stocks were subsequently defrosted in

ice and inoculated into 2xYT media [1.6 % (w/v) tryptone, 1 % (w/v) yeast extracts and 0.5 % (w/v) NaCl, pH 7.4] containing 100 µg/mL (final concentration) of ampicillin. The culture was grown overnight (16 hours) at 37°C with shaking at 230 rpm.

Plasmids were extracted from the T7 Express *E. coli* cells using a GeneJET™ plasmid mini preparation (mini-prep) kit (ThermoScientific, USA) which uses the alkaline-lysis method. Two rounds of 1 mL cell culture were used to harvest cells by centrifugation at 12000 x g. These cells were subsequently re-suspended by aspiration in 250 µL of Resuspension Buffer using a pipette tip. The Resuspension Buffer contains EDTA ions which chelate divalent cations to prevent enzymatic cleavage of plasmid DNA in preparation for cell lysis. The buffer also helps in maintaining optimal pH during the process of cell lysis. The cells were then lysed by addition of 250 µL of Lysis Solution. The alkaline based Lysis Solution contains SDS (sodium dodecyl sulfate) and NaOH. The SDS disturbs the membrane by denaturing the proteins embedded in the membrane while the NaOH denatures the plasmid and genomic DNA. Following lysis, the reaction was neutralised by the addition of 350 µL Neutralisation Solution containing sodium acetate. The solution helps in precipitating out proteins and larger genomic DNA. This process is essential as it helps in limiting the association between the longer strands of genomic DNA to assist the genomic DNA to reanneal correctly. The cell debris was separated from the plasmid DNA by centrifugation at 12000 x g. The cell debris pelleted and the plasmid DNA remained soluble in the supernatant. The supernatant containing the plasmid DNA was decanted into a silica-membrane based spin column which can bind the plasmid DNA. The spin column and supernatant were then centrifuged at 12000 x g and the flow through discarded. The spin column was rinsed twice with 500 µL Washing Solution containing ethanol to get rid of any contaminants. The plasmid DNA was eluted from the column using 50 µL MilliQ water and stored at -20 °C. Thereafter, the purified DNA was sent for Sanger sequencing at Inqaba Biotechnical Industries Pty (Ltd) (Pretoria, South Africa).

The extracted plasmid DNA samples of Prdx6 and GSTP1-1 were sequenced to verify that the sequences encoding for both proteins was correct and to make sure that there were no changes to the DNA sequences that may have been introduced during synthesis and transformation.

2.4. Over-expression of Prdx6 and GSTP1-1

The over-expression of the proteins was carried out following transformation of the T7 Express *E. coli* cells with the pET-11a and pET-15b vectors containing the DNA insert of interest for Prdx6 and GSTP1-1 respectively. The expression of the proteins was induced using isopropyl-beta-D-1-thiogalactopyranoside (IPTG), an expression inducer in transformed cells which contain the T7 promoter (Marbach and Bettenbrock, 2012).

Expression of Prdx6 was conducted following the conditions similar to those outlined by Tavender and Bulleid (2010). The T7 Express *E. coli* cells containing the pET-11a plasmid were grown in 2xYT media [1.6 % (w/v) tryptone, 1 % (w/v) yeast extracts and 0.5 % (w/v) NaCl, pH 7.4] at 37 °C to an OD₆₀₀ of 0.6 in a shaking incubator at 230 rpm. Protein over-expression was induced by the addition of IPTG to a final concentration of 1 mM and the induced cells were allowed to incubate further for 3 hours prior to harvesting.

GSTP1-1 was over-expressed following a protocol outlined by Chang *et al.* (1999). The T7 Express *E. coli* cells containing the pET-15b plasmid were grown using the same media and conditions as above. However, IPTG to the final concentration of 0.2 mM was used to induce expression and the cells were incubated for 6 hours before harvesting.

2.4.1. Cell harvesting and lysis

After the proteins were over-expressed, both Prdx6 and GSTP1-1 needed to be extracted from the bacterial cells. The cells were harvested by centrifugation at 5000 x g for 40 minutes and re-suspended in lysis buffer [20 mM sodium phosphate pH 7.4, 0.02 % sodium azide, 0.5 M NaCl, 30 mM imidazole, for Prdx6 and 50 mM Tris-HCl pH 8.4, 0.02 % sodium azide, 0.5 M NaCl, 30 mM imidazole, for GSTP1-1]. Cells obtained from 1 litre media were re-suspended in 25 mL lysis buffer supplemented with 1 mg/mL lysozyme and 5 µg/mL DNase and left to incubate for 20 minutes at 20 °C. The lysozyme was added to facilitate the procedure of protein extraction through lysis of the bacterial cell wall while the DNase was added to degrade cellular DNA to prevent it from contaminating the protein during extraction. Thereafter, the cells were further lysed by sonication using 6 pulses at maximum voltage for 30 seconds and allowed to cool for 30 seconds on ice to dissipate heat. Sonication uses ultrasonic sound waves to agitate cells, therefore lysing them and facilitating the process of protein extraction. The sonicated cells were centrifuged for 30

minutes at 12000 rpm. The supernatant (soluble fraction) obtained from the centrifugation was harvested and filtered using a 0.45 micron filter to remove large particles and aggregates, this also reduces contaminants and prevents clogging of the tubing when conducting purification of the protein. The pellet (insoluble fraction) was re-suspended in lysis buffer at the same volume as the supernatant and sonicated. The soluble and insoluble fractions were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to determine if the protein expression was successful and if the cells were efficiently lysed.

2.5. Purification of Prdx6 and GSTP1-1

Purification of the Prdx6 and GSTP1-1 was carried out using immobilised metal ion affinity chromatography (IMAC). The principle of the technique was developed by Professor Jeker Porath (Porath *et al.*, 1975). The known binding affinities of the transition metals (copper, nickel, cobalt and zinc) for histidine and cysteine in aqueous solutions form the basis of the principle of the technique (Porath *et al.*, 1975; Block *et al.*, 2009). IMAC allows for the fractionation of specific target proteins which have been “tagged” by specific amino acid motifs either at the N- or C-terminus; the fractionation of the proteins is achieved using metal ions fixed to a support as ligands (Block *et al.*, 2009). The most commonly used protein tag is the histidine tag more commonly known as the His-tag; this tag has affinity for cobalt and nickel metals. The proteins in this investigation were hexa-His-tagged at their N-terminus ends. The use of IMAC proved to be advantageous because it allowed for mild eluting conditions and the tags used allowed for specific and strong binding (Block *et al.*, 2009). The process of IMAC protein purification was carried out using the ÄKTA Prime liquid handling system (American biosciences, USA).

2.5.1. Purification of Prdx6

A HisTrap™ (GE healthcare, UK) 5 mL nickel column was used for purification. The column was equilibrated with 5 column volumes of binding buffer [20 mM sodium phosphate pH 7.4, 0.5 M NaCl, 30 mM imidazole, 0.02 % sodium azide]. The supernatant obtained from cell lysis was loaded onto the column and washed with binding buffer. The binding buffer washes out any loosely bound contaminants from the column. The His-tagged Prdx6 was eluted using elution buffer [20 mM sodium phosphate pH 7.4, 0.5 M NaCl, 500 mM imidazole, 0.02% sodium azide]. The His-tagged protein is able to elute off the column because imidazole has an affinity for the metal

charged column and thus it competes with the His-tag for binding to the column and eventually displaces the tagged protein from the column allowing elution of the target protein. The purified target protein was then collected and its purity analysed using SDS-PAGE.

2.5.2. Purification of GSTP1-1

A 5 mL cobalt HisTrap™ column (GE healthcare, UK) was used for purification of GSTP1-1. The column was equilibrated with 5 column volumes of binding buffer [50 mM Tris-HCl pH 8.0, 0.5 M NaCl, 50 mM imidazole, 0.02 % sodium azide]. The same buffer was used for the wash step after the application of the protein solution. The His-tagged target protein was eluted from the column with elution buffer [50 mM Tris pH 8.0, 0.5 M NaCl, 300 mM imidazole, 0.02% sodium azide buffer]. The purified target protein was then collected and analysed using SDS-PAGE.

2.6. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE technique uses the anionic detergent sodium dodecyl sulfate (SDS) which dissociates oligomeric proteins to their individual polypeptide subunits by disrupting the non-covalent interactions between the polypeptide chains of the protein, giving a uniform net negative charge along each polypeptide (Reed *et al.*, 2003). Electrophoresis is based on the migration of charged particles in an electric field and since the SDS-protein complexes are uniformly negatively charged the separation is based solely on size without protein shape and charge interference because all the proteins will be linear with a uniform negative charge (Campbell, 1991). The SDS-PAGE yields molecular masses of the protein's subunits rather than that of intact protein unless the subunits are inter-disulfide linked or monomeric. To prevent inter-disulfide linkage, β -mercaptoethanol was added to reductively cleave the disulfide links (Voet and Voet, 2004). All samples were analysed using a polyacrylamide discontinuous electrophoresis technique based on the Laemmli method (Laemmli, 1970).

The SDS-PAGE gels consisted of 4 % acrylamide stacking gels [4 % (w/v) acrylamide, 0.36 % (w/v) bis-acrylamide, 50 mM Tris-HCl pH 6.8, 0.1 % (w/v) SDS, 0.005 % (w/v) ammonium per sulphate and 0.2 % (v/v) TEMED] and 12.5 % acrylamide separating gels [12.5 % (w/v) acrylamide, 1.08 % (w/v) bis-acrylamide, 250 mM Tris-HCl pH 8.8, 0.1 % (w/v) SDS, 0.05 % (w/v) ammonium per sulphate and 0.2 % TEMED]. The samples to be analysed were diluted with

an equal amount of reducing sample buffer [25 mM Tris-HCl pH 6.8, 4 % (w/v) SDS, 20 % (v/v) glycerol, 10 % (v/v) β -mercaptoethanol and 3.5 μ g/mL bromophenol blue] and heated at 90 °C for 10 minutes to complete protein denaturation.

The samples were then subjected to electrophoresis in a 12.5 % acrylamide/bis-acrylamide gel using a Bio-Rad™ mini-PROTEAN tetra cell electrophoresis system, set at 180 V 60 mA for $\pm$ 1 hour. The electrophoresis system used the electrode buffer (anode and cathode) [192 mM glycine, 0.1 % (w/v) SDS, 250 mM Tris-HCl pH 8.3]. The gels were then stained in coomassie stain solution [0.1 % (w/v) coomassie dye in 1:5:4 (v/v/v) acetic acid: methanol: water] for 1-3 hours and destained overnight using destaining solution [1: 5: 4 (v/v/v) acetic acid: methanol: water]. An unstained molecular mass ladder from Fermentas™ was used as a reference; the molecular mass ladder covered the molecular mass range of 14 kDa to 116 kDa. The gel images were captured using the Bio-Rad™ GelDoc imager.

2.7. Protein concentration determination

The concentration of the proteins was determined by subjecting each protein to a series of dilutions and measuring the absorbance of each dilution at 280 nm. The side chains of the aromatic residues absorb maximally at this wavelength. These absorbance values were used to construct a standard curve. The absorbance value of the undiluted stock solution was obtained by extrapolating to a 1x dilution on the standard curve. Then the extrapolated absorbance at 280 nm was applied to the Beer-Lambert equation:

$$A_{280} = \epsilon \cdot c \cdot l \quad \text{Equation 1}$$

where A_{280} is the absorption value at 280 nm, ϵ is the extinction coefficient/ molar absorption coefficient ($M^{-1} \text{ cm}^{-1}$) at 280 nm, c is the concentration (M) value to be determined and l is the cell path-length (cm). The extinction coefficients of both proteins were determined using the Expasy ProtParam tool (<http://web.expasy.org/protparam/>) (Gasteiger *et al.*, 2005). The ProtParam tool uses the amino acid sequence of the protein as an input to compute and deduce its physico-chemical properties (Gasteiger *et al.*, 2005). The extinction coefficient for dimeric Prdx6 was determined to be $44920 \text{ M}^{-1} \text{ cm}^{-1}$ and the extinction coefficient for dimeric GSTP1-1 was determined to be $55760 \text{ M}^{-1} \text{ cm}^{-1}$. The concentration of each protein was determined with the Jasco V-630 spectrophotometer using 10 mm path-length quartz cuvette.

2.8. Mass spectrometry

The identity of the proteins was confirmed using peptide mass finger printing which was performed using liquid chromatography-mass spectrometry/mass spectrometry (LC-MSMS). This is a highly sensitive technique which combines the physical separation capabilities of liquid chromatography with the mass-charge analysis of mass spectrometry. The technique provides fast spectral acquisition rates with high resolving power and mass measurement accuracy (Andrews *et al.*, 2012).

Purified Prdx6 and GSTP1-1 samples were subjected to electrophoresis using SDS-PAGE, the gel obtained was then sent for LC-MS/MS analysis at the Council for Scientific and Industrial Research (CSIR, Pretoria, South Africa). The proteins bands were destained [50 mM ammonium bicarbonate/ 50 % methanol] followed by in-gel reduction [50 mM DTT suspended in 25 mM ammonium bicarbonate] and subjected to alkylation [55 mM iodoacetamide dissolved in 25 mM ammonium bicarbonate]. The bands were then extracted from the gel using in-gel trypsin digestion (Shevchenko *et al.*, 2007); in this process the gel was digested overnight at 37 °C with 5 -50 ng/μL trypsin depending on the size of the gel piece. Thereafter, the digested peptides were re-suspended in 2 % acetonitrile/ 0.2 % formic acid after which they were desalted on an Acclaim PepMap C18 trap column for 2 minutes at 15 μL/min using 2 % acetonitrile/ 0.2 % formic acid and separated on an Acclaim PepMap C18 RSLC (2 μm particle size) column. The peptides were eluted using a flow-rate of 8 μL/min with a 4-60 % gradient of 80 % acetonitrile/ 0.1 % formic acid. A Dionex Ultimate 3000 RSLC system coupled to an AB Sciex6600 TripleTOF mass spectrometer was used to analyse the peptides. The TripleTOF mass spectrometer was operated in Data Dependant Acquisition mode. The eluted peptides now suspended in 80 % acetonitrile/ 0.1 % formic acid solution were subsequently ionised by electrospray ionisation (electrospray voltage of 5.5 kV). The MS scans were acquired from m/z 400-1500 using an accumulation time of 250 ms followed by 30 MSMS scans, acquired from m/z 100-1800 at 100 ms each, for a total scan time of 3.3 sec. Multiply charge ions (2^+ - 5^+ , 400 -1500 m/z) were automatically fragmented in Q2 collision cells using nitrogen as the collision gas. Collision energies were chosen automatically as function of m/z and charge.

Protein Pilot v5 using the Paragon search engine (AB Sciex) was used for comparison of the obtained MS/MS spectra with a custom database (UniSwiss) containing known protein sequences, the peptide sequences and the sequences of the contaminants. The Paragon search engine is a novel database search engine used for the identification of peptides from mass spectrometry data (Shilov *et al.*, 2007). Only protein sequence matches identified with confidence of above 95 % were reported to eliminate any false positive sequence matches.

2.9. Structural characterisation of Prdx6 and GSTP1-1

2.9.1. Circular dichroism (secondary structure)

Circular dichroism (CD) spectroscopy measures the difference in absorption of left and right circularly polarised light which is generated by asymmetric molecules when they interact with the light (Greenfield, 2006). This differential absorption of the circularly polarised light results in the formation of an ellipse.

The secondary structure of proteins can be evaluated in the far-UV CD region (180 – 240 nm) because the peptide backbone of proteins can absorb circularly polarised light in this region due to the presence of the asymmetric peptide bonds that exist in the peptide backbone (Corrêa and Ramos, 2009). The secondary structural elements of protein such as the alpha helices and beta sheets produce characteristic spectra when they interact with circularly polarised light. The alpha helices give negative troughs at 222 nm and 208 nm and a large positive peak at 190 nm whereas the beta sheets produce negative troughs at 218 nm and positive peaks at 195 nm (Woody, 1995; Greenfield, 2006).

CD was conducted with 4 μ M Prdx6 and GSTP1-1 samples. All spectra represent an average of 5 accumulations recorded in the 250-180 nm wavelength range with a scan speed of 200 nm.min⁻¹ using a Jasco J-1500 CD spectrometer. The spectra were recorded at a constant temperature of 20 °C with the bandwidth set at 1 nm with a 2 mm path-length cuvette; the data interval was set to 0.5 nm with 1 second response. The samples were prepared by dialysing the proteins into 5 mM sodium phosphate buffer pH 7.4 with 0.02 % sodium azide overnight at 4 °C. The data was buffer corrected and converted to mean residue ellipticity [θ] using the equation:

$$[\theta] \text{ (deg.cm}^2\text{.dmol}^{-1}\text{)} = \theta_{\text{obs}} \text{ (mdeg).}10^6 / l \text{ (mm). [Protein](}\mu\text{M). n} \quad \text{Equation 2}$$

where θ_{obs} is the ellipticity raw data obtained from the instrument; l is the path-length and n is the number of residues in the protein (Sharpe, 2012). The experimental data obtained was then analysed using DichroWeb (Whitmore & Wallace, 2008) to determine the percentage of alpha helices and beta strands in the protein.

2.9.2 *Intrinsic tryptophan fluorescence (local tertiary structure)*

Molecules usually occupy the lowest vibrational level of the electronic states known as the ground state. Upon excitation by electromagnetic radiation these molecules absorb the radiation energy and become elevated to higher vibrational levels, this is known as the excited state. This excited state can last for a few nanoseconds after which the molecules begin to rapidly lose their excess vibrational energy due to collisions with each other until they fall back to their ground state. The molecules emit light as they transition from the excited state to the ground state; fluorescence measures this light emitted by these molecules (Gordon, 1994). The emitted light appears as band spectra which is usually a mirror image of the absorption spectra which is then studied to understand the structure of the associated molecule (Gordon, 1994).

Fluorescence uses fluorescent moieties known as fluorophores as probes for detection. Fluorophores enable a molecule to absorb radiation energy at specific wavelength and to emit the energy again at a longer but specific wavelength (Greenfield, 2006). Proteins contain three aromatic residues which are able to serve as fluorophores, these being tyrosine, phenylalanine and tryptophan. Tryptophan is the most commonly used probe because it is the dominant source of intrinsic protein fluorescence as it is present in higher concentration and it is more abundant as compared to phenylalanine and tyrosine (Greenfield, 2006). The indole ring of tryptophan is highly sensitive to its local environment such that changes in polarity, hydrogen bonding and other non-covalent interaction result in the change of tryptophan fluorescence making it a good probe of local tertiary structure (Gordon, 1994; Greenfield, 2006). Tryptophan residues can absorb light at 295 nm thus they are excitable at that wavelength (Gordon, 1994). Tryptophan fluorescence was used in this to study the local tertiary structure of the proteins using fluorescence spectroscopy.

Prdx6 and GSTP1-1 were expected to produce strong and reliable tryptophan fluorescence spectra as the Prdx6 homodimer contains 3 tryptophan residues in each subunit and the GSTP1-1 contains 2 tryptophan residues in each subunit. The position of the tryptophan residues in both proteins may

provide some basic information on their local tertiary structure. Figure 4 shows the positions of the tryptophan residues in the folded structure of both the proteins. The proteins were studied in their native and denatured forms to get a basic idea of how the local tertiary structure is affected by folding.

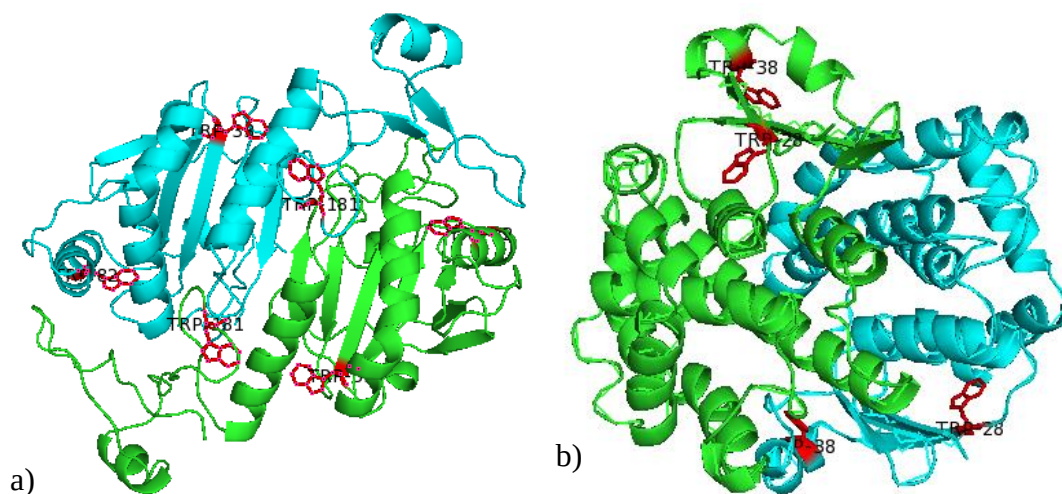


Figure 4: The positions of the tryptophan residues (red) in the folded structure of: a) the Prdx6 (PDB ID: 1PRX) and b) the GSTP1-1 (PDB ID: 9GSS). The residues are shown both in chain A (cyan) and in Chain B (green) of each protein. Image was generated using PyMol (www.pymol.org).

Fluorescence measurements were conducted with 2 μ M Prdx6 and GSTP1-1 samples. The samples for this procedure were prepared by dialysing the proteins into 5 mM sodium phosphate buffer pH 7.4 with 0.02 % sodium azide overnight at 4 °C, these samples were used for the native protein spectra. Denatured protein was obtained by incubating the required volume of native protein into 8 M spectroscopic grade urea (Merck, USA) overnight at 20 °C. This was then followed by analysis using a Jasco FP-6300 spectrofluorometer. The studies were conducted using a micro-fluorescence cuvette with a 10 mm path-length. The excitation wavelength was set at 295 nm to selectively excite the tryptophan residues. The emission was set over the wavelength range of 250 – 500 nm using 5 nm excitation and emission slit widths. All the spectra were recorded at scan speed of 200 nm.min⁻¹ over 3 accumulations at 20 °C and all data was buffer corrected.

2.9.3. Size exclusion-HPLC (quaternary structure)

The analysis of the quaternary structure was carried out using SE-HPLC (size exclusion-high pressure liquid chromatography). SE-HPLC separates proteins based on their hydrodynamic size, diffusion coefficient, and surface properties, with the larger proteins in the gel filtration column eluting first and the smallest proteins eluting last (Tayyab *et al.*, 1991). Protein retardation in the column depends on the pore/channel size of the stationary phase of the column. The column is usually made of glass or steel and the stationary phase is made of chemically inactive particles packed in a 3-dimensional arrangement within the column. The mobile phase can be a buffer, water, an organic solvent or a mixture of these solutions (Tayyab *et al.*, 1991); in this case the mobile phase was a buffer. Eluted fractions were monitored at 280 nm, which is the wavelength at which the side chains of the aromatic residues absorb light maximally.

The Shimadzu UFLC SPD-20A size exclusion-HPLC system was used for the analysis of the proteins. The column was calibrated using SEC-HPLC buffer [20 mM sodium phosphate pH 7.0, 0.02 % sodium azide, 150 mM NaCl] in which the proteins were stored. The calibration and operations of the system were set as per manufacturer's instructions. The column used for the analysis was the TSK gel superSW2000, 4.6 mm ID × 30 cm, 4 µm (Tosoh Biosciences, LLC). The list of the gel filtration protein molecular mass standards (6.5- 66 kDa) used is provided in Table 1. The standards enabled the sizing of the target proteins.

Table 1: The list of protein standards used for SEC-HPLC analysis.

Reagent	Amount (mg)/vial	Approximate Molecular mass (kDa)
Blue Dextran*	50	2000
Albumin, bovine serum (with ~0.3 % DTT)	50	66
Cabornic Anhydrase from bovine erythrocytes	15	29
Cytochrome C from horse heart	10	12.4
Aprotinin from bovine lung	15	6.5

*Blue Dextran is eluted in the void volume of the column thus only 4 of the 5 standards are seen in the elution profile (see section 3.4.3).

2.10. Functional characterisation of Prdx6 and GSTP1-1

2.10.1. GST enzyme activity assay for GSTP1-1

The activity of the GSTP1-1 was assayed using the 1-chloro-2, 4-dinitrobenzene (CDNB)/GSH assay (Habig *et al.*, 1974). The assay exploits the ability of the enzyme to conjugate the thiol group of GSH to electrophilic substrates as part of its cellular detoxification mechanism (Reinemer *et al.*, 1991). In the assay the thiol group of the GSH is conjugated to the CDNB substrate, this conjugation results in an increase in absorbance at 340 nm (Habig *et al.*, 1974). The change in absorbance at 340 nm is then measured using a visible light spectrophotometer. The reaction is as follows:



Image is adapted from <http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Bulletin/cs0410bul.pdf>

The assay was carried out in a 3 mL reaction buffer [100 mM sodium phosphate pH 6.5, 1 mM EDTA, 1 mM GSH, 1 mM CDNB (dissolved in 100 % ethanol) and 0.02 % sodium azide] with enzyme concentration range of 10 nM – 30 nM. The reactions were carried out in triplicates for each concentration and each reaction was monitored for 60 seconds on the visible light spectrophotometer using a 10 mm path-length cuvette.

2.10.2. Peroxidase activity assay for Prdx6

The GSH-reductase/NADPH-coupled GSH peroxidase assay was carried out to assess the peroxidase activity of Prdx6. The assay measured the enzyme activity using a system in which the oxidation of NADPH by glutathione reductase is coupled to the oxidation of reduced glutathione (GSH) (Carmagnol *et al.*, 1983). NADPH can absorb light at 340 nm whereas its oxidised counterpart NADP⁺ does not; this enables the change in NADPH concentration to be readily determined at 340 nm (Carmagnol *et al.*, 1983). This occurrence forms the basis of this assay as the change in concentration of NADPH is monitored as the glutathione reductase uses it to catalyse the reduction of the oxidised glutathione (GSSG) to reduced glutathione (2GSH) as follows:



Image adapted from: http://www.bioxys.com/i_zeptometrix/0805004.htm

Initially, the standard reaction buffer (3 mL) used was [50 mM Tris-HCl pH 8.0, 2 mM NaN₃, 0.1 mM EDTA, 0.3 mM NADPH, 0.36 mM GSH, and 0.23 units/mL GSH reductase] (Fisher *et al.*, 1999) but after several failed reactions the assay buffer (1 mL) was adjusted to 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.1 mM NADPH, 1 mM GSH, and 0.009 units/mL GSH reductase. The assay was monitored for 5 minutes using the visible light spectrophotometer (Jasco V630) at a wavelength of 340 nm with a 10 mm path-length cuvette.

CHAPTER 3: RESULTS

3.1. Over-expression of Prdx6 and GSTP1-1 proteins using *Escherichia coli* (*E. coli*) cells as the expression system

Sanger sequencing performed by Inqaba Biotechnical Industries Pty (Ltd) (Pretoria, South Africa) on the pET-11a and pET-15b plasmids confirmed that the gene inserts were encoding for the correct proteins without mutations. The DNA sequences obtained from Inqaba (Figure 5) were translated into protein sequences using the Expasy translate tool (<http://web.expasy.org/translate/>). Thereafter, the protein sequences were checked against the UniProt database (<http://www.uniprot.org/>) to confirm that the protein sequences obtained correspond to the protein sequences of Prdx6 and GSTP1-1. The results of the sequence alignments are presented in Figures 6 and 7.

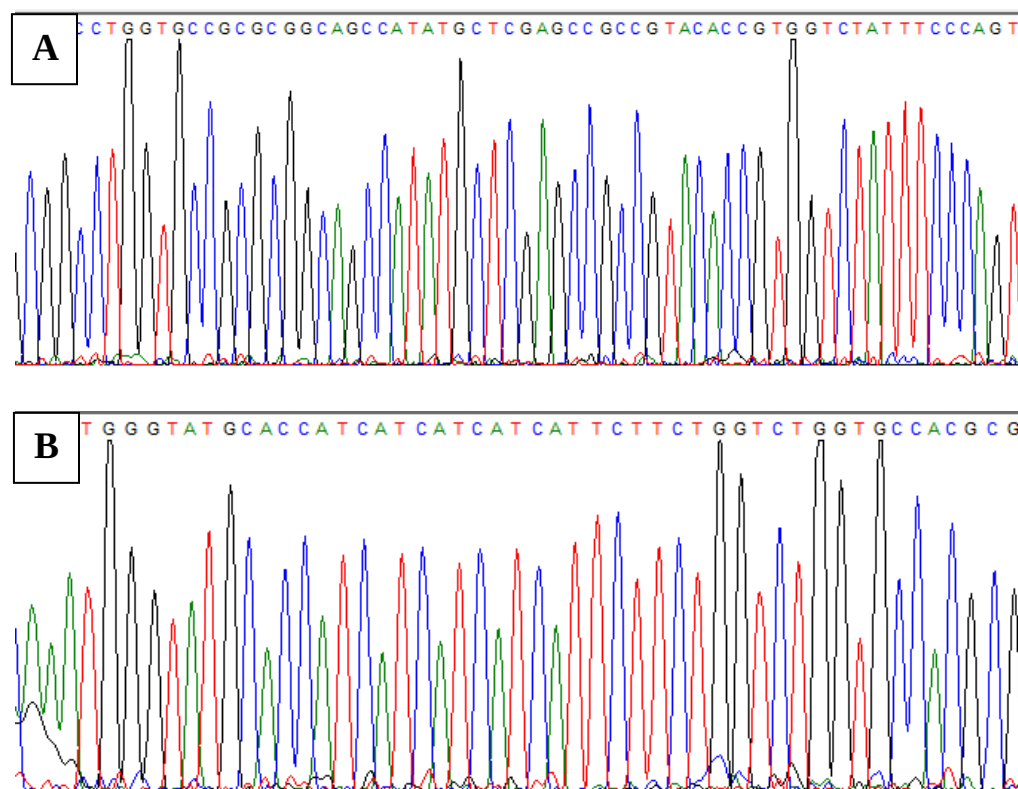


Figure 5: The plasmid sequencing results for a selected segment of the A) GSTP1-1 and B) Prdx6 genes. The sequencing results were viewed using the program Finch TV version 1.4.0 (<http://www.geospiza.com/FinchTV>: Geospiza Inc.).

Alignment

P30041	PRDX6_HUMAN - Peroxiredoxin-6 Homo sapiens (Human)
E-value: 7.3e-159 Score: 1162 Ident.: 99.6% Positives : 99.6% Query Length: 251 Match Length: 224	
	
Query	28 MPGGLLLGDVAPNFEANTTVGRIRFHDFLGDSWGILFSHPRDFTPVCTTELGRAAKLAPE 87
P30041 PRDX6_HUMAN	1 MPGGLLLGDVAPNFEANTTVGRIRFHDFLGDSWGILFSHPRDFTPVCTTELGRAAKLAPE 60
Query	88 FAKRNVKLIALSIDSVEDHLAWSKDINAYNSEEPTKLPFPPIIDRNRELAILLGMLDPA 147
P30041 PRDX6_HUMAN	61 FAKRNVKLIALSIDSVEDHLAWSKDINAYNSEEPTKLPFPPIIDRNRELAILLGMLDPA 120
Query	148 EKDEKGMPTARVVFVFGPDKKLKSILYPATTGRNFDEILRVVISLQLTAEKRVATPVD 207
P30041 PRDX6_HUMAN	121 EKDEKGMPTARVVFVFGPDKKLKSILYPATTGRNFDEILRVVISLQLTAEKRVATPVD 180
Query	208 WKDGD SVMVLPTIPEEEAKKLFPGKVFTKELPSGKKYLRYPQP 251
P30041 PRDX6_HUMAN	181 WKDGD SVMVLPTIPEEEAKKLFPGKVFTKELPSGKKYLRYPQP 224

Figure 6: The sequence alignment results for Prdx6. The input sequence (query sequence) matches the sequence of Prdx6 in the database by 99.6 %. The 0.4 % difference may be attributed to the fact the sequence in protein database has a cysteine in position 91 whereas the crystal structure (PDB ID: 1PRX) has a serine at that position.

Alignment

P09211	GSTP1_HUMAN - Glutathione S-transferase P Homo sapiens (Human)
E-value: 2.9e-149 Score: 1093 Ident.: 100.0% Positives : 100.0% Query Length: 212 Match Length: 210	
	
Query	4 PPYTVVYFPVRGRCAALRMLLADQGQSWKEEVVTVETWQEGSLKASCLYGQLPKFQDGL 63
P09211 GSTP1_HUMAN	2 PPYTVVYFPVRGRCAALRMLLADQGQSWKEEVVTVETWQEGSLKASCLYGQLPKFQDGL 61
Query	64 TLYQSNITLRHLGRTLGLYGKDQQAALVDMVNDGVEDLRCKYISLIYTNYEAGKDDYVK 123
P09211 GSTP1_HUMAN	62 TLYQSNITLRHLGRTLGLYGKDQQAALVDMVNDGVEDLRCKYISLIYTNYEAGKDDYVK 121
Query	124 ALPGQLKPFETLLSQNGGKTFIVGDQISFADYNLLDLLLIEVLAPGCLDAFPLLSAYV 183
P09211 GSTP1_HUMAN	122 ALPGQLKPFETLLSQNGGKTFIVGDQISFADYNLLDLLLIEVLAPGCLDAFPLLSAYV 181
Query	184 GRLSARPKLKAFLASPEYVNLPIINGNGKQ 212
P09211 GSTP1_HUMAN	182 GRLSARPKLKAFLASPEYVNLPIINGNGKQ 210

Figure 7: The sequence alignment results for GSTP1-1. The input sequence (query sequence) matches the sequence of GSTP1-1 in the database by 100 %.

3.1.1. Over-expression of Prdx6

Over-expression induction trials of Prdx6 were carried out at 25 °C and 37 °C over a period of 3 or 6 hours. The results of the trials showed that Prdx6 was best expressed at 37 °C over 3 hours with induction using IPTG to a final concentration of 1 mM (Figure 8).

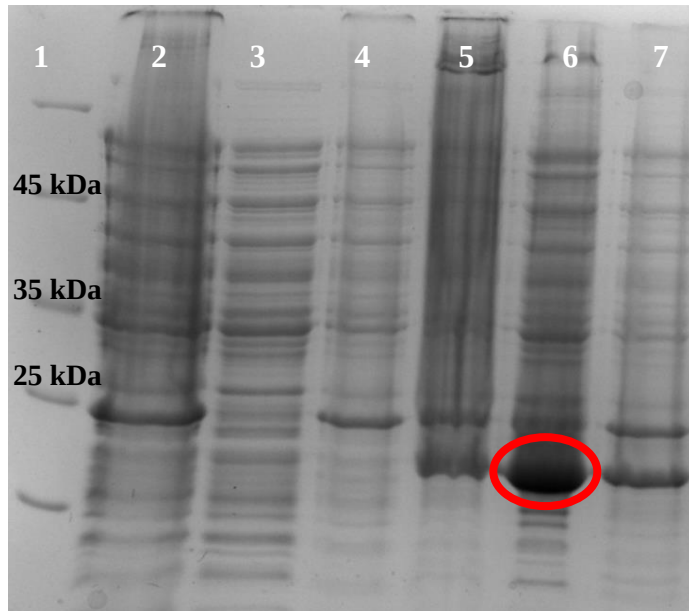


Figure 8: The SDS-PAGE gel showing the results of the over-expression of Prdx6. Each monomeric subunit of Prdx6 is ~ 25 kDa. Lanes: (1-4 Uninduced samples) 1- Molecular mass marker; 2- Cell lysate; 3- Pellet of lysed cells; 4- Supernatant; (5-7 Induced samples) 5- Cell lysate; 6-Supernatant; 7- Pellet of lysed cells. Image shows that the protein is over-expressed in the soluble form in the *E. coli* cells. The red circle is indicating the band of Prdx6.

3.1.2. Over-expression of GSTP1-1

Over-expression of GSTP1-1 was carried out at 37 °C over 6 hours with induction using IPTG to a final concentration of 0.2 mM (Figure 9). These conditions were used as they have been established and shown to work successfully in our laboratory.

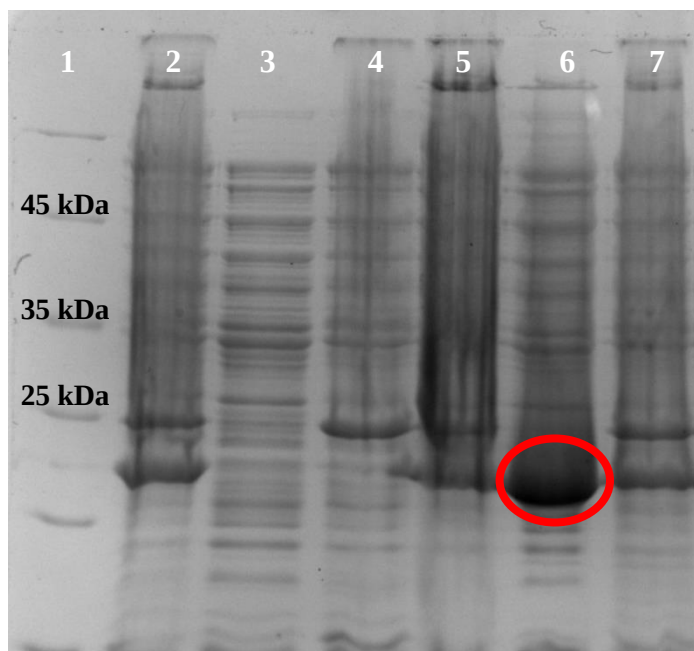


Figure 9: The SDS-PAGE analysis of the over-expression of GSTP1-1. Each subunit of GSTP1-1 is ~ 25 kDa. Lanes: (1-4 Uninduced samples) 1- Molecular mass marker; 2- Cell lysate; 3- Pellet of lysed cells; 4- Supernatant; (5-7 Induced samples) 5- Cell lysate; 6- Supernatant; 7- Pellet of lysed cells. Image shows that the protein is over-expressed in the soluble form in the *E. coli* cells. The band of GSTP1-1 is circled in red.

3.2. Purification of the His-tagged Prdx6 and GSTP1-1 using immobilised metal ion affinity chromatography (IMAC)

3.2.1. The purification of His-tagged Prdx6

The purification of Prdx6 was performed using IMAC with a nickel charged column because the His-tag on the protein has affinity for the nickel ions. The elution profile is shown alongside the SDS-PAGE analysis of the samples obtained from the purification (Figure 10).

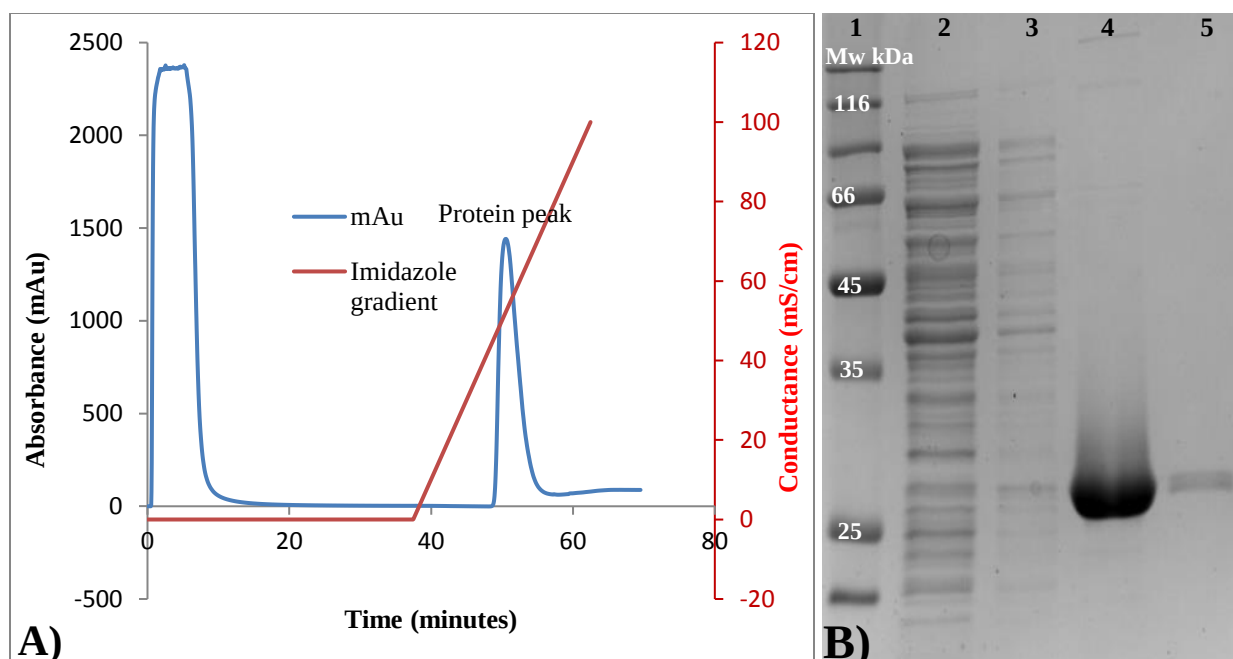


Figure 10: A) The elution profile of Prdx6 using IMAC on an AKTA Prime purification system. B) SDS-PAGE analysis following IMAC. Lanes: 1- Molecular mass marker; 2- Flow through from the column when loading supernatant; 3- Flow through from the column washing step; 4- Protein peak; 5- End of protein peak. Lane 4 shows that protein yield is high and pure.

3.2.2. The purification of GSTP1-1

The purification of GSTP1-1 was carried out using cobalt charged column and the elution profile is presented in Figure 11. Following the purification procedure, a 12.5 % SDS-PAGE gel was run to determine purity of the protein eluted from the column.

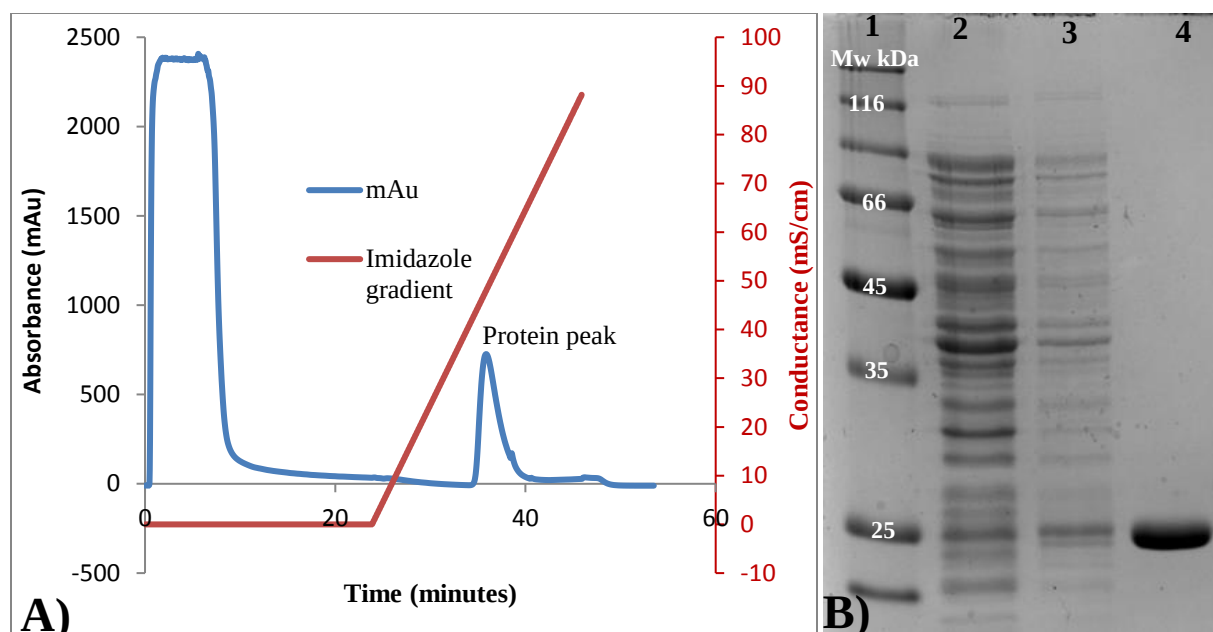


Figure 11: A) The elution profile of GSTP1-1. The protein was eluted from the column using an imidazole gradient shown in dark red. Following purification of GSTP1-1, the samples obtained were analysed using SDS-PAGE shown in B). Lanes: 1- Molecular mass marker; 2- Flow through from the column when loading supernatant; 3- Flow through from the column wash step; 4- Protein peak. Lane 4 shows that protein obtained is pure.

The absorbance during purification (of both Prdx6 and GSTP1-1) was monitored at 280 nm. The initial increase in absorbance on the elution profiles is due to the elution of non-specific proteins which do not bind the affinity columns. Protein for downstream analysis, of each protein, was pooled from the main protein peak on the elution profiles (labelled lane 4 on the SDS-PAGE).

3.3. Protein identity confirmation using mass spectrometry

Proteins were sent for sequencing at the Council of Scientific and Industrial Research (CSIR, Pretoria). The mass spectrometry analysis was carried out by Dr Stoyan Stoychev. An AB Sciex6600 TripleTOF mass spectrometer was used for the analysis and only proteins with threshold above ≥ 95 % confidence were reported. The confidence indicates the probability that the fragment consists of specific sequence of amino acid residues. Figure 12 shows the reports of the peptides and the level of peptide confidence coverage obtained from the analysis.

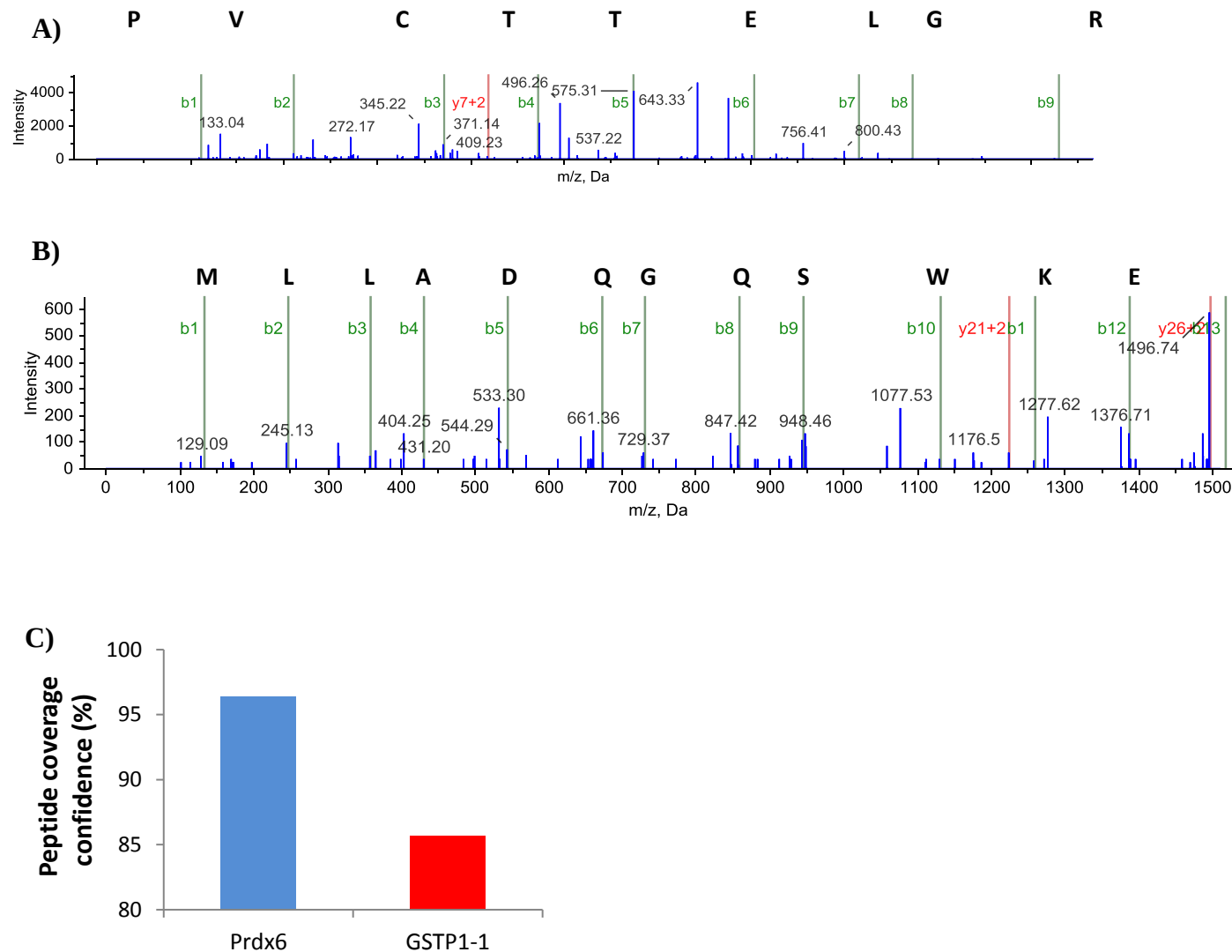


Figure 12: Mass spectrometry data analysis. Segments of the peptides forming part of A) the active centre of Prdx6 and B) GSTP1-1. C) Graphical report indicating that the peptides were identified with > 95 % confidence to be from Prdx6 and > 85 % to be from GSTP1-1. These data support the sequence alignment data obtained previously (see section 1) which confirms the identity of both Prdx6 and GSTP1-1.

The LC-MS/MS data indicated that the peptide fragments belonged to Prdx6 and GSTP1-1 with > 95 % confidence. The peptide analysis showed that the catalytically active cysteine residue of the Prdx6 was not oxidised prior to analysis as it was carbamidomethylated during the alkylation step in the analysis. Oxidised cysteine residues cannot be carbamidomethylated (Steen & Mann, 2000).

3.4. Structural analysis of Prdx6 and GSTP1-1

3.4.1. Circular dichroism (CD) spectropolarometry

The secondary structure of the proteins was analysed using far-UV CD (180- 250 nm), the spectrum obtained for Prdx6 is depicted in Figure 13 and the spectrum obtained for GSTP1-1 is presented in Figure 14. The experimental data obtained were further analysed using DichroWeb (Whitmore & Wallace, 2008) in order to establish the alpha helical and beta sheet content of the proteins. The DichroWeb programme calculates the amount of alpha helical and beta sheet content of the protein based on a spectrum of best fit generated from its reference spectra of protein. The goodness of the fit is measured based on the NRMSD (normalised root-mean-square deviation) value, the lower this value the better the fit. The analysis programme used for the generated fit is the SELCON3 (Sreerema & Woody, 1993). The NRSMD value obtained for the fit was 0.064 for GSTP1-1 and 0.114 for Prdx6.

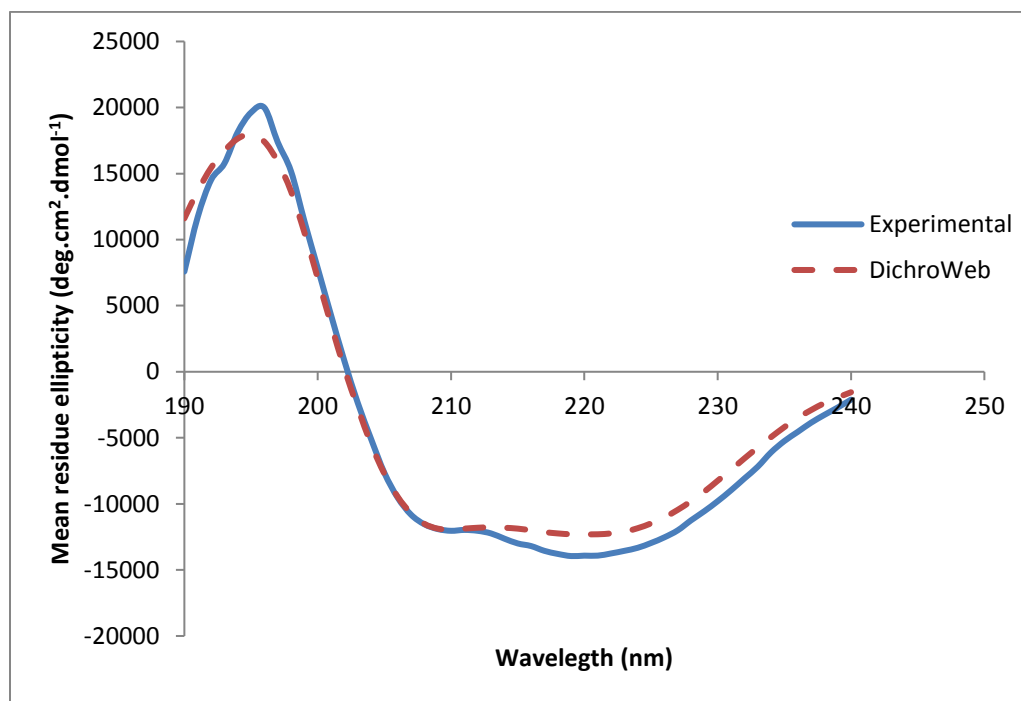


Figure 13: The CD spectra of Prdx6 obtained experimentally along with the model generated by DichroWeb (<http://dichroweb.cryst.bbk.ac.uk/html/home.shtml>). This shows that the protein is mainly alpha helical characterised by the negative troughs at 222 nm and 208 nm and a large positive peak at 190 nm.

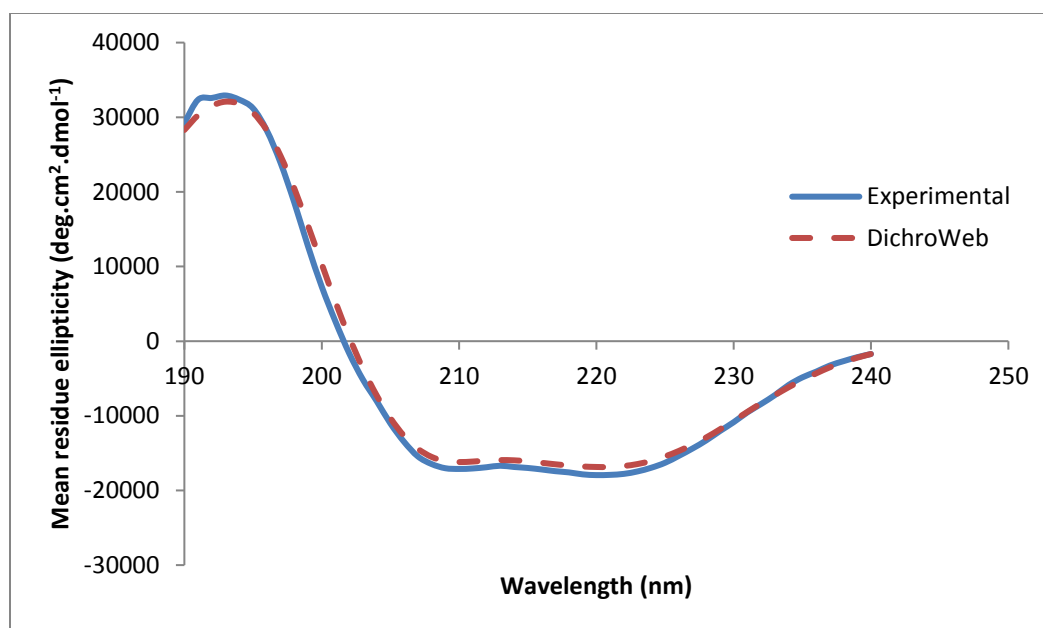


Figure 14: The CD secondary structural analysis of GSTP1-1 showing spectra obtained experimentally along with the model spectrum generated by DichroWeb (<http://dichroweb.cryst.bbk.ac.uk/html/home.shtml>). The protein is predominantly alpha helical due to the large alpha helical content in its structure.

The proteins have high amount of helical content which is evident based on the troughs at 280 nm and 222 nm and the large positive peak at 195 nm in the spectra, this is characteristic of alpha helical behaviour. Analysis of the crystal structure and DichroWeb generated data of GSTP1-1 and Prdx6 (Table 2) clearly show the abundant presence of alpha helices.

Table 2: Summary of the amount of structural content based on the DichroWeb computed data and crystal structure data from the database of secondary structure assignments (DSSP).

Structural motif	Content (%)			
	Crystal structure*		DichroWeb	
	Prdx6	GSTP1-1	Prdx6	GSTP1-1
Helix	29	61	46	52
Strands	22	8	10	9

*The data on the structural content of the crystal structure was obtained from database of secondary structure assignments (DSSP) (Kabsch and Sander, 1983).

The DSSP and crystal structure data of GSTP1-1 are not very different as both show that the protein has high helical content ($> 50\%$) with minimal beta sheet content ($< 10\%$). The data for the Prdx6 varies considerably between the two databases. This difference may be because these two databases use different algorithms to assess structural content. However, it is clear from both databases that Prdx6 is also flagged by high presence of helical content.

3.4.2. Fluorescence spectroscopy

Intrinsic tryptophan fluorescence was used as a probe to study the local tertiary structure of the proteins and the spectra obtained from this analysis are depicted in Figures 15 and 16 for Prdx6 and GSTP1-1 respectively. The spectra were generated for the native and denatured forms of the proteins. The spectra of the denatured protein served as a control to show how the tryptophans behave when the protein is in the unfolded state and exposed to solvent/buffer.

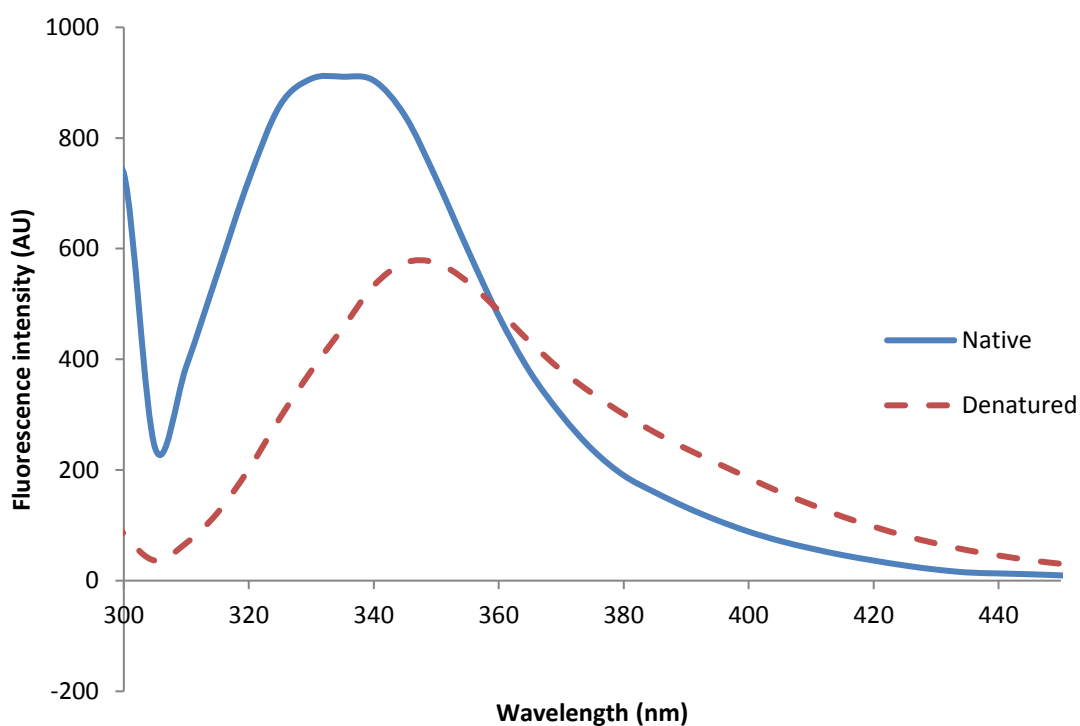


Figure 15: The fluorescence emission spectra of $2\ \mu\text{M}$ Prdx6 in the native and denatured forms when excited at 295 nm. The emission wavelength is at 335 nm for the native form whereas for the denatured form the emission is at 345 nm.

There is a bathochromic shift (spectrum red shift) observed in the spectra when the protein is denatured. This allows us to infer that the tryptophan residues become exposed to solvent when the protein is denatured suggesting that these tryptophan residues are buried in the native structure. Prdx6 had 3 tryptophan residues (Trp33, 82 and 181) per subunit which are responsible for the intrinsic tryptophan fluorescence. Trp33 and 82 are located within the thioredoxin fold while Trp181 is located at the connector loop which connects domain I and domain II. The buried nature of the two tryptophan residues (Trp33 and 82) resulted in the observed changes in spectra. Trp181 is not buried in the structure; it is exposed and would thus not experience significant changes when protein is in the unfolded state.

The fluorescence emission wavelength of free tryptophan is at 350 nm (Ghisaidoobe and Chung, 2014); this is considerably close to the emission wavelength of the denatured protein which was at 345 nm which shows that in the native form the tryptophan residues are buried into the hydrophobic core. Tryptophan is a hydrophobic amino acid which also accounts for why it would be buried into the hydrophobic core of the native protein. The hypochromic shift in intensity suggests that tryptophan fluorescence is quenched when exposed to the buffer hence the decrease in fluorescence intensity.

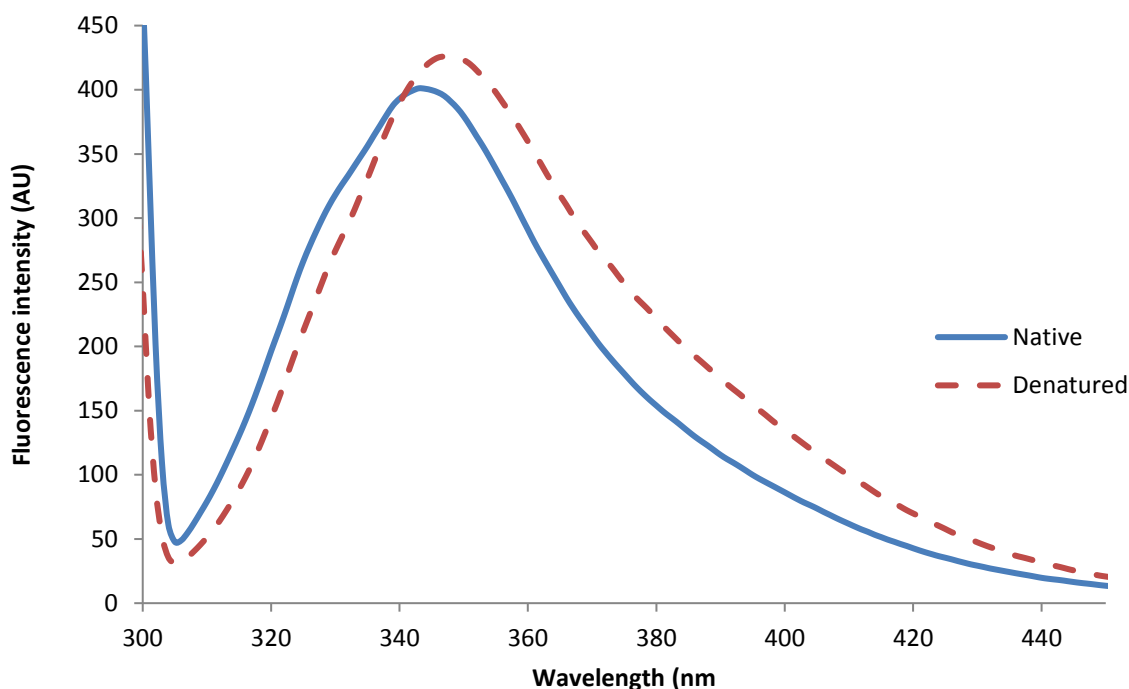


Figure 16: The fluorescence emission spectra of the native and denatured GSTP1-1. The emission of the native form is at 343 nm and the denatured form is at 347 nm, there is a 4 nm difference. A slight increase in intensity of 24 A.U is observed for the denatured form.

There is slight hyperchromic shift observed in intensity when the protein is denatured. This change in intensity indicates that the tryptophans are slightly exposed to solvent in the native structure and become more exposed as the structure unfolds. GSTP1-1 has two tryptophan residues (Trp28 and 38), both located in domain I of each subunit. Trp38 is located in the flexible and exposed region of the G-site, this account for the exposed nature of the tryptophans residues. The slight bathochromic shift of 4 nm observed can be attributed to the change in environment the tryptophan residues experience as the protein unfolds.

3.4.3. Size exclusion-high pressure liquid chromatography (SE-HPLC)

SE-HPLC was carried out on GSTP1-1 and Prdx6 to analyse the quaternary structure of both proteins. The proteins were sized against gel filtration protein standards; the results of the analysis are presented in Figure 17.

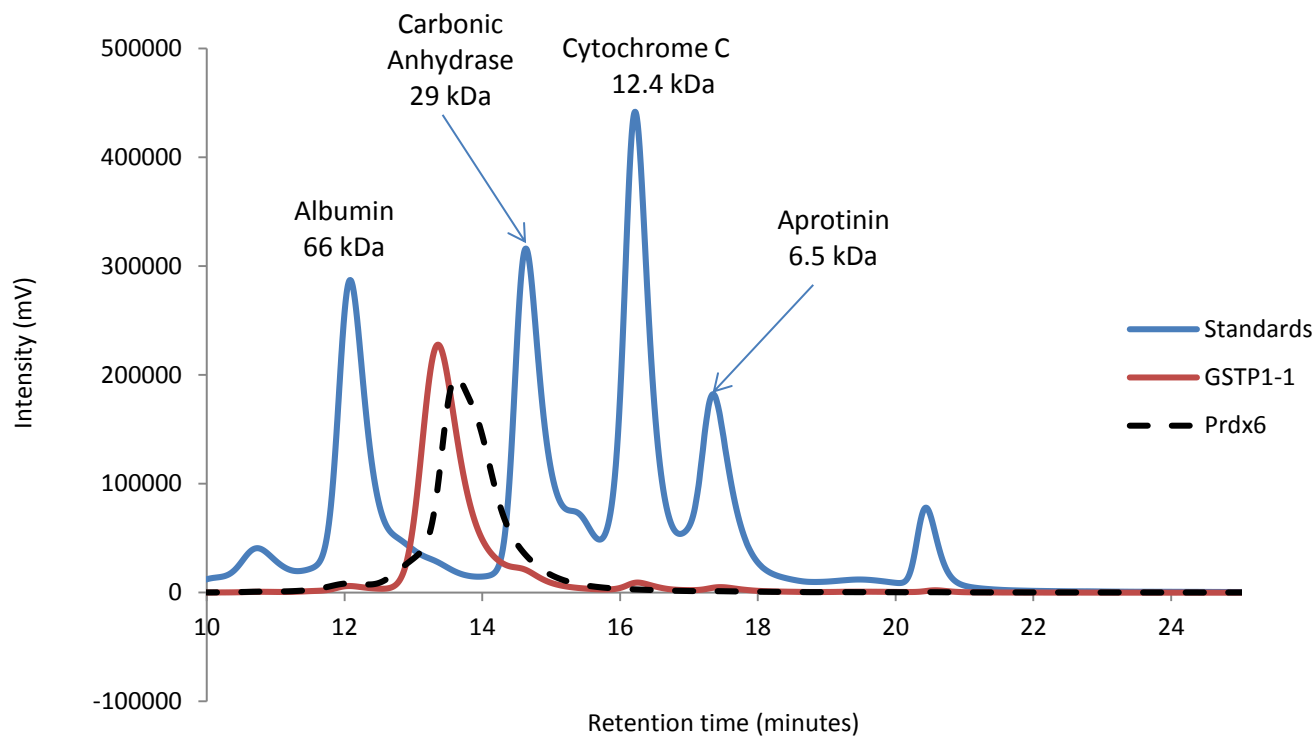


Figure 17: The SE-HPLC elution profile of Prdx6 and GSTP1-1 relative to those of the gel filtration protein standards. Prdx6 and GSTP1-1 are eluted at approximately the same time (13.62 and 13.36 minutes respectively) indicating that they are relatively the same size.

The elution profile of the standards (Figure 17) was used to draw a standard curve (Figure 18) in order to determine the sizes of the Prdx6 and GSTP1-1 as eluted from the SE-HPLC column.

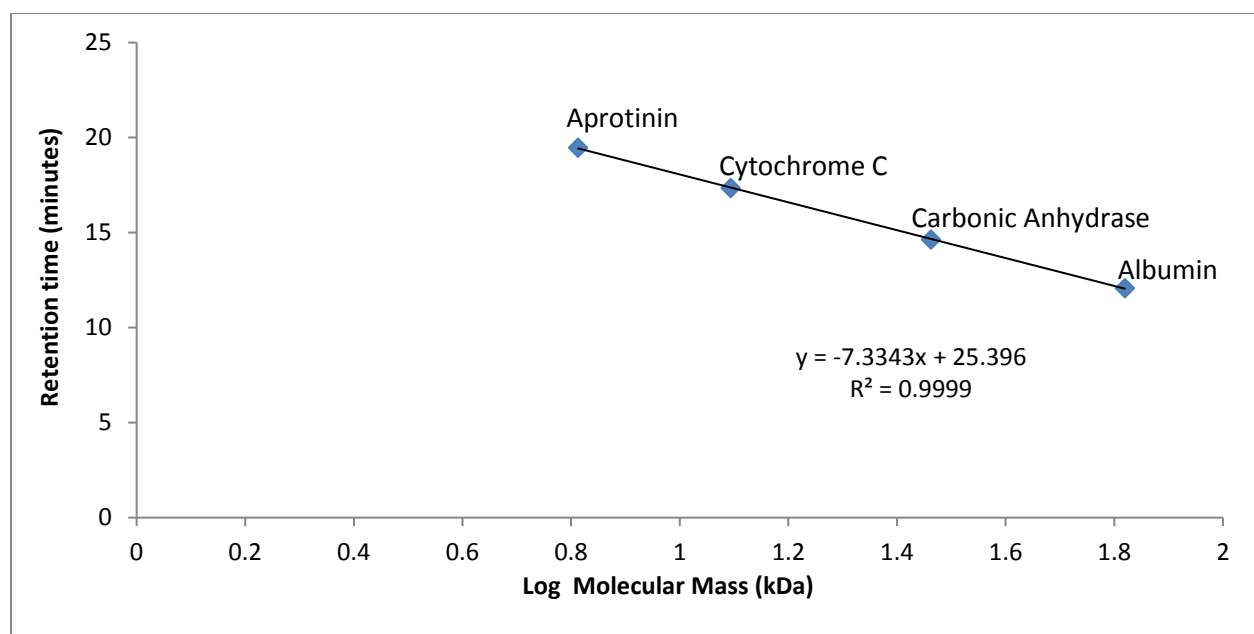


Figure 18: The standard curve constructed from the elution times and molecular masses of the gel filtration protein standards. This was used to extrapolate the sizes of the Prdx6 and GSTP1-1 as eluted from the SE-HPLC.

The molecular masses of Prdx6 and GSTP1-1 were determined by use of extrapolation from the standard curve in Figure 18. The extrapolated molecular masses of GSTP1-1 and Prdx6 as eluted from the column are depicted in Table 3.

Table 3: The extrapolated molecular mass (kDa) of Prdx6 and GSTP1-1.

Protein species	Log Molecular Mass (kDa)	Mass (kDa)
GSTP1-1	1.64	43.8
Prdx6	1.61	40.3

The molecular masses obtained from the SE-HPLC analysis are different from the expected masses reported in literature which are 50 kDa for Prdx6 and 47 kDa for GSTP1-1. This is usually due to the hydrodynamic volume of the proteins when in solution (see section 4.2.3).

3.5. Functional characterisation of GSTP1-1 and Prdx6

3.5.1. GST activity assay for GSTP1-1

The GSTP1-1 activity was assayed using the 1-chloro-2, 4-dinitrobenzene (CDNB)/GSH assay. Assaying the activity of the protein provides an indication whether the protein is folded into the correct native state as it would in the native cell environment. Figure 19 shows the plot of the specific activity of GSTP1-1.

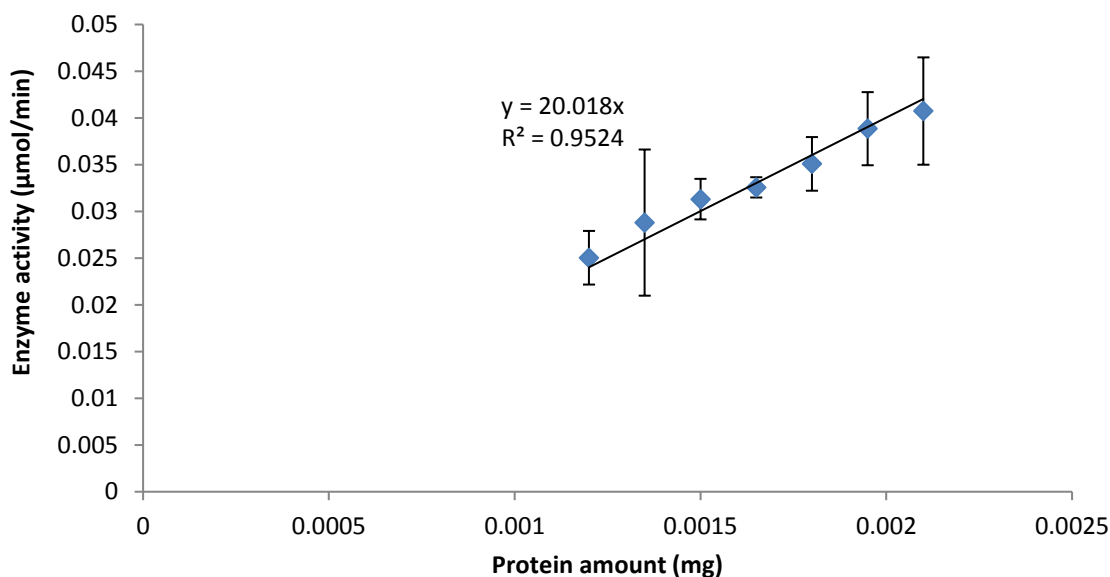


Figure 19: The specific activity of GSTP1-1. The activity was measured with respect to CDNB as the electrophilic substrate. It was determined to be 20 μmol/min/mg protein.

GSTP1-1 was active towards CDNB. This is an indication that the protein indeed folded into the correct native state.

3.5.2. Peroxidase enzyme activity assay for Prdx6

The glutathione reductase/NADPH coupled GSH peroxidase assay was carried out to assess the peroxidase activity of Prdx6. The assay was initially conducted with the standard assay conditions (as stipulated in section 2.9.2). The progress curves obtained from this standard assay showed that Prdx6 was inactive (Figure 20). After several failed reactions, the best way forward was to try and optimise the assay. The failure of the assay could be due to multiple factors such as pH, temperature, and enzyme concentration used. The conditions applied kept the pH and temperature constant, we therefore opted to verify if the enzyme concentrations used were optimal. When

taking into consideration that the assay employed a few coupled reactions to be carried out, we also investigated the coupled reaction to ensure that they were functional.

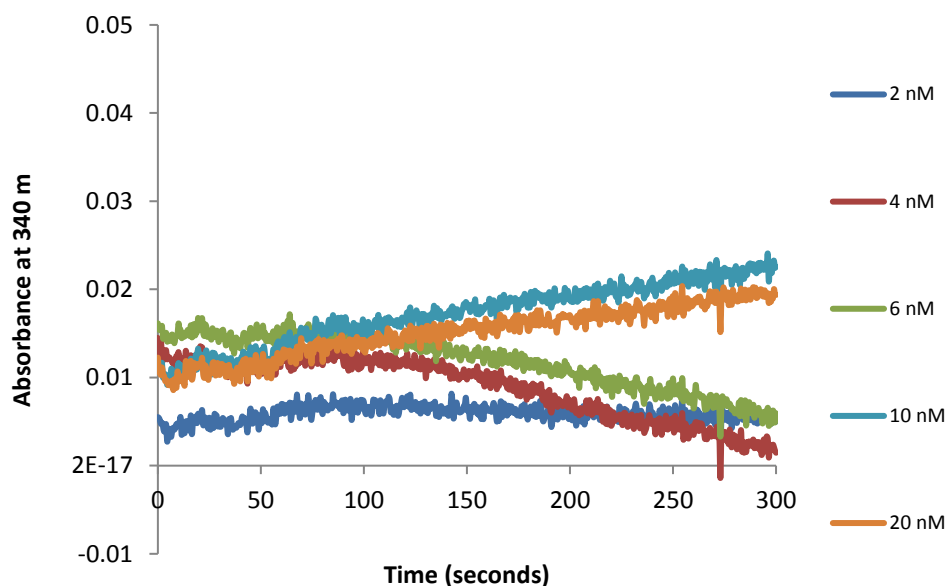


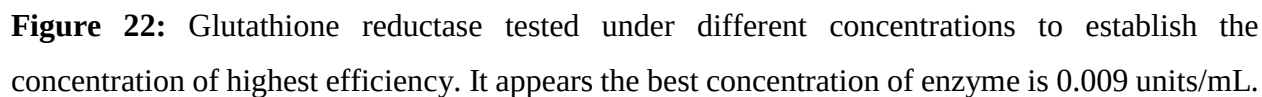
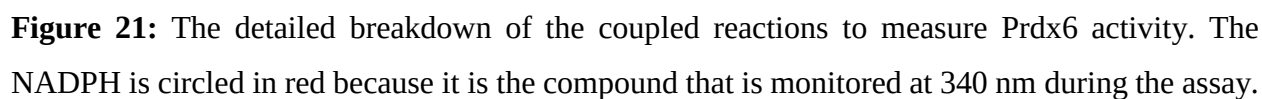
Figure 20: The progress curves obtained using the standard Prdx6 assay conditions. Concentrations between 2 nM and 20 nM of Prdx6 were used however the enzyme appears to be inactive.

3.5.2.1. Optimisation of the Prdx6 standard assay

The detailed breakdowns of the coupled reactions used to measure Prdx6 activity are illustrated in Figure 21. This figure enables us to see the crucial point in the assay which is the point where glutathione reductase reaction comes into play when monitoring NADPH depletion. The role of glutathione reductase is important because the progression of the reaction is dependent on the efficient functioning of the glutathione reductase; this makes it a crucial point in the assay.

3.5.2.1.1 Optimisation of glutathione reductase activity

This glutathione reductase was the main target for optimisation and was therefore the first component to be tested for activity. The activity of glutathione reductase was tested under different concentrations (Figure 22) and it was discovered that the concentrations used (0.23 units/mL) were too high and thus the concentration was lowered to 0.009 units/mL where the enzyme was

$$2\text{H}_2\text{O}_2 + 2\text{GSH} \xrightarrow{\text{PRDX6}} 2\text{H}_2\text{O} + \text{GSSG} + \text{O}_2$$


3.5.2.1.2 Optimisation of Prdx6 and GSTP1-1 concentrations

In order for Prdx6 to complete its catalytic cycle, GSTP1-1 is required for its reactivation. This was taken into consideration and the assay was carried out with GSTP1-1 present to help with reactivating inactive Prdx6.

The assay was then performed in the absence and in the presence of GSTP1-1 over a range of Prdx6 and GSTP1-1 concentrations. The highest concentration used in the assay was 100 nM which was greater than the concentrations previously reported in literature of 1-10 nM range (Fisher *et al.*, 1999). The progress curves depicted in Figure 23 show the progression of the Prdx6 enzymatic activity in the presence and in the absence of GSTP1-1. The progress curves are not blank corrected for non-enzyme related activity but it can already be seen that there is no significant difference between the experimental reaction and the blank (control) reaction.

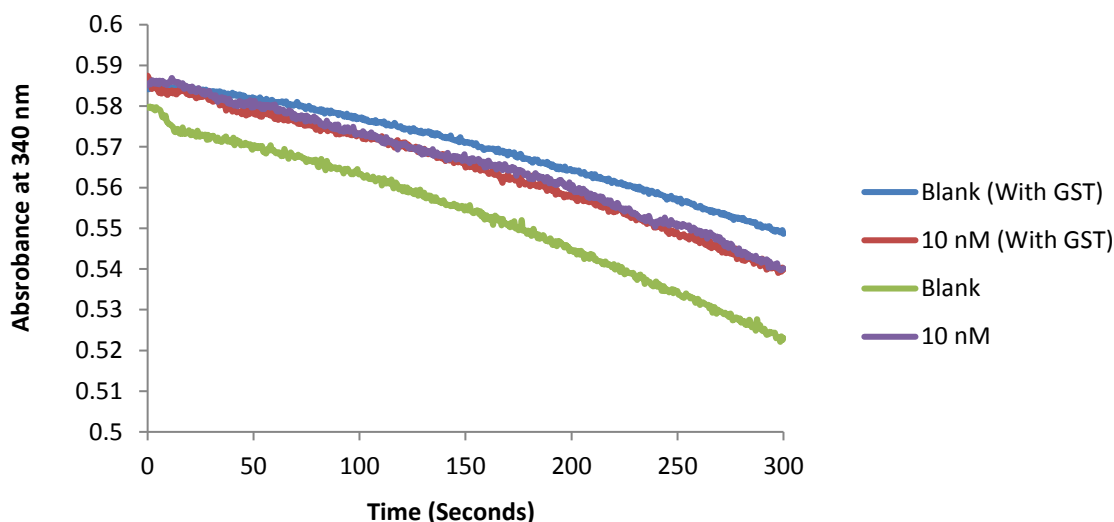


Figure 23: The progress curves showing the progression of the Prdx6 assay in the presence and in the absence of GSTP1-1.

3.5.2.1.3 Optimisation of H_2O_2 concentration

When assessing the blank (control) reactions, it can be seen that there is oxidation of NADPH by H_2O_2 even in the absence of Prdx6. An attempt was made to minimise the effects of this phenomenon by reducing the concentration of H_2O_2 from the 250 μ M to 50 μ M while keeping all the other components of the reaction constant. These changes in concentration did not make a

difference in the oxidation of NADPH (Figure 24). The concentration of H_2O_2 was then used at the original concentration of 250 μM .

It was expected that upon addition of enzyme there would be a decrease in absorbance at 340 nm but however it can be seen that this is not the case. The decrease in absorbance would have been an indication that the NADPH was being depleted in the reaction process however this is not evident.

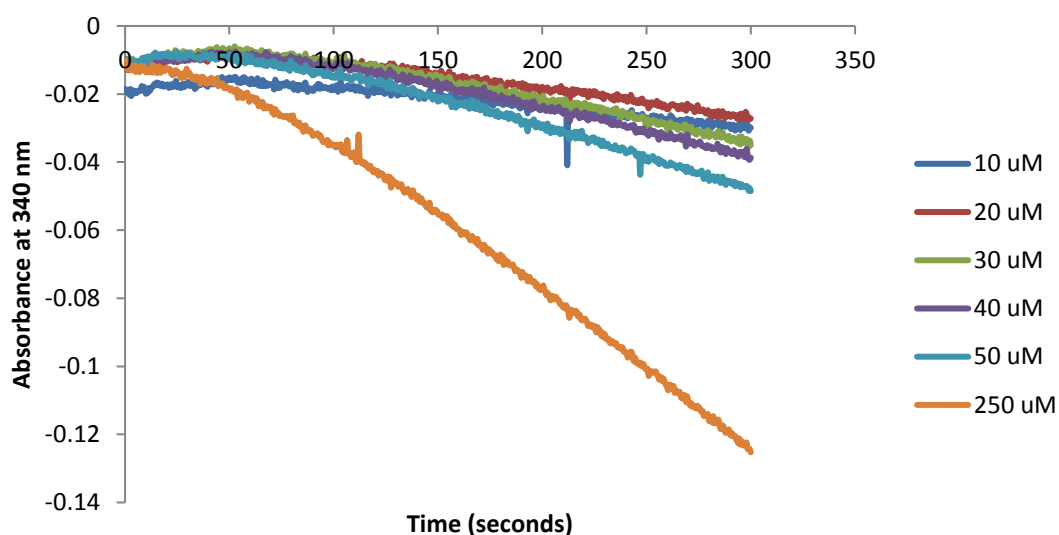


Figure 24: The progress curves showing the progression of the oxidation of NADPH under varying H_2O_2 concentrations.

CHAPTER 4: DISCUSSION

Oxidative stress is a phenomenon that every living organism is faced with. It can induce cellular injury in the organism through damage of DNA, peroxidation of membrane lipids, proteins and carbohydrates. Due to the debilitating results of oxidative stress, it is of utmost importance to study enzymes such as GSTP1-1 and Prdx6 which possess antioxidant defence against reactive oxygen species which are sources of oxidative stress. These enzymes have been linked to the removal of reactive oxygen species because their expression and activity levels are elevated during periods of oxidative stress in the cells (Hayes *et al.*, 2005; Manevich and Fisher, 2005); this shows their important role in cell survival and therefore the overall survival of the organism.

It has been shown recently that GSTP1-1 interacts with Prdx6, through formation of a heterodimer, to assist in the completion of the catalytic cycle of Prdx6 (Ralat *et al.*, 2006). This interaction has proven to be vital for the functioning of the Prdx6. The GSTP1:Prdx6 heterodimer is of great importance for the peroxidase enzymatic activity of Prdx6, if this dimerisation does not occur the catalytic cycle will not be completed and the Prdx6 will remain inactive. It will remain inactive because the Cys47 catalytic residue will be in the sulfenic form which renders the enzyme catalytically inactive (Ralat *et al.*, 2008).

The function of a protein/enzyme is dependent on its structure which in turn determines the protein's physical and chemical parameters. Thus, to understand the context in which the biochemical properties of these enzymes are used we needed to study the structure and function of the enzymes individually. In this study, we looked at the structure and the function of the Prdx6 and GSTP1-1 to elucidate how these proteins can carry out their function and how this relates to their structures.

4.1. Transformation, over-expression and purification

The transformation, over-expression and purification of GSTP1-1 and Prdx6 were successful. The proteins were purified to homogeneity and were used for downstream characterisation techniques without the need for further purification. Sanger sequencing was used to confirm the encoding region for the proteins in their respective plasmids and mass spectrometry was employed to confirm that the correct proteins were purified. Downstream analysis techniques were carried out with the His-tagged present for both proteins. The His-tag was not removed for either protein

because literature has shown that the presence of the His-tag does not affect the structure and function of both proteins (Ralat *et.al.*, 2008; Chang *et.al.*, 1999).

4.2. Structural Characterisation

The structural aspects of GSTP1-1 and Prdx6 were investigated using an array of biophysical techniques. The techniques used probed various aspects (secondary, tertiary and quaternary) of the structure of each protein. This type of analysis provides a basic understanding of the structural and functional attributes of the proteins.

4.2.1. Secondary Structure: Circular Dichroism

GSTP1-1 and Prdx6 are predominantly alpha helical as indicated by the deep troughs at 222 nm (Figures 14 and 15), with GSTP1-1 having more of the alpha helical structure than Prdx6. 64 % of the amino acids in GSTP1-1 form part of the alpha helices and only 30 % of the amino acids in Prdx6 form part of the alpha helices indicating the presence of high alpha helical content in GSTP1-1. This high alpha helical content in GSTP1-1 can be attributed to the presence of the strictly alpha helical C-terminal domain which contains 5 alpha helices (Dirr *et al.*, 1994). 46 % of the total amino acids constitute the helices of this domain with most of the amino acids forming part of the H-site which is binding site of electrophilic substrates such as the CDNB used in this study (Mannervik *et al.*, 1985; Reinemer *et al.*, 1991). The low content of alpha helices in the N-terminal domain contributes towards the alpha helical signature of the CD spectrum because of the alpha helices found within the evolutionary conserved thioredoxin fold contained in this domain (Dirr *et al.*, 1994). The fold contains 3 alpha helices which flank the 4 beta strands on either side; two on one side and one on the other side (Dirr *et al.*, 1994) (Figure 2). 17 % of amino acids in the structure contribute towards the formation of this domain.

Prdx6 contains higher beta strand content as compared to GSTP1-1. 30 % of the amino acids in Prdx6 compose the beta strands whereas only 15 % of the amino acids in GSTP1-1 form part of the beta strands. The N-terminal domain of Prdx6 contains the thioredoxin fold which is relatively similar to that of the GSTP1-1 i.e. it contains 4 beta strands flanked by 3 alpha helices (Figure 1). However, in addition to the thioredoxin fold, Prdx6 has 3 beta strands and 2 alpha helices inserted within its N-terminal domain (Choi *et al.* 1990), these insertions are responsible for the higher beta

strand contents. The N-terminal domain is the site of the proteins peroxidase and calcium-independent phospholipase A₂ activities (Choi *et al.*, 1990; Zheng *et al.*, 2010; Fisher, 2011). The C-terminal domain of Prdx6 would not contribute significantly to the alpha helical signature of the CD spectrum, but would however, contribute to the beta strand content of the protein as it consists of 3 beta strands and only 1 alpha helix. This report of structure is also observed from the data reported by Manevich *et al.* (2007).

The data regarding the structural content of the proteins obtained from the database of secondary structure assignments (DSSP) and the DichroWeb deconvolution tool (Table 2) are generally in agreement with the findings deduced from the analysis of the crystal structure obtained from PDB. The data suggest that > 50 % of GSTP1-1 is alpha helical and 8-10 % is beta stranded which is quite similar to the findings that 64 % is alpha helical and 15 % is beta strand. The beta strands were under estimated in the DSSP and DichroWeb databases which could be due to the different analysis algorithms used by each database. The Prdx6 DSSP and DichroWeb data showed that 29-46 % of the protein is alpha helical and 10-22 % is beta strand whereas the analysis from the crystal structure showed that 30 % is alpha helical and 30 % is beta stranded. There is a notable difference in the amount of structural content estimation between the data sets however the DSSP and crystal structure results seem to agree on that the alpha helices and beta strands are almost equal. The DichroWeb data seems to underestimate the beta strand content while overestimating the helical content, this may be due to the absence of a protein that accurately represents Prdx6 in the database. The structural signatures obtained from the CD data for both proteins agree with their respective crystal structures.

Although these proteins contain a similar conserved thioredoxin fold, their functions differ in the cell. As aforementioned in Chapter 1 (section 1.1), Prdx6 functions in the removal of reactive oxygen species and GSTP1-1 performs xenobiotic removal processes which secondarily contribute to antioxidant defence. The differences in their thioredoxin fold containing N-terminal domains, in addition to their C-terminal domain, indicate how these proteins are structured differently to display the different enzymatic properties that they possess.

4.2.2. Tertiary Structure: Fluorescence Spectroscopy

The sensitivity of this technique allows for probing of the microenvironment around the tryptophan residues which gives a general idea of the tertiary structure of the proteins. The tryptophan environment of Prdx6 gives an indication that the tryptophans are buried into the core as the protein folds. There is shift in wavelength and fluorescence intensity observed between the folded and unfolded states of the protein (Figure 15) which suggests that the tryptophans become exposed upon unfolding. The fluorescence emission wavelength of free tryptophan is at 350 nm (Ghisaidoobe and Chung, 2014); this is considerably close to the emission wavelength of the denatured protein which was at 345 nm (Figure 15) which shows that in the native form the tryptophan residues are buried into the hydrophobic core. Tryptophan is a hydrophobic amino acid which also accounts for why it would be buried into the hydrophobic core of the native protein. There is a hypochromic shift observed in intensity (Figure 15), this suggests that tryptophan fluorescence is quenched when exposed to the buffer hence the decrease in fluorescence intensity. Two of the three tryptophans in Prdx6 are found buried within the thioredoxin fold in the N-terminal domain of the protein with Trp33 being minimally solvent accessible (Figure 25) which explains the observed changes.

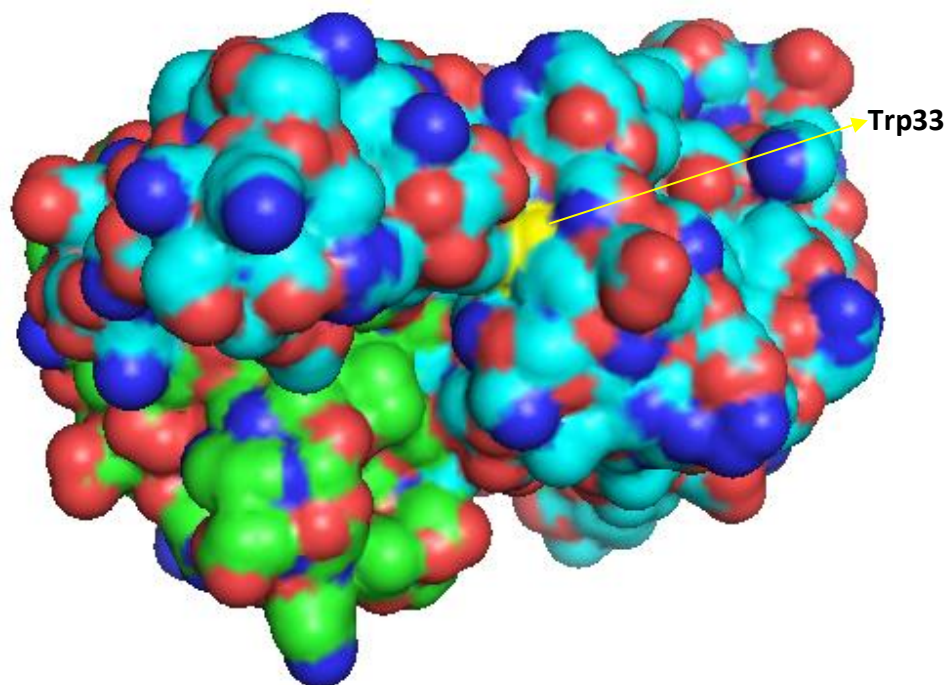


Figure 25: The solvent accessible surface of Prdx6 (PDB ID: 1PRX) computed using PyMol depicting the solvent accessible tryptophan residues in the structure. The tryptophan residues are coloured in yellow and only 1 of 3 appears to be accessible on the solvent accessible surface. Chain A is coloured cyan whereas Chain B is coloured green.

GSTP1-1 contains two tryptophan residues (Trp28 and Trp38) and the environment around these residues appeared to be less shielded when compared to that of Prdx6. There were minimal wavelength and intensity changes in the free and folded protein fluorescence spectra of GSTP1-1 (Figure 16). Analysis of the crystal structure of GSTP1-1 showed that the tryptophan residues of GSTP1-1 are in the solvent accessible regions of the structure (Figure 26) hence the minimal wavelength and intensity changes observed between the folded and unfolded protein states.

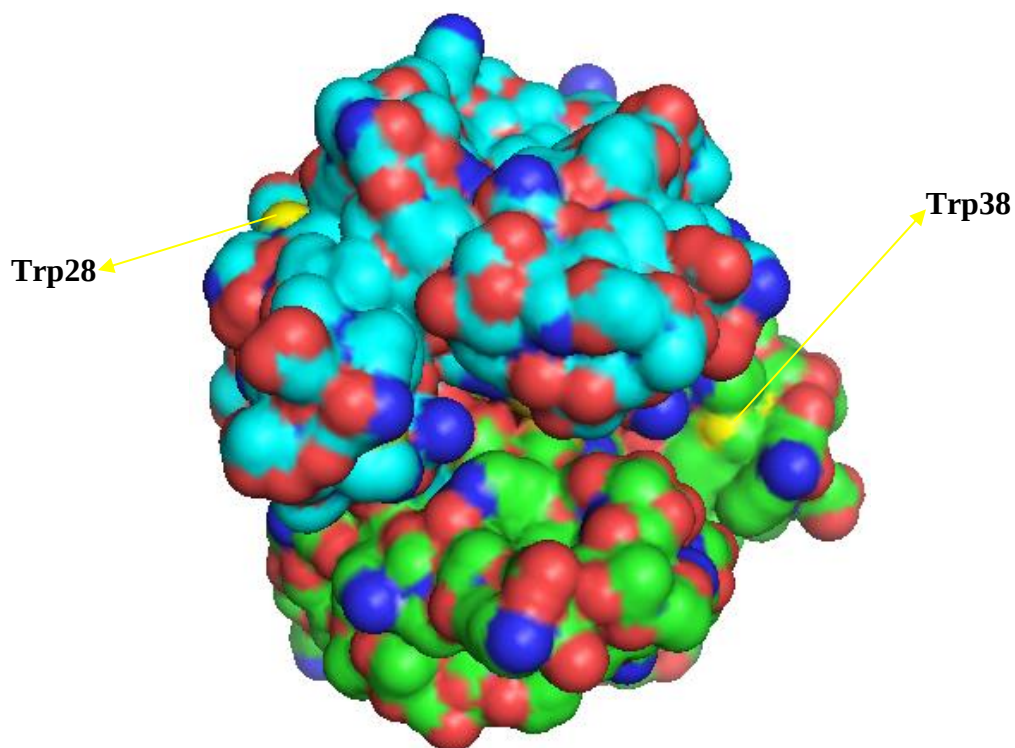


Figure 26: The image depicting the solvent accessible tryptophan residues on the surface of GSTP1-1 (PDB ID: 9GSS) generated using PyMol. The tryptophan residues are coloured in yellow and both appear to be on the solvent accessible surface. Chain A is coloured cyan whereas Chain B is coloured green.

4.2.3. Quaternary Structure: Size exclusion-high pressure liquid chromatography (SE-HPLC)

Both proteins are predominantly homodimeric. The reported monomeric size of Prdx6 is ~25.9 kDa (with a hexa His-tag) and thus the dimeric form in this case would be expected to be ~52 kDa (Ralat *et al.*, 2008). In contrast, the SE-HPLC data shown in Table 3 indicate the molecular mass of Prdx6 is slightly lower (40.3 kDa) than that expected for a fully dimeric species (52 kDa). There is also a difference in the expected and experimentally observed molecular mass of GSTP1-1. The expected size of the dimeric GSTP1-1 is ~50 kDa (Reinemer *et al.*, 1991; Dirr *et al.*, 1994) and the experimentally observed size was 43.8 kDa. The observed size difference can be attributed to the proteins tumbling volume and hydrodynamic interaction which could be smaller than the theoretically expected tumbling volumes for a 50 kDa protein. Most proteins as with most biological macromolecules follow a hydrodynamic interaction model of a ring polymer when in

solution (Chen *et al.*, 2015). These proteins are likely to follow the ring polymer hydrodynamic model due to their globular/spherical shape. This hydrodynamic model normally leads these molecules to have smaller size and faster self-diffusion in different matrices (Chen *et al.*, 2015) as observed with GSTP1-1 and Prdx6 on the SE-HPLC column.

4.3. Functional Characterisation

4.3.1. Assessment of GST activity of GSTP1-1

GSTP1-1 showed catalytic activity towards the intended substrate (CDNB). This implies that the protein is correctly folded hence it could carry out its cellular functions. The activity of GSTP1-1 has been reported ~100 $\mu\text{mol}/\text{min}/\text{mg}$ protein (Hegazyt *et al.*, 2003). The value reported in this study is very low compared to the reported value and thus implies that the enzyme was not at maximal efficiency. The ability of the enzyme to effectively catalyse the CDBN and GSH relies on the structural integrity of the protein as well the environment around it. For example, the helix alpha-2 influences the catalytic activity of the enzyme through modulation of GSH binding and catalysis (Hegazyt *et al.*, 2003). Whereas environmental changes such as pH and temperature can also affect the catalytic efficiency.

4.3.2. Assessment of Prdx6 peroxidase activity

Prdx6 conveys two enzymatic activities, the peroxidase activity was tested. The results obtained from the assay of Prdx6 showed that it does not convey the peroxidase activity although the mass spectrometry data had indicated that the Cys47 residue was not oxidised prior to analysis. The assay conditions used in this study were the same as those reported in literature and as aforementioned, the optimised conditions. The assay was also conducted in the presence of GSTP1-1 as it is reported in literature that GSTP1-1 interacts with Prdx6 in the cell to reactivate Prdx6 during its catalytic cycle (Ralat *et al.*, 2006; Manevich *et al.*, 2007; Lien *et al.*, 2012). The addition of the GSTP1-1 did not aid in the reactivation of the Prdx6 as the enzyme remained inactive.

As already emphasised the structure of a protein is crucial for the functioning of the protein. Analysing the crystal structure of Prdx6 indicates that the catalytic residue Cys47 is buried in the hydrophobic core of the structure; this makes it inaccessible to the GSH tripeptide. Small structural

changes could render the active site completely inaccessible. It has been hypothesised that the folding of Prdx6 requires a cofactor in order to deliver the GSH tripeptide into the active site to complete the catalytic cycle (Fisher *et al.*, 2011). It is possible that the protein may have slightly misfolded during synthesis in the *E. coli* cells thus making the active site inaccessible. This may have been induced by the presence of the His-tag which has once been reported to shield the PLA₂ activity of the protein (Chen *et al.*, 2000). It was not anticipated that the His-tag could present a problem as previous studies were conducted with the tag attached and the protein was active (Ralat *et.al.*, 2008). There have been reports of some Prdx6 recombinant proteins which do not use GSH as reductant but use other reductants such as dithiothreitol (Fisher and Dodia, 1996; Chen *et al.*, 2006). However, since dithiothreitol is not a physiological reductant it was assumed that the use of GSH would be compatible. This knowledge along with published literature was the basis of the selected experimental conditions.

Another factor that could have resulted in inactive Prdx6 is the possibility of the GSTP1-1 being oxidised by the hydrogen peroxide and thus not being able to reactive Prdx6. The residues of the GSTP1-1 that are involved in reactivating Prdx6 are unknown thus it cannot be said with certainty that the hydrogen peroxide did not affect its interaction with Prdx6.

4.4. Future work

This study could have been improved by using the phospholipase A₂ activity as an assessment for the functional activity of the protein in addition to that of the peroxidase activity since Prdx6 conveys both catalytic activities. It would be advantageous to determine the activity of GSTP1-1 after it has been incubated with hydrogen peroxide in the Prdx6 assay to ascertain that the inactivity of Prdx6 not due to GSTP1-1 oxidation by the hydrogen peroxide.

CHAPTER FIVE: REFERENCES

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